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MINIREVIEWS

Diet as an epigenetic factor in inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) has as a main characteristic the exacerbation of the immune system against enterocytes, compromising the individual's intestinal microbiota. This inflammatory cascade causes several nutritional deficiencies, which further compromise immunological functioning and, as a result, worsen the prognosis. This vicious cycle can be interrupted as the patient's dietary pattern meets their needs according to their clinical condition, acting directly on the inflammatory process of IBD through the interaction of food, intestinal microbiota, and epigenome. Specific nutritional intervention for IBD has a crucial role in pre-

venting and managing disease activity. This review addresses epigenetic modifications through dietary compounds as a mechanism for modulating the intestinal microbiota of patients with IBD.

Key Words: Inflammatory bowel disease; Epigenetics; Nutrition; Nutrigenetics

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Core Tip: Inflammatory bowel disease is an autoimmune disease that oscillates between phases of active disease and remission. In any of these situations there are epigenetic mechanisms involved, which are modified by lifestyle, with diet being one of the factors of epigenetic modulation.

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INTRODUCTION

Epigenetic modifications are heritable alterations in gene expression that do not involve changes in DNA sequence but affect how genes are turned on and off, modulating the risk and severity of many diseases, including inflammatory bowel disease (IBD)[1,2]; such modifications include DNA methylation, histone modifications, and non-coding RNA molecules [such as microRNAs (miRNAs)][3].

These epigenetic mechanisms can be activated and modified through the individual's genetic inheritance and/or environmental factors, including the individual's diet, which requires additional attention[4]. Alongside an exponential growth in IBD cases, we observe obesity and overweight individuals due to a Westernized diet, rich in sugars and fats but deficient in vitamins and minerals[5]. The nutritional status and disease lead to malnutrition, commonly observed in patients with IBD[6,7]. Given the role of diet as a modulator of epigenetic parameters in patients with IBD, this review aims to present some micronutrients and their importance in preventing and/or treating malnutrition in these individuals.

Epigenetics in the pathogenesis of IBD

Epigenetics is classically defined as the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence. However, this broad definition allows several mechanisms to be classified as epigenetic or 'above the genome'[4]. Some events are defined as fixed epigenetic mechanisms, including DNA methylation, histone modifications, and non-coding RNA molecules (such as miRNAs), which translate to phenotypes and can be transmitted down the cell lineage or across generations. These types of epigenetic preservations represent common patterns of epigenetic inheritance.

Environmental factors, including diet, gut microbiota composition, and exercise, can elicit phenotype changes through gene expression without changing the genetic sequence[8]. DNA methylation and histone modifications are two central epigenetic mechanisms that impact gene transcription and cell fate. On the other hand, the impact on the gene transcription and translation by non-coding RNAs, including miRNAs, is also linked to epigenetic control associated with a widely complex system of non-coding RNA regulation[9]. MiRNAs are a class of small, non-coding RNA molecules that play a crucial role in epigenetics. These tiny molecules, typically consisting of 18 to 25 nucleotides, do not encode proteins themselves but act as potent post-transcriptional regulators[10]. These small molecules can influence gene expression by binding to specific messenger RNA (mRNA) molecules, leading to either degradation or translational repression of the target mRNA. Through this mechanism, miRNAs can fine-tune the expression of a wide array of genes, thereby having significant control over various cellular processes[11].

The role of miRNAs in regulating transcriptional processes is paramount to maintaining cellular homeostasis and responding to external stimuli. When miRNAs bind to their target mRNA sequences, they prevent the translation of those mRNA molecules into functional proteins, effectively downregulating the expression of the corresponding genes. This regulation is essential in diverse biological phenomena, including development, cell proliferation, differentiation, and immune responses[12]. By modulating gene expression, miRNAs influence various signaling pathways and cellular networks, acting as crucial epigenetic regulators. Dysregulation of miRNAs has been associated with numerous diseases, such as cancer, gastrointestinal tract diseases, neurodegenerative disorders, and cardiovascular conditions, highlighting their significance in understanding the molecular basis of complex human biology[13].

DNA methylation is a genetic modification that influences gene activity through cytosine methylation and transformation at the 5-carbon position. Single-base resolution maps of DNA methylation in human cell lines indicate that approximately 5% of all cytosines are methylated under normal physiological conditions[14]. Cytosine methylation predominantly occurs in the CpG dinucleotides sequence, where the guanidine base follows methylated cytosines. The primary function of DNA methylation is to actively silence genes and DNA regions, repressing the gene transcription in

the gene promoter region by inhibiting the binding of transcription factors or transcriptional events[15]. The regulation of DNA methylation occurs by the *de novo* DNA methyltransferases DNMT3a and DNMT3b. Across cell divisions, DNA methylation events are maintained by DNMT1. Active removal of methyl groups from cytosines is also hypothesized to occur by specific but yet to be identified factors[15].

The mechanism of DNA methylation in IBD includes specific patterns such as global DNA hypomethylation. Studies have shown that patients with Crohn's disease (CD) and ulcerative colitis (UC) exhibit global DNA hypomethylation in various cell types, including intestinal epithelial cells and immune cells[16]. This hypomethylation can lead to genomic instability and altered gene expression, contributing to disease progression. On the other hand, promoter hypermethylation occurs when there is increased DNA methylation in specific promoter regions, which can result in gene silencing. Several genes involved in inflammation regulation, immune response, and epithelial barrier function in bowel diseases were found to be hypermethylated[17,18]. For example, genes encoding anti-inflammatory cytokines, such as interleukin-10, have been observed to have increased promoter methylation in IBD patients, potentially impairing their ability to control inflammation. However, some genes, including the nuclear transcription factor-kappa B signaling pathway, present hypomethylation status in IBD, leading to their overexpression, contributing to increased bowel inflammation[19].

In IBD patients, alterations in DNA methylation patterns have been identified in genes related to intestinal permeability, mucin production, and tight junction proteins[19,20]. These changes can disrupt the integrity of the gut barrier, leading to increased immune activation and inflammation.

Regarding histone modifications, another frequent epigenetic mechanism, the chromatin structure, has the function to protect and maintain the DNA organization. Histones are essential chromatin proteins and facilitate DNA packaging into nucleosomes[21]. Histone proteins can be subdivided into five major classes in humans (H1, H2A, H2B, H3, and H4), all of which possess tails that can be post-translationally modified to alter the accessibility of the DNA. Histone modifications include acetylation, methylation, phosphorylation, ADP-ribosylation, and ubiquitination in the histone tail which can occur mainly by lysine modifications[22]. The effect of histone modifications on transcription is highly diverse and depends on the type of modification, either silencing or activating gene transcription. Histone proteins and the adjacent tail can be modified to orchestrate DNA transcription, but the extension of this modification results in transcriptional activation or inactivation, depending on the type of modification[23]. Methylation of H3 is associated with transcriptional activation, whereas lysine 36 methylation results in transcriptional repression. Histone lysine acetylation activates gene transcription by histone lysine acetyltransferases (HATs), whereas histone deacetylases (HDACs) remove acetyl groups from lysine residues[22].

Phosphorylation of histone proteins at specific serine or threonine residues can affect gene expression by influencing chromatin structure and protein-protein interactions. In bowel diseases, abnormal histone phosphorylation events have been observed, particularly in inflammation. For instance, phosphorylation of histone H3 at serine 10 (H3S10ph) has been associated with increased expression of pro-inflammatory genes in colonic epithelial cells during colitis[24]. Regarding the histone acetylation pathways, inflammatory disturbances are mediated by the dysregulation of HDACs and HATs, directly impacting the balance of histone deacetylation and acetylation status. Increased expression and activity of HDACs have been observed in IBD, leading to histone deacetylation and transcriptional repression of anti-inflammatory genes, contributing to the perpetuation of chronic inflammation. On the other hand, reduced expression or function of HATs in IBD decreases histone acetylation, suppressing genes involved in maintaining intestinal barrier integrity, immune homeostasis, and immunoregulation[25]. Thus, altered activities of HDACs and HATs induce aberrant cytokine production, imbalanced T cell responses, and impaired epithelial barrier function in IBD, highlighting their crucial roles in disease pathogenesis[26].

In IBD, increased histone acetylation in certain gene promoters has also been observed, leading to the upregulation of pro-inflammatory genes, contributing to chronic inflammation[27]. Furthermore, alterations in histone methylation marks, such as H3K4me3 and H3K27me3, have been associated with altered gene expression profiles in the intestinal epithelium of patients with IBD[28].

It is important to note that specific histone modifications in IBD can vary depending on the individual, the disease subtype, and the stage. The effects of these modifications may depend on the genetic context and environmental factors, influencing several aspects of the pathophysiology of the disease, including inflammation, epithelial barrier integrity, and immune response.

Nutritional aspects of IBD

The incidence of IBD appears to be increasing worldwide in both developed and developing countries[6]. Today, 0.1% of Brazilians live with this chronic condition, as verified through the temporal analysis performed by Quaresma *et al*[29], which also showed a decrease in the incidence of CD but an increase in UC. Although we recognize genetic predisposition as a determining factor in the pathogenesis of IBD, other factors, such as environmental ones, are increasingly recognized as contributing to the risk of developing IBD. Notably, the incidence of IBD follows global trends in terms of lifestyle, industrialization, and the Western diet[7].

Alongside the rise of new cases of IBD in adults and children, malnutrition is also a prevalent nutritional characteristic, which varies from 65%-75% in CD and 18%-62% in UC. Furthermore, we often see overweight and obesity associated with malnutrition in patients with IBD.

This panorama regarding the nutritional condition of patients with IBD reflects the Westernized dietary pattern and the numerous deficiencies in the absorptive process and intestinal selectivity. Among the risk factors related to diet, the intake of ultra-processed foods, additives, and emulsifiers[30-32] is mentioned, since these reduce bacterial diversity and increase intestinal permeability and inflammatory mechanisms[33]. Considering these dietary risk factors associated with a higher incidence of IBD, it is important to prioritize preventive interventions through existing dietary guides, which

warn to avoid ultra-processed foods and increase the intake of fresh and minimally processed foods. The latter promotes diet-microbiome interaction through food groups and short-chain fatty acid (SCFA)-producing bacteria, which benefit from ingesting these foods, thus contributing to the necessary dietary changes in preventing IBD[33].

The key element that links diet to the emergence or worsening of IBD is dysbiosis. The chronic consumption of increased amounts of animal protein, saturated fats, and refined carbohydrates, a Westernized dietary pattern, results in dysbiosis and changes in the microbiota, with an increase in pathogenic bacteria, such as *Bacterioides spp.* and *Ruminococcus torques*[34].

Dysbiosis disrupts the intestinal barrier, making the mucous layer thinner and more permeable to pathogens and antigens, resulting in persistent inflammation. On the other hand, a diet rich in vegetables and fiber reduces intestinal pH and prevents the growth of potentially pathogenic bacteria, ensuring membrane selectivity[32].

People living with IBD should be encouraged to make dietary changes, as these play a significant role in the etiology and management of the disease, promoting the induction and maintenance of clinical remission associated with IBD therapies, in addition to preventing malnutrition and/or obesity, thus avoiding severe nutritional deficiencies, which compromise the course of the disease[35-38].

The most common nutritional deficiencies in IBD patients are folate and fat-soluble vitamins. Patients who undergo extensive bowel resection have an increased risk of vitamin B12 malabsorption. Other key micronutrients that can be deficient and should be monitored are calcium, selenium, magnesium, zinc, and iron[39,40].

Nutri-epigenetic modulator in IBD

Nutri-epigenetics is a field that explores the interaction between nutrition, gene expression, and disease development [41]. In the context of IBD, which includes conditions like CD and UC, there is emerging evidence suggesting that certain dietary factors can influence the epigenetic modifications that contribute to the development and progression of the disease. These modifications are believed to result from a complex interplay between environmental, immunological, and genetic factors[42] (Figure 1).

While diet alone may not be the underlying cause of IBD, it plays a crucial role in developing and managing this complex disease, significantly influencing the symptoms, flare-ups, and overall disease management[43]. Several dietary factors have been investigated in the context of IBD and their potential impact on epigenetic modifications, biased gene expression, and impact on health outcomes. Here are some critical points regarding diet as an epigenetic modulator of IBD (Table 1).

Methyl donor micronutrients

Certain nutrients found in the diet can act as methyl donors or cofactors for enzymes involved in epigenetic processes. Methyl donors provide methyl groups (-CH3) for various biochemical reactions in the body, including DNA methylation, histone modifications, and RNA processing. These nutrients are involved in one-carbon metabolism and are crucial for maintaining proper epigenetic regulation, gene expression, and overall health[44,45]. Some essential methyl donor nutrients are discussed below.

Vitamin B9 (folate): Vitamin B9 (folate) is an essential water-soluble vitamin found in foods such as leafy green vegetables (spinach, cabbage, lettuce, and broccoli), legumes (bean, lentil, and pea), citrus fruits (orange and grapefruit), liver (chicken and beef), and fortified grains (wheat flour). Folate is an important component in metabolism and crucial in various biological processes, including DNA synthesis, repair, and methylation[46,47]. Regarding IBD, folate deficiency has been associated with alterations in DNA methylation patterns. Studies have shown that patients with IBD often exhibit lower levels of folate, and this deficiency can contribute to the dysregulation of DNA methylation in the intestinal epithelial cells[48]. Impaired methylation can lead to the aberrant expression of genes involved in inflammation, immune response, and intestinal barrier function, which are all critical aspects in the pathogenesis of IBD. By influencing DNA methylation, folate supplementation may restore usual gene expression patterns and improve the symptoms of IBD[49]. However, it is important to note that the relation between folate and IBD is complex, and the effects of folate supplementation may vary depending on individual factors. While folate deficiency can be detrimental, excessive folate intake may also have adverse effects, particularly in individuals with specific genetic variations.

Vitamin B12 (cobalamin): Vitamin B12 (cobalamin) is another water-soluble vitamin found primarily in animal-based foods like meat, fish (especially cold-water fish like salmon, trout, tuna, and sardines), shellfish, eggs, dairy products like milk, cheese, and yogurt, and fortified products like morning cereal and vegetable milk (soy, almond, or oat). Vitamin B12 contributes with folate to donate methyl groups for DNA methylation and other essential cellular processes[50].

Methionine: Methionine is an essential amino acid found in protein-rich foods such as meat, fish, eggs, seafood, nuts, soy-based food like tofu, legumes (beans, lentils, and chickpeas), dairy products, oats, and whole grains. It acts as a precursor for S-adenosylmethionine (SAM), the primary methyl donor for DNA and histone methylation[50]. SAM is a molecule formed from methionine and adenosine triphosphate. It is the primary methyl donor in numerous cellular processes, including DNA, RNA, protein, and histone methylation[51].

Betaine (trimethylglycine): Betaine (trimethylglycine) is a naturally occurring compound found in foods like beets, spinach, broccoli, shellfish, and whole grains (wheat and rye) that acts as a methyl donor in reactions that convert homocysteine to methionine, which can be used for DNA and protein methylation[52].

Choline: Choline is an important nutrient involved in various biological processes, including lipid metabolism and cell membrane structure. Choline can be converted to betaine, and both choline and betaine behave as methyl donors in the

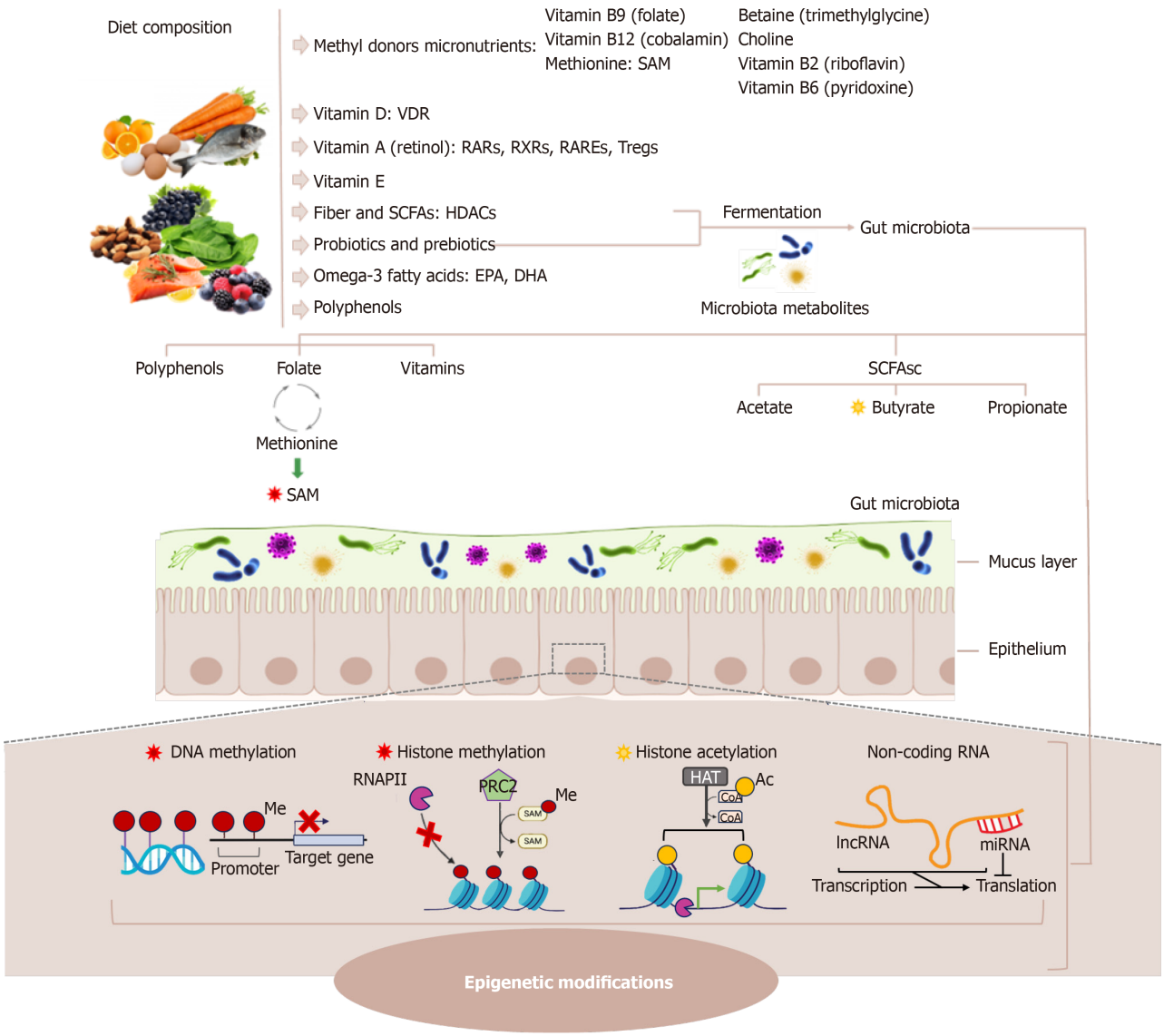
Table 1 Nutrients and their effect on epigenetic modifications in the context of inflammatory bowel disease

Nutrient(s)	Dietary sources	Biological activities	Epigenetic modifications	Ref.
Vitamin B (folate)	Leafy green vegetables, legumes, citrus fruits, and fortified grains	Methyl donor, DNA synthesis and repair	DNA methylation	[47-50]
Vitamin B12 (cobalamin)	Animal-based foods like meat, fish, eggs, and dairy products	Methyl donor	DNA methylation	[51]
Methionine	Protein-rich foods such as meat, fish, and dairy products	Methyl donor	DNA and histone methylation	[51, 52]
Betaine (trimethylglycine)	Beets, spinach, and whole grains	Methyl donor	DNA and protein methylation	[53]
Choline	Eggs, liver, peanuts, and cruciferous vegetables	Methyl donor, lipid metabolism, and cell membrane structure	DNA methylation	[53]
Vitamins B2 (riboflavin) and B6 (pyridoxine)	Whole grains, nuts, seeds, poultry, fish, and leafy green	FAD and FMN precursors	DNA methylation	[54]
Vitamin D	Fatty fish, eggs	VDRs ligand	DNA and histone methylation	[55-60]
Vitamin A	Leafy green vegetables, orange and yellow vegetables, tomato, fruits, and vegetable oils	RARs and RXRs ligand	DNA methylation, histone methylation and acetylation	[61-64]
Vitamin E	Plant-based oils, nuts, seeds, fruits, and vegetables	Antioxidant	DNA and histone methylation	[64-68]
SCFAs	High-fiber diets	Inhibition of HDACs activity	Histone acetylation	[69-73]
Lactobacillus rhamnosus GG	Fermented foods	Anti-inflammatory	DNA methylation	[78]
EPA and DHA	Salmon, mackerel, and sardines, flaxseeds, and walnuts	Anti-inflammatory	DNA methylation, histone methylation and acetylation, non-coding RNA	[81-83]
Polyphenols	Plant-based foods, such as fruits (berries), vegetables, nuts, seeds, green tea, cocoa, and olive oil	Anti-inflammatory and antioxidant	DNA methylation and non-coding RNA	[84, 85]

FAD: Flavin adenine dinucleotide; FMN: Flavin mononucleotide; VDRs: Vitamin D receptors; RARs: Retinoic acid receptors; RXRs: Retinoid X receptors; SCFAs: Short-chain fatty acids; HDACs: Histone deacetylases; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid.

body. Good sources of choline include eggs, liver, cold-water fish such as salmon and tuna, peanuts, dairy products, walnuts, almonds, sunflower seeds, and cruciferous vegetables[52].

Vitamins B2 (riboflavin) and B6 (pyridoxine): Vitamins B2 (riboflavin) and B6 (pyridoxine) are not primary donors like folate, vitamin B12, betaine, or choline, but they indirectly contribute to one-carbon metabolism and influence methylation reactions. B2 is an essential water-soluble vitamin, as it is a precursor for two important coenzymes, flavin adenine dinucleotide and flavin mononucleotide. These coenzymes are involved in redox reactions, energy metabolism, and various enzymatic reactions, including those related to the methylation process. Moreover, B2 plays an indirect role by synthesizing and recycling the methyl donor methionine. Conversion of homocysteine to methionine requires a methyl group from 5-methyltetrahydrofolate derived from folate metabolism. Additionally, vitamin B2 is necessary for converting folate to its active form, 5-methyltetrahydrofolate, thus indirectly supporting one-carbon metabolism and methylation reactions. Regarding B6, it represents a group of water-soluble compounds that include pyridoxine, pyridoxal, and pyridoxamine. These compounds are converted to their active form, pyridoxal 5′-phosphate (PLP), in the body. PLP is a coenzyme involved in various enzymatic reactions, including those related to amino acid metabolism. Similar to B2, vitamin B6 indirectly supports methylation reactions by converting methionine to SAM, the primary methyl donor for DNA and histone methylation. PLP is specifically required for the enzymatic reaction that converts methionine to SAM. Hence, a balanced diet that includes sources of B2 and B6, such as whole grains, nuts, seeds, avocado, potatoes, banana, dairy products, poultry, fish, and leafy green[45] vegetables, can help ensure sufficient levels of these nutrients [45]. Although methyl donors can alter DNA methylation patterns, little is known about the necessary doses and the exact period of dietary exposure or depletion that contributes to changes in epigenetic marks[45]. Therefore, more systematic studies are needed to provide more consistent findings.



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Figure 1 Schematic representation of the interplay of specific dietary constituents with the gut microbiota that interacts with the mammalian epigenome through the production of epigenetic substrates or regulators of chromatin-modifying enzymes. This process leads to epigenetic modifications that affect the immune response, compromising the epithelial barrier and defense mechanisms, resulting in chronic inflammation, as observed in inflammatory bowel disease. VDR: Vitamin D receptor; SCFAs: Short-chain fatty acids; HDACs: Histone deacetylases; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; RARs: Retinoic acid receptors; RXRs: Retinoid X receptors; RAREs: Retinoic acid response elements; Tregs: Regulatory T cells.

VITAMIN D

Vitamin D is a fat-soluble vitamin that is crucial in various physiological processes. Adequate levels of vitamin D, which can be obtained through sun exposure and dietary sources like fatty fish, cod liver oil, eggs, liver, and fortified foods (milk, orange juice, and morning cereals), have been linked to a lower risk of IBD[53,54]. Growing evidence suggests that vitamin D can affect DNA methylation patterns and histone modifications, leading to changes in gene expression profiles. Vitamin D acts as a ligand for the vitamin D receptor (VDR), which is present in various cell types, including immune cells. Upon binding to the VDR, vitamin D can influence the activity of enzymes involved in DNA methylation and histone modification, thereby altering gene expression[55,56].

Recent findings demonstrated that vitamin D deficiency is prevalent in individuals with IBD, and low vitamin D levels have been associated with increased disease activity and a higher risk of flare-ups[57]. Supplementing vitamin D in IBD patients has been shown to benefit disease activity and reduce inflammation. Furthermore, studies revealed that vitamin D supplementation can modulate epigenetic processes, altering IBD patients' DNA methylation patterns in immune cells and intestinal tissues. Thus, these changes in DNA methylation can affect the expression of genes involved in inflammation and immune regulation[58]. However, the exact mechanisms through which vitamin D influences epigenetic processes in IBD are still being elucidated.

VITAMIN A

Vitamin A, also known as retinol, is a crucial nutrient that plays a significant role in various biological processes, including immune function and inflammation. This fat-soluble vitamin is found in the form of beta-carotene in food sources like liver, carrots, sweet potato, pumpkin, spinach, kale, mango, melon, egg yolks, fatty fish, dairy products, and other orange vegetables (peppers and yellow zucchini). Their effects on gene expression through both genomic and non-genomic pathways were already demonstrated previously[59]. Retinoic acid, the active form of vitamin A, acts as a ligand for nuclear receptors known as retinoic acid receptors and retinoid X receptors. When retinoic acid binds to these receptors, it can regulate gene expression by interacting with specific DNA sequences called retinoic acid response elements in the promoter regions of targeted genes[60].

Recent findings demonstrated that retinoic acid affects DNA methylation patterns, leading to changes in gene expression profiles associated with immune regulation and inflammation. Additionally, retinoic acid can influence histone modifications, such as acetylation and methylation, which can further impact gene expression and cellular processes involved in IBD[61]. Furthermore, vitamin A has been shown to promote the development and function of regulatory T cells (Tregs) in the gut. Tregs are crucial in maintaining immune tolerance and preventing excessive inflammatory responses. Vitamin A helps to induce the expression of the transcription factor Foxp3, which is essential for Treg development and function. Epigenetic modifications, including DNA methylation and histone modifications, have been implicated in regulating Foxp3 expression and Treg function[62]. The therapeutic benefits of vitamin A as an epigenetic modulator, concerning its continuous use as a nutritional supplement, depend on our further understanding of its epigenetic effects on health and disease through different generations.

VITAMIN E

Vitamin E is a fat-soluble vitamin with antioxidant properties that protects cells from oxidative damage. While vitamin E is primarily known for its antioxidant effects, it also has some influence on epigenetic processes[62]. There were limited direct studies on the influence of vitamin E on epigenetic processes in IBD. However, research in other contexts suggests that vitamin E could impact epigenetic mechanisms.

Some studies in different disease models and cellular systems have shown that vitamin E can affect DNA methylation and histone modifications, both of which are crucial epigenetic mechanisms. These changes in epigenetic marks could lead to altered gene expression patterns and potentially influence disease processes[63-66].

While these studies provide preliminary evidence of the potential influence of vitamin E on epigenetic processes in IBD, additional research is needed to fully understand the mechanisms involved and establish a clear cause-and-effect association. Further studies, including clinical trials, are necessary to determine the optimal dosage and period of vitamin E supplementation and its effects on epigenetic modifications in individuals with IBD. It is important to emphasize that vitamin E supplementation should be approached with caution, especially at high doses, as excessive intake may have adverse effects. On the other hand, the consumption of foods rich in vitamin E can be encouraged, such as sunflower seeds, almonds, nuts, vegetable oils (wheat oil, sunflower oil, and wheat germ oil), avocado, hazelnuts, salmon, kiwi, man-go, and tomatoes.

Fiber and SCFAs

High-fiber diets have been associated with several beneficial effects in IBD, such as reduced inflammation, improved gut barrier function, and modulation of the gut microbiota[67]. Soluble fiber can dissolve in water, forming thick gels, and is subject to fermentation by colonic bacteria. Examples of highly fermentable soluble fibers with significant solubility and viscosity are beta-glucans and pectin, naturally present in whole grains like oats and barley and fruits such as apples. Non-viscous soluble fibers that undergo fermentation by the gut microbiota include inulin, gum acacia, resistant starch, polydextrose, and corn fiber. Fructans of the inulin-type are naturally found in agave (a succulent plant), artichokes, asparagus, bananas, chicory root, garlic, onions, and leeks. Resistant starches (found in legumes, seeds, raw and cooked potatoes, green bananas, and whole grains) and polydextrose are not absorbed in the small intestine due to their specific physical and chemical characteristics, rendering them inaccessible to α-amylase[35].

One of the mechanisms by which high-fiber diets can influence epigenetic processes is the production of SCFAs. SCFAs are generated by the fermentation of dietary fiber by gut bacteria, such as butyrate, propionate, and acetate. They have been shown to have anti-inflammatory effects and can act as signaling molecules that modulate gene expression through epigenetic modifications[28].

Butyrate, a type of SCFA, has been shown to inhibit the activity of HDACs, enzymes involved in epigenetic regulation. HDACs remove acetyl groups from histone proteins, leading to a more condensed chromatin structure and reduced gene expression. By inhibiting HDACs, butyrate and other SCFAs can promote a more relaxed chromatin structure, allowing increased gene expression of anti-inflammatory genes[62,28,68]. Furthermore, high-fiber diets can also influence the gut microbiota composition. Certain beneficial bacteria in the gut, such as *Faecalibacterium prausnitzii*, have been associated with the production of anti-inflammatory metabolites and a healthy gut environment[69,70].

Probiotics and prebiotics

Probiotics are live microorganisms that can be consumed through fermented foods or supplements and, when administered in adequate amounts, provide health benefits to the host. Some foods are naturally rich in probiotics or are fermented to contain these microorganisms like yogurt, kefir, kimchi, sauerkraut, pickles, aged cheese, and some

fermented soy products such as miso and tempeh. Probiotics have been extensively studied for their potential therapeutic effects in various health conditions, including IBD. While probiotics have shown promise in improving symptoms and reducing inflammation in IBD, their specific influence on epigenetic processes in this context is an area of ongoing research[71].

Some studies have explored the effects of probiotics on epigenetic modifications in the context of IBD. They found that the probiotic treatment led to changes in DNA methylation patterns at specific gene loci, suggesting a potential epigenetic mechanism underlying the beneficial effects of probiotics in IBD[72-74].

Other studies evaluated the effects of a specific probiotic strain, *Lactobacillus rhamnosus* GG, on DNA methylation patterns in a mouse model of colitis. They observed that the probiotic treatment altered DNA methylation in the colon tissue, potentially contributing to its anti-inflammatory effects[75].

Although these studies provide insights into the potential epigenetic effects of probiotics in IBD, it is important to note that the exact mechanisms by which probiotics influence epigenetic processes are still not fully understood. Further research is needed to elucidate the specific molecular pathways involved to determine the clinical implications of these findings.

In addition to probiotics, there also are prebiotics, which are non-digestible fibers that promote the growth of beneficial gut bacteria. Both have shown promise in modulating epigenetic processes and reducing inflammation associated with IBD[76]. A recent review showed that dietary prebiotics, as well as their microbial metabolites, directly target enzymatic activities and/or modulate the expression of enzymes involved in epigenetic gene regulation, with potential implications for health status and susceptibility to diseases[77].

Although the exact mechanisms by which prebiotics influence epigenetic processes in IBD are not yet fully understood, these studies provide evidence of their potential to modulate gene expression through epigenetic modifications. Further research is needed to elucidate the specific mechanisms and identify the most effective prebiotic interventions for individuals with IBD.

Omega-3 fatty acids

Omega-3 fatty acids, particularly eicosapentaenoic acid and docosahexaenoic acid, are polyunsaturated fatty acids commonly found in fatty fish (such as salmon, mackerel, and sardines), flaxseeds, and walnuts. They have anti-inflammatory properties and have been studied for their potential therapeutic effects on IBD. Several studies have indicated that omega-3 fatty acids can modulate epigenetic processes associated with inflammation in IBD by reducing DNA methylation, potentially upregulating the expression of anti-inflammatory genes and downregulating the expression of pro-inflammatory genes, leading to beneficial effects on IBD[78,79].

In addition to DNA methylation, omega-3 fatty acids can also affect other epigenetic mechanisms, such as histone modi-fications and expression of miRNAs, which are small RNA molecules that can post-transcriptionally regulate gene expression, although the specific mechanisms are still being investigated[80].

Overall, omega-3 fatty acids have the potential to modulate epigenetic processes in IBD, leading to reduced inflammation and improved outcomes. However, further research is needed to fully understand the precise mechanisms and determine the optimal dosages and treatment strategies for using omega-3 fatty acids in managing IBD.

Polyphenols

Polyphenols are a group of bioactive compounds found in various plant-based foods, such as fruits (berries, apple, and grape), green leafy vegetables (spinach, kale, and broccoli), onion, garlic, nuts, hazelnuts, almonds, seeds, green tea, black tea, coffee, cocoa, whole grains (oats, barley, and buckwheat), and olive oil. These compounds have been studied for their potential health benefits, including their ability to modulate epigenetic processes and potentially exert beneficial effects in IBD.

These compounds have been shown to have anti-inflammatory and antioxidant properties, and they can modulate various signaling pathways involved in immune response and inflammation. One of the well-studied epigenetic effects of polyphenols is their ability to influence DNA methylation. Polyphenols can affect DNA methyltransferase enzymes, which are responsible for adding or removing methyl groups from DNA molecules. By modulating these enzymes, polyphenols can potentially alter DNA methylation patterns, leading to changes in gene expression[81,82].

Moreover, polyphenols can also affect histone modifications, which regulate chromatin structure and gene expression. They can influence enzymes responsible for adding or removing acetyl, methyl, or other chemical groups to histone proteins, thereby modulating the accessibility of DNA and influencing gene expression patterns. Additionally, polyphenols have been shown to regulate the expression of miRNAs[83-85].

While promising evidence suggests the epigenetic influence of polyphenols in IBD, it is important to note that research in this area is still evolving, and more studies are needed to fully understand the specific mechanisms and effects of different polyphenols in IBD. It is important to note that the field of nutri-epigenetics in IBD is still in its early stages. Understanding the interplay between dietary factors and epigenetic mechanisms in IBD is an active area of research. While evidence suggests their involvement, more studies are needed to elucidate the specific mechanisms and determine the potential therapeutic implications of targeting these factors in managing IBD. It is essential for individuals with IBD to work closely with healthcare professionals, such as gastroenterologists and registered dietitians, to ensure optimal nutrient intake and personalized management strategies.

CONCLUSION

In conclusion, evidence has shown that dietary composition should be the primary basis for managing patients with IBD due to its role in intestinal health. Moreover, the impact of diet on the epigenetic process of microbiota modulation is clear. However, further clinical trials involving diets, supplements, and epigenetic and inflammatory markers are still needed.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Cohort Study

Application of extended criteria donor grafts in liver transplantation for acute-on-chronic liver failure: A retrospective cohort study

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Abstract

BACKGROUND

There is no consensus on the usage of extended criteria donor (ECD) grafts in liver transplantation (LT) for acute-on-chronic liver failure (ACLF) patients.

AIM

To summarize the experience of using ECD livers in ACLF-LT.

METHODS

A retrospective cohort study was conducted, enrolling patients who underwent LT at the First Affiliated Hospital of Sun Yat-Sen University from January 2015 to November 2021. The patients were divided into ECD and non-ECD groups for analysis.

RESULTS

A total of 145 recipients were enrolled in this study, of which ECD and non-ECD recipients accounted for 53.8% and 46.2%, respectively. Donation after cardiac death (DCD) recipients accounted for the minority compared with donation after brain death (DBD) recipients (16.6% *vs* 83.4%). Neither overall survival nor graft survival significantly differed between ECD and non-ECD and DCD and DBD recipients. ECD grafts were associated with a significantly higher incidence of early allograft dysfunction (EAD) than non-ECD grafts (67.9% *vs* 41.8%, P = 0.002). Postoperative outcomes between DCD and DBD recipients were compa-

rable (P > 0.05). ECD graft (P = 0.009), anhepatic phase (P = 0.034) and recipient gamma glutamyltransferase (P = 0.016) were independent risk factors for EAD. Recipient preoperative number of extrahepatic organ failures > 2 (P = 0.015) and intraoperative blood loss (P = 0.000) were independent predictors of poor post-LT survival.

CONCLUSION
Although related to a higher risk of EAD, ECD grafts can be safely used in ACLF-LT. The main factors affecting post-LT survival in ACLF patients are their own severe preoperative disease and intraoperative blood loss.

Key Words: Extended criteria donor; Acute-on-chronic liver failure; Liver transplantation

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Core Tip: This manuscript is intended to summarize a Chinese single center experience of using extended criteria donor (ECD) grafts in liver transplantation (LT) for acute-on-chronic liver failure (ACLF) patients. In this paper, we found that under ECD grafts are associated with a higher risk of early allograft dysfunction than non-ECD grafts but can be safely used in ACLF recipients as they do not affect post-LT survival. The main factors affecting the prognosis of ACLF recipients are the severity of their own preoperative disease and intraoperative blood loss.

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INTRODUCTION

Acute-on-chronic liver failure (ACLF) is a complex clinical syndrome characterized by the failure of extrahepatic organ(s) that has an extremely high short-term mortality and a 90-d transplant-free mortality above 50%[1]. Liver transplantation (LT) is the only curative treatment option for various end-stage liver diseases and has been reported to bring strong survival benefits to ACLF patients[2]. However, the current supply of acceptable donor livers is far from sufficient to meet the demands of the growing number of recipients. In an effort to reduce waiting list mortality, extended criteria donor (ECD) livers, also known as marginal livers, are increasingly being used in LT[3,4].

Usually, ECD livers are mainly defined as livers from donors with advanced age, macrovesicular steatosis, donation after cardiac death (DCD), and other unfavorable characteristics that indicate suboptimal quality[5,6]. The use of livers with ECD in ACLF-LT remains controversial. On the one hand, ECD livers were historically considered to be related to poor graft function and even poor survival; on the other hand, although transplanted with ECD grafts, recipients with high model for end-stage liver disease (MELD) scores or severe ACLF also obtained strong survival benefits, with 1-year post-LT survival rates reaching 78.1%[7,8]. The impact of the increased use of ECD grafts in ACLF patients needs to be further researched[9].

To our knowledge, there are still no studies published based on experiences at a Chinese hospital. In this study, we aimed to investigate the perioperative and long-term outcomes of ACLF patients in terms of whether they were ECD or non-ECD recipients.

MATERIALS AND METHODS

Patients

A retrospective cohort study was conducted. We recruited patients who underwent LT at the First Affiliated Hospital of Sun Yat-Sen University from January 2015 to November 2021 for our study. The inclusion criteria were as follows: Underwent LT; met the ACLF diagnostic criteria; and age ≥ 18 years. The exclusion criteria were complicated with hepatocellular carcinoma or other hepatobiliary cancer, combined transplantation with other organ (s), cases of ischemia-free LT (IFLT)[10] in a prospective randomized controlled study (registration number: ChiCTR1900021158) conducted during the same period, living donor LT, and incomplete medical records. Of note, there is still no unified definition of ACLF. Considering the unique epidemiological background in our country in which ACLF is mainly caused by hepatitis B virus infection, we adopted the diagnostic criteria recently proposed by the Chinese Group on the Study of Severe Hepatitis B[11] in this study; that is, regardless of the presence of cirrhosis, patients with chronic hepatitis B, total bilirubin (TB) ≥ 12 mg/dL and international normalized ratio ≥ 1.5 should be diagnosed with ACLF.

Donor and recipient clinical characteristics

The clinical parameters of both donors and recipients were extracted from electronic medical records. The baseline

characteristics of recipients were based on the last examination before LT. The severity of ACLF was measured by the MELD score and the number of extrahepatic organ failures (OFs) at the time before LT. Extrahepatic OF was defined by previous reports and included kidney[12], coagulation[13], circulatory system[14], respiratory system[15], and hepatic encephalopathy[16]. The baseline characteristics of donors were based on the last examination before organ procurement. There is still no precise definition of an ECD liver; with reference to previous reports[5,6] and the experience of our center, ECD was defined in this study as meeting any of the following criteria: Age > 65 years, body mass index (BMI) > 30 kg/m^2, macrovesicular steatosis ≥ 30%, serum sodium > 165 mmol/L, serum alanine aminotransferase (ALT)/aspartate aminotransferase (AST) > 120 U/L, serum TB > 51 μmol/L, cold-ischemia time (CIT) > 12 ho, split, DCD.

Outcomes

The primary endpoint events of interest were graft survival (from LT to re-LT or death) and overall survival (OS), from LT to death. The patients were followed up until December 2021. The secondary outcomes mainly included rates of early allograft dysfunction (EAD)[17], acute kidney injury (AKI)[17] and other perioperative characteristics [intraoperative blood loss/transfusion, operative time, and intensive care unit (ICU) stay].

Statistical analysis

The patients were divided into ECD and non-ECD recipient groups for analysis. As the main factor of concern in ECD, DCD recipients were also analyzed as a subgroup for comparison with donation after brain death (DBD) recipients. Statistical analyses were conducted using SPSS (version 23.0, IBM). Continuous measurement data are expressed as the mean ± SD, and differences between groups were detected by Student's t test. Enumeration data are expressed as numbers (percentages), and differences were detected by the χ^2 test. OS and graft survival were calculated by the Kaplan-Meier method and compared through the log-rank test. Univariate and multivariate logistic/Cox regression analyses were performed to identify the risk factors and independent risk factors for EAD/OS, and variables showing univariate significance or considered clinically relevant were entered into multivariate analysis[18]. Statistical significance was defined as $P < 0.05$.

RESULTS

Baseline characteristics of recipients

Ultimately, 145 patients were enrolled in our study, of whom 78 (53.8%) were in the ECD group and 67 (46.2%) were in the non-ECD group (Table 1). The severity of ACLF was quantified by the MELD score and OFs, and both quantitative tools showed no significant difference in the severity of the preoperative disease between these two groups ($P = 0.579$ and 0.547 and 0.591 and 0.547, respectively). Other demographic indicators, such as age, sex, blood type, and preoperative biochemical tests, also proved to have nonsignificant differences ($P > 0.05$). In addition, the numbers of IFLT cases were approximately similar in both groups ($P = 0.170$).

This finding indicates that there is no significant bias between these two groups in the allocation and usage of ECD grafts, and the probability of being assigned an ECD liver is approximately equal for patients with severe or mild ACLF.

Baseline characteristics of donor livers

Compared with non-ECD, ECD accounted for more than half of the total grafts [Table 2, 67 (46.2%) *vs* 78 (53.8%)]. The specific types of ECD grafts are shown in Table 2. Liver grafts were defined as ECD mainly because ALT/AST was greater than 120 U/L (29.5%/46.2%), followed by DCD (30.8%) and high serum sodium (17.9%). A total of 11.5% of grafts were classified as ECD due to BMI > 30 kg/m^2, and 9% were classified due to TB > 51 μmol/L. Notably, macrovesicular steatosis (2.6%), advanced age (1.3%), prolonged CIT (6.4%), and split (1.3%) only accounted for a very small proportion of ECD livers.

Primary outcomes

During the follow-up period, only one patient underwent retransplantation. Neither OS nor graft survival significantly differed between patients in the ECD and non-ECD groups and in the DCD and DBD groups (Figure 1). In ECD *vs* non-ECD recipients, the 1-, 3-, and 5-year OS rates were 87.0%, 83.7%, and 81.4% *vs* 86.0%, 83.8% and 83.8% ($P = 0.901$), respectively, and the 1-, 3-, and 5-year graft survival rates were 87.0%, 83.8% and 81.5% *vs* 86.0%, 83.8% and 83.8% ($P = 0.902$). In DCD and DBD recipients, the 1-, 3-, and 5-year OS rates were equal to the graft survival rates (83.3%, 83.3%, and 75.8% *vs* 87.1%, 83.5% and 83.5%, respectively) ($P = 0.631$ and 0.633, respectively).

Secondary outcomes

ECD recipients demonstrated a significantly higher postoperative EAD incidence than non-ECD recipients (67.9% *vs* 41.8%, $P = 0.002$). Except for EAD, there were no significant differences ($P > 0.05$) in the other secondary endpoints between these two groups (Table 3).

Then, we divided the patients into DCD and DBD groups, and all secondary endpoints, including EAD, AKI, blood loss and other perioperative indicators, showed no significant differences (Table 4, $P > 0.05$).

Identification of independent risk factors for EAD and OS

We enrolled all potential clinical parameters in univariable and multivariable logistic regression analyses to identify risk

Table 1 Baseline characteristics of recipients, *n* (%)

	ECD (*n* = 78)	Non-ECD (*n* = 67)	*P* value
MELD	30.73 ± 5.96	30.19 ± 5.60	0.579
MELD			0.547
> 30	40 (51.3)	31 (46.3)	
≤ 30	38 (48.7)	36 (53.7)	
OFs			0.591
0	25 (32.1)	28 (41.8)	
1	33 (42.3)	20 (29.9)	
2	11 (14.1)	9 (13.4)	
3	7 (9)	8 (11.9)	
4	2 (2.6)	2 (3)	
OFs			0.547
> 2	9 (11.5)	10 (14.9)	
≤ 2	69 (88.5)	57 (85.1)	
Age (yr)			0.365
> 60	4 (5.1)	6 (9)	
≤ 60	74 (94.9)	61 (91)	
Sex			0.730
Male	69 (88.5)	58 (86.6)	
Female	9 (11.5)	9 (13.4)	
BMI (kg/m^2)			0.611
> 30	5 (6.4)	3 (4.5)	
≤ 30	73 (93.6)	64 (95.5)	
Blood group			0.411
O	29 (37.2)	28 (41.8)	
A	24 (30.8)	20 (29.9)	
B	22 (28.2)	13 (19.4)	
AB	3 (3.8)	6 (9)	
WBC (× 10^9/L)	6.84 ± 3.56	6.35 ± 3.52	0.411
N/L	6.27 ± 6.34	7.00 ± 6.12	0.482
Hb (g/L)	94.27 ± 22.29	89.45 ± 19.06	0.167
GGT (U/L)	49.51 ± 26.39	50.12 ± 32.49	0.901
ALB (g/L)	36.87 ± 4.94	38.37 ± 4.99	0.071
ALT (U/L)	85.33 ± 96.36	60.88 ± 75.02	0.088
AST (U/L)	114.47 ± 103.80	92.42 ± 82.42	0.163
PLT (× 10^9/L)	63.50 ± 33.83	62.49 ± 38.57	0.867
Fib (g/L)	1.02 ± 0.47	1.12 ± 0.43	0.176
IFLT	2 (2.6)	5 (7.5)	0.170

MELD: Model for end-stage liver disease; ECD: Extended criteria donor; OFs: Organ failures; BMI: Body mass index; WBC: White blood cell; Hb: Hemoglobin; GGT: Gamma glutamyltransferase; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PLT: Platelets; Fib: Fibrinogen; IFLT: Ischemia-free liver transplantation.

Table 2 Baseline characteristics of donor livers, *n* (%)

	ECD (*n* = 78)	Non-ECD (*n* = 67)	*P* value
DCD/DBD	24 (30.8)/54 (69.2)	0/ 67 (100)	0.000
Split	1 (1.3)	0	0.352
Macrovesicular steatosis			0.187
≥ 30	2 (2.6)	0	
< 30	76 (97.4)	67 (100)	
Age (yr)			0.352
> 65	1 (1.3)	0	
≤ 65	77 (98.7)	67 (100)	
BMI (kg/m^2)			0.004
> 30	9 (11.5)	0	
≤ 30	69 (88.5)	67 (100)	
Na (mmol/L)			0.000
> 165	14 (17.9)	0	
≤ 165	64 (82.1)	67 (100)	
ALT (U/L)			0.000
> 120	23 (29.5)	0	
≤ 120	55 (70.5)	67 (100)	
AST (U/L)			0.000
> 120	36 (46.2)	0	
≤ 120	42 (53.8)	67 (100)	
TB (μmol/L)			0.012
> 51	7 (9.0)	0	
≤ 51	71 (91.0)	67 (100)	
CIT (h)			0.035
> 12	5 (6.4)	0	
≤ 12	73 (93.6)	67 (100)	
Reason of death			0.969
Trauma	41 (52.6)	35 (52.2)	
Other	37 (47.4)	32 (47.8)	

DCD: Donation after cardiac death; DBD: Donation after brain death; ECD: Extended criteria donor; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TB: Total bilirubin; CIT: Cold-ischemia time.

factors and independent risk factors for EAD. The results showed that ECD graft (P = 0.002), anhepatic phase (P = 0.003), operation time (P = 0.005) and recipient gamma glutamyltransferase (GGT), (P = 0.027) were risk factors for EAD. Then, ECD graft (P = 0.009), anhepatic phase (P = 0.034) and recipient GGT (P = 0.016) were shown to be independently associated with EAD (Table 5).

All potential factors that may be related to OS were included in the Cox regression analysis. Univariable analysis showed that blood loss (P = 0.000), EAD (P = 0.048), and OFs > 2 (P = 0.011) were risk factors for OS; then, multivariable analysis further demonstrated that blood loss (P = 0.000) and OFs > 2 (P = 0.015) were independent risk factors for OS (Table 6). Patients complicated with preoperative OFs > 2 (P = 0.007) or intraoperative blood loss > 2 L (median = 2 L, P = 0.038) were associated with significantly poorer post-LT survival (Figure 2).

Table 3 Secondary outcomes of recipients between extended criteria donor and non-extended criteria donor grafts, *n* (%)

	ECD (*n* = 78)	Non-ECD (*n* = 67)	*P* value
EAD	53 (67.9)	28 (41.8)	0.002
AKI	34 (43.6)	30 (44.8)	0.886
Blood loss (mL)	2.55 ± 1.70	2.82 ± 2.93	0.489
RBC transfused (U)	7.89 ± 3.93	9.13 ± 6.18	1.148
Plasma transfused (U)	9.77 ± 4.28	10.23 ± 4.51	0.534
Operative time (h)	7.48 ± 1.23	7.35 ± 1.37	0.554
ICU stay (d)	4.68 ± 4.95	4.63 ± 6.27	0.958

EAD: Early allograft dysfunction; ECD: Extended criteria donor; AKI: Acute kidney injury; ICU: Intensive care unit; RBC: Red blood cell.

Table 4 Secondary outcomes of recipients between donation after cardiac death and donation after brain death grafts, *n* (%)

	DCD (*n* = 24)	DBD (*n* = 121)	*P* value
EAD	17 (70.8)	64 (52.9)	0.106
AKI	11 (45.8)	53 (43.8)	0.855
Blood loss (mL)	2.53 ± 1.81	2.70 ± 2.44	0.747
RBC transfused (U)	8.61 ± 3.77	8.43 ± 5.35	0.876
Plasma transfused (U)	9.17 ± 5.03	10.14 ± 4.24	0.319
Operative time (h)	7.67 ± 1.46	7.37 ± 1.26	0.298
ICU stay (d)	5.25 ± 5.00	4.54 ± 5.70	0.571

DCD: Donation after cardiac death; DBD: Donation after brain death; ECD: Extended criteria donor; AKI: Acute kidney injury; ICU: Intensive care unit; RBC: Red blood cell.

DISCUSSION

The impact of the increased use of ECD grafts in ACLF patients has not yet been evaluated well[9]. To our knowledge, this is the first report from China that summarizes the experiences of using ECD grafts in ACLF-LT patients.

In our study, both OS and graft survival between ECD and non-ECD recipients and DCD and DBD recipients were not significantly different. This was approximately consistent with the conclusions of recent studies based on adult recipients (regardless of primary disease)[19] and high-acuity patients (MELD ≥ 35)[20]. For severe ACLF recipients[8], a marginal donor liver (donor risk index[21] above 1.7) was considered an independent risk factor for 1-year post-LT survival. However, as the authors pointed out in this article, although transplanted with marginal livers, it was clear that patients still obtain strong survival benefits, with 1-year post-LT survival rates reaching 78.1%. In our study, ECD recipients had a 5-year post-LT survival rate above 80%. Compared with the poor prognosis of 90-d transplant-free mortality above 50% [1], ECD grafts undoubtedly provide an important life-saving option for ACLF patients. Moreover, refusing ECD and continuing to wait for an ideal graft means a prolonged waiting time, which also means an increased risk of worse preoperative disease and higher post-LT mortality[22]. Consequently, it may be better for ACLF patients to accept an existing ECD graft rather than waiting for a prospective ideal liver.

In our opinion, the reason why there was no significant difference in survival between ECD and non-ECD patients was mainly due to the inevitable selection bias in clinical practice. As shown in Table 2, advanced age, prolonged CIT and macrovesicular steatosis, which have been widely recognized as the strongest prognostic risk factors[23], only accounted for 1.3%, 6.4% and 2.6% of our ECD grafts, respectively. This indicates that the ECD grafts actually adopted in our clinical practice may be relatively safe, and those grafts empirically judged as "high risk" were abandoned. Nevertheless, ECD recipients still showed a significantly higher incidence of EAD than non-ECD recipients.

ECD grafts, anhepatic phase and recipient GGT were proven to be significantly associated with EAD in our further research. The importance of shortening the anhepatic phase in transplantation is self-evident and has been proven by previous studies[24,25]. Our research emphasized the importance of surgical techniques once again. Traditionally, high serum GGT has been considered a biomarker of hepatobiliary diseases. Recent studies have shown its predictive role in carcinogenesis, tumor progression and many other life-threatening diseases[26]. The potential of donor GGT in predicting EAD[27] and graft survival[28] has also been reported, but few studies have focused on its role in recipients. Our study found that a high preoperative recipient serum GGT level was significantly correlated with post-LT EAD but did not

Table 5 Univariable and multivariable logistic analysis of risk factors for early allograft dysfunction

Variables	OR	Univariate analysis		OR	Multivariate analysis	
		HR (95%CI)	*P* value		HR (95%CI)	*P* value
Donor characteristics						
HBV (positive *vs* negative)	1.076	0.522-2.220	0.843			
Death of trauma (yes *vs* no)	1.331	0.689-2.568	0.395			
ECD (yes *vs* no)	2.953	1.497-5.826	0.002	2.712	1.286-5.720	0.009
Operation characteristics						
Blood loss (L)	1.080	0.925-1.262	0.330			
Anhepatic phase (min)	1.036	1.012-1.060	0.003	1.031	1.002-1.060	0.034
Operation time (h)	1.487	1.124-1.967	0.005	1.271	0.915-1.767	0.153
IFLT (yes *vs* no)	0.121	0.014-1.031	0.053	0.114	0.011-1.218	0.072
Recipient characteristics						
BMI (> 30 *vs* ≤ 30)	0.779	0.187-3.244	0.732			
MELD (> 30 *vs* ≤30)	1.455	0.753-2.812	0.265			
OFs (> 2 *vs* ≤ 2)	0.861	0.327-2.264	0.761			
WBC ($\times 10^9$/L)	1.039	0.945-1.142	0.432			
N/L	0.997	0.946-1.051	0.906			
Hb (g/L)	1.006	0.990-1.022	0.444			
GGT (U/L)	1.015	1.002-1.029	0.027	1.017	1.003-1.032	0.016
ALB (g/L)	1.052	0.984-1.126	0.139			
ALT (U/L)	1.002	0.998-1.006	0.288			
AST (U/L)	1.001	0.997-1.004	0.650			
PLT ($\times 10^9$/L)	1.001	0.992-1.010	0.800			
Fib (g/L)	0.692	0.332-1.441	0.325			

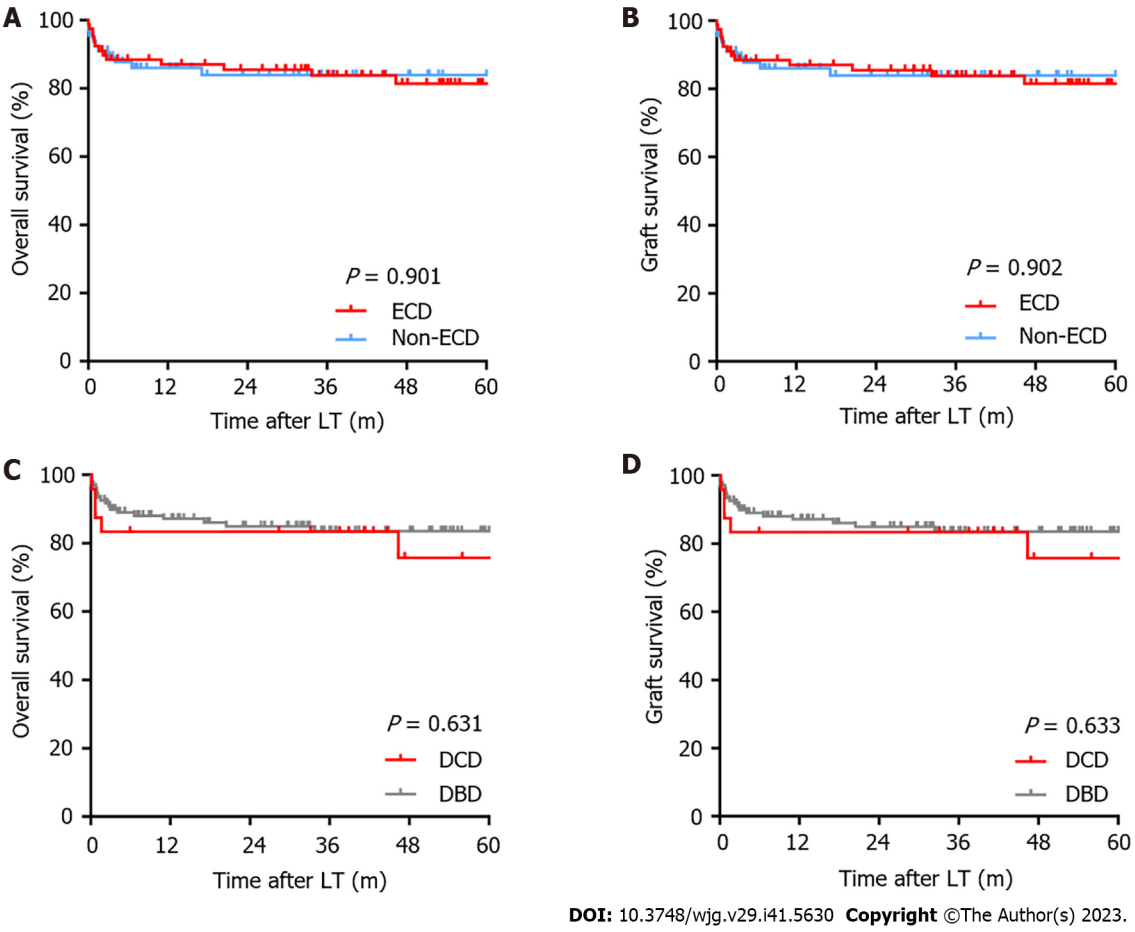
OR: Odds ratio; CI: Confidence interval; HBV: Hepatitis B virus; ECD: Extended criteria donor; BMI: Body mass index; IFLT: Ischemia-free liver transplantation; MELD: Model for end-stage liver disease; OFs: Organ failures; WBC: White blood cell; Hb: Hemoglobin; GGT: Gamma glutamyltransferase; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PLT: Platelets; Fib: Fibrinogen.

affect survival. This may be due to its critical role in the modulation of redox equilibria[29], and high serum GGT may reflect worse preoperative disease in recipients. At the same time, it should be noted that the determinants of prognosis in LT are numerous and complex.

Our analysis showed that only preoperative recipient OFs and intraoperative blood loss were independently associated with OS. This finding indicates that under our current ECD experience, the post-LT survival of ACLF patients mainly depends on the severity of their own preoperative disease and intraoperative conditions. The MELD score is widely accepted as a tool to quantify the severity of end-stage liver disease and to allocate donor livers. However, in recent years, a growing number of studies have found that MELD or MELD-Na underestimates the severity of ACLF, mainly because it fails to capture the two key pathophysiological features of ACLF: Extrahepatic OF and systemic inflammation[30,31]. Our study also showed that OFs may reflect the severity of ACLF more accurately than MELD.

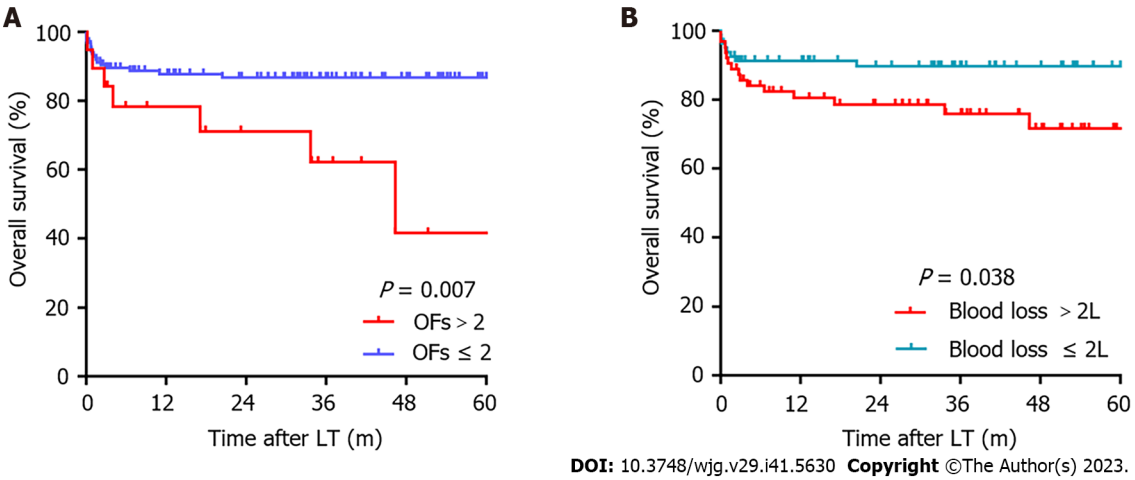
There are limitations in our study. The first is that the boundary of ECD remains undetermined. It should be recognized that the definition of ECD is not a simple concept of yes or no but should be linearly quantified. The application value of the Donor Risk Index[21] in China is limited due to unique ethnic characteristics. What is the safe boundary of an acceptable ECD graft? Unfortunately, we have not been able to establish a quantitative formula thus far in our country, and this will be the focus of our future research. Second, our results need to be further confirmed by a larger sample study.

In conclusion, our experience suggests that ECD grafts are associated with a higher risk of EAD than non-ECD grafts but can be safely used in ACLF recipients, as they do not affect post-LT survival. The main factors affecting the prognosis of ACLF recipients are the severity of their own preoperative disease and intraoperative blood loss.



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Figure 1 Kaplan-Meier analysis. A: Overall survival between extended criteria donor (ECD) and non-ECD groups; B: Graft survival between ECD and non-ECD groups; C: Overall survival between donation after cardiac death (DCD) and donation after brain death (DBD) groups; D: Graft survival between DCD and DBD groups. LT: Liver transplantation; ECD: Extended criteria donor; DCD: Donation after cardiac death; DBD: Donation after brain death.



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Figure 2 Kaplan-Meier analysis of overall survival. A: Organ failures; B: Blood loss. OFs: Organ failures.

CONCLUSION

In conclusion, our experience suggests that ECD grafts are associated with a higher risk of EAD than non-ECD grafts but can be safely used in ACLF recipients as they do not affect post-LT survival. The main factors affecting the prognosis of ACLF recipients are the severity of their own preoperative disease and intraoperative blood loss.

Table 6 Univariable and multivariable Cox analysis of risk factors for overall survival

Variables	OR	Univariate analysis		OR	Multivariate analysis	
		HR (95%CI)	*P* value		HR (95%CI)	*P* value
Donor characteristics						
HBV (positive *vs* negative)	1.478	0.646-3.383	0.355			
Death of trauma (yes *vs* no)	0.848	0.379-1.898	0.688			
ECD (yes *vs* no)	1.053	0.465-2.386	0.901			
No. of ECD features (≥ 2 *vs* < 2)	0.840	0.279-2.531	0.757			
Operation characteristics						
Blood loss (L)	1.271	1.137-1.421	0.000	1.276	1.123-1.449	0.000
Anhepatic phase (min)	1.009	0.989-1.028	0.381			
Operation time (h)	1.002	0.746-1.345	0.990			
IFLT (yes *vs* no)	0.822	0.111-6.107	0.848			
EAD (yes *vs* no)	2.726	1.009-7.365	0.048	2.481	0.914-6.737	0.075
AKI (yes *vs* no)	1.882	0.830-4.269	0.130			
Recipient characteristics						
BMI (> 30 *vs* ≤ 30)	0.831	0.112-6.166	0.856			
MELD (> 30 *vs* ≤ 30)	1.528	0.678-3.444	0.307			
OFs (> 2 *vs* ≤ 2)	3.191	1.309-7.780	0.011	3.042	1.245-7.432	0.015
WBC ($\times 10^9$/L)	1.007	0.897-1.129	0.912			
N/L	1.040	0.992-1.090	0.102			
Hb (g/L)	0.984	0.963-1.005	0.128			
GGT (U/L)	0.986	0.968-1.005	0.142			
ALB (g/L)	1.044	0.963-1.131	0.300			
ALT (U/L)	0.992	0.984-1.001	0.070			
AST (U/L)	0.998	0.992-1.004	0.463			
PLT ($\times 10^9$/L)	0.999	0.987-1.010	0.813			
Fib (g/L)	0.874	0.345-2.211	0.775			

OR: Odds ratio; CI: Confidence interval; HBV: Hepatitis B virus; ECD: Extended criteria donor; EAD: Early allograft dysfunction; BMI: Body mass index; IFLT: Ischemia-free liver transplantation; AKI: Acute kidney injury; MELD: Model for end-stage liver disease; OFs: Organ failures; WBC: White blood cell; Hb: Hemoglobin; GGT: Gamma glutamyltransferase; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PLT: Platelets; Fib: Fibrinogen.

ARTICLE HIGHLIGHTS

Research background

There is no consensus on the usage of extended criteria donor (ECD) grafts in liver transplantation (LT) for acute-on-chronic liver failure (ACLF) patients.

Research motivation

It was intended to summarize the experience of using ECD livers in ACLF-LT.

Research objectives

Our study aimed to summarize the experience of using ECD livers in ACLF-LT, and to provide reference for clinical practice.

Research methods
We conducted a retrospective cohort study to analyze outcomes between ECD and non-ECD recipients.

Research results
There was no significant difference ($P > 0.05$) in survival between ECD and non-ECD recipients after LT, although ECD grafts were associated with a significantly higher incidence of early allograft dysfunction. The most important factors affecting post-LT survival of ACLF patients were extrahepatic organ failures (OFs) > 2 ($P = 0.015$) and intraoperative blood loss ($P = 0.000$).

Research conclusions
ECD grafts can be safely used in ACLF-LT, although related to a higher risk of early allograft dysfunction.

Research perspectives
Due to the unavoidable selection bias in clinical practice, there were only 2 cases of donor liver have diagnosed as macrovesicular steatosis more than 30%. This indicates that the ECD grafts actually adopted in our clinical practice may be relatively safe, and those grafts empirically judged as "high risk" were abandoned. Admittedly, this is a major limitation of our current study, and the next step will be to try to compensate it by including more cases in our study over a longer period or in conjunction with other transplant centers.

FOOTNOTES

Author contributions: Gong JL, Tang YH, and Zhu XF conceived study; Gong JL, Yu J and Wang TL collected data and analyzed data; Yu J and Wang TL prepared figures and tables; Gong JL and Yu J wrote manuscript; He XS, Tang YH, and Zhu XF reviewed manuscript; All authors read and approved the final manuscript.

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Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

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Data sharing statement: The datasets of the current study are available from the corresponding author upon reasonable request.

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ORIGINAL ARTICLE

Retrospective Study

Long-term efficacy and predictors of pembrolizumab-based regimens in patients with advanced esophageal cancer in the real world

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Abstract

BACKGROUND

Pembrolizumab combined with chemotherapy has been proven effective as first-line therapy in patients with advanced esophageal cancer. Few trials have assessed the safety and efficacy of this treatment in patients with locally advanced disease.

AIM

To analyze long-term outcomes of pembrolizumab in locally advanced or metastatic esophageal squamous cell carcinoma (ESCC) in the real world.

METHODS

Patients with advanced ESCC admitted to our center from October 2019 to October 2021 were enrolled in this study. Clinical staging of the patients was based on the 8th edition of the American Joint Committee on Cancer TNM staging system. The patients received different treatments based on clinical stage. In brief, patients with locally advanced and resectable ESCC received neoadjuvant therapy combined with surgery. For those who were not candidates for resection, radical concurrent chemoradiotherapy plus pembrolizumab was more preferable. Patients with metastatic ESCC or who were unsuitable for radiotherapy underwent chemotherapy in combination with pembrolizumab. Long-term survival outcomes such as overall survival (OS), progression-free survival, disease-free survival, long-term adverse effects (AEs), immune maintenance therapy and predictors of immune checkpoint inhibitors (ICIs) efficacy were evaluated.

RESULTS

A total of 55 patients with advanced ESCC were enrolled in this retrospective, observational study. The median age was 61 years (range 44-74), with 47.3% (26/55) of the patients in stage IV and 45.5% of the patients had the tumor (25/55) located in the middle third of the esophagus. The median OS in all patients was not reached. The 12-mo OS rate among all patients was 78.8% and the 18-mo OS rate was 72.7%. 9 patients died due to tumor progression and 7 patients died due to treatment-related complications. The therapeutic effect evaluated at the interim evaluation was significantly reflected in the long-term outcome. Patients with complete response or partial response in all patients (P = 0.005) and in the chemoradiotherapy plus pembrolizumab group (P = 0.007) obtained a better prognosis than non-responders. A total of 20 patients (20/55, 36%) received immune maintenance therapy. Baseline peripheral blood biomarkers of the neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio, and neutrophil-to-(leukocyte-neutrophil) ratio did not predict the efficacy of ICIs.

CONCLUSION

Pembrolizumab combined with chemotherapy or radiotherapy resulted in favorable long-term survival in patients with locally advanced or metastatic ESCC, with safe and manageable long-term AEs.

Key Words: Esophageal cancer; Pembrolizumab; Radiotherapy; Long-term survival; Chemotherapy; Real-world

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Core Tip: Pembrolizumab combined with chemotherapy has been proven effective as first-line therapy in patients with metastatic esophageal cancer. Few trials have assessed the safety and efficacy of this treatment in patients with locally advanced disease. Our study showed that this treatment in patients with locally advanced or metastatic esophageal squamous cell carcinoma resulted in favorable long-term survival and manageable long-term adverse effects. Randomized phase III trials should be carried out for further verification.

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URL: https://www.wjgnet.com/1007-9327/full/v29/i41/5641.htm
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INTRODUCTION

Esophageal cancer is the seventh most common and the sixth leading cause of malignant tumor death worldwide[1]. In China, the incidence and mortality risk of esophageal cancer rank sixth and fourth, respectively[2]. The majority of esophageal cancer patients in China have esophageal squamous cell carcinoma (ESCC) which accounts for 90% of tissue types, and less than 10% have adenocarcinoma[3,4]. Most patients initially diagnosed with esophageal cancer have advanced disease, some patients have locally advanced disease which is inoperable, and some patients have metastases to other sites. Neoadjuvant chemoradiotherapy (nCRT) followed by surgery is the main treatment for resectable ESCC[5,6]. Radical concurrent chemoradiotherapy is an important treatment strategy for locally advanced unresectable patients[7]. For metastatic ESCC, systemic chemotherapy is the only treatment option[8,9]. In fact, for locally advanced or metastatic ESCC, treatment modalities are limited, progress in long-term survival is slow, and the efficacy is unsatisfactory. Data shows that the 5-year survival rate for locally advanced ESCC is no more than 30%. The 5-year survival rate for metastatic ESCC is less than 10%[10].

In recent years, immune checkpoint inhibitors (ICIs) combined with chemotherapy has made significant progress in the first-line treatment of advanced esophageal cancer[11-15]. In the randomized phase III KEYNOTE-590 study, ICIs therapy targeting programmed cell death protein 1 (PD-1), pembrolizumab combined with chemotherapy showed a significant survival advantage over chemotherapy alone in the first-line therapy. The median overall survival (OS) was more than 12 mo and the median progression-free survival (PFS) was 6.3 mo, significantly better than the median OS of 9.8 mo and the median PFS of 5.8 mo in the chemotherapy alone group. In addition, the safety was reliable[12]. For locally advanced patients treated with neoadjuvant therapy, a multicenter real-world study in China showed that the R0 resection rate reached 97.7% in combination with ICIs, and 25.5% of patients in the ICIs plus chemotherapy group and 42.3% of patients in the ICIs plus chemoradiotherapy group achieved pathologic complete response (pCR)[16]. Furthermore, some single-arm clinical trials have also investigated the application of ICIs combined with chemotherapy or concurrent chemoradiotherapy in the field of neoadjuvant therapy[17-20]. To date, the evidence for neoadjuvant treatment combined with ICIs remains inadequate, and results from large phase III clinical trials and long-term follow-up data are lacking. In unresectable locally advanced ESCC, a recent phase IB clinical study examined the efficacy and safety of the PD-1 inhibitor camrelizumab combined with radical radiotherapy for locally advanced ESCC which was intolerant to concurrent chemoradiotherapy[21]. The results showed that median OS and PFS were 16.7 mo and 11.7 mo, respectively. The 24-mo OS rate and PFS rate were 31.6% and 35.5%, respectively. The objective response rate (ORR) was 74%. Three

randomized phase III studies (KEYNOTE-975, ESCORT-CRT and RATIONALE 311) are currently being conducted to further confirm the value of ICIs combined with concurrent chemoradiotherapy. Although these studies have demonstrated the benefit of ICIs plus chemotherapy or chemoradiotherapy in locally advanced or metastatic ESCC, there is currently a lack of reliable predictors of the efficacy of ICIs in esophageal cancer. Several retrospective studies have explored the predictors of efficacy in subsequent-lines for ESCC, and the results showed that blood cell composition can predict the efficacy of ICIs[22-24]. However, predictive results for first-line treatment of locally advanced or metastatic ESCC are still lacking.

Based on this, our center conducted a real-world clinical study to examine the efficacy and safety of pembrolizumab in neoadjuvant therapy, concurrent chemoradiotherapy and first-line therapy for ESCC[25]. Early results showed that the combination with pembrolizumab demonstrated considerable ORR and acceptable adverse effects (AEs). We here report the long-term survival such as OS, disease-free survival (DFS) ,PFS, long-term toxicities and ICIs completion rates. We also assess the relationship between baseline blood cell composition indices such as the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), neutrophil-to-(leukocyte-neutrophil) ratio (dNLR) and long-term survival in order to determine the predictive factors of ICIs.

MATERIALS AND METHODS

Study design

This single-arm, single-center, retrospective clinical study was conducted in the First Medical Center of Chinese PLA General Hospital. Patients who were initially diagnosed with locally advanced or metastatic esophageal cancer from October 1, 2019 to October 1, 2021 were included. Clinical staging of the patients was based on the 8th edition of the American Joint Committee on Cancer TNM staging system. According to the different clinical stages and treatment modalities, the patients were divided into different subgroups. ORR was defined as the proportion of total patients with CR or partial response (PR). OS was considered the time from definitive diagnosis to death by any cause. DFS was regarded as the period from definitive diagnosis to disease recurrence or death in operable patients. PFS was defined as the period from definitive diagnosis to disease recurrence or death in patients with inoperable locally advanced or metastatic esophageal cancer. Blood samples were obtained at baseline. The NLR was the total number of neutrophils divided by the lymphocyte count. The PLR was the platelet count divided by the lymphocyte count. The dNLR represented the total number of neutrophils divided by the difference between the total number of white blood cells and neutrophils. The study was approved by the Ethics Committee of the Chinese PLA General Hospital in line with the Declaration of Helsinki (as revised in 2013).

Therapeutic regimen

Neoadjuvant therapy plus surgery: Eligible patients were aged 18-75 years, initially diagnosed with operable locally advanced ESCC (T2-4N0M0 or T2-4N+M0), with Eastern Cooperative Oncology Group performance status score of 0 or 1, and life expectancy of at least 6 mo. Patients with active autoimmune disease, and a history of ICIs or chemotherapy treatment were excluded. The patients underwent neoadjuvant chemotherapy (lobaplatin combined with albumin-paclitaxel) plus ICIs (pembrolizumab), prior to surgery once every 3 wk for 2 or 3 cycles. Surgery was then performed after physical examination, laboratory tests, contrast-enhanced chest computed tomography (CT) and pulmonary function tests.

Chemoradiotherapy plus pembrolizumab: Patients were aged 18-75 years, locally advanced and inoperable esophageal cancer or limited to supraclavicular lymph node metastasis, with Eastern Cooperative Oncology Group performance status score of 0 or 1, life expectancy of at least 6 mo were included. Patients with active autoimmune disease, and a history of ICIs or chemotherapy treatment were excluded. They received radical chemoradiotherapy plus pembrolizumab. The patients underwent 2-4 cycles of induction therapy with a chemotherapy regimen (lobaplatin combined with albumin-paclitaxel) plus pembrolizumab. Radical radiotherapy or chemoradiotherapy was then given and an external irradiation technique was used. The total dose of radiotherapy was 54 Gy/30 F, 1.8 Gy each time, 5 times a week. On this basis, primary esophageal lesions and metastatic lymph nodes received 63 Gy/30 F. Pembrolizumab could be discontinued during radiotherapy due to safety concerns. After radiotherapy, pembrolizumab was used as maintenance therapy for a total of 2 years. Treatment was suspended if disease progression or intolerable toxicity occurred.

Chemotherapy plus pembrolizumab: Patients were aged 18-75 years, diagnosed with metastatic esophageal cancer or unsuitable for radiotherapy, with adequate organ function, Eastern Cooperative Oncology Group performance status score of 0 or 1, life expectancy of at least 6 mo were included. Patients with active autoimmune disease, and a history of ICI or chemotherapy treatment were excluded. A chemotherapy regimen (lobaplatin combined with albumin-paclitaxel) plus pembrolizumab was administered every 3 wk for a total of 4 cycles, and then pembrolizumab was given as maintenance therapy for 2 years.

Follow-up

Follow-up began at the time of the patient's diagnosis and treatment in our hospital. The last follow-up was on December 1, 2022. Contrast-enhanced chest and abdominal CT, upper gastrointestinal contrast, ultrasound, and laboratory tests were routinely performed during the follow-up. Gastroscopy, positron emission tomography-CT and chest magnetic resonance imaging were also performed when necessary. Follow-up was conducted every 3 mo during the first 2 years

and then every 6 mo thereafter. The patient's physical condition and long-term AEs were assessed by consultation, telephone and other methods.

Statistical analysis

SAS 9.4 was used for all statistical analyses. The Kaplan-Meier method was used to estimate OS, DFS, PFS and their corresponding 95% confidence intervals (CIs). We divided patients into 3 subgroups according to the different treatment modalities (neoadjuvant treatment plus ICIs, radical chemoradiotherapy plus ICIs, chemotherapy plus ICIs). For the analysis of predictors of immunotherapy efficacy, we used a median cutoff value of 2.43 for NLR, 139.7 for PLR, and 1.72 for dNLR. The group with a larger cutoff value than the median cutoff value was defined as the high group, while the group with a smaller value than the median cutoff value was defined as the low group.

RESULTS

Patient characteristics

A total of 55 patients with ESCC were enrolled in this study from October 1, 2019 to October 1, 2021 (Table 1). The majority of patients were male (43/55, 78.2%) and 12 patients were female. The median age was 61 years (range 44-74), with 47.3% (26/55) of the patients in stage IV and 45.5% of the patients had the tumor (25/55) located in the middle third of the esophagus.

Therapeutic regimen received

Patients received different therapeutic regimens according to clinical stage. Among them, 21 patients received neoadjuvant treatment plus pembrolizumab followed by surgery. 20 patients with locally advanced inoperable and partial stage IV with supraclavicular lymph node metastasis were treated with radical chemoradiotherapy combined with pembrolizumab. The remaining patients who had metastatic esophageal cancer or were unsuitable for chemoradiotherapy received chemotherapy plus pembrolizumab.

Long-term efficacy

The median OS in all patients was not reached. The 12-mo OS rate in all patients was 78.8% and the 18-mo OS rate was 72.7% (Figure 1A). 9 patients died due to tumor progression and 7 died due to treatment-related complications. In the subgroup analysis, the 12-mo OS rate was 65% and the 18-mo OS rate was 60% in the chemoradiotherapy plus pembrolizumab group (Figure 1B). The 12-mo OS rate in the neoadjuvant treatment plus pembrolizumab group was 95% and the 18-mo OS rate was 89.7% (Figure 1C). In the chemotherapy plus pembrolizumab group, the 12-mo OS rate was 75% and the 18-mo OS rate was 66.7% (Figure 1D). The median OS for the 3 subgroups was not reached (Table 2). The 12-mo PFS rate was 55%, the 18-mo PFS rate was 50% and the median PFS was 17 mo in the chemoradiotherapy plus pembrolizumab group (Figure 2A). The 12-mo DFS rate was 85%, the 18-mo DFS rate was 75% and the median DFS was not reached in the neoadjuvant treatment plus pembrolizumab group (Figure 2B). The 12-mo PFS rate was 67.7%, the 18-mo PFS rate was 67.7% and the median PFS was not reached in the chemotherapy plus pembrolizumab group (Figure 2C, Tables 3 and 4).

In addition, the therapeutic effect assessed at the interim evaluation was significant in the long-term outcome. Patients with ORR (CR or PR) in all patients (P = 0.005) (Figure 3A) and in the chemoradiotherapy plus pembrolizumab group (P = 0.007) (Figure 3B) obtained a better prognosis than non-responders. However, we did not find a tendency for benefit in the neoadjuvant therapy followed by surgery group (Figure 3C) and chemotherapy plus pembrolizumab group (Figure 3D).

Safety and patterns of recurrence

In the chemoradiotherapy plus pembrolizumab group, 8 patients died (4 due to esophageal fistula, 1 due to liver failure, 2 due to tumor progression, and 1 due to lung infection). 5 patients developed disease progression (4 patients had recurrence in the radiotherapy targeted area of supraclavicular lymph node metastasis, esophageal lesion, mediastinal lymph node and 1 patient had liver metastasis). In the neoadjuvant treatment plus pembrolizumab group, 4 patients died, including 3 patients who died from tumor progression and 1 patient from a treatment-related complication. 7 patients had disease recurrence and metastasis, among whom 2 patients had local recurrence and 5 patients developed distant metastases. In the chemotherapy plus pembrolizumab group, 4 patients died (two from lung metastases and two from liver metastases) (Table 5).

10 patients in the chemoradiotherapy plus pembrolizumab group (10/20, 50%), 3 patients in the neoadjuvant treatment plus pembrolizumab group (3/21, 14.3%) and 7 patients in the chemotherapy plus pembrolizumab group (7/14, 50%) received immune maintenance therapy. Rash occurred in 3 patients (3/20, 15%), 2 patients developed hypothyroidism (2/20, 10%), and 3 patients experienced pneumonia (3/20, 15%). To date, 6 patients have stopped immune maintenance therapy due to AEs (6/20, 30%) (Table 5).

Impact of NLR, PLR, and dNLR on clinical outcomes

Figure 4 showed the relationship between the baseline NLR (Figure 4A), PLR (Figure 4B), dNLR (Figure 4C) and long-term survival outcomes following ICIs. These results suggested that baseline NLR < 2.43, dNLR < 1.72 and PLR < 139.7 indicated a trend in OS benefit compared with NLR > 2.43, dNLR > 1.72, and PLR > 139.7, although there were no statist-

Table 1 Patient baseline characteristics (*n* = 55)

Characteristics	*n* (%)
Age (yr)	
Median	61
Range	44-74
Sex	
Male	43 (78.2)
Female	12 (21.8)
Tumor location	
Upper esophagus	19 (34.5)
Middle esophagus	25 (45.5)
Lower esophagus	11 (20)
Clinical stage	
II	10 (18.2)
III	19 (34.5)
IV	26 (47.3)
Subgroups	
Chemoradiotherapy plus pembrolizumab (group A)	21 (38.2)
Neoadjuvant therapy plus surgery (group B)	20 (36.4)
Chemotherapy plus pembrolizumab (group C)	14 (25.5)

Table 2 Summary of overall survival

	All patients (n = 55)	Group A (*n* = 20)	Group B (*n* = 21)	Group C (*n* = 14)
Patients with event	16 (29.1%)	8 (40.0%)	4 (19.0%)	4 (28.6%)
Patients without event	39 (70.9%)	12 (60.0%)	17 (81.0%)	10 (71.4%)
Time to event (mo)				
Median	-	-	-	-
95%CI	27.0, -	9.0, -	27.0, -	12.0, -
25% and 75%-ile	15.50, -	10.50, -	27.00, -	12.50, -
Min-max	0.5-39	5-26	2.6-39	0.5-35
12 mo probability (95%CI)	78.8 (65.1-87.7)	65.0 (40.3-81.5)	95.0 (69.5-99.3)	75.0 (40.8-91.2)
18 mo probability (95%CI)	72.7 (58.3-82.9)	60.0 (35.7-77.6)	89.7 (64.8-97.3)	66.7 (33.7-86.0)

Group A: Chemoradiotherapy plus pembrolizumab. Group B: Neoadjuvant therapy plus pembrolizumab. Group C: Chemotherapy plus pembrolizumab. CI: Confidence interval.

ically significant differences. The *P* values were 0.457, 0.474 and 0.238, respectively.

DISCUSSION

Our previous results showed that PD-1 inhibitor plus chemotherapy or chemoradiotherapy had a good ORR and manageable safety[25]. We used lobaplatin and albumin-paclitaxel as the chemotherapy regimen instead of cisplatin, as cisplatin has AEs on renal function. The trial proved that lobaplatin had favorable results in ESCC[26]. The present study reported the results of long-term follow-up.

Table 3 Summary of progression-free survival

	Group A (*n* = 20)	Group C (*n* = 14)
Patients with event	11 (55.0%)	4 (28.6%)
Patients without event	9 (45.0%)	10 (71.4%)
Time to event (mo)		
Median	17	-
95%CI	8.0, -	9.0, -
25% and 75%-ile	8.50, -	12.0, -
Min-max	5-26	0.5-35
12 mo probability (95%CI)	55.0 (31.3-73.5)	67.7 (34.9-86.5)
18 mo probability (95%CI)	50.0 (27.1-69.2)	67.7 (34.9-86.5)

Group A: Chemoradiotherapy plus pembrolizumab. Group C: Chemotherapy plus pembrolizumab. CI: Confidence interval.

Table 4 Summary of disease-free survival

	Group B (*n* = 21)
Patients with event	7 (33.3%)
Patients without event	14 (66.7%)
Time to event (mo)	
Median	-
95%CI	5.0, -
25% and 75%-ile	17.50, -
Min-max	2.6-27
12 mo probability (95%CI)	85.0 (60.4-94.9)
18 mo probability (95%CI)	75.0 (50.0-88.7)

Group B: Neoadjuvant therapy plus pembrolizumab. CI: Confidence interval.

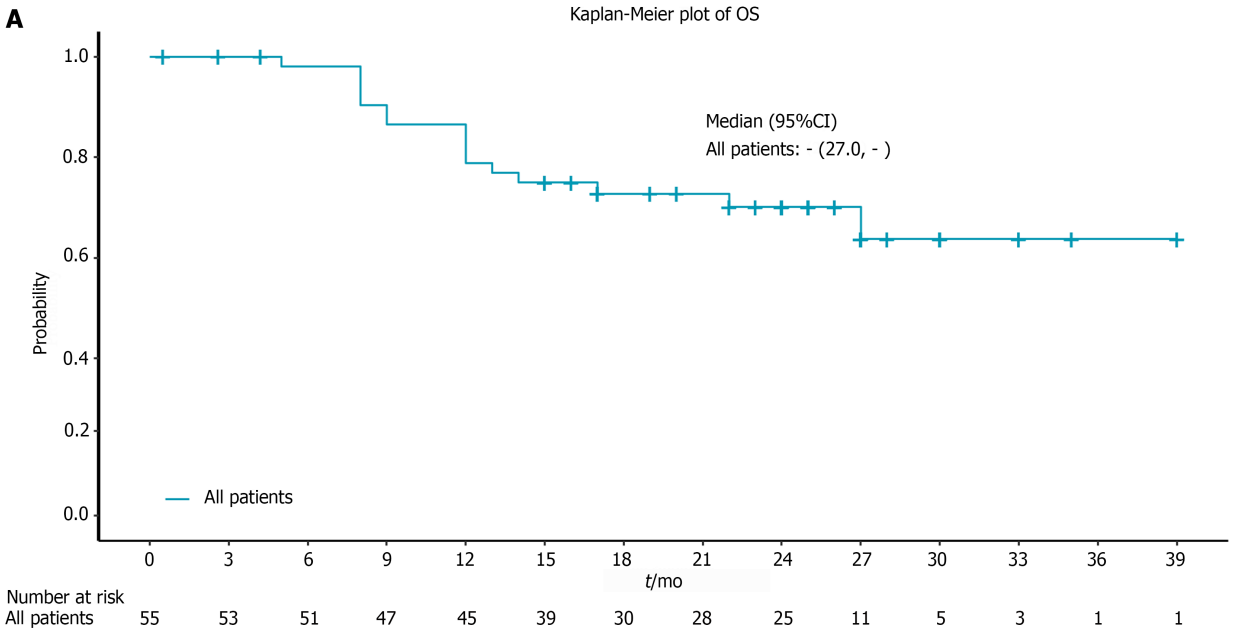
Table 5 Patterns of recurrence and immune maintenance therapy

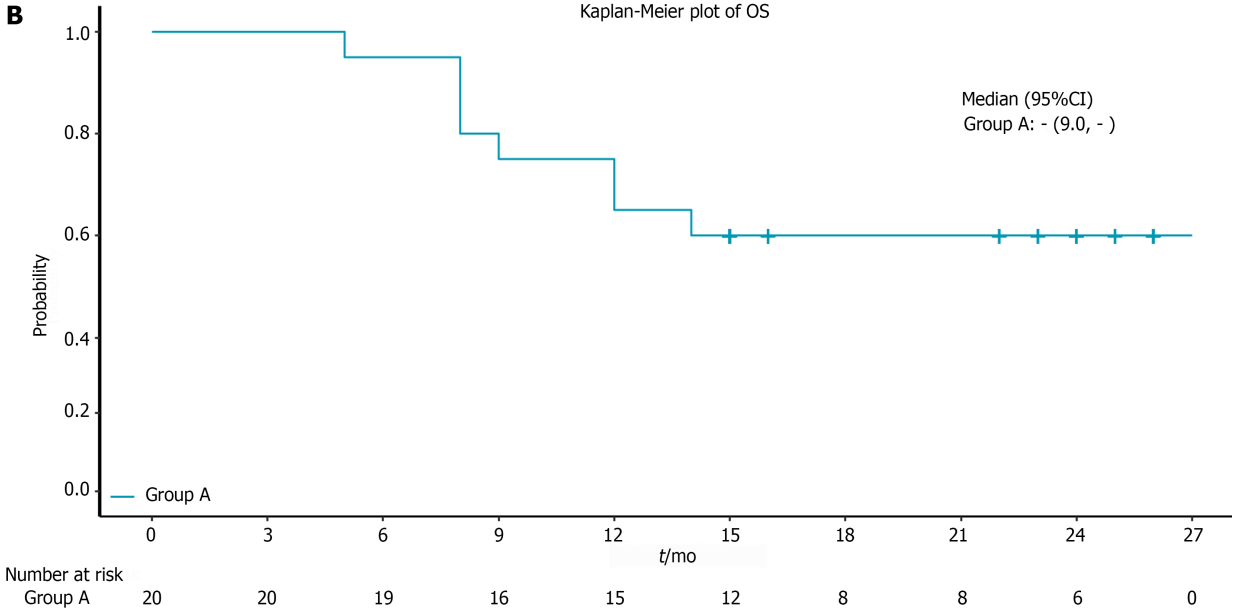
	Patterns of recurrence		Immune maintenance therapy
	Local	Distant organ	
Group A (20)	4 (20%)	1 (5%)	10 (50%)
Group B (21)	2 (9%)	5 (24%)	3 (14.3%)
Group C (14)	2 (14.3%)	4 (28.6%)	7 (50%)

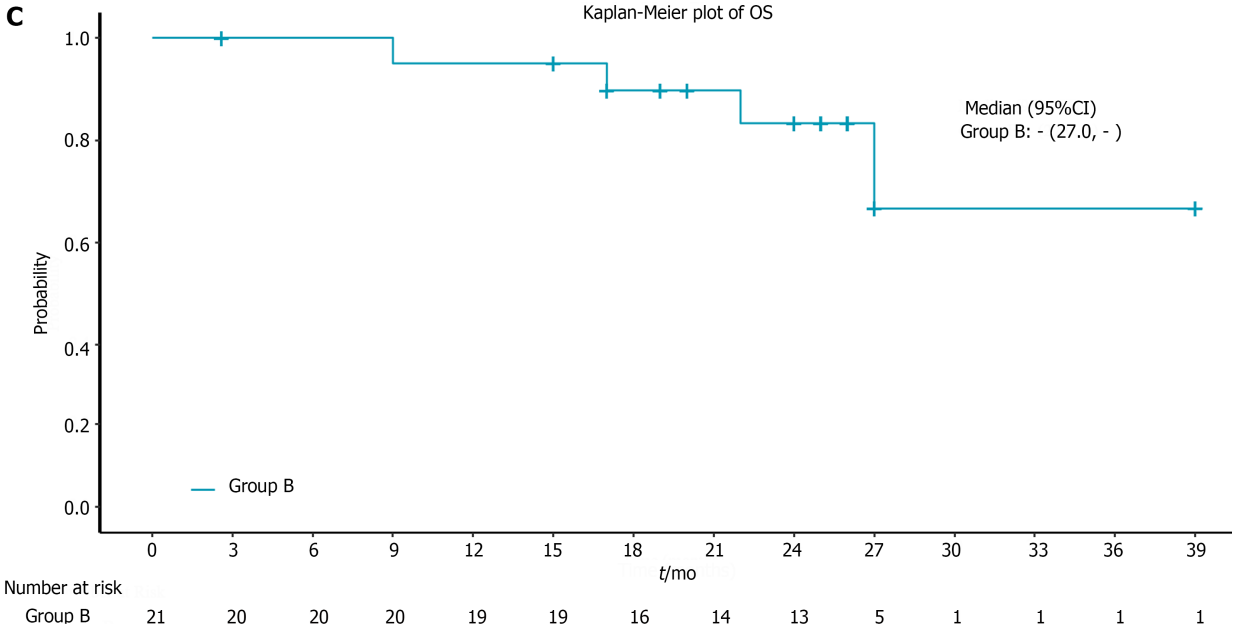
Group A: Chemoradiotherapy plus pembrolizumab. Group B: Neoadjuvant therapy plus pembrolizumab. Group C: Chemotherapy plus pembrolizumab.

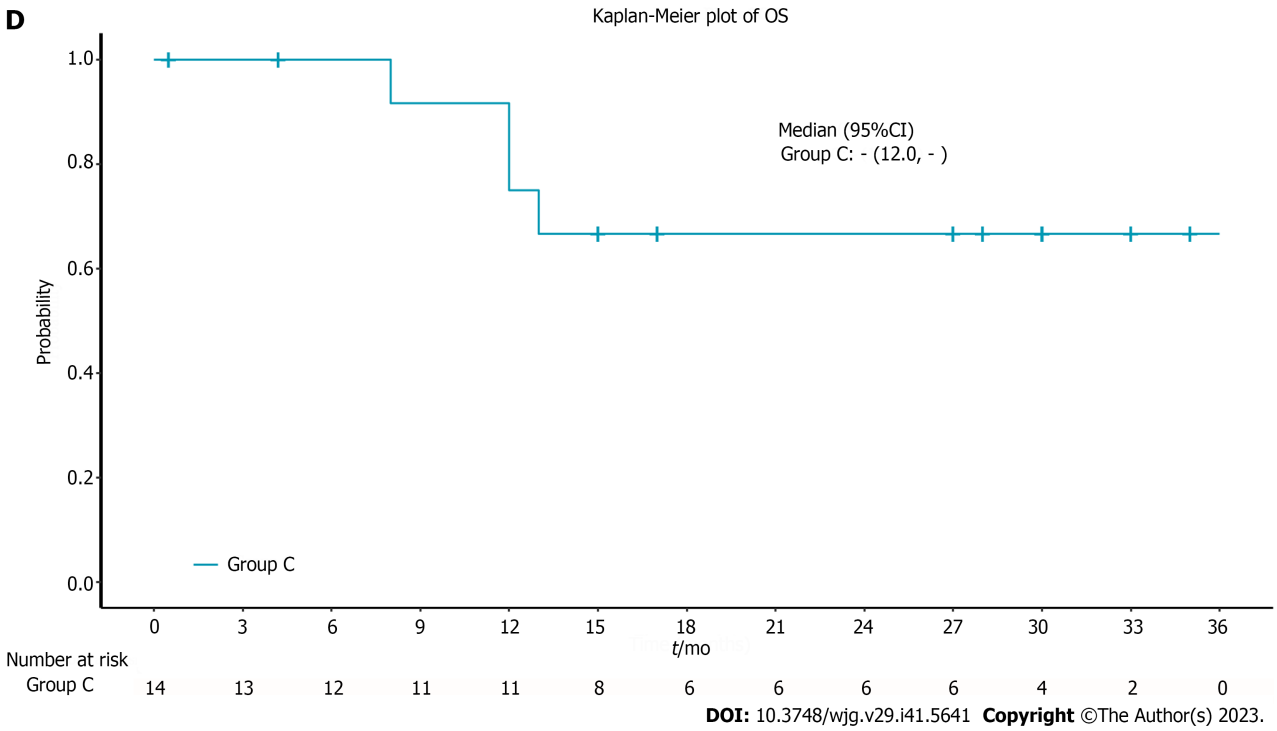
For advanced ESCC, especially locally advanced disease, neoadjuvant chemotherapy plus immunotherapy followed by surgery or chemoradiotherapy combined with immunotherapy warrants further studies, as current clinical studies are confined to phase I-II trials, and long-term follow-up data are lacking. In the present study, relatively good long-term outcomes were achieved with tolerable side effects, and evidence for PD-1 inhibitor combined with chemotherapy or radiotherapy used in ESCC has been provided.

In this study, 21 patients received neoadjuvant therapy plus pembrolizumab followed by surgery. The results demonstrated that the 12-mo DFS rate was 85%, the 18-mo DFS rate was 75%, the 12-mo OS rate was 95% and the 18-mo OS rate was 89.7%. The median OS or DFS was not reached. The results of the NEOCRTEC 5010 study indicated that the 1-year OS rate in the nCRT group was 90% and the 2-year OS rate was 75.1%[5]. Our results were similar to those of the NEOCRTEC 5010 trial. However, during a median follow-up of 24 mo in our study, patients were found to have local


A
Kaplan-Meier plot of OS
Median (95%CI)
All patients: - (27.0, -)
Probability
All patients
t/mo
Number at risk
All patients 55 53 51 47 45 39 30 28 25 11 5 3 1 1

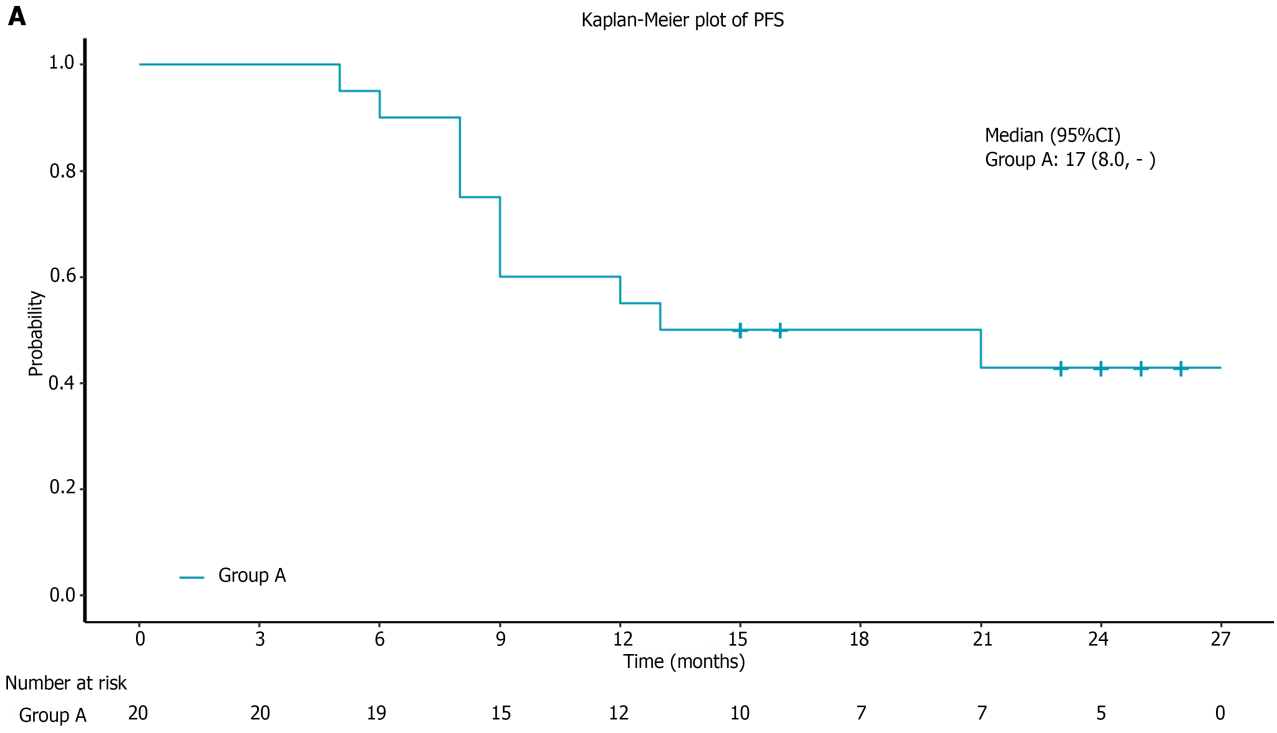

B
Kaplan-Meier plot of OS
Median (95%CI)
Group A: - (9.0, -)
Probability
Group A
t/mo
Number at risk
Group A 20 20 19 16 15 12 8 8 6 0

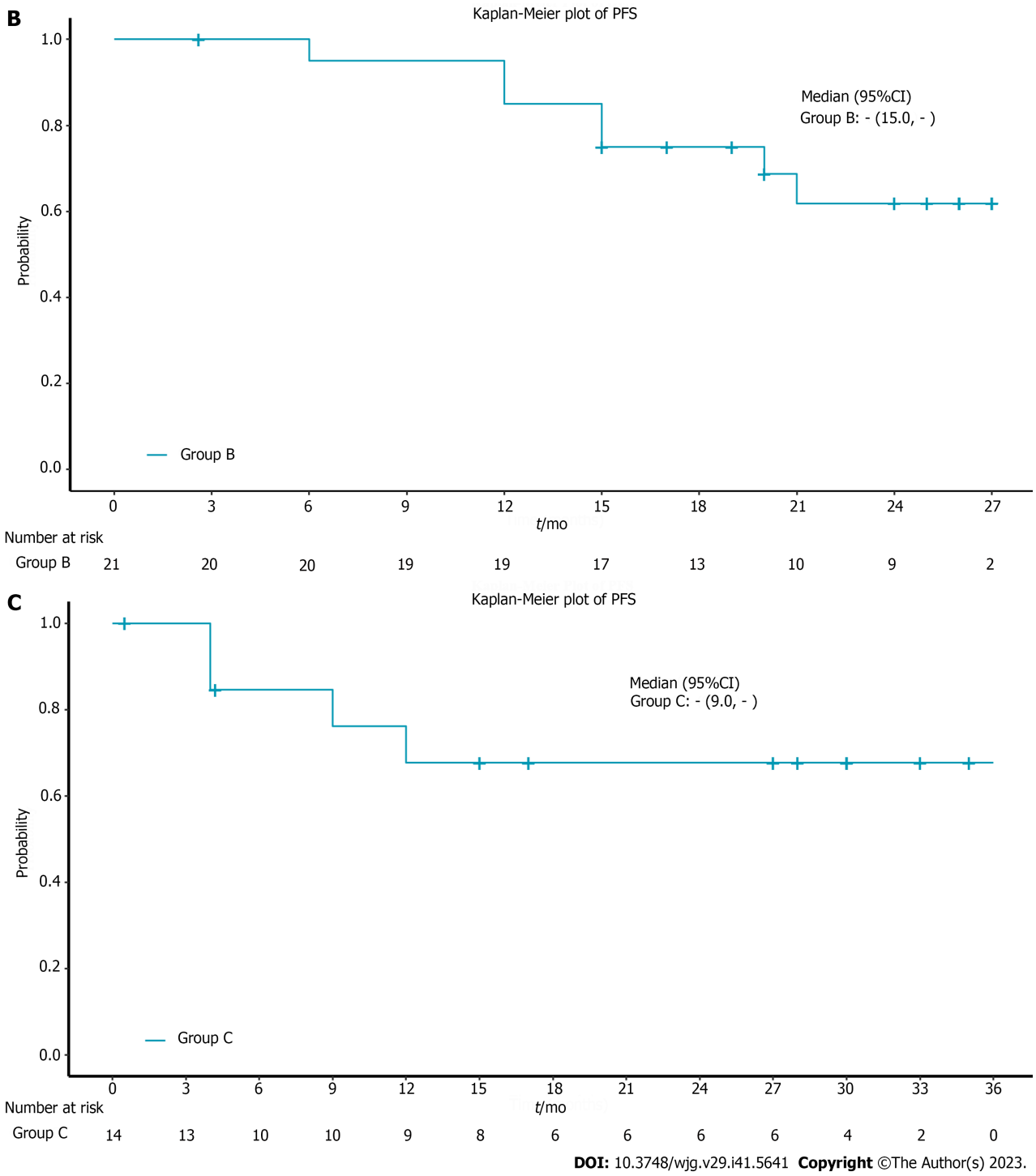

C
Kaplan-Meier plot of OS
Median (95%CI)
Group B: - (27.0, -)
Probability
Group B
t/mo
Number at risk
Group B 21 20 20 20 19 19 16 14 13 5 1 1 1 1



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Figure 1 Kaplan-Meier plot of overall survival. A: Kaplan-Meier plot of overall survival (OS) in all patients; B: Kaplan-Meier plot of OS in group A; C: Kaplan-Meier plot of OS in group B; D: Kaplan-Meier plot of OS in group C. Group A: Chemoradiotherapy plus pembrolizumab; Group B: Neoadjuvant therapy plus pembrolizumab; Group C: Chemotherapy plus pembrolizumab; OS: Overall survival; CI: Confidence interval.

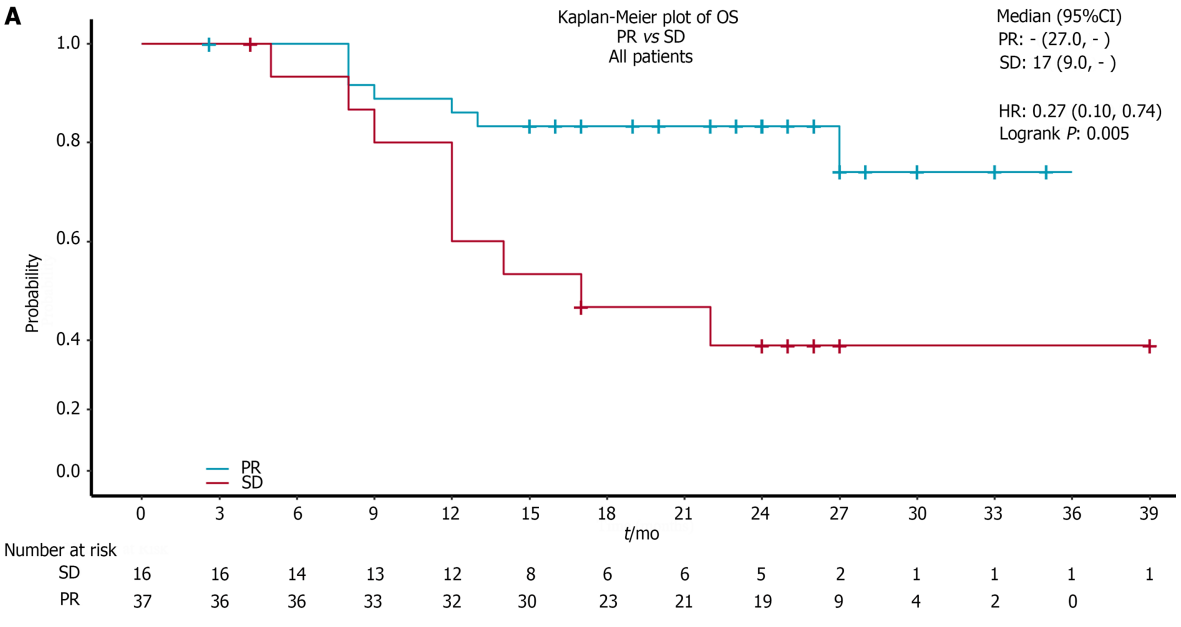


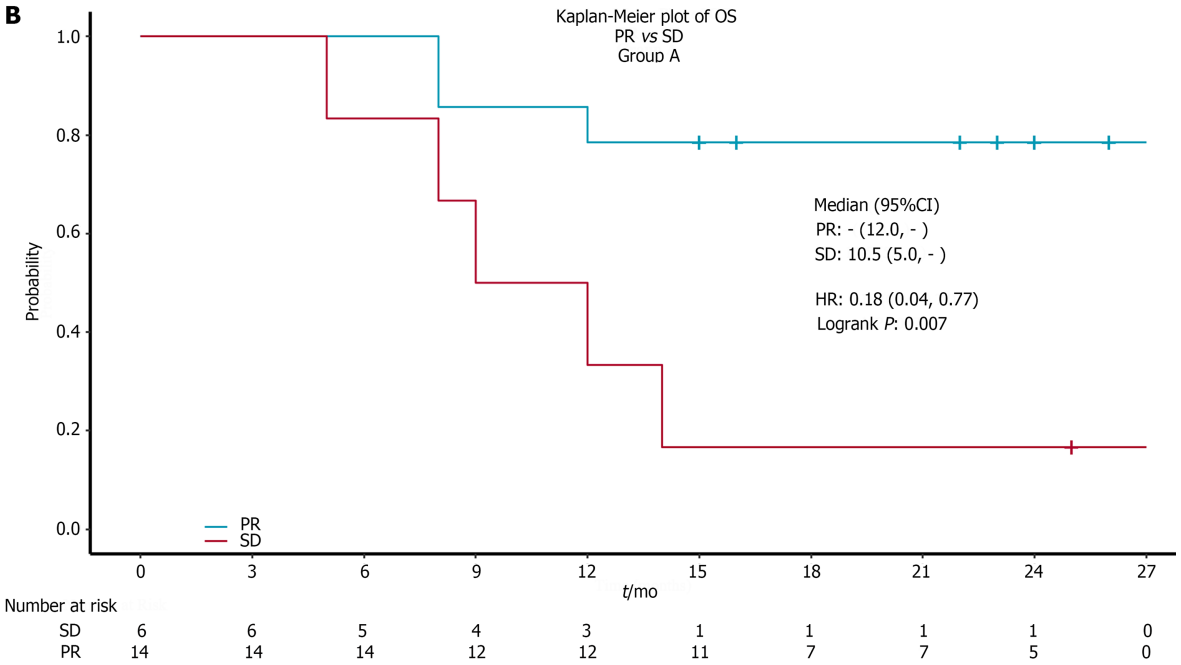


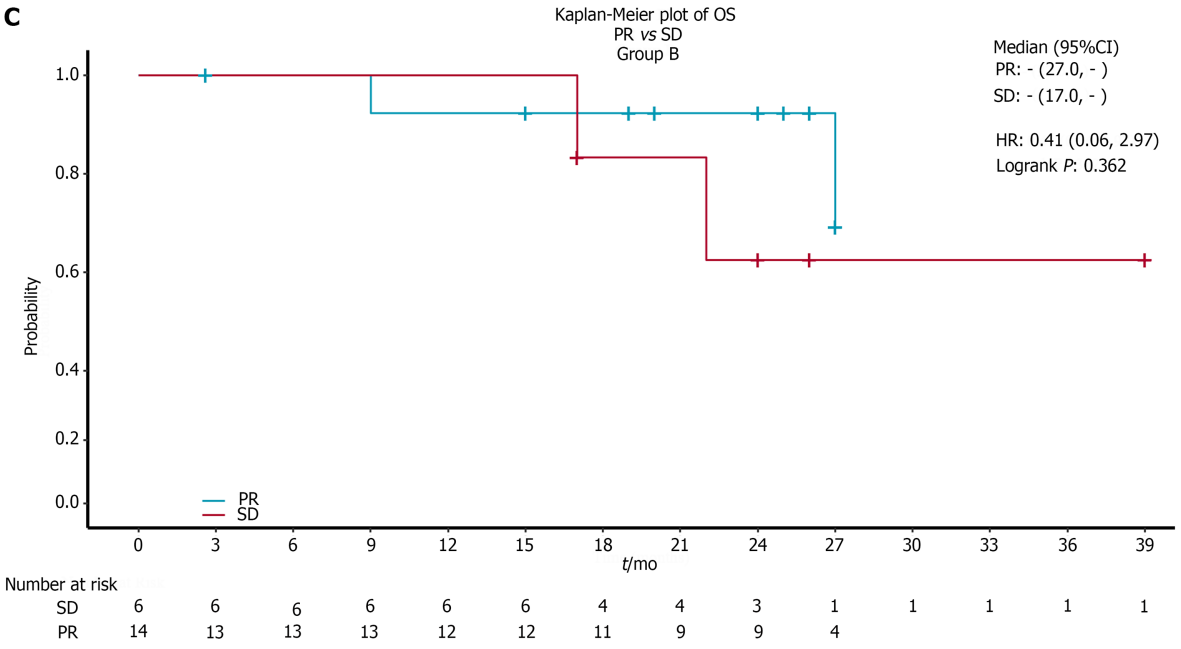
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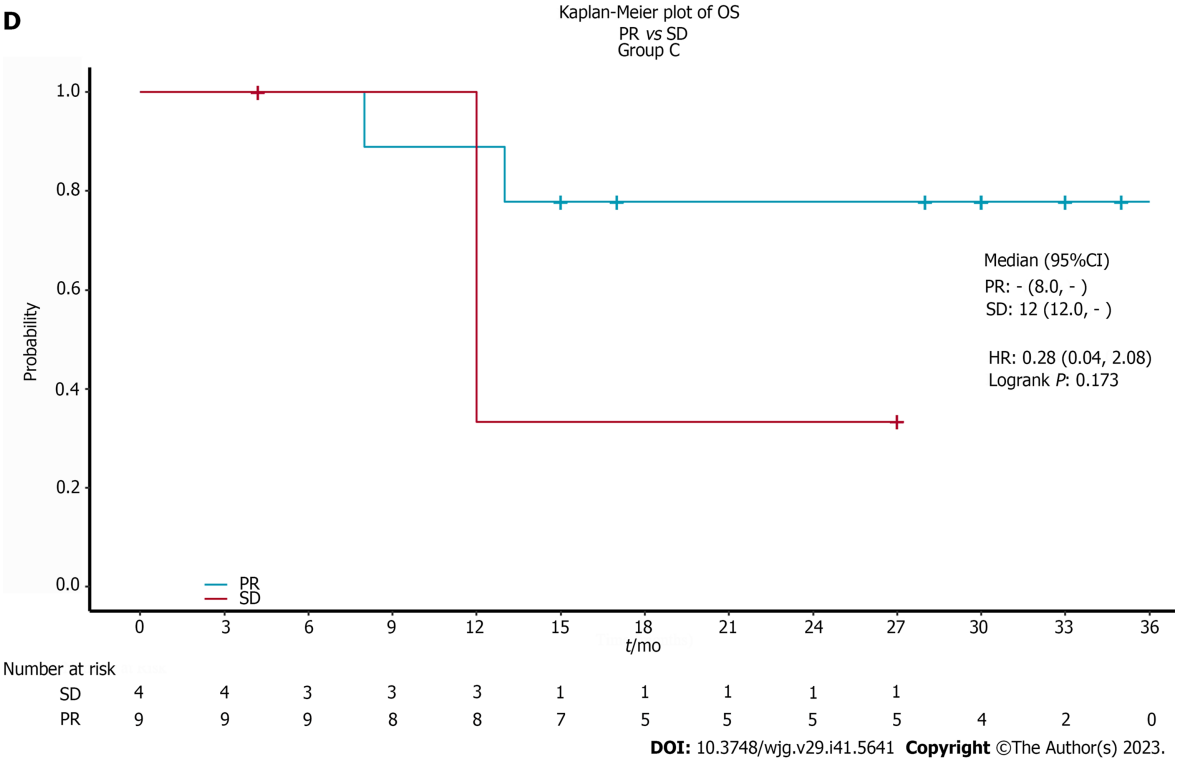
Figure 2 Kaplan-Meier plot of progression-free survival. A: Kaplan-Meier plot of progression-free survival (PFS) in group A; B: Kaplan-Meier plot of disease-free survival in group B; C: Kaplan-Meier plot of PFS in group C. Group A: Chemoradiotherapy plus pembrolizumab; Group B: Neoadjuvant therapy plus pembrolizumab; Group C: Chemotherapy plus pembrolizumab; PFS: Progression-free survival; CI: Confidence interval; DFS: Disease-free survival.

recurrence in mediastinal lymph nodes, anastomotic stoma and retroperitoneal lymph nodes. Lung, pleural effusion, and supraclavicular lymph node metastases were found in 23.8% of patients (5/21). The 10-year pattern of recurrence and metastasis in the CROSS study showed that the proportion of isolated local recurrence in the neoadjuvant group was 8% (15/178). The percentage of patients with both local recurrence and distant metastasis were 13% (23/178). In addition, the ratio of patients with simple distant metastasis was 27% (48/178)[27]. In our study, the recurrence pattern was dominated by distant metastasis, but there was still a high local recurrence rate. Therefore, it remains unclear whether the local recurrence risk with neoadjuvant chemotherapy plus ICIs is non-inferior to neoadjuvant concurrent chemoradiotherapy. Furthermore, the relatively high recurrence rate in the short follow-up period in our study requires further verification in large clinical trials. In the CHECKMATE-577 trial, the median DFS for patients who did not reach pCR after nCRT was significantly better in the maintenance treatment group with nivolumab than in the placebo group[28]. In our study, the postoperative immune maintenance rate was only 14.3% (3/21), which may also be one of the reasons for the increased


A
Kaplan-Meier plot of OS
PR vs SD
All patients
Median (95%CI)
PR: - (27.0, -)
SD: 17 (9.0, -)
HR: 0.27 (0.10, 0.74)
Logrank P: 0.005
Probability
PR
SD
t/mo
Number at risk
SD 16 16 14 13 12 8 6 6 5 2 1 1 1 1
PR 37 36 36 33 32 30 23 21 19 9 4 2 0


B
Kaplan-Meier plot of OS
PR vs SD
Group A
Median (95%CI)
PR: - (12.0, -)
SD: 10.5 (5.0, -)
HR: 0.18 (0.04, 0.77)
Logrank P: 0.007
Probability
PR
SD
t/mo
Number at risk
SD 6 6 5 4 3 1 1 1 1 0
PR 14 14 14 12 12 11 7 7 5 0


C
Kaplan-Meier plot of OS
PR vs SD
Group B
Median (95%CI)
PR: - (27.0, -)
SD: - (17.0, -)
HR: 0.41 (0.06, 2.97)
Logrank P: 0.362
Probability
PR
SD
t/mo
Number at risk
SD 6 6 6 6 6 6 4 4 3 1 1 1 1 1
PR 14 13 13 13 12 12 11 9 9 4



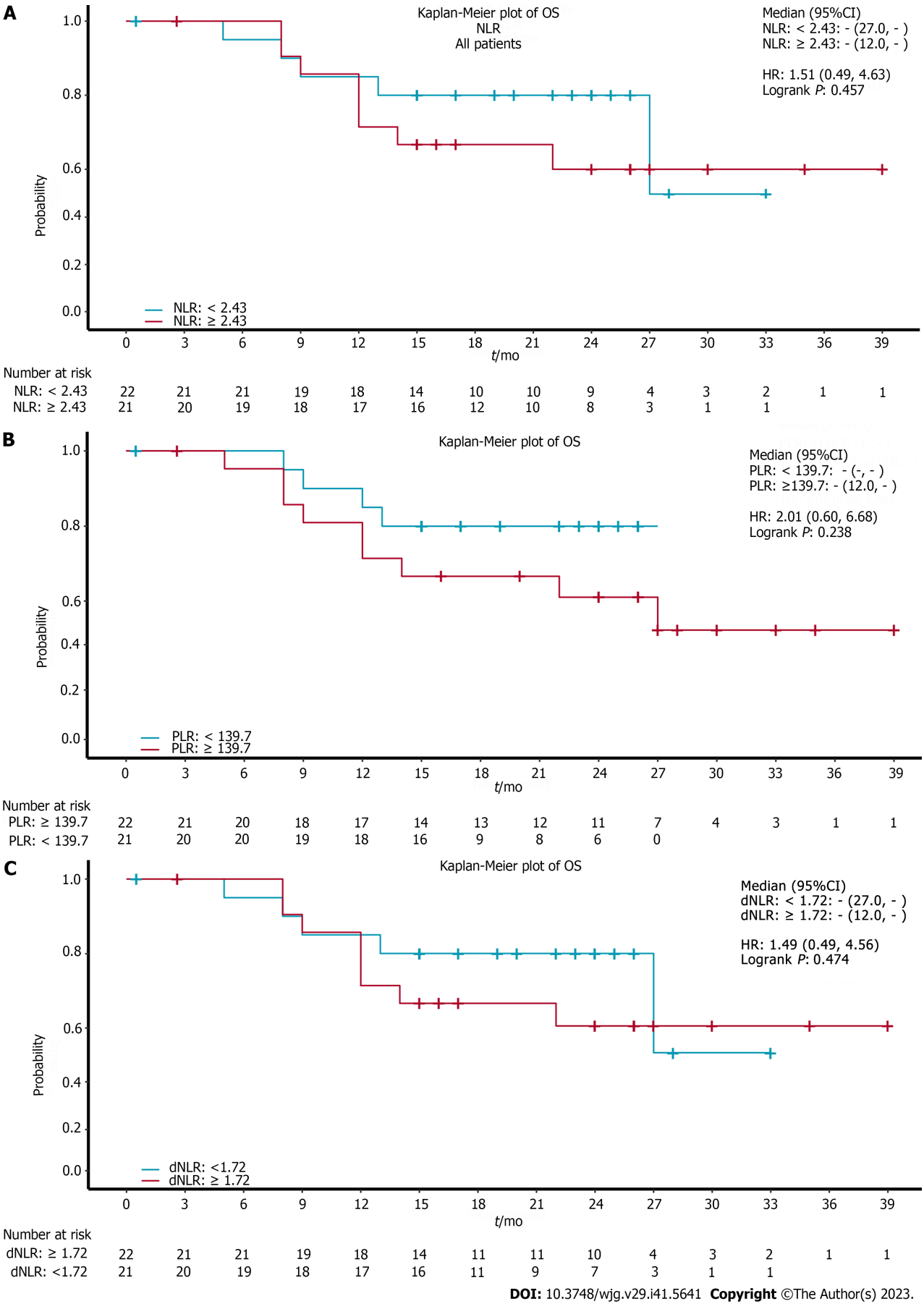
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Figure 3 Kaplan-Meier plot of overall survival in partial response and stable disease. A: Kaplan-Meier plot of overall survival (OS) in partial response (PR) and stable disease (SD) at the midterm evaluation in all patients; B: Kaplan-Meier plot of OS in PR and SD at the midterm evaluation in group A; C: Kaplan-Meier plot of OS in PR and SD at the midterm evaluation in group B; D: Kaplan-Meier plot of OS in PR and SD at the midterm evaluation in group C. PR: Partial response; SD: Stable disease; HR: Hazard ratio; OS: Overall survival; group A: Chemoradiotherapy plus pembrolizumab; group B: Neoadjuvant therapy plus pembrolizumab; group C: Chemotherapy plus pembrolizumab.

rate of distant metastasis. In the future, neoadjuvant therapy for locally advanced esophageal cancer requires continuous optimization of protocols to reduce the risk of distant metastasis and improve survival. In addition, on the premise of ensuring local control, eliminating radiotherapy to reduce AEs is also worth further exploration.

20 patients with unresectable locally advanced or limited supraclavicular lymph node metastases received chemoradiotherapy combined with pembrolizumab. The results showed that the 12-mo OS rate was 65%, the 18-mo OS rate was 60% and the median OS was not reached. The 12-mo PFS rate was 55%, the 18-mo PFS rate was 50% and the median PFS was 17 mo. The median survival time following radical concurrent chemoradiotherapy recommended by current guidelines was 18 mo, and the 2-year survival rate was about 40%[29]. The long-term survival in the radical chemoradiotherapy plus pembrolizumab group in our study was slightly better than that in the standard radical concurrent chemoradiotherapy group. The addition of ICIs to chemoradiotherapy likely increased the efficacy and prolonged survival. However, randomized phase III studies are needed to verify this. A phase IB clinical study is currently examining the efficacy and safety of the PD-1 inhibitor camrelizumab combined with radical radiotherapy in patients with locally advanced ESCC who are intolerant to concurrent chemoradiotherapy[21]. The median OS and PFS were 16.7 mo and 11.7 mo, respectively. The 24-mo OS rate and PFS rate were 31.6% and 35.5%, respectively. The ORR rate was 74%. Our results showed a more beneficial outcome, probably because we used the combination of radiotherapy and chemotherapy, which strengthened the intensity of treatment and improved survival outcomes. Studies have shown that the incidence rate of esophageal fistula caused by radiotherapy and chemotherapy is approximately 15%, of which T4 and esophageal stenosis increase the risk of fistula with a poor prognosis[30]. In our study, 20% (4/20) patients died from esophageal fistula. The patients with fistula in the radiotherapy plus pembrolizumab group were all T4 and the tumor was closely related to the trachea, which was suspected to have invasion. Furthermore, the radiotherapy dose in this group was 63 Gy, and the high radiotherapy dose was also the main cause of fistula. Studies have shown that higher 60 Gy did not improve long-term survival and simultaneously increased AEs[31]. Therefore, in the era of ICIs, for locally advanced patients with T3-T4, radiotherapy dose should be carefully selected and safety should be taken into account in the absence of clear evidence of benefit. The data and results of randomized phase III studies on the combination therapy of radiotherapy and ICIs are lacking at present. There are still some problems to be solved such as the timing of combination therapy, selection of the combination chemotherapy regimen, clinical target volume and so on. The results of KEYNOTE-975, ESCORT-CRT, RATIONALE-311 and other randomized phase III studies are expected.

A total of 14 patients in our study received chemotherapy combined with pembrolizumab. The 12-mo OS rate was 75% and the 18-mo OS rate was 66.7%. The 12-mo PFS rate and 18-mo PFS rate were 67.7%. In the randomized phase III JUPITER-06 study, toripalimab combined with chemotherapy significantly prolonged PFS in patients with a 42% reduction in the risk of disease progression and resulted in a significant benefit in median OS (17 mo *vs* 11 mo) compared with placebo plus chemotherapy. The 1-year PFS rate was 27.8% and the 1-year OS rate was 66% in the toripalimab-based



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Figure 4 Kaplan-Meier plot of overall survival at baseline. A: Kaplan-Meier plot of overall survival (OS) at baseline (neutrophil-to-lymphocyte ratio < 2.43 *vs* > 2.43); B: Kaplan-Meier plot of OS at baseline (platelet-to-lymphocyte ratio < 139.7 *vs* > 139.7); C: Kaplan-Meier plot of OS at baseline [neutrophil-to-(leukocyte-

neutrophil) ratio < 1.72 *vs* > 1.72]. OS: Overall survival; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; dNLR: Neutrophil-to-(leukocyte-neutrophil) ratio; CI: Confidence interval; HR: Hazard ratio.

group[11]. The long-term survival results in our study were better than those in the JUPITER-06 study, and even better than those in the radiotherapy plus pembrolizumab group. There are several possible reasons for this result: (1) The tumor burden in the chemotherapy combined with pembrolizumab group was relatively low. Some patients with stage III refused radiotherapy due to toxicity, while others had single liver metastasis or small nodules in lung metastasis; (2) In the radiotherapy plus pembrolizumab group, 4 patients died due to esophageal fistula after radiotherapy and survival outcomes were negatively affected; and (3) Small sample size and short follow-up time may have led to deviations in the results.

As indicators of systemic inflammation, the NLR, PLR and dNLR can reflect the microenvironment of inflammation. Neutrophils can promote tumor invasion and progression by secreting cytokines, vascular endothelial cell growth factors and chemokines[32]. However, lymphocytes play an important role in the immune system and can inhibit tumor proliferation[33]. Studies have reported that in patients with non-small cell lung cancer and a higher baseline NLR, ICIs had poor efficacy, which had a negative predictive value on PFS and OS[34]. Our study found that low baseline NLR, dNLR and PLR showed a trend for OS benefit, but a statistically significant difference was not observed. This result may have been limited by the small sample size. Thus, a larger sample size is needed to examine this issue in the future.

This study also had some limitations: (1) This was a single-arm, single-center retrospective clinical study, with a small number of patients and did not include a control group; (2) The follow-up period should have been longer, as there is a lack of 3-year and 5-year long-term survival outcomes; and (3) Prospective randomized controlled studies with long-term follow-up data are needed.

CONCLUSION

Our real-world results revealed that pembrolizumab combined with chemotherapy or radiotherapy resulted in a favorable long-term survival outcome in patients with locally advanced and metastatic esophageal cancer. Long-term toxicities associated with these regimens were manageable.

ARTICLE HIGHLIGHTS

Research background

Although pembrolizumab combined with chemotherapy has been proven effective as first-line therapy in patients with advanced esophageal cancer, few trials have assessed the safety and efficacy of this treatment in patients with locally advanced disease.

Research motivation

Progress has been made in the immune checkpoint inhibitors combined with chemotherapy as the first-line treatment of advanced esophageal cancer. The efficacy and safety of pembrolizumab in locally advanced or metastatic esophageal squamous cell carcinoma (ESCC) in the real world were worth studying.

Research objectives

To analyze the long-term outcomes of pembrolizumab in locally advanced or metastatic ESCC in the real world.

Research methods

This was a single-arm, single-center, retrospective clinical study. Patients who were initially diagnosed with locally advanced or metastatic esophageal cancer from October 1, 2019 to October 1, 2021 were included. According to the different clinical stages and treatment modalities, the patients were divided into different subgroups. Long-term survival outcomes were evaluated.

Research results

A total of 55 patients with ESCC were enrolled in this study from October 1, 2019 to October 1, 2021. The median overall survival (OS) in all patients was not reached. The 12-mo OS rate was 78.8% and the 18-mo OS rate was 72.7%. Nine patients died due to tumor progression and 7 patients died due to treatment-related complications.

Research conclusions

Pembrolizumab combined with chemotherapy or radiotherapy resulted in favorable long-term survival in patients with locally advanced or metastatic ESCC, with safe and manageable long-term adverse effects.

Research perspectives

It is necessary to explore the efficacy of pembrolizumab combined with chemotherapy or radiotherapy in patients with locally advanced or metastatic ESCC. Randomized phase III trials should be carried out for further verification of the efficacy.

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FOOTNOTES

Author contributions: Liu F, Wang HC, and Huang X were involved in the study conception and design; Wang HC, Huang X, and Feng SQ drafted the article and interpreted the data; Chen J, Li Y, and Cong Y collected and analyzed the data; Qu BL supervised the report.

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ORIGINAL ARTICLE

Observational Study

Colorectal motility patterns and psychiatric traits in functional constipation and constipation-predominant irritable bowel syndrome: A study from China

Chao-Lan Lv, Geng-Qing Song, Jie Liu, Wei Wang, Yi-Zhou Huang, Bo Wang, Jia-Shuang Tian, Meng-Qing Yin, Yue Yu

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Abstract

BACKGROUND

Functional constipation (FC) and constipation-predominant irritable bowel syndrome (IBS-C) represent a spectrum of constipation disorders. However, the majority of previous clinical investigations have focused on Western populations, with limited data originating from China.

AIM

To determine and compare the colorectal motility and psychiatric features of FC and IBS-C in an Eastern Chinese population.

METHODS

Consecutive chronic constipation patients referred to our motility clinic from December 2019 to February 2023 were enrolled. FC and IBS-C diagnoses were established using ROME IV criteria, and patients underwent high-resolution anorectal manometry (ARM) and a colonic transmit test using the Sitz marker

study. Constipation-related symptoms were obtained through questionnaires. Anxiety and depression were assessed by the Hamilton anxiety rating scale and the Hamilton Depression Rating Scale-21. The clinical characteristics and colorectal motility patterns of FC and IBS-C patients were compared.

RESULTS

No significant differences in sex, age or abdominal discomfort symptoms were observed between IBS-C and FC patients (all $P > 0.05$). The proportion of IBS-C patients with delayed colonic transit was higher than that of patients with FC (36.63% *vs* 15.91%, $P < 0.05$), while rectosigmoid accumulation of radiopaque markers was more common in the FC group than in the IBS-C group (50% *vs* 26.73%, $P < 0.05$). Diverse proportions of these dyssynergic patterns were noted within both the FC and IBS-C groups by ARM. IBS-C patients were found to have a higher prevalence of depression than FC patients (66.30% *vs* 42.42%, $P < 0.05$). The scores for feelings of guilt, suicide, psychomotor agitation, diurnal variation, obsessive/compulsive disorder, hopelessness, self-abasedment and gastrointestinal symptoms were significantly higher in IBS-C patients than that in FC patients ($P < 0.05$). For IBS-C ($\chi^2 = 5.438$, $P < 0.05$) but not FC, patients with normal colon transit time were significantly more likely to have anxiety than those with slow colon transit time. For IBS-C patients but not FC patients, the threshold of first constant sensation, desire to defecate and sustained urgency were all weakly correlated with the degree of anxiety ($r = 0.414$, $r = 0.404$, and $r = 0.418$, respectively, $P < 0.05$). The proportion of patients with a low threshold of desire to defecate among IBS-C patients with depression was lower than that in those without depression (69.6% *vs* 41.9%, $\chi^2 = 4.054$, $P < 0.05$).

CONCLUSION

Our findings highlight both overlapping and distinctive patterns of colon transit, dyssynergic patterns, anorectal sensation, psychological distress, and associations of psychiatric and colorectal motility characteristics in FC and IBS-C patients in an Eastern Chinese population, providing valuable insights into the pathophysiological underpinnings of these disorders.

Key Words: Functional constipation; Constipation-predominant irritable bowel syndrome; High-resolution anorectal manometry; Colonic transmit test; Anxiety; Depression

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Core Tip: Functional constipation (FC) and constipation-predominant irritable bowel syndrome (IBS-C) are the two primary subtypes of constipation. Previous clinical studies that attempted to illuminate distinctive physiological mechanisms between FC and IBS-C patients were predominantly from Western countries, with limited data originating from China. Our study has revealed distinctive elements of FC and IBS-C across multifaceted parameters, namely colonic transmit time, psychological distress, and dyssynergic patterns, and the relationship among these parameters. These findings extend our comprehension of the intricate pathophysiological mechanisms underlying FC and IBS-C. These findings could provide guidance for constipation patients to choose appropriate colorectal tests.

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INTRODUCTION

Chronic constipation (CC) represents a prevalent health challenge globally, impacting approximately 4% to 10% of the population in China[1,2]. The process of fecal evacuation is governed by a complex interplay of brain-gut axis interactions, intestinal peristalsis, and the function of the pelvic floor muscles and anal sphincter. The multifaceted pathogenesis of CC involves a dynamic interplay between biological and psychosocial factors. Two principal contributors to the pathophysiology of CC are colonic sensorimotor disturbances and pelvic floor dysfunction[3,4]. A multitude of diagnostic tools, including high-resolution anorectal manometry (HR-ARM), the colonic transmit test (CTT), barium and magnetic resonance defecography, and the balloon expulsion test (BET) can provide valuable insights into the underlying mechanisms of CC. In a clinical setting, the assessment of colorectal motility and psychiatric evaluations are often essential when patients with CC do not respond to conventional laxative treatments[5].

Functional constipation (FC) and constipation-predominant irritable bowel syndrome (IBS-C) are the two primary subtypes of CC. As per the Rome IV criteria, an IBS-C diagnosis requires the presence of abdominal pain or discomfort, a criterion that is not required for FC. The Rome IV guidelines also suggest that IBS-C and FC are not distinct disorders but

rather exist on a continuum of constipation disorders. Despite overlaps in symptoms, pathophysiological mechanisms and treatment responses, distinctions between IBS-C and FC exist[6]. A thorough understanding of these physiological mechanisms could enhance our ability to differentiate between IBS-C and FC more effectively than relying solely on symptoms. While several clinical studies, predominantly from Western countries, have attempted to illuminate this issue [7-9], data from Chinese populations remain scarce.

In China, the first choice of treatment for patients with treatment-resistant constipation is often ARM, the only minimally invasive tool available to measure anorectal pressures. Due to its affordability, ease of execution, and ready availability, the radiopaque marker technique is widely utilized to assess colonic transit[10]. Therefore, in our study, we selected these two modalities to examine the physiological mechanisms of CC. However, controversy persists regarding the correlation between these two tests, and it remains unclear which test provides more meaningful data for IBS-C or FC [7-11]. Thus, our study aimed to compare the psychiatric (depression and anxiety) and colorectal motility (colonic transit and anorectal motility and sensation) characteristics between FC and IBS-C patients in an Eastern Chinese population. We also sought to investigate the correlations between psychiatric and colorectal motility characteristics in both FC and IBS-C patients.

MATERIALS AND METHODS

Patients

For this study, we recruited patients diagnosed with FC and IBS-C from the Anhui Provincial Hospital's motility clinic between December 2019 and February 2023. The diagnoses of FC and IBS-C were made according to the Rome IV criteria [6]. The exclusion criteria included pregnancy or lactation; diabetes; thyroid dysfunction; and cardiovascular, hepatic or renal disease. All patients underwent standardized HR-ARM and CTT. The study received approval from the Ethics Committee of Anhui Provincial Hospital.

HR-ARM

HR-ARM was conducted using a water-perfusion HR-ARM device (GAP-08A, Maida Instruments, Ningbo, China) according to the London consensus protocol[12]. Patients remained in the left lateral decubitus position during simulated evacuation. Four patterns of dyssynergia were classified according to the Rao classification, along with a normal and an unclassified pattern. Type I dyssynergia showed an adequate increase in rectal pressure (≥ 45 mmHg) accompanied by a paradoxical simultaneous increase in anal pressure; type II dyssynergia showed an inadequate increase in rectal pressure of (< 45 mmHg) (poor propulsive force) accompanied by a paradoxical simultaneous increase in anal pressure; type III dyssynergia showed an adequate increase in rectal pressure (≥ 40 mmHg) accompanied by failure of reduction in anal pressure (≤ 20% baseline pressure); and type IV dyssynergia showed an inadequate increase in rectal pressure of (< 45 mmHg) (poor propulsive force) accompanied by failure of reduction in anal pressure (≤ 20% baseline pressure). The normal pattern showed an adequate increase in rectal pressure (≥ 45 mmHg) accompanied by a simultaneous reduction in anal pressure. The unclassified pattern showed anorectal pressure changes not consistent with any patterns mentioned above[13]. The rectal sensory test was subsequently performed, recording sensory thresholds based on balloon volumes at first constant sensation, desire to defecate, maximum tolerance, and sustained urgency[10].

CTT

Colonic transit time was assessed using radiopaque marker techniques (Sitzmarks; Konsyl Pharmaceuticals, TX, United States). Medications that might affect gastrointestinal transmission were discontinued for 1 wk before and during the CTT study. Patients were instructed to adhere to their regular diet and avoid laxatives throughout the study. The patients ingested a single capsule containing 24 radiopaque markers on day 1, and an abdominal X-ray was obtained 48 h later. The X-ray analysis determined the number and distribution of the markers as per the protocol described by Metcalf *et al* [14]. Spinal processes and imaginary lines from the fifth lumbar vertebra to the pelvic outlets served as landmarks by which the right colon (RC), left colon (LC) and rectosigmoid (RS) colon were defined. Patients were classified as positive for evidence of normal transit constipation (NTC) when less than 10% of the markers were visible throughout the colon at 48 h. Slow transit constipation (STC) was defined as retention of more than 50% of the markers in the RC and LC on imaging. RS accumulation of radiopaque markers (RSARM), defined as retention of more than 50% of the markers in the RS region, suggested the possibility of functional defecation disorders[10,14,15].

Questionnaires

All patients were asked to complete the clinical symptoms questionnaires capturing data such as age, sex, stool frequency, Bristol stool form scale score, abdominal pain, abdominal bloating, relationship between abdominal discomfort and defecation, straining during a bowel movement, feeling of incomplete emptying, sensation that stool cannot be passed and feeling of defecation urgency. The Hamilton anxiety rating scale (HAMA) and the Hamilton Depression Rating Scale (HAMD)-21 were used to evaluate patients' mental health, with higher scores signifying more severe anxiety and depression. A score ranging from 7 to 13 was indicative of possible anxiety, a score ranging from 14 to 20 was indicative of anxiety, and a score ≥ 21 was indicative of severe anxiety. A score ranging from 8 to 19 was indicative of mild depression, a score ranging from 20 to 34 was indicative of moderate depression, and a score ≥ 35 was indicative of severe depression[16,17].

Table 1 Comparison of baseline characteristics between constipation-predominant irritable bowel syndrome and functional constipation patients

Variable	IBS-C	FC	*P* value
Age (yr)	42.32 ± 14.41	43.95 ± 18.04	> 0.05
Sex			> 0.05
Male	17	8	
Female	84	33	
Stool frequency			> 0.05
> 6 d	23	6	
< 6 d	72	27	
Feeling of incomplete emptying			> 0.05
Yes	76	26	
No	18	13	
Sensation that stool cannot be passed			> 0.05
Yes	54	16	
No	16	11	
Feeling of defecation urgency			> 0.05
Yes	39	7	
No	56	24	
Straining during a bowel movement			> 0.05
Yes	91	28	
No	5	5	

The values are expressed as numbers (*n*) or mean ± SD. IBS-C: Constipation-predominant irritable bowel syndrome; FC: Functional constipation.

Statistical methods

Baseline demographic, clinical, ARM, and CTT variables were compared between IBS-C patients and FC patients. The proportions of each variable were compared using the chi-square test, while means and medians were evaluated using the Student's *t* test and the Wilcoxon rank-sum test, respectively. The association between variables was determined using the Pearson correlation coefficient. All data were analyzed using SPSS 19.0. $P < 0.05$ was considered statistically significant.

RESULTS

Patient demographics

Our study comprised 230 patients with CC, of whom 149 were diagnosed with IBS-C and 81 with FC. Females represented a larger portion of both groups (82.39%) compared to males (17.61%). No statistically significant differences were observed in terms of sex, age, or abdominal discomfort symptoms between the IBS-C and FC cohorts (Table 1; $P > 0.05$).

Colonic transit test results

IBS-C patients demonstrated a higher prevalence of delayed colonic transit than FC patients (36.63% *vs* 15.91%, $P < 0.05$). Conversely, RSARM was more common among FC patients (50% *vs* 26.73%, $P < 0.05$). No significant differences were observed in the proportions of patients with a normal colon transit time between the two groups (36.63% *vs* 34.09%, $P > 0.05$) (Table 2).

Anorectal motility and sensation

There were no significant differences in the prevalence of dyssynergic patterns (I-IV) observed on HR-ARM between the FC (81%) and IBS-C (85.2%) groups. Moreover, diverse proportions of these dyssynergic patterns were noted within both groups. In FC patients, the type I pattern was most prevalent (41.94%), while the type II pattern was most commonly observed in IBS-C patients (34.55%). The type III pattern was found to be the least common in both groups (3.23% in FC

Table 2 Comparison of psychiatric and colorectal motility characteristics between constipation-predominant irritable bowel syndrome and functional constipation patients

Variable	IBS-C	FC	*P* value
ARM			
Dyssynergic patterns			> 0.05
I	19	12	
II	18	8	
III	5	1	
IV	14	10	
Normal	6	1	
Unclassified	7	1	
Anorectal sensation thresholds			
First constant sensation			> 0.05
Low	2	1	
High	38	14	
Normal	25	10	
Desire to defecate			> 0.05
Low	30	10	
High	4	1	
Normal	22	10	
Sustained urgency			> 0.05
Low	30	12	
High	4	1	
Normal	29	14	
CTT			< 0.05
STC	37	7	
RS accumulation	27	22	
Normal	37	15	
Psychiatric characteristics			
Scores			> 0.05
HAMA	11.71 ± 9.48	12.00 ± 9.15	
HAMD	11.51 ± 8.61	9.41 ± 0.33	
Anxiety			> 0.05
No	34	13	
Possible	24	7	
Yes	35	13	
Depression			< 0.05
No	31	19	
Possible	46	9	
Yes	15	5	

The values are expressed as numbers (*n*) or mean ± SD. ARM: Anorectal manometry; CTT: Colonic transmit test; IBS-C: Constipation-predominant irritable bowel syndrome; FC: Functional constipation; HAMA: Hamilton anxiety rating scale; HAMD: Hamilton Depression Rating Scale.

Table 3 Comparison between psychiatric and colorectal motility characteristics in constipation-predominant irritable bowel syndrome and functional constipation patients

Variable	IBS-C				FC			
	Anxiety	Without anxiety	Depression	Without depression	Anxiety	Without anxiety	Depression	Without depression
STC	21	16	25	12	4	1	3	2
NTC	24	6	21	9	8	3	5	6
First sensation								
High	26	8	21	11	10	1	5	6
Normal	14	9	11	12	4	5	4	5
Desire to[a]								
Low	19	11	13	16	6	4	6	4
Normal	16	6	15	6	8	2	4	6
High	4	0	3	1	1	0	0	1
Urgency								
Low	15	12	14	12	4	4	5	3
Normal	21	5	15	10	10	2	5	7
High	3	0	2	1	1	0	0	1

[a]$\chi^2 = 4.054$, $P < 0.05$ for the association of the threshold of desire to defecate and depression in constipation-predominant irritable bowel syndrome patients. The values are expressed as numbers (*n*). STC: Slow transmit constipation; NTC: Normal transmit constipation; IBS-C: Constipation-predominant irritable bowel syndrome; FC: Functional constipation.

and 10.91% in IBS-C). Although a marginal increase in the prevalence of type II and type IV patterns was noticed in FC patients compared to IBS-C patients, the difference was not statistically significant (56.3% *vs* 55.9%, $P > 0.05$).

In the assessment of sensory thresholds, a majority of both IBS-C (58.46%) and FC (56%) patients exhibited high thresholds for the first constant sensation. Low thresholds for the desire to defecate were more commonly observed in IBS-C patients (53.57%) and in approximately half of the FC patients (47.62%). Slightly higher proportions of high thresholds for the first constant sensation, low thresholds for the desire to defecate, and anxiety or potential anxiety (63.44% *vs* 60.61%) were observed in IBS-C patients compared to FC patients, although these differences lacked statistical significance ($P > 0.05$) (Table 3).

Psychiatric characteristics

A significantly higher prevalence of depression was observed among IBS-C patients compared to their FC counterparts (66.30% *vs* 42.42%, $P < 0.05$). Although the incidence of anxiety was slightly higher among IBS-C patients, this difference was not statistically significant (63.44% *vs* 60.61%, $P > 0.05$). The mean scores for HAMA and HAMD were similar for both groups (Table 1; $P > 0.05$). However, IBS-C patients reported significantly higher scores for specific symptoms, such as feelings of guilt, suicidal ideation, psychomotor agitation, diurnal variation, obsessive-compulsive tendencies, hopelessness, self-abasement, and gastrointestinal symptoms (Figure 1; $P < 0.05$).

Correlation between psychiatric and colorectal motility characteristics

In both the IBS-C and FC patient cohorts, a significant percentage of individuals with NTC or STC demonstrated symptoms of anxiety or depression. However, the incidence of depression was comparable among patients with NTC and STC (70% *vs* 67.57% for IBS-C, 45.45% *vs* 60% for FC, $P > 0.05$). Interestingly, in the IBS-C group (but not the FC group), patients with NTC exhibited a notably higher prevalence of anxiety than those with STC (80% *vs* 56.76%, $\chi^2 = 5.438$, $P < 0.05$).

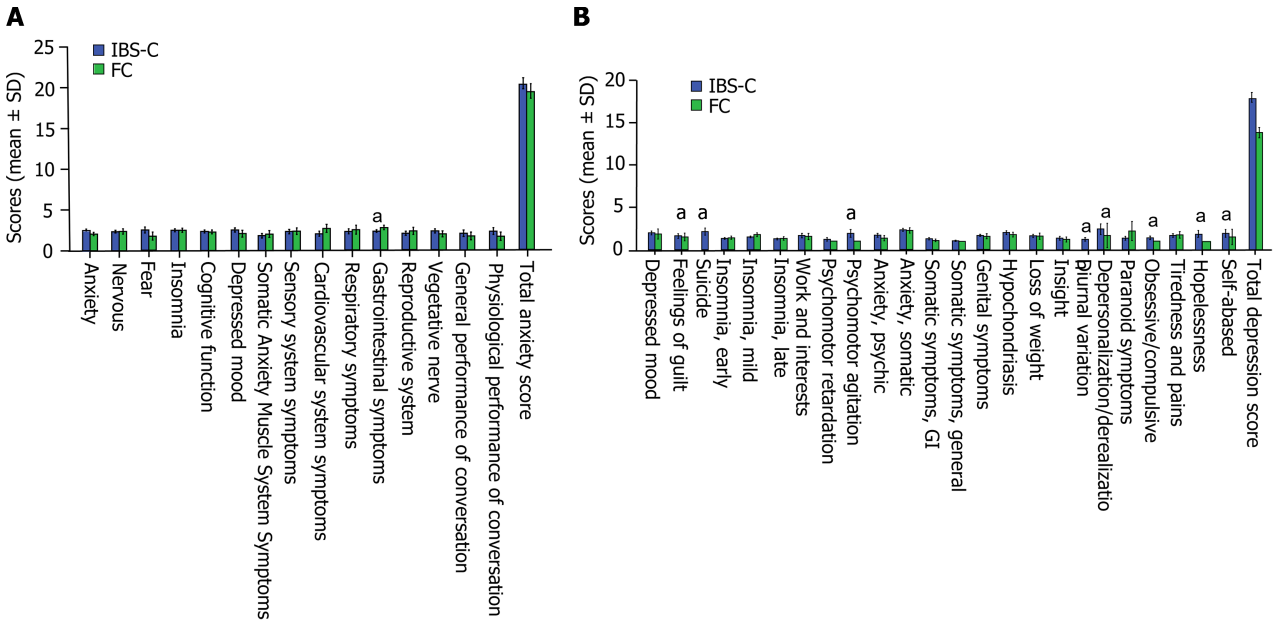
In the IBS-C group, again not observed in the FC group, weak correlations were identified between the degrees of anxiety and the thresholds for first constant sensation, desire to defecate, and sustained urgency ($r = 0.414$, $r = 0.404$, and $r = 0.418$, respectively, $P < 0.05$). Furthermore, IBS-C patients suffering from depression exhibited a lower prevalence of low thresholds for the desire to defecate than those without depression (69.6% *vs* 41.9%, $\chi^2 = 4.054$, $P < 0.05$). No substantial correlation was observed between depression and other anorectal sensations ($P > 0.05$) (Table 3 and 4).

The probability of normal patterns in ARM was 9.68% for patients who exhibited RSARM during CTT tests. Conversely, a 16.67% probability of RSARM was found in patients devoid of type I-IV patterns. RSARM was prominent in 77.78% of FC patients with a type IV pattern, while only 16.67% of IBS-C patients with a type I pattern exhibited RSARM. Notably, all patients with a type III pattern demonstrated RSARM in both the IBS-C and FC cohorts.

Table 4 Correlations of psychiatric characteristics and anorectal sensation thresholds in constipation-predominant irritable bowel syndrome and functional constipation patients

		Thresholds of first sensation				Thresholds of desire to defecate					Thresholds of urgency				
		High	Normal	*r*	*P* value	Low	Normal	High	*r*	*P* value	Low	Normal	High	*r*	*P* value
IBS-C	Anxiety degree			0.414	< 0.05				0.404	< 0.05				0.407	< 0.05
	Depression degrees			0.803	> 0.05				0.019	> 0.05				-0.018	> 0.05
FC	Anxiety degree			0.241	> 0.05				0.111	> 0.05				0.242	> 0.05
	Depression degrees			-0.098	> 0.05				-0.166	> 0.05				-0.233	> 0.05

IBS-C: Constipation-predominant irritable bowel syndrome; FC: Functional constipation.



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Figure 1 Comparison of psychological symptom scores between constipation-predominant irritable bowel syndrome and functional constipation patients. A: Hamilton anxiety rating scale; B: Hamilton Depression Rating Scale-21. $^{a}P < 0.05$ for comparison between the functional constipation and constipation-predominant irritable bowel syndrome groups. IBS-C: Constipation-predominant irritable bowel syndrome; FC: Functional constipation; GI: Gastrointestinal.

DISCUSSION

We observed high proportions of dyssynergic patterns in both IBS-C and FC patients. Interestingly, these patterns also occurred in a substantial proportion of asymptomatic individuals, potentially due to the unnatural posture adopted during simulated defecation[18]. A previous study by Grossi *et al*[19] suggested that the type IV pattern could be helpful for differentiating FC patients from healthy volunteers, with rectal pressure measurements proving more indicative than anal pressure measurements. Furthermore, a study conducted in India highlighted a higher prevalence of insufficient rectal force in FC patients than in those with IBS-C[9]. Consistent with these findings, our study detected a marginal increase in the incidence of insufficient rectal force and type IV dyssynergia among FC patients compared with their IBS-C counterparts, although these differences were not statistically significant. The potential for abnormal rectal pressure shifts during a push maneuver to serve as an effective discriminator between IBS-C or FC and a healthy state within the Chinese population requires further investigation.

Beyond the evaluation of anorectal pressure, ARM also offers the opportunity to gather additional physiological data relating to anorectal sensation[20]. Abnormal visceral sensitivity has been linked with intestinal dysfunction. It is common for constipation patients to exhibit rectal hyposensitivity, whereas rectal hypersensitivity often accompanies IBS [3,4]. In our study, we observed a substantial proportion of both IBS-C and FC patients displaying high thresholds for

initial sensation, potentially contributing to fecal retention. This occurrence was slightly more prevalent in IBS-C patients than in FC patients; however, the difference between the two patient cohorts was not statistically significant.

Psychiatric conditions have been suggested to influence constipation[5]. In our study, we observed a higher incidence of depression among IBS-C patients than among FC patients, a finding consistent with prior studies conducted in China [21]. Moreover, our results showed a positive correlation between anxiety levels and anorectal sensory thresholds in IBS-C patients, suggesting that elevated anxiety may decrease rectal sensitivity, potentially leading to constipation in this patient group.

Impaired peristaltic motility within the intestine is another potential factor contributing to CC. Utilizing radiopaque markers, we evaluated colonic transit in our study population. Our results showed comparable proportions of NTC in IBS-C and FC patients and an increased prevalence of STC among IBS-C patients. Previous studies reported varied findings regarding the prevalence of STC and NTC in IBS-C and FC patients. For example, research by Lam *et al*[22] indicated that STC was more common in FC patients than in IBS-C patients, a finding corroborated by Patcharatrakul and Gonlachanvit[23]. Conversely, Shekhar *et al*[24] did not observe a significant difference in STC prevalence between the two patient groups. The discrepancies in these findings may be attributable to variations in diet, ethnicity, or methodology across studies. Psychological stress may also affect colonic motor activity[25]. We further explored the relationship between CTT and psychological stress. Our data showed that IBS-C patients with NTC were more likely to experience anxiety compared to those with STC. However, we found no significant correlations between psychological stress and colonic motility in FC patients. This difference indicates that emotional factors may have varying effects on colonic motility between these two patient groups.

Dyssynergic defecation (DD) is a condition characterized by a patient's inability to effectively coordinate abdominal and pelvic floor muscles to eliminate stool, commonly seen in functional defecatory disorders. According to the ROME IV classification, DD can be diagnosed in both FC and IBS-C patients. Given the high diagnostic sensitivity of ARM for DD, a normal ARM result can be quite effective in excluding DD[26]. Radiopaque transit studies not only assess colonic transit but can also indicate outlet obstruction based on marker accumulation in the RS region. RSARM has been proven to distinguish DD from STC and NTC, with specificity ranging between 81.2% and 88.2%[27-29]. In our study, most CC patients with normal ARM showed no RS marker accumulation during CTT, with two IBS-C patients being exceptions. We hypothesize that these two patients might have structural anorectal abnormalities causing difficulty in eliminating the radiopaque markers. Alternatively, overlapping colon segments might have made marker separation challenging, potentially leading to classification errors. The relationship between DD and IBS-C and FC is currently a subject of debate. Our study indicated a higher prevalence of RSARM and elevated anal resting pressure in FC patients than in IBS-C patients. High resting pressures associated with anal dyssynergia have been proposed as a useful diagnostic tool to distinguish DD patients from healthy individuals[19,30]. Hence, our findings suggest that DD might be more prevalent among Chinese FC patients. Furthermore, we observed that nearly half of the FC patients with RSARM exhibited type IV dyssynergia, nearly double the prevalence of IBS-C patients, implying distinct pathogeneses of DD in FC and IBS-C patients.

Recognizing DD is crucial because it is a substantial indicator that patients may benefit from biofeedback therapy[13]. The Rome IV criteria define DD according to CC symptoms and at least two abnormal anorectal tests, such as BET, ARM, or defecography. However, no single method is sufficient to diagnose DD[20,31-33]. It is important to note that many institutions may not have access to these diagnostic tools. In our study, we found that most FC patients with type IV dyssynergia and IBS-C patients with type III dyssynergia displayed RSARM. Given that RSARM indicates a possibility of DD, we hypothesize that FC patients with type IV dyssynergia and IBS-C patients with type III dyssynergia are more likely to have DD. Consequently, further BET or defecography might not be necessary for these patients. However, the exact mechanisms underlying the absence of RSARM in most IBS-C patients with type I or II dyssynergia remain elusive. Although RSARM shows a fair correlation with DD, the absence of markers in the RS region does not conclusively rule out DD due to high false-negative rates[31]. Therefore, type I or II dyssynergia cannot rule out the need for CTT in CC patients. On the other hand, it might not be necessary for FC patients with type IV dyssynergia and IBS-C patients with type III dyssynergia to undergo CTT. Although RSARM can somewhat differentiate STC from DD, it cannot distinguish NTC from DD[29,34]. Therefore, further BET or defecography may still be necessary in all aforementioned scenarios.

Our study has several limitations. The clinical features of hospital-based constipation patients are somewhat different from those of community-based patients[35]. As all patients were from one tertiary hospital, our results may represent more severe cases of constipation, and these psychosocial profiles and colorectal motility patterns may not be generalizable to a broader population. Future studies adding community-based patients will be more informative. Deeper and wider psychosocial studies should also be performed in the future. Additionally, our sample size was relatively small, which may have limited our ability to detect significant differences.

CONCLUSION

In conclusion, our study revealed elements that can distinguish between FC and IBS-C across multifaceted parameters, namely colonic transmit time, psychological distress, the association between colonic transit and psychiatric conditions, variations in dyssynergic patterns, and the relationship between RSARM and these patterns. Clinical manifestations, manometric evidence of dyssynergia, and elevated thresholds for first sensation, although valuable, have limited discriminatory power in distinguishing FC from IBS-C. These findings extend our comprehension of the intricate pathophysiological mechanisms underlying FC and IBS-C and could potentially guide the formulation of more precise diagnostic protocols and personalized treatment methodologies.

ARTICLE HIGHLIGHTS

Research background

The comparation of colorectal motility, psychiatric features, and the association of colorectal motility patterns and psychiatric traits between functional constipation (FC) and constipation-predominant irritable bowel syndrome (IBS-C) groups, especially in the Chinese population has not been fully studied.

Research motivation

Controversy persists regarding the correlation between high-resolution anorectal manometry (HR-ARM) and the colonic transmit test (CTT), and it remains unclear which test provides more meaningful data for IBS-C or FC.

Research objectives

We aimed to compare the psychiatric and colorectal motility characteristics between FC and IBS-C patients in an Eastern Chinese population. We also sought to investigate the correlations between psychiatric and colorectal motility characteristics in both FC and IBS-C patients.

Research methods

Colorectal motility patterns were obtained by HR-ARM and CTT. Anxiety and depression were assessed by the Hamilton anxiety rating scale (HAMA) and the Hamilton Depression Rating Scale (HAMD)-21.

Research results

Our study indicated a higher prevalence of rectosigmoid accumulation of radiopaque markers (RSARM) and elevated anal resting pressure in FC patients compared to IBS-C patients. Furthermore, we observed that nearly half of the FC patients with RSARM exhibited type IV dyssynergia, a prevalence nearly double that of IBS-C patients. Our data also showed that IBS-C patients with normal transit time were more likely to experience anxiety compared to those with slow transit time. However, we found no significant correlations between psychological stress and colonic motility in FC patients. FC patients with type IV dyssynergia and IBS-C patients with type III dyssynergia are more likely to have dyssynergic defecation. Type I or II dyssynergia cannot rule out the need for CTT in chronic constipation patients, while it might not be necessary for FC patients with type IV dyssynergia and IBS-C patients with type III dyssynergia to undergo CTT, but further balloon expulsion test or defecography might still be necessary.

Research conclusions

The associations of psychological stress and colonic motility in our study are discrepant from results of Western studies, indicating that emotional factors may have varying effects on colonic motility between these two patient groups. The associations we found between CTT results and dyssynergia patterns by ARM could provide guidance for different constipation groups to choose appropriate colorectal tests.

Research perspectives

We compared not only colorectal motility and psychiatric features, but also the correlations between psychiatric and colorectal motility characteristics in FC and IBS-C patients. What we found could provide guidance for constipation patients to choose appropriate colorectal tests.

FOOTNOTES

Author contributions: Yu Y designed the study and critically revised the article for important intellectual content; Lv CL participated in the analysis and interpretation of the data, and drafted the initial manuscript; Song GQ participated in project supervision and editing the manuscript; Liu J, Wang W, Huang YZ, Wang B, Tian JS, and Yin MQ participated in acquisition of the data.

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STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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ORIGINAL ARTICLE

Observational Study

Inflammatory bowel diseases patients suffer from significant low levels and barriers to physical activity: The "BE-FIT-IBD" study

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Abstract

BACKGROUND

The place regular physical activity (PA) should occupy in managing patients with inflammatory bowel diseases (IBD) is unclear.

AIM

To assess PA levels and barriers in a southern Italian IBD population.

METHODS

IBD patients with non-severe disease activity [assessed with partial Mayo score for ulcerative colitis (UC) and Harvey-Bradshaw index for Crohn's disease] were approached to receive an anonymous online questionnaire to assess PA levels using the International Physical Activity Questionnaire (IPAQ) and to assess disease activity as patient-reported outcomes 2 (PRO-2) and finally to assess habits, beliefs and barriers in conducting regular PA. Clinical, anthropometric and demographic data of patients were also collected. PA was expressed as continuous units of resting metabolic rate (Met) in min/wk. Three PA groups were identified: Inactive (< 700 Met min/wk), sufficiently active (700-2500 Met min/wk) and health enhancing PA (HEPA) (*i.e.*, HEPA active, > 2500 Met min/wk) patients.

RESULTS

Included patients (219) showed overall PA levels of 834.5 Met min/wk, with a large proportion (94, 42.9%) classified as inactive while only a minority (9, 4.1%)

as health-enhancing PA. Patients without dyslipidaemia ($P < 0.0001$) or on biologics therapy ($P = 0.022$) showed better IPAQ scores in moderate activities. UC PRO-2 correlated negatively with IPAQ intense activities scores ($\tau = -0.156$, $P = 0.038$). PRO-2 did not show notable sensitivity/specificity in predicting IPAQ inactivity (AUC < 0.6). IBD activity did not differ between active and inactive patients ($P > 0.05$). Active patients expressed the need to discuss PA with their gastroenterologist. Some barriers (*e.g.*, diagnosis of IBD and fear of flare-ups after PA) are significantly more reported by inactive patients.

CONCLUSION

A significant rate of physical inactivity was recorded in this setting. IPAQ showed good feasibility. PA should be an element of discussion in IBD visits assessed quickly with non-invasive questionnaires.

Key Words: Crohn's disease; Inflammatory bowel disease; International Physical Activity Questionnaire; Physical activity; Ulcerative colitis

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Core Tip: The place regular physical activity (PA) should occupy in managing patients with inflammatory bowel diseases (IBD) is unclear. IBD patients were approached to receive an anonymous questionnaire to assess PA levels using the International Physical Activity Questionnaire (IPAQ), assess disease activity as patient-reported outcomes 2, and assess habits, beliefs and barriers in conducting regular PA. A large proportion of included IBD patients were classified as inactive. Patients on biologics therapy showed better IPAQ scores in moderate activities. Inactive patients report some barriers. PA should be an element of discussion in IBD gastroenterology visits.

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INTRODUCTION

Inflammatory bowel diseases (IBD), mainly comprising Crohn's disease (CD) and ulcerative colitis (UC), underlie sustained and chronic gastrointestinal inflammation[1] associated with varying disabilities, including those in the psychological sphere[2,3], impacting patients' quality of life (QoL)[4]. Physical activity (PA) is part of and positively affects QoL [5]. A recent consensus encouraged regular PA (consistent with individual tolerance level) to ameliorate the IBD course [6]. PA is, by definition, the use of skeletal muscles with energy expenditure and can promote an anti-inflammatory phenotype in various tissues (such as muscle, adipose tissue, and heart)[7]. It is also widely framed as a modifiable risk factor for several chronic diseases, such as cardiovascular and metabolic as well as neoplastic ones and, in some, such as rheumatoid arthritis, has even shown the ability to associate with a milder disease course[8].

Moreover, low-intensity PA can positively impact mild/remission CD patients' QoL[9], and no change in CD activity nor predisposition to flare-ups had been observed[10]. Finally, a further study on the IBD population also showed how higher PA associates with better QoL, excluding sweat-inducing exercises[11].

In addition, IBD patients seem less likely to perform PA, and, despite the potential benefits of it, precise recommendations and guidelines on how to approach this subject in IBD have not yet been codified[12]. IBD patients often suffer from sarcopenia, which is, among other things, also a predictive factor for the need to incur surgery and worsening the risk of postoperative complications[13]. PA improves muscle mass and poses as an ameliorative measure of sarcopenia [14,15]. In addition, the World Health Organization recommends, generally for every age group as well as for adults with disabilities, regular PA stigmatizing its multidimensional benefits (from cardioprotective effects to beneficial psychological effects)[16].

Ultimately, the epidemiologic data regarding PA levels in IBD is severely lacking. The barriers that block patients from practising regular PA and what factors instead are facilitators of this are unknown. Even less evidence is definitive on what impact PA (splitting the data even by intensity) may have on IBD activity. Therefore, this study aimed to assess self-reported PA levels in an IBD population and to examine whether there are differences in that setting concerning clinical/demographic, patient-reported IBD activity and reported barriers to regular PA.

MATERIALS AND METHODS

Study design

This cross-sectional observational study was conducted at the Hepatogastroenterology Unit of the University of Campania Luigi Vanvitelli in the first half of 2023. IBD participants included were given an online questionnaire to fill out anonymously. This study was written following the "strengthening the reporting of observational studies in epidemiology" (*i.e.*, STROBE) checklist. The study was conducted in compliance with the Declaration of Helsinki and received approval from the Ethics Committee of the University of Campania Luigi Vanvitelli (protocol number 7892, 15 March 2023).

Inclusion and exclusion criteria

Patients with an established histologic diagnosis of IBD (*i.e.*, CD or UC) were included. Patients with known psychiatric conditions and severe comorbidities, recent surgery, clinically significant infection (*e.g.*, *Clostridioides difficile*), hospitalised or who had received contraindications to performing any form and degree of PA were, instead, excluded. In addition, patients with severe disease activity assessed (within one month before the inclusion) by partial Mayo score[17] for UC patients *(i.e.*, score > 7) and by Harvey-Bradshaw index for CD patients (*i.e.*, score > 16)[18] were also excluded.

Collected variables

Through the questionnaire, several variables were collected. First, demographic and anthropometric data were, in detail, collected, such as sex, age, weight (in Kg), height (in cm), body mass index (BMI) (in Kg/m^2), level of education, employment, smoking status, alcohol consumption (the patient was defined as an alcohol user if daily consumption was ≥ 20 g in female or ≥ 30 g in the male)[19] as well as, finally, having or not having a partner. Concerning IBD, the type (*i.e.*, CD or UC), age at diagnosis of IBD, the current treatments, and previous biologic failure were collected. IBD disease activity was assessed with patient-reported outcomes 2 (PRO-2) for both CD[20] and UC[21]. Therefore, the sub-score on stool frequency (SF) and abdominal pain (AP) for patients with CD was evaluated. On the other hand, the subscore, SF and that on rectal bleeding (RB) were examined for UC patients.

Finally, patients were also asked whether they had comorbidities (such as diabetes, hypertension, nephropathies, dyslipidaemia, or pneumopathies) or extra-intestinal manifestations.

PA Assessment

The international PA questionnaire (IPAQ) showed good validity and reliability characteristics[22,23] and was used to assess PA in this study. IPAQ evaluate the PA type and amount performed by the compiler by referring to the past 7 d. IPAQ contemplates intense activities (such as aerobic activities like running), moderate activities (such as carrying light weights) and mild activities (walking for at least 10 min). IPAQ identifies three categories of respondents based on PA levels: Type 1 (*i.e.*, inactive), type 2 (*i.e.*, minimally active), and, finally, type 3, also defined as health enhancing PA (HEPA) (*i.e.*, HEPA active).

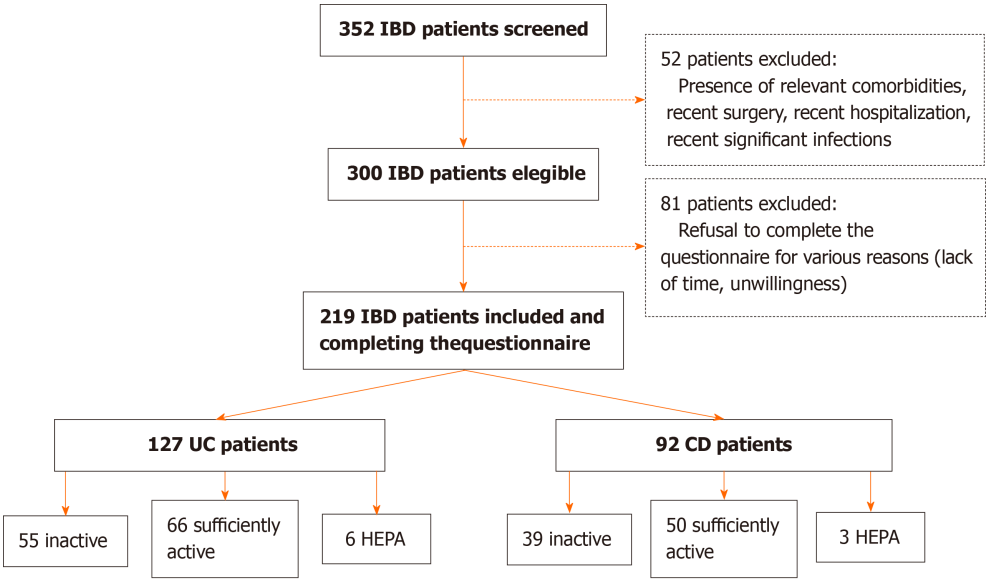
The IPAQ score was made continuous using multiples of the resting metabolic rate [*i.e.*, metabolic rate (Met)] as units. Therefore, the Met of PA was calculated by level and specifically for intense (minutes × days × 8 Met), moderate (minutes × days × 4 Met) and, finally, for mild/walking (minutes × days × X Met) activities. The value of X for the last activities is a function of a multiplier based on the steep grade. Specifically, for an intense stride that gave the compiler a marked perceived increase in respiratory rate relative to normal, the multiplier is 3.3; for a moderate stride that increased respiratory rate at a rate only moderately higher than usual, the multiplier is 3 while, finally, for a slow stride with no change in respiratory rate the multiplier is 2.5. PA levels were, therefore, ultimately expressed as Met min/wk. IPAQ has, moreover, already been employed in IBD[24]. IPAQ was scored according to the available guidelines (http://www.ipaq.ki.se/) using the Italian-validated IPAQ version[25]. Patients were finally considered inactive (< 700 Met min/wk), sufficiently active (700-2500 Met min/wk), or active/HEPA (> 2500 Met min/wk)[23,26].

In addition, the link provided to patients included additional questions to weigh the possible presence of barriers/facilitators to performing regular PA. These questions associate with five levels of agreement (with extremes from completely agree to disagree) responses. Finally, an 11-point Likert scale question was administered to understand how important the patient thought it was from 0 to 10 to discuss PA with their gastroenterologist during outpatient visits.

Statistical analysis

Descriptive statistics were used for data presentation. Continuous variables are presented as a median and relative interquartile range, while categorical and ordinal variables as a percentage of the total (%) for each degree of freedom. The Kolmogorov-Smirnov test preliminarily evaluated variables distribution to choose between parametric and nonparametric analyses for data analysis according to study outcomes. The Chi-square and Fisher's exact test were used for the relationship between categorical variables. The Mann-Whitney U-test compared ordinal continuous variables with two-level categorical independent variables. In the case of ordinal variables with multiple degrees of freedom, Kruskal-Wallis's test was used instead. The strength of correlations between the variables of interest was probed with Kendall's tau-b test. If it was necessary to categorize PA levels dichotomously (active/inactive), that of 699 Met wk/min (according to IPAQ scoring) was chosen as the threshold, defining active as those who had a PA > of this threshold.

To evaluate the predictors of physical inactivity, the independent variables of the implemented logistic regression model coincided with the other continuous and/or categorical variables deemed relevant. The regression model was evaluated according to the goodness of fit according to Hosmer-Lemeshow (as well as according to Cox and Snell R^2 and Nagelkerke R^2 values) by expressing the data as an exponential value of B, *i.e.*, exp (B). The latter was presented as the



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Figure 1 Flow chart summarizing the inclusion in the study patients, divided by type of inflammatory bowel disease and resulting physical activity levels. HEPA: Health enhancing physical activity; IBD: Inflammatory bowel disease; CD: Crohn's disease.

odds ratio, and the risk measure was expressed as the OR and its 95% confidence interval (95%CI).

The receiver operating characteristic (ROC) method was used to weigh the specificity/sensitivity of any variables regarding PA levels. These assessments were performed after checking for the existence of an adequate area under the ROC curve (AUC) > 0.699, which was calculated along with its 95%CI.

To evaluate the internal reliability of the questions in our survey to assess patient barriers to PA, we analysed Cronbach's alpha coefficient, recording a value of 0.7. In addition, a statistical significance value was accepted for $P < 0.05$ (two-tailed) values, placing an alpha error of 0.05. Statistical analyses were performed with IBM® SPSS® software, graphs with GraphPad PRISM®, and sample size calculation with G*Power software.

RESULTS

Sample characteristics

Figure 1 describes the steps for the enrolment of all patients. Two hundred nineteen patients were finally included, and Table 1 summarizes their characteristics by stratifying by type of IBD. Most of the sample, 127 (58%) patients, had UC.

Some differences emerged between IBD subgroups. CD patients had a higher rate of biologics use than the UC ones (*i.e.*, 82.6% *vs* 61.4%, $P = 0.001$) and unemployment (60.9% *vs* 40.2%, $P = 0.009$). In addition, females had a significantly lower age than males [38 (28-50) *vs* 45 (31-56), $P = 0.017$].

Most of the sample was on subcutaneous biologic drug therapy (113, 51.6%), while a minority were taking intravenous (24, 11%) or oral (17, 7.8%) biological treatment, while the remainder (65, 29.7%) were not taking biologics. The overall failure rate of a previous biologic was 21.5% (47/219).

PA levels

Most of the sample (116, 53%) met the IPAQ criteria for sufficiently active, while only a minority (9, 4.1%) met the criteria for HEPA activity. On the contrary, a large sample portion was classified as inactive (94, 42.9%). The overall IPAQ total score was 834.5 (384.5 – 1424) Met min/wk. Gender did not seem particularly impactful concerning PA (Figure 2A). The other variables in Table 1 showed no variations when stratified by PA grade (Table 2). IBD type did not result in variations in PA levels (see Table 3 and Figure 2B) since both the type of PA (*i.e.*, intense, moderate, or mild) and the class of PA (*i.e.*, inactive, sufficiently or HEPA active) did not vary particularly differentially between CD and UC patients. In addition, the comorbidities most represented in our sample, hypertension ($P = 0.095$), arthritis ($P = 0.101$), or Hashimoto's thyroiditis ($P = 0.540$), did not particularly impact IPAQ total score levels. In contrast, PA levels differed according to dyslipidaemia ($P < 0.0001$). In detail, dyslipidaemia patients presented higher [956 (325 - 1622)] levels of PA than those without dyslipidaemia [811 (393.75 - 1358.77)]. However, in contrast, the moderate activity score was higher in healthy patients than in those with dyslipidaemia [176 (0-567) *vs* 160 (0-480), $P < 0.0001$].

Patients on biological therapy showed some advantage over those on standard therapy [246 (0-642) *vs* 56 (0-394), $P = 0.022$], as shown in Figure 2C. At bivariate analysis, neither age ($\tau = -0.27$, $P = 0.550$) nor BMI ($\tau = 0.75$, $P = 0.100$) showed correlations with IPAQ total score.

Table 1 Sample characteristics concerning the type of inflammatory bowel disease

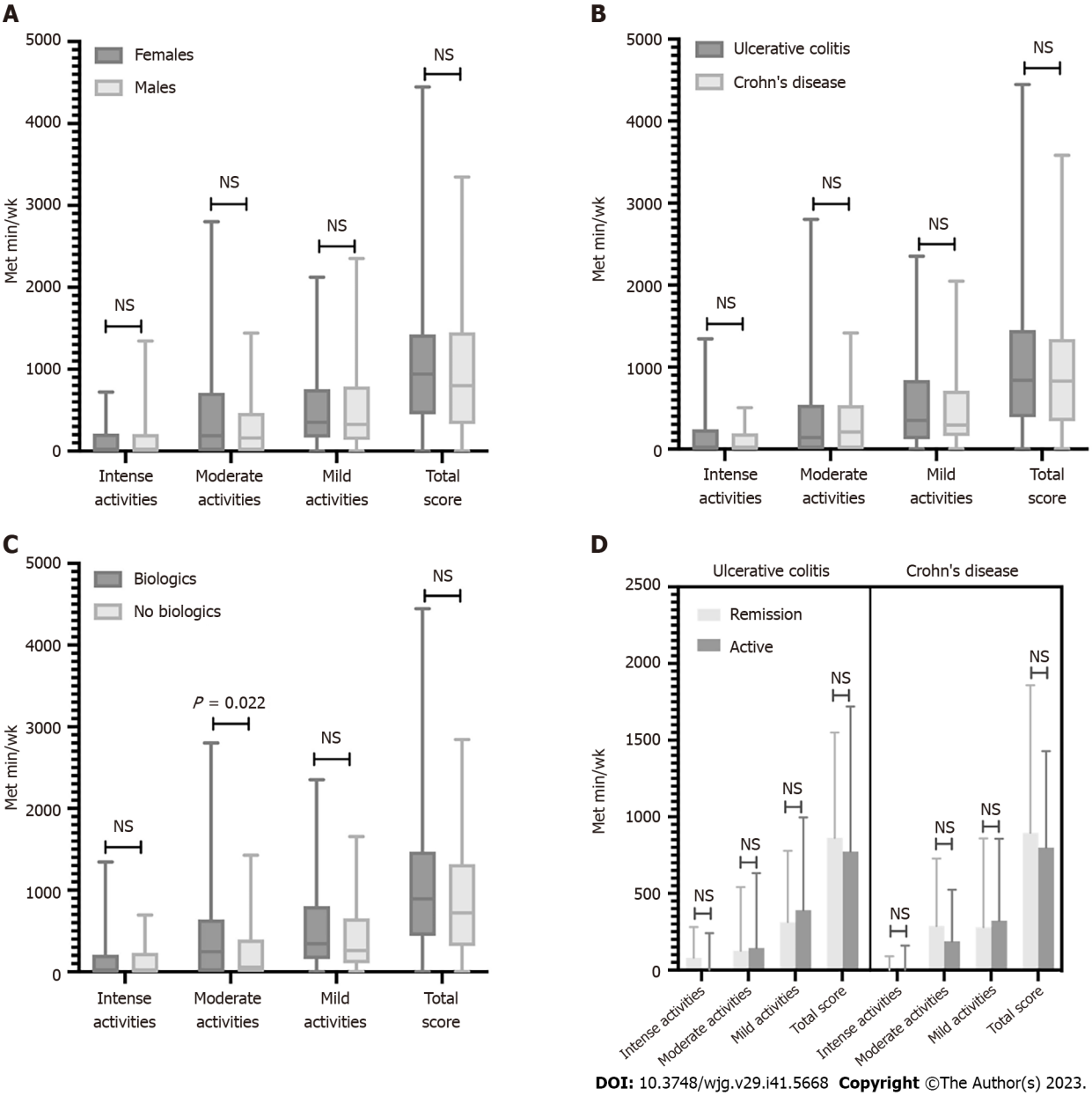
Variable	Crohn's disease (*n* = 92)	Ulcerative colitis (*n* = 127)	*P* value[1]
Age	43.5 (29-53)	41 (30-55)	0.871
BMI	23.8 (20.8-26.4)	23.8 (21.4-25.9)	0.787
Gender			0.157[2]
Male	44 (47.8%)	73 (57.5%)	
Female	48 (52.5%)	54 (42.5%)	
Education			0.192
Primary school	32 (24.8%)	27 (21.3%)	
Secondary school	52 (56.5%)	78 (61.4%)	
Degree	8 (8.7%)	22 (17.3%)	
Job			**0.009**
Unemployed	56 (60.9%)	51 (40.2%)	
Employee	16 (17.4%)	38 (29.9%)	
Entrepreneur	8 (8.7%)	16 (12.6%)	
Worker	4 (4.3%)	7 (5.5%)	
Student	8 (8.7%)	15 (11.8%)	
Smoking status			**0.003**
Active smoker	16 (17.4%)	20 (15.7%)	
Past smoker	32 (34.8%)	16 (12.6%)	
Non-smoker	44 (47.8%)	91 (71.7%)	
Alcohol consumer			0.827[2]
Yes	8 (8.7%)	10 (7.9%)	
No	84 (91.4%)	117 (92.1%)	
Comorbidity			0.256
Diabetes	8 (8.7%)	4 (3.1%)	
Hypertension	20 (21.7%)	10 (7.9%)	
Recurrent UTI	2 (2.2%)	4 (3.1%)	
Chronic renal failure	1 (1.1%)	2 (1.6%)	
Nephrolithiasis	3 (3.3%)	1 (0.8%)	
Asthma	3 (3.3%)	-	
COPD	2 (2.2%)	1 (0.8%)	
Previous pneumonia	1 (1.1%)	1 (0.8%)	
Dyslipidaemia	12 (13%)	35 (27.6%)	
Arthritis	32 (34.8%)	34 (26.8%)	
Hashimoto's thyroiditis	7 (7.6%)	15 (11.8%)	
Partner			0.322[2]
Yes	68 (73.9%)	86 (67.7%)	
No	24 (26.1%)	41 (32.3%)	
Biologics (yes)	76 (82.6%)	78 (61.4%)	**0.001**[2]
Steroids (yes)	4 (4.3%)	8 (6.3%)	0.531[2]

[1]The *P* value was calculated by checking the difference in the distribution of different variables between the two identified groups (*i.e.*, Crohn's disease and

ulcerative colitis).

[2]The Chi-square test or Fisher's exact test was employed for evaluation.

BMI: Body mass index; COPD: Chronic obstructive pulmonary disease. Data are expressed for continuous variables as median (interquartile range) and, for categorical and ordinal variables, as numerosity (%). Significant *P* values are indicated in bold.



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Figure 2 Physical activity levels in the main subgroups examined. A-D: Physical activity levels observed in males and females (A), patients with ulcerative colitis and Crohn's disease (B), patients on treatment and not on biologics (C), and, finally, patients physically active or inactive concerning baseline disease activity (D). Met: Metabolic rate; NS: Not significant.

In this work, we detected a significantly higher unemployment rate in patients with CD on bivariate analysis. This finding led us to consider whether work occupation could impact PA levels. By processing the specific variable categorically over the entire sample (employed/unemployed), PA levels (as IPAQ total score) were not found to be impacted by employment rate (P = 0.851). This trend was also preserved when filtering by IBD type in both UC (P = 0.654) and CD (P = 0.481). Furthermore, the result was also maintained by comparing the employment and PA rates using the Chi-square test (χ^2 = 0.321, P = 0.588).

Disease activity and PA levels

PA levels (*i.e.*, as IPAQ total score in Met min/wk) were not different concerning PRO-2 measured IBD activity. In detail, this trend was confirmed by stratifying by PA intensity (*i.e.*, intense, moderate and mild/walking) and total score (Figure 2D). PRO-2 data are summarized in Table 4 and related to the PA intensity.

Table 2 Sample characteristics concerning the levels of physical activity

Variable	Physically active (*n* = 125)	Physically inactive (*n* = 94)	*P* value[1]
IBD			0.892[2]
Crohn's disease	53 (42.4%)	39 (41.5%)	
Ulcerative colitis	72 (57.6%)	55 (58.5%)	
Age	39 (29.5-52)	44 (29-55.25)	0.506
BMI	24.25 (21.47-26.28)	22.72 (20.95-25.71)	0.185
Gender			0.952[2]
Male	67 (53.6%)	50 (53.2%)	
Female	58 (46.4%)	44 (46.8%)	
Education			0.903
Primary school	34 (27.2%)	25 (26.6%)	
Secondary school	75 (60%)	55 (58.5%)	
Degree	16 (12.8%)	14 (14.9%)	
Job			0.432
Unemployed	59 (47.2%)	48 (51.1%)	
Employee	30 (24%)	24 (25.5%)	
Entrepreneur	15 (12%)	9 (9.6%)	
Worker	6 (4.8%)	5 (5.3%)	
Student	15 (12%)	8 (8.5%)	
Smoking status			0.607
Active smoker	21 (16.8%)	15 (16%)	
Past smoker	29 (23.2%)	19 (20.2%)	
Non-smoker	75 (60%)	60 (63.8%)	
Alcohol consumer			0.892[2]
Yes	10 (8%)	4 (4.3%)	
No	115 (92%)	90 (95.7%)	
Comorbidity			0.899
Diabetes	8 (6.4%)	4 (4.3%)	
Hypertension	20 (16%)	10 (10.6%)	
Recurrent UTI	3 (3.2%)	2 (2.1%)	
Chronic renal failure	-	1 (1.1%)	
Nephrolithiasis	3 (2.4%)	2 (2.1%)	
Asthma	2 (1.6%)	2 (2.1%)	
COPD	1 (0.8%)	1 (1.1%)	
Previous pneumonia	2 (1.6%)	-	
Dyslipidaemia	28 (22.4%)	19 (20.2%)	
Arthritis	32 (25.6%)	34 (36.2%)	
Hashimoto's Thyroiditis	12 (9.6%)	10 (10.6%)	
Partner			0.570[2]
Yes No	86 (68.8%) 39 (31.2%)	68 (72.3%) 26 (27.7%)	
Biologics *(yes)*	91 (72.8%)	63 (67%)	0.354[2]

Steroids *(yes)*	10 (8%)	2 (2.1%)	0.059[2]

[1]The *P* value was calculated by checking the difference in the distribution of different variables between the two identified groups (*i.e.*, physically active or inactive).
[2]The Chi-square test or Fisher's exact test was employed for evaluation.
BMI: Body mass index; COPD: Chronic obstructive pulmonary disease; UTI: Urinary tract infections. Data are expressed for continuous variables as median (interquartile range) and, for categorical and ordinal variables, as numerosity (%).

Table 3 Physical activity concerning the type of inflammatory bowel disease

PA variable	Crohn's disease (*n* = 92)	Ulcerative colitis (*n* = 127)	*P* value[1]
Intense activities (Met min/wk)	0 (0-192)	0 (0-240)	0.099
Moderate activities (Met min/wk)	208 (0-536)	140 (0-540)	0.590
Mild activities (Met min/wk)	293.75 (158.12-711.6)	350 (120-840)	0.940
Sitting time at work (min)	210 (113-292.5)	215 (125-292)	0.719
Sitting time at home (min)	174 (118.75-221.75)	177 (115-229)	0.855
Total score (Met min/wk)	828.25 (339.37-1343.5)	839 (390-1451)	0.678
PA level			0.995
Inactive	39 (42.4%)	55 (43.3%)	
Sufficiently active	50 (54.3%)	66 (52%)	
HEPA active	3 (3.3%)	6 (4.7%)	

[1]The *P* value was calculated by checking the difference in the distribution of different variables between the two identified groups (*i.e.*, Crohn's disease and ulcerative colitis).
HEPA: Health enhancing physical activity; PA: Physical activity. Data are expressed for continuous variables as median (interquartile range) and, for categorical and ordinal variables, as numerosity (%).

Table 4 Disease activity and physical activity levels expressed as total International Physical Activity Questionnaire score

PA variable	*n* (%)	PA active (Met min/wk)	PA inactive (Met min/wk)	*P* value[1]
PRO-2 CD	***n* = 92**	***n* = 53**	***n* = 39**	
Remission	27 (29.3%)	1353 (1026.5-2064)	210.75 (101.25-313.75)	0.303
Mild	24 (26.1%)	1213.65 (1039.52-1534.75)	346.25 (229.62-566.37)	
Moderate	41 (44.6%)	1240.75 (879.67-1950)	352.5 (45-468.75)	
Overall	**92 (100%)**	**1234 (981.75-1769.25)**	**280 (157.5-465)**	
PRO-2 UC	***n* = 127**	***n* = 72**	***n* = 55**	
Remission	74 (58.3%)	1345.35 (1057.5-1766.75)	321.25 (204.25-535.87)	0.994
Active	53 (41.7%)	1457 (952-1964.75)	350 (0-574)	
Overall	**127 (100%)**	**1373.35 (990-1792.62)**	**325 (111.5-538.5)**	

[1]The *P* value was calculated by checking the difference in the distribution of different variables between the two identified groups (*i.e.*, active and inactive patients).
PRO: Patient reported outcome; CD: Crohn's disease; UC: Ulcerative Colitis; IPAQ: International Physical Activity Questionnaire; PA: Physical activity. Data are expressed for continuous variables as median (interquartile range) and, for categorical and ordinal variables, as numerosity (%).

Considering the whole sample, the SF of CD patients showed a median of 4.5 (2 - 8) bowel movements, while AP was reported as absent in 56 (60.9%) patients, as mild in 24 (26.1%) and finally, as moderate in 12 (13%) patients. UC patients, on the other hand, reported normal SF in most cases (92, 72.4%), increased 1-2 times in 17 (13.4%) and increased 3-4 times in 18 (14.2%) cases. Moreover, UC patients concerning RB reported no visible blood in most cases (91, 71.7%), traces in less than half of bowel movements in 27 (21.3%) cases and, finally, visible blood in most bowel movements in 9 (7.1%) cases. In general, as shown in Table 4, even in the absence of significance, CD patients in remission with regular PA had better disease activity scores than those with mild and moderate activity, while this trend was not superimposable in the

Table 5 Beliefs about physical activity of patients included

Question (*n* = 219)	Completely agree	I think it is irrelevant	Partially agree	Partially disagree	Completely disagree	*P* value[1]
I think my IBD is a block to doing regular PA	12 (5.5%)	65 (29.7%)	58 (26.5%)	28 (12.8%)	56 (25.6%)	0.957
The treatment I am taking for my IBD is a block to performing regular PA	5 (2.3%)	78 (35.6%)	15 (6.8%)	16 (7.3%)	105 (47.9%)	0.520
I believe that engaging in regular PA may reactivate my IBD or, if already active, make it worse	61 (27.9%)	43 (19.6%)	18 (8.2%)	19 (8.7%)	78 (35.6%)	**< 0.001**
I believe that performing regular PA may result in complications in my IBD (*e.g.*, fistula formation, abscesses or other)	13 (5.9%)	45 (20.5%)	21 (9.6%)	26 (11.9%)	114 (52.1%)	0.527
I believe that performing regular PA can improve my IBD	28 (12.8%)	55 (25.1%)	87 (39.7%)	6 (2.7%)	43 (19.6%)	0.942
I believe that performing regular PA can protect me from new IBD recurrence	25 (11.4%)	74 (33.8%)	87 (39.7%)	18 (8.2%)	15 (6.8%)	0.538
My family doctor adequately informed me regarding the possibility of performing regular PA	52 (23.7%)	45 (20.5%)	51 (23.3%)	18 (8.2%)	53 (24.2%)	0.936
My gastroenterologist adequately informed me regarding the possibility of performing regular PA	76 (34.7%)	26 (11.9%)	80 (36.5%)	10 (4.6%)	27 (12.3%)	0.871
People close to me (*e.g.*, relatives and friends) have repeatedly urged me to conduct a regular PA	91 (41.6%)	22 (10%)	69 (31.5%)	18 (8.2%)	19 (8.7%)	0.795
People close to me (relatives, friends) have repeatedly advised/banned me from conducting regular PA	0 (%)	35 (16%)	29 (13.2%)	24 (11%)	131 (59.8%)	0.291
Before receiving the diagnosis of IBD, I was more inclined to perform regular PA, but now, upon receiving the diagnosis, I feel less convinced to perform PA	41 (18.7%)	45 (20.5%)	45 (20.5%)	13 (5.9%)	75 (34.2%)	**< 0.001**

[1]The *P* value was calculated by checking the difference in the distribution of different variables between the two identified groups (*i.e.*, active and inactive patients).

IBD: Inflammatory bowel disease; PA: Physical activity. Data are expressed as numerosity (%). Significant *P* values are indicated in bold.

case of UC. At bivariate analysis, the UC PRO-2 score negatively correlated with the IPAQ intense activity subscore (τ = -0.156, P = 0.038). This correlation was not met by CD patients (τ = 0.114, P = 0.160). PRO-2 showed no other relationships with other IPAQ parameters (P > 0.05). Finally, on ROC analysis, neither PRO-2 in the UC (AUC = 0.512, 95%CI 0.409-0.614) nor CD (AUC = 0.431, 95%CI 0.311-0.551) showed notable AUCs.

IBD patient's beliefs and barriers toward PA

The sample felt, on average, essential to discuss PA with their gastroenterologist during outpatient visits, as evidenced by a median of 6 (4-8) on the 11-point Likert scale administered to patients (Figure 3A and B) and active patients tended to respond more frequently with scores at the positive extreme (*i.e.*, 9, 10, P = 0.044). Figure 3C resumes sports practised by patients, and differences in the chosen sport between active and inactive in terms of PA were not found (P = 0.445). In addition, several IBD-related barriers to PA were reported (Figure 3D), with some reported more frequently by inactive patients, specifically diarrhoea and evacuation urgency (P = 0.004). Table 5 reports the central beliefs of our patients about several PA aspects. In this context, 63.8% (60/94) were wholly convinced that PA could reactivate/worsen the clinical activity of their IBD. Sixty percent (75/125) of PA active patients thoroughly reported the opposite (P < 0.001). In addition, 46.8% (44/94) of PA inactive believed (entirely or partially) that the diagnosis of IBD was the starting point of their distrust of PA. The trend was predictably opposite in PA active patients (P < 0.001).

A traceable element, in general, is how the patients' social network in majority urged the patient to practice regular PA. In contrast, less than half of the patients felt adequately informed by their family doctor or gastroenterologist about the possibility of performing regular PA.

Finally, a binary logistic regression analysis was conducted to investigate physical inactivity predictors among all the study variables, not recording any significant predictor (Figure 4 and Table 6).

DISCUSSION

This study weighed patient-reported PA in a group of IBD European patients. In this study, IBD adults showed a particularly worrying rate of physical inactivity (*i.e.*, 42.9%), with only 4.1% of the sample meeting the HEPA criteria. Median overall PA levels (*i.e.*, 834.5 Met min/wk) were just above the IPAQ threshold for inactivity (*i.e.*, 700 Met min/wk).

In this experience, PA showed no relationship with IBD activity (employing the PRO-2 tool), except for a negative relationship between UC PRO-2 and moderate PA levels. Clinical PROs, as moreover measured by PRO-2, have been

Table 6 Predictors of physical inactivity analysis among clinical and demographic variables evaluated by binary logistic regression

Variable	Exp (B)/Odds ratio	95%CI	*P* value
Age (yr)	0.996	0.976-1.018	0.743
Weight (Kg)	1.017	0.992-1.041	0.180
Height (cm)	1.018	0.989-1.048	0.231
BMI (Kg/m^2)	0.999	0.998-1.001	0.867
IBD (CD)	0.931	0.503-1.721	0.819
IBD (UC)	1.074	0.581-1.987	
Gender (Male)	1.196	0.642-2.225	0.573
Gender (Female)	0.836	0.449-1.556	
Partner (Yes)	1.343	0.681-2.652	0.395
Partner (No)	0.744	0.377-1.469	
Biologics (Yes)	0.816	0.430-1.549	0.534
Biologics (No)	1.225	0.646-2.325	
Arthritis (Yes)	1.710	0.892-3.278	0.106
Arthritis (No)	0.585	0.305-1.121	
Dyslipidaemia (Yes)	0.747	0.343-1.627	0.463
Dyslipidaemia (No)	1.338	0.615-2.913	
Diabetes (Yes)	1.074	0.209-5.517	0.932
Diabetes (No)	0.931	0.181-4.786	
Hypertension (Yes)	0.622	0.204-1.893	0.403
Hypertension (No)	1.608	0.528-4.893	
Hashimoto's thyroiditis (Yes)	1.121	0.462-2.717	0.800
Hashimoto's thyroiditis (No)	0.892	0.368-2.162	
Importance PA discussion (Likert 10-point scale)	0.911	0.823-1.008	0.072
Employed	0.857	0.501-1.464	0.571
Unemployed	1.167	0.683-1.994	

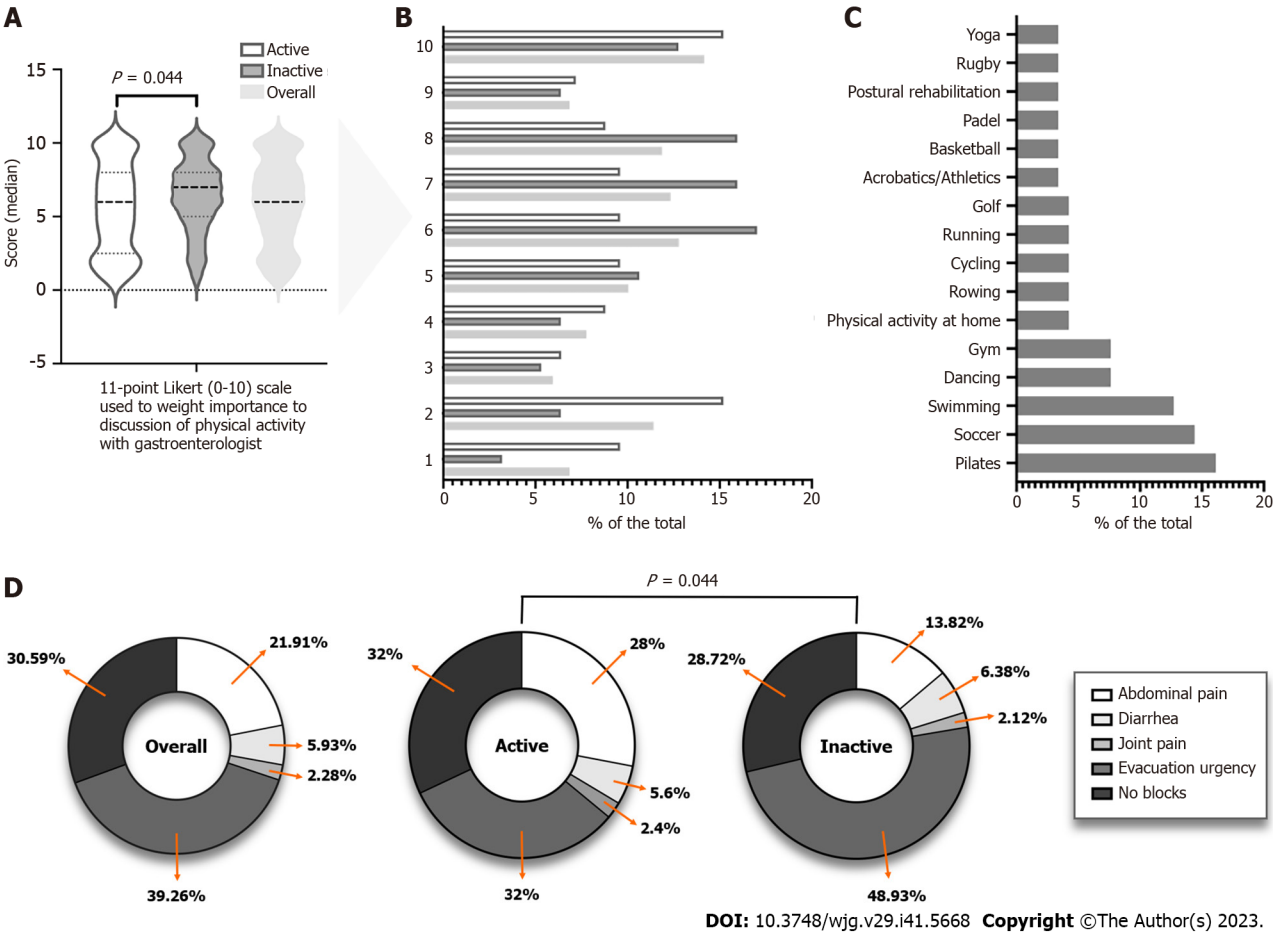
Risk is expressed as the exponential value of B, or Exp(B), presented in odds ratio with a relative 95% confidence interval and relative *P* value. 95%CI: 95% confidence interval; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative Colitis; PA: Physical activity.

shown in a recent cross-sectional study to associate with daily activities impairment compared with physician-reported ones[27]. PA and IBD relationship is still highly controversial and under study. Khalili *et al*[28], in a cohort study, showed an inverse association between PA and the risk of CD but not UC.

In contrast, another Japanese study showed an inverse association between intense-type PA and mucosal healing but not with clinical remission[29]. Much of the available evidence, albeit little, seems to suggest in IBD a moderate and, in a significant minority, mild PA intensity, and it appears that this increased PA is associated with better management of symptoms (including fatigue) as well as better psychological outcomes and QoL[30]. Despite this, there are still no detailed recommendations on the best sport to suggest, at what intensity and for how long for IBD patients.

Complicating the picture, there are vast geographical differences in conceptions of PA[31]. A previous New Zealand survey (which examined a smaller sample of 77 patients) found a higher rate of PA (*i.e.*, 66%) and PA levels (1613 *vs* 834.5 Met min/wk) than ours[32]. In each case, however, this study also found similar barriers reported by patients with IBD to PA (*i.e.*, evacuation urgency). These data were also similar to that of Tew *et al*[24].

Whether regular PA can give tangible benefits to IBD activity is still not completely clear. However, it appears that PA may increase the T-regulatory lymphocytes expression, reduce the immunoglobulins secretion by negatively regulating T helper 1 Lymphocytes, and increase the anti-inflammatory cytokine IL-10 production[8]. However, as in our study, all in all, not a strong relationship between PA and IBD activity was also obtained from another American sample of about 250 patients with an average age similar to ours (*i.e.*, 39.6 years)[33]. In contrast, in another study, more marked differences in PA had emerged between patients with active disease and in remission, postulating a negative role of disease activity [24]. These differences may be partially explained by the fact that a higher rate of patients in our study was on biologics, which have a pronounced impact on the course of the disease[34]. Not surprisingly, as written before, we observed how



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Figure 3 Importance given by patients to discuss physical activity with their gastroenterologist, major sports played by them, and barriers to physical activity related to inflammatory bowel disease. A and B: The importance given by patients to discuss with their gastroenterologist physical activity stratified by physical activity level (A) and detailed by individual Likert scale score (B); C: Main sports stated by participants; D: Factors related to inflammatory bowel disease hinder regular physical activity.

being on biological therapy provided an advantage toward moderate PA activities ($P = 0.022$, Figure 2C).

This study also examined the potential impact of work employment on PA levels by leaning toward the little impact of the former on the latter. Although not detailed in IBD, this finding contrasts with what is already reported in the general population[35]. This matter is difficult to interpret in a population (*i.e.*, IBD) already heavily impacted by unemployment [36]. Not coincidentally, our unemployment rate was high (*i.e.*, 48.9%, 107/219), so studies including a larger sample of employed people probably need to verify a real difference in PA levels.

The comorbidities we noted did not have much effect on PA levels. However, to assess arthritis, we did not evaluate clinical activity because of the study design and purpose. Therefore, although having or not having this comorbidity did not impact PA levels in our setting, the limitation of not grading joint disease/disability activity must be considered. We also found better PA levels in dyslipidaemia-affected patients. However, we believe the small number of dyslipidaemia-affected patients must weigh this result compared to healthy ones, so a subgroup analysis should be considered merely exploratory. In any case, IBD patients without dyslipidaemia tolerated higher activity levels better than those with dyslipidaemia, as reported in a non-IBD setting[37].

The problem of physical inactivity in IBD is relevant because an inactive patient risks losing the potential benefits that PA can provide in several aspects already undergoing impairment in such patients (such as mood disorders[38], metabolic syndrome[39], and sarcopenia[14]).

To recover inactive patients (in terms of PA), our study offers several insights. The first point is probably, to discuss with the patient of PA during the gastroenterology visit and identify the patient's fears. Secondly, providing the patient with a cognitive intervention is necessary by discussing possible solutions to the barriers for PA (*i.e.*, evacuation urgency, thinking that there may be disease reactivation and the like).

In view also that our inactive patients significantly identified the core of their PA-related fears at the diagnosis of IBD (Table 5, $P < 0.001$) compared with active patients, this suggests that these complementary aspects should be discussed at diagnosis before patients integrate misconceptions into the management of their IBD and PA.

In addition, as exhibited in Table 5 (questions 7, 8), a not insignificant percentage of patients feel uninformed about the IBD-PA relationship by their family physician and gastroenterologist. Therefore, training courses that aim to provide general knowledge about the possibilities of practising PA in patients with chronic digestive diseases should solve this unmet patient need.

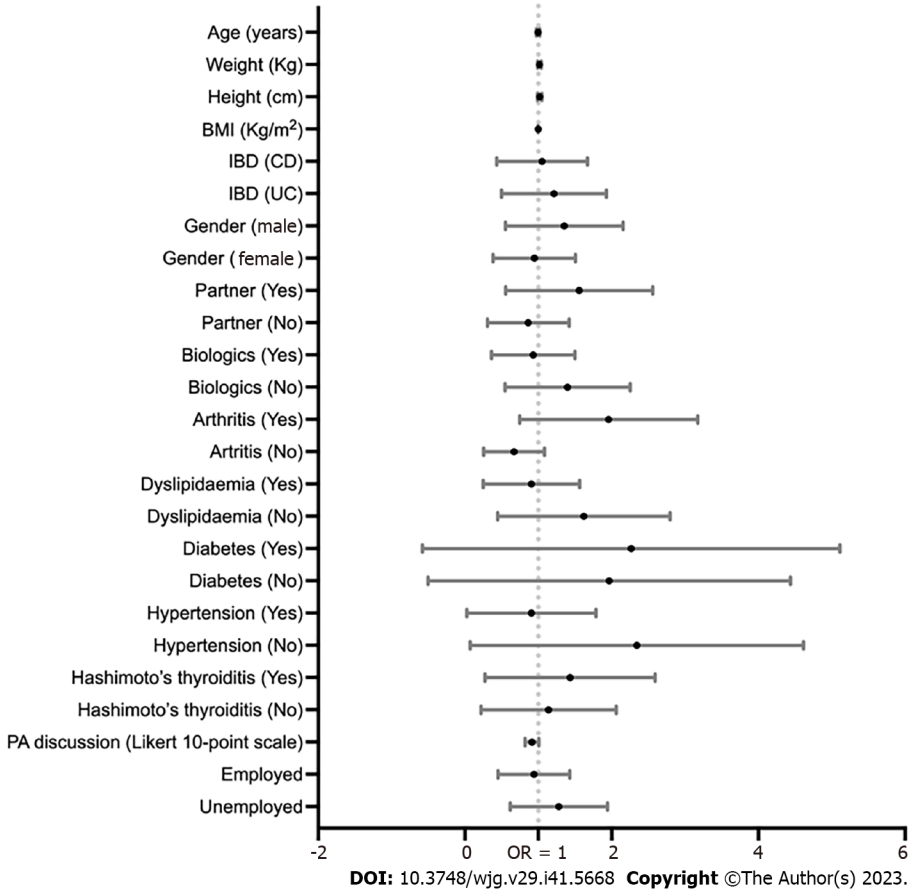


Figure 4 Forest plot showing predictors analysis of physical inactivity analysis among clinical and demographic variables evaluated by binary logistic regression. CD: Crohn's disease; UC: Ulcerative colitis; OR: Odds ratio.

Moreover, regular PA can pose a valuable strategy for reducing inflammatory burden, especially in diseases with inflammatory pathogenesis, such as IBD. Exercise can contribute to the promotion of an anti-inflammatory phenotype in several ways.

In fact, at the level of fatty tissue, it can downregulate several pro-inflammatory cytokines such as IL-1, IL-16 and tumour necrosis factor (TNF), and, in addition, it can promote the M2 cytotype of macrophages (*i.e.*, their anti-inflammatory cytotype) and act against oxidative stress[7]. In the context of muscle tissue, moreover, these actions are, to a large extent, repeated[7] with an increase also in peroxisome proliferator-activated receptor γ co-activator 1α, a molecule that in knockout mice for the same, results in the promotion of IL-6 and TNF[40]. Repeated exercise also appears to induce adaptive changes in the immune system by predisposing to lower neutrophil recruitment[41]. For these reasons, exercise has been repeatedly proposed to counter chronic inflammation[42].

In addition to the above, regular PA can improve vascular endothelial balance by ameliorating oxidative stress and nitric oxide availability[43].

Although set in a research context severely lacking solid evidence already available, this study has several limitations. The data are from a single-centre experience, and future multicentre evidence would be desirable; our subgroup analyses are, by definition, exploratory; therefore, studies of larger sample sizes are desirable to confirm them. In addition, it will be preferable to confirm and strengthen our data even more a multicentre, prospective study design to bring out more differences in population subgroups.

CONCLUSION

IBD southern Italian patients seem physically inactive and may be exposed to all the complications of not practising regular PA. This does not seem totally dependent on disease activity but is affected by patients' beliefs about PA's impact on underlining IBD. Using validated and feasible questionnaires (*e.g.*, IPAQ) could be a strategy to weigh patient-reported PA levels and get an initial idea about which patients have insufficient PA levels.

ARTICLE HIGHLIGHTS

Research background

Patients with inflammatory bowel diseases (IBD) often experience reduced quality of life (QoL) and disability. Regular physical activity (PA) determines QoL. Initial studies have shown that mild PA seems safe in IBD and is not associated with an increased risk of flare-ups.

Research motivation

There are no precise guidelines on what type of PA and the intensity to recommend for patients with IBD. Epidemiological levels of PA in the IBD population are not yet fully known, nor are the barriers that block patients from practising regular PA.

Research objectives

This study aimed to weigh PA levels with standardised instruments in an Italian IBD population to examine PA's relationship with IBD disease activity and identify barriers to PA.

Research methods

This cross-sectional study employed the standardised International Physical Activity Questionnaire (IPAQ) to weigh PA and the patient-reported outcome 2 (PRO-2) to assess IBD disease activity. PA was expressed as multiples of resting metabolic rate (Met) in Met min/wk. This study included only patients with confirmed, excluding patients with severe or hospitalised activity.

Research results

Two hundred nineteen patients were included. Fifty-three per cent were found to be sufficiently active, 42.9% as inactive, and only 4.1% as health-enhancing PA active. Median overall PA levels were 834.5 Met min/wk, just above the threshold for inactivity (*i.e.*, 700 Met min/wk). Ulcerative colitis PRO-2 showed a negative correlation with intense PA activities. Several barriers to PA were identified (*e.g.*, fear of IBD flare-up, fears initiated as early as IBD diagnosis).

Research conclusions

Patients with IBD were found in this setting to be burdened by a significant rate of physical inactivity. Barriers persist on which to act to regain adherence to regular PA. As measured by the PRO-2, disease activity did not drastically affect PA. The IPAQ questionnaire showed excellent feasibility and ease of completion and interpretation.

Research perspectives

Regular PA has multiple benefits (from cardiovascular health to psychological health), and it is necessary to make sure that patients with IBD practice it so that these benefits are not lost. It is appropriate for gastroenterologists to pay more attention to this aspect during medical visits. IPAQ can be a potential tool for recognising and monitoring physically inactive patients.

FOOTNOTES

Author contributions: Gravina AG, Pellegrino R, and Federico A designed the study; all authors participated in the acquisition and interpretation of the data and drafted the initial manuscript; Pellegrino R performed the analysis; all the authors revised the article critically for important intellectual content.

Institutional review board statement: The study was conducted in compliance with the Declaration of Helsinki and received approval from the Ethics Committee of the University of Campania Luigi Vanvitelli (protocol number 7892, 15 March 2023).

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

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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ORIGINAL ARTICLE

Basic Study

First report on establishment and characterization of the extrahepatic cholangiocarcinoma sarcoma cell line CBC2T-2

Ning-Zu Jiang, Ming-Zhen Bai, Chong-Fei Huang, Ze-Long Ma, Ru-Yang Zhong, Wen-Kang Fu, Long Gao, Liang Tian, Ning-Ning Mi, Hai-Dong Ma, Ya-Wen Lu, Zi-Ang Zhang, Jin-Yu Zhao, Hai-Ying Yu, Bao-Ping Zhang, Xian-Zhuo Zhang, Yan-Xian Ren, Chao Zhang, Yong Zhang, Ping Yue, Yan-Yan Lin, Wen-Bo Meng

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Abstract

BACKGROUND

Extrahepatic cholangiocarcinoma sarcoma is extremely rare in clinical practice. These cells consist of both epithelial and mesenchymal cells. Patient-derived cell lines that maintain tumor characteristics are valuable tools for studying the molecular mechanisms associated with carcinosarcoma. However, cholangiocarcinoma sarcoma cell lines are not available in cell banks.

AIM
To establish and characterize a new extrahepatic cholangiocarcinoma sarcoma cell line, namely CBC2T-2.

METHODS
We conducted a short tandem repeat (STR) test to confirm the identity of the CBC2T-2 cell line. Furthermore, we assessed the migratory and invasive properties of the cells and performed clonogenicity assay to evaluate the ability of individual cells to form colonies. The tumorigenic potential of CBC2T-2 cells was tested *in vivo* using non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. The cells were injected subcutaneously and tumor formation was observed. In addition, immunohistochemical analysis was carried out to examine the expression of epithelial marker CK19 and mesenchymal marker vimentin in both CBC2T-2 cells and xenografts. The CBC2T-2 cell line was used to screen the potential therapeutic effects of various clinical agents in patients with cholangiocarcinoma sarcoma. Lastly, whole-exome sequencing was performed to identify genetic alterations and screen for somatic mutations in the CBC2T-2 cell line.

RESULTS
The STR test showed that there was no cross-contamination and the results were identical to those of the original tissue. The cells showed round or oval-shaped epithelioid cells and mesenchymal cells with spindle-shaped or elongated morphology. The cells exhibited a high proliferation ratio with a doubling time of 47.11 h. This cell line has migratory, invasive, and clonogenic abilities. The chromosomes in the CBC2T-2 cells were polyploidy, with numbers ranging from 69 to 79. The subcutaneous tumorigenic assay confirmed the *in vivo* tumorigenic ability of CBC2T-2 cells in NOD/SCID mice. CBC2T-2 cells and xenografts were positive for both the epithelial marker, CK19, and the mesenchymal marker, vimentin. These results suggest that CBC2T-2 cells may have both epithelial and mesenchymal characteristics. The cells were also used to screen clinical agents in patients with cholangiocarcinoma sarcoma, and a combination of paclitaxel and gemcitabine was found to be the most effective treatment option.

CONCLUSION
We established the first human cholangiocarcinoma sarcoma cell line, CBC2T-2, with stable biogenetic traits. This cell line, as a research model, has a high clinical value and would facilitate the understanding of the pathogenesis of cholangiocarcinoma sarcoma.

Key Words: Carcinosarcoma; Drug resistance; Xenograft; Cell line; Establishment

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Core Tip: The study established and characterized the CBC2T-2 cell line as a potential model for studying human extrahepatic cholangiocarcinoma sarcomas. The cells exhibited both epithelial and mesenchymal characteristics and demonstrated high proliferation, migration, invasion, and clonogenic abilities. Chromosomal analysis revealed polyploidy with varying chromosome numbers and *in vivo* tumorigenicity was confirmed in non-obese diabetic/severe combined immunodeficient mice. Immunohistochemistry indicated positive expression for both epithelial marker CK19 and mesenchymal marker vimentin. The cell line was also used to screen clinical agents, and paclitaxel and gemcitabine exhibited optimal effects. Whole-exome sequencing further revealed genetic insights.

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INTRODUCTION

Extrahepatic cholangiocarcinoma sarcomas are extremely rare clinically because they represent a mixture of carcinomas and sarcomas, accounting for < 0.1% of cholangiocarcinoma[1]. Polypoid growth and ossification within the carcinoma are representative features of extrahepatic cholangiocarcinoma sarcoma[2]. It is composed of epithelial and stromal cells, with a sarcomatous component that differentiates from the spindle or pleomorphic cells[3]. Carcinosarcoma is commonly observed in the elderly, and it is mostly diagnosed in the lungs, bladder, pancreas, ovary, esophagus, thyroid gland, and breast and rarely occurs in the liver or biliary system[4,5]. Extrahepatic cholangiocarcinoma sarcoma with chondrogenic differentiation is a poorly differentiated and aggressive cancer with poor prognosis. The effectiveness of radiotherapy and chemotherapy for cholangiocarcinoma sarcoma is limited; hence, complete surgical resection of the tumor is the ultimate

treatment option for cholangiocarcinoma sarcoma[6-8]. However, most patients experience local recurrence even after extensive local excision. Many patients receive treatment for jaundice or abnormal liver function without significant increases in the levels of tumor markers, and their prognosis is worse than that of patients with common bile duct cancer, with a low survival rate of 1 mo to 5 years[9-11]. Therefore, the development of novel therapeutic strategies for cholangiocarcinoma sarcoma is crucial.

Patient-derived cell lines can maintain tumor characteristics and are valuable tools for studying the molecular mechanisms of carcinosarcoma, disease progression, and biological features[12,13]. Owing to the rarity of cholangiocarcinoma sarcoma and its incompletely elucidated oncogenicity, its clinical and therapeutic significance remain uncertain. Basic research models are essential for thorough analysis of the developmental processes of cholangiocarcinoma sarcoma. Therefore, we established CBC2T-2, the first histologically confirmed cell line for cholangiocarcinoma sarcoma and comprehensively described its application in research.

MATERIALS AND METHODS

Patient's background

A 62-year-old female, was admitted to the First Hospital of Lanzhou University in July 2022 with intermittent pain in her right upper abdomen and lower back. The results of physical examination were unremarkable, except for slight pressure pain in the upper right abdomen. Preoperative computed tomography and magnetic resonance imaging revealed the dilatation of the common bile duct and intrahepatic bile duct. This was accompanied by a soft tissue shadow in the upper part of the common bile duct showing arterial phase enhancement, indicative of bile duct cancer (Figures 1A and B; arrow). Radical surgery was performed under general anesthesia without any treatment prior to surgery. Intraoperative frozen section confirms negative margins. However, the postoperative pathological diagnosis revealed cholangiocarcinoma sarcoma of the porta hepatis, comprising 90% adenocarcinoma and 10% chondrosarcoma. The American Joint Committee on Cancer staging system of the tumor was T2aN2Mx (Figures 1C and G; arrow). The tumor markers were within the normal range, with alpha-fetoprotein at 2.1 U/mL, carbohydrate antigen 19-9 (CA19-9) at 9.4 U/mL, and carcinoembryonic antigen (CEA) at 0.7 U/mL (Figure 1G). Hematoxylin and eosin (HE) staining revealed microscopic epithelial heterogeneous hyperplasia with striated, nested, and glandular arrangements; increased cell volume; large deep-stained nuclei with pathological nuclear abnormalities; localized mucus and mucous cartilage; and infiltrative growth of cancer cells (Figures 1D and E; arrow).

This study was approved by the Ethics Committee of the First Hospital of Lanzhou University (LDYYLL-2022-489) and informed consent was obtained from all the patients. This study adhered to the 1964 Declaration of Helsinki and its subsequent amendments and similar ethical standards[14].

Cell culture

The specimens were collected under the guidance of a pathologist to ensure that the diagnosis in the pathology report was unaffected. The tumor specimens were rinsed thrice with phosphate buffer saline (PBS; BI), cut into 1-2 mm^3 pieces using a sterile blade, and then digested with collagenase type IV (0.1 mg/mL; Gibco) for 10 min in a 37 °C incubator. The cell pellet was maintained in DMEM/F-12 (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco). It was cultured at 37 °C under humidified air containing 5% CO_2. For continuous culture, the cells were periodically passaged and frozen in liquid nitrogen at regular intervals. No contamination with other cells or foreign microorganisms was observed during the culture process. No external growth factors or stimulatory cytokines were added during the establishment of the cell lines.

Short tandem repeat analysis for cell line validation

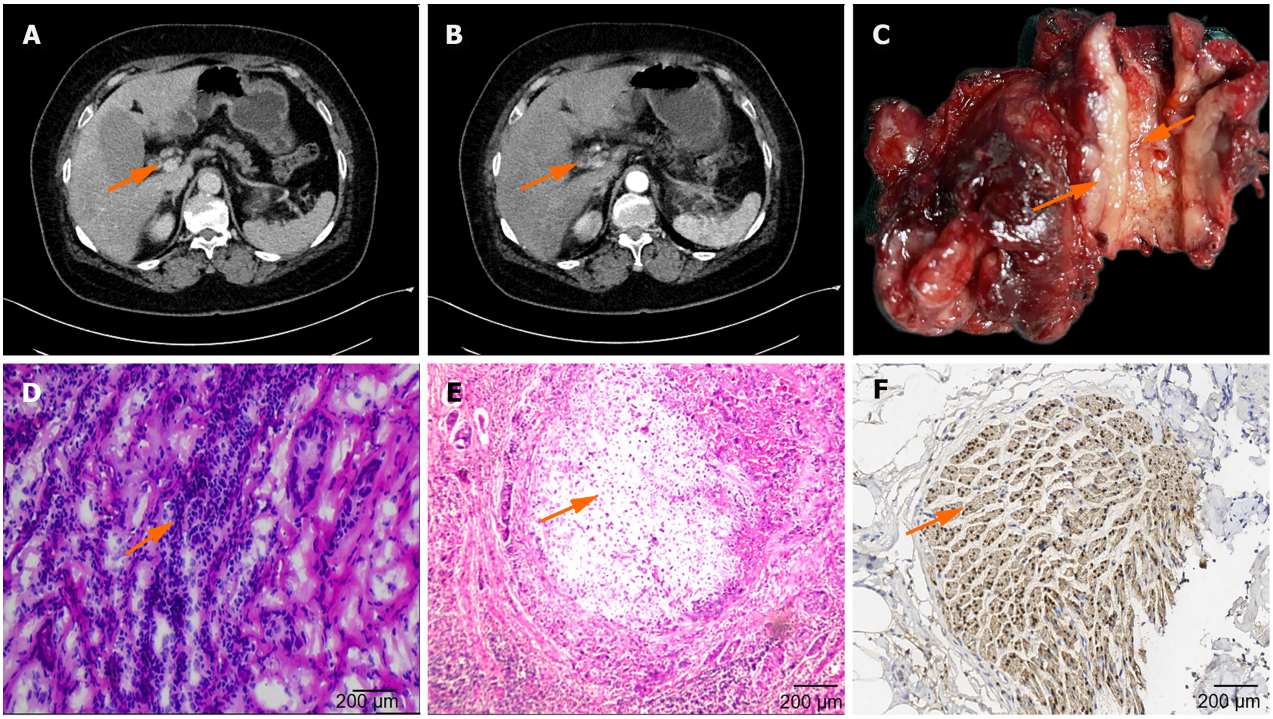
Freshly cultured human cholangiocarcinoma sarcoma cell line CBC2T-2 (p25) and frozen tumor tissues were collected following the manufacturer's instructions. The genomic DNA of the cells was extracted using an Animal Genome Extraction Kit and then subjected to polymerase chain reaction with fluorescently labeled primers at the 5′ end. The resulting products were sequenced and analyzed for sequence repeats of 21 short tandem repeat (STR) loci, including AMEL, D19S433, D5S818, D21S11, D18S51, D6S1043, D3S1358, D13S317, D7S820, D16S539, CSF1PO, Penta D, D2S441, vWA, D8S1179, TPOX, Penta E, TH01, D12S391, D2 S1338, and FGA. The obtained STR profiles were compared with reference STRs from public cell repositories, including the American Type Culture Collection (ATCC), Deutsche Sammlung Mikroorganismenund Zelkulturen (DSMZ), and the CELLOSAURUS cell database.

Chromosome analysis

Cells in the logarithmic growth phase (p25) were incubated with 10 μg/mL of colchicine for 2 h in a cell culture incubator. Chromosomes were prepared using standard methods and their numbers were mostly distributed across the G-dominant band. Representative images of chromosomes were obtained for karyotype analysis. Karyotype interpretation was based on the International System for Human Cytogenetic Nomenclature (1995)[15].

Spheroid formation assay

In total, 1 × 10^5 cells in the logarithmic growth phase (p25) were digested and inoculated into ultra-low-attachment 96-well plates (Corning). Sphere formation was monitored on days 3, 7, 10, and 14 after inoculation to assess the ability of the cells to form spheres.



G

Cell line	Patient age/ethicity	Gender	Current Status (days)	Edmondson -Steiner (grade)	Tumor size (cm)	Prior therapy	Culture date	Serum		
								AFP (IU/mL)	CEA (ng/mL)	CA199 (U/mL)
CBC2T-2	62/Asian	Female	NA	Ⅲ	8×4	None	2022-6-20	2.1	4.0	9.4

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Figure 1 Clinical and pathological profile of CBC2T-2. A and B: Contrast-enhanced computed tomography of the abdomen showing a high-grade occupying lesion of the common bile duct with high level obstruction of the common bile duct (orange arrows); C: Specimen of postoperative carcinosarcoma of the extrahepatic bile duct with thickened duct wall and polyp-like protrusion into the lumen; D and E: Hematoxylin and eosin and immunohistochemistry staining of primary tumor tissue. Scale bars, 200 μm; F: Immunohistochemistry staining of primary tumor tissue. Antibodies against Vimentin, Scale bars, 200 μm; G: Clinicopathological characteristics of patients. AFP: Alpha-fetoprotein; CEA: Carcinoembryonic antigen; CA199: Carbohydrate antigen199.

Cell counting kit-8 cell growth assay

CBC2T-2 cells were inoculated into 96-well plates at a density of 5000 cells per 100 μL. Cell counting kit 8 (CCK-8) (APEx BIO) was added at time points of 0, 24, 48, 72, 96, and 120 h at a ratio of 1:10. The cell growth curve was plotted with time on the horizontal axis and absorbance on the vertical axis. The ploidy doubling time (PDT) software was used to calculate the cell doubling time (http://www.doubling-time.com).

Live cell imaging

CBC2T-2 cells were inoculated into 96-well plates at a density of 4000 cells per 100 μL. The plate was then placed in a Cytation 1 imaging system (Biotek) under a 4 × objective, and the field of view was selected for each well. Images were taken every 2 h for 120 h and processed using the Gen5 Software.

Flow cytometry

Tumor cells (p25) in the logarithmic growth phase were washed with PBS, digested with ethylenediaminetetraacetic acid-free trypsin, and prepared as a single-cell suspension. The CBC2T-2 cell precipitates were washed twice with PBS and the density was adjusted to 1×10^7/mL. The cells were fixed by adding pre-cooled 75% ice-cold ethanol to each tube for 1-2 h. After incubating away from light for 15-30 min, the cells were stained with 0.5 mL of propidium iodide stain [consisting of 0.5 mL staining buffer, 25 μL propidium iodide staining solution, and 10 μL RNaseA (50 ×)] (Bioscience).

Migration and invasion abilities

To assess the migratory ability of the cells, a suspension of 200 μL of cells (1×10^5/mL) was evenly distributed in the upper chamber of a transwell without matrix gel (BD). To evaluate the invasive ability of the cells, a suspension of 200 μL of cells (1×10^5/mL) was evenly distributed in the upper chamber of a transwell with matrix gel (Corning). The lower chamber was filled with medium containing 15% FBS and incubated in a cell culture incubator. After 24 and 48 h of incubation, the contents of the upper lumen were removed. After staining the cells with 0.1% crystal violet solution (Beyotime) for 20 min, they were rinsed with PBS; next, they were observed under an inverted microscope and the images were captured. This procedure was repeated using TFK-1 cells from the extrahepatic bile duct as controls for the CBC2T-2

cells.

Wound healing assay

CBC2T-1 and TFK-1 cells were seeded into 6-well plates. When the cells reached 95% confluence, the cell monolayers were scraped into a cross-shape using a pipette tip and gently washed thrice with PBS. Medium containing 10% FBS was added to continue the culture. The images were captured at 0, 24, and 48 h, and the entire scratch area was measured using the ImageJ software.

Colony formation assay

The cells were digested with 0.25% trypsin, and the cell density was adjusted to 700 cells/well before inoculation into 6-well plates. Colony formation was monitored at 3, 7, 10, and 14 d after inoculation. On day 14, the colonies were fixed with 4% paraformaldehyde, stained with 0.5% crystal violet for 20 min, photographed, and analyzed using ImageJ software.

Histology and immunostaining

Patient and mouse tumor tissues and crawling cells were fixed in formaldehyde solution. Subsequently, the specimens were subjected to baking, decolonization, hydration, antigen retrieval, peroxidase blocking, and goat serum closure. The slides were incubated with antibodies against CK19, Vimentin, S100, Desmin, CD56, CD117, and S100. Drops of DAB liquid were added, followed by reaction for 1-5 min before color development was promptly terminated.

Whole-exome sequencing

Sequencing and data analyses were conducted using the BGI software (Wuhan, China). The exome region DNA was captured using probe capture technology and the target region DNA was sequenced using high-throughput sequencing technology. Genomic DNA was extracted from CBC2T-2 cells and compared with that from normal tissues adjacent to the patient's resected tumor. Library construction and whole-exome capture of genomic DNA were performed using SureSelect Human All Exon V6 (Agilent Clara, Technologies, Santa CA, United States) and the captured DNA library was sequenced on the DNBSEQ platform. This procedure uses FACETS[16] software to detect somatic copy number variation (CNV) in tumor and normal paired samples, the depth distribution of reads to compare copy number variation with the reference genome, and Ensemble VEP[17] to annotate the CNV. We performed InDel detection using the results of the GATK[18] comparison, followed by the annotation of the detected InDels. We used the GATK MuTect2 tool to identify somatic single-nucleotide variant (SNV) loci and the GATK Funcotator tool to annotate these loci.

Tumor driver gene analysis

We compared somatic mutations with known driver genes from databases and the literature and screened for known driver genes in tumor samples. The reference data sources were Integrative OncoGenomics (IntOGen), the Cancer Gene Census (CGC), three highly cited articles, and pan-cancer data[19-22].

Tumorigenesis in mice

To study the *in vivo* tumorigenicity of CBC2T-2 cells, cells at a concentration of 1×10^7/mL were injected into the axillae of three 4-wk-old non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. The mice were housed in a specific pathogen-free laminar flow animal facility and observed for 8 wk. Tumor diameters were measured every 3 d during this period. After 8 wk, the mice were euthanized, the tumors were excised and photographed, and the samples portions were immersed in a 10 % formalin solution for routine processing.

Screening of anticancer drugs

CBC2T-2 cells (p30) at the logarithmic growth phase were washed with PBS and digested with 0.25% trypsin. The concentration of the cell suspension was adjusted to 10000 cells/100 μL. After culturing the cells for 24 h, different concentrations of chemotherapeutic drugs, including gemcitabine, oxaliplatin, cisplatin, paclitaxel, and 5-fluorouracil were added. Following a drug exposure period of 72 h, the viability of each group was measured by incubating the cells with CCK-8 reagent for 2 h.

Statistical analysis

The results are presented as the mean ± standard error of at least three independent experiments. Graphs were created using GraphPad Prism 9.0, and a one-way analysis of variance was used to compare the two groups. Statistical significance was set at $P < 0.05$.

RESULTS

Establishment and identification of the CBC2T-2 cell line

We successfully established the cell line, namely CBC2T-2, from a patient with cholangiocarcinoma sarcoma. The cells were cultured in monolayers for > 13 mo and subjected to > 100 generations. The cell line was established from the original tumor tissue by comparing the 21 STR loci of both the tissue and cell lines using the assay currently

Table 1 Short tandem repeat profile of tumor tissue and cell line

STR loci	CBC2T-2	Tumor tissue
AMEL	X, X	X, X
D19S433	13	13
D5S818	11, 13	11, 13
D21S11	30, 31.2	30, 31.2
D18S51	13, 13	13, 13
D6S1043	12, 18	12, 18
D3S1358	16, 16	16, 16
D13S317	8, 11	8, 11
D7S820	9, 10	9, 10
D16S539	9	9
CSF1PO	10, 12	10, 12
Penta D	9, 11	9, 11
D2S441	11, 12	11, 12
vWA	17, 18	17, 18
D8S1179	14, 15	14, 15
TPOX	10, 11	10, 11
Penta E	12, 14	12, 14
TH01	9	9
D12S391	19, 20	19, 20
D2S1338	19, 22	19, 22
FGA	23, 23.2	23, 23.2

STR: Short tandem repeat.

recommended by the ATCC. The genomic characteristics of the CBC2T-2 cell line and the originating tumor tissue are presented in Table 1. The assay measurements showed that the cholangiocarcinoma sarcoma cell line was of human origin, and its genetic information was comparable with those from three prestigious culture collections (ATCC, DSMZ, and CELLOSAURUS). No sequences matching other cells were found, indicating that the cells were not contaminated. The cells were maintained at the China Center for Type Culture Collection (CCTCC No. C2022273).

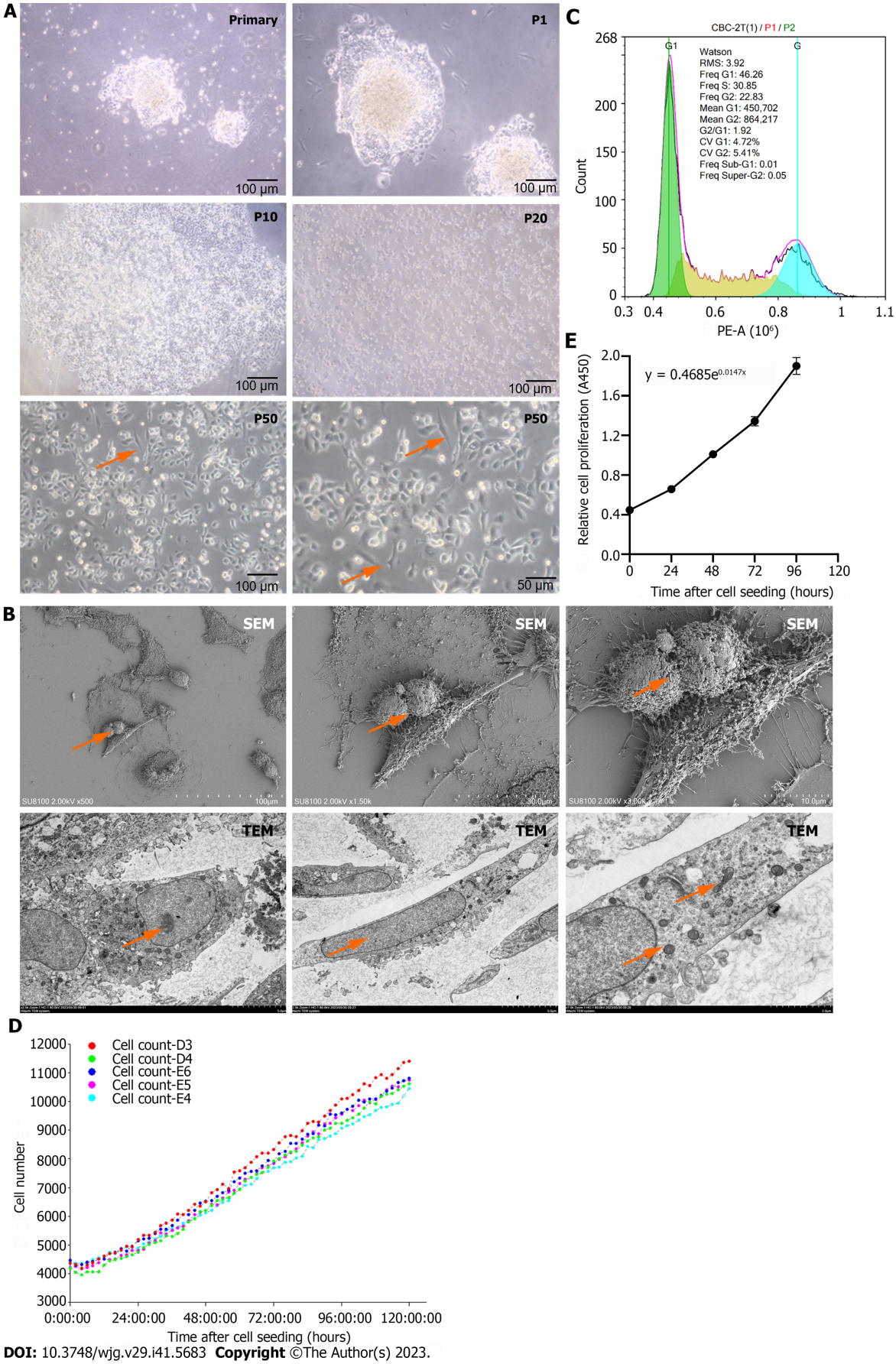
Phenotypic characterization, doubling time, and cell cycle

CBC2T-2 cells (P1, P10, P20, and P50) were observed microscopically as they actively proliferate and grow in a monolayer against the wall. Two types of cell morphology were observed: Typical polygonal cholangiocarcinoma cell morphology and long spindle-shaped chondrosarcoma cell morphology (Figure 2A). Transmission electron microscopy revealed the presence of numerous mitochondria, rough endoplasmic reticulum, ribosomes, and irregular nuclei with deep indentations in the nuclear membrane. Scanning electron microscopy demonstrated microvilli-like protrusions on the cell surface as well as tight junctions and intercellular bridges (Figure 2B).

Flow cytometry was used to detect cell cycle progression in CBC2T-2 cells. Diploid cells were observed in G0/G1 phase, tetraploid cells in G2/M phase, and hyperdiploid cells in S phase (Figure 2C). The PDT of CBC2T-2 cells was calculated to be approximately 47.11 h using both live cell imaging and CCK-8 with the PDT software (PDT) (Figures 2D and E). A short PDT indicated active cell proliferation.

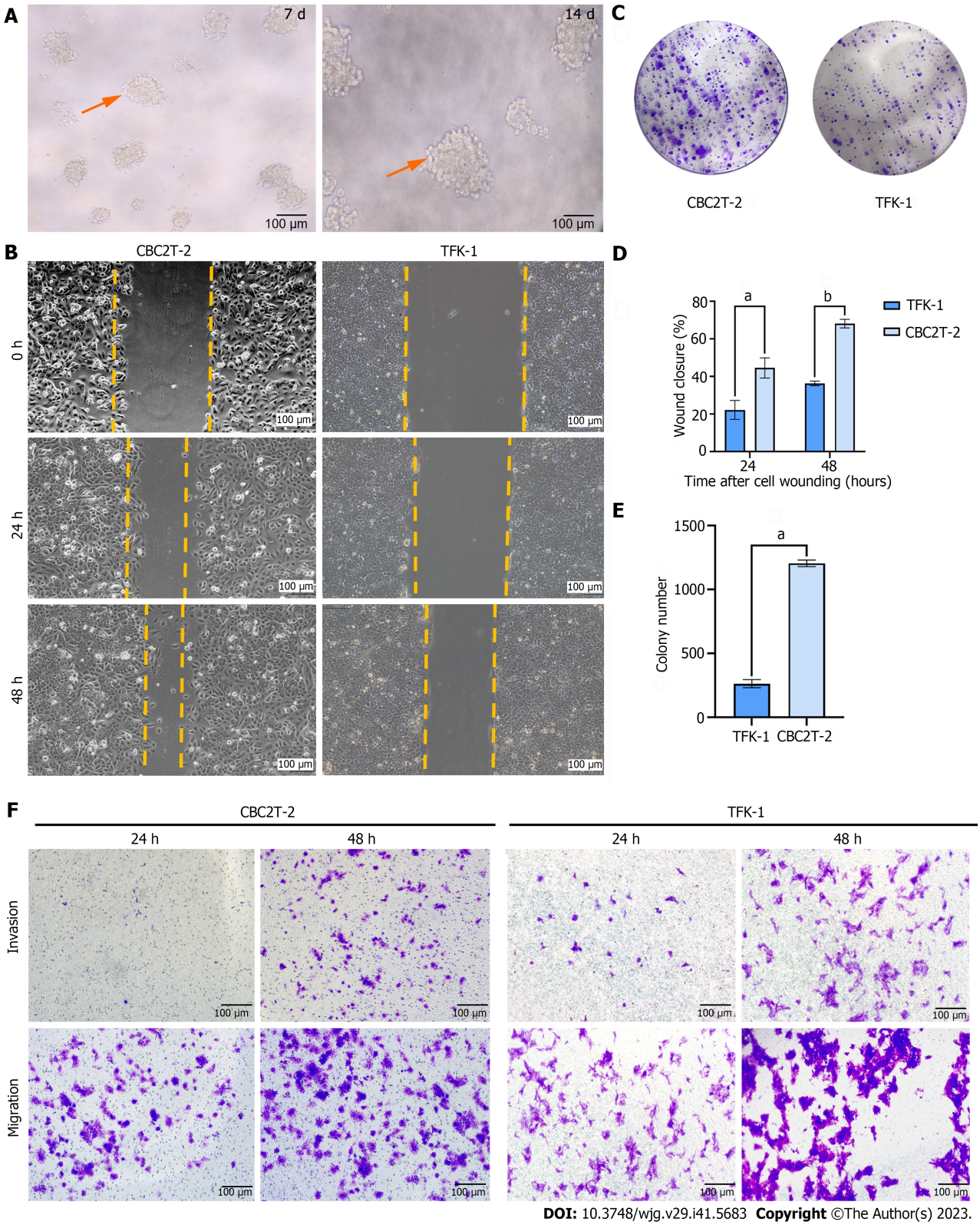
Sphere formation, wound healing assay, and migration, invasion, and clonogenic abilities

The novel cell line CBC2T-2 was inoculated into an ultra-low-attachment 96-well plate and observed for 14 d. The cells demonstrated their ability to form spheroids (Figure 3A). The results of the wound healing assay showed that CBC2T-2 cells showed higher levels of wound repair at both 24 and 48 h (Figures 3B and C). Furthermore, compared with TFK-1 cells, CBC2T-2 cells exhibited greater migratory and invasive capacities (Figure 3D). Moreover, CBC2T-2 cells exhibited significantly stronger clonogenic ability than TFK-1 cells (Figures 3E and F). These findings indicate that the cell line CBC2T-2 has a high capacity for healing, invasion, migration, and sphere formation, making it a valuable tool for studying the pathogenesis of cholangiocarcinoma sarcoma.



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Figure 2 Morphological features, doubling time and cell cycle of CBC2T-1 cells. A: Bright field morphology of CBC2T-2 cells in the primary,1st, 10th, 20th and 50th passages (scale bars, 100 μm); B: SEM and TEM electron microscopy were used to observe the cellular ultrastructure (scale bars, 100, 30, 10 μm); C: Flow cytometry of CBC2T-2 cells; D and E: Cumulative growth curve of CBC2T-2 cells.

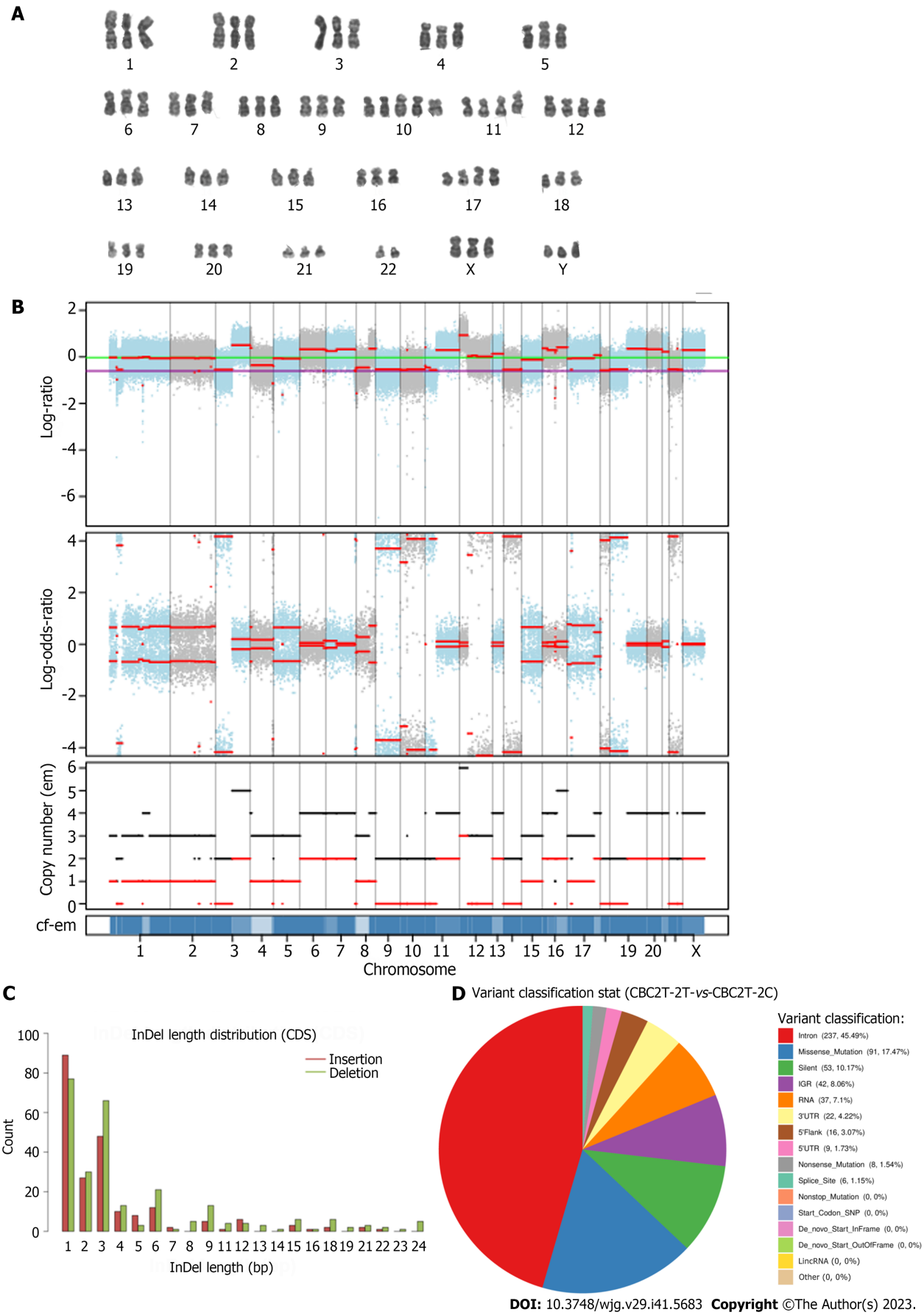


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Figure 3 Characterization of CBC2T-2 cell behavior. A: Representative images of the ability of CBC2T-2 cells to form spheres at days 7 and 14; B and C: Wound healing assay of CBC2T-2 and TFK-1 cells after 24 and 48 h; D: The migratory and invasive abilities of CBC2T-1 and TFK-1 cells were detected by transwell assay; E and F: Representative images showing the colony formation of CBC2T-1 and TFK-1 cells. Scale bars, 100 μm. [a]*P* < 0.01; [b]*P* < 0.001.

Cytogenetic analysis and genomic characteristics

Karyotype analysis of representative single cells from the CBC2T-2 cell line revealed abnormalities in both chromosome number and structure, with polyploidy chromosomes ranging from 69 to 79 and structural aberrations, including gain, deletion, and translocation (Figure 4A). When the value of the black line exceeds 2, it indicates an increase in the copy number, whereas a value below 2 suggests a decrease. Copy number alterations occurred throughout almost the entire chromosomal section of the cells and tumor tissues (Figure 4B). Single-nucleotide polymorphism analysis showed that the



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Figure 4 Cytogenetic analysis and genomic characteristics. A: Chromosome karyotype analysis shows that the cells are triploid and there is variation in the number of structures; B: Cells and tumor tissues have copy number increases and decreases in almost the entire chromosome section; C: Histogram of the distribution of somatic cell InDel annotation results; D: Somatic somatic single-nucleotide variant annotation result distribution map.

Table 2 Comparison of somatic mutant genes detected with the Cancer Gene Census database

Gene name	Chrom	Pos	Ref	Alt	CGC-cancers
ALK	Chr2	29221210	G	T	Neuroblastoma
BARD1	Chr2	214809500	G	A	Ovariancancer; breastcancer; endometrioid cancer
KDR	Chr4	55106807	T	A	Melanoma
FAT1	Chr4	186621601	T	C	Pancreatic
WRN	Chr8	31167138	T	C	Osteosarcoma; meningioma; other tumour types
HNF1A	Chr12	120999418	T	C	Hepatic adenoma; hepatocellular carcinoma
BRCA1	Chr17	43071077	T	C	Breast; ovarian
BRCA1	Chr17	43092418	T	C	Breast; ovarian
AXIN2	Chr17	65535650	G	A	Colorectal carcinoma
SETBP1	Chr18	44876688	G	A	Neuroepithelial tumours

Chrom: Chromosome; Pos: Position on chromosome; Ref: Reference base on the genome; Alt: Mutated base; CGC-Cancers: Tumour types with driver genes included in the Cancer Gene Census database.

cell line CBC2T-2 had extensive insertions and deletions in almost the entire chromosomal fraction (Figure 4C). Somatic SNVs are single nucleotide variants of somatic mutations. On average, 521 SNVs were found in all samples, with an average of 91 missense mutations, eight nonsense mutations, and zero SNV invalidating the termination codon (Figure 4D).

A comparison of the detected mutated genes with the CGC database was used to screen for possible cancer susceptibility genes (Table 2). The major tumor susceptibility genes in CBC2T-2 cells included BALK, BARD1, KDR, FAT1, WRN, HNF1A, BRCA1, AXIN2, and SETBP1. We compared somatic mutations in CBC2T-2 with known driver genes from databases and the literature and identified known driver genes (Table 3). TP53 and ARID1A are the most frequently reported genetic alterations in extrahepatic cholangiocarcinoma[23].

Subcutaneous tumorigenesis assay and immunophenotyping

Xenograft tumor formation assays were performed and tumor growth was monitored weekly to confirm the tumorigenic ability of CBC2T-2 cells in NOD/SCID mice. Within 3 wk, tumors developed in all the three mice, indicating that the established cell lines exhibited good tumorigenicity and could be used to establish an *in vivo* model (Figures 5A-C).

After 8 wk, the mice were euthanized, and their tumors were removed for measurement and photography. HE and immunohistochemical staining were performed on the tumor xenografts. We observed tumors in mouse tumor grafts, CBC2T-2 (P30) cells, and the patient's primary tumor tissue using HE staining. The histological consistency was confirmed by the staining procedure (Figure 5D, first row). In addition, CK19, S-100, Vimentin, Desmin, CD117, and CD68 were positively expressed. These findings demonstrated that the primary tumor tissue had both an epithelial component (carcinoma) and a mesenchymal component (chondrosarcoma). Furthermore, *in vivo* and *in vitro* experiments were conducted to demonstrate histological concordance through immunohistochemistry (Figure 5D).

Sensitivity to anticancer drugs

The sensitivity of CBC2T-2 cells to the first-line anticancer drugs used for the treatment of cholangiocarcinoma was assessed. Among the chemotherapeutic agents tested, oxaliplatin (IC_{50} = 77.51 μM), paclitaxel (IC_{50} = 0.002 μM), 5-fluorouracil (IC_{50} = 7.516 μM), cisplatin (IC_{50} = 19.24 μM), and gemcitabine (IC_{50} = 0.009 μM) were evaluated. Our results demonstrated that among the five anticancer drugs, paclitaxel was the most sensitive, followed by gemcitabine. These findings provide clinical guidelines for the treatment of patients with cholangiocarcinoma sarcoma, suggesting a preference for combined paclitaxel and gemcitabine as the most effective treatment option (Figure 6).

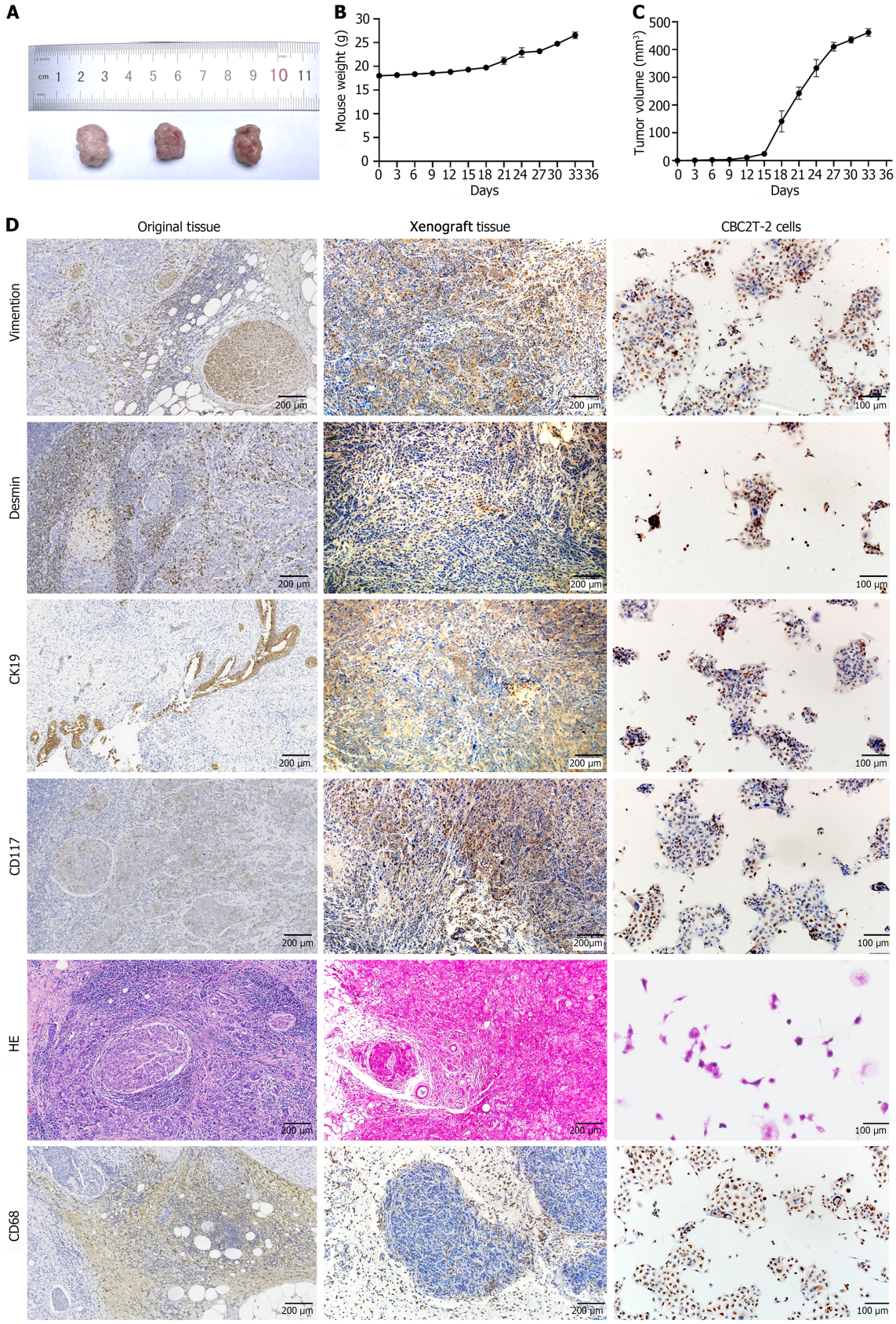
DISCUSSION

Bile duct cancer is a highly aggressive malignant tumor that originates in the epithelial cells of the bile ducts. Its incidence and mortality rates are increasing worldwide and account for approximately 2% of all cancers, with autopsy detection rates ranging from 0.01% to 0.046%[24]. There are a few bile duct tumors with specific histological manifestations, among which carcinosarcomas are observed in the liver, gallbladder, pancreas, and jugular abdomen[25,26]. The pathological features of carcinosarcomas include tumor tissues with both epithelial and mesenchymal components[27,28]. The sarcomatous component typically comprises undifferentiated spindle cells and a variety of heterogeneous elements, including cartilage, bone, smooth muscle, and rhabdomyosarcoma cells[29]. The carcinoma component usually consists of adenocarcinoma and, rarely, squamous, small cell, and undifferentiated carcinomas. Moreover, the tissues are well

Table 3 Results of screening for known driver genes

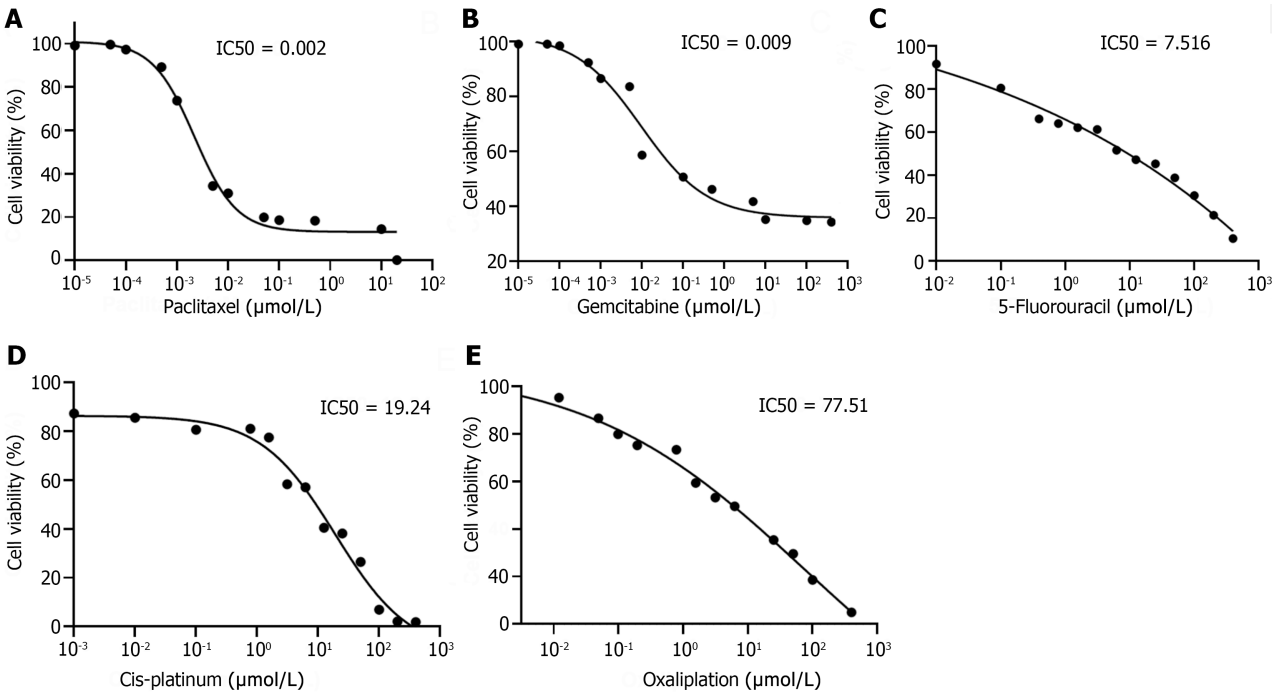
Gene name	Chrom	Pos	Ref	Alt
ARID1A	Chr1	26780184	-	A
SFPQ	Chr1	35192164	G	C
DNMT3A	Chr2	25244384	G	A
CAD	Chr2	27237546	G	A
FBXO11	Chr2	47839654	C	T
LRP1B	Chr2	140683563	C	T
NEB	Chr2	151529162	G	A
NEB	Chr2	151540532	G	A
HOXD13	Chr2	176093701	C	T
MECOM	Chr3	169112718	C	A
MECOM	Chr3	169112734	C	G
ETV5	Chr3	186081304	G	C
NSD2	Chr4	1955726	C	T
NSD1	Chr5	177135451	G	C
AKAP9	Chr7	92097135	G	A
KMT2C	Chr7	152182489	G	A
BRD3	Chr9	134040128	C	T
FAS	Chr10	89014231	G	C
KIF20B	Chr10	89738070	C	G
NUP98	Chr11	3683422	G	T
NUP98	Chr11	3683472	C	T
TNKS1BP1	Chr11	57317951	T	C
KMT2D	Chr12	49040044	G	A
NAV3	Chr12	78159123	C	A
BTG1	Chr12	92145413	G	C
SRCAP	Chr16	30698165	G	A
CNOT1	Chr16	58538927	G	T
CNOT1	Chr16	58576526	C	T
TP53	Chr17	7661876	C	T
TP53	Chr17	7675217	T	C
PRKACA	Chr19	14092743	G	C
ASXL1	Chr20	32359040	C	G
CLTCL1	Chr22	19210202	G	A
MYH9	Chr22	36321935	G	C
DMD	ChrX	33077488	C	T
KDM5C	ChrX	53211367	C	T

Chrom: Chromosome; Pos: Position on chromosome; Ref: Reference base on the genome; Alt: Mutated base.



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Figure 5 Verification the tumorigenic ability of CBC2T-2 cells in non-obese diabetic/severe combined immunodeficient mice. A: Tumor formation in non-obese diabetic/severe combined immunodeficient mice; B: Body weight gain curves in xenograft mice; C: Tumor size over time in three xenograft mice; D: Hematoxylin and eosin staining and immunohistochemical (CK19, CD68, CD117, Vmention, Desmin) results of primary tumors, CBC2T-2 xenografts and CBC2T-2. Scale bars, 50 and 100 μm. HE: Hematoxylin and eosin.



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Figure 6 Sensitivity of CBC2T-2 cell line to anticancer drugs. A-E: Sensitivity of CBC2T-2 cells to paclitaxel, gemcitabine, 5-fluorouracll, cisplatin, and oxaliplatin.

defined and not transformed[30]. Recently, a new hypothesis of epithelial-mesenchymal transition (EMT) suggested that during EMT, cancer cells lose their epithelial properties and intercellular adhesion, transform into mesenchymal cells, and acquire the ability to migrate[31,32]. In contrast, cholangiocarcinoma sarcoma with chondrocytes is a very rare malignancy that has rarely been reported in domestic and international literature[33].

The patient-derived cell lines were consistent with the principles of precision medicine and translational research[34]. Although cell lines derived from rare tumors have significant utility in research, they are difficult to obtain from public cell banks. The development and evaluation of new therapeutic approaches for cholangiocarcinoma sarcoma are limited by the lack of available models. A tumor model is necessary for gaining a deeper understanding and developing effective solutions to overcome the poor prognosis of patients diagnosed with this disease[35]. Herein, we report the successful establishment of a new cell line, CBC2T-2, derived from a patient with cholangiocarcinoma sarcoma, along with detailed clinical and pathological data from the donor patient. This newly established cell line is a breakthrough in the establishment of cholangiocarcinoma sarcoma cell lines and fills a gap in the sarcoma cell lines. This cell line may provide a valuable tool for studying the mechanisms of cholangiocarcinoma progression and developing new therapeutic approaches.

We confirmed that CBC2T-2 cells can proliferate, form spheroids and clones, and migrate for invasion. The short ploidy time and high migratory aggressiveness observed *in vitro* may have contributed to the poor clinical outcomes of cholangiocarcinoma sarcoma. It is biologically stable in culture and capable of undergoing stable passages, which now exceed 100 generations. To determine the tumorigenic potential of CBC2T-2 cells, they were xenografted into NOD/SCID mice. The resulting solid tumors were histologically identical to the original surgical specimens, indicating that CBC2T-2 may serve as a reliable tool for preclinical *in vivo* studies.

The cell line CBC2T-2 (P30) and the primary tumor tissue of the patient were subjected to immunohistochemistry and immunocytochemistry to verify histological consistency. Sarcoma cells are locally positive for keratin (CK19), an epithelial marker[36]. The sarcoma component markers, Vimentin and Desmin, and the mesenchymal cartilage differentiation marker, S-100, are positively expressed[37-41]. This confirmed that the tumor was composed of adenocarcinoma and sarcomatous components with chondroid differentiation[42]. The sarcomatous component was mixed with and was adjacent to the carcinomatous component. Based on the histological findings, it is possible that the sarcomatous component resulted from an invasive carcinomatous component[43]. However, determining the histogenesis of this tumor remains challenging, similarly to the histogenesis of carcinosarcomas in other organs[43].

We performed whole-exome sequencing (WES) on CBC2T-2 cells and normal tissues adjacent to primary tumors to screen for somatic mutations. In this study, we identified the cancer susceptibility genes associated with cholangiocarcinoma, including BALK, BARD1, KDR, FAT1, WRN, HNF1A, BRCA1, AXIN2, and SETBP1. Additionally, we identified the tumor driver genes, TP53 and ARID1A, in the CBC2T-2 cell line, which are common driver genes for extrahepatic cholangiocarcinoma and play important roles in the CBC2T-2 cell line. The identification of the most commonly mutated genes associated with cholangiocarcinoma using WES in the CBC2T-2 cell line makes the cells a potential model for studying the pathogenesis of this disease.

We characterized the response of CBC2T-2 cells to treatment with anticancer drugs. Furthermore, we calculated the semi-inhibitory concentration of the drugs on CBC2T-2 cells. Among the tested compounds, paclitaxel exhibited the lowest semi-inhibitory concentration, followed by gemcitabine. Although other anticancer drugs exhibited significant antiproliferative effects, their semi-inhibitory concentrations were lower than those of paclitaxel and gemcitabine. This drug sensitivity analysis can provide clinical treatment information and guide the clinical use of paclitaxel in combination with gemcitabine.

CONCLUSION

We established the first human cholangiocarcinoma sarcoma cell line, CBC2T-2, with stable biogenetic traits. This cell line, as a research model, has a high clinical value and would facilitate the understanding of the pathogenesis of cholangiocarcinoma sarcoma.

ARTICLE HIGHLIGHTS

Research background

Cholangiocarcinoma sarcoma is a rare tumor composed of epithelial and mesenchymal cells, and the sarcomatous component in carcinosarcoma is differentiated from spindle cells or pleomorphic cells.

Research motivation

There is no extrahepatic cholangiocarcinoma sarcoma cell line.

Research objectives

To establish and characterize a new extrahepatic cholangiocarcinoma sarcoma cell line.

Research methods

Establish cell lines through primary culture and subculture, and identify their biological characteristics.

Research results

In this study, we successfully established and characterized of a extrahepatic cholangiocarcinoma sarcoma cell line CBC2T-2, from the primary tumor of a patient with cholangiocarcinoma sarcoma.

Research conclusions

To date, first report of the extrahepatic cholangiocarcinoma sarcoma cell line CBC2T-2.

Research perspectives

The establishment of extrahepatic cholangiocarcinoma sarcoma cell line provides suitable experimental models for further study of cholangiocarcinoma sarcoma.

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FOOTNOTES

Author contributions: Jiang NZ and Ren YX interpreted the study design; Bai MZ and Gao L supervised our study; Yue P, Lin YY, and Meng WB obtained the research fund; Huang CF, Ma ZL, and Mi NN screened the publications, performed statistics, and drafted the manuscript; Ma HD and Lu YW helped perform statistics; Fu WK, Tian L, Zhao JY, Zhang BP, Zhang XZ, Zhang C, and Zhang Y revised the manuscript; and all authors contributed to the article and approved the submitted version.

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Institutional review board statement: This study was approved by the Ethics Committee of the First Hospital of Lanzhou University (LDYYLL-2022-489) and informed consent was obtained from all the patients. This study adhered to the 1964 Declaration of Helsinki and its subsequent amendments and similar ethical standards.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the

Institutional Animal Care and Use Committee of the First Hospital of Lanzhou University.

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

Data sharing statement: The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

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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P-Editor: Cai YX

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REVIEW

Role of exosomes in metastasis and therapeutic resistance in esophageal cancer

Xing-Yu Ning, Jin-Hu Ma, Wei He, Jun-Ting Ma

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Abstract

Esophageal cancer (EC) has a high incidence and mortality rate and is emerging as one of the most common health problems globally. Owing to the lack of sensitive detection methods, uncontrollable rapid metastasis, and pervasive treatment resistance, EC is often diagnosed in advanced stages and is susceptible to local recurrence. Exosomes are important components of intercellular communication and the exosome-mediated crosstalk between the cancer and surrounding cells within the tumor microenvironment plays a crucial role in the metastasis, progression, and therapeutic resistance of EC. Considering the critical role of exosomes in tumor pathogenesis, this review focused on elucidating the impact of exosomes on EC metastasis and therapeutic resistance. Here, we summarized the relevant signaling pathways involved in these processes. In addition, we discussed the potential clinical applications of exosomes for the early diagnosis, prognosis, and treatment of EC.

Key Words: Exosomes; Esophageal cancer; Tumor microenvironment; Metastasis; Signaling pathway; Therapeutic resistance

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Core Tip: Esophageal cancer (EC) is a highly malignant type of cancer, and its early diagnosis and effective treatment are lacking. We highlighted the following: (1) Exosomes are involved in altering the number or function of cells in the tumor microenvironment of EC; (2) all steps of EC metastasis and various therapeutic resistance are closely related to exosomes; and (3) exosomes can serve as reliable biomarkers and effective treatment tools for EC. Finally, we presented the urgent challenges and described the future research directions for exosome application in the diagnosis and treatment of EC.

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INTRODUCTION

Esophageal cancer (EC) is a common malignancy of the digestive system, ranking seventh in incidence and sixth in mortality worldwide. According to the GLOBOCAN Project, approximately 604000 new cases of EC were reported, and more than 544000 patients died worldwide in 2020[1]. EC has two main histological subtypes, esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). ESCC is the primary subtype of EC found in East and Central Asia, whereas EAC is prevalent in Western Europe and North America. Early EC is difficult to diagnose because of its insidious onset, as most patients are diagnosed at stage 4 or an advanced stage. Surgery and systematic drug therapy play an important role in preventing further spread of cancer cells. A distant metastasis or therapeutic resistance also occurs in approximately half of patients after treatment, and the 5-year survival rate for patients is less than 25%. Therefore, rapid metastasis and therapeutic resistance have emerged as two major issues that need to be addressed in EC.

Extracellular vesicles (EVs) were first described by Chargaff and West as "procoagulant platelet-derived particles" in 1946[2]. Subsequently, Wolf distinguished minute particulate materials (platelet dust) from intact platelets[3]. With in-depth investigation, the biology of EVs has gradually been revealed and their functions have been explored[4-7]. Research on EVs has increased markedly over the past decade, with exosomes being the most widely discussed topic[8]. According to the canonical classification scheme, EVs can be divided into exosomes (< 150 nm in diameter) and microvesicles (≤ 1000 nm in diameter) based on their size and biogenesis route[9]. With a diameter of 30-150 nm, exosomes carry abundant cargo, including selected RNAs [miRNAs, regulatory mRNAs, and long noncoding RNAs (lncRNAs)], specific DNA sequences, proteins, lipids, glycoconjugates, and metabolites[10,11]. Exosomes secreted by all cell types, such as fibroblasts, tumor cells, and various immune cells, can be found in blood and other biological fluids, including urine[12], saliva[13], cerebral spinal fluid[14], breast milk[15] amniotic fluid[16], and gastric acid[17]. Multiple studies have revealed that exosomes play an important role in processes related to cancer progression, including carcinoma cell proliferation[18], apoptosis[19], invasion[20], epithelial-mesenchymal transition (EMT)[21], angiogenesis [22], immunosuppression[23], and tumor implantation[24] (Table 1).

The aim of this review was to provide a brief introduction to the underlying mechanisms through which exosomes affect oncogenesis, metastasis, and resistance to different therapies in EC. Here, we summarized the applications of exosomes as diagnostic and/or prognostic biomarkers and therapeutic tools to enhance the treatment efficacy of EC.

BIOGENESIS AND CHARACTERIZATION OF EXOSOMES

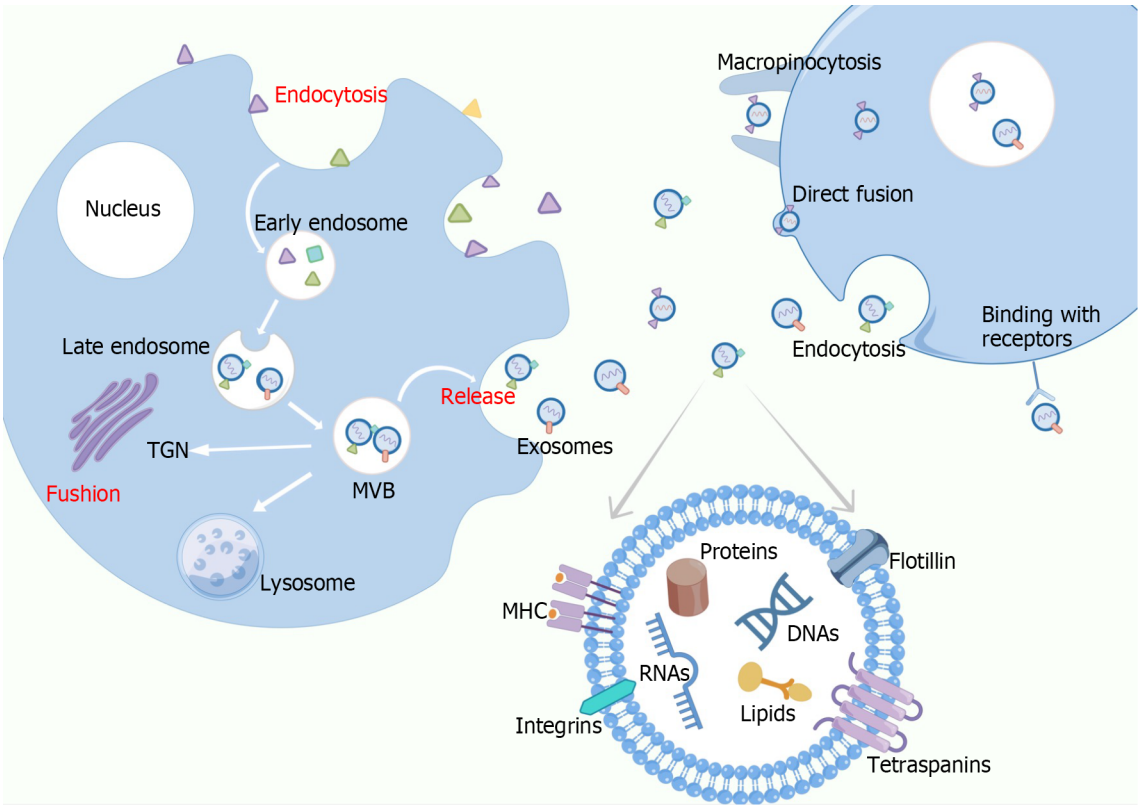
Content of exosomes

Exosomes are phospholipid bilayer-encapsulated nanosized vesicles formed *via* the endocytic pathway[25]. There are multiple ways in which exosomes are transported from donors to recipient cells (Figure 1). Depending on the cell source, physiological conditions, and sorting mechanisms, exosomal contents can be divided into two main categories: those related to the exosome formation mechanism and those related to exosome functions[26]. The cargos of exosomes play important roles in cancer metastasis, modulating stromal reactions, inducing angiogenesis, immune response, and other biological processes through paracrine, autocrine, and endocrine effects. The fact that exosomes have substantial effects on the abovementioned processes explains their essential role in cell-to-tissue communication as well as intercellular communication, which robustly alters the function of cells and promotes the development of cancer and drug resistance [27,28]. Extensive research has proposed that proteins in exosomes not only participate in the biogenesis of exosomes, but also mediate signal transduction and immunogenic regulators, including the transferrin receptor, epidermal growth factor receptor (EGFR), major histocompatibility complex-I (MHC-I), and MHC-II[29]. Protein studies have revealed that exosomes have their own conserved components that may serve as biomarkers for other exosomes[30]. The main RNAs carried by exosomes, including miRNAs, mRNA, rRNAs, tRNA, lncRNAs, and circular RNAs, have been revealed to play a crucial role in tumorigenesis by modulating gene expression and regulating inter-organ communication during disease progression[31-34]. Another type of nucleic acid, DNA, is an exosomal cargo. Jeppesen *et al*[35] observed the presence of a large fraction of cell-free DNA in plasma exosomes during the qualitative analysis of plasma exosomes. Lipids are abundant in exosomes, and their expression level and status vary with exosome type, suggesting that lipids are important

Table 1 Typical studies on tumor-associated exosomes that affect esophageal cancer progression

Molecules in exosomes	Biological process	Targets	Ref.
lncRNA ZFAS1	Regulate proliferation, invasion, migration, and apoptosis	MicroRNA-124, STAT3	[2]
miR-103a-2-5p	Promote proliferation and migration of ESCC cells	-	[3]
miR-93-5p	Promote the proliferation of esophageal cancer cells	PTEN, p21, CCND1	[4]
miR-200a	Promote the proliferation, migration, and invasion of esophageal cells and inhibits apoptosis	KEAP1, NRF2	[5]
miR-19b-3p	Inhibit apoptosis, promote cell migration and invasion	PTEN	[6]
LINC01410	Promote metastasis and epithelial-mesenchymal transition	miR-122-5p, PKM2	[7]
lncRNA FAM225A	Accelerate progression and angiogenesis	miR-206, NETO2, FOXP1	[8]
miRNA-21-5p	Promote angiogenesis and malignant progression	-	[9]
miR-154-5p	Attenuate progression and angiogenesis	KIF14	[10]
miR-301a-3p	Promote angiogenesis	PTEN	[11]
uc.189	Promote proliferation and lymph angiogenesis of human lymphatic endothelial cells and thus facilitate lymph node metastasis	EPHA2	[12]

ESCC: Esophageal squamous cell carcinoma.



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Figure 1 Exosome biogenesis, uptake, and contents. Briefly, the biogenesis of exosomes involves the inward invagination of the cytoplasmic membrane, the transition from early endosomes to late endosomes, the maturation of late endosomes into multivesicular bodies, the generation of ILVs, and the release of ILVs into the extracellular environment to become exosomes. Exosomes are structured with proteins, lipids, and nucleic acids. Recipient cells mainly uptake exosomes through the manner of endocytosis, direct fusion, macropinocytosis and binding with receptors. MHC: Major histocompatibility complex; TGN: Trans-Golgi network; MVB: Multivesicular body.

tools that can be used to improve cancer diagnosis. Studies on prostate cancer have revealed that urinary exosomes in patients with cancer contain lipids, such as phosphatidylserine, lactosylceramide, and cholesterol, which are notably different from those in healthy controls[36].

Isolation and identification of exosomes

As exosomes are more representative of their originating cells, are more abundant, and have higher biological stability in body fluids than other cellular factors, developing accurate methods for exosome isolation is critical[37]. Current separation methods for exosomes have limitations such as low reproducibility, low accuracy, complicated extraction procedures, low exosome yield, extensive duration, and high cost. The small size and intrinsic heterogeneity of exosomes limit their applications[38]. Although ultracentrifugation is usually applied as the standard method and is the most commonly used protocol for exosome isolation, it requires expensive machinery and considerable time. Nonetheless, purity requirements are met with this technology, albeit at the expense of recovery.

Ultrafiltration operates based on the specific sizes and molecular weights of membranes, obtaining exosomes in less time and with lower equipment and reagent costs; however, its purity is lower than that of ultracentrifugation[39-41]. Size-exclusion chromatography has proven to outperform other technologies in the following areas: purity, efficiency, integrity, and retention of major characteristics of exosomes[42,43]. However, size-exclusion chromatography involves high equipment costs and additional methods for exosome extraction[44]. As mentioned above, each method for isolating exosomes has its advantages and disadvantages, and the choice must be made according to the specific situation. Further efforts are warranted to create a high-purity, high-utilization, and low-cost method of exosome isolation that can be widely used in the future.

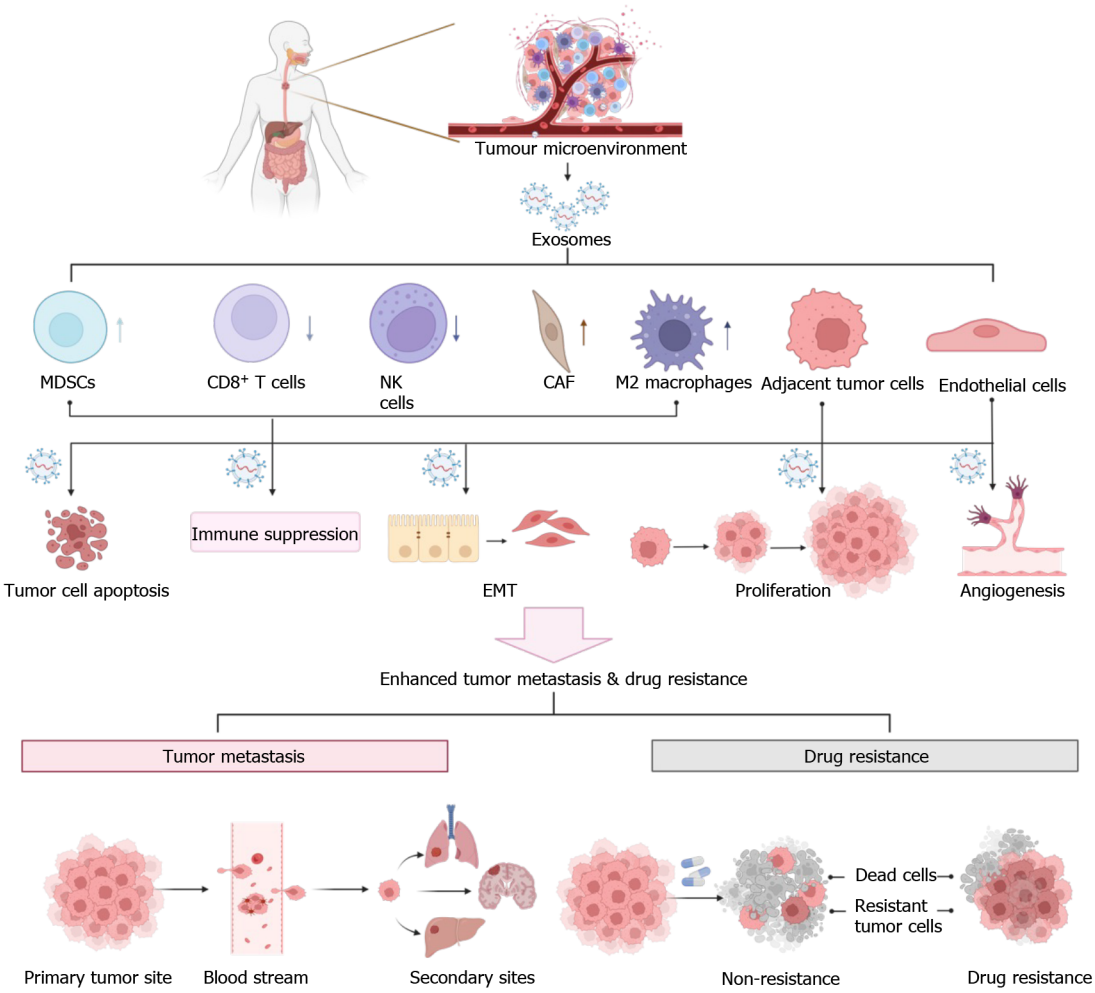
CELLS IN THE TUMOR MICROENVIRONMENT ASSOCIATED WITH METASTASIS AND THERAPEUTIC RESISTANCE OF EC

The tumor microenvironment (TME), comprising malignant cells, peripheral blood vasculature, noncancer host cells, and noncellular components, plays a central role in tumor invasion and metastasis[45,46]. According to the "seed and soil" theory, metastasized tumor cells invade tissue and modulate it to create a suitable "soil" called the TME, which is amenable to the ectopic growth and survival of metastasized cancer cells[47]. In addition to the therapeutic resistance caused by tumor cell changes, emerging evidence has revealed the role of TME in therapeutic resistance[48]. Nontumor cell components in the TME can also drive tumor cell resistance by secreting cytokines, chemokines, growth factors, and exosomes[49,50]. Extracellular matrix (ECM) modeling can influence drug delivery, promote immune escape, and mediate signal transduction, consequently diminishing the therapeutic effects of antitumor agents[51]. The acidic and hypoxic status of the TME can lead to resistance to anticancer treatments[52]. We focused on how cells in the TME influence EC development and the role of exosomes in this process (Figure 2).

Endothelial cells play a vital role in angiogenesis, thereby initiating tumors and facilitating distant metastasis. EC angiogenesis involves growth factors, cytokines, and ECM, among which vascular endothelial growth factor acts as a key factor[53,54]. Studies on EC have demonstrated that the expression of vascular endothelial growth factor is negatively correlated with the degree of tissue differentiation and positively correlated with the degree of cell malignancy and angiogenic ability[55]. Exosomes derived from ESCC cells cultured under hypoxic conditions promote angiogenesis by altering the phenotype and transcriptome of endothelial cells[56]. Exosomes derived from ESCC cells cultured under hypoxia have been reported to promote angiogenesis by altering the phenotype and transcriptome of endothelial cells [57]. Therefore, exosomes may participate in angiogenesis by altering the levels of angiogenesis-related factors in the EC.

T cells, which can generally be divided into CD4+, CD8+, helper, and regulatory T cells (Tregs), play both pro- and anti-tumorigenic roles in the TME[58]. Fibroblast growth factor 2 produced by tumor fibroblasts can impair the activation of $CD8^+$ T cells in EC, increasing the growth and metastasis of cancer cells *in vitro* and *in vivo* by regulating the expression of recombinant sprouty homologue 1[59]. Irradiated esophageal carcinoma-infiltrating T-cell derived exosomes can directly upregulate β-catenin, nuclear factor-κB (NF-κB), and snail in cancer cells, which is related to EC development, indicating a protumorigenic role of T-cell derived exosomes in EC[60].

Under the influence of exosomes, fibroblasts involved in tissue remodeling and repair transform into cancer-associated fibroblasts (CAFs)[61]. CAFs can provide suitable conditions for metastatic tumor cells and are thus an important determinant of EC. A study involving the analysis of clinical samples and establishment of an orthotopic metastasis model of EC indicated that CAFs could promote lymph node metastasis[62]. CAFs accelerate the progression and metastasis of tumors by secreting different tumor-promoting factors and regulating communication between tumor cells and microenvironment components[63]. CAFs can promote the growth and migration of ESCC cells by generating exosomes containing sonic hedgehog factors; inhibition of the Hedgehog signaling pathway can partly neutralize this phenomenon, suggesting a new strategy for ESCC treatment[64]. In 2022, Shi *et al*[65] designed *in vitro* experiments that revealed that CAF-derived exosomes facilitate the metastasis and EMT of ESCC cells *via* the LINC01410/miR-122-5p/PKM2 axis. CAFs enhance tumor cell evasion during immune surveillance by inducing M2 polarization of macrophages and apoptosis of T and NK cells[66]. Additionally, Zhang *et al*[67] observed a role of CAFs in establishing resistance of ESCC cells to chemotherapeutic drugs, which they accomplished *via* exosome-mediated FOXO1/TGF1 signaling. In addition to being associated with the formation of CAFs, exosomes play an indispensable role in immune dysfunction in patients with EC, which may lead to disease progression and poor response to therapy.



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Figure 2 Cells promote metastasis and drug resistance of esophageal cancer in the tumor microenvironment. Esophageal cancer (EC)-cell derived exosomes can promote metastasis and drug resistance of EC by influencing cells in the tumor microenvironment (TME). Exosomes are involved in altering the number or function of immune cells in the TME, resulting in more EC cells evading the immune system. And, EC-cell derived exosomes may promote the proliferation of endothelial cells to form neovascularization and the proliferation of adjacent tumor cells. Moreover, EC-cell derived exosomes could launch the migration of EC cells from the primary tumor site to the secondary site. Additionally, originally sensitive EC cells could appear therapeutic resistance under the influence of exosomes secreted by neighboring drug-resistant cells. MDSCs: Myeloid-derived suppressor cells; CAF: Cancer-associated fibroblasts; EMT: Epithelial-mesenchymal transition.

Owing to the high variability of tumor-associated macrophages (TAMs), different stimuli in the TME, including exosomes and cytokines secreted by tumor cells, can drive the tilt differentiation of TAMs towards M1 or M2 macrophages[68]. The transfer of miR-21-5p from EC109 or EC9706 cells to M0 macrophages *via* exosomes resulted in M2 macrophage polarization and EMT in EC cells[69]. Shou *et al*[70] verified that another EC-derived exosomal cargo, miR-301a-3p, could also facilitate angiogenesis by inducing macrophage polarization The transfer of hsa-circ-0048117 from hypoxic ESCC cells to macrophages *via* exosomes results in M2 macrophage polarization[71]. EC-derived exosomes can alter the function of macrophages; similarly, exosomes secreted by macrophages can also affect EC progression. Emerging evidence indicates that M2 macrophages are associated with the migration, invasion, poor prognosis of ESCC, and the subsequent induction of EMT[72]. By transmitting lncRNA AFAP1-AS1 and microRNA-26a, exosomes derived from M2 macrophages promoted the migration, invasion, and lung metastasis of EC cells. Mechanistically, M2 macrophage-derived exosomes increase EC metastasis by transferring lncRNA AFAP1-AS1 to upregulate ATF2 and downregulate miR-26a, revealing that targeting M2 macrophages and the lncRNA AFAP1AS1/miR-26a/ATF2 signaling axis may be a useful therapeutic strategy for EC[73].

Myeloid-derived suppressor cells (MDSCs) can suppress various types of immune responses and participate in the formation of the premetastatic niche and are immature myeloid cells closely linked to tumor development and prognosis [74,75]. Chen *et al*[76] found that the level of MDSCs, which cause immune escape and promote metastasis by eliciting T cell dysfunction, is considerably related to ESCC formation. Notably, MDSC differentiation has been reported to aggravate immunosuppression in EC through transcriptomic alterations induced by the lncRNA Lnc-17Rik[77]. Based on the alterations in cells in the TME, the use of exosomes to intervene in this process to regulate TME is worth studying.

EXOSOME FUNCTION IN METASTASIS

High metastatic rate is a major issue in EC, resulting in high mortality and poor prognosis. Tumor metastasis is a complex and multistep process that includes the growth of primary tumors, neovascularization, interactions between tumor and stromal cells, EMT, intravasation of tumor cells, extravasation of surviving tumor cells, and colonization and growth at secondary sites[78], which are affected by a number of signaling pathways. Existing studies on EC have revealed that exosomes derived from tumor and nontumor cells participate in various steps of tumor metastasis by mediating intercellular communication (Figure 3).

Exosomes in EMT

EMT, which involves epithelial cells losing their apical polarity, gaining anteroposterior polarity, and losing cell adhesion to transform into mesenchymal cells, is a key driver of tumor metastasis[79]. Primary tumors undergo EMT through the regulation of various factors, including EMT transcription factors, paired related homeobox protein 1 (Prrx1), cancer stem cells, N-cadherin, vimentin, E-cadherin, occludin, cytokeratin, and claudin[80]. Through the transmission of these elements, exosomes can cause epithelial cells to undergo morphological and functional changes and acquire invasive properties that facilitate EMT.

We summarized the relevant literature on exosomes and EMT process, focusing on changes in signaling pathways. Qiao *et al*[81] compared 50 paired ESCC tumor tissues and matched normal adjacent tissues and found that the expression of EMT-related proteins was notably upregulated in ESCC cells in the presence of miR-106b-3p. Then, the researchers performed gene and protein expression analyses, and the results revealed that Wnt, GSK3, and β-catenin related to Wnt/β-catenin pathway were increased in the miR-106b-3p mimic group compared with the control group. In ESCC, miR-106b-3p may be transported by exosomes to induce these effects. By transmitting miR-10527-5p, exosomes derived from human ESCC cell lines notably downregulated N-cadherin and vimentin (mesenchymal markers) and upregulated the epithelial marker E-cadherin by activating the Wnt/β-catenin pathway[82]. Additionally, one study emphasized the function of CAF-derived exosomal miR-3656 in the TME, which could serve as a biomarker for EC diagnosis. MiR-3656 in CAF-derived exosomes promotes the proliferation, migration, and invasion of EC cells by triggering the ACAP2/phosphatidylinositol 3-kinase (PI3K/AKT) signaling pathway[83].

Exosomes extracted from T cells after radiotherapy significantly promote the EMT of EC cells by increasing the expression of β-catenin, NF-κB, and snail, as revealed using western blot analysis[60]. Li *et al*[84] demonstrated that FMR1-AS1 was increased in ESCC tissues, and a comparison of lncRNA levels between 179 pairs of ESCC tissues and their adjacent normal tissues showed that the upregulation of FMR1-AS1 was associated with poor prognosis in female patients. According to further experiments and analyses, ESCC-derived exosomal FMR1-AS1 maintains the interconversion state of cancer stem cells by activating the TLR7-NF-κB signaling pathway and upregulating c-Myc levels in target cells. All these studies demonstrate that when studying the relationship between exosomes and EC, we can focus on changes in the molecules of the relevant signaling pathways, which opens up possibilities for diagnosis and subsequent treatment.

Exosomes in ECM degradation

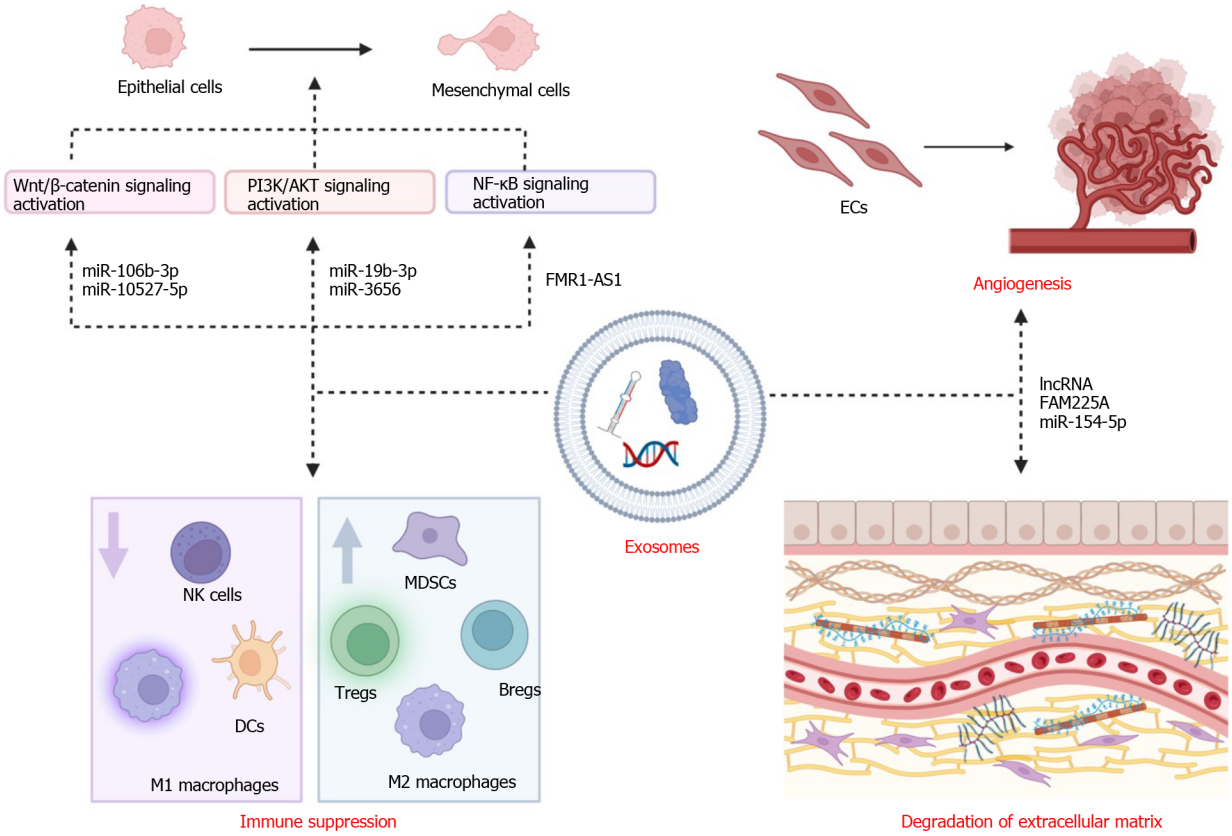
The ECM is composed of collagen, glycoprotein, proteoglycans, and glucosamine and is a constantly changing part of the TME, supporting the survival and activity of cells, mediating the intercellular signal transduction system, and maintaining homeostasis. ECM remodeling depends on the functions of MMPs, adamalysins, meprins, growth factors secreted by tumor cells, monocytes, CAFs, polymorphonuclear cells, and leukocytes[85,86]. During tumor metastasis, collagen decomposes and destroys the basement membrane[79]. CAFs can generate exosomes that transmit miR-451 to the esophageal TME, mediating changes in the ECM and degradation of basement membrane collagen. As miR-451 is overexpressed in the serum of patients with EC compared with healthy individuals, it may serve as a potential diagnostic biomarker for EC[80].

Exosomes in angiogenesis

The importance of exosomes in EC angiogenesis has been demonstrated in three reports. Zhang *et al*[87] showed that the exosome-encapsulated lncRNA FAM225A was highly expressed in ESCC tissues and cell lines and could sponge miR-206, which targets NETO2 and FOXP1 to accelerate angiogenesis. Exosomal cation-dependent mannose-6-phosphate receptors from SRGN-overexpressing ESCC cells facilitate neovascularization[88]. Tumor cell-derived exosomes can negatively regulate ESCC progression and angiogenesis by transmitting miR-154-5p, which downregulates kinesin family member 14[89].

Exosomes in immune suppression

Immune cells constitute an essential part of the immune system and play diverse protumor and antitumor roles in the genesis and metastasis of tumors. Tumor-derived exosomes can mediate the expansion of Tregs, MDSCs, and regulatory B cells (Bregs); inhibit the function of NK cells; and control the differentiation of myeloid progenitors, lymphoid progenitors, and dendritic cells (DCs) by delivering contents obtained from parent tumor cells to recipient cells[90]. Yuan *et al*[91] observed that exosomes produced from EC stem cells could transfer the nutrient sensor O-GlcNAc transferase to neighboring CD8+ T cells, silencing antitumor immune responses by increasing PD-1 expression in CD8+ T cells. These studies will bring new promise for immunotherapy of EC.



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Figure 3 The role of exosomes in esophageal cancer metastasis. Exosomes are associated with esophageal cancer (EC) metastasis by transferring their contents. The activation of several signaling pathways, including Wnt/β-catenin, PI3K/AKT and NF-κB signaling pathways, is closely to the exosome-mediated tumor microenvironment in EC. Exosomes can mediate immune suppression by downregulating antitumor cells (including NK cells, dendritic cells and M1 macrophages) and upregulating protumor cells (including myeloid-derived suppressor cells, Tregs, Bregs and M2 macrophages). Additionally, in primary EC sites, degradation of extracellular matrix and angiogenesis are also promoted by exosomes. MDSCs: Myeloid-derived suppressor cells; ECs: Esophageal cancers; DCs: Dendritic cells.

Exosomes in the formation of the premetastatic niche

The steps underlying the formation of a premetastatic niche in target organs, which is a fundamental process required for tumor metastasis, include the arrival of tumor cell-secreted components, infiltration of bone marrow-derived cells, and alteration of the matrix microenvironment[92]. In addition to the vascular metastatic niche, the formation of a lymphatic metastatic niche in the local lymph nodes that induces immunosuppression, reduces proliferation, and enhances apoptosis is vital for metastasis in patients with ESCC[93]. Available evidence suggests that exosomes play an indispensable role in mediating signals between the primary tumor and target organs to construct a premetastatic niche in breast[94], pancreatic[95], and ovarian cancers[96]. As mentioned above, it is reasonable to hypothesize that exosomes participate in the generation of a premetastatic niche in ESCC.

EXOSOMES FUNCTION IN THERAPEUTIC RESISTANCE

Various types of treatment, including surgery, endoscopy, chemotherapy, radiotherapy, and immunotherapy, can be employed for EC; they can be used as monotherapy or in combination and should be selected carefully according to the cancer stage[96]. However, the use of multitarget drugs and the presence of cellular heterogeneity and immunoediting have led to the emergence of widespread treatment resistance, which greatly affects the survival rate of patients with EC [97,98]. Although detailed mechanisms of tumor therapy resistance have rarely been explored, exosomes, as tools for cell-to-cell communication, are speculated to play a vital role in the transmission of therapy resistance. In addition to the role of exosomes in tumor angiogenesis and immunosuppression, a large number of studies have reported that exosomes can mediate changes in signal transduction, drug efflux, and drug sequestration, leading to the development of tumor treatment resistance[99]. Therapeutic resistance remains the main factor that limits the application and efficacy of therapeutic measures for EC. In the following sections, we explain the role of exosomes in therapeutic resistance using specific examples.

Exosome-mediated tumor chemotherapy resistance

When receiving chemotherapy, patients with EC can develop multidrug resistance *via* several mechanisms, including

increase in drug efflux, attenuation of drug influx, alteration of cell apoptosis machinery, changes in molecular drug targets, and enhancement of drug metabolism, ultimately leading to treatment failure[98,100-102]. Exosomes derived from drug-resistant cells can induce sensitive tumor cells to acquire antitumor characteristics by transmitting proteins and nucleic acid cargo to regulate oncogene expression[61]. Additionally, exosomes can lead to drug resistance by directly or indirectly regulating immune cells in the EC microenvironment[103,104]. Owing to the complex and unknown mechanisms of chemotherapy resistance, it is difficult to find an effective solution.

Recently, CAF-derived exosomes have been reported to participate in EC resistance by establishing a physical barrier that inhibits the delivery of drugs and rays to tumor cells[105]. A previous study demonstrated that CAFs can generate monocytic myeloid-derived suppressor cells by activating signal transducer and activator of transcription 3 (STAT3) by secreting interleukin-6 and exosomal miR-21. Monocytic myeloid-derived suppressor cells not only dampen the function and promote apoptosis of some immune cells but also strengthen the cisplatin resistance of EC cells[106]. In 2020, a series of experiments conducted by Tong *et al*[59] suggested that ESCC cell-derived exosomal lncRNA POU3F3 affects the proliferation of tumor cells and cisplatin resistance of ESCC by activating normal fibroblasts.

Considering the importance of signaling pathways in mediating the development of drug resistance and their potential in treating EC, we focused on signaling pathways related to the exosome-based modulation of chemotherapy resistance. The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, which includes more than 50 cytokines and growth factors, plays an important role in immune fitness, inflammation, and cell apoptosis; the pathway begins with the activation of JAK, which leads to the phosphorylation of STAT[107,108]. In 2020, a study reported 189 overexpressed and 304 downregulated miRNAs in exosomes secreted by cisplatin-resistant EC cells. Analysis of the correlation between small RNA-seq and RNA-seq revealed that exosomes derived from drug-resistant cells decreased the inhibitory effects of cisplatin on the cell cycle in sensitive cells and thus increased their cisplatin resistance in a manner that was potentially related to JAK/STAT signaling[109]. Exosomal circ_0000337 overexpression in cisplatin-resistant EC tissues and cells promoted cisplatin resistance by regulating the miR-377-3p/JAK2 axis[110]. JAK2 is a crucial component of the JAK2/STAT3 signaling pathway and its activation has been reported to mediate resistance to chemotherapy and radiotherapy[111]. Exosomal miR-21 was significantly overexpressed in EC cells with low sensitivity to cisplatin[112]. Further experiments indicated that exosomal miR-21 can inhibit the expression of PTEN, ultimately activating STAT3 signaling[106]. In summary, exosomes mediate chemotherapy resistance, especially cisplatin resistance, in EC through the JAK/STAT signaling pathway. However, whether JAK/STAT signaling participates in other forms of therapy resistance remains unknown. A recent study demonstrated that exosomal PD-L1 Leads to paclitaxel resistance in EC cells by targeting the STAT3/miR-21/PTEN/Akt axis[113]. CAF-derived exosomes induce cell proliferation, suppress cell apoptosis, and decrease tumor chemosensitivity to cisplatin in ESCC, and Cui *et al*[114] reported that RIG-I/IFN-β signaling plays a crucial role in these processes. Exploring additional signaling pathways that play an essential role in EC drug resistance and focusing on the relationship between exosomes and these pathways may help develop strategies to overcome the widespread problem of chemotherapy resistance[115].

Exosome-mediated tumor radiotherapy resistance

Radiation can directly or indirectly kill cells within a limited range and restrict tumor growth by damaging DNA or generating free radicals and ions. However, tumors tend to exhibit radiation resistance *via* EMT, immunosuppression, repair of DNA damage, abnormal expression of noncoding RNAs, activation of autophagy and initiation of related signaling pathways[116]. The importance of exosomes in radiotherapy resistance in EC has been demonstrated in two reports. In vitro assays and xenograft models showed that hypoxic cell-derived exosomes transfer miR-340-5p to normoxic cells, thereby inducing the proliferation of radiotherapy-resistant normoxic ESCC cells by influencing the KLF10/UVRAG axis. The level of plasma exosomal miR-340-5p is upregulated and is associated with the in-field recurrence-free survival of patients with ESCC, suggesting that plasma exosomal miR-340-5p can serve as a biomarker for prognostic evaluation[117]. Additionally, NORAD is associated with local recurrence in patients with ESCC after radiotherapy, and *in vitro* experiments further confirmed that cell-to-cell transfer of exosome-encapsulated miR-199a-5p improved the radiosensitivity of radioresistant cells[118]. Further, miR-26b-5p contained in EVs derived from dying tumor cells after irradiation promotes cancer cell metastasis by potentiating the deposition of premetastatic niche components and the expansion and activation of MDSCs in the TME[119]. After radiotherapy, EC cells may acquire radiation resistance by secreting specific exosomes, and it is crucial to identify effective interference targets to improve the efficacy of radiotherapy and reduce the recurrence rate.

Exosome-mediated tumor immunotherapy resistance

Immunotherapy exerts antitumor effects by strengthening the body's immune response and disrupting immune tolerance by employing tumor cells, nucleic acids, cytokines, antibodies, and/or immune cells[120]. Recently, based on the role of immune cells in the TME during EC development, immune checkpoint blockade, chimeric antigen receptor T-cell therapy, and tumor vaccines have been used for EC treatment. Despite significant breakthroughs in these immunotherapies, primary resistance lowers the overall response rate of immunotherapy and acquired resistance leads to a gradual decrease in immunotherapy efficacy in EC treatment[121]. Sensitive EC cells can acquire resistance *via* the downregulation of tumor antigens to escape the attack of T cells, the acquisition of immune escape ability *via* mutation, interferon-γ (IFN-γ) signaling, and neoantigen consumption[122]. Programmed death 1 (PD-1), which can interact with programmed death-ligand 1 (PD-L1), is a critical immune checkpoint that helps tumor cells escape T cell attack by disguising malignant cells as normal cells and promoting T cell depletion. PD-1/PD-L1 blockade strategies have been developed to overcome this problem by decreasing T cell suppression and activation[123]. However, changes in PD-L1 expression, decreased presentation of tumor neoantigens, and immune suppression may be responsible for the failure of anti-PD-1/PD-L1 immunotherapy. Exosomes derived from M2 TAMs regulate PD-1/PD-L1 expression and mediate PD-

1/PD-L1 immunosuppression[124]. Tumor cell-derived exosomal PD-L1 can be used to supplement PD-1 on the cell surface, thereby antagonizing the effects of PD-1/PD-L1-blocking antibodies[125]. Exosomes may participate in immunotherapy resistance by disseminating immune checkpoint molecules, inhibitory molecules, cytokines, and growth factors, resulting in a poor clinical response[126]. In addition to PD-1/PD-L1, the relationship between exosomes and other immunotherapy approaches for EC remains to be explored. Identifying a reliable and universal relationship may help address immunotherapy resistance, although this remains a severe challenge.

APPLICATION OF EXOSOMES AS BIOMARKERS IN ESCC

As some exosomes can be found in the early stages of EC and change specifically as malignancy progresses, it is reasonable to use exosomes for the early diagnosis of ESCC to prevent EC progression, which will improve the cure rate of EC[127,128]. Compared with RNAs in plasma, exosomal RNAs have higher applicability and specificity in diagnosing EC for the following reasons: exosomes are stable in the body fluids of patients and can be used as an effective transport tool for RNAs; the level of exosomes correlates with that of donor cells; and exosomes can protect RNAs from degradation[127,129]. In addition, studies on EAC have reported that serum is a better sample type to test exosome content than plasma because it has fewer interfering components, and miRNAs of non-vesicle origins exist in plasma [130].

Recent studies have demonstrated that exosome-encapsulated miR-21 is prominently upregulated in patients with ESCC. In addition, the level of exosomal miR-21 was related to the clinicopathological features, clinical stage, and prognosis of patients with ESCC. In summary, exosomal miR-21 may serve as a clinical biomarker for human EC[131]. In a multicenter prospective study in 2022, researchers found that the newly discovered "small RNA identified in exosome from saliva of patients with ESCC" could be combined with tRNA-GlyGCC-5 to diagnose and determine the prognosis of EC. These two types of small RNAs can be found in saliva-derived exosomes, indicating the possible application of saliva-derived exosomal small RNAs as accurate and noninvasive predictive markers of EC[132]. Searching for ideal diagnostic molecules to be applied in combination may greatly improve diagnostic accuracy and reduce the false-positive rate. As the levels of circulating exosomal miRNAs differ between patients with Barrett's esophagus (BE) who develop EAC and those who do not and between patients with EAC and those with BE, they may predict and distinguish the prognosis of BE/EAC and distinguish them[27,133]. Previous reports suggest that the expression level of miR-93-5p is notably upregulated in patients with ESCC compared to healthy controls[134]. CircFNDC3B encapsulated in exosomes participates in the migration and invasion of EC cells by sponging miR-490-5p and regulating thioredoxin reductase 1 expression and is abundant in the tissues and cells of EC samples[135].

Currently, the diagnosis of EC mainly depends on medical imaging and endoscopy; laboratory examinations are insufficient and inaccurate. Owing to the disadvantages of imaging and endoscopy, such as being time consuming, expensive, and invasive, laboratory examinations should be the focus of research to identify strategies for screening and diagnosing EC. The application of exosome-based liquid biopsy for the early diagnosis of cancer has pioneering significance; however, this strategy cannot be widely applied because of its high false-positive rate and high cost[136]. Consequently, finding accurate and readily available contents of exosomes as a new strategy for diagnosing EC is complicated but crucial and urgent (Table 2).

EXOSOMES ARE USED IN EC TREATMENT

Only a limited number of patients can receive direct esophagectomy, the most effective treatment, whereas others need to choose an alternative comprehensive therapy to prolong survival if they have middle- or advanced-stage disease[137, 138]. Although esophagectomy is the primary method of EC treatment, it can lead to a loss of appetite, dysphagia, reflux, and other complications, thereby seriously damaging the quality of life of patients[139]. Furthermore, patients treated with esophagectomy are susceptible to local recurrence owing to the presence of cells resistant to radiotherapy, immunotherapy, or chemotherapy. Therefore, there is an urgent need to develop novel therapeutic strategies for EC. Exosomes can serve as delivery tools by selectively transporting miRNAs, mRNAs, lncRNAs, and proteins to target cells, thereby providing new avenues for EC treatment[140]. Although no direct evidence has shown that exosomal miRNAs can alter target cell functions in BE/EAC, several studies have provided evidence supporting the application of exosomal miRNAs as therapeutic modalities to inhibit the progression from BE to EAC[141,142]. Exosome-derived miR-154-5p attenuates the invasion of EC cells and inhibits their angiogenic capability *in vitro*, curbing the malignant progression of ESCC[89]. Whether specific miRNAs regulate radiation resistance or can be used as tumor radiosensitizers in ESCC remains unclear. Luo *et al*[143] observed that some miRNAs, such as miR-339-5p, selectively secreted by exosomes, can promote sensitivity to radiation therapy by downregulating the cell division cycle 25 A (Cdc25A), and high levels of miR-339-5p in tumor tissues and serum indicated good prognosis, providing a theoretical basis for developing new tumor radiosensitizers based on the use of exosomal miRNAs.

In addition, exosomes derived from immune cells can exert antitumor effects by displaying specific surface antigens that initiate the human immune system, thus helping kill tumor cells[144]. Evidence suggests that MHC molecules and CD86 expressed on the surface membranes of exosomes derived from DCs are associated with T cell activation[145]. The activation of T cells, including CD4+ and CD8+ T cells, is vital for tumor immunity. Exosomes derived from tumor antigen-stimulated DCs carrying anti-CD3 and anti-EGFR antibodies promote the interaction of T cells with cancer cells [146]. We believe that exosomes can achieve similar regulatory effects in EC, suggesting new approaches for EC

Table 2 Clinical application of exosome in esophageal cancer

Clinical application	Biofluids	Exosomes or exosomal cargos	Expression	Ref.
Diagnosis	Plasma	miR-106a, miR-18a, miR-20b, miR-486-5p, miR-584	High	[155]
	Serum	lncRNA UCA1, POU3F3, ESCCAL-1 and PEG10	High	[156]
	Plasma	lncRNAs NR_039819, NR_036133, NR_003353, ENST00000442416.1, and ENST00000416100.1	High	[157]
	Plasma	miR-223-3p	Low	[122]
Prognosis	Serum	miR-182	High	[158]
	Serum	miR-766-3p	High	[159]
	Serum	hsa_circ_0026611	High	[160]
	Serum	lncRNA-POU3F3	High	[156]
	Plasma	hsa_circ_0001946	Low	[161]

treatment.

Synthetic drugs must pass through the cell membrane and enter target cells to function[147,148]. Concomitantly, exosomes are better for delivering synthetic drugs than other materials, including liposomes, polymers, and dendrimers, as they are more biocompatible, biodegradable, less toxic, better at penetrating the blood-brain barrier, better at traveling deep into tissues, and have better target specificity for recipient cells[149,150]. In addition, exosomes cause minimal acute immune reactions, more accurately identify tumors, and are not targeted by early attack by circulating immune cells, ultimately prolonging the half-life of drugs and reducing drug resistance[148,151]. Similar to exosome isolation, there are multiple techniques for loading drugs into exosomes, including sonication, transfection, incubation, transgenesis, pH gradient loading, extrusion, and hypotonic dialysis[148].

When considering the application of exosomes as drug delivery systems, it is necessary to focus on engineered exosomes. Engineered exosomes are exosomes with particular molecules attached to their surface or loaded with specific molecules for delivery, which enables the exosomes to target cells or tissues and enhances the local enrichment of the delivered substance. In addition, the engineered exosomes considerably increased the output and shortened the time of exosome production. Engineered exosomes of different origins have different lipid and surface protein compositions that may affect their functions. For example, tumor cell-derived exosomes may mediate immune responses, immune cell-derived exosomes may induce immune evasion, and exosomes isolated from fruits, milk, or plants are usually safe, inexpensive, and scalable[152]. Therefore, it is important to select a suitable source of exosomes based on their applications and available conditions. Existing evidence shows that engineered M1 macrophage-derived exosomes can inhibit tumor growth and transform M2-type TAMs into M1-like macrophages by targeting IL4 receptors on the surfaces of M2 macrophages[153]. In summary, we can expect engineered exosomes to have great potential for use in EC treatment.

Based on the above data, the components of exosomes affect the progression of EC through some key signaling pathways, and we propose that we can not only use exosomes as vessels for the delivery of signaling pathway inhibitors or activators but also change the content of key substances in exosomes to affect downstream signaling. Additionally, exosomes can act as carriers to deliver critical components for gene therapy and cancer vaccines. Exogenous exosomes also hold tremendous potential for optimizing the therapeutic outcomes of the surgical treatment of EC. Notably, there are challenges that urgently need to be overcome for the successful application of exosomes in EC treatment. These may be addressed by developing strategies to enable the controlled or continuous release of therapeutic drugs, increasing the ability of exosomes to carry therapeutic agents, determining what cells should be used to produce exosomes, and determining how exosomes can escape cell and/or enzyme[154]. More efforts are needed to address these problems in order to apply exosomes in clinical therapy.

CONCLUSION

Valuable biomarkers with high sensitivity and availability for the diagnosis and prognosis of EC, as well as effective therapeutic strategies for EC to confine the disease to a manageable stage and improve disease-free survival in patients, need to be urgently identified. Increasing evidence has shown that exosomes are robustly involved in the metastasis and treatment resistance of EC by facilitating carcinoma cell proliferation, EMT, angiogenesis, and immunosuppression. Factors in the TME, especially cells, have an important impact on tumor growth and development. Furthermore, exosomes are differentially expressed and widely present in various biofluids, indicating that they can be used as novel biomarkers for the diagnosis and prognosis of EC and delivery of antitumor drugs. However, there remain several challenges to overcome for the clinical application of exosomes. Exosomes with considerable specificity and high sensitivity for discriminating patients from healthy individuals and EC from other cancers are urgently needed. New biomarkers should not only be easy and inexpensive to detect but should also show changes at an early stage of EC. Based on the findings of previous studies, we concluded that specific signaling pathways are associated with the exosome regulation of metastasis and drug resistance in EC. To identify suitable and precise targets for EC treatment, it is worth

exploring more relevant signaling pathways. Overall, there is still a long way to go before exosomes can be used for clinical diagnosis, treatment, and prognosis of EC.

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FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study

Time series analysis-based seasonal autoregressive fractionally integrated moving average to estimate hepatitis B and C epidemics in China

Yong-Bin Wang, Si-Yu Qing, Zi-Yue Liang, Chang Ma, Yi-Chun Bai, Chun-Jie Xu

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Abstract

BACKGROUND

Hepatitis B (HB) and hepatitis C (HC) place the largest burden in China, and a goal of eliminating them as a major public health threat by 2030 has been set. Making more informed and accurate forecasts of their spread is essential for developing effective strategies, heightening the requirement for early warning to deal with such a major public health threat.

AIM

To monitor HB and HC epidemics by the design of a paradigmatic seasonal autoregressive fractionally integrated moving average (SARFIMA) for projections into 2030, and to compare the effectiveness with the seasonal autoregressive integrated moving average (SARIMA).

METHODS

Monthly HB and HC incidence cases in China were obtained from January 2004 to June 2023. Descriptive analysis and the Hodrick-Prescott method were employed to identify trends and seasonality. Two periods (from January 2004 to June 2022 and from January 2004 to December 2015, respectively) were used as the training sets to develop both models, while the remaining periods served as the test sets to evaluate the forecasting accuracy.

RESULTS

There were incidents of 23400874 HB cases and 3590867 HC cases from January 2004 to June 2023. Overall, HB remained steady [average annual percentage change (AAPC) = 0.44, 95% confidence interval (95%CI): -0.94-1.84] while HC was increasing (AAPC = 8.91, 95%CI: 6.98-10.88), and both had a peak in March and a trough in February. In the 12-step-ahead HB forecast, the mean absolute deviation (15211.94), root mean square error (18762.94), mean absolute percentage error (0.17), mean error rate (0.15), and root mean square percentage error (0.25) under the best SARFIMA (3, 0, 0) $(0, 0.449, 2)_{12}$ were smaller than those under the best SARIMA (3, 0, 0) $(0, 1, 2)_{12}$ (16867.71, 20775.12, 0.19, 0.17, and 0.27, respectively). Similar results were also observed for the 90-step-ahead HB, 12-step-ahead HC, and 90-step-ahead HC forecasts. The predicted HB incidents totaled 9865400 (95%CI: 7508093-12222709) cases and HC totaled 1659485 (95%CI: 856681-2462290) cases during 2023-2030.

CONCLUSION

Under current interventions, China faces enormous challenges to eliminate HB and HC epidemics by 2030, and effective strategies must be reinforced. The integration of SARFIMA into public health for the management of HB and HC epidemics can potentially result in more informed and efficient interventions, surpassing the capabilities of SARIMA.

Key Words: Hepatitis; Seasonal autoregressive fractionally integrated moving average; Seasonal autoregressive integrated moving average; Prediction; Epidemic; Time series analysis

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Core Tip: This retrospective study used a seasonal autoregressive fractionally integrated moving average (SARFIMA) to monitor hepatitis B (HB) and hepatitis C (HC) epidemics, and its forecasting potential was then compared to that of the seasonal autoregressive integrated moving average (ARIMA) (SARIMA). The resulting forecast error rates under the SARFIMA were less than those under the SARIMA. The integration of SARFIMA into public health decision-making for the management of HB and HC epidemics can result in more informed interventions. The predicted HB totaled 9865400 [95% confidence interval (95%CI): 7508093-12222709] cases and HC totaled 1659485 (95%CI: 856681-2462290) cases in 2030, resulting in major challenges to eliminate hepatitis in China by 2030.

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INTRODUCTION

Hepatitis is a condition characterized by liver inflammation, which can be caused by various infectious viruses and noninfectious agents, resulting in a variety of health complications[1]. There are five primary strains of the hepatitis virus, namely, types A, B, C, D, and E. Hepatitis B (HB) and hepatitis C (HC), in particular, can progress into chronic diseases affecting millions of individuals, and they are the leading causes of liver cirrhosis, liver cancer, and mortality related to viral hepatitis[1,2]. Although significant achievements have been made in the prevention of HB and HC through the implementation of various strategies, these infections still pose significant health risks globally, affecting millions of people and causing a substantial burden on healthcare systems[3,4]. According to the World Health Organization (WHO), an estimated 296 and 58 million people were living with HB and HC, respectively, in 2019, and HB- and HC-related complications cause 820000 and 290000, respectively, deaths each year[1]. These numbers indicate the scale of the issue and emphasize the need for effective prevention and control strategies. Also, the WHO has set a goal of ending HB and HC as major public health threats by 2030 (compared with the 2015 baseline, new infections decreased by 30% by 2020 and 90% by 2030)[2]. China bears the greatest burden of HB virus (HBV) and HC virus (HCV) infections globally and is expected to play a pivotal role in achieving the global goal of eliminating HB and HC by 2030[4]. Accurate prediction aids in anticipating prospective scenarios and making proactive judgments, allowing policymakers to make educated decisions, develop strategies, and prepare for potential challenges and opportunities[5]. It has served as a valuable tool for mitigating risks, forming policies, and optimizing outcomes in different domains.

Time series analysis plays a crucial role in formulating hypotheses to comprehend the epidemics of different diseases and estimating the dynamics of observed phenomena, ultimately contributing to the establishment of a high-quality control system[5]. Various statistical methods, including generalized regression neural network[6], back propagation neural network[6], grey prediction[7], and Elman neural network[8], have been applied to predict the epidemics of infectious diseases. However, disease epidemics are frequently influenced by different factors, resulting in a combination of trends, seasonality, and randomness in the spread. Accordingly, the aforementioned statistical techniques may not adequately capture the complex and dynamic nature of epidemics, and it is challenging to generalize the models above

[5].

The seasonal autoregressive integrated moving average (SARIMA) has been the most commonly used model in different domains thanks to its simplicity, fast applicability, and ability to explain data[5,7,8]. Previous studies have also demonstrated the successful application of the SARIMA in estimating the prevalence, morbidity, and mortality of hepatitis[7], coronavirus disease 2019 (COVID-19)[5], hemorrhagic fever with renal syndrome (HFRS)[9], hand-foot-and-mouth disease (HFMD)[10], and tuberculosis[11]. The SARIMA can effectively capture the dynamic dependency structure by considering secular trends, periodic fluctuations, and random variations within a time series simultaneously[5]. Despite the attractive attributes of SARIMA, it is a representative of modelling short fluctuations in a series instead of the long term[12]. Additionally, using an integer difference in SARIMA for a series exhibiting long memory can cause over-differencing and the removal of valuable features, which harms forecasts[13]. In contrast, the seasonal autoregressive fractionally integrated moving average (SARFIMA) considers not only short memory, but also long memory, and such benefits as irregular fluctuations and complex seasonality by use of fractional differencing[12,14,15]. Also, the user-friendly nature of SARFIMA is relatively easy to explain to end-users and does not involve advanced mathematics or statistics. This enhances understanding and enables users to rely on the model for decision-making. The epidemiology of HB and HC is impacted by various factors, contributing to the epidemic patterns of long memory and complex dynamics [16]. Notwithstanding the advantages of SARFIMA, there is still a lack of exploration into how SARFIMA contributes to estimating HB and HC epidemics. Therefore, this study aimed: (1) To evaluate the usefulness of SARFIMA in monitoring HB and HC epidemics (projection into 2030) in mainland China; and (2) to assess the forecasting potential of SARFIMA compared to SARIMA.

MATERIALS AND METHODS

Data collection

The monthly incidence cases of HB and HC from January 2004 to June 2023 were provided by the Chinese Center for Disease Control and Prevention (https://www.chinacdc.cn/). The population numbers were obtained from the National Statistical Yearbook. All HB and HC cases were confirmed according to the HB and HC diagnosis criteria issued by The National Health Commission of the People's Republic of China (http://www.nhc.gov.cn/wjw/s9491/wsbz.shtml). HB and HC are notifiable diseases in China, and the confirmed cases required to be reported within 24 h. Any duplicate records were removed at the end of the same month. The SARIMA and SARFIMA were constructed using the data from January 2004 to June 2022, while the rest was the test set to indicate the predictive ability of both models. Because we required projection into December 2030 from July 2023 (90 data), the models were developed using the data between January 2004 and December 2015 to project the trends between January 2016 and June 2023 (90 data) to confirm the forecasting reliability.

SARIMA development

ARIMA is a widely used method that incorporates three components to model a time series: Autoregressive, differencing, and moving average models[17]. SARIMA is an extension of the ARIMA that includes a seasonal component, encompassing all the components of ARIMA and adding seasonal autoregressive, seasonal differencing, and seasonal moving average[18]. The model structure is represented as SARIMA (p, d, q) $(P, D, Q)_s$, where (p, d, q) represents the orders of the autoregressive, differencing, and moving average, respectively, (P, D, Q) represents the seasonal counterparts, and S is the seasonality. Selecting the appropriate values for p, d, q, P, D, and Q in SARIMA involves four procedures. First, HB and HC data must be stationary. If the data is found to be non-stationary through an Augmented Dickey-Fuller (ADF) test, differencing should be applied to obtain stationary series[19]. Second, the initial orders for p, q, P, and Q were identified by doing autocorrelation function (ACF) and partial ACF (PACF) analyses[17]. A series of attempts were then employed to identify the optimal combination by comparing Akaike's information criterion (AIC), corrected AIC (CAIC), Bayesian information criterion (BIC), and log-likelihood (LL)[18]. The one with lower values for AIC, BIC, and CAIC, along with higher LL was considered the preferred. Third, the errors from the best-fitting SARIMA were analyzed to verify if they behaved like a white noise series, which was done by the Ljung-Box *Q* test, ACF, and PACF[7]. Finally, once the model passed all the required statistical diagnoses, it could project into future periods.

SARFIMA development

Earlier values often significantly influence subsequent ones present in time series, demonstrating a concept known as hyperbolic decay (HD) time series[14]. By understanding the underlying mechanisms and developing accurate models for an HD series, researchers can extract important insights into complex systems and make data-driven predictions[13,14]. The SARIMA assumes an exponential decay of ACF, while the SARFIMA considers an HD pattern[9,13], indicating the presence of long-term memory. For this reason, the SARFIMA, with its added fractional differencing (df and Df), has been paid to considerable attention owing to its potential to capture persistent data[13,14]. df or Df ranges from -1 to 1[15]: (1) A value < 0.5 shows a stationary series; (2) a value between -1 and -0.5 means an invertible series; (3) a value between -0.5 and 0 shows an anti-persistent series; (4) a value of 0 shows short memory and mean-reverting process in the series; (5) a value between 0 and 0.5 illustrates long-term positive dependence in the series; (6) a value between 0.5 and 1 suggests mean reverting but not stationarity in the series; and (7) a value of 1 signifies a unit root process[15]. The model structure is denoted as SARFIMA (p, d^1, q) (P, D^1, Q) s, where $d^1 = d + df$ and $D^1 = D + Df$, whereby df or Df lies within (-1, 0.5) as the fractional term and d or D ≥ 0 is the integer term. The Hurst exponent (H), a measure of long-range dependency, often denotes the fractional differencing as df or Df = H - 0.5[15]. The value of H falls between 0 and 1, with H < 0.5 showing an

intermediate-memory series, H = 0.5 showing an uncorrelated series, and H > 0.5 indicating a long-memory series[15]. The H was computed using the rescaled range (R/S) method in this study[15]. As Veenstra's finding[14], the SARFIMA uses multiple starting values during its initial modelling phase. Consequently, it results in multiple modes, and the selection of the optimal one becomes a key step. From all possible modes, the one with the highest LL, alongside the lowest AIC and BIC, was the most considered[14]. The remaining steps of constructing the SARFIMA, such as parameter estimation and model diagnostics, were executed as described in SARIMA.

Statistical analysis

The Hodrick-Prescott method was employed to decompose the series into its trend and seasonality components[20]. The seasonal factor (SF) denotes the extent to which the incidence for a specific period deviates from the mean level (SR > 1 indicating a high-risk season; otherwise, a low-risk)[21], which was computed by multiplicative decomposition. The annual percentage change (APC) and average APC (AAPC) with a 95% confidence interval (95%CI) were calculated using Joinpoint (Version 4.8.0.1) to assess the changing trends[22]. The SARIMA and SARFIMA were implemented using the "forecast" and "arfima" packages in R (version 4.2.0, R Development CoreTeam, Vienna, Austria). Additionally, considering the significant impact of the COVID-19 pandemic on disease epidemics, a variable was thus created that was assigned a value of "1" between January 2020 and March 2023 and "0" between January 2004 and December 2019 to control for its impact on forecasting ability.

Evaluating the effectiveness of both models involved comparing the predicted values with the actual values of the test data using various performance metrics such as the mean absolute deviation (MAD), root mean square error (RMSE), mean absolute percentage error (MAPE), mean error rate (MER), and root mean square percentage error (RMSPE)[7]. A lower value on these metrics shows a more accurate forecast.

RESULTS

Data description

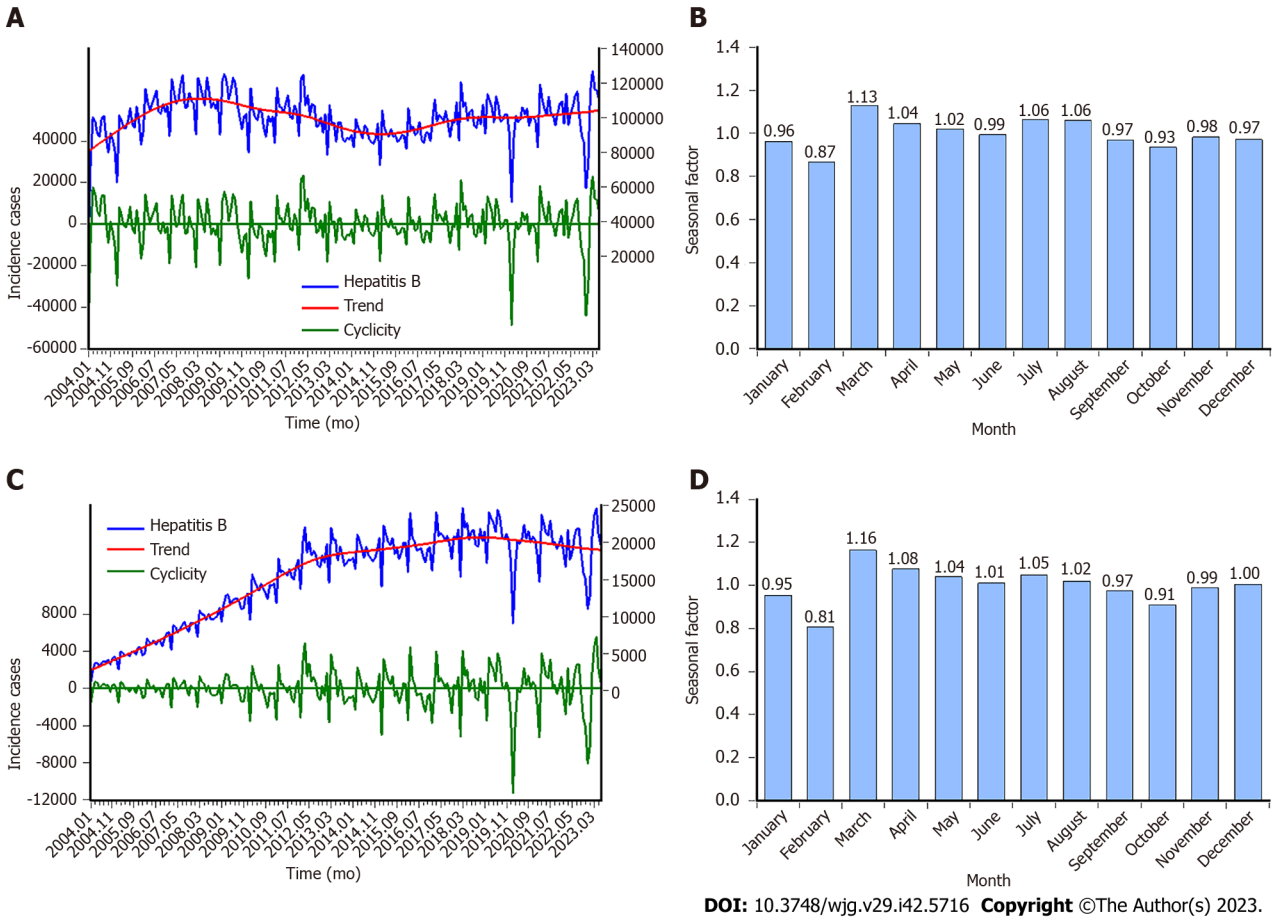
Between January 2004 and June 2023, China reported 23400874 HB cases (with incidence rates of 87.92 per 100000 persons per year and 7.33 per 100000 persons per month) and 3590867 HC cases (with incidence rates of 13.41 per 100000 persons per year and 1.12 per 100000 persons per month). Figure 1 illustrates that HB exhibited an overall trend of stabilization (AAPC = 0.44, 95%CI: -0.94-1.84) but we observed a rise during 2004-2007 (APC = 9.13, 95%CI: 2.02-16.74), a decline during 2007-2014 (APC = -3.53, 95%CI: 2.02-16.74), and a steady level during 2014-2022 (APC = 0.86, 95%CI: -0.61-2.35) (Figure 1A). HC showed an overall trend of escalation (AAPC = 8.91, 95%CI: 6.98-10.88) but we noted an unpredictable upsurge during 2004-2007 (APC = 32.05, 95%CI: 20.31-44.94), a considerable increase during 2007-2012 (APC = 15.48, 95%CI: 11.34-19.78), a slight upturn during 2012-2019 (APC = 1.65, 95%CI: 0.08-3.24), and a reduction during 2019-2022 (APC = -4.30, 95%CI: -8.7-0.32) (Figure 1C). Based on Figure 1B and D, it is evident that HBV and HCV infections could occur all the year round. However, there were notable fluctuations in infection rates. March showed the highest infection rates, with an SF of 1.13 for HB and 1.16 for HC, signifying a peak. In contrast, February recorded the lowest infection rates, with SF values of 0.87 for HB and 0.81 for HC, indicating a trough. The infection rates for the other months remained relatively stable.

The preferred SARIMA

A stationary test for the HB incidence series between January 2004 and June 2022 generated an ADF = -0.29 (P = 0.52), and thus a non-stationary series was demonstrated. Given seasonality in the series, it was then seasonally differenced once (ADF = -6.12, P < 0.001), showing its stationarity. By observing the ACF and PACF plots of the differenced series (Supplementary Figure 1), a series of attempts were done, and we finally identified eight possible models with significant parameters (Supplementary Table 1). To ensure the selection of the optimal model, the "auto.arima" function in R was also run, ultimately leading to the automatic choice of SARIMA (1, 0, 2) $(2, 0, 0)_{12}$. By comparing the resulting nine models, it could be seen that SARIMA (3, 0, 0) $(0, 1, 2)_{12}$ was preferred as it gave the least values of AIC (4363.97), CAIC (4364.53), and BIC (4387.4), alongside the greatest value of LL (-2174.99). For the diagnoses of residuals, no correlations toughed the significant bounds besides the ones at delays of 11 and 22 in the ACF and PACF plots (Supplementary Figure 2A) and P = 0.49 for the Ljung-Box Q statistic indicated an unrelated series of residuals, meaning that this preferred model sufficiently fits the data, which could be used to project into the next 12 data (Figure 2A). Likewise, following the modelling methods, SARIMA (1, 0, 2) $(2, 1, 0)_{12}$ (AIC = 2748.41, CAIC = 2749.08, BIC = 2765.71, and LL = -1368.21), SARIMA (3, 0, 0) $(0, 1, 1)_{12}$ (AIC = 3636.75, CAIC = 3637.17, BIC = 3656.84, and LL = -1812.38), and SARIMA (3, 1, 0) $(2, 1, 0)_{12}$ (AIC = 2022.09, CAIC = 2022.77, BIC = 2219.34, and LL = -1095.05) were determined as the optimal specifications for the 90-step-ahead forecasts of HB (Figure 2B), 12-step-ahead forecasts of HC (Figure 3A), and 90-step-ahead forecasts of HC (Figure 3B), respectively (statistical checks for the forecast errors are illustrated in Supplementary Figure 2B-D).

The preferred SARFIMA

The resulting R/S = 0.73 for the HB incidence series between January 2004 and June 2022 indicated that the series exhibited long-range dependence, and thus it was well suited to build SARFIMA. Subsequently, based on the fitting steps, the SARFIMA (3, 0, 0) $(0, 0.449, 2)_{12}$ with one mode tended to be indicated to be the preferred because it yielded the least values of AIC (4014.66) and BIC (4048.69), along with the maximum value of LL (-1997.33). Supplementary



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Figure 1 Time series graphs displaying the incidence cases, trend, cyclicity, and seasonality of hepatitis B and C. A: Hepatitis B incidence cases and the decomposed trend and cyclicity by Hodrick-Prescott (HP) method; B: Seasonal factor for hepatitis B series; C: Hepatitis C incidence cases and the decomposed trend and cyclicity by HP method; D: Seasonal factor for hepatitis C series. It is clear that hepatitis B and C have the same seasonal patterns, a peak in March and a trough in February, and the other months remain relatively stable.

Figure 3A presents the ACF and PACF analyses for the residuals, showing that all spikes were within the significance bounds and P = 0.48 for the Ljung-Box Q statistic pinpointed that the forecast errors belonged to a white noise series. Accordingly, this preferred model could make a forecast for the 12-holdout data (Figure 3A). In the same way, SARFIMA (1, 0, 2) (2, 0.454, 0)$_{12}$ (R/S = 0.76, AIC = 2610.36, BIC = 2637.09, and LL = -1296.18), SARFIMA (3, 0, 0) (0, 0.428, 1)$_{12}$ (R/S = 0.84, AIC = 3257.01, BIC = 3287.63, and LL = -1619.5), and SARFIMA (3, -0.155, 0) (2, -0.274, 0)$_{12}$ (R/S = 0.83, AIC = 2036.61, BIC = 2063.34, and LL = -1009.3) were identified as the best model for the 90-step-ahead forecasts of HB (Figure 2B), 12-step-ahead forecasts of HC (Figure 3A), and 90-step-ahead forecasts of HC (Figure 3B), respectively (the resulting modes and the required diagnoses for the forecast errors are given in Supplementary Tables 2-4 and Supplementary Figure 3B-D).

Assessing predictive accuracy and reliability

The data in Table 1 reveals the forecasting accuracy of both SARIMA and SARFIMA. Notably, the SARFIMA exhibited smaller values of MAD, MAPE, RMSE, MER, and RMSPE. Figure 2A and B and Figure 3A and B demonstrate a comparison between the forecasts generated by both models and the observed values. The SARFIMA depicted a closer resemblance to the actual trends and seasonality in comparison to the SARIMA. These results meant that the SARFIMA outperformed the SARIMA. Moreover, two sensitivity analyses were conducted to examine the influence of the age of the affected population and the cultural patterns during the spring season on the predictive quality of SARFIMA. These analyses indicated that the SARFIMA consistently produced lower forecasting error rates compared to the SARIMA (Supplementary Tables 5 and 6). This reinforces the robustness of the SARFIMA. Consequently, a projection into 2030 for HB and HC epidemics was done by identifying the optimal SARFIMA (1, 0, 1) (0, 0.438, 1)$_{12}$ and SARFIMA (1, 0.429, 1) (2, 0, 0)$_{12}$, respectively, on the whole data. The resulting results indicated that HB would reach a plateau in the upcoming years (Figure 2C), and the forecasts totaled 9865400 (95%CI: 7508093-12222709) incidents, with a yearly average of 1233175 (95%CI: 938512-1527839) incidents (Table 2); HC would begin to recede in the next years (Figure 3C), and the forecasts totaled 1659485 (95%CI: 856681-2462290) incidents, with an annualized average of 207436 (95%CI: 107085-307786) incidents (Table 2).

Table 1 Comparison of the forecasting ability under the seasonal autoregressive integrated moving average and the seasonal autoregressive fractionally integrated moving average

Metrics	Hepatitis B		Hepatitis C	
	SARIMA	SARFIMA	SARIMA	SARFIMA
12-step ahead forecasts				
MAD	16867.708	15211.939	3355.664	3245.308
MAPE	0.191	0.173	0.208	0.201
RMSE	20775.123	18762.935	4093.553	3940.966
MER	0.165	0.149	0.178	0.172
RMSPE	0.267	0.248	0.285	0.279
90-step ahead forecasts				
MAD	13423.963	10446.219	2246.348	1586.973
MAPE	0.134	0.106	0.133	0.096
RMSE	15289.986	12577.033	3379.293	2482.687
MER	0.134	0.105	0.112	0.079
RMSPE	0.155	0.137	0.241	0.192

SARIMA: Seasonal autoregressive integrated moving average; SARFIMA: Seasonal autoregressive fractionally integrated moving average; MAD: Mean absolute deviation; MAPE: Mean absolute percentage error; RMSE: Root mean square error; MER: Mean error rate; RMSPE: Root mean square percentage error.

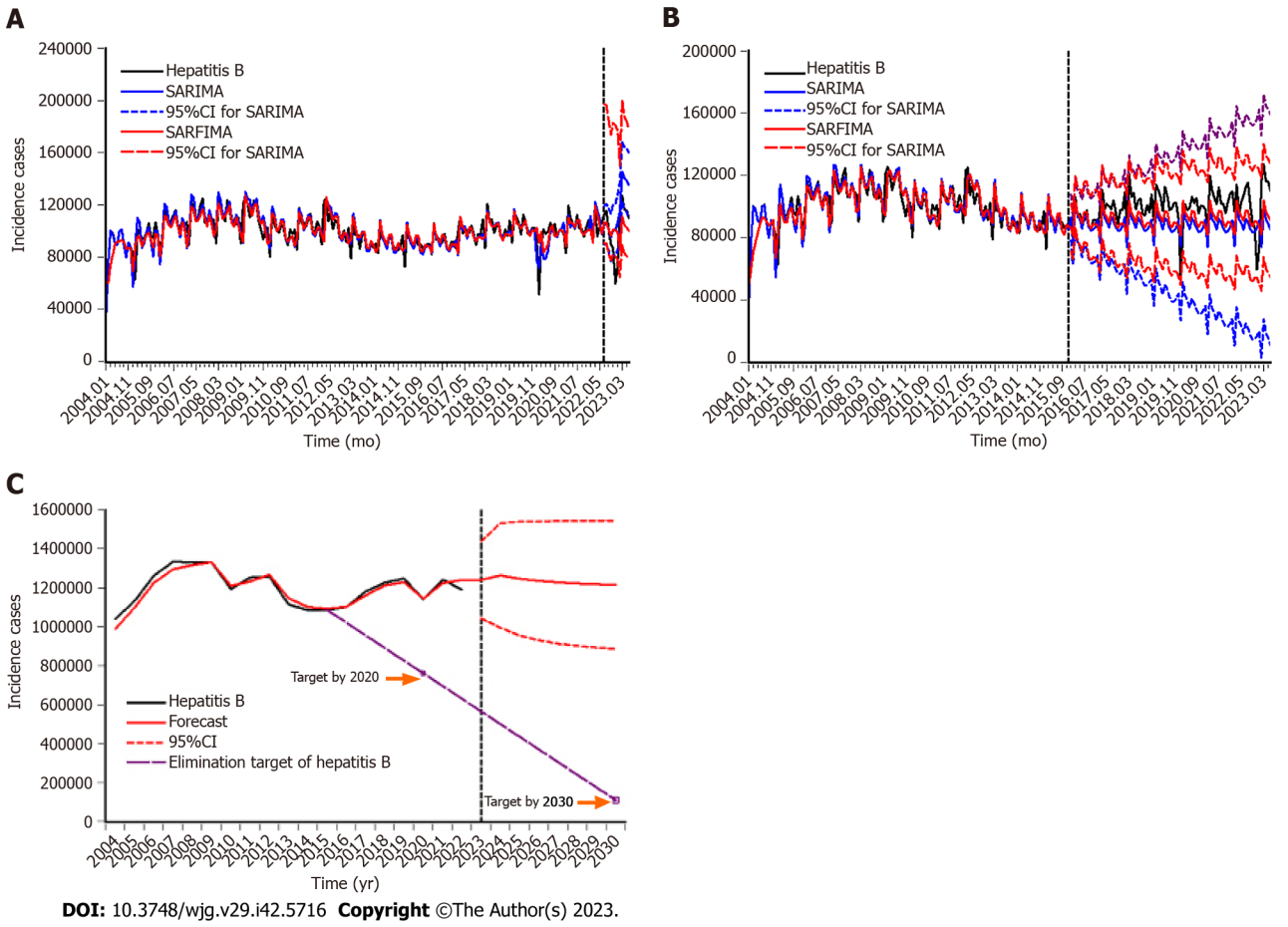
Table 2 Projection into 2030 for hepatitis B and C incidence cases

Time (yr)	Hepatitis B		Hepatitis C	
	Forecasts	95%CI	Forecasts	95%CI
2023	1240031	1041413-1438650	210742	141933-279552
2024	1262930	994670-1531191	209337	149760-268915
2025	1245681	953797-1537566	213123	138049-288198
2026	1234951	929339-1540563	209276	117296-301256
2027	1227438	912670-1542207	208070	101135-315004
2028	1221949	900585-1543314	205350	84304-326395
2029	1217813	891418-1544208	203073	69221-336926
2030	1214607	884203-1545011	200514	54983-346044
Mean	1233175	938512-1527839	207436	107085-307786
Total	9865400	7508093-12222709	1659485	856681-2462290

95%CI: 95%confidence interval.

DISCUSSION

Hepatitis poses a major threat to public health globally[2,16]. Analyzing and predicting epidemics are of great importance for the prevention and control. This study represents an important contribution to the field as it is the first to explore the efficacy of SARFIMA in monitoring HB and HC epidemics, while also comparing its predictive accuracy with that of SARIMA. The results supported our initial hypothesis, demonstrating the SARFIMA as a more comprehensive approach for capturing the epidemic dynamics of HB and HC compared to the SARIMA. Importantly, the forecasting robustness was confirmed by our further sensitivity analyses (Supplementary Tables 5 and 6). Also, previous work indicated that the SARFIMA showcased a good performance in forecasting costs[13], road fatality rate[15], CO_2 emission[23], temperature [14], and stock markets[24]. These findings provide further validation for the effectiveness of SARFIMA as a promising alternative in monitoring the spread of HB and HC. Moreover, the application of SARFIMA can superbly contribute to

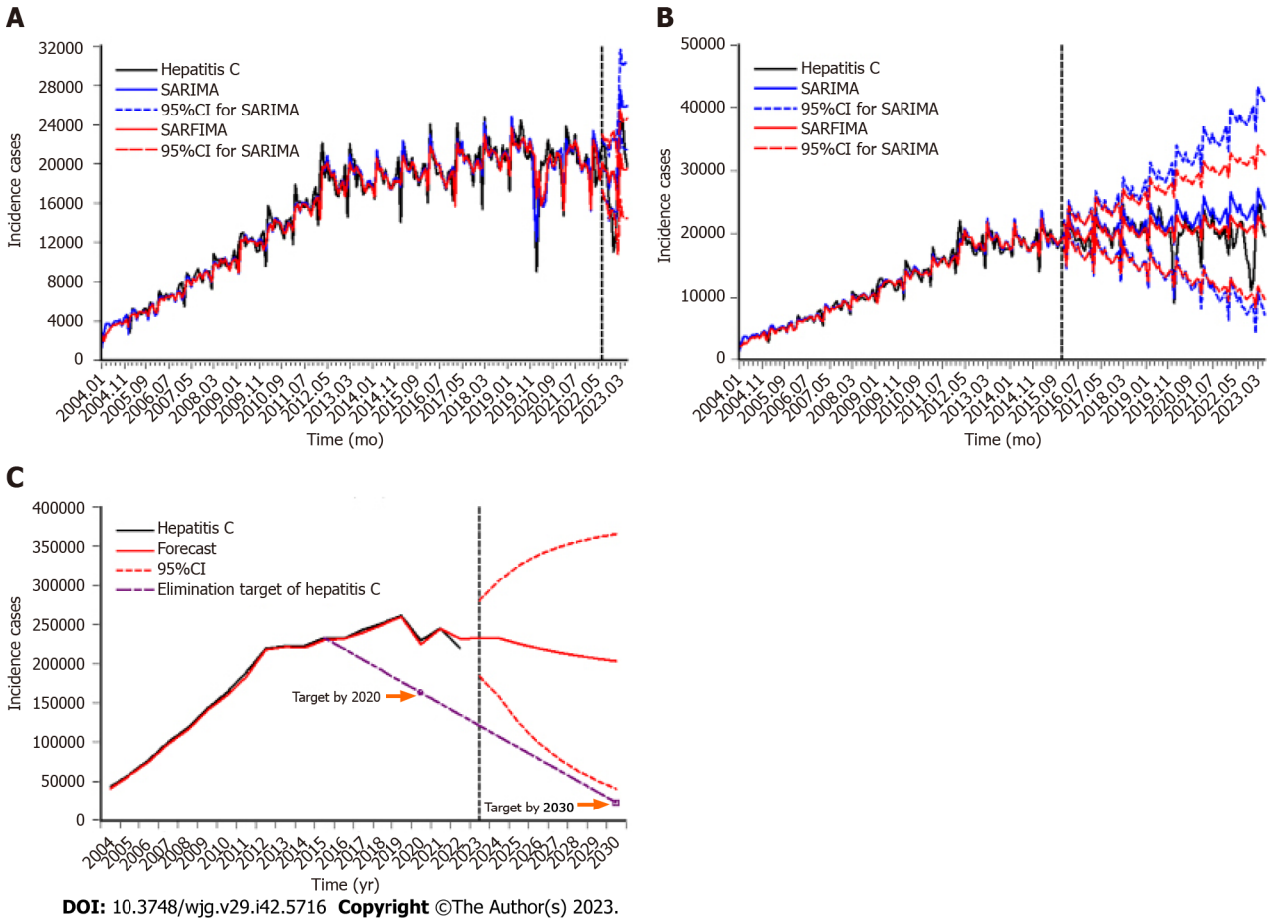


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Figure 2 Comparison of the hepatitis B incidence cases with the forecasts under the seasonal autoregressive integrated moving average and seasonal autoregressive fractionally integrated moving average. A: Comparison of the 12-step ahead forecasts under the seasonal autoregressive integrated moving average (SARIMA) and seasonal autoregressive fractionally integrated moving average (SARFIMA); B: Comparison of the 90-step ahead forecasts under the SARIMA and SARFIMA; C: Projection into 2030 under the SARFIMA. The predictive results are plotted in the right of the virtual vertical line. It seemed that the forecasts under the SARFIMA are in closer proximity to the actual hepatitis B incidence values and the predictive hepatitis B incidence curves would reach a plateau in the upcoming years. 95%CI: 95% confidence interval; SARFIMA: Seasonal autoregressive fractionally integrated moving average; SARIMA: Seasonal autoregressive integrated moving average.

guiding the intensity and type of public health measures. For example, if the model clearly showed an upsurge in the midst of receding HB and HC epidemics, it would suggest the effectiveness of measures currently in place. Instead, if the SARFIMA predicted a decline despite increasing HB and HC epidemics, the need for further or optimized measures should be heightened. These practical and actionable insights hold great promise for SARFIMA in monitoring and controlling HB and HC epidemics.

The SARIMA, as a reliable method for analyzing and forecasting time series data, has gained significant recognition within the fields of economics, finance, meteorology, and healthcare[11,17,18]. It can adapt to different time series patterns by adjusting the orders of nonseasonal and seasonal terms that incorporate trend, seasonal, and random components into the modelling framework, and can often generate acceptable forecasts[5,7,8]. As evidenced by our study, the SARIMA generated a relatively accurate forecast despite its inferiority to the SARFIMA in terms of predictive quality. The SARIMA has been indicated to be satisfactory in predicting the spread of various infectious diseases such as hepatitis [7], COVID-19[5], HFRS[9], HFMD[10], and tuberculosis[11]. Also, analysts well-versed in the underlying principles and the procedural steps involved with the SARIMA have been able to generate informed and precise forecasts, thereby assisting decision-making and planning efforts in containing the spread of diseases. Notwithstanding this, the SARIMA has indicated to be effective in capturing regular short-run dynamics and simple seasonality, and it often generates over-differencing[13,18]. By contrast, the SARFIMA combining the strengths of SARIMA and fractional integration can capture long-range dependence, handle complex seasonal patterns, accommodate both stationarity and non-stationarity, provide reliable parameter estimates, and offer robustness and stability[14,15,23], enabling it an invaluable tool for monitoring HB and HC epidemics, contributing to more informed decision-making and improved understanding of complex temporal dynamics. Considering that time series analysis is a crucial aspect of forecasting that combines various factors and the comprehensive effects of uncertain variables into a time variable, which is cost-effective and widely applicable in practice [25], promoting the adoption of SARFIMA can contribute to the improved accuracy and reliability of modelling and forecasting other infectious diseases. However, it is crucial to further validate its generalization. It is also worth mentioning that recent studies have unveiled satisfactory applications of alternative models such as Bayesian structural



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Figure 3 Comparison of the hepatitis C incidence cases with the forecasts under the seasonal autoregressive integrated moving average and seasonal autoregressive fractionally integrated moving average. A: Comparison of the 12-step ahead forecasts under the seasonal autoregressive integrated moving average (SARIMA) and seasonal autoregressive fractionally integrated moving average (SARFIMA); B: Comparison of the 90-step ahead forecasts under the SARIMA and SARFIMA; C: Projection into 2030 under the SARFIMA. The predictive results are plotted in the right of the virtual vertical line. It appeared that the forecasts under the SARFIMA are in closer proximity to the actual hepatitis C incidence values and the predictive hepatitis C incidence cases would begin to recede in the next years. 95%CI: 95% confidence interval; SARFIMA: Seasonal autoregressive fractionally integrated moving average; SARIMA: Seasonal autoregressive integrated moving average.

time series and innovation state-space framework for assessing the epidemics of diseases[26,27]. Accordingly, additional studies focus on comparing and confirming the forecasting performance of these models alongside the SARFIMA.

Different from the global declined trend in HB and HC incidences[2], an overall increase at an average rate of 0.44% for HB and 8.91% for HC per year was noted in our study, also consistent with earlier studies in Guangxi[8], China[4], and Pakistan[28]. There are several possible explanations for this trend. First, a study revealed that only around 20% of individuals infected with HBV in China were diagnosed[29]. While the gradual improvements in surveillance systems and improved diagnostic capabilities contribute to the detection of more cases[30]. Second, despite the successful implementation of the neonatal HB vaccination program, which has led to a noticeable decline in HB incidence and transmission, the prevalence of HB and HC remains alarmingly high in China. This is primarily due to the extensive population, approximately 296 and 58 million individuals, living with HB and HC, respectively. Of particular concern is the about 5% prevalence of HB surface antigen (HBsAg) and 2% prevalence of anti-HCV in women of reproductive age [31,32], with mother-to-child transmission being a significant risk. This poses a substantial challenge in terms of advancing diagnostic coverage, eradicating HBV and HCV infections, and reducing mortality. Third, despite the high curative rate achieved by direct-acting antiviral agents (DAAs) treatment for patients with HC[33], China's actual diagnostic rate for HC is only 2.1%[34]. Due to this low diagnostic rate, a smaller number of HC patients receive antiviral therapy, contributing to the ongoing spread of HC from high-risk populations to the general population. Lastly, coinfections of sexually transmitted diseases (STDs), HB, and HC are common and significantly contribute to long-term morbidity and mortality. In recent years, there has been a rapid increase in the incidence of STDs in China[35]. The high rates of HB and HC among individuals with STDs are predominantly driven by injection drug use (IDU) and sexual transmission, particularly among men who have sex with men (MSM)[36]. A significant decline in HB incidence during 2007-2014 can be attributed to the incorporation of HB prevention and treatment into the "Eleventh Five-Year" and "Twelfth Five-Year" plans in China. Comprehensive measures such as strengthening vaccination, enhancing public awareness and education, and conducting training programs were implemented[4,37]. Previous studies indicated that there was an upturn in HB cases in China since 2016/2017[7,8], aligning with our results, possibly attributable to the

accelerated urbanization process, a significant increase in the migrant population, a rapid rise in co-infections with HB, and the heavy economic burden. HC has insidious onset and nonspecific symptoms, making it difficult to be detected in the early stages. Although there is no effective vaccine for HC so far, with the gradual expansion of monitoring and testing coverage and the reporting of several outbreaks, more people have undergone screening, resulting in a rapid increase in confirmed cases during 2004-2012[30], and this matched well with our findings. Recent years have witnessed a decline in HC, which may be associated with the strict screening of blood donors and the standardized management of blood products, the comprehensive implementation of monitoring, early warning, intervention, and assessment measures for infectious diseases, the improvement in public knowledge-attitude-behavior regarding hepatitis, the continued optimization of policies, and the improvement of medical insurance[4,30]. Besides, according to the predicted figures for 2030, HB reached a plateau and HC receded. Thus, it can be said that the elimination of HB and HC by 2030 under current interventions faces enormous challenges. Therefore, addressing these challenges requires a comprehensive approach that encompasses prevention, diagnosis, and treatment[2,4]. First, efforts should be focused on expanding the reach of the neonatal HB vaccination program to ensure that all infants are protected from HBV infection[2,4]. Additionally, targeted vaccination campaigns should be implemented to reach vulnerable populations, such as women of reproductive age, in order to reduce the risk of mother-to-child transmission[3]. Second, it is imperative to improve the diagnostic rate of both HBV and HCV infections in China. This can be achieved through increased awareness and education among healthcare professionals, as well as the general population, regarding the importance of early detection and screening[2,4]. Furthermore, the establishment of screening programs in high-risk areas and the provision of affordable and accessible diagnostic tests can significantly contribute to improving the diagnostic rates. Third, it is crucial to strengthen the healthcare infrastructure and expand access to antiviral therapies for both HB and HC[2,4]. This can be achieved by training healthcare professionals in the latest treatment guidelines and protocols, as well as ensuring the availability and affordability of antiviral drugs. Lastly, efforts should be made to reduce the stigma associated with these diseases, as this can act as a barrier to seeking medical help and receiving appropriate treatment[2,4].

HB and HC show a seasonal profile in this study, with a trough in February and a peak in March, consistent with prior reports[7]. The seasonal trough during Spring Festival is largely attributed to people's reluctance to seek medical treatment and under-reporting that is more severe than in other months. However, the seasonal peak is associated with large-scaled population movement after Spring Festival and increased participation in various entertainment activities during the holiday. In China, although the primary mode of HB and HC transmission has shifted from drug use to sexual transmission, injecting drugs and engaging in unprotected high-risk sexual activities following drug use are common practices[38]. People's behavior is evolving to recognize the potential transmission risks of HCV/HBV, such as unprotected sexual contacts and IDU[38]. This shift is resulting in a growing demand for testing, as individuals are becoming more conscious of the need to check their status after festive events, leading to an increase in patients during this period. However, some studies have also indicated that HB and HC incidences follow the epidemiological characteristics of blood-borne and sexually transmitted diseases, with less pronounced seasonality[30].

Shortcomings also need to be considered in this study. First, data was taken from a passive monitoring system, and under-reporting is inevitable. Second, regional heterogeneity varied greatly in HB and HC incidences in China, and the region-specific forecasting ability of SARFIMA may require additional validation. Third, to capture timely information, the model requires integrating new data duly. Fourth, limited data may not exhibit long-term dependencies, and thus a series comprising 100 or more samples is recommended in application[17]. Fifth, due to the lack of available data pertaining to the nutritional status of the population, comorbidities such as diabetes and hypertension, the specifics of daily water consumption, and immunological resistance related to the genetic traits of the Chinese population, we are unable to provide a more detailed analysis of how these factors may impact the observed results. Lastly, as to whether the SARFIMA is transferable to monitor HB and HC in other study regions or other infectious diseases, verification is warranted.

CONCLUSION

Overall, HB remains steady while HC is rising in China, and both exhibit a seasonal pattern, a peak in March and a trough in February. Under current interventions, additional feasible and effective control strategies (e.g., expanding the scope of adult HB vaccination, a breakthrough in vaccination for HC, preventing mother-to-child transmission, investigating high-risk factors, implementing standardized antiviral treatment in rural areas, and enhancing health education and promotion) require to be designed to ensure the elimination of HB and HC by 2030. The SARFIMA provides a more sophisticated and adaptable framework for capturing intricate patterns and interdependencies in monitoring HB and HC epidemics, as opposed to SARIMA. This ultimately leads to enhanced forecasting capabilities and a deeper comprehension of the underlying process. Consequently, the integration of SARFIMA into public health decision-making for the management of HB and HC epidemics can result in more informed and efficacious interventions.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B (HB) and hepatitis C (HC) have the largest burden in China, and a goal of eliminating them as a major public health threat by 2030 has been raised.

Research motivation

Accurate prediction helps to anticipate possible scenarios and make proactive choices, enabling policymakers to make informed decisions, plan strategies, and prepare for potential challenges and opportunities.

Research objectives

This study aimed to evaluate the usefulness of seasonal autoregressive fractionally integrated moving average (SARFIMA) in monitoring HB and HC epidemics (projection into 2030) in mainland China and to assess the forecasting potential of SARFIMA compared to seasonal autoregressive integrated moving average (SARIMA).

Research methods

The monthly incidence cases of HB and HC from January 2004 to June 2023 were obtained. Then, the two periods (from January 2004 to June 2022 and from January 2004 to December 2015, respectively) were used as the training sets to build the SARFIMA and SARIMA models, while the remaining periods served as the test sets to evaluate the forecasting accuracy of both models.

Research results

During the study period, a total of 23400874 HB cases and 3590867 HC cases were reported. In the 12-step-ahead HB, 90-step-ahead HB, 12-step-ahead HC, and 90-step-ahead HC forecasts, the best SARFIMA generated lower error rates compared with the best SARIMA. The predicted HB incidents totaled 9865400 [95% confidence interval (95%CI): 7508093-12222709] and HC totaled 1659485 (95%CI: 856681-2462290) during 2023-2030.

Research conclusions

The SARFIMA provides a more sophisticated and adaptable framework for capturing intricate patterns and interdependencies in monitoring HB and HC epidemics compared with the SARIMA. This ultimately leads to enhanced forecasting capabilities and a deeper comprehension of the underlying process.

Research perspectives

The integration of SARFIMA into public health decision-making for managing HB and HC epidemics can result in more informed and efficacious interventions.

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FOOTNOTES

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ORIGINAL ARTICLE

Observational Study

Location-based prediction model for Crohn's disease regarding a novel serological marker, anti-chitinase 3-like 1 autoantibodies

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Abstract

BACKGROUND

Defective neutrophil regulation in inflammatory bowel disease (IBD) is thought to play an important role in the onset or manifestation of IBD, as it could lead to damage of the intestinal mucosal barrier by the infiltration of neutrophils in the inflamed mucosa and the accumulation of pathogens. Like neutrophils in the context of innate immune responses, immunoglobulin A (IgA) as an acquired immune response partakes in the defense of the intestinal epithelium. Under normal conditions, IgA contributes to the elimination of microbes, but in connection with the loss of tolerance to chitinase 3-like 1 (CHI3L1) in IBD, IgA could participate in CHI3L1-mediated improved adhesion and invasion of potentially pathogenic microorganisms. The tolerance brake to CHI3L1 and the occurrence of IgA autoantibodies to this particular target, the exact role and underlying mechanisms of CHI3L1 in the pathogenesis of IBD are still unclear.

AIM

To determine the predictive potential of Ig subtypes of a novel serological marker,

anti-CHI3L1 autoantibodies (aCHI3L1) in determining the disease phenotype, therapeutic strategy and long-term disease course in a prospective referral cohort of adult IBD patients.

METHODS

Sera of 257 Crohn's disease (CD) and 180 ulcerative colitis (UC) patients from a tertiary IBD referral center of Hungary (Division of Gastroenterology, Department of Internal Medicine, Faculty of Medicine, University of Debrecen) were assayed for IgG, IgA, and secretory IgA (sIgA) type aCHI3L1 by enzyme-linked immunosorbent assay using recombinant CHI3L1, along with 86 healthy controls (HCONT).

RESULTS

The IgA type was more prevalent in CD than in UC (29.2% *vs* 11.1%) or HCONT (2.83%; $P < 0.0001$ for both). However, sIgA subtype aCHI3L1 positivity was higher in both CD and UC patients than in HCONT (39.3% and 32.8% *vs* 4.65%, respectively; $P < 0.0001$). The presence of both IgA and sIgA aCHI3L1 antibodies was associated with colonic involvement ($P < 0.0001$ and $P = 0.038$, respectively) in patients with CD. Complicated disease behavior at sample procurement was associated with aCHI3L1 sIgA positivity (57.1% *vs* 36.0%, $P = 0.009$). IgA type aCH3L1 was more prevalent in patients with frequent relapse during the disease course in the CD group (46.9% *vs* 25.7%, $P = 0.005$). In a group of patients with concomitant presence of pure inflammatory luminal disease and colon involvement at the time of diagnosis, positivity for IgA or sIgA type aCH3L1 predicted faster progression towards a complicated disease course in time-dependent models. This association disappeared after merging subgroups of different disease locations.

CONCLUSION

CHI3L1 is a novel neutrophil autoantigenic target in IBD. The consideration of antibody classes along with location-based prediction may transform the future of serology in IBD.

Key Words: Chitinase 3-like 1 autoantibodies; Crohn's disease; Ulcerative colitis; Disease progression; Immunoglobulin subtypes; Enzyme-linked immunosorbent assay

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Core Tip: The tolerance brake to chitinase 3-like 1 (CHI3L1), a novel neutrophil autoantigenic target in inflammatory bowel disease (IBD), the presence of immunoglobulin A (IgA) autoantibodies against this specific target, as well as the precise function and underlying processes of CHI3L1 in the development of IBD, continue to be uncertain. In the present prospective observational study, we first reported an enhanced formation of IgA and secretory IgA (sub)type against CHI3L1 in adult patients with Crohn's disease, which was associated with the clinical phenotype and development of a complicated disease course during follow-up in a tertiary referral IBD center in Hungary. By taking into account the classes of antibodies and utilizing location-based predictions, serology in IBD may undergo a significant transformation in the future.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory condition involving all or parts of the gastrointestinal tract with a potential to present extraintestinal manifestations (EIM). The two main clinical entities are Crohn's disease (CD) and ulcerative colitis (UC), both of which have heterogeneous presentations and disease courses. Their significance lies in their increasing incidence, which mainly affects the young, still active, and working-age population[1].

The exact pathophysiology of the disease has not yet been fully elucidated. Partially unknown environmental factors are thought to trigger an uncontrolled immune-mediated inflammatory process in genetically predisposed individuals, in which altered microbiome, dysfunction of the intestinal mucosal barrier and dysregulation of the mucosal immune response to commensal or pathogenic microbes of the intestinal microbiota are key players[2-5]. This concept is well represented in the new immunological classification of autoimmune and autoinflammatory diseases, where mixed-pattern conditions such as IBD are acknowledged. The latter are considered to belong to the group of immune-mediated inflammatory disorders (IMIDs) in this autoimmune-autoinflammatory spectrum[6-9]. Like other IMIDs (*e.g.*, rheumatoid arthritis), IBD also exhibits features of autoimmunity with a dysregulated adaptive immune response (the breakdown of immune tolerance, the identification of self-antigens, and the activation of T cells and B cells, resulting in the creation of

specific autoantibodies) and autoinflammatory characteristics with the activation of the innate immune system (involvement of neutrophils, macrophages, as well as dendritic-, natural killer-, and mast cells, different granulocyte subsets, and the complement system as major players)[6,10,11].

Crosstalk between the gut microbiota and the host occurs at the intestinal mucosal level[12]. The mucosal immune system maintains the balance between tolerance to commensal bacteria and the immune response against pathogens and is responsible for mucosal homeostasis[13]. Mechanical and/or immunological disruption of the gut barrier can lead to the enhanced uptake of bacteria and bacterial products from the gut lumen [bacterial translocation (BT)], which can trigger and perpetuate chronic inflammation [14,15]. However, whether this phenomenon in IBD is a primary defect or consequence is still under debate[14,16]. Supported by robust scientific data, impaired sensing of intestinal bacteria by cytoplasmic and membrane-bound pattern recognition receptors (NOD-like and Toll-like receptors, respectively) is thought to play a role in IBD development[17]. Currently, evidence is accumulating, that the formation of auto- and antimicrobial antibodies in IBD appears to be the result of enhanced microbial load[18].

Several types of innate immune cells are involved in IBD immunopathogenesis[14]. Dysregulated neutrophil function can lead to damage to the intestinal mucosal barrier due to the infiltration of neutrophils into the inflamed mucosa and the accumulation of harmful pathogens[19,20]. In UC, the infiltration and accumulation of neutrophils were found to be elevated and associated with disease activity, whereas in CD, impaired recruitment of neutrophils, reduced production of cytokines and phagocytosis, and delayed/defective antimicrobial clearance were described[21,22]. Clinical utility of blood-based and fecal neutrophil-related biomarkers has been studied extensively in IBD. In the latter group, fecal calprotectin has been proven to be the most helpful in monitoring disease activity and is recommended by clinical practice guidelines in various clinical settings (initial diagnosis, diagnosis of relapse, and response to treatment)[22,23]. Other fecal neutrophil-derived biomarkers are lactoferrin, lysozyme, polymorphonuclear neutrophil elastase, myeloperoxidase, human neutrophil peptides, neutrophil gelatinase-associated lipocalin, and chitinase 3-like 1 (CHI3L1)[24-26]. Similarly to fecal calprotectin, fecal CHI3L1 appears to correlate well with endoscopic activity in patients with CD[27,28].

Recently, loss of tolerance to a novel neutrophil autoantigenic glycoprotein target, CHI3L1 has been confirmed by our group in IBD patients[21,29,30]. Evaluation of autoantibodies against CHI3L1 (aCHI3L1) was performed in a pediatric IBD cohort[30]. Immunoglobulin A (IgA), a key player in acquired immune responses, contributes to the defense of the intestinal epithelium, similarly to how neutrophils are involved in innate immune responses. Under typical circumstances, IgA plays a crucial role in eliminating microbes through its antibacterial and antiviral activities. However, in the context of diminished tolerance to CHI3L1 in IBD, IgA may participate in the enhanced adhesion and invasion of potentially pathogenic microorganisms, as facilitated by CHI3L1. Given the current state of knowledge, the precise function and mechanism of CHI3L1 in the development of IBD, loss of tolerance to this antigen, formation of IgA autoantibodies against it, and their role in the pathogenesis of IBD remain elusive[21,30].

The main objective of this research was to identify the frequency and predictive value of Ig subtypes of a newly discovered serological marker, aCHI3L1, in relation to the development of disease-specific complications or the need for surgery in a large group of adult IBD patients with a prospective follow-up. Additionally, we aimed to investigate: The long-term stability of aCHI3L1 autoantibodies; the relationship between aCHI3L1 formation and the clinical, serological, and genetic characteristics of CD.

MATERIALS AND METHODS

Patient population

We performed a cohort study among adult CD and UC patients in a tertiary IBD referral center in Hungary (Department of Gastroenterology, Institute of Internal Medicine, University of Debrecen). The baseline clinical data regarding this cohort overlap with those of our previous studies[29,31,32]; however, we present an extended follow-up time with nearly 2.5 years and re-evaluation of the outcomes. We used the same step-by-step thorough statistical evaluations; therefore, the text appeared to reproduce the information reported in detail elsewhere. The clinical characteristics of patients at diagnosis are presented in Table 1 in detail. The original CD cohort consisted of 271 patients, however 5 of them should have been excluded from the statistical analysis after performing total Ig measurements due to decreased or absent serum IgA levels equivalent of international consensus laboratory criteria of severe selective IgA deficiency (serum IgA level less than 7 mg/dL or 0.07 g/L - the lowest detectable limit established by most of the laboratories)[33-35].

The diagnosis of IBD was based on a combination of clinical, biochemical, stool, endoscopic, cross-sectional imaging, and histological investigations equivalent to the recently published European Crohn's and Colitis Organization (ECCO) and the European Society of Gastrointestinal and Abdominal Radiology guidelines[23,36]. Disease phenotype (age at onset, duration, location/extent, and behavior including perianal involvement as a modifier) was determined according to the Montreal Classification[37]. Blood samples and detailed clinical phenotypes were captured at inclusion. Clinical data were determined by a thorough review of the patients' medical records, which were collected in a uniform format. Medical records documenting the disease phenotype, presence of EIM, frequency of flare-ups (frequent flare-up: > 1 clinical relapse/year)[38], medication use (*e.g.*, steroid, immunosuppressive and/or biological use at any time), need for IBD-related surgery (resection and surgical intervention due to perianal disease complications in CD and colectomy in UC), and IBD-related risk factors (the presence of familial IBD, smoking habits and previous appendectomy) were retrospectively analyzed for the period prior to prospective follow-up. At enrolment, clinical disease activity was calculated according to the Harvey-Bradshaw Index (HBI)[39] for CD and the Partial Mayo Score (PMS) for UC[40]. In this study, we followed the ECCO guidelines[41] and defined HBI ≤ 4 as a state of remission and ≥ 5 as a state of active disease. In case of UC, PMS ≤ 3 was defined as a state of remission and PMS > 4 as a state of active disease. Endoscopic

Table 1 Clinical characteristics of inflammatory bowel disease patients, *n* (%)

	CD (*n* = 266)	UC (*n* = 187)
Age at presentation (yr), median (IQR)	25 (19-33)	33 (23-43)
Male/female (*n*)	112/154	86/101
Follow-up (mo) from, median (IQR)		
Diagnosis[1]	143 (99-214)	135 (84-213)
Time of serum collection, median (IQR)	97 (77-118)	78 (51-102)
Location/extent at diagnosis		
L1	58 (21.8%)	E1 30 (16.0%)
L2	86 (32.3%)	E2 104 (55.6%)
L3	121 (45.5%)	E3 53 (28.3%)
L4 only	1 (0.4%)	
Behavior at diagnosis/last follow up		
B1	213 (80.1%)/117 (44.8%)	
B2	32 (12.0%)/54 (20.7%)	
B3	21 (7.9%)/90 (34.5%)	
Perianal disease		
At diagnosis/last follow-up[1]	48 (18.0%)/100 (38.3%)	
Extraintestinal manifestations		
PSC	8 (3.0%)	8 (4.3%)
Arthritis	49 (18.4%)	26 (13.9%)
Skin	35 (13.2%)	16 (8.6%)
Ocular	65 (24.4%)	12 (6.4%)
Smoking habits		
Never	215 (80.8%)	167 (89.3%)
Yes	46 (17.3%)	18 (9.6%)
Previous	5 (1.9%)	2 (1.1%)
Familial IBD	12 (4.4%)	6 (3.2%)
Frequent relapse	52 (20.9%)	
Cumulative exposure of medication and surgeries during follow-up		
Steroid use/refractory	219 (82.3%)/30 (13.7%)	117 (63.9%)/11 (7.6%)
Azathioprine use	198 (74.4%)	70 (38.3%)
Biological use	112 (42.1%)	25 (13.4%)
Resective surgery/multiple in CD	117 (44.8%)/ 33 (12.6%)	
Colectomy in UC		11 (6.0%)
Surgery due to perianal complication/multiple	61 (23.4%)/33 (12.6%)	

[1]261 Crohn's disease and 183 ulcerative colitis patients had follow-up.

Location: L1: Ileal, L2: Colonic, L3: Ileocolonic, L4: Upper gastrointestinal disease. Behavior: B1: Inflammatory (non stricturing/non penetrating), B2: Stenosing (stricturing), B3: Penetrating. Disease extent: E1: Proctitis; E2: Left sided colitis; E3: Extensive colitis. Surgery: Crohn's disease-related abdominal surgery and colectomy in ulcerative colitis. CD: Crohn's disease; IQR: Interquartile range; UC: Ulcerative colitis; PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease.

activity was determined according to the Simple Endoscopic Score for Crohn's Disease (SES-CD) for CD[42] and the endoscopic component of the Mayo score for UC[43]. SES-CD defines endoscopic activity ≥ 3 points and inactive disease ≤ 2 in CD; meanwhile in UC, state of active disease is defined as an invasive PMS ≥ 1.

Phenotypical characterization of IBD patients during prospective follow-up

A total of 261 of 266 patients with CD and 183 of 187 patients with UC were enrolled in a prospective follow-up study. During regular and extraordinary outpatient follow-up visits and inpatient stays, the treating IBD physicians registered laboratory data, endoscopic and imaging results, disease activity, medical treatment regimens, and information on disease-specific complications and surgeries. In Hungary, a follow-up visit is typically scheduled every six months at a specialized gastroenterology center, which can vary between three and six months depending on the center. The medical and surgical treatment algorithms are aligned and conform to the ECCO guidelines[41,44-47]. The decision regarding the need for surgery and its timing involves a multidisciplinary approach, with the collaboration of gastroenterologists, radiologists, and surgeons. The data that was collected was transferred and saved in a database for the purpose of further evaluation. On October 11, 2016, all patient records and databases were revised and updated with the relevant data points. The patient's follow-up was discontinued if there were no additional records. The median duration of follow-up from diagnosis for CD patients was 143 mo [with an interquartile range (IQR) of 99 to 214], while for UC patients, it was 135 mo (with an IQR of 84 to 213). In CD, complicated disease behavior is characterized by the presence of stenosis (stricture) or internal penetration (interintestinal fistula, inflammatory conglomerate, and/or abscess(es) formation). A distinction was made between perianal fistulizing disease and internal penetrating disease, and they were evaluated separately. The need for surgery in CD was defined as CD-related abdominal- (resection) or perianal surgery (oncotomy). Complicated disease behavior in UC was defined as the progression of disease extent or the need for colectomy.

The healthy controls (HCONT) included 86 age- and sex-matched healthy individuals. Of these, 52 were male and 34 were female, with a median age of 28 years at disease onset (range: 24-41). The control group comprised of individuals who were free from gastrointestinal and liver diseases and were obtained from invent Diagnostica GmbH (Hennigsdorf, Germany).

Serological analysis

At enrollment, blood samples were obtained from each participant and were subsequently frozen at a temperature of -80 °C until further testing. All serological assays were conducted in a blinded manner, without prior knowledge of the patient's diagnosis or any other clinical information. To determine the stability of different serologic antibodies, we analyzed samples from the same patient taken at various arbitrary timepoints during the course of their disease. Most patients with CD (n = 165) had at least two serum samples taken and re-tested for all serological antibodies.

Detection of antibodies to CHI3L1 by enzyme-linked immunosorbent assays

Presence of IgG, IgA and secretory (sIgA) (sub)type aCHI3L1 in serum samples of IBD patients (n = 257 in CD and n = 180 in UC) and HCONT (n = 86) were determined by an "in house" enzyme-linked immunosorbent assay technique using recombinant human CHI3L1 as solid-phase antigen. A detailed description of the methodology is available in the authors' previous paper on pediatric IBD[30]. The levels of aCHI3L1 IgG, IgA, or sIgA were read as the ratio of OD_{sample}/OD_{cutoff} (OD_{cutoff}, mean + 3 SD of HCONT) for standardization. Serum samples with a ratio of ≥ 1 were considered positive [30].

Detection of classic auto- and antimicrobial antibodies and NOD2/CARD15 SNP8, 12, 13 mutations

The detection of classic auto- [anti-neutrophil cytoplasmic antibodies (ANCA), anti-pancreatic autoantibodies (PAbs), antiphospholipid antibodies (APLAs)], and antimicrobial [anti-*Saccharomyces cerevisiae* antibodies (ASCA), anti-OMP PlusTM IgA] antibodies has been described in detail by the authors in previous publications[29,31,32]. NOD2/CARD15 SNP8, SNP12, and SNP13 genotyping was performed previously[48] in patients with CD (n = 235). A detailed description of the methodology has been provided in the cited manuscript.

Ethical permission

The study was granted ethical approval by the regional and national committees for research ethics (DE RKEB/IKEB: 4773-2017; DE OEC RKEB/IKEB: 3515-2011; TUKEB: ad.3880/2012-EKU). All patients were informed about the study's details and provided their informed consent.

Statistical analysis

We utilized GraphPad Prism 6 (San Diego,1 CA) and SPSS 22.0 (SPSS Inc., Chicago, IL) for statistical analysis. The study's statistical methods were reviewed by Elek Dinya, from Semmelweis University's Institute of Health Informatics, Development, and Further Training. The normality of variables was evaluated using the Shapiro-Wilk's W test. The continuous variables were presented in the form of means ± SD or medians and IQR based on their consistency. To determine distinctions between the IBD and HCONT groups, as well as among subgroups of patients with IBD, the following statistical methods were employed: Categorical variables were compared using Fisher's exact test or χ^2 test with Yates correction, depending on the situation. To compare continuous variables, we used Student's t-test, one-way analysis of variance (ANOVA), Mann-Whitney's U test, or Kruskal-Wallis H test with post hoc analysis (Dunn's multiple comparison test). The Spearman's nonparametric rank correlation test was used to determine correlations.

The Kaplan-Meier survival curves were used to examine the impact of categorical clinical variables or serologic antibodies on unfavorable disease outcomes during follow-up. To determine the association, the log-rank test or Cox regression analysis was conducted in time-dependent models. Cox regression analysis and a backward elimination method were used to investigate the predictive value of serological antibodies and all clinical variables as cofactors for the development of a complicated disease course in CD patients. The associations were expressed as odds ratio (OR) and hazard ratio (HR) accompanied by 95% confidence interval (CI). A probability value of less than 0.05, indicating a 2-tailed test, was deemed statistically significant.

RESULTS

Frequency of aCHI3L1 antibodies in IBD

The frequencies of different Ig (sub)types of aCHI3L1 antibodies in patients with IBD and controls are summarized in Table 2. The occurrence of IgA-type aCHI3L1 was higher in CD than in UC (29.2% *vs* 11.1%) or HCONT (2.83%; $P <$ 0.0001 for both). The sIgA subtype aCHI3L1 was more prevalent among both CD and UC patients than in the HCONT group (39.3% and 32.8% *vs* 4.65%, respectively; $P < 0.0001$). No differences were found in IgG antibodies in either group. Information on the sensitivity and specificity of aCHI3L1 antibodies as diagnostic tests is available in Supplementary Table 1.

The stability of aCHI3L1 antibodies in CD and its correlation with the overall disease duration

In a subset of CD patients ($n = 165$), more than one serum sample per patient was available at different time points to assess the stability of antibody levels and statuses (positive or negative for the respective antibodies) over time. The median time between sample procurements was 44.4 mo (IQR: 23.3-66.6). Comparing the results of the first and last available serum samples using the Wilcoxon test, no significant differences in aCHI3L1 antibody levels were found for any Ig type. The aCHI3L1 antibody status was equally stable for all Ig types, with an average of < 10% of patients showing a change in antibody status over time. The IgA type antibody status showed a slightly greater change (20.6%). The stability data for different serological antibodies are summarized in Table 3. There were no clinically significant differences in the evaluated study endpoints when considering stability data. Further studies with sequential sampling and serial measurements both before and after the study endpoint events are needed for a more detailed evaluation of aCHI3L1 antibody stability in CD, which is beyond the limits of the current work.

No correlation was found between antibody status and clinical or endoscopic disease activity (as measured by either HBI or SES-CD) or disease duration at the time of sample acquisition (Supplementary Table 2). aCHI3L1 IgG levels were not analyzed further because of the low prevalence of antibody positivity in the CD cohort. IgA and sIgA aCHI3L1 antibody levels did not differ according to clinical activity ($P = 0.385$ and 0.6830, respectively). Nevertheless, the actual CDAI, HBI, and SES-CD indices were also not correlated with IgA and sIgA aCHI3L1 antibody levels, as determined by Spearman correlation analysis (Supplementary Table 3). The levels of aCHI3L1 antibodies were not associated with disease duration (Kruskal-Wallis test).

Association of aCHI3L1 antibody formation with clinical, serological, and genetic characteristics of CD

A summary of the results is presented in Table 4.

Correlation with clinical features: The presence of both IgA and sIgA aCHI3L1 antibodies was associated with colonic involvement (IgA: 35.5% *vs* 7.7%, $P < 0.0001$; sIgA: 43.5% *vs* 26.9%, $P < 0.038$) in CD. The occurrence of aCHI3L1 sIgA was higher in patients with complicated disease behavior at diagnosis compared to those with uncomplicated disease behavior (57.1% *vs* 36.0%, $P = 0.009$). Additionally, a higher prevalence of IgA-type aCH3L1 was observed in patients with frequent relapses during the course of CD than in those without (46.9% *vs* 25.7%, $P = 0.005$).

Association with serological and genetic characteristics: Significant associations were found between the presence of certain IgA antimicrobial (ASCA, anti-OMP), antiphospholipid (anti-PS/PT), and autoantibody [anti-glycoprotein 2 (GP2)] types, and aCHI3L1 IgA and sIgA positivity. The following correlations were found when examining these antibodies. Anti-ASCA, anti-OMP, and anti-PS/PT IgA showed a significantly higher prevalence of anti-CHI3L1 antibody IgA and sIgA in patients positive for the above-mentioned antibodies. For anti-GP2 positive patients, this association was only seen for anti-CHI3L1 sIgA, with no significant difference for anti-CHI3L1 IgA. However, anti-GP2 positive patients had the highest prevalence of anti-CHI3L1 sIgA. In our study, the prevalence of different aCHI3L1 antibodies was not associated with the presence or absence of major NOD2/CARD15 mutations (data not shown).

Significance of aCHI3L1 antibodies in predicting the development of a complicated disease course in CD

In time-dependent univariate models, a trend was observed between the presence of IgA or sIgA aCHI3L1 and the time to development of a complicated disease behavior with borderline significance (Figure 1).

Considering that the prevalence of antibodies against CHI3L1 was significantly higher in patients with colonic involvement (aCHI3L1 IgA positivity: L2/L3 35.5% *vs* L1 7.7%; sIgA: 43.5% *vs* 26.9%), we performed subgroup analysis. Kaplan-Meier analysis was used to analyze a group of patients with CD who had concomitant presence of pure inflammatory luminal disease (B1) and colon involvement at the time of diagnosis. The results showed that positivity for IgA or sIgA type aCH3L1 predicted faster progression towards a complicated disease course in time-dependent models (Figure 2). The association was no longer present after merging the subgroups of different disease locations (as shown in

Table 2 Prevalence of autoantibodies against chitinase 3-like 1 in inflammatory bowel disease patients

Serum aCHI3L1	CD (*n* = 257)		UC (*n* = 180)		HCONT (*n* = 86)	
	n	%	*n*	%	*n*	%
IgG	21	8.2%[b]	3	1.7%[b]	2	2.83%
IgA	75	29.2%[a,c]	20	11.1%[c,d]	2	2.83%[a,d]
sIgA	101	39.3%[a]	59	32.8%[e]	4	4.65%[a,e]

[a]$P \leq 0.0001$. Crohn's disease *vs* controls.
[b]$P = 0.003$. Crohn's disease *vs* ulcerative colitis.
[c]$P \leq 0.0001$. Crohn's disease *vs* ulcerative colitis.
[d]$P = 0.016$. Ulcerative colitis *vs* controls.
[e]$P \leq 0.0001$. Ulcerative colitis *vs* controls.
Using χ^2-test with Yates correction. CD: Crohn's disease; UC: Ulcerative colitis; IgA: Immunoglobulin A; sIgA: Secretory immunoglobulin A; aCHI3L1: Anti-chitinase 3-like 1 autoantibodies; HCONT: Healthy controls.

Table 3 Stability of anti-chitinase 3-like 1 autoantibodies status over time in patients with Crohn's disease during the disease course

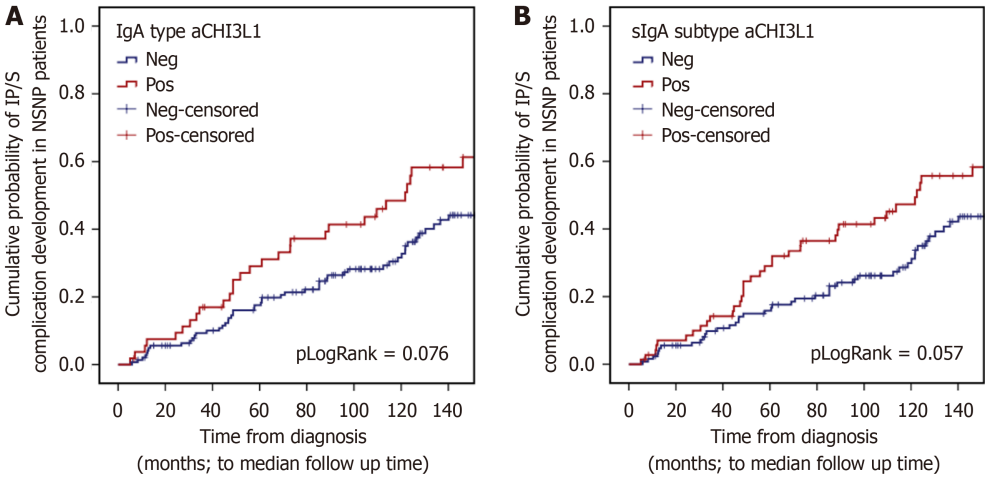
Serologic antibodies	(Sub)type	*n*	Stable negative, *n* (%)	Stable positive, *n* (%)	Negative to positive, *n* (%)	Positive to negative, *n* (%)
aCHI3L1	IgG	165	152 (92.1)	2 (1.2)	2 (1.2)	9 (5.5)
	IgA	165	92 (55.8)	39 (23.6)	17 (10.3)	17 (10.3)
	sIgA	165	83 (50.3)	51 (30.9)	14 (8.5)	17 (1.1)

aCHI3L1: Anti-chitinase 3-like 1 autoantibodies.

Table 4 Association of anti-chitinase 3-like 1 autoantibodies antibody formation with clinical, serological, and genetic characteristics of Crohn's disease at diagnosis and last follow-up

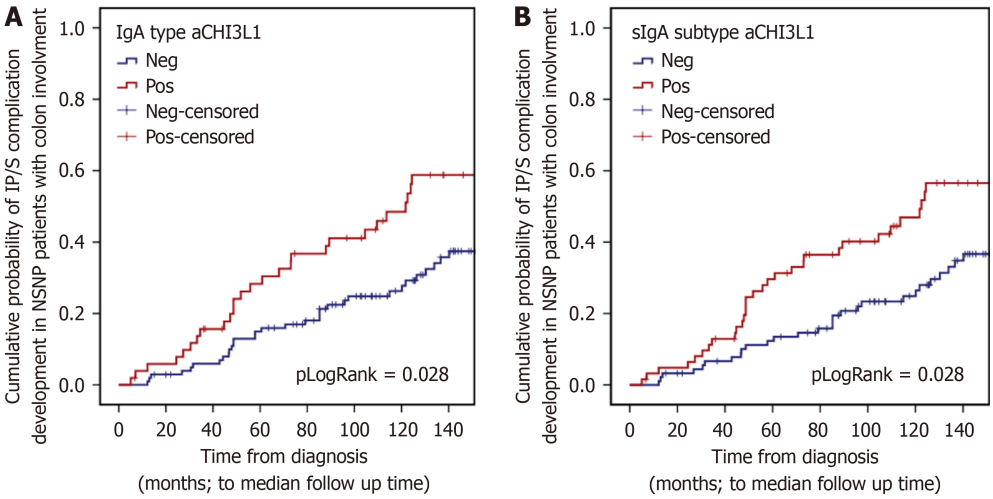
Anti-CHI3L1	IgA		sIgA	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
At diagnosis				
Complicated disease behaviour	1.86 (0.97-3.55)	0.081	2.37 (1.26-4.48)	0.009
Colon involvement	6.61 (2.29-19.07)	< 0.0001	2.09 (1.07-4.10)	0.038
ASCA IgA	4.11 (2.24-7.55)	< 0.0001	4.16 (2.40-7.19)	< 0.0001
OMP IgA	3.16 (1.79-5.57)	< 0.0001	2.64 (1.57-4.44)	< 0.0001
Anti-PS/PT IgA	3.46 (1.49-8.04)	0.005	2.56 (2.15-14.58)	< 0.0001
Anti-GP2 IgA	4.76 (0.87-26.18)	0.108	9.47 (1.12-79.52)	0.018
At last visit				
Frequent relapse	2.56 (1.34-4.91)	0.005	1.89 (1.00-3.56)	0.047

Column corresponding to immunoglobulin G type anti-chitinase 3-like 1 autoantibodies and rows corresponding to age at onset, perianal disease at diagnosis, extraintestinal manifestations, NOD2/CARD15 mutations and therapy were omitted because statistically significant differences for a given parameter were not obtained. Positive associations are indicated in bold and negative associations are in italics (*P* values, odds ratio and 95% confidence interval). IgA: Immunoglobulin A; sIgA: Secretory immunoglobulin A; aCHI3L1: Anti-chitinase 3-like 1 autoantibodies; OR: Odds ratio; CI: Confidence interval; ASCA: Anti-*Saccharomyces cerevisiae* antibody; OMP: Outer membrane protein; Anti-PS/PT: Anti-phosphatidylserine/prothrombin antibody; Anti-GP2: Anti-glycoprotein 2 antibody.



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Figure 1 Kaplan-Meier survival analysis for the probability of internal penetrating/stricturing complication development in Crohn's disease patients with non-stricturing and non-penetrating disease at diagnosis. A: Comparing survival curves of immunoglobulin A (IgA)-type anti-CHI3L1 autoantibody (aCHI3L1) positive *vs* negative subgroups; B: Comparing survival curves of secretory IgA-type aCHI3L1 positive *vs* negative subgroups. IP/S: Internal penetrating/stricturing; NSNP: Non-stricturing and non-penetrating; IgA: Immunoglobulin A; sIgA: Secretory immunoglobulin A; aCHI3L1: Anti-CHI3L1 autoantibodies.



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Figure 2 Kaplan-Meier survival analysis for the probability of internal penetrating/stricturing complication development in Crohn's disease patients with non-stricturing and non-penetrating disease and colonic involvement at diagnosis. A: Comparing survival curves of immunoglobulin A (IgA)-type anti-CHI3L1 autoantibody (aCHI3L1) positive *vs* negative subgroups; B: Comparing survival curves of secretory IgA-type aCHI3L1 positive *vs* negative subgroups. IP/S: Internal penetrating/stricturing; NSNP: Non-stricturing and non-penetrating; IgA: Immunoglobulin A; sIgA: Secretory immunoglobulin A; CHI3L1: Chitinase 3-like 1.

Figure 1). This relationship is shown in Tables 5-8, which summarizes the time-dependent univariate subgroup analyses for colon involvement.

The results of the Kaplan-Meier and univariate Cox regression analyses regarding associations between clinical factors and the development of complications are summarized in Tables 5-8. The risk of the development of internal penetrating and/or stenotic complications was associated with location, including extensive disease, which remained significant in the subgroup of patients with colonic involvement. In contrast, colon involvement was a protective factor (Table 5). Based on this, the occurrence of perianal penetrating complications in P0 patients was associated with early onset disease, colon involvement, and frequent relapses. Within the subgroup of patients with colonic involvement, this association remained significant in those with frequent relapses (Table 6).

Resective surgery was not associated with any of the clinical factors studied, neither in the B1 patient group nor in patients with colonic involvement (Table 7). Patients with a previous surgery were more likely to undergo a new operation in patients who relapsed frequently, which was also true for the subgroup with colonic involvement (Table 8).

In multivariate Cox regression analysis, the subgroup of B1 patients with colonic involvement, IgA-type aCHI3L1 positivity independently predicted a faster progression towards a complicated disease course in time-dependent models

Table 5 Univariate and multivariate Cox regression analyses evaluating the association between clinical and serologic variables and the study end-point events (complicated disease course) in Crohn's disease patients. Subgroup analysis of B1 patients with colonic involvement is shown in the second half of the table

		Development of internal penetrating and/or stenotic complication in B1 patients at diagnosis							Development of internal penetrating and/or stenotic complication in B1 patients at diagnosis with colonic involvement						
		n of subjects	CP of event (%)[1]	pLogRank	HR (95%CI)[2]	*P* value[2]	HR (95%CI)[3]	*P* value[3]	*n* of subjects	CP of event (%)[1]	pLogRank	HR (95%CI)[2]	*P* value[2]	HR (95%CI)[3]	*P* value[3]
Overall population		209	47.5						160	44.4					
Clinical factors															
Age at onset	A1	27	47.7	0.137	1.95 (0.74-5.14)	0.175			26	47.7	0.095	4.12 (0.93-18.28)	0.062	7.26 (0.94-55.89)	0.057
	A2	157	51.0		2.26 (0.98-5.21)	0.055			118	46.7		4.20 (1.02-17.23)	0.046	7.79 (1.07-56.52)	0.042
	A3	25	25.9			0.152			16	19.8			0.137		0.127
Gender	Male	91	47.7	0.494	1.15 (0.77-1.74)	0.495			69	44.8	0.489	1.18 (0.74-1.90)	0.490	1.10 (0.68-1.79)	0.697
	Female	118	47.3						91	43.9					
Location	L1 + L3	129	57.3	0.002	1.98 (1.27-3.08)	0.003			80	55.5	0.013	1.82 (1.13-2.95)	0.014	1.83 (1.12-2.99)	0.016
	L2	80	34.5						80	34.5					
	L2 + L3	160	44.4	0.027	0.59 (0.37-0.95)	0.029									
	L1	49	62.9												
Frequent relapse	No	154	44.8						115	41.7					
	Yes	45	53.3	0.489	1.18 (0.74-1.89)	0.490			38	52.7	0.533	1.19 (0.69-2.04)	0.534		
Antibodies against CHI3L1															
aCHI3L1 IgG	Negative	182	49.7						141	45.5					
	Positive	17	33.9	0.219	0.57 (0.23-1.41)	0.226			14	38.6	0.561	0.76 (0.31-1.90)	0.562		
aCHI3L1 IgA	Negative	145	44.1						103	37.4					
	Positive	54	58.3	0.076	1.49 (0.96-	0.078			52	58.8	0.028	1.71 (1.05-	0.030	1.67 (1.02-	0.041

					2.31)						2.78)		2.71)	
aCHI3L1 sIgA	Negative	127	43.7				92	36.6						
	Positive	72	55.7	0.057	1.50 (0.99-2.29)	0.059	63	56.6	0.028	1.70 (1.05-2.75)	0.031	1.59 (0.98-2.58)	0.061

[1]Cumulative probability of event (%) corresponds to the median follow-up values.
[2]Univariate analysis.
[3]Multivariate analysis.
Rows corresponding to perianal disease according to the Montreal classification at diagnosis, smoking habits, and familial inflammatory bowel diseases were omitted because statistically significant differences for a given parameter were not obtained. HR: Hazard ratio; Age at onset: A1: ≤ 16 years, A2: 17-40 years, A3: > 40 years; Location: L1: Ileal, L2: Colonic, L3: Ileocolonic, L1 + L3: Ileal involvement, L2 + L3: Colonic involvement; Behavior: B1: Inflammatory/non-stricturing and non-penetrating; B2: Stenosing; B3: Internal penetrating; P0: Lack of perianal disease/involvement; P1: Perianal disease/involvement. IBD: Inflammatory bowel diseases; Ig: Immunoglobulin; sIgA: Secretory IgA; aCHI3L1: Anti-chitinase 3-like 1 autoantibodies; CP: Cumulative probability; CI: Confidence interval; HR: Hazard ratio.

(HR = 1.67; 95%CI: 1.02-2.71; P = 0.041). Among the clinical factors, the same was observed for extensive disease (HR = 1.83; 95%CI: 1.12-2.99; P = 0.016). None of the other clinical factors and or serologic antibodies studied were independently associated with the development of unfavorable disease outcomes (Tables 5-8).

DISCUSSION

CHI3L1 (also known as human cartilage glycoprotein-39 or YKL-40 or 40-kDa mammary gland protein) is a 40 kDa heparin-, chitin-, hyaluronan-, and collagen-binding glycoprotein expressed by various cell types such as synovial cells, chondrocytes, endothelial cells, smooth muscle cells, hepatic stellate cells, cancer cells, fibroblast-like cells, macrophages, neutrophils, and colonic epithelial cells (CEC)[21,49]. It belongs to the 18-glycosylhydrolase family and lacks chitinase activity owing to an amino acid change in the catalytic region[21,49-51]. Besides oncogenesis[49] previous studies proposed its role in numerous systemic-[52-57], respiratory-[58-69], digestive-[21,27,28,70-78], cardiovascular-[79-85], endocrine-[80,84,86,87], neurological-[88,89], urinary-[90-94], skeletal-[84,95], autoimmune-[96], and dermatological[97,98] conditions with features of either acute or chronic inflammation.

According to a comprehensive review by Tizaoui *et al*[99], 14 studies were conducted on the role of CHI3L1 in IBD. Our literature search identified only three additional papers from 2019 on the topic in PubMed[100-102]. Elevated serum CHI3L1 levels have been reported in most of those studies; however, there are controversial results regarding their correlation with disease activity[99]. In a study by Vind *et al*[103], serum CHI3L1 levels tended to be elevated in 30% of patients with CD, even when the disease became inactive. In UC, they correlated well with the laboratory and clinical markers of disease activity. These distinctions can be attributed to the different characteristics of inflammation in CD and UC [transmural *vs* (sub)mucosal][104].

Most of the studies were cross-sectional without a detailed prospective follow-up; therefore, it is very difficult to draw any conclusions regarding the usefulness of serum CHI3L1 in monitoring disease activity, even in UC alone. More promising information is available on the use of CHI3L1 as a fecal biomarker in pediatric[27] and adult[28] IBD cohorts as well, since it showed a good correlation with endoscopic activity in both CD and UC, with comparable performance to fecal calprotectin.

Table 6 Univariate and multivariate Cox regression analyses evaluating the association between clinical and serologic variables and the study end-point events (development of perianal complications) in Crohn's disease patients. Subgroup analysis of B1 patients with colonic involvement is shown in the second half of the table

		Development of perianal penetrating complication in P0 patients at diagnosis							Development of perianal penetrating complication in P0 patients at diagnosis with colonic involvement						
		n of subjects	CP of event (%)[1]	pLogRank	HR (95%CI)[2]	*P* value[2]	HR (95%CI)[3]	*P* value[3]	*n* of subjects	CP of event (%)[1]	pLogRank	HR (95%CI)[2]	*P* value[2]	HR (95%CI)[3]	*P* value[3]
Overall population		215	24						157	27.9					
Clinical factors															
Age at onset	A1	25	34.9	0.011	5.05 (1.44-17.7)	0.012			24	34.9	0.109	3.22 (0.92-11.31)	0.068		
	A2	158	23.9		2.46 (0.76-8.01)	0.134			113	28.5		1.94 (0.59-6.33)	0.274		
	A3	30	7			0.016			20	10.5			0.122		
Gender	Male	90	23.1	0.584	0.85 (0.48-1.51)	0.585			68	27.2	0.474	0.81 (0.45-1.45)	0.475		
	Female	123	24						89	28.7					
Location	L1 + L3	147	20.5	0.066	0.60 (0.35-1.04)	0.640			91	27.1	0.634	0.87 (0.49-1.54)	0.635		
	L2	66	28.8						66	28.8					
	L2 + L3	157	27.9	0.002	4.25 (1.53-11.79)	0.001									
	L1	56	8.9												
Frequent relapse	No	160	18						115	22.3					
	Yes	45	40.9	0.000	2.94 (1.68-5.15)	0.000			33	44.2	0.001	2.61 (1.45-4.68)	0.001		
Antibodies against CHI3L1															
aCHI3L1 IgG	Negative	187	22.3						138	25.0					
	Positive	15	33.3	0.988	0.99 (0.36-2.76)	0.988			12	37.5	0.933	0.96 (0.34-2.68)	0.933		
aCHI3L1 IgA	Negative	145	21.7						96	25.2					
	Positive	57	25.8	0.624	1.16 (0.64-	0.624			54	26.7	0.862	0.95 (0.51-	0.862		

					2.11)						1.75)	
aCHI3L1 sIgA	Negative	122	22.0				83	24.9				
	Positive	80	24.7	0.746	0.91 (0.51-1.62)	0.746	67	27.6	0.645	0.87 (0.48-1.58)	0.645	

[1]Cumulative probability of event (%) corresponds to the median follow-up values.
[2]Univariate analysis.
[3]Multivariate analysis.
Rows corresponding to behaviour according to the Montreal classification at diagnosis, smoking habits, and familial inflammatory bowel disease were omitted because statistically significant differences for a given parameter were not obtained. HR: Hazard ratio; Age at onset: A1: ≤ 16 years, A2: 17-40 years, A3: > 40 years; Location: L1: Ileal, L2: Colonic, L3: Ileocolonic, L1 + L3: Ileal involvement, L2 + L3: Colonic involvement; Behavior: B1: Inflammatory/non-stricturing and non-penetrating; B2: Stenosing; B3: Internal penetrating; P0: Lack of perianal disease/involvement; P1: Perianal disease/involvement. IBD: Inflammatory bowel diseases; Ig: Immunoglobulin; sIgA: Secretory IgA; aCHI3L1: Anti-chitinase 3-like 1 autoantibodies; CP: Cumulative probability; CI: Confidence interval; HR: Hazard ratio.

CHI3L1 was first described as an autoantigenic target in rheumatoid arthritis in 1997[105,106] and in IBD in 2019[30]. The latter was confirmed by the authors of this manuscript[30]. To date, autoantibodies against CHI3L1 have only been detected in these two entities[30,107-110]. aCHI3L1 were evaluated in only one cross-sectional study in IBD on a pediatric population (CD: n = 110, UC: n = 95)[30]. To our knowledge, this is the first prospective study on aCHI3L1 as a biomarker with predictive potential in an adult cohort of IBD patients. Similar prevalence rates were reported by Deutschmann *et al* [30] for IgA and sIgA aCHI3L1 in our adult IBD cohort. IgG-type positivity was more prevalent in children. In the pediatric population, ileal-, whilst in adults colonic involvement was associated with higher antibody positivity. The discrepancies between the two studies may be attributed to presumably different immune responses owing to the different maturity of the immune system, age-related changes in the microbiome, and differences in the onset and course of IBD in children[111].

It is suspected that, unlike UC, CD patients are more prone to developing a tolerance break against CHI3L1 due to persistent elevation of CHI3L1 independent of disease activity[30,103,112]. This hypothesis is also supported by our findings regarding the differences in aCHI3L1 autoantibody prevalence among pediatric[30] and adult IBD subgroups. In our study, similar to CHI3L1, no correlation was found between aCHI3L1 positivity and disease activity in CD patients. Regarding autoantibodies against CHI3L1, we did not find any association between aCHI3L1 formation and response to therapy, which is consistent with previous IBD studies. Unlike other disorders, the levels of serum auto- and antimicrobial antibodies in IBD remain largely unchanged regardless of the inflammatory burden or the effects of anti-inflammatory therapy or surgical resection. Unlike other autoimmune diseases such as ANCA-associated vasculitides, classic and newly discovered autoantibodies (*e.g.*, ANCA, PAbs, APLAs, aCHI3L1), and antimicrobial antibodies (ASCA, anti-OMP PlusTM IgA) have no established role in monitoring the efficacy of IBD treatment[18].

The prevalence of IgA-type aCHI3L1 antibodies was significantly higher in CD patients than in UC patients and HCONT. A similarly elevated prevalence was also observed for the sIgA subtype, with the difference that this was independent of IBD subtype. In our previous studies, significant differences in favor of IgA and sIgA (sub)types were also detected in the evaluated classic auto- (PAB, ACA, aPS/PT) and antimicrobial antibodies (ASCA, anti-OMP) in IBD[29,31,113,114]. In parallel, in the aforementioned studies, we found associations between complications in CD for IgA and not IgG types[29,31,113,114]. The mucosal immune system in the gut plays a central role in IgA antibody production. In previous studies, the increased IgA_2 subtype ratio and concomitant presence of the secretory component (SC) were considered as evidence for a mucosal origin of IgA secretion[115,116]. Our research group previously reported the

Table 7 Univariate and multivariate Cox regression analyses evaluating the association between clinical and serologic variables and the study end-point events (need for resective surgery) in Crohn's disease patients. Subgroup analysis of B1 patients with colonic involvement is shown in the second half of the table

		Need for resective surgery in B1 patients at diagnosis							Need for resective surgery in B1 patients at diagnosis with colonic involvement						
		n of subjects	CP of event (%)[1]	pLogRank	HR (95%CI)[2]	*P* value[2]	HR (95%CI)[3]	*P* value[3]	*n* of subjects	CP of event (%)[1]	pLogRank	HR (95%CI)[2]	*P* value[2]	HR (95%CI)[3]	*P* value[3]
Overall population		209	38						160	36					
Clinical factors															
Age at onset	A1	27	29.1	0.937	0.94 (0.38-2.35)	0.896			26	29.1	0.989	0.96 (0.33-2.80)	0.945		
	A2	157	39.8		1.06 (0.50-2.22)	0.887			118	37		1.01 (0.40-2.58)	0.980		
	A3	25	40.4			0.937			16	43			0.989		
Gender	Male	91	34.2	0.958	0.99 (0.62-1.57)	0.958			69	33.7	0.977	0.99 (0.59-1.66)	0.977		
	Female	118	40.8						91	37.7					
Location	L1 + L3	129	31.1	0.109	1.48 (0.91-2.40)	0.112			80	41.5	0.174	1.45 (0.85-2.43)	0.176		
	L2	80	43.7						80	31.1					
	L2 + L3	160	50.2	0.382	0.78 (0.44-1.38)	0.384									
	L1	49	36.0												
Frequent relapse	No	154	36.9	0.950					115	35.8					
	Yes	45	38.7		0.98 (0.58-1.68)	0.950			38	32.4	0.602	0.85 (0.47-1.56)	0.852		
Antibodies against CHI3L1															
aCHI3L1 IgG	Negative	182	38.9						141	36.4					
	Positive	17	34.7	0.997	1.00 (0.43-2.32)	0.997			14	39.9	0.623	1.24 (0.53-2.89)	0.624		
aCHI3L1 IgA	Negative	145	34.1						103	30.3					
	Positive	54	48.5	0.246	1.34 (0.82-2.18)	0.248			52	47.6	0.255	1.36 (0.80-2.29)	0.257		

aCHI3L1 sIgA	Negative	127	33.8				92	29.8			
	Positive	72	46.3	0.159	1.40 (0.88-2.24)	0.161	63	46.4	0.227	1.37 (0.82-2.31)	0.230

[1]Cumulative probability of event (%) corresponds to the median follow-up values.

[2]Univariate analysis.

[3]Multivariate analysis.

Rows corresponding to perianal disease according to the Montreal classification at diagnosis, smoking habits, and familial inflammatory bowel diseases were omitted because statistically significant differences for a given parameter were not obtained. HR: Hazard ratio; Age at onset: A1: ≤ 16 years, A2: 17-40 years, A3: > 40 years; Location: L1: Ileal, L2: Colonic, L3: Ileocolonic, L1 + L3: Ileal involvement, L2 + L3: Colonic involvement; Behavior: B1: Inflammatory/non-stricturing and non-penetrating; B2: Stenosing; B3: Internal penetrating; P0: Lack of perianal disease/involvement; P1: Perianal disease/involvement. IBD: Inflammatory bowel diseases; Ig: Immunoglobulin; sIgA: Secretory IgA; aCHI3L1: Anti-chitinase 3-like 1 autoantibodies; CP: Cumulative probability; CI: Confidence interval; HR: Hazard ratio.

mucosal origin of ANCA IgA in a group of patients with liver cirrhosis indirectly by demonstrating the presence of SC [117]. Similarly, we were able to demonstrate the mucosal origin of ASCA IgA in CD using flow cytometry (unpublished results, first presented at the 12th Congress of the ECCO)[114]. In the present study, we also demonstrated the presence of aCHI3L1 antibodies of mucosal origin in IBD. The higher prevalence of aCHI3L1 IgA in CD than in UC may be due to the different nature of mucosal inflammation (degree of intestinal wall involvement more pronounced in CD - transmural *vs* mucosal)[104], as well as its localisation. In CD, serum total sIgA levels were also significantly higher (median: 51 *vs* 29 μg/mL; $P < 0.001$), which was more pronounced in the presence of certain antimicrobial antibody IgA types[114]. This phenomenon, namely the tendency for IgA-dominant antibody formation in IBD, is thus not specific for antibodies produced against CHI3L1 and may reflect an immune response to an enhanced microbial load. This hypothesis is also supported by the significant associations between the presence of certain IgA antimicrobial (ASCA, anti-OMP), antiphospholipid (anti-PS/PT), and autoantibody (anti-GP2) types and aCHI3L1 IgA and sIgA positivity found in our study. Meanwhile, IgA-type autoantibodies are viewed as an indication of an immune response to enteric antigens, which in other ailments has been linked to a rise in BT[29]. BT refers to the increased uptake of bacteria or bacterial products from the intestinal lumen into systemic circulation[118,119]. In IBD, the combination of structural and functional intestinal barrier damage due to chronic intestinal inflammation, reduced intestinal mucosal defenses, altered microbiome, and dysregulated immune response to specific bacterial components is thought to lead to chronic BT. The uncontrolled uptake of various luminal bacteria and/or bacterial products exacerbates the local and systemic proinflammatory processes already underway, thereby contributing to disease exacerbation and complications[104,120-125]. However, it remains unclear whether these alterations can directly contribute to the development of IBD or whether they are just an epiphenomenon.

Roggenbuck *et al*[126] first hypothesized the pathophysiological role of anti-pancreatic antibodies in CD in inducing and maintaining increased BT[127]. Following loss of tolerance to GP2, plasma cells synthesize anti-GP2 IgA, which is transported by intestinal epithelial cells into the intestinal lumen. Secreted anti-GP2 IgA can form a bridge between GP2-derived pancreatic GP2-opsonized FimH-positive bacteria and membrane-bound GP2 on the surface of M cells. These processes lead to microbial overload of the mucosa due to increased transcytosis in CD, which amplifies the inflammatory processes in the gut[29]. In the case of CHI3L1 and antibodies against it, very similar mechanisms can enhance BT if an increased tendency to form antibodies against this glycoprotein develops as a consequence of loss of CHI3L1 tolerance.

Under normal conditions, CHI3L1 levels are extremely low, but under inflammatory conditions, they are also expressed at elevated levels in CEC and macrophages[51,128]. Mizoguchi[129] demonstrated an upregulated expression of CHI3L1 in the lamina propria and CECs of IBD patients and animal models. This upregulation, along with the loss of

Table 8 Univariate and multivariate Cox regression analyses evaluating the association between clinical and serologic variables and the study end-point events (reoperation after resection) in Crohn's disease patients. Subgroup analysis of B1 patients with colonic involvement is shown in the second half of the table

		Need for resective surgery in B1 patients with previous CD-related abdominal surgery							Need for resective surgery in B1 patients with previous CD-related abdominal surgery and colonic involvement						
		n of subjects	CP of event (%)[1]	pLogRank	HR (95%CI)[2]	*P* value[2]	HR (95%CI)[3]	*P* value[3]	*n* of subjects	CP of event (%)[1]	pLogRank	HR (95%CI)[2]	*P* value[2]	HR (95%CI)[3]	*P* value[3]
Overall population		73	28.2						58	26.6					
Clinical factors															
Age at onset	A1	11	60.2	0.186	4.47 (0.52-38.57)	0.173			11	60.2	0.122	3.16 (0.36-27.59)	0.298		
	A2	54	23.8		1.94 (0.25-15.25)	0.530			42	17.9		0.97 (0.12-8.13)	0.976		
	A3	8	12.5			0.214			5	20.0			0.150		
Gender	Male	32	29.5		1.03 (0.38-2.77)	0.956			26	19.8	0.368	0.58 (0.17-1.93)	0.374		
	Female	41	28.4	0.956					32	33.2					
Location	L1 + L3	45	31.0	0.339	1.67 (0.58-4.82)	0.345			30	29.5	0.454	1.55 (0.49-4.92)	0.457		
	L2	28	23.2						28	23.2					
	L2 + L3	58	26.6	0.430	0.64 (0.20-1.98)	0.434									
	L1	15	35.8												
Familial IBD	No	68	25.5						55	23.8					
	Yes	5	60.0	0.010	4.59 (1.29-16.36)	0.019			3	66.7	0.002	8.19 (1.64-40.86)	0.010		
Frequent relapse	No	51	14.9						40	9.0					
	Yes	19	61.0	0.006	3.79 (1.36-10.55)	0.011			15	73.3	0.000	7.94 (2.03-31.00)	0.003		
Antibodies against CHI3L1															
aCHI3L1 IgG	Negative	64	27.3						51	23.7					
	Positive	4	40.0	0.595	1.50 (0.34-	0.597			6	40.0	0.485	1.72 (0.37-	0.490		

					6.69)						8.04)	
aCHI3L1 IgA	Negative	46	21.5				34	16.3				
	Positive	24	42.1	0.238	1.83 (0.66-5.04)	0.245	23	39.3	0.220	2.07 (0.63-6.80)	0.230
aCHI3L1 sIgA	Negative	40	17.2				31	13.9				
	Positive	30	46.2	0.052	2.68 (0.95-7.58)	0.062	26	41.0	0.086	2.82 (0.82-9.68)	0.100

[1]Cumulative probability of event (%) corresponds to the median follow-up values.
[2]Univariate analysis.
[3]Multivariate analysis.
Rows corresponding to perianal disease according to the Montreal classification at diagnosis, smoking habits were omitted because statistically significant differences for a given parameter were not obtained. HR: Hazard ratio; Age at onset: A1: ≤ 16 years, A2: 17-40 years, A3: > 40 years; Location: L1: Ileal, L2: Colonic, L3: Ileocolonic, L1 + L3: Ileal involvement, L2 + L3: Colonic involvement; Behavior: B1: Inflammatory/non-stricturing and non-penetrating; B2: Stenosing; B3: Internal penetrating; P0: Lack of perianal disease/involvement; P1: Perianal disease/involvement. IBD: Inflammatory bowel diseases; Ig: Immunoglobulin; sIgA: Secretory IgA; aCHI3L1: Anti-chitinase 3-like 1 autoantibodies; CP: Cumulative probability; CI: Confidence interval; HR: Hazard ratio.

tolerance to CHI3L1, enhances the adhesion and invasion of certain commensal and/or pathogenic bacteria *via* carbohydrate binding [chitin binding protein (CBP) 21] or homologous motifs (for example, ChiA)[21,30]. Significantly higher prevalence of IgA and sIgA (sub)type antibodies against CHI3L1 in patients with colonic involvement (aCHI3L1 IgA positivity: L2/L3 35.5% *vs* L1 7.7%; sIgA: 43.5% *vs* 26.9%) along with a positive predictive value for complicated disease course in only this subgroup of CD patients could be considered as an indirect confirmation of colonic origin of these antibodies. Further studies to assess the presence of aCHI3L1 antibodies in different parts of the gut mucosa of IBD patients are needed to confirm this hypothesis.

CHI3L1 is recognized as a biomarker of fibrosis due to its contribution to tissue remodeling and fibrosis by hindering the degradation of type I collagen and hyaluronic acid, and by regulating the enzymatic activity of matrix metalloproteinases, ultimately affecting cell adhesion and migration[39,40,101]. Previously it has been shown by Erzin *et al*[130] that increased serum CHI3L1 was associated with intestinal stricture formation in CD patients. Here, we present confirmatory results that autoantibodies against CHI3L1 may be involved in stricture and fistula formation in CD patients. In pediatric IBD patients with complicated disease behavior, a trend toward higher prevalence of IgA and sIgA (subtype) aCHI3L1 was observed (38.5% *vs* 22.0% and 53.8% *vs* 35.6%, respectively)[30]. The lack of statistical significance in this case can be explained by the relatively low number of patients in these subgroups of patients (aCHI3L1 IgA- and sIgA-positive patients with complicated disease behavior: n = 5 and 7, respectively). In adults aCHI3L1 sIgA positivity was associated with complicated disease behavior at diagnosis (OR = 2.37, 95%CI: 1.26-4.48). The results of cross-sectional single-time point studies should be interpreted with caution[18]. After more than sixty years longitudinal follow-up studies are still considered rare in the field of IBD serology[18]. This study offers, for the first time, longitudinal and prospective data regarding the predictive capabilities of aCHI3L1 antibodies in identifying disease-specific complications and surgical requirements. The presence of IgA or sIgA aCH3L1 antibodies in patients with both inflammatory luminal disease and colon involvement at the time of diagnosis indicates a faster progression towards a complicated disease course in time-dependent models. This association remained significant in multivariate models as well in case of IgA type. This observation, together with our previous results, highlights the need to consider location in addition to antibody class when predicting disease progression. Based on both pathogenic and clinical heterogeneity of IBD, instead of "the one size

fits all" model, an increasing amount of data as this point towards the need for the development and application of prognostic matrix models[131]. As no data are available on the prognostic potential of aCHI3L1 antibodies in IBD, a literature search was performed in this regard in rheumatoid arthritis (RA), the only other entity where the presence of aCHI3L1 was described. Based on these findings, CHI3L1 was not considered a long-term prognostic biomarker for RA [99]. As for autoantibodies against this particular target, no data are available on the predictive potential of RA[107-110].

Similar to other diseases, where CHI3L1 is involved in pathogenesis, CHI3L1 could also serve as a potential therapeutic target in IBD. Kawada *et al*[132] found that by inhibiting CHI3L1 with anti-CHI3L1 antibodies or CHI3L1-specific small interfering RNA, the adhesion of *E. coli* cells overexpressing CBP to CECs was reduced. Ongoing clinical trials are evaluating the use of antibodies against the active region of CHI3L1, and recombinant nasal CHI3L1 (Org39141) to reach tolerance in RA[49].

In conclusion, the integration of IgA and sIgA against CHI3L1 with newly applied CD-specific antibodies (*e.g.*, anti-GP2) in laboratory screening of patients suspected of having CD may improve the sensitivity and specificity of the screening process. Further research is required to determine whether antibodies against CHI3L1 have a diagnostic potential and whether elevated aCHI3L1 levels are only a concomitant feature of CD or contribute to the development and presentation of IBD[30].

A drawback of our study was that the prevalence of antibodies against CHI3L1 was not high. Moreover, these antibodies do not play an exclusive role in prognostics. Although the number of cohorts was significant, we could detect significant differences only in a subgroup of patients, so it would be important to investigate these parameters in a larger group of patients. It would be necessary to establish a validation cohort, also of adults, with a large number of cases, followed prospectively, whose results could confirm or refute our findings.

CONCLUSION

In conclusion, we observed increased IgA autoantibody production in CD patients in an adult IBD cohort by testing autoantibodies against a novel neutrophil autoantigen target, CHI3L1. Antibodies against CHI3L1 showed long-term stability over the course of the disease. It was associated with the clinical phenotype of the disease and identified patients with colonic involvement, complicated disease course, or ASCA IgA and/or anti-OMP positivity. It was correlated with a more rapid onset of a complicated disease (B2 and/or B3) during disease progression in the subgroup of CD patients with no complications at diagnosis (B1) and with colonic involvement. In conclusion, the combined consideration of antibody classes and location in predicting disease progression may revolutionize IBD serology. IgA-type anti-CHI3L1 antibodies may interfere with innate immunity against intestinal bacteria or may reflect an immune response against microbial overload in the intestinal barrier.

ARTICLE HIGHLIGHTS

Research background

Chitinase 3-like 1 (CHI3L1) is a 40 kDa heparin-, chitin-, hyaluronan-, and collagen-binding glycoprotein expressed by various cell types, including fibroblast-like cells, macrophages, neutrophils, and colonic epithelial cells (CEC). Under normal conditions, CHI3L1 levels are extremely low; however, under inflammatory conditions, they are also expressed at elevated levels in CEC and macrophages. Upregulated expression of CHI3L1 in the lamina propria and CECs has been described in patients with inflammatory bowel disease (IBD). This upregulation, along with the loss of tolerance to CHI3L1, can enhance the adhesion and invasion of certain commensal and/or pathogenic bacteria *via* carbohydrate binding (chitin binding protein 21) or homologous motifs (for example, ChiA). Microbial overload, along with mechanical and/or functional derangement of the gut barrier, can lead to the uncontrolled uptake of various luminal bacteria and/or bacterial products. As a result, enhanced bacterial translocation exacerbates local and systemic proinflammatory processes already underway, thereby contributing to disease exacerbation and complications.

Research motivation

Given the current state of knowledge, the precise function and mechanism of CHI3L1 in the development of IBD, loss of tolerance to this antigen, formation of immunoglobulin A (IgA) autoantibodies against it, and their role in the pathogenesis of IBD remain elusive. Concomitantly, there is an unmet need to identify new biomarkers to identify patients with IBD with a complicated disease course.

Research objectives

To evaluate the predictive value of different immunoglobulin subtypes of the novel serological marker, anti-CHI3L autoantibodies (aCHI3L1), in terms of their ability to define the disease phenotype, therapeutic strategy, and long-term disease course in a group of adult IBD patients with prospective follow-up.

Research methods

A total of 257 patients with Crohn's disease (CD) and 180 patients with ulcerative colitis (UC) from a tertiary IBD referral center in Hungary (Division of Gastroenterology, Department of Internal Medicine, Faculty of Medicine, University of

Debrecen) were tested for IgG, IgA, and secretory IgA (sIgA) type aCHI3L1 using enzyme-linked immunosorbent assay with recombinant CHI3L1, along with 86 healthy controls.

Research results

Enhanced formation of IgA and sIgA (sub)-type against CHI3L1 was first detected in adult patients with CD, which was associated with the clinical phenotype in a tertiary referral IBD center in Hungary. This observational study presents, for the first time, longitudinal and prospective results on the predictive ability of aCHI3L1 antibodies to identify disease-specific complications and surgical requirements, which are critical for patient care. The presence of IgA or sIgA aCH3L1 antibodies in CD patients with both inflammatory luminal disease and colon involvement at the time of diagnosis indicates a faster progression towards a complicated disease course in time-dependent models.

Research conclusions

CHI3L1 is a newly discovered neutrophil autoantigenic target in IBD. By taking into account the classes of antibodies and utilizing location-based predictions, serology in IBD may undergo a significant transformation in the future.

Research perspectives

Based on both the pathogenic and clinical heterogeneity of IBD, instead of the "the one size fits all" model, an increasing amount of data points towards the need for the development and application of prognostic matrix models. Therefore, identifying new biomarkers for this purpose is of utmost importance. A significantly higher prevalence of IgA and sIgA (sub)type antibodies against CHI3L1 in patients with colonic involvement, along with a positive predictive value for complicated disease course in only this subgroup of CD patients, could be considered as an indirect confirmation of colonic origin of these antibodies. Further studies to assess the presence of aCHI3L1 antibodies in different parts of the gut mucosa of IBD patients are needed to confirm this hypothesis. Unravelling the role of CHI3L1 and aCHI3L1 autoantibodies in IBD pathogenesis can help identify new potential therapeutic targets for IBD.

FOOTNOTES

Author contributions: Sipeki N, Roggenbuck D, and Papp M made the concept and designed the present study; Sipeki N and Kovats PJ were responsible for clinical data acquisition; Deutschmann C, Schierack P, and Roggenbuck D made the experimental work and were responsible for laboratory data acquisition; Sipeki N, Kovats PJ, and Papp M made the analysis and interpretation of the data, and wrote paper; Deutschmann C, Schierack P, and Roggenbuck D supervised the work, provided expert insights and made critical revisions related to important intellectual content of the manuscript; all authors have read and approved the final manuscript.

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ORIGINAL ARTICLE

Observational Study

Global burden of inflammatory bowel disease 1990-2019: A systematic examination of the disease burden and twenty-year forecast

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Abstract

BACKGROUND

Inflammatory bowel disease (IBD) is an idiopathic intestinal disease with various levels and trends in different countries and regions. Understanding the current burden and trends of IBD in various geographical locations is essential to establish effective strategies for prevention and treatment. We report the average annual percentage change (AAPC) and estimated annual percentage change (EAPC) in age-standardized rates (ASR) of IBD in different regions based on the Global Burden of Disease (GBD) study from 1990-2019, and the relationships between IBD and the human development index (HDI) and socio-demographic index (SDI). The prevalence trends of IBD were predicted by gender from 2019-2039.

AIM

To comprehensively investigate IBD data, providing further insights into the management of this chronic disease.

METHODS

We collected the information on the incidence of IBD from the GBD study from 1990-2019 to calculate the AAPC and EAPC in ASR of IBD in different regions. The relationships between IBD, HDI, and SDI were analyzed. The Nordpred and Bayesian age-period-cohort models were used to predict the prevalence trends of IBD by gender from 2019-2039, and the reliability of the results was validated.

Statistics of all the data in this study were performed using R software (version 4.2.1).

RESULTS

North America consistently had the highest IBD ASR, while Oceania consistently had the lowest. East Asia had the fastest average annual growth in ASR (2.54%), whereas Central Europe had the fastest decline (1.38%). Countries with a low age-standardized incidence rates in 1990 showed faster growth in IBD while there was no significant correlation in 2019. Additionally, IBD increased faster in countries with a low age-standardized death rates in 1990, whereas the opposite was true in 2019. Analysis of SDI and IBD ASR showed that countries with a high SDI generally had a higher IBD ASR. Finally, the projections showed a declining trend in the incidence of IBD from 2019-2039, but a gradual increase in the number of cases.

CONCLUSION

As the global population increases and ages, early monitoring and prevention of IBD is important to reduce the disease burden, especially in countries with a high incidence of IBD.

Key Words: Inflammatory bowel disease; Incidence; Average annual percentage change; Age-standardized rates; Prevalence trends; Global Burden of Disease

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Core Tip: This study comprehensively analyzed the burden of inflammatory bowel disease (IBD) from 1990 to 2019 at the global, regional, and national levels. The association and significance of various demographic indicators were analyzed in different areas. Furthermore, the number and incidence rate of IBD for the next twenty years (from 2019 to 2039) were predicted and validated based on the R software. This study provides new hypotheses for the management of IBD to alleviate the global burden of this chronic disease.

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INTRODUCTION

Inflammatory bowel disease (IBD) is an idiopathic, chronic, intestinal inflammatory condition that includes Crohn's disease and ulcerative colitis. It is a relapsing and life-threatening disease with increasing incidence and prevalence worldwide[1,2]. Typically, IBD has a tendency for recurrence and remission, with exacerbations characterized by diarrhea, abdominal pain, and enterorrhagia. The treatment can generally relieve symptoms effectively, but does not cure the patient completely[3]. A considerable proportion of patients diagnosed with IBD ultimately required surgical intervention and experienced various complications, including extraintestinal manifestations, and complications inherent to the disease itself, such as strictures, fistulas, and abscesses[4]. This caused significant suffering for patients and affected their overall quality of life. Although the exact causes of IBD remain unclear, some common features have been observed in IBD susceptibility factors, including genetic predisposition, dysbiosis of the gut microbiota, environmental triggers, and immune abnormalities[5]. Furthermore, due to recurrent episodes of chronic intestinal inflammation, patients with IBD are at a high risk of developing colorectal cancer and extraintestinal malignancies[6,7], which is a negative factor that contributes to poor outcomes. Despite the emergence of novel biologics and therapeutic approaches for the treatment of IBD that has mitigated the potential adverse effects of traditional treatments such as corticosteroids and immunosuppressants, the clinical needs of patients have not been fully met due to the complexity and clinical heterogeneity of IBD[8,9]. Currently, new strategies that have focused on IBD personalized treatment, microbiota research, immunotherapy, digital health and remote monitoring, and collaborative disease research are expected to expand the prevention and treatment options for IBD[10-14].

IBD initially emerged in Western countries and is now considered a Western disease. Over the past few decades, the incidence of IBD has increased rapidly in developing countries in Asia, Eastern Europe, and Africa. However, in developed Western countries that previously had high prevalence of IBD, the incidence of IBD is now stabilizing[15]. The changing trends are parallel to alterations in dietary habits, such as the introduction of processed foods, increased intake of sugar and fat, excessive use of antibiotics, and overall improvements in sanitation[16]. Meanwhile, with the rapid development of economy, the increase in psychological issues and socio-economic burdens is also correlated with IBD [17]. This comprehensive analysis of the global burden and trends of IBD from 1990 to 2019 was conducted to determine its incidence, prevalence, and prognosis in different regions to provide researchers with valuable insights into the etiology of the disease. By comprehensively understanding the specific disease burden of IBD in different regions, future healthcare policies, medical resource allocations, and healthcare service systems can be established to satisfy the health

needs of the population. According to the Global Burden of Disease (GBD) 2019, we reported the burden of IBD in 204 countries and territories from 1990-2019, including the age-standardized rates (ASR), average annual percentage change (AAPC), and estimated annual percentage change (EAPC). The relationship between the socio-economic status of the regions and incidence and mortality of IBD were also analyzed. Additionally, the global number of IBD cases, incidence, and prevalence trends for the next 20 years (2019-2039) were predicted. The aim of our study is to comprehensively investigate and give adequate attention to the IBD data, providing further insights into the IBD trends to guide future policy-making efforts in alleviating the global burden of IBD.

MATERIALS AND METHODS

Data sources

Data regarding the incidence, mortality, and age-standardized incidence rates (ASIR) for IBD in 204 countries and territories from 1990-2019 were extracted from the Global Health Data Exchange Query Tool (http://ghdx.healthdata.org/gbd-results-tool)[18]. We selected "all countries or territories" or "all GBD regions" from the database as the location, "inflammatory bowel disease" as the cause, "death" and "incidence" as the measure, "number", "percent" and "rate" as metric, and selected "female", "male" and "both" as gender. For age, in addition to "all ages" and "age-standardized," the following age groups were also used: "< 1 year", "1-4 years", "5-9 years", "10-14 years", "15-19 years", "20-24 years", "25-29 years", "30-34 years", "35-39 years", "40-44 years", "45-49 years", "50-54 years", "55-59 years", "60-64 years", "65-69 years", "70-74 years", "75-79 years", "80-84 years", "85-89 years", "90-94 years", and "95+ years". Based on the above criteria, we finally selected the data from 1990-2019 for follow-up analysis.

Country-level human development index (HDI) data were collected from the World Bank to explore the correlation between HDI and IBD (https://hdr.undp.org/data-center/human-development-index#/indicies/HDI). Finally, to further predict the future development of IBD, global population estimates (2017-2100) were obtained from the GBD database (https://ghdx.healthdata.org/record/ihme-data/global-population-forecasts-2017-2100).

Statistical analysis

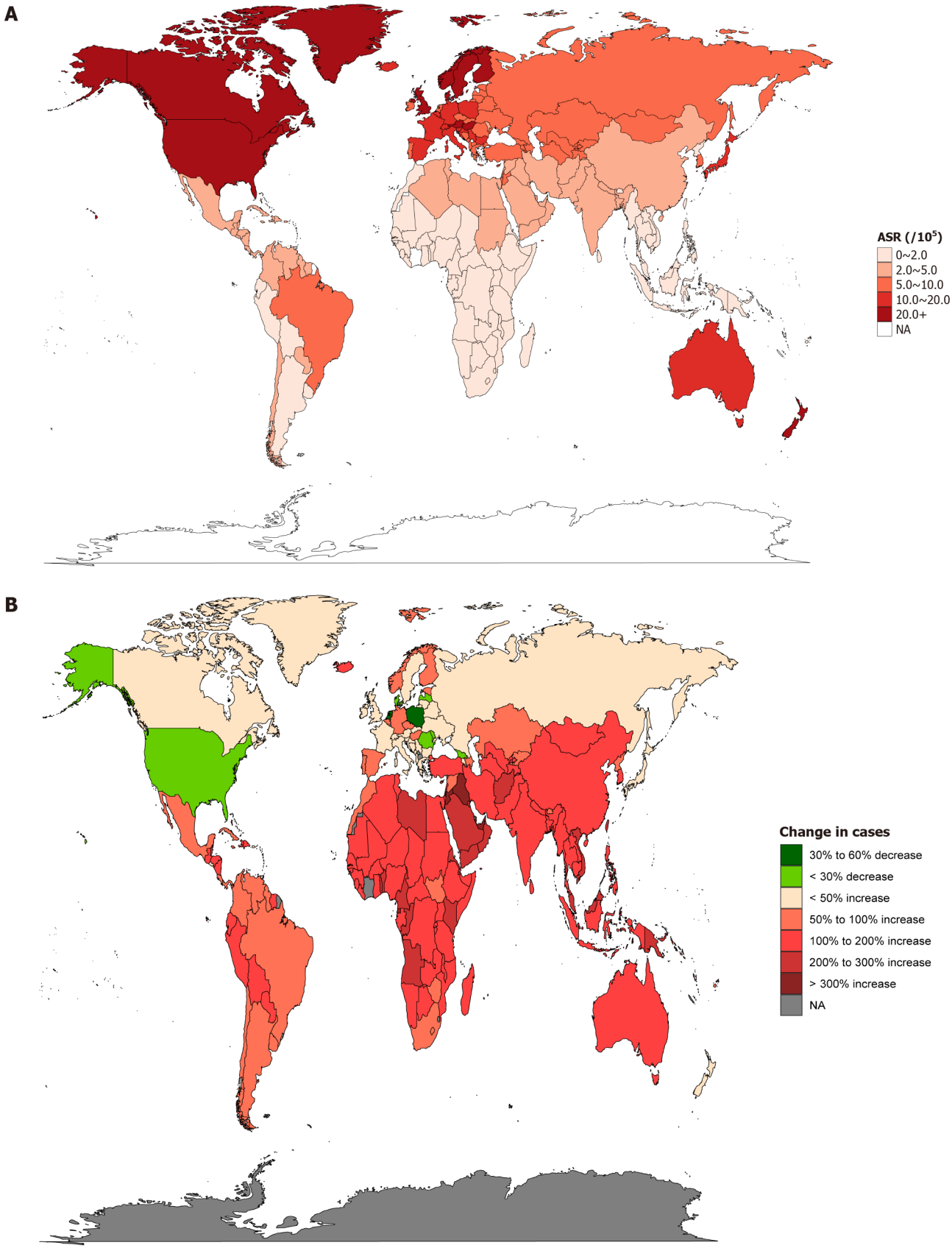
The ASR, EAPC, and AAPC were used to quantify the trends in the incidence of IBD. As the included data were from multi-group populations of different age groups and changed over time, the data must be standardized and analyzed to avoid errors. The ASR was used as the main indicator for estimating disease burden, and its calculation formula is as follows: ASR = ΣiA ai wi / ΣiA wi × 100000, where i represents the ith age group, and ai and wi represent the age-specific ratio of each age group and the standard population of the world, respectively. The EAPC is a widely used summary of the ASR trends over a certain period. The regression line is fitted to the natural logarithm of the ratio, $y = \alpha + \beta x + e$, where y = ln (ASR) and x = calendar year. EAPC is calculated as 100 × [exp(β)-1], and its 95% confidence interval (CI) was obtained from the linear regression model[19]. Similarly, the AAPC was calculated using Joinpoint software (version 4.9.1.0, https://surveillance.cancer.gov/joinpoint) to measure the trends in IBD from 1990 to 2019, calculated as follows: AAPC = {exp (Σwibi / Σwi) - 1} × 100, where bi is the slope coefficient for each segment in the desired year range, and wi is the length of each segment in the year range. Next, the relationships between the EAPCs, ASRs (1990 and 2019), and HDI (2022) in various countries were determined. In addition, a hierarchical clustering analysis was used to cluster the EAPCs of morbidity and mortality, and countries with similar trends in EAPCs were identified. Additionally, socio-demographic index (SDI) data from 1950-2019 from the GBD database (https://ghdx.healthdata.org/record/ihme-data/gbd-2019-socio-demographic-index-sdi-1950-2019) were used to analyze the relationship between the socioeconomic development status of regions and countries and the age-standardized incidence and mortality of IBD. The value of the SDI ranges from 0 to 1, with 0 being the worst and 1 being the best. This index consists of the total fertility rate of women under 25 years of age, the average education level of individuals aged 15 years and older, and the lagging distribution of per capita income. In addition, the country's economic development level was divided according to the quintiles of the SDI value into low, low-medium, medium, high-medium, and high SDI.

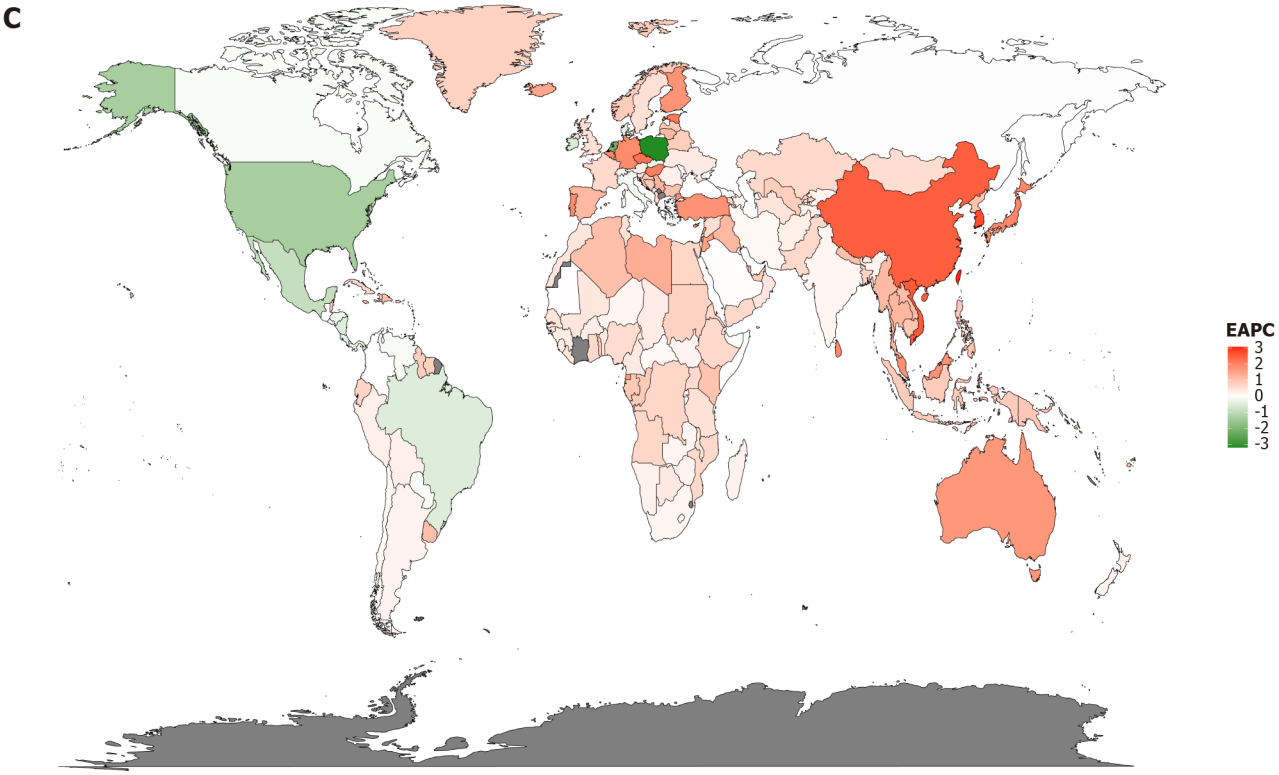
Finally, the Nordpred package of R software (version 4.2.1) was used to predict the number, incidence, and ASR of IBD from 2019-2039 by sex. To verify the reliability of the predicted results, the Bayesian age-period-cohort (BAPC) and Integrated nested Laplace approximation packages of the R software were used for analysis. All statistical analyses were conducted using R software (version 4.2.1). The level of significance was set at $P < 0.05$.

RESULTS

Global disease burden of IBD

After normalizing the data for IBD incidence, Canada [37 per 100000, 95% uncertainty interval (UI): 35.6-38.3] had the highest ASR followed by Norway (36.6 per 100000, 95%UI: 31.7-42.1). Meanwhile, Timor-Leste, Cambodia, Maldives, Papua New Guinea, Laos, and Thailand had the lowest ASR (all 0.5 per 100000, 95%UI: 0.4-0.6) (Figure 1A and Supplementary Table 1). Furthermore, we counted the incidence of IBD in each country in 1990 and 2019 to analyze the changes in IBD. It was found that the highest incidence rate increase in Qatar was 862.7 % from 13.0 in 1990 to 125.1 in 2019. However, Poland saw the most pronounced decline, from 14031.4 in 1990 to 5805.2 in 2019, a drop of 58.6% (Figure 1B and Supplementary Table 1). In order to better reflect the changes in the incidence of IBD, the results of EAPC analysis based on ASR showed that Taiwan (China)'s ASR increased the fastest, with an average annual rate of 3.07%,


A
ASR (/10^5)
0~2.0
2.0~5.0
5.0~10.0
10.0~20.0
20.0+
NA
B
Change in cases
30% to 60% decrease
< 30% decrease
< 50% increase
50% to 100% increase
100% to 200% increase
200% to 300% increase
> 300% increase
NA



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Figure 1 Inflammatory bowel disease burden in 204 countries and territories worldwide. A: Inflammatory bowel disease (IBD) age standardized rate (ASR) in 2019; B: Changes in the incidence of IBD from 1990-2019; C: Estimated annual percent change analysis of IBD ASR from 1990-2019. IBD: Inflammatory bowel disease; ASR: Age-standardized rate; EAPC: Estimated annual percent change.

while Poland's ASR decreased the fastest, with an average annual rate of 3.19% (Figure 1C and Supplementary Table 1). In addition, the United States of America had the highest incidence of IBD in both 1990 and 2019 (1990: 96513.1 per 100000, 95%UI: 83182-112506.7; 2019: 85388 per 100000, 95%UI: 77956.1-94653.4), followed by China (1990: 17221.4 per 100000, 95%UI: 14326.5-20399.5; 2019: 51462 per 100000, 95%UI: 43933-60474.5) (Supplementary Table 2). The results obtained from the AAPC analysis were consistent with the EAPC analysis. The fastest-growing ASR was observed in Taiwan (China), with an average annual growth rate of 3.107% (95%CI: 2.939-3.274), whereas Poland's ASR declined the fastest, with an average annual average of 3.198% (95%CI: -3.325 to -3.071) (Supplementary Table 2).

Through the analysis of ASR in different regions, it was found that the regions with the highest incidence of IBD in 1990 and 2019 were both High-income North America (1990: 34.9 per 100000, 95%UI: 30.6-40; 2019: 24.5 per 100000, 95%UI: 22.6-26.8) and North America (1990: 34.9 per 100000, 95%UI: 30.6-40; 2019: 24.5 per 100000, 95%UI: 22.6-26.8); at the same time, the Oceania region had the lowest ASR (1990: 0.4 per 100000, 95%UI: 0.4-0.5; 2019: 0.6 per 100000, 95%UI: 0.5-0.7). However, the EAPC analysis found that the ASR in the East Asia region had the fastest average annual growth rate of 2.54% (95%CI: 2.4-2.68), while the Central Europe region had the fastest decline, with an average annual ASR decline of 1.38% (95%CI: -1.75 to -1.01) (Table 1).

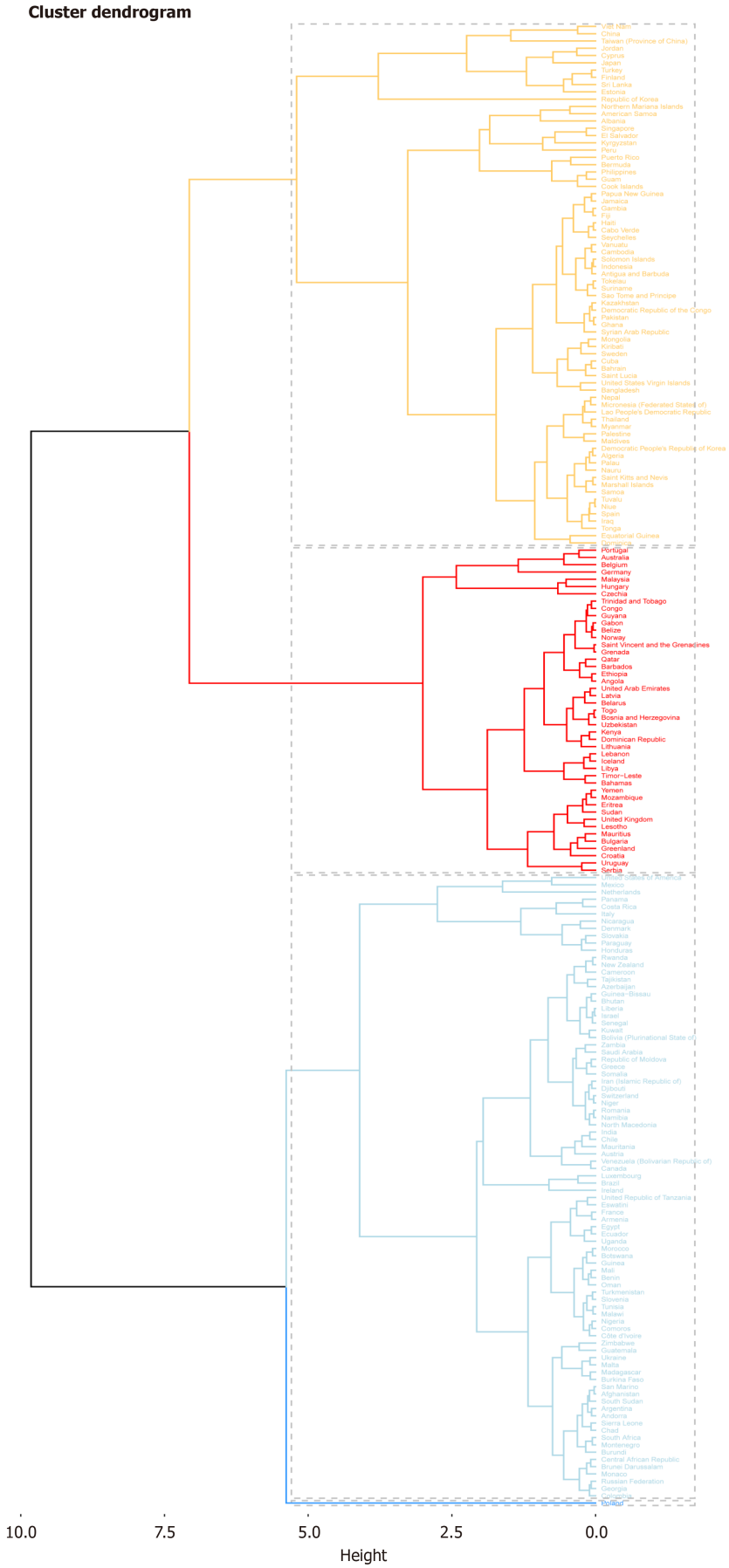
Seventy-two countries (or regions) including Vietnam, China, Taiwan (China), and Jordan were classified as the "significantly increased" group in the cluster analysis (Figure 2). Forty-five countries (or territories), including Portugal, Australia, Belgium, and Germany, were placed in the "small growth" group. Eighty-six countries (or territories), including the United States of America, Mexico, the Netherlands, and Panama, were included in the "stable or slightly declining" group. Only one country, Poland, was included in the "significant decrease" group (Supplementary Figure 1).

Correlation analysis of EAPC with ASR and HDI

To determine whether there was a potential association among EAPC, ASR, and HDI, a correlation analysis was performed. HDI data were obtained from Human Development Reports (http://hdr.undp.org/en/composite/HDI). As shown in Figure 3A, in 1990, the EAPC was significantly negatively correlated with both ASIR (ρ = -0.33, P < 0.001) and age-standardized death rates (ASDR) (ρ = -0.35, P < 0.001). By 2019, Figure 3B showed there was no significant correlation between EAPC and ASIR (ρ = -0.05, P = 0.46); more interestingly, at this time, EAPC and ASDR (ρ = 0.30, P < 0.001) showed a significant positive correlation. This indicates that the incidence of IBD has changed significantly over the past 30 years. In addition, the analysis found that there was no significant correlation between HDI and EAPC (both P > 0.05).

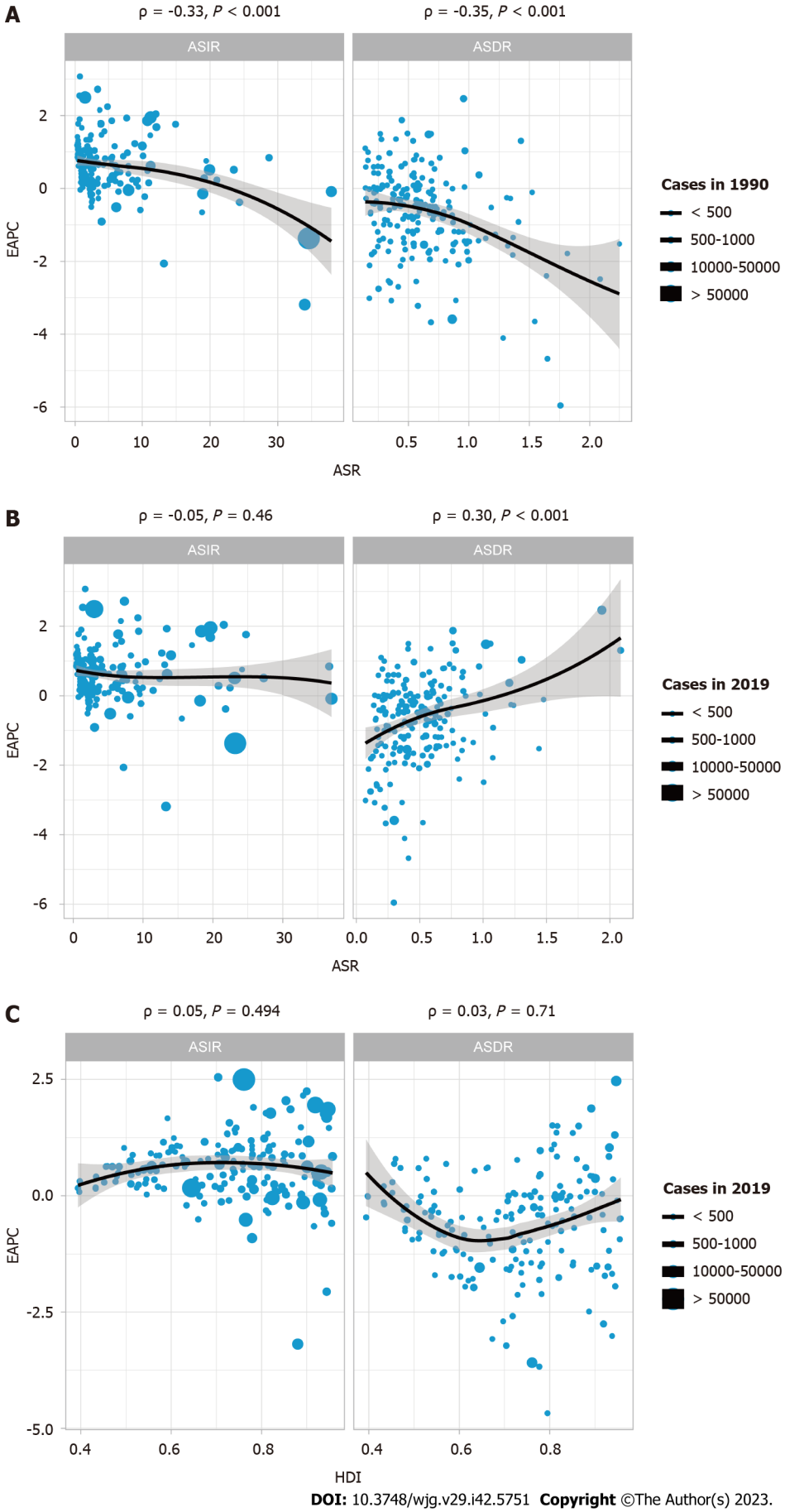
Trends of IBD ASR in various regions based on SDI

As shown in Figure 4, we investigated the relationship between ASR and SDI, and further analyzed the expected levels of SDI-based ASR at different locations. Research shows that Eastern, Western, and Central sub-Saharan Africa, East Asia,



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Figure 2 Cluster analysis: Estimated annual percent change data combining incidence and deaths.



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Figure 3 Correlation of estimated annual percent change with inflammatory bowel disease age standardized rate and human

development index. A: Correlation of estimated annual percent change (EAPC) with inflammatory bowel disease (IBD) age-standardized incidence rates (ASIR) and age-standardized death rates (ASDR) in 1990; B: Correlation of EAPC with IBD ASIR and ASDR in 2019; C: Correlation of EAPC with human development index in 2019. The circles in the figure represent countries, and the size of the circles represents the number of IBD cases. *ρ* and *P* values were obtained using the Pearson correlation coefficient analysis. EAPC: Estimated annual percent change; ASR: Age standardized rate; HDI: Human development index.

Andean Latin America, and the Caribbean are closely related to the expected ASR trends. In the mid-rear region of the SDI, the ASR is highly variable. On the one hand, some regions were much lower than expected and the ASR remained basically unchanged throughout the study period, other regions were much higher than expected with significant fluctuations in the ASR (Figure 4A). According to the analysis of each country, it is found that the ASR of IBD was significantly and positively correlated with the SDI in 2019, and the ASR of IBD in countries with higher SDI levels was also higher (Figure 4B).

Prediction of global IBD incidence

To further understand the future incidence of IBD globally, we used the Nordpred and BAPC packages of R software to predict it, respectively. The incidence of IBD in all age groups increased annually. Based on the Nordpred analysis, the total number of cases is predicted to increase from 298412 in 1990-1994 to 490887 in 2035-2039, with the incidence among men being consistently higher than that among women (Figure 5A and Supplementary Table 3). The analysis results based on the BAPC model also proved the same conclusion, and the total number of cases is predicted to increase from 298393 to 476402 with a higher incidence among males than that of females (Figure 5B and Supplementary Table 3).

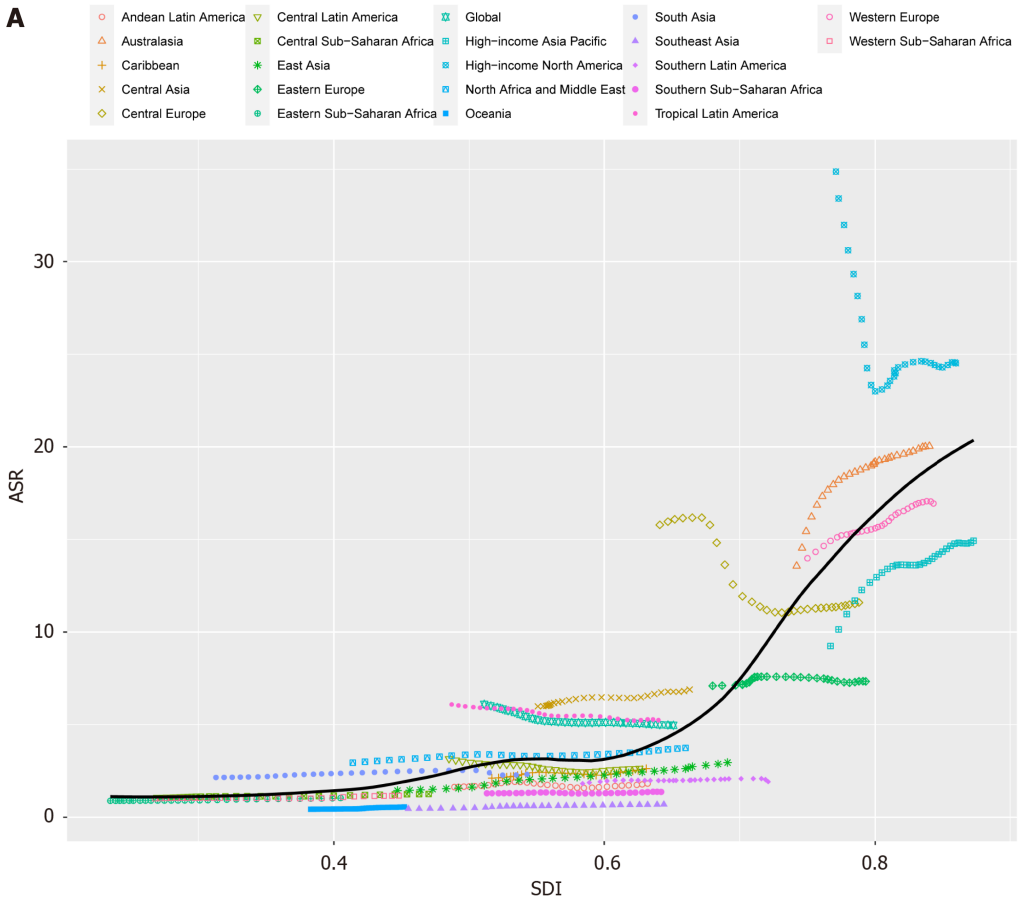
The number and incidence of IBD in each age group were analyzed. Overall, the total number of women and men with IBD showed an upward trend, but the incidence showed a downward trend, and the overall trend increase was higher in males than in females (Figure 6, Supplementary Tables 4 and 5). To assess the robustness of the predicted results, the BAPC model was used to predict the future burden of IBD. The predicted results of the BAPC model showed a consistent trend with the above results (Supplementary Figure 1, Supplementary Tables 6-8).

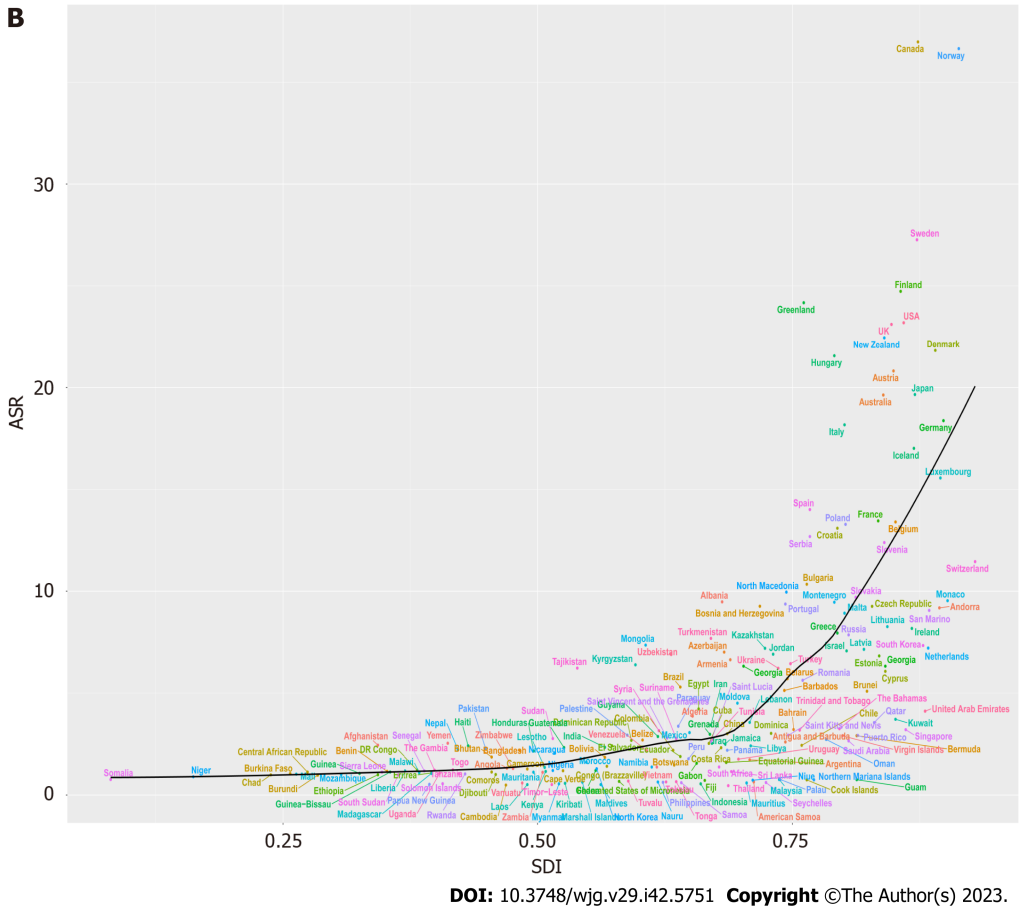
In order to better predict the future incidence of IBD, we divided the patients by gender and, analyzed and predicted the changes in the ASR of IBD. According to the Nordpred analysis, the ASR of females is predicted to decrease from 5.63 per 100000 in 1990-1994 to 4.51 in 2035-2039; and the ASR of males is predicted to decrease from 6.22 per 100000 to 5.19 during the same period (Figure 7 and Supplementary Table 9). The prediction results of the BAPC model also showed that the ASR of females decreased from (5.79983 ± 1.52e-07) per 100000 in 1990 to (4.19518 ± 1e-05) in 2039 and a decreased ASR in males from (6.38098 ± 1.64e-07) per 100000 to (5.14319 ± 1.24e-05) (Figure 7 and Supplementary Table 10). Therefore, combined with the previous analysis results for each age group (Figure 6), we can confirm that the overall incidence of IBD is declining, and the incidence of males is higher than that in females.

DISCUSSION

In this study, we conducted a comprehensive analysis on the burden of IBD at the global, regional, and national levels. The GBD 2019 report estimated that there were approximately 4.9 million cases of IBD worldwide, and the number of cases is on the rise. In our study, we found that the regions with the highest ASIR of IBD were predominantly high-income regions of North America, Canada, and Norway, which was consistent with previous findings[20,21]. Conversely, Oceania, East Timor, Cambodia, the Maldives, Papua New Guinea, Laos, and Thailand had the lowest ASR of IBD, suggesting a possible correlation among urban industrialization, environmental factors, and hygiene conditions in these areas. Additionally, different dietary habits in various countries, such as a high-fat, low-fiber, high-sugar diet or the consumption of processed foods, may contribute to the occurrence of the disease[22-24]. Moreover, the incidence of IBD is related to income level. In 2019, the ASR of IBD and the SDI were significantly and positively correlated on a global scale. Countries with higher SDI also exhibited a higher incidence of IBD, which could be explained by individuals with higher social status undergoing more routine and specialized examinations, resulting in higher diagnostic rates[25]. The delayed or limited exposure to common infectious diseases during early childhood may also account for this correlation. Consequently, in genetically susceptible individuals, the immune response underwent alterations, leading to an increased incidence of IBD[26,27]. Piovani *et al*[28] also reported a significant increase in the prevalence of IBD in countries with low and medium-low SDI. This shift may be attributed to increasing Westernization trends in these regions, which have introduced new environmental exposures that contributed to the increasing incidence of IBD[20,29,30]. In countries with a low SDI, disease-related premature mortality was a major component of the IBD burden, whereas in countries with a high SDI, the proportion was relatively low[28]. This may reflect variations in healthcare services, infrastructure, disease diagnoses, and awareness among different countries.

IBD has long been regarded as a disease that is primarily prevalent in industrialized, Western countries. However, the EAPC analysis included in this study revealed that the average annual growth rate of the ASR was highest in East Asia, whereas Central Europe had the most rapidly decreasing ASR. Furthermore, a significant decrease in ASR was observed in the high-income regions of North America, which was consistent with the previous reports[20,31] and indicated a noteworthy shift in the epidemiological stage of IBD in Western countries. As Western countries experienced a widespread aging population, the number of individuals diagnosed with IBD is projected to increase despite the stable or declining incidence rates of IBD[32]. Economic improvements have resulted in improvements to healthcare infrastructure, including advances in medical resources such as colonoscopy examinations. Consequently, the diagnostic rate of IBD has

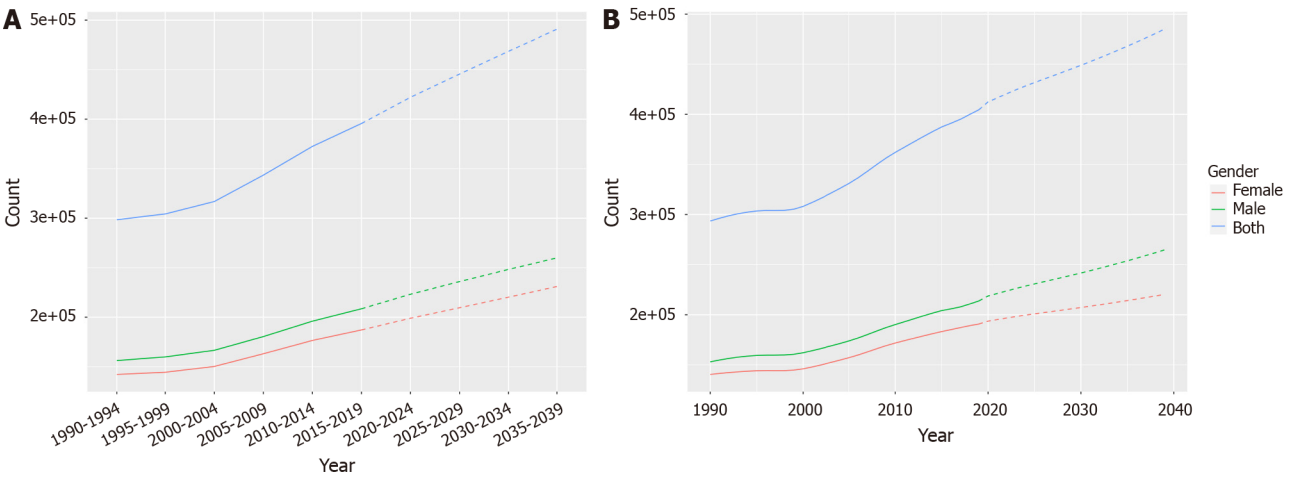




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Figure 4 Trends in inflammatory bowel disease age standardized rate by sociodemographic index from 1990-2019. A: Twenty-one Global

Burden of Diseases regions are shown; B: 204 countries and territories are shown. The expected values for sociodemographic indices and disease incidence based on all sites are shown as black lines. ASR: Age standardized rate; SDI: Socio-demographic index.



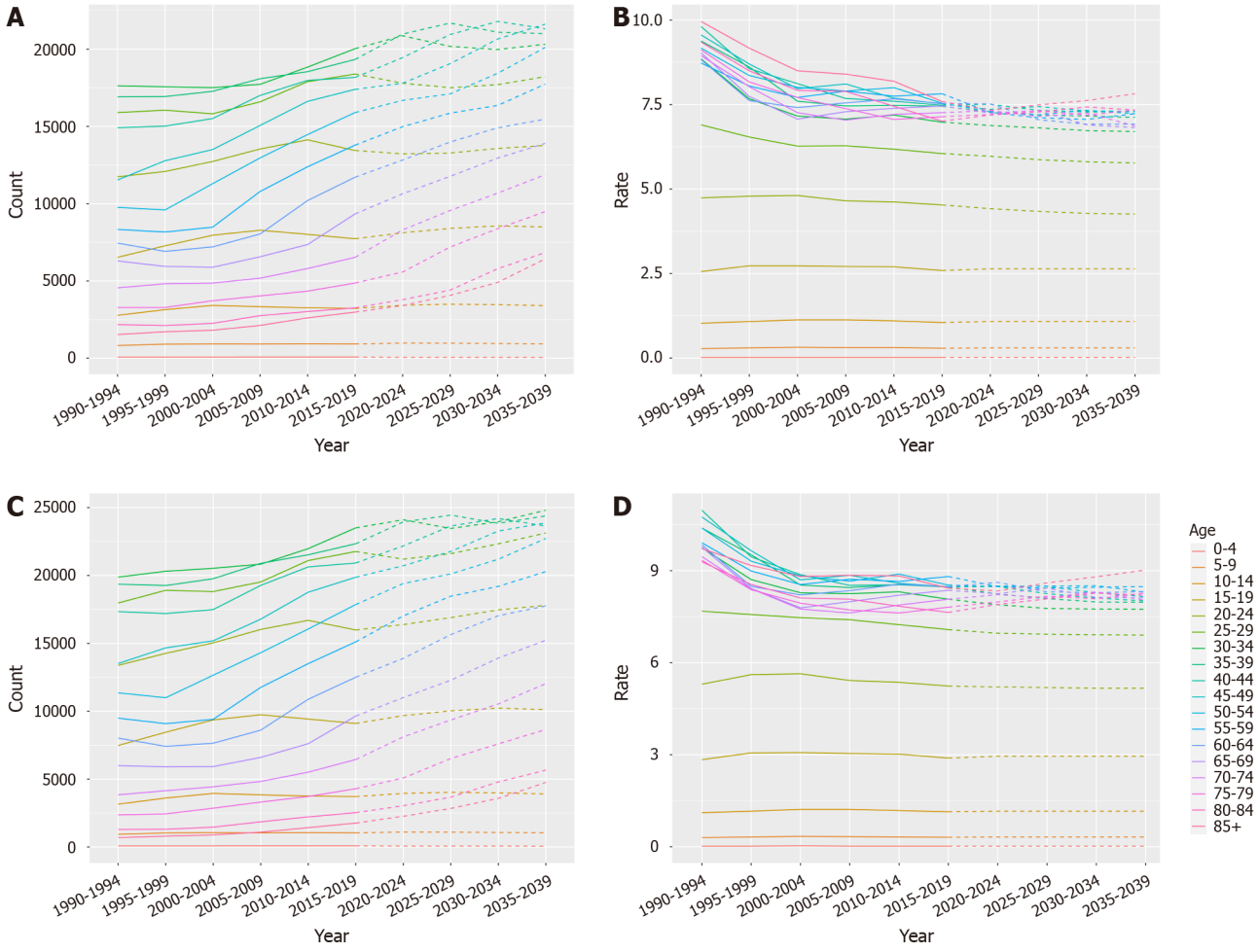
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Figure 5 Statistics and predictions of the number of cases in all age groups. A: Prediction based on Nordpred model; B: Prediction based on Bayesian age-period-cohort model.

increased in low-income regions, leading to an increasing incidence of IBD in emerging industrialized nations such as Asia. The cluster analysis results of the current study further support this trend, revealing a significant surge in the incidence of IBD in Asian regions, such as Vietnam, China, Taiwan, and Jordan. This transformation may be attributed to urbanization, industrialization, and cultural Westernization in societies. In addition, the actual increase in IBD incidence may be due to an increased number of individuals with genetically-susceptible genes to an environment influenced by Westernization[33]. In densely populated areas, residents experience the influence of Westernization, leading to changes in diet with higher fat and refined sugar consumption. Lifestyles changes as well including increased smoking, reduced breastfeeding, greater exposure to antibiotics, and improvements in personal hygiene and sanitation, may also play a role [34]. All of these factors have contributed to the increase in the incidence of IBD, which exerted significant pressure on the populations. The reported IBD incidence varied among emerging industrialized countries in Asia and within different regions of Asian countries. For example, from 2011-2013, the IBD incidence was 1.14 per 100000 (95%CI: 0.91-1.41) in Singapore, while in India was 9.31 per 100000 (95%CI: 8.38-10.31)[35]. Overall, the highest incidences rates have been observed in India and China, as the published incidence rates are approaching the Coalescing Incidence Range in the Western world[20,35,36]. Due to the large population proportions and continuously increasing gross domestic product of the economy, IBD may become a substantial healthcare burden in these countries. Furthermore, the current study revealed that the increasing trend of IBD incidence has gradually extended to East and Southeast Asia.

While previous studies have reported that the global IBD incidence was higher in females than males[37], our analysis of IBD cases from different age groups revealed that both females and males had an increasing trend in the number of cases, but a declining trend in incidence. The overall incidence was higher in males than in females in this study. The IBD incidence predictions for the next 20 years by BAPC and Nordpred models indicate a decreasing trend in ASR for both males and females, with a higher incidence in males than in females. Based on the heterogeneity among different geographical regions, significant disparities exist in the incidence of IBD between genders. These disparities were influenced not only by biological factors but also by variations in environmental exposure and access to healthcare services[38,39]. Males exhibited more severe disease symptoms of IBD compared to females, and premature mortality confirmed that this constituted a significant portion of the burden in males[28]. Additionally, sex hormones may influence the pathogenesis of IBD and the function of the intestinal epithelial barrier[37,40]. Moreover, some certain genetic loci associated with IBD are located on the X chromosome, indicating the potential involvement of sex-related factors in the development of the disease[41].

This study revealed a continuous increase in IBD cases paired with a declining trend in the global ASIR of IBD. The population is aging over time, resulting in more elderly IBD individuals in 2020 compared to 1990. For instance, the annual increase in IBD prevalence among elderly individuals between 1999 and 2008 in Ontario, Canada was 5.2%[42]. Furthermore, in terms of life expectancy, the elderly population with IBD experienced a slightly increased risk of mortality compared to both the age-related comorbidity group (such as cardiovascular diseases and cancer) and the general control group in previous studies[43,44]. Therefore, with an aging population and extended life expectancies, healthcare systems face a significant demographic burden. While the IBD population in the Western world is expected to eventually transition into a phase of balanced disease prevalence and even decline in some regions[33]. Furthermore, there were significant variations in the ASIR among the GBD regions, and we found that East Asia exhibited the largest upward trend, while Central Europe showed the most rapid decline, which were consistent with the previous report[20].



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Figure 6 Inflammatory bowel disease incidence from 1990-2019 and projections for the next 20 years, stratified by gender. A: Statistics and projections of the number of female patients with inflammatory bowel disease (IBD) are shown; B: Statistics and predictions of the female incidence of IBD are shown; C: Statistics and projections of the number of male patients with IBD are shown; D: Statistics and predictions of the male incidence of IBD are shown. Prediction of changes over the next 20 years are based on the Nordpred model.

The increasing incidence of IBD in emerging industrialized nations and the heterogeneity of reported values may be attributed to several factors. First, economic progress has improved awareness regarding IBD and provided greater access to healthcare, leading to the identification of undiagnosed cases. Second, the Westernization of society has contributed to an increase in the prevalence of IBD. With advancements in healthcare facilities, the epidemiological surveillance strategies towards electronic medical databases, and the implementation of endoscopic examinations, the detection and diagnosis of IBD have increased. Over time, there has been a steady increase in the reported incidence of IBD. However, studies have shown that age-standardized disability-adjusted life years and mortality rates had a declining trend globally [28], which might be attributed to improved treatment strategies and the implementation of patient support programs. The introduction of biological agents has significantly improved IBD treatment strategies, enhanced patient quality of life, and reduced the rates of surgical intervention[45]. Recent emerging therapies showed immense potential for the treatment of IBD, for example, the targeted adhesion molecule therapies, cytokine inhibitors, stem cell therapies, gut-brain axis modulation, and host-microbiome interaction regulation have enhanced the efficacy of IBD treatment and reduced the adverse events and overall mortality[46]. However, the cost of these biological therapies was considerably high, imposing a substantial financial burden on patients[47]. Therefore, it is crucial to focus more on the inclusion of early stage patients, optimization of drug dosage, and determination of the appropriate time for trial discontinuation to alleviate such financial burden.

This study has several strengths. First, it included a large, population-based dataset covering numerous countries and regions, including Asia. Second, the associations and significances of various demographic indicators in different areas were analyzed. We forecast the number and incidence of IBD from 2019-2039 based on the R software and validated the reliability of the results. Variations in the incidence of IBD may reflect differences in urbanization rates, living conditions, or dietary factors[48]. The data in this study may guide research targeting specific environmental risk factors for IBD and be used to determine the burden of IBD on society.

However, the study also has some limitations. First, the data obtained from the GBD 2019 is limited. As for regions with scarce data, we have to rely on predictive covariates or global trends, which leads to poor representativeness. Therefore, further population-based high-quality research is needed, especially in countries with scarce data. Second, we

Table 1 Incidence and age-standardized incidence of inflammatory bowel disease in various regions of the world from 1990-2019 and temporal trends from 1990-2019

Region	1990		2019		1990-2019
	Incident cases, No. (95%UI)	ASR per 100000, No. (95%UI)	Incident cases, No. (95%UI)	ASR per 100000, No. (95%UI)	EAPC, No. (95%CI)
Africa	6350.4 (5449.9-7409.6)	1.4 (1.2-1.6)	16261.3 (13488.9-19647.2)	1.6 (1.4-2)	0.41 (0.35-0.47)
African Region	3832.8 (3242.9-4587.4)	1.1 (1-1.3)	10258.1 (8583.6-12449.3)	1.3 (1.1-1.6)	0.52 (0.46-0.57)
America	121311.1 (106225.2-139803.3)	17.8 (15.6-20.4)	122924.6 (112507.2-135597.1)	11.1 (10.1-12.2)	-1.3 (-1.64 to -0.96)
Andean Latin America	472.1 (401.7-549.7)	1.6 (1.4-1.9)	1128.2 (974.6-1314.4)	1.8 (1.6-2.1)	-0.05 (-0.28-0.19)
Asia	64767.7 (54186.2-76818.5)	2.3 (2-2.8)	145560.7 (124960.3-170895)	2.9 (2.5-3.4)	0.78 (0.69-0.86)
Australasia	2979 (2555.6-3481.3)	13.6 (11.6-15.8)	6446.3 (5754.6-7211.5)	20 (17.8-22.6)	0.94 (0.73-1.15)
Caribbean	676.8 (580.4-796)	2.1 (1.8-2.5)	1311.8 (1121-1571.1)	2.6 (2.2-3.1)	0.49 (0.34-0.63)
Central Asia	3514.1 (2995-4146.2)	6 (5.1-7.1)	6461.3 (5487.2-7636.7)	6.9 (5.9-8.1)	0.5 (0.46-0.53)
Central Europe	20996.8 (18082.3-24169.9)	15.8 (13.6-18.2)	14808.9 (13442.8-16390.5)	11.6 (10.5-12.8)	-1.38 (-1.75 to -1.01)
Central Latin America	3967.5 (3401.5-4617.8)	3.2 (2.7-3.7)	6753.6 (5833.6-7825.9)	2.6 (2.3-3.1)	-0.7 (-0.86 to -0.54)
Central Sub-Saharan Africa	381.4 (318.5-468.6)	1 (0.9-1.3)	1149.7 (954.9-1399.8)	1.3 (1.1-1.5)	0.64 (0.59-0.69)
Commonwealth High Income	26861.8 (24786.3-29244.5)	22.3 (20.6-24.2)	37806 (34948.3-41071.2)	24.5 (22.6-26.6)	0.42 (0.33-0.52)
Commonwealth Low Income	1939.8 (1595.5-2362)	1.4 (1.1-1.7)	5032.5 (4152.5-6067.4)	1.6 (1.4-2)	0.61 (0.56-0.66)
Commonwealth Middle Income	18395 (15168.4-22494.1)	2 (1.7-2.4)	39643.1 (33048-48435)	2.1 (1.8-2.6)	0.44 (0.3-0.58)
East Asia	17567.3 (14626.9-20802.4)	1.4 (1.2-1.7)	52318.8 (44680.4-61454.8)	3 (2.5-3.4)	2.54 (2.4-2.68)
East Asia & Pacific - WB	40537 (34343.4-47828.7)	2.3 (2-2.7)	90853.2 (79210-104418.9)	3.4 (2.9-3.9)	1.16 (1.06-1.26)
Eastern Europe	18149.7 (15600.1-21209.1)	7.1 (6.1-8.3)	19499.8 (16838-22671)	7.3 (6.3-8.5)	0.07 (-0.01-0.16)
Eastern Mediterranean Region	6925.3 (5871.5-8131.4)	2.5 (2.1-2.9)	18957.5 (15653.5-22992.9)	2.9 (2.4-3.5)	0.54 (0.49-0.59)
Eastern Sub-Saharan Africa	1092.2 (908-1315.3)	0.9 (0.8-1.1)	2937.7 (2448.6-3538.4)	1 (0.9-1.3)	0.55 (0.49-0.61)
Europe	100967 (89900.9-113361.1)	11.5 (10.3-13)	119525.9 (108023.4-132990.6)	12.5 (11.3-14)	0.18 (0.11-0.26)
Europe & Central Asia - WB	103166.8 (91767-115892)	11.2 (10-12.7)	124122.3 (111829.4-138334.3)	12 (10.8-13.5)	0.13 (0.06-0.2)
European Region	103678.4 (92211.4-116480.8)	11.2 (10-12.6)	125055.7 (112703.8-139366)	12 (10.8-13.4)	0.12 (0.05-0.2)
High-income Asia Pacific	18105.1 (15391.4-21421.8)	9.2 (7.9-10.9)	27083.8 (23870.3-30621.1)	14.9 (13.1-16.9)	1.1 (0.85-1.36)
High-income North America	107616.3 (94146.8-123884.4)	34.9 (30.6-40)	99611.1 (91945.2-108999.8)	24.5 (22.6-26.8)	-0.82 (-1.18 to -0.47)
Latin America & Caribbean - WB	13797.5 (11970.2-15892.3)	3.8 (3.3-4.3)	23456.2 (20522.6-26876.2)	3.4 (3-3.9)	-0.41 (-0.47 to -0.35)
Middle East & North Africa - WB	5823.7 (5004.6-6721.7)	2.9 (2.5-3.4)	15826.6 (13170.5-19047.6)	3.4 (2.9-4.1)	0.5 (0.43-0.58)
North Africa and Middle East	8106.7 (7039.5-9203.5)	2.9 (2.6-3.4)	22722.3 (19277.1-26951.9)	3.7 (3.2-4.5)	0.62 (0.53-0.72)
North America	107604.1 (94136.5-123870)	34.9 (30.6-40)	99596.5 (91933-108983)	24.5 (22.6-26.8)	-0.82 (-1.18 to -0.47)

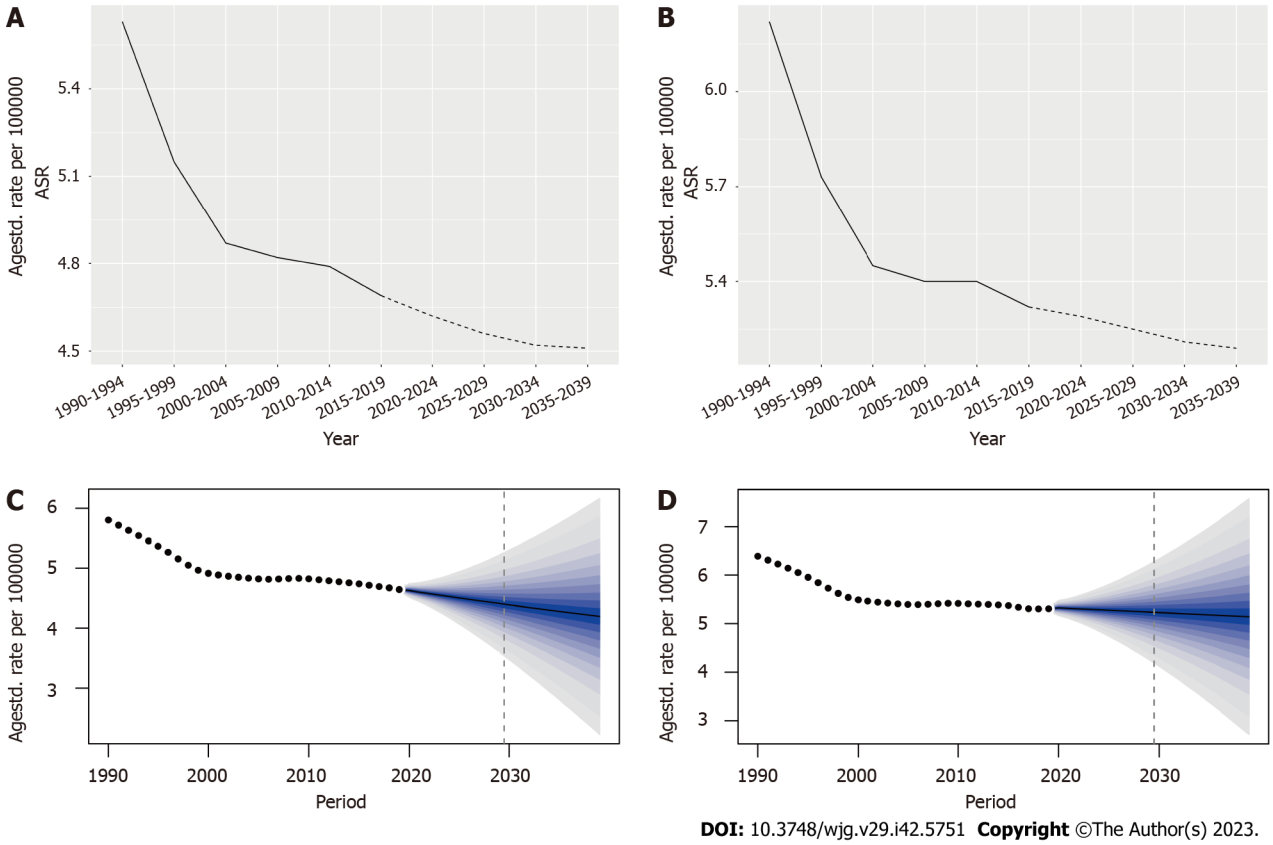
Oceania	22.5 (18.3-26.8)	0.4 (0.4-0.5)	63.2 (51.8-76.5)	0.6 (0.5-0.7)	0.87 (0.77-0.98)
Region of the Americas	121311.1 (106225.2-139803.3)	17.8 (15.6-20.4)	122924.6 (112507.2-135597.1)	11.1 (10.1-12.2)	-1.3 (-1.64 to -0.96)
South-East Asia Region	18276.9 (15080.6-22303.3)	1.7 (1.4-2.1)	38664.5 (32256.8-47052.4)	1.9 (1.6-2.3)	0.57 (0.42-0.73)
South Asia	18439.4 (15168.6-22581.9)	2.2 (1.8-2.6)	39704.7 (32943.4-48453.5)	2.3 (1.9-2.8)	0.47 (0.32-0.62)
South Asia - WB	18738.6 (15412.5-22942.5)	2.1 (1.8-2.6)	40659.5 (33741.9-49588.8)	2.3 (1.9-2.8)	0.49 (0.34-0.64)
Southeast Asia	1882 (1527.1-2270.3)	0.5 (0.4-0.6)	4980 (4181.8-5972.3)	0.7 (0.6-0.8)	1.45 (1.28-1.62)
Southern Latin America	879.4 (741.6-1029.7)	1.8 (1.5-2.1)	1382 (1177.4-1619.7)	1.9 (1.6-2.3)	0.24 (0.15-0.33)
Southern Sub-Saharan Africa	503.7 (422.7-607.5)	1.3 (1.1-1.6)	988.7 (837.3-1188)	1.4 (1.2-1.6)	0.13 (0.07-0.2)
Sub-Saharan Africa - WB	3626.5 (3031.8-4397.1)	1.1 (0.9-1.3)	9635 (8053-11630.3)	1.2 (1-1.5)	0.42 (0.34-0.5)
Tropical Latin America	7825.7 (6858.8-8975.6)	6.1 (5.4-6.9)	12927.7 (11333.6-14873.9)	5.2 (4.6-6)	-0.55 (-0.59 to -0.51)
Western Europe	59080.5 (53732.6-65528.7)	14 (12.7-15.5)	78644.7 (71611.1-86708.9)	16.9 (15.3-18.7)	0.6 (0.56-0.65)
Western Pacific Region	39273.1 (33180.5-46324.7)	2.6 (2.2-3.1)	88120.1 (76953.2-101217.2)	4 (3.5-4.5)	1.25 (1.15-1.35)
Western Sub-Saharan Africa	1313.7 (1101.9-1598.6)	1 (0.9-1.2)	3628.3 (3028.5-4400.8)	1.2 (1-1.4)	0.36 (0.22-0.49)
World Bank High Income	206040 (182079.4-234728.1)	18.8 (16.6-21.4)	225597.7 (206227.4-249141.7)	17.1 (15.6-18.9)	-0.24 (-0.39 to -0.08)
World Bank Low Income	2388.3 (1998.4-2891.8)	1 (0.9-1.3)	6306.6 (5252.9-7655.6)	1.2 (1.1-1.5)	0.56 (0.52-0.6)
World Bank Lower Middle Income	30142.4 (25192.1-36034.9)	2 (1.7-2.4)	63401.3 (53275.5-76198.6)	2.1 (1.8-2.6)	0.29 (0.2-0.37)
World Bank Upper Middle Income	54916.9 (47252.2-63473.1)	2.8 (2.5-3.3)	109097 (94356.3-126861)	3.5 (3.1-4.1)	0.74 (0.67-0.8)

ASR: Age standardized rate; CI: Confidence interval; EAPC: Estimated annual percentage change; UI: Uncertainty interval.

cannot establish a causal relationship between the population data and the incidence of IBD as they are ecological associations. We also did not investigate the dose-response effects to demonstrate whether the risk of IBD increases with the rising population size or levels of multiple exposure factors. In regions and countries with a lower SDI, patients may be less likely to be diagnosed with IBD due to limited access to healthcare, resulting in an underestimation and underreporting of the disease burden. Therefore, the reported incidence may be lower than the actual incidence in these areas. Further studies should focus on regional variations in IBD incidence, taking into account the potential influences of healthcare conditions, lifestyles, and the environment with an emphasis on the associations between IBD and certain risk factors. In conclusion, based on the global burden of IBD from 1990-2019 and the prediction for the next 20 years trend, the latest global epidemiological estimates for IBD and new hypotheses are provided in this study to improve the management of IBD and alleviate the global burden of this chronic disease.

CONCLUSION

This study provides insights into monitoring the burden and trends of IBD at global, regional, and national levels, which is crucial for implementing interventions to address the increasing burden of IBD. The findings revealed that IBD continues to pose a significant disease burden worldwide, particularly in regions with higher SDI, where the incidence of IBD is higher. However, in recent years, the incidence of IBD has rapidly increased in emerging industrialized and developing countries. Therefore, it is imperative to prioritize the prevention, management, and recurrence of IBD with a focus on closely-related factors such as infections, diet, biomarkers, disease treatments, lifestyle, psychology, and genetics. Emphasizing personal hygiene and health education, improving nutritional status, and implementing early treatment are essential methods to alleviate the burden of IBD. Further research is needed to determine more effective public health interventions.



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Figure 7 Statistics and predictions of inflammatory bowel disease age standardized rate for different genders. A and B: The age standardized rate (ASR) statistics and predictions based on the Nordpred model for female and male, respectively; C and D: The ASR statistics and predictions for female and male based on the Bayesian age-period-cohort model, respectively. ASR: Age standardized rate.

ARTICLE HIGHLIGHTS

Research background

Inflammatory bowel disease (IBD) is an intestinal inflammatory disorder of unknown origin with a prevalence that varies across different countries and regions. To develop effective strategies for the prevention and treatment of IBD, a comprehensive understanding of the current burden and distinct trends in various geographical areas are crucial. There is a scarcity of research for the prediction of the global burden of IBD and future epidemiological trends.

Research motivation

Data from the Global Burden of Disease (GBD) database 2019 were used to assess the burden of IBD across 204 countries and regions using various indicators from 1990-2019. Previous studies lacked the use of specific models for forecasting future trends in IBD and did not validate the reliability of their findings. Our findings offer new insights regarding the management of IBD.

Research objectives

This study aimed to conduct a thorough investigation of IBD data and predict future epidemiological trends to provide the latest estimates of the global burden of IBD and improve the management strategies.

Research methods

The incidence data for IBD were collected from the GBD study from 1990-2019. The average annual percentage change and estimated annual percentage change (EAPC) in age-standardized rates (ASR) of IBD were calculated for various regions. The relationships among IBD, the human development index, and the socio-demographic index (SDI) were analyzed. The Nordpred and Bayesian age-period-cohort models were used to predict the prevalence trends of IBD from 2019-2039.

Research results

North America consistently maintained the highest IBD ASR, while Oceania consistently had the lowest ASR. East Asia had the fastest average annual growth in ASR (2.54%), whereas Central Europe had the most rapid decline (1.38%) in ASR. Nations that had low age-standardized incidence rates in 1990 exhibited accelerated IBD growth, although no significant correlation was observed in 2019. Additionally, IBD grew faster in countries with low age-standardized death

rates in 1990, whereas the opposite was true in 2019. An examination of the SDI and IBD ASR revealed that countries with a high SDI generally exhibited a higher IBD ASR. Finally, the projections indicated a decreasing trend in IBD incidence from 2019-2039 but a gradual increase in the total number of cases.

Research conclusions

IBD poses a substantial disease burden globally, particularly in regions with higher SDI scores, which are associated with increased IBD incidence. Nevertheless, in recent years, the incidence of IBD has rapidly increased in emerging industrialized and developing nations. Therefore, it is imperative to prioritize the prevention, management, and reduction of IBD cases, with particular consideration for the associated factors.

Research perspectives

The investigation of regional variations in the incidence of IBD is required, and it is imperative to investigate the associations between IBD and various risk factors.

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FOOTNOTES

Author contributions: Li CJ and He SX conceptualized and designed the study; Zhang SM and Ren MD collected the data; Li CJ and Wang YK analyzed and interpreted the data; Li CJ wrote this paper; and all authors have read and approved the final manuscript.

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Institutional review board statement: The study was wavied by the IRB of the First Affiliated Hospital of Xi'an Jiaotong University due to all the data used in this study were from the Global Burden of Diseases (GBD) 2019 database. This database comprises publicly accessible data, freely available in an open-access repository, ensuring that all information used was already in the public domain.

Informed consent statement: There was no requirement to give informed consent in this study, because all the data used in this study were from the Global Burden of Diseases (GBD) 2019 database. This database comprises publicly accessible data, freely available in an open-access repository, ensuring that all information used was already in the public domain.

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Data sharing statement: Dataset can be available.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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ORIGINAL ARTICLE

Observational Study

Predicting disease progression in cirrhotic patients after transjugular intrahepatic portosystemic shunt implantation: A sex-stratified analysis

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Abstract

BACKGROUND

Transjugular intrahepatic portosystemic shunt (TIPS) has been extensively used to treat portal hypertension-associated complications, including cirrhosis. The prediction of post-TIPS prognosis is important for cirrhotic patients, as more aggressive liver transplantation is needed when the post-TIPS prognosis is poor.

AIM

To construct a nutrition-based model that could predict the disease progression of cirrhotic patients after TIPS implantation in a sex-dependent manner.

METHODS

This study retrospectively recruited cirrhotic patients undergoing TIPS implantation for analysis. Muscle quality was assessed by measuring the skeletal muscle index (SMI) by computed tomography. Multivariate Cox proportional hazard models were utilized to determine the association between SMI and disease progression in cirrhotic patients after TIPS implantation.

RESULTS

This study eventually included 186 cirrhotic patients receiving TIPS who were followed up for 30.5 ± 18.8 mo. For male patients, the 30-mo survival rate was significantly lower and the probability of progressive events was higher (3.257-fold) in the low-level SMI group than in the high-level SMI group. According to the multivariate Cox analysis of male patients, SMI < 32.8 was an independent risk factor for long-term adverse outcomes after TIPS implantation. A model was constructed, which involved creatinine, plasma ammonia, SMI, and acute-on-chronic liver failure and hepatic encephalopathy occurring within half a year after surgery. This model had an area under the receiver operating characteristic curve of 0.852, sensitivity of 0.926, and specificity of 0.652. According to the results of the DeLong test, this model outperformed other models (Child-Turcotte-Pugh, Model for End-Stage Liver Disease, and Freiburg index of post-TIPS survival) ($P < 0.05$).

CONCLUSION

SMI is strongly associated with poor long-term outcomes in male patients with cirrhosis who underwent TIPS implantation.

Key Words: Transjugular intrahepatic portosystemic shunt; Skeletal muscle index; Nutrition; Cirrhosis; Sex stratified; Prognosis

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Core Tip: Our study highlights the strong association between the skeletal muscle index and long-term adverse outcomes in male patients with cirrhosis who received transjugular intrahepatic portosystemic shunt (TIPS) implantation, reflecting the necessity for adequate sex-stratified analysis. Additionally, nutritional indicator-based models hold tremendous promise for effectively predicting the prognosis of cirrhotic patients undergoing TIPS implantation and assisting clinicians in closely monitoring high-risk populations.

Citation: Zhang Q, Long L, Zhu HL, Peng H, Luo XH, Zhu KS, Wang RP. Predicting disease progression in cirrhotic patients after transjugular intrahepatic portosystemic shunt implantation: A sex-stratified analysis. *World J Gastroenterol* 2023; 29(42): 5768-5780
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INTRODUCTION

Cirrhosis is frequently complicated by esophagogastric variceal bleeding and ascites, two decompensating events, which are closely associated with the presence of portal hypertension[1]. Transjugular intrahepatic portosystemic shunt (TIPS) has been extensively applied for the treatment of portal hypertension-related complications, which reduces the portal pressure gradient by creating a conduit between the portal and systemic circulations. At present, TIPS can be used for the treatment of cirrhosis complicated by refractory ascites or for secondary prevention of upper gastrointestinal hemorrhage in patients who cannot be treated by endoscopy[2-4].

Although TIPS implantation techniques have improved, there is still a subset of patients with a poor prognosis because such patients typically develop advanced cirrhosis with more than one complication when TIPS implantation is needed. Hence, it is warranted to screen patients with a potentially poor prognosis after TIPS for closer follow-up, as they may require more aggressive liver transplantation. Currently, the post-TIPS survival of patients is commonly predicted with models including Child-Turcotte-Pugh (CTP) and Model for End-Stage Liver Disease (MELD)[5,6]. In addition, recent studies have also elaborated that the post-TIPS survival of patients can be effectively predicted with the bilirubin-platelet and Freiburg index of post-TIPS survival (FIPS) models[7,8]. Nevertheless, the use of these models is limited by two factors. First, these models are constructed based on studies of Western patients who have different pathogeneses from Eastern patients. Consequently, the predictive value of these models for Eastern patients has yet to be verified. Second, these models focus on the short-term prognosis of patients after surgery and cannot assess the long-term survival of patients. Thus, a new alternative model is needed to accurately predict the long-term outcome of Asian patients with ascites- or variceal bleeding-induced end-stage liver disease who receive TIPS implantation.

In addition, the basic nutritional status of patients is also an important factor that affects the long-term survival of patients with end-stage liver disease[9,10]. Notably, measuring the skeletal muscle index (SMI) of the third lumbar spine (L3) by abdominal computed tomography (CT) is the most objective method for assessing nutritional status[11]. Intriguingly, prior studies demonstrated that SMI was more strongly associated with end-stage liver disease in men than in women[12,13]. Therefore, this study sought to establish a nutritional indicator-based model to predict the long-term survival of Asian patients with cirrhosis after TIPS implantation and stratify long-term survival by sex.

MATERIALS AND METHODS

This retrospective study recruited 264 patients undergoing TIPS implantation for refractory ascites or major gastroesophageal bleeding at the Guizhou Provincial People's Hospital from January 2016 to December 2021. Among these patients, we excluded those with incomplete data (n = 54), Budd-Chiari syndrome (n = 7), noncirrhotic portal hypertension (n = 6), hepatocellular carcinoma (n = 6), right heart diastolic dysfunction (the ratio of early-to-late diastolic flow velocity < 0.75) (n = 2), left ventricular ejection fraction of less than 50% evaluated by echocardiography within 1 mo before TIPS implantation (n = 2), or pulmonary hypertension (n = 1). Finally, 186 patients were included (Figure 1).

Refractory ascites referred to the failure to reduce ascites by sodium restriction and high-dose diuretics (spironolactone at a dose up to 400 mg/d and furosemide at a dose of 160 mg/d) or an untreatable condition due to complications[14]. Based on the Baveno VI criteria, major gastroesophageal bleeding was defined as a high risk of rebleeding after the first episode of gastroesophageal variceal bleeding was treated[15].

The indication for TIPS implantation was determined through the careful clinical evaluation of each patient by a panel of hepatologists and interventional specialists at our hospital. CT-guided puncture of the right internal jugular vein was used as peripheral vascular access for TIPS, and the patient underwent portal vein puncture guided by real-time CT. An expandable polytetrafluoroethylene-covered stent (10 mm; Viatorr TIPS endoprosthesis; Gore, Flagstaff, Arizona, USA) was initially expanded to 8 mm. The patency rate of TIPS in patients was monitored by Doppler ultrasound every 3-6 mo.

Demographic data and laboratory parameters of all cirrhotic patients were extracted from medical records. Laboratory parameters included blood biochemistry, routine blood tests, alpha-fetoprotein, and plasma ammonia. Prognostic scores (including MELD and CTP scores) were calculated with the latest test results before TIPS implantation. SMI at L3 was evaluated according to abdominal CT scans within one month before TIPS implantation. SMI was calculated according to our previous research[16]. The primary outcome was adverse progression within 30 mo after TIPS implantation, including liver transplantation or death from complications such as gastrointestinal bleeding, liver failure, and hepatic encephalopathy (HE).

Ethics statement

This study was carried out in accordance with recommendations in the ethical guidelines of the latest version of the Declaration of Helsinki and those provided by the Guizhou Provincial People's Hospital. The protocol was approved by the Ethics Committee of the Guizhou Provincial People's Hospital. Individual consent was waived for this retrospective analysis.

Statistical methods

Data were processed with SPSS 25.0 statistical analysis software (IBM Corp., Armonk, NY, United States) and R statistical analysis software (version 3.6.0; R Foundation for Statistical Computing, Vienna, Austria). A difference significantly differed at $P < 0.05$ (two-sided). Continuous variables were compared by the Student's t test or the Mann-Whitney U test and are expressed as the mean ± standard error in the presence of a normal distribution or as the median in the absence of a normal distribution. Categorical data are presented as numbers (percentages) and were compared by the chi-square test. Spearman's correlation coefficients (r_s) were used for correlation analyses. Independent predictors of disease progression were evaluated with univariate and multivariate Cox proportional hazard models, followed by the assessment of model calibration by analyzing the C-index. The area under the receiver operating characteristic (ROC) curve (AUC) was compared by the DeLong test. Cumulative transplant-free survival rates were plotted as Kaplan-Meier curves and compared by the log-rank test.

RESULTS

Characteristics of patients

This study ultimately included 186 cirrhotic patients receiving TIPS implantation who were followed up for 30.5 ± 18.8 mo on average. The main characteristics of the cohorts at baseline are presented in Table 1. These participants comprised 142 males (115 in the transplantation-free survival group and 27 in the progression group) and 44 females (34 in the transplantation-free survival group and 10 in the progression group), suggesting that the transplantation-free survival rate was 80.1% in total, 81.0% for male patients, and 77.3% for female patients. Subsequently, various baseline characteristics of the male and female patients were compared between the two groups. It was found that patients with poor outcomes were older with a higher neutrophil-to-lymphocyte ratio. For male patients, the SMI and albumin levels were lower but ammonia and the CTP scores were higher in the progression group than in the transplantation-free survival group. However, these trends were not obvious for female patients.

Survival analysis

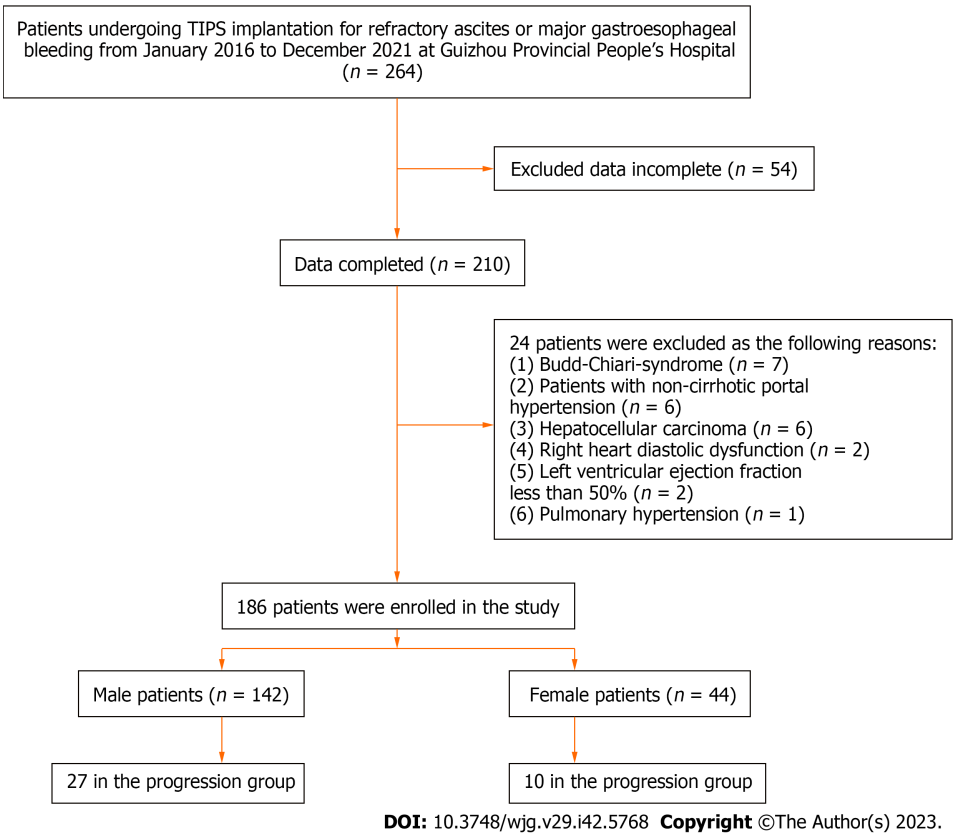
To further identify the impact of SMI on the outcomes of patients, we used SMI to plot ROC curves and selected the optimal cutoff value (SMI = 32.8) based on the Youden index. Based on the cutoff value, patients were arranged into low-level SMI and high-level SMI groups, followed by the comparison of the long-term cumulative survival rates between the two groups. For male patients, the 30-mo survival rate was significantly lower in the low-level SMI group (58.0%) than in the high-level SMI group (86.6%) ($P < 0.001$), accompanied by a higher probability (3.257-fold) of progressive events (liver transplantation or death), as shown in the Cox proportional hazard regression analysis. Conversely, no significant

Table 1 Patient characteristics in the cohorts

Variable	All (*n* = 186)	Male (*n* = 142)			Female (*n* = 42)		
		Transplantation-free survival (*n* = 115)	Progression (*n* = 27)	*P* value	Transplantation-free survival (*n* = 34)	Progression (*n* = 10)	*P* value
Age (yr)	51 (44-57)	48.0 (43.0-53.0)	56.0 (45.0-63.0)	0.017	55.0 (47.0-62.0)	64.0 (62.0-66.0)	0.001
BMI (kg/m^2)	23.0 (21.4-24.0)	23.0 (21.7-24.3)	23.0 (20.8-23.0)	0.098	22.1 (21.4-23.5)	21.9 (19.4-23.7)	0.73
SMI (cm^2/m^2)	38.9 (31.2-45.3)	41.9 (36.6-48.1)	37.6 (30.8-44.2)	0.036	29.4 (25.4-33.4)	28.68 (26.49-35.36)	0.901
SATI (cm^2/m^2)	27.1 (14.1-45.6)	24.8 (13.1-38.6)	20.9 (10.4-36.5)	0.252	43.79 (19.23-55.96)	51.51 (32.58-81.8)	0.128
VATI (cm^2/m^2)	37.6 (24.5-49.7)	37.3 (22.7-53.1)	39.0 (24.8-48.1)	0.592	32.45 (24.58-41.92)	39.90 (34.25-69.58)	0.075
MELD score	9.0 (6.0-12.0)	8.0 (5.0-11.0)	11.0 (7.0-14.0)	0.070	9.0 (6.0-12.0)	10.0 (9.75-11.25)	0.354
CTP score	7.0 (6.0-9.0)	7.0 (6.0-9.0)	9.0 (7.0-9.0)	0.014	7.5 (6.0-8.0)	7.0 (6.0-8.0)	0.572
CTP classification				0.148			0.513
A (%)	55 (29.6)	38 (33.0)	4 (14.8)		10 (20.4)	3 (30.0)	
B (%)	113 (60.8)	67 (58.3)	19 (70.4)		20 (58.8)	7 (70.0)	
C (%)	18 (9.7)	10 (8.7)	4 (14.8)		4 (11.8)	0 (0)	
Etiology (%)				0.051			0.09
HBV/HCV	104 (55.9)	69 (60.0)	12 (44.4)		20 (58.8)	3 (30.0)	
Alcohol	51 (27.4)	40 (34.8)	10 (37.0)		1 (2.9)	0 (0)	
Others	31 (16.7)	6 (5.2)	5 (18.6)		13 (38.2)	7 (70.0)	
Ascites (%)				0.43			0.993
None	48 (25.8)	28 (24.3)	4 (14.8)		12 (35.3)	4 (40.0)	
Mild	51 (27.4)	35 (30.4)	7 (25.9)		7 (20.6)	2 (20.0)	
Moderate	45 (24.2)	29 (25.2)	7 (25.9)		7 (20.6)	2 (20.0)	
Massive	42 (22.6)	23 (20.0)	9 (33.3)		8 (23.5)	2 (20.0)	
Portal vein thrombosis (%)	37 (19.9)	23 (20.0)	6 (22.2)	0.797	8 (23.5)	0 (0)	
Portal vein pressure (mm Hg)	32.0 (28.0-36.8)	32.3 (28.4-36.9)	33.8 (30.1-38.4)	0.141	30.5 (24.4-33.1)	30.5 (27.0-32.3)	0.553
Albumin (g/L)	30.45 (26.80-33.90)	30.85 (26.95-33.93)	28.60 (24.90-30.80)	0.041	31.05 (27.33-34.68)	29.90 (23.43-33.45)	0.464
International normalized ratio	1.28 (1.16-1.38)	1.29 (1.18-1.37)	1.34 (1.15-1.50)	0.295	1.23 (1.07-1.37)	1.18 (1.13-1.38)	0.61
Total bilirubin (mol/L)	22.60 (14.90-31.65)	20.7 (13.6-29.9)	28.3 (12.6-43.6)	0.236	24.15 (17.88-37.18)	28.35 (22.20-32.68)	0.649
Creatinine (mol/L)	70.5 (59.0-82.3)	72.55 (62.38-83.18)	79.60 (64.80-114.10)	0.069	57.05 (49.75-65.88)	58.50 (51.50-80.00)	0.499
Sodium (mmol/l)	138.45 (136.0-140.8)	138.25 (136.00-140.48)	138.20 (135.00-141.00)	0.985	139.00 (135.75-140.85)	139.00 (133.90-140.80)	0.585
Alanine aminotransferase (U/L)	21.0 (15.0-32.3)	21.0 (15.0-30.0)	24.0 (14.0-45.0)	0.274	19.5 (15.8-32.8)	28.4 (22.2-32.7)	0.989
Plasma ammonia (mol/L)	47.8 (34.7-54.6)	44.84 (33.80-49.20)	50.56 (44.40-69.07)	0.005	47.80 (34.46-54.84)	48.18 (46.20-62.12)	0.354
White blood cells (×	3.76 (2.60-	3.71 (2.60-5.50)	4.78 (3.20-8.44)	0.108	3.32 (2.17-5.55)	3.79 (3.07-6.22)	0.312

10^9/L)	5.66)						
Platelets (× 10^9/L)	64.5 (46.0-91.3)	65.0 (47.0-92.0)	70.0 (49.0-92.0)	0.888	66.0 (46.5-83.5)	51.5 (39.0-80.8)	0.354
Neutrophils (× 10^9/L)	2.60 (1.58-4.23)	2.49 (1.61-3.99)	3.93 (2.12-6.79)	0.048	2.11 (1.34-4.00)	2.66 (2.28-5.03)	0.273
Lymphocytes (× 10^9/L)	0.67 (0.49-0.97)	0.68 (0.49-0.96)	0.65 (0.46-1.08)	0.944	0.64 (0.54-1.09)	0.52 (0.40-0.78)	0.108
NLR	3.45 (2.52-5.79)	3.38 (2.41-5.64)	5.22 (3.16-7.28)	0.046	2.70 (1.99-4.50)	6.38 (3.97-8.59)	0.001
PLR	94.25 (67.80-133.51)	95.39 (68.91-133.33)	86.36 (64.12-153.33)	0.670	100.00 (67.18-127.60)	101.80 (78.06-149.53)	0.591
Alpha-fetoprotein, mmol/L	4.10 (2.19-5.36)	4.09 (2.10-5.36)	3.92 (2.40-5.36)	0.796	3.48 (2.07-5.36)	5.36 (5.36-5.53)	0.016

Continuous variables are presented as median in the absence of normal distribution. Categorical variables are presented as absolute value and relative frequency [*n* (%)]. Differences in continuous variables were calculated using Mann-Whitney U test and differences in categorical variables were assessed using the chi-square test. BMI: Body mass index; SMI: Skeletal muscle index; SATI: Subcutaneous adipose tissue index; VATI: Visceral adipose tissue index; MELD: Model of end-stage liver disease; CTP: Child-Turcotte-Pugh; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio.



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Figure 1 Flow diagram. TIPS: Transjugular intrahepatic portosystemic shunt.

difference was observed in the long-term cumulative survival rate of female patients between the two groups (Figure 2).

Factors associated with disease progression

In the entire cohort, significantly different indicators in the univariate analysis and body mass index were combined for further multivariate Cox proportional hazard regression analyses. The results revealed that etiology, creatinine, plasma ammonia, alpha-fetoprotein, SMI < 32.8, and HE occurring within half a year after surgery were independent risk factors for 30-mo adverse outcomes in cirrhotic patients undergoing TIPS. In male patients, SMI < 32.8 was also found to be an independent risk factor for long-term adverse outcomes after TIPS but not in female patients (Table 2). Further analyses exhibited a negative correlation between SMI and CTP scores (r = -0.302, P < 0.001), as confirmed by the lower SMI in

Table 2 Univariable and multivariable Cox regression analyses for prognostic factors in patients with transjugular intrahepatic portosystemic shunt implantation

Death or liver transplantation	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
All (*n* = 186)				
Age (yr)	1.073 (1.039-1.108)	< 0.001		
BMI	0.914 (0.814-1.026)	0.128		
CTP score	1.212 (1.012-1.451)	0.037		
Etiology (%)				
HBV/HCV	Reference		Reference	
Alcohol	1.421 (0.638-3.165)	0.39	1.205 (0.503-2.888)	0.675
Others	2.862 (1.337-6.125)	0.007	3.807 (1.680-8.623)	0.001
Creatinine (mol/L)	1.008 (1.005-1.012)	< 0.001	1.006 (1.002-1.011)	0.005
Plasma ammonia (mol/L)	1.027 (1.013-1.041)	< 0.001	1.025 (1.009-1.041)	0.003
Alpha-fetoprotein (mmol/L)	1.030 (1.017-1.044)	< 0.001	1.021 (1.005-1.036)	0.009
NLR	1.088 (1.036-1.142)	0.001		
SMI < 32.8	2.250 (1.179-4.291)	0.014	2.192 (1.008-4.769)	0.048
Postoperative ACLF	2.927 (1.218-7.037)	0.016		
Postoperative HE	10.115 (4.851-21.091)	< 0.001	8.721 (4.065-18.710)	< 0.001
Male (*n* = 142)				
Age (yr)	1.062 (1.024-1.100)	0.001		
BMI	0.897 (0.781-1.030)	0.897		
CTP score	1.294 (1.069-1.566)	0.008		
MELD score	1.122 (1.034-1.217)	0.006		
Etiology (%)				
HBV/HCV	Reference			
Alcohol	1.453 (0.627-3.367)	0.384		
Others	3.396 (1.193-9.670)	0.022		
Creatinine (mol/L)	1.009 (1.005-1.013)	< 0.001	1.008 (1.004-1.013)	0.001
Plasma ammonia (mol/L)	1.028 (1.012-1.044)	< 0.001	1.028 (1.011-1.046)	0.001
Alpha-fetoprotein (mmol/L)	1.032 (1.015-1.049)	< 0.001		
NLR	1.080 (1.018-1.146)	0.011		
SMI < 32.8	3.275 (1.516-7.073)	0.003	3.277 (1.306-8.226)	0.011
Postoperative ACLF	10.066 (3.249-31.187)	< 0.001	5.621 (1.685-18.752)	0.005
Postoperative HE	6.697 (3.015-14.876)	< 0.001	5.110 (2.153-12.126)	< 0.001

Variable selection for the multivariable Cox regression model was performed using stepwise forward selection. CI: Confidence interval; HR: Hazard ratio; BMI: Body mass index; CTP: Child-Turcotte-Pugh; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NLR: Neutrophil-to-lymphocyte ratio; SMI: Skeletal muscle index; ACLF: Acute-on-chronic liver failure; HE: Hepatic encephalopathy.

patients with CTP grades B and C than in the patients with CTP grade A ($P < 0.05$) (Figure 3).

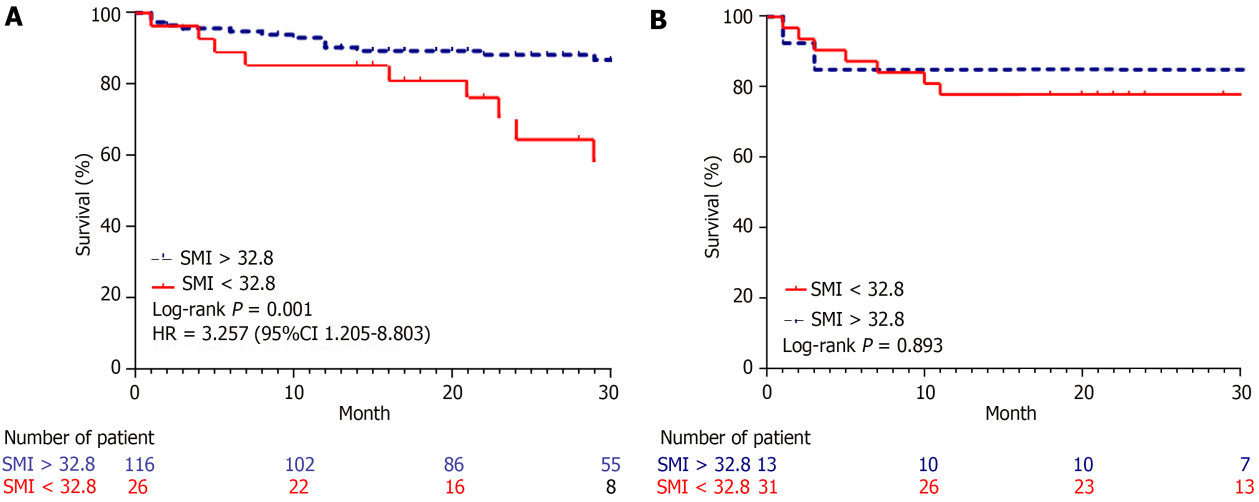
A prognostic model for patients receiving TIPS implantation

To further analyze the predictive value of SMI for the long-term prognosis of male patients, we used 5-fold cross-validation to classify male patients into five folds, among which four folds were used as the training set and a fold as the validation set. As demonstrated in Table 3, SMI was included as a predictor in all five models. The results showed that the other models had more stable prediction performance in both the training and validation sets (the AUC values were

Table 3 Predictive performance of different models in the training set and test set

Model	Variable	Training set AUC (95%CI)	Sensitivity	Specificity	Test set AUC (95%CI)	Sensitivity	Specificity
Model 1	Creatinine, plasma ammonia, SMI, ACLF, HE	0.842 (0.763-0.921)	0.957	0.652	0.875 (0.672-1.000)	0.75	0.962
Model 2	Creatinine, plasma ammonia, SMI, ACLF, HE	0.864 (0.785-0.942)	0.864	0.707	0.809 (0.631-0.986)	1.000	0.609
Model 3	Creatinine, SMI, HE	0.804 (0.689-0.918)	0.857	0.674	0.768 (0.581-0.955)	0.833	0.696
Model 4	Creatinine, plasma ammonia, SMI, ACLF, HE	0.842 (0.761-0.923)	0.783	0.813	0.948 (0.844-1.00)	1.000	0.792
Model 5	Creatinine, plasma ammonia, SMI, HE	0.840 (0.760-0.921)	0.957	0.636	0.823 (0.659-0.987)	1.000	0.667

We used 5-fold cross-validation to classify male patients into five parts, among which four parts were used as the training set and one part as the validation set. AUC: Area under the receiver operating characteristic curve; CI: Confidence interval; SMI: Skeletal muscle index; ACLF: Acute-on-chronic liver failure; HE: Hepatic encephalopathy.



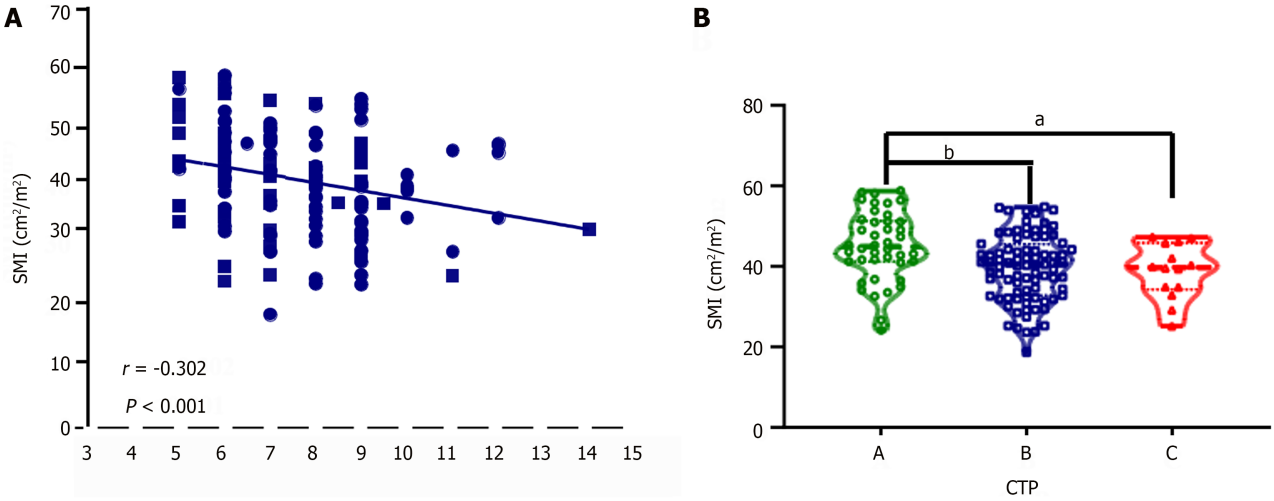
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Figure 2 Actuarial survival rates of cirrhotic patients undergoing transjugular intrahepatic portosystemic shunt implantation according to sex-stratified analysis. A: Actuarial survival in male patients with cirrhosis after transjugular intrahepatic portosystemic shunt implantation; B: Actuarial survival in female patients with cirrhosis after transjugular intrahepatic portosystemic shunt implantation. CI: Confidence interval; SMI: Skeletal muscle index; HR: Hazard ratio; TIPS: Transjugular intrahepatic portosystemic shunt.

all > 0.8), except for model 3, which had slightly worse prediction performance. To perform a comprehensive assessment of the entire male data set, we reconducted the multivariate Cox proportional hazard regression analysis of the characteristics screened for each submodel in the 5-fold cross-validation across the entire male data set. Eventually, a model was constructed by including creatinine, plasma ammonia, SMI, and acute-on-chronic liver failure (ACLF) and HE occurring within half a year after surgery, which had an AUC of 0.852 [95% confidence interval (CI): 0.782-0.923], sensitivity of 0.926, and specificity of 0.652.

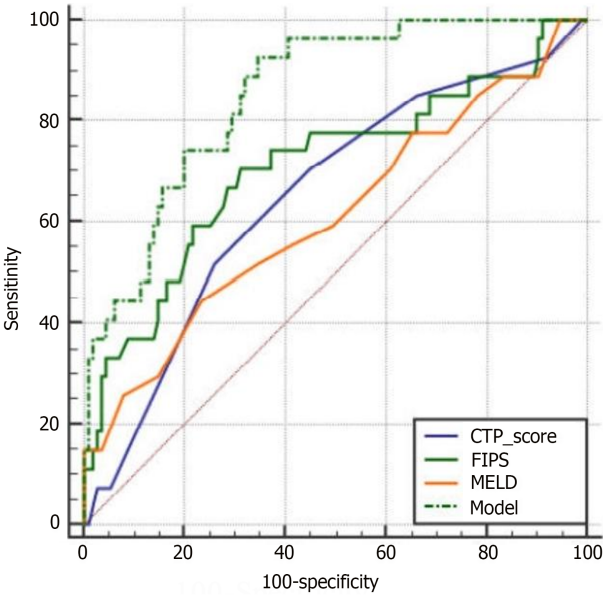
Comparative analysis with the existing models

Finally, our model was compared with other models, including MELD, CTP, and FIPS, in terms of the C-index, AUC, and survival rate. The C-index and AUC of our model for 30-mo survival were 0.890 and 0.852 (95%CI = 0.782-0.923), respectively, significantly higher than those of MELD [C-index = 0.603, AUC = 0.612 (P < 0.001)], CTP [C-index = 0.644, AUC = 0.651 (P = 0.003)], and FIPS [C-index = 0.737, AUC = 0.709 (P = 0.015)] (Figure 4). Moreover, the 30-mo cumulative survival rates were compared between the low- and high-risk groups in the different models. For the high-risk group, the survival rate was extremely low, at 59.5%, according to our model, 67.0% according to CTP, 71.2% according to MELD, and 69.5% according to FIPS. Similarly, the median survival was 22.9 mo based on our model (95%CI: 20.3-25.5), 24.1 mo based on CTP (95%CI: 21.1-27.2), 24.8 mo based on MELD (95%CI: 21.6-27.9), and 24.0 mo based on FIPS (95%CI: 21.3-



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Figure 3 Relationship between changes in skeletal muscle index levels and Child-Turcotte-Pugh scores in cirrhotic patients undergoing transjugular intrahepatic portosystemic shunt implantation. A: Correlation between Child-Turcotte-Pugh (CTP) scores and skeletal muscle index (SMI) in male patients with cirrhosis who underwent transjugular intrahepatic portosystemic shunt implantation. Spearman's correlation coefficients (*r*) are indicated; B: Comparisons of SMI levels among patients with different CTP grades. CTP: Child-Turcotte-Pugh; SMI: Skeletal muscle index; TIPS: Transjugular intrahepatic portosystemic shunt. $^{a}P < 0.05$; $^{b}P < 0.01$.



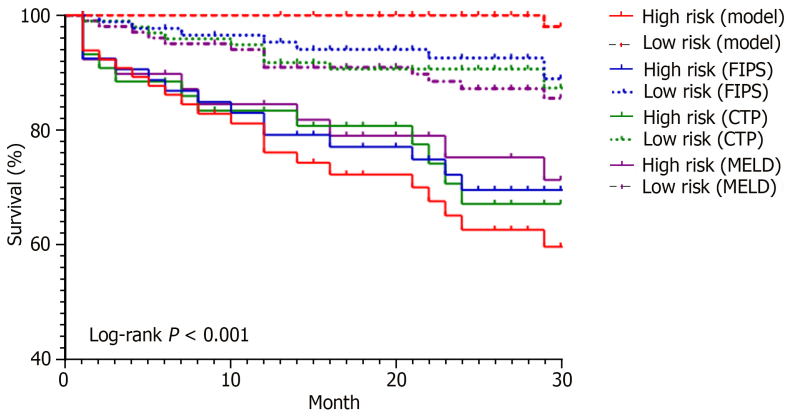
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Figure 4 Comparisons of the newly constructed model against previous models including Child-Turcotte-Pugh, Freiburg index of post-transjugular intrahepatic portosystemic shunt survival, and Model for End-Stage Liver Disease in terms of the value in predicting 30-mo mortality. In the DeLong test, $P < 0.05$ was considered statistically significant. The constructed model involved creatinine, plasma ammonia, skeletal muscle index at baseline, and acute-on-chronic liver failure and hepatic encephalopathy occurring within half a year after surgery. CTP: Child-Turcotte-Pugh; FIPS: Freiburg index of post-transjugular intrahepatic portosystemic shunt survival; MELD: Model for End-Stage Liver Disease.

26.8). The survival curves were significantly different among the groups ($P < 0.001$) (Figure 5).

DISCUSSION

Our data demonstrated that among 186 cirrhotic patients receiving TIPS implantation, approximately 20% experienced liver transplantation or death within 30 mo after implantation, largely consistent with some previous studies[17,18]. Interestingly, our results also exhibited a significant association between SMI and long-term poor prognoses only in male patients with cirrhosis. Subsequent multivariate regression analyses elucidated that a low level of SMI in males was an independent risk factor for long-term adverse outcomes in cirrhotic patients receiving TIPS. Under the low level of SMI,



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Figure 5 Kaplan-Meier plots showing the cumulative probability of liver transplant-free survival in different risk groups according to different models. The constructed model involved creatinine, plasma ammonia, skeletal muscle index at baseline, and acute-on-chronic liver failure and hepatic encephalopathy occurring within half a year after surgery. CTP: Child-Turcotte-Pugh; FIPS: Freiburg index of post-TIPS survival; MELD: Model for End-Stage Liver Disease.

the 30-mo postoperative adverse outcome rate was 42.0% in male patients and 22.6% in female patients. These findings essentially reveal the differential impacts of SMI on the long-term prognosis of patients undergoing TIPS implantation in the context of sex differences. Therefore, it is necessary to raise awareness of sex-specific therapeutic interventions in clinical practice.

A low level of SMI has been reported to share an association with various adverse outcomes in extrahepatic and intrahepatic diseases[19-21]. Meanwhile, some studies have also described the relationship between SMI and TIPS implantation in cirrhotic patients under a wide spectrum of pathological conditions and the possible related pathogenesis. For instance, in a recent study involving 224 patients undergoing TIPS, SMI was measured preoperatively and at 2, 5, and 12 mo postoperatively. The results showed that sarcopenia was associated with an increased risk of post-TIPS mortality and that the reversal of sarcopenia after TIPS may reduce the risk of mortality[22]. Other studies have also displayed an association of reduced skeletal muscle with HE, ACLF, and increased mortality after TIPS implantation[23-25]. Of note, a prior study was conducted in 2019 to investigate sarcopenia and frailty in patients with cirrhosis, which emphasized the importance of sex-stratified analyses[12]. This study revealed that two-thirds of male patients with frailty showed signs of sarcopenia, while only a quarter of female patients with frailty exhibited signs of sarcopenia, indicating that frailty-inducing factors might vary by sex. Another study in 2021 also demonstrated that SMI level reduction was associated with multidimensional frailty in male patients with cirrhosis[13]. Accordingly, sex-specific therapeutic strategies are needed to reduce frailty in this population. In this context, our follow-up cohort study was performed for in-depth analysis. Interestingly, our study illustrated SMI as an independent risk factor for long-term adverse outcomes in male patients with cirrhosis following TIPS implantation. Furthermore, the newly constructed prediction model based on SMI had a higher prediction efficiency than previous models. We believe that this finding is plausible and informative since a growing body of research has demonstrated a strong correlation of nutritional status with disease progression and outcomes in cirrhotic patients.

In addition, our findings also suggested sex differences in the relationship between a low level of SMI and long-term prognosis after TIPS implantation, concordant with findings in other phenotypic studies of chronic liver disease[12,13]. This difference can be explained by two facts. First, cirrhotic patients usually have hormonal changes, such as a substantial diminishment in testosterone levels in male patients[26]. Second, muscle homeostasis varies by sex, and androgen may be the main sex hormone regulating male muscle homeostasis[27]. Overall, the prognostic value of low SMI levels in male patients receiving TIPS implantation may be partly related to sex-specific hormonal differences.

Subsequently, we noted that the poor prognosis of cirrhotic patients undergoing TIPS implantation was also closely related to elevated plasma ammonia levels. Reportedly, hyperammonaemia is not only a primary cause of HE but is also associated with damage to multiple systems, causing numerous complications of decompensated cirrhosis, including bacterial infection, variceal bleeding, and ascites[28]. For instance, plasma ammonia can promote the progression of liver injury, liver fibrosis, and portal hypertension by activating hepatic stellate cells through direct induction of hepatocyte death[29,30]. In addition, hyperammonaemia exhibits an association with innate immune dysfunction in patients with cirrhosis[31]. More importantly, progressive skeletal muscle loss leads to the deficiency of compensatory pathways for extrahepatic ammonia detoxification *via* glutamine synthetase, and ammonia accumulation has been documented to contribute to skeletal muscle loss through a variety of mechanisms[32]. Of note, previous studies have also confirmed that sarcopenia is associated with the increased mortality of patients with cirrhosis and is attributable to hyperammonemia [33]. Furthermore, hyperammonaemia in patients with cirrhosis may be the result of liver dysfunction, portosystemic shunts, skeletal muscle loss (reduced extrahepatic ammonia clearance), renal dysfunction, and intestinal dysbacteriosis [34]. Hence, the occurrence of hyperammonaemia reflects a complex and multiorgan interaction in cirrhosis that involves the dysfunction of the liver, kidneys, nerves, immunity, and skeletal muscle, and it is a critical biomarker for predicting the prognosis of patients with cirrhosis.

In addition to SMI levels and hyperammonaemia, creatinine levels also exert effects on the prognosis of cirrhosis. It has been reported that some patients with cirrhosis may also develop hepatorenal syndrome. Prior studies have indicated that renal dysfunction can be considered the result of hemodynamic damage associated with the severity of liver diseases, illustrating that renal dysfunction may also affect the long-term prognosis of patients receiving TIPS implantation[8]. Importantly, the postoperative level of creatinine, a fairly reliable index of renal function, can reflect renal function. ACLF and HE are also two frequent complications of TIPS implantation, which can result in the deterioration of liver function and are tightly related to poor prognoses in patients. In summary, the long-term survival of patients after TIPS implantation can be more accurately predicted by prognostic models constructed based on SMI, creatinine, and plasma ammonia levels, as well as the short-term postoperative complications of ACLF and HE in patients.

Our study has some limitations. First, because of the nature of observational studies, we only observed an association between SMI level reduction and long-term prognosis in male patients with cirrhosis after TIPS implantation. Nevertheless, it is not clear whether changes in muscle mass are a cause of disease progression, an aggravating factor of persistent pathological progression, or an incidental phenomenon reflecting a general adverse condition in patients with cirrhosis. Second, our study did not analyze and assess changes in the sex hormone levels of each patient in the cohort. Therefore, the different potential roles of this factor in muscle formation between men and women is only speculative. Third, our model lacked external validation in similar populations in other centers. Accordingly, further multicenter studies are warranted to validate our findings.

CONCLUSION

Our study highlights the strong correlation between SMI and long-term adverse outcomes in male patients with cirrhosis after TIPS implantation, favoring adequate sex-stratified analyses. Moreover, models constructed based on nutritional indicators hold promise for the effective prediction of prognosis in cirrhotic patients receiving TIPS implantation, assisting clinicians in closely monitoring the dynamics of high-risk populations.

ARTICLE HIGHLIGHTS

Research background

Transjugular intrahepatic portosystemic shunt (TIPS) has been extensively applied for the treatment of portal hypertension-related complications. However, there is still a subset of patients with a poor prognosis because such patients typically develop advanced cirrhosis with more than one complication when TIPS implantation is needed. Hence, it is warranted to screen patients with a potentially poor prognosis after TIPS for closer follow-up, as they may require more aggressive liver transplantation.

Research motivation

Currently, the post-TIPS survival of patients is commonly predicted by models including Child-Turcotte-Pugh and Model for End-Stage Liver Disease. Nevertheless, the use of these models is limited by two factors. First, these models are constructed based on studies of Western patients who have different pathogeneses from Eastern patients. Second, these models focus on the short-term prognosis of patients after surgery and cannot assess the long-term survival of patients. Thus, a new alternative model is needed to accurately predict the long-term outcome of Asian patients with ascites- or variceal bleeding-induced end-stage liver disease who receive TIPS implantation.

Research objectives

This study aimed to construct a nutrition-based model that could predict the disease progression of cirrhotic patients after TIPS implantation in a sex-dependent manner.

Research methods

According to the inclusion and exclusion criteria, 186 patients were eventually enrolled in this retrospective study. Skeletal muscle index (SMI) at the third lumbar spine was evaluated according to abdominal computed tomography scans within one month before TIPS implantation. A difference significantly differed at $P < 0.05$. Continuous variables were compared by the Student's t test or the Mann-Whitney U test. Categorical data are presented as numbers (percentages) and were compared by the chi-square test. Spearman's correlation coefficients (r_s) were used for correlation analyses. Independent predictors of disease progression were evaluated with univariate and multivariate Cox proportional hazard models, followed by the assessment of model calibration by analyzing the C-index. The area under the receiver operating characteristic curve was compared by the DeLong test. Cumulative transplant-free survival rates were plotted as Kaplan-Meier curves and compared by the log-rank test.

Research results

Our data demonstrated that among 186 cirrhotic patients receiving TIPS implantation, approximately 20% experienced liver transplantation or death within 30 mo after implantation. Interestingly, our results also exhibited a significant association between SMI and long-term poor prognoses only in male patients with cirrhosis. Subsequent multivariate

regression analyses elucidated that a low level of SMI in males was an independent risk factor for long-term adverse outcomes in cirrhotic patients receiving TIPS. Under the low level of SMI, the 30-mo postoperative adverse outcome rate was 42.0% in male patients and 22.6% in female patients. These findings essentially reveal the differential impacts of SMI on the long-term prognosis of patients undergoing TIPS implantation in the context of sex differences. Further multicenter studies are warranted to validate our findings.

Research conclusions

Our study highlights the strong correlation between SMI and long-term adverse outcomes in male patients with cirrhosis after TIPS implantation, favoring adequate sex-stratified analyses.

Research perspectives

Models constructed based on nutritional indicators hold promise for the effective prediction of prognosis in cirrhotic patients receiving TIPS implantation, assisting clinicians in closely monitoring the dynamics of high-risk populations. Therefore, it is necessary to raise awareness of sex-specific therapeutic interventions in clinical practice.

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FOOTNOTES

Author contributions: Wang RP and Zhu KS conceptualized and designed the study; Long L and Zhu HL acquired the data; Zhang Q and Long L analyzed and interpreted the data; Zhang Q drafted the manuscript; Peng H and Luo XH critically revised the manuscript for important intellectual content; Zhang Q, Peng H, and Luo XH provided administrative, technical, or material support; Wang RP supervised the study; all authors made a significant contribution to this study and approved the final manuscript.

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Institutional review board statement: This study was carried out in accordance with recommendations in the ethical guidelines of the latest version of the Declaration of Helsinki and those provided by the Guizhou Provincial People's Hospital. The protocol was approved by the Ethics Committee of the Guizhou Provincial People's Hospital.

Informed consent statement: Individual consent was waived for this retrospective analysis.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: The clinical data used to support the findings of this study are available from the corresponding author at wangrongpin@126.com upon request.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of item.

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SCIENTOMETRICS

Research status and hotspots of autoimmune gastritis: A bibliometric analysis

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Abstract

BACKGROUND

As an emerging potential risk factor for gastric cancer, autoimmune gastritis (AIG) has garnered increasing attention from researchers.

AIM

To analyze the research overview and popular topics in the field of AIG using bibliometrics.

METHODS

Relevant publications on AIG in the Web of Science Core Collection were collated, and data visualization and analysis of the number of publications, countries, institutions, journals, authors, keywords, and citations were performed using software such as VOSviewer, CiteSpace, and Scimago Graphic.

RESULTS

In total, 316 relevant articles were included in the analysis. From 2015 to 2022, the number of publications increased annually. The countries, institutions, authors, and journals with the highest number of publications in this field were Italy, Monash University, Toh BH, and Internal Medicine. The main keywords used in this field of research were pathogenesis, *Helicobacter pylori*, autoantibody, parietal cell antibody, atrophic gastritis, classification, diagnosis, autoimmune disease,

risk, cancer, gastric cancer, vitamin B12 deficiency, and pernicious anemia. The following directions may be popular for future research: (1) The role of *Helicobacter pylori* in the pathogenesis of AIG; (2) diagnostic criteria for AIG and reference values for serum antibodies; (3) comorbidity mechanisms between AIG and other autoimmune diseases; (4) specific risks of AIG complicating gastric and other cancers; and (5) the role of vitamin B12 supplementation in patients with early-stage AIG.

CONCLUSION
This bibliometric analysis reported on popular topics and emerging trends in AIG, with diagnosis and prognosis being research hotspots in this field.

Key Words: Autoimmune gastritis; Autoimmune diseases; Bibliometric; CiteSpace; VOSviewer; *Helicobacter pylori*

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Core Tip: The pathogenesis, diagnostic criteria and prognosis of autoimmune gastritis (AIG) have been controversial. This study is expected to provide valuable insights for AIG. It uses bibliometrics to scrutinize factors such as publication years, countries, institutions, authors, journals, and keywords, while also revealing key areas for future research: (1) The role of *Helicobacter pylori* in the pathogenesis of AIG; (2) diagnostic criteria for AIG and reference values for serum antibodies; (3) comorbidity mechanisms between AIG and other autoimmune diseases; (4) specific risks of AIG complicating gastric and other cancers; and (5) the role of vitamin B12 supplementation in patients with early-stage AIG.

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INTRODUCTION

Autoimmune gastritis (AIG) is a chronic inflammatory disease characterized by the destruction of gastric parietal cells, loss of intrinsic factors, and atrophy of the gastric mucosa[1]. Epidemiological investigations indicate that the prevalence of AIG ranges from 0.1% to 2% in the general population, and it can be as high as 2%-3% in females and those older than 60 years of age[2]. The pathogenesis of AIG has not been fully elucidated, and current theories suggest that CD4+ T lymphocytes target the H+/K+- ATPase of parietal cells, stimulating plasma cells to secrete autoantibodies, which may constitute the main pathogenesis[3]. The autoantibodies secreted by plasma cells mainly include parietal cell antibodies (PCA) and intrinsic factor antibodies (IFA)[4]; the former plays a pivotal role in parietal cell destruction and glandular atrophy[5], while the latter is an internal mechanism that triggers vitamin B12 deficiency and pernicious anemia[6]. Due to the nonspecific clinical symptoms of AIG, its diagnosis primarily relies on digestive endoscopy, histopathological manifestations, and serum antibodies[7]. Although recently published "Diagnostic criteria and endoscopic and histological findings of autoimmune gastritis in Japan" offer guidance for the clinical diagnosis of AIG[8], ongoing controversy remains regarding the diagnostic criteria for AIG among different countries, and as yet, there are no uniform diagnostic criteria. Research progress has provided increasing evidence in recent years that AIG may be a preneoplastic disease, which increases the patient's risk of developing gastric cancer and neuroendocrine tumors[9]. Furthermore, AIG is often comorbid with other autoimmune diseases, and it has been reported that approximately 53% of patients with AIG suffer from autoimmune thyroid disease[10]. Moreover, AIG may be a risk factor for other autoimmune diseases[11]. There are ongoing controversies surrounding the pathogenesis, diagnostic criteria, and prognosis of AIG, which may characterize future research in this field.

Bibliometrics is an emerging method of literature analysis that can quantitatively and qualitatively analyze articles in related fields[12] and summarize various characteristics of the included literature, such as countries, institutions, journals, authors, citation rates, and keywords, thereby revealing popular research topics and trends in related fields that have been widely applied across multiple fields[13-15]. In recent years, research in the field of AIG has progressed rapidly, with an increasing number of scholars focusing on the pathogenesis, diagnosis, and prognosis of AIG; however, there are no bibliometric studies related to AIG. Therefore, this study uses bibliometric analysis to explore the current research status and popular topics of AIG to provide directions for future research.

MATERIALS AND METHODS

Data sources and search strategy

The Web of Science Core Collection (WoSCC) was chosen as the data source for this study because of its coverage, citation

analysis, journal quality, standardized data, and interdisciplinary research[16,17]. The proposed search formula was "translational science = Autoimmune gastritis OR Autoimmune atrophic gastritis". The search was conducted until May 2023, the restricted language was English, and the types of studies included were articles and review articles.

Data collection and analysis

Yu YF and Tong KK screened the documents and excluded non-journal papers, papers not written in English, and those that were not relevant to the research topic. The remaining documents were exported in "plain text file" and "delimited file" formats. Disagreements were resolved by Yang XY.

The data were imported into Microsoft Excel 2021 to construct a bibliometric database. VOSviewer 1.6.19, CiteSpace 6.2.2, Pajek 5.16, and Scimago Graphica 1.0.34 were used for the bibliometric analysis of the research data, which included the countries, institutions, authors, journals, references, and keywords of the selected literature. Bibliometric indicators such as average citations per item (ACI), H-index, centrality, and sum of times cited (SOTC) were used for data analysis [18,19]. The ACI metric was used to measure the academic impact of a researcher, organization, or scholarly journal, with higher values indicating a greater academic impact. The H-index is the highest number of papers that received at least one citation. A higher H-index indicates greater influence. Centrality is an important metric used to identify the relative importance of a node within a network. The SOTC summed all citations of a set of papers, providing a holistic view of the overall impact, with a higher value indicating greater academic influence.

RESULTS

The trend of publication output

We searched a total of 1014 articles and ultimately included 316 articles after screening (Figure 1). As shown in Figure 2, the study of AIG commenced in 1969, and 53.48% of the total research articles were published in the last decade. The number of articles published each year after 2018 has consistently increased compared to the previous year, with the number of articles published in 2019 being 1.31 times that in 2018, the number of articles published in 2020 being 1.35 times that in 2019, and the number of articles published in 2021 being 1.17 times that in 2020. In 2022, there were 39 articles, which was 1.44 times that in 2021. For 2023, our data cover only the first five months; thus, a year-to-year comparison is not yet available.

Analysis of country/region distribution

Thirty countries published studies on AIG. Italy had the highest number of publications, accounting for 22.15% of the total, followed by Japan (21.52%), the United States (18.67%), and Australia (15.19%); the remaining countries contributed 5.7% or less (Table 1).

To visualize the collaboration among countries/regions, this study employed Scimago Graphica 1.0.34, in which a larger node area corresponds to a larger number of publications from that country or region. Nodes with a redder color represent a larger number of collaborative publications between that country/region and other countries/regions, whereas a thicker connecting line between nodes indicates closer collaboration between the two countries/regions, as shown in Figure 3. Among these, the United States had the highest number of collaborative publications with other nations. Italy and the United States exhibited the closest cooperation, followed by Italy and the Netherlands. In terms of the H-index, Italy led the way with an H-index of 25, followed by Australia (H-index = 22) and the United States (H-index = 19). Regarding ACI, Sweden (ACI = 57.3) took the lead, followed by the Netherlands (ACI = 50.63) and Australia (ACI = 33.52).

Analysis of institution distribution

A total of 409 institutions conducted research on AIG. The institution with the highest number of publications was Monash University in Australia, with 38 articles, which is twice as many as the second-ranked University of Pavia (n = 19). Kyoto University in Japan ranked third with 17 publications. Half of the top 10 institutions were based in Italy, while the remaining institutions were from Australia (n = 2), Japan (n = 2), and Turkey (n = 1), as shown in Table 2.

Institutional co-occurrence analysis illustrates the collaborative relationships among organizations, as demonstrated in Figure 4. The size of the node indicates the volume of publications, and the color intensity of the node represents the level of collaboration with other institutions. Among the top 10 institutions, Monash University had the highest number of collaborative publications with other organizations, indicating a strong cooperative relationship.

Analysis of authors and authors' collaborations

A total of 1428 authors participated in AIG-related research, with an average of 4.5 authors per paper. Notably, the top four authors with the highest number of publications were from Australia. Among them, Toh had the highest publication volume, with 31 articles published, followed by Vandriel (n = 25), Gleeson (n = 23), and Alderuccio (n = 19). Among the top 10 authors, three were from Italy, two were from Turkey, and one was from Japan. In terms of the ACI, Gleeson topped the list with a score of 41.96, closely followed by Toh (ACI = 41.35) and Lenti (ACI = 34.17), as shown in Table 3.

Author co-occurrence networks, as illustrated in Figure 5, provide insight into the collaborative relationships between authors. Larger nodes signify more publications, node colors represent different countries, and connecting lines denote collaborative relationships between individual authors. In terms of collaborative relationships, four large working groups consisting of 10 or more authors were observed. Among the top 10 authors by publication volume, Australian authors

Table 1 Top 10 productive countries

Rank	Country	Quantity	Proportion (%)	ACI	H-index	Centrality
1	Italy	70	22.15	29.16	25	0.49
2	Japan	68	21.52	14.65	16	0.03
3	United States	59	18.67	26.32	19	0.80
4	Australia	48	15.19	33.52	22	0.04
5	Germany	18	5.70	18.5	11	0.06
6	Turkey	16	5.06	6.44	6	0.00
7	Sweden	10	3.16	57.3	9	0.01
8	China	9	2.85	1.78	2	0.00
9	Netherlands	8	2.53	50.63	6	0.00
10	Finland	7	2.22	11.57	5	0.29

ACI: Average citations per item.

Table 2 Top 10 productive institutions

Rank	Institution	Country	Quantity	SOTC	ACI	H-index
1	Monash University	Australia	38	1413	37.18	20
2	University of Pavia	Italy	19	433	22.79	10
3	Kyoto of University	Japan	17	593	34.88	12
4	Ankala University	Turkey	14	95	6.79	6
5	Sapienza University Rome	Italy	14	284	20.29	9
6	University of Melbourne	Australia	14	367	26.21	11
7	Irccs Fondazione San Matteo	Italy	13	375	28.85	7
8	University of Padua	Italy	12	467	38.92	8
9	Azienda Ospedaliera Sant Andrea	Italy	11	192	17.45	7
10	Kawasaki Medical School	Japan	11	92	8.36	6

SOTC: Sum of times cited; ACI: Average citations per item.

tended to collaborate more frequently with their domestic counterparts, whereas Italian authors exhibited higher degrees of international collaboration.

Analysis of journal distribution

Table 4 lists the top 20 journals that have published the highest number of articles in the field of AIG. The journal with the highest number of articles published is "Internal Medicine" with 11 relevant articles, followed by "Gastroenterology" (n = 10). The journal with the highest impact factor (IF) is "Gastroenterology" with an IF of 33.883, followed by "Autoimmunity Reviews" (IF = 17.390), while the IF of all other journals is below 10. In terms of journal partition, two of the top three journals belong to the Q4 region, and one is in the Q1 region. Among the top 20 journals, 13 fall within Q1 and Q2 regions, while the remaining seven are in Q3 and Q4 regions.

Analysis of popular topics and frontiers

The keywords included in the literature were statistically analyzed using CiteSpace, and the top 20 keywords in terms of frequency are listed in Table 5. Apart from the subject and free words related to AIG, keywords such as "pernicious anemia", "*Helicobacter pylori*", "classification", "diagnosis", "*Helicobacter pylori* infection", "autoantibody", and "cancer" have a high frequency. Among them, "*Helicobacter pylori*", "*Helicobacter pylori* infection" and "autoantibody" are related to the pathogenesis of AIG, while "classification" and "diagnosis" are linked to its clinical diagnosis. "Pernicious anemia", "cancer", and "vitamin B12 deficiency" are associated with AIG prognosis, indicating that researchers in this field should pay close attention to its pathogenesis, diagnosis, and prognosis.

Table 3 Top 10 authors

Rank	Author	Country	Institution	Quantity	ACI	H-index
1	Toh BH	Australia	Monash University	31	41.35	19
2	Vandriel IR	Australia	University of Melbourne	25	33.88	17
3	Gleeson PA	Australia	University of Melbourne	23	41.96	16
4	Alderuccio F	Australia	Monash University	19	33.89	12
5	Miceli E	Italy	Irccss S Matteo Hosp Fdn	14	24.07	8
6	Soykan I	Turkey	Ankara University	14	6.79	6
7	Di Sabatino A	Italy	University of Pavia	13	11.62	6
8	Haruma K	Japan	Kawasaki Medical School	12	7.83	6
9	Lenti MV	Italy	University of Pavia	12	34.17	10
10	Kalkan C	Turkey	City Hospital Ankara	10	6.1	4

ACI: Average citations per item.

Table 4 Top 20 journals

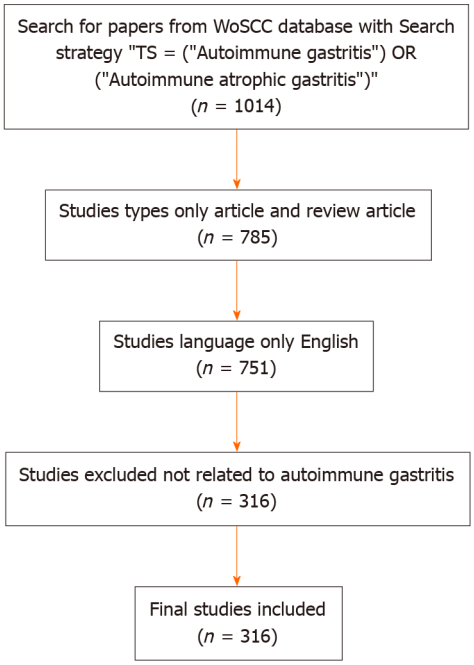
Rank	Journal	Quantity	ACI	IF (2022)	JCR
1	*Internal Medicine*	11	6.73	1.282	Q4
2	*Gastroenterology*	10	39.6	33.883	Q1
3	*Autoimmunity*	9	21.33	2.957	Q4
4	*Journal of Immunology*	9	44.11	5.426	Q2
5	*World Journal of Gastroenterology*	8	29.25	5.374	Q2
6	*Digestive and Liver Disease*	7	11.29	5.165	Q2
7	*Frontiers in Immunology*	7	1.57	8.786	Q1
8	*International Reviews of Immunology*	7	17.43	5.078	Q2
9	*Alimentary Pharmacology & Therapeutics*	6	35.5	9.524	Q1
10	*Clinical and Experimental Immunology*	6	22.83	5.732	Q2
11	*Digestion*	6	17.33	3.672	Q3
12	*Autoimmunity Reviews*	5	58	17.390	Q1
13	*Clinical Journal of Gastroenterology*	5	2.8	0	Q3
14	*Journal of Gastroenterology*	5	6.6	6.772	Q2
15	*Scandinavian Journal of Gastroenterology*	5	18.4	3.027	Q4
16	*American Journal of Physiology-Gastrointestinal and Liver Physiology*	4	28.75	4.871	Q1/Q2
17	*Archives of Pathology & Laboratory Medicine*	4	14.25	5.686	Q1/Q2
18	*BMC Gastroenterology*	4	14.5	2.847	Q4
19	*Digestive Diseases and Sciences*	4	8	3.487	Q3
20	*European Journal of Immunology*	4	41.25	6.688	Q2

IF: Impact factor; JCR: Journal citation reports; ACI: Average citations per item.

Burst detection of keywords can indicate current popular research topics and trends in a field, as shown in Figure 6. The red bars represent the start, end, and duration of citation bursts. "Follow up" is the earliest keyword to appear, with a burst strength of 2.8 and a duration of 4 years, indicating that it is a topic of close attention in the early stage. "Prevalence" has the longest duration, appearing in 2015 and continuing for seven years, and is a topic that researchers continue to focus on. Excluding the subject and free words of AIG, "pathogenesis" is the keyword with the highest burst

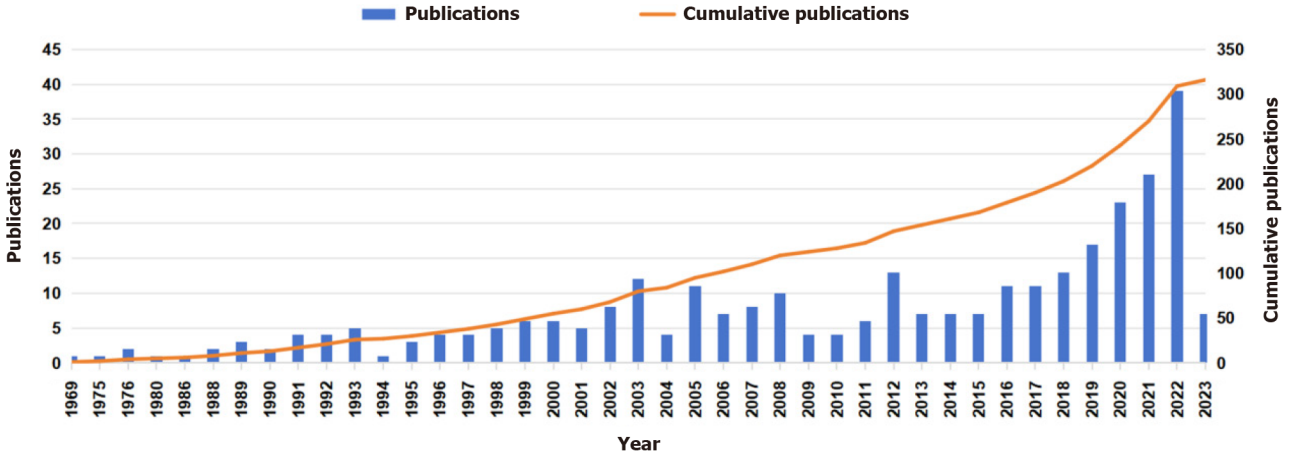
Table 5 Top 20 Keywords

Rank	Keywords	Count	Centrality
1	Autoimmune gastritis	96	0.25
2	Pernicious anemia	64	0.12
3	Atrophic gastritis	46	0.12
4	Helicobacter pylori	44	0.14
5	Classification	40	0.16
6	Diagnosis	29	0.07
7	Helicobacter pylori infection	27	0.18
8	Disease	26	0.26
9	Autoantibody	21	0.10
10	Cancer	21	0.13
11	Prevalence	20	0.04
12	Parietal cell antibody	20	0.03
13	Follow up	16	0.08
14	Atrophic body gastritis	15	0.06
15	Pathogenesis	15	0.03
16	Management	15	0.03
17	Risk	12	0.04
18	Autoimmune atrophic gastritis	11	0.08
19	Gastric cancer	11	0.07
20	Body gastritis	10	0.08



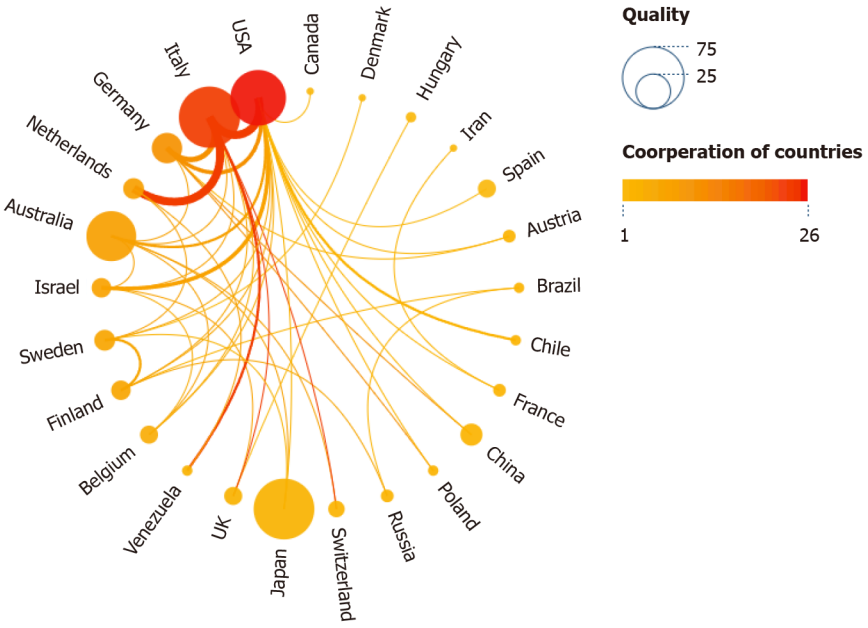
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Figure 1 Flow diagram of the included papers. TS: Translational science; WoSCC: Web of Science Core Collection.



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Figure 2 Trends of the related annual publications.



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Figure 3 Scimago Graphic collaboration visualization map of country/region. In this visual map, each node is a country, and the links between countries represent cooperative relations. The size of each node is proportional to the total number of publications. The red nodes indicate that there are more cooperative publications between the country/region and other countries/regions, while the thicker connecting lines between the nodes indicate that the cooperation between the two countries/regions is closer.

intensity, indicating that pathogenesis is a topic of great concern for researchers. "Risk", "vitamin B12 deficiency", "atrophic gastritis", and "gastric cancer" are the keywords that have remained in the burst to the present, and they are currently popular topics that researchers are still focusing on.

CiteSpace clusters the keywords and presents them in a timeline view; Figure 7 shows the clustering results for the first 11 clusters. The differently colored horizontal lines represent the clusters formed by the keywords, the nodes on the horizontal lines represent the keywords, and the positions of the nodes represent the year in which the documents containing the keywords first appeared. "Atrophic body gastritis" was the largest keyword cluster, followed by "*Helicobacter pylori*". "Vitamin B12 deficiency" appeared the latest, while "vaccine candidates" and "expression" appeared later, and the remaining keyword clusters appeared early. Over time, nodes for "*Helicobacter pylori*", "parietal cell antibody", and "autoimmune disease" continue to exist and increase, signifying that they remain popular topics for future research in this field.

Analysis of cited references and reference burst

Table 6 lists the top 20 cited articles in the field. "Reappraisal of nature and significance of chronic atrophic gastritis" authored by Strickland and Mackay[20] in 1973 was the earliest published article and has been cited 505 times. It was the first AIG-related article to be recognized and cited by many researchers. The most cited article is "Classification and

Table 6 Top 20 cited references

Rank	Title	Journal	Author	Year	Citation
1	Classification and grading of gastritis - The updated Sydney System	*American Journal of Surgical Pathology*	Dixon M	1996	4036
2	Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation	*Journal of Experimental Medicine*	Asano M	1996	1067
3	Prevalence of undiagnosed pernicious anemia in the elderly	*Archives of Internal Medicine*	Carmel R	1996	798
4	Reappraisal of nature and significance of chronic atrophic gastritis	*American Journal of Digestive Diseases*	Strickland RG	1973	505
5	Mechanisms of disease: Pernicious anemia	*New England Journal of Medicine*	Toh BH	1997	381
6	Genetic susceptibility to post-thymectomy autoimmune-diseases in mice	*Immunogenetics*	Kojima A	1981	244
7	Autoimmune atrophic gastritis-pathogenesis, pathology and management	*Nature Reviews Gastroenterology & Hepatology*	Neumann WL	2013	198
8	Major parietal-cell antigen in autoimmune gastritis with pernicious-anemia is the acid-producing H+,K+-adenosine triphosphatase of the stomach	*Journal of Clinical Investigation*	Karlsson FA	1988	183
9	Molecular mimicry between Helicobacter pylori antigens and H+,K+-adenosine triphosphatase in human gastric autoimmunity	*Journal of Experimental Medicine*	Amedei A	2003	180
10	Variable hematologic presentation of autoimmune gastritis: age-related progression from iron deficiency to cobalamin depletion	*Blood*	Hershko C	2006	157
11	An autoimmune-disease with multiple molecular targets abrogated by the transgenic expression of a single autoantigen in the thymus	*Journal of Experimental Medicine*	Alderuccio F	1993	148
12	Autoimmune gastritis in type 1 diabetes: A clinically oriented review	*Journal of Clinical Endocrinology & Metabolism*	De Block C	2008	121
13	H+, K+-ATPase (proton pump) is the target autoantigen of Th1-type cytotoxic T cells in autoimmune gastritis	*Gastroenterology*	D'elios MM	2001	109
14	Systematic review: gastric cancer incidence in pernicious anaemia	*Alimentary Pharmacology & Therapeutics*	Vannella L	2013	109
15	Reassessment of Intrinsic Factor and Parietal Cell Autoantibodies in Atrophic Gastritis With Respect to Cobalamin Deficiency	*American Journal of Gastroenterology*	Lahner E	2009	95
16	The parietal-cell autoantibodies recognized in neonatal thymectomy-induced murine gastritis are the alpha-subunit and beta-subunit of the gastric proton pump	*Gastroenterology*	Jones CM	1991	92
17	Immunological and clinical-studies on murine experimental autoimmune gastritis induced by neonatal thymectomy	*Gastroenterology*	Fukuma K	1988	85
18	Diagnosis and classification of autoimmune gastritis	*Autoimmunity Reviews*	Toh BH	2014	83
19	Analysis of mononuclear cell infiltrate and cytokine production in murine autoimmune gastritis	*Gastroenterology*	Martinelli TM	1996	75
20	Common Features of Patients with Autoimmune Atrophic Gastritis	*Clinical Gastroenterology and Hepatology*	Miceli E	2012	73

grading of gastritis - The updated Sydney System", published by Dixon *et al*[21] in 1996, with a citation frequency of 4036. This count is significantly higher than other articles, indicating that the concepts and grading involved in the article are widely recognized by the academic community. These highly cited articles were predominantly concentrated within the 20-year period from 1990 to 2010, with only three highly cited articles published in the last decade. Among the top 20 cited articles, only Toh published two articles, while the remainder were authored by different researchers.

Reference burst detection can be used to identify specific works or authors that have received significant attention during a specific period, as shown in Figure 8. The red bars represent the start, end, and duration of citation bursts. Burst detection reveals that the article "Autoimmune gastritis" by Lenti *et al*[22] in 2020 has the highest burst intensity, indicating that the results have received focused attention from researchers. Additionally, the articles "Multicenter study of autoimmune gastritis in Japan: clinical and endoscopic characteristics" by Terao *et al*[23] in 2020, "Natural history of autoimmune atrophic gastritis: a prospective, single centre, long-term experience" by Miceli *et al*[2] in 2019, and "Prevalence of autoimmune gastritis in individuals undergoing medical checkups in Japan" by Notsu *et al*[24] in 2019 have bursts that have continued to the present, indicating that they remain popular topics in the field.

Analysis of co-cited references

Over the last two decades, 6625 co-cited references on AIG have been published. Table 7 shows the top 20 cocited references, all of which were co-cited at least 33 times. "Classification and grading of gastritis. The updated Sydney

Table 7 Top 10 co-cited references

Rank	Title	Journal	Author	Year	Citation
1	Classification and grading of gastritis - The updated Sydney System	*American Journal of Surgical Pathology*	Dixon MF	1996	78
2	Mechanisms of disease: Pernicious anemia	*New England Journal of Medicine*	Toh BH	1997	71
3	Reappraisal of Nature And Significance of Chronic Atrophic Gastritis	*American Journal of Digestive Diseases*	Strickland RG	1973	69
4	Autoimmune Atrophic Gastritis-Pathogenesis, Pathology and Management	*Nature Reviews Gastroenterology & Hepatology*	Neumann WL	2013	67
5	Analysis of Mononuclear Cell Infiltrate and Cytokine Production in Murine Autoimmune Gastritis	*Gastroenterology*	Martinelli TM	1996	46
6	An Autoimmune-Disease with Multiple Molecular Targets Abrogated by the Transgenic Expression of A Single Autoantigen In the Thymus	*Journal of Experimental Medicine*	Alderuccio F	1993	44
7	Autoimmune Gastritis in Type 1 Diabetes: A Clinically Oriented Review	*Journal of Clinical Endocrinology & Metabolism*	De Block CEM	2008	44
8	Major Parietal-Cell Antigen In Autoimmune Gastritis With Pernicious-Anemia is The Acid-Producing H+,K+-Adenosine Triphosphatase of The Stomach	*Journal of Clinical Investigation*	Karlsson Fa	1988	42
9	Variable Hematologic Presentation of Autoimmune Gastritis: Age-Related Progression From Iron Deficiency To Cobalamin Depletion	*Blood*	Hershko C	2006	41
10	Common Features of Patients With Autoimmune Atrophic Gastritis	*Clinical Gastroenterology and Hepatology*	Miceli E	2012	41
11	Immunologic and clinical studies on murine experimental autoimmune gastritis induced by neonatal thymectomy	*Gastroenterology*	Fukuma K	1988	40
12	Genetic susceptibility to post-thymectomy autoimmune diseases in mice	*Immunogenetics*	Kojima A	1981	40
13	H(+),K(+)-atpase (proton pump) is the target autoantigen of Th1-type cytotoxic T cells in autoimmune gastritis	*Gastroenterology*	D'Elios Mm	2001	37
14	Prevalence of undiagnosed pernicious anemia in the elderly	*Arch Intern Med*	Carmel R	1996	36
15	The parietal cell autoantigens recognized in neonatal thymectomy-induced murine gastritis are the alpha and beta subunits of the gastric proton pump [corrected]	*Gastroenterology*	Jones Cm	1991	36
16	Diagnosis and classification of autoimmune gastritis	*Autoimmun Rev*	Toh Bh	2014	36
17	Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation	*J Exp Med*	Asano M	1996	34
18	Reassessment of intrinsic factor and parietal cell autoantibodies in atrophic gastritis with respect to cobalamin deficiency	*Am J Gastroenterol*	Lahner E	2009	34
19	Molecular mimicry between Helicobacter pylori antigens and H+, K+ --adenosine triphosphatase in human gastric autoimmunity	*J Exp Med*	Amedei A	2003	33
20	Autoimmune gastritis: Pathologist's viewpoint	*World J Gastroentero*	Coati I	2015	33

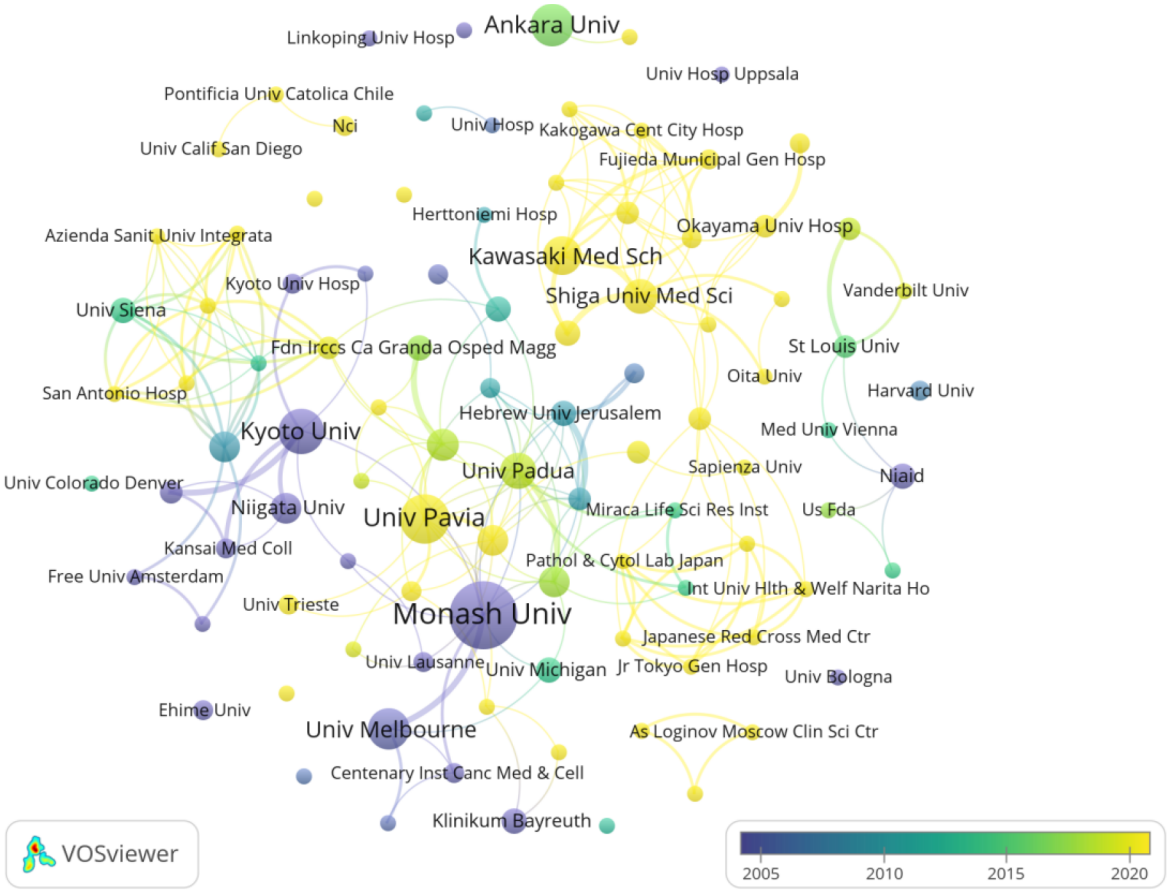
System" by Dixon *et al*[21] published in 1996, is the most co-cited reference, with 78 co-citations. This count is significantly higher than that of other articles, indicating that the concepts and grading involved in the article are widely recognized by the academic community.

We chose references with 15 or more co-citations to construct a co-citation network graph, as shown in Figure 9. The size of the nodes was proportional to the number of co-citations. The lines between nodes represent co-citation relationships, and the thickness and number of connections between nodes indicate the strength of the links between citations. "Dixon *et al*[21], 1996, *Am J Surg Pathol*" and "Strickland and Mackay[20], 1973, *Am J Dig Dis*", as well as "Dixon *et al*[21], 1996, *Am J Surg Pathol*" and "Toh *et al*[25] 1997, *New Engl J Med*" show a strong association, suggesting that they are universally recognized and relevant studies in the AIG field.

DISCUSSION

General information

With the growing awareness of AIG, an increasing number of doctors are focusing on this often-overlooked and undiagnosed disease. The pathogenesis, diagnosis, and treatment of AIG are gradually becoming popular research topics across multiple disciplines[26]. This study aims to utilize bibliometric analysis to examine the major countries,



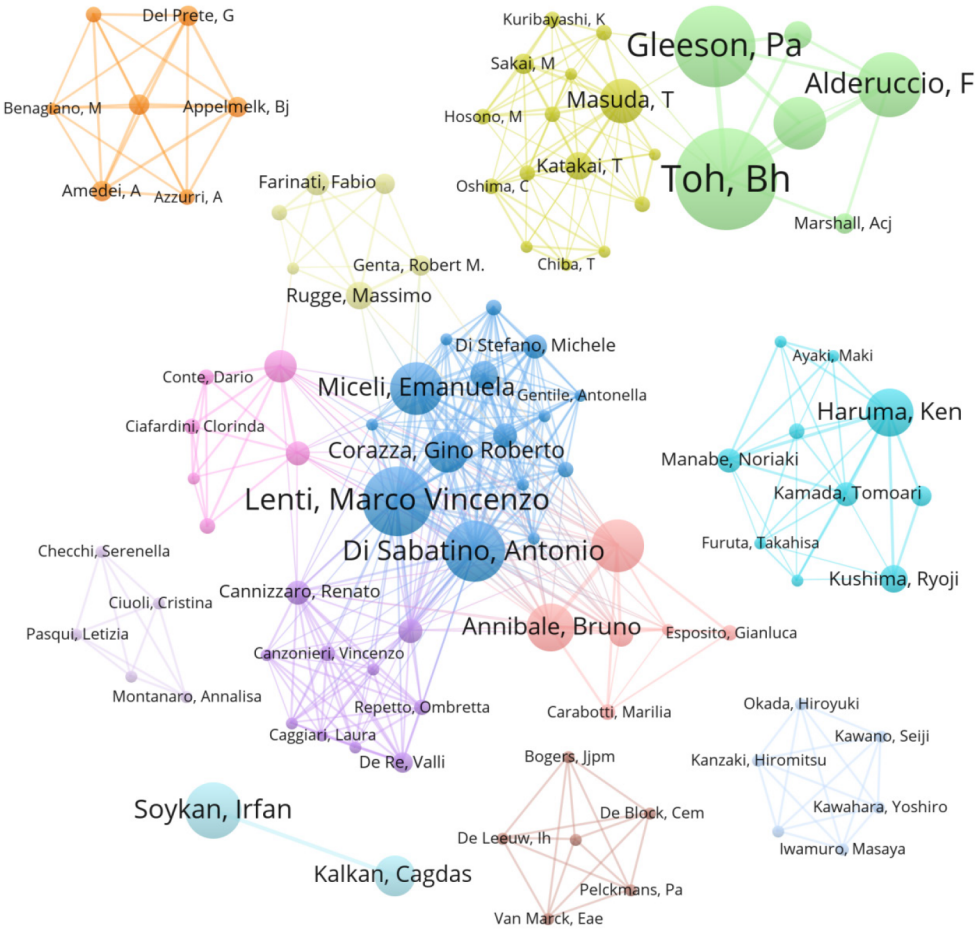
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Figure 4 Scimago Graphic collaboration visualization map of institutions. In this network visualization map, each node is an institution. The size of the node indicates the volume of publications, and the color intensity of the node represents the level of collaboration with other institutions. The node color reflects the corresponding average appearing year according to the color gradient in the lower right corner.

institutions, journals, authors, *etc.*, that have studied the AIG field and explore the popular research topics and trends in this field.

Research on AIG originated in the mid-20th century, and its publication volume grew slowly in the early years. However, since 2018, there has been explosive growth in terms of publications. Approximately 40% of the articles were published in 2018 or after, and the number of articles issued annually has shown a growing trend. In the 1960s, with the widespread use of gastroscopy and the discovery of PCA and IFA, AIG emerged as a special disease characterized by gastritis caused by autoimmune mechanisms[27,28]. In 1973, Strickland and Mackay[20] proposed categorizing gastritis into type A and type B based on whether serum PCA was positive. They pointed out that type A gastritis is associated with an autoimmune response characterized by highly restrictive atrophy of the body and fundus of the stomach[20]. Since then, AIG and type A gastritis have become synonymous. In the 1990s, the Sydney System proposed by the World Congress of Gastroenterology categorized atrophic gastritis into two types: Predominantly gastric body gastritis associated with autoimmunity, known as AIG, and multifocal gastritis associated with environmental factors[21]. In the 20 years that followed, research progress on AIG was relatively modest because researchers' understanding of AIG was mostly inseparable from pernicious anemia and *Helicobacter pylori* infection. The harmful effects of *Helicobacter pylori* were further elaborated by the Kyoto Global Consensus in 2015[29]. As the rate of *Helicobacter pylori* eradication increases, an increasing number of patients with AIG are being clinically diagnosed, leading people to realize that AIG is an underestimated disease. Since 2018, an increasing number of researchers have focused on AIG, resulting in an explosive surge in publications, with the annual number of articles reaching 39 in 2022.

Analyzing the countries/regions contributing to published articles helps us understand the influence and contributions of each country/region in the field. Italy ranked the highest in publication volume, with 70 articles, accounting for 22.15% of the total. In terms of influence, Italy also holds the highest H-index, while the Netherlands has the highest ACI. In terms of cooperative relationships between countries, Italy engages in close cooperation with the United States and the Netherlands. These results show that Italy is an indispensable and important country in this field of research, not only contributing significantly to the research progress of AIG but also playing a crucial role in international collaboration. Among the top 10 countries in terms of publication volume, Italy, Germany, Sweden, the Netherlands, and Finland are all European countries, suggesting that research on AIG is more popular in the European region, which may be related to the high prevalence of pernicious anemia among Europeans[30]. Pernicious anemia is the result of a deficiency in internal factors caused by the autoimmune mechanism of AIG[31], and its diagnosis relies on atrophy of the gastric acid gland mucosa. This makes AIG research in European countries both necessary and urgent. It is worth noting that Japan, ranked



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Figure 5 Analysis of author collaborations. In this network visualization map, each node is an author, and the links between the authors represent the cooperative relationship. The size of each node is proportional to the total number of publications, the colors of nodes represent different countries, and the connecting lines represent the cooperative relationship between individual authors.

second in publication volume, has an average citation rate of only 14.65, whereas China, which is also an Asian country, has a much lower H-index and publication volume than European and American countries. This suggests a large gap between the international influence of Asian countries in the field of AIG and that of European and American countries. From a collaborative perspective, there is a lack of collaboration between Japan and China, which could be detrimental to the long-term development of academia. The disease characteristics of the Chinese and Japanese populations may be similar because they are both of Asian descent, indicating that strengthening the collaboration between the two countries in this field would be meaningful.

The institution with the highest number of publications was Monash University in Australia, with 38 articles, twice the number of second-ranking institutions. Monash University collaborates closely with the University of Melbourne within the same country and with a number of institutions abroad, indicating that Monash University has conducted in-depth research in this field and can promote progress in this field internationally. Half of the top 10 institutions by volume are from Italy, which shows that there is a strong interest in AIG among Italian researchers; however, these institutions lack close collaboration among themselves. Thus, they should focus on exchanges with domestic institutions to enhance their breadth and depth of cooperation. Similarly, Kyoto University in Japan, which ranks third in publication volume, has collaborations with institutions from various countries but lacks collaboration with the second-ranking domestic institution, which is detrimental to research on AIG in Japan as well as to the long-term development of both institutions. It is advisable for Kyoto University to deepen collaboration with domestic institutions and, at the same time, strengthen ties with institutions in other Asian countries, such as China, to increase its international influence while increasing the output of its achievements. It is worth noting that the United States, which ranks third in publication volume, lacks an institution in the top 10 rankings, suggesting that while there might be numerous American institutions engaged in research on AIG, there is a lack of leading institutions with high output in the field. Research institutions in the US should provide stronger support to establish leading institutions that can guide research efforts, thereby fostering coordinated collaboration among various institutions.

The four authors with the highest number of publications were from Australia. Toh and Alderuccio are from Monash University, while Vandriel and Gleeson are from the University of Melbourne. Their research focuses on cell biology, immunology, pathology, and gastroenterology. Toh, Vanriel, and Gleeson are a team, and Toh and Alderuccio work closely together; their research focuses on exploring the pathogenesis of AIG in mouse models. They conducted a series of

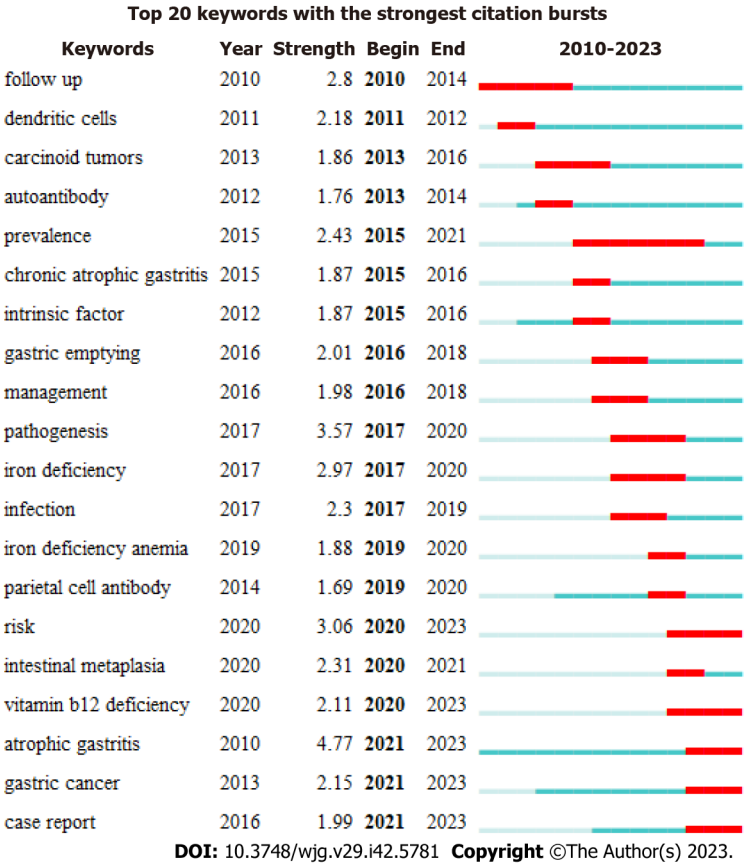
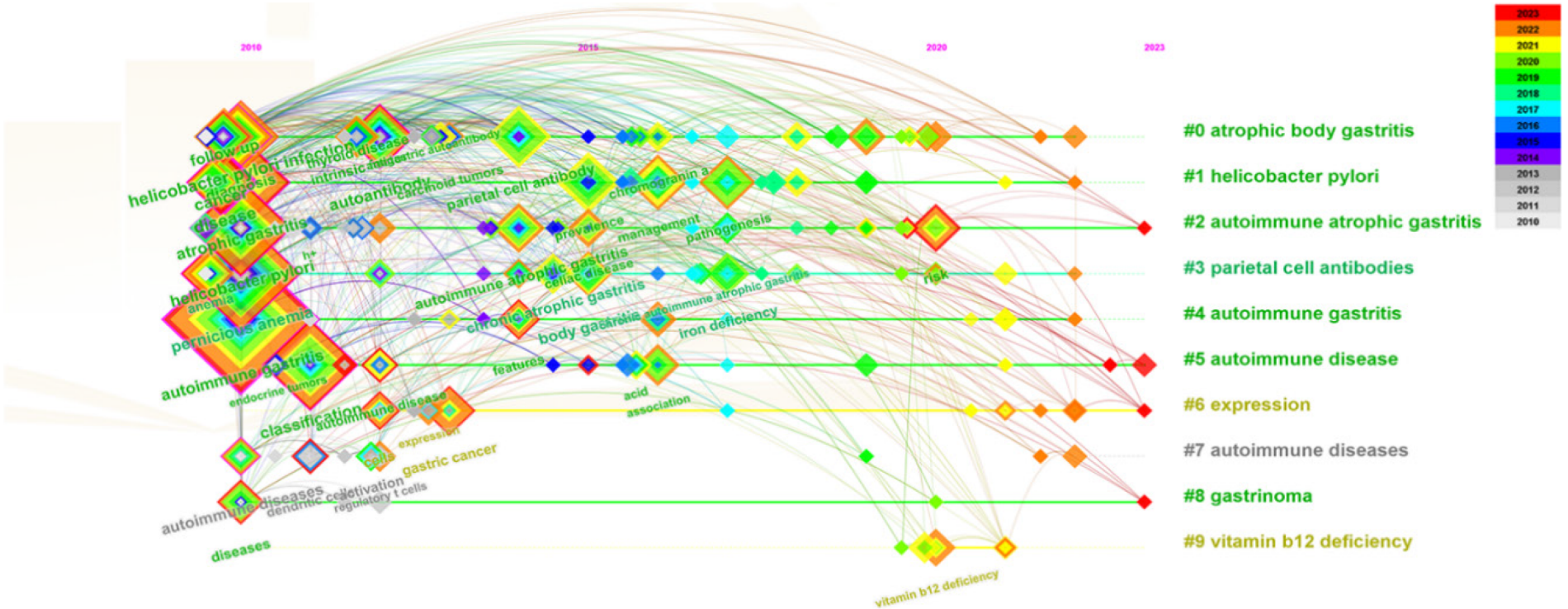


Figure 6 Top 20 keywords with the strongest keyword bursts. The red bars represent the starting and ending, and the duration of citation bursts.

studies on BALB/c mice induced by neonatal thymectomy and found that the autoimmune response to AIG is caused by TH1-type CD4 T cells recognizing the β-subunit of the gastric H+/K+ ATPase[32] and indicates that Fas is necessary for the development of gastric mucosal cell damage in AIG[33]. These authors' articles rank among the top in terms of quantity and quality, and they collaborate closely with each other, jointly promoting research in the field of AIG pathogenesis in Australia. However, Australia has fewer authors engaged in this field, and it is hoped that more Australian scholars will engage in AIG research in the future. Among the top 10 authors, there are also three from Italy: Miceli from Fondazione IRCCS Policlinico San Matteo and Di Sabatino and Lenti from the University of Pavia. They also constitute a tightly collaborating team, primarily engaging in laboratory serology studies and clinical research related to AIG and focusing on controversial aspects of AIG, such as its relationship with *Helicobacter pylori*[34], its course[2], and its potential risk factors[35,36]. In terms of cooperation, they not only closely cooperate with domestic scholars but also actively cooperate with foreign researchers, which enriches their clinical trial data, thus promoting the development of AIG clinical research. It is anticipated that more multicenter clinical trials will be conducted in the future to provide an inexhaustible impetus for further advancement in AIG research. The two authors from Turkey, Soykan and Kalkan, predominantly study complications and laboratory serology in relation to AIG[37,38], and their achievements are primarily found in cooperation with each other, while they lack of collaboration with the outside world. Haruma from Japan promoted endoscopic research on AIG[23,39] and identified endoscopic clues for early AIG by accumulating cases [40]. Overall, AIG research has been conducted in various ways, but each country focuses on different directions, and academic exchanges should be strengthened in the future to provide a better environment for the development of this field.

In analyzing the publication journals, the journal with the highest IF is *Gastroenterology* (IF=33.883, Q1), which has published a total of 10 articles in the field of AIG. Articles published before 2002 mainly focused on foundational research on AIG pathogenesis[3,41], whereas in recent years, the tendency has been to publish high-quality clinical studies[42]. This shift indicates that the AIG research trend is evolving from basic research to clinical investigation, with an increasing number of researchers attempting to explore the pathogenesis, clinical diagnosis, and treatment of AIG through clinical studies. The journal with the largest number of published articles is *Internal Medicine* (IF=1.282, Q4), which mainly publishes case reports related to AIG, and most of the topics are related to digestive endoscopy. This suggests that the journal has a strong interest in case reports of endoscopic manifestations of AIG and that the accumulation and summary of these case reports play an important role in promoting clinical research on AIG. The top 10 journals in terms of publication volume mostly belong to the Q1 and Q2 regions, indicating that AIG research is still influential in the relevant disciplines in this field.



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Figure 7 CiteSpace visualization timeline view of keywords clustering analysis. The different colored horizontal lines represent the clusters formed by the keywords, the nodes on the horizontal lines represent the keywords, and the position of the nodes represents the year in which the documents containing the keywords first appeared.

Popular topics and frontiers

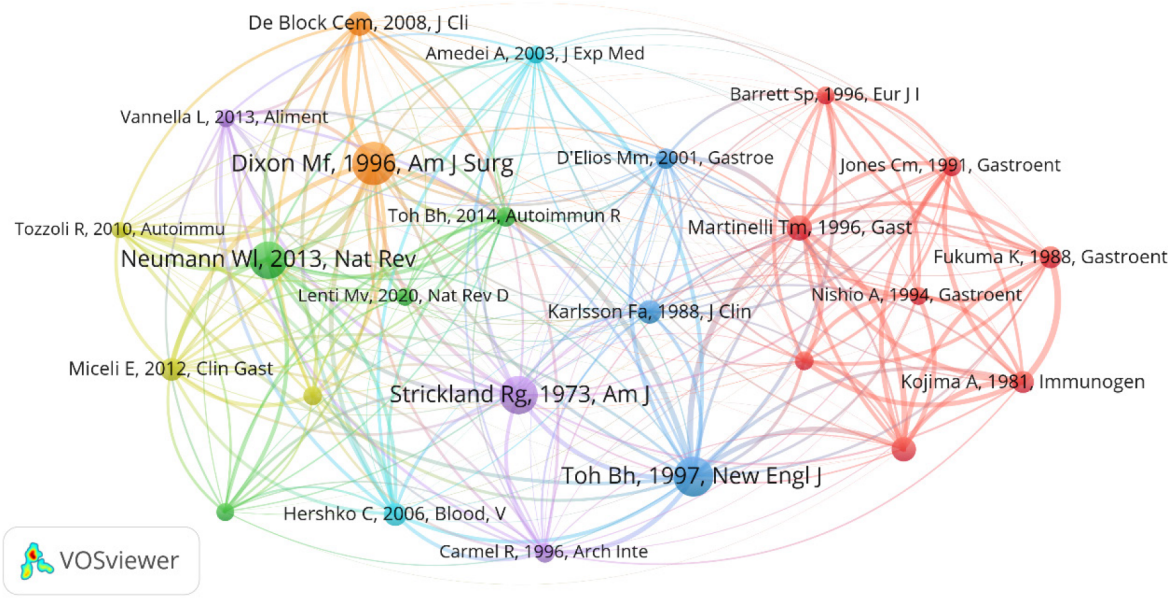
To gain a clearer understanding of the research focal points in the realm of AIG, we reviewed primary literature pertaining to popular topics implicated in frequency, burst, and temporal clustering analyses. Subsequently, we distilled from these pivotal keywords the current quintet of popular research topics in the domain of AIG: (1) The role of *Helicobacter pylori* in AIG pathogenesis (keywords: *Helicobacter pylori*, *Helicobacter pylori* infection): The role of *Helicobacter pylori* infection in AIG pathogenesis is the current focal point of investigation. Most scholars posit that *Helicobacter pylori* is implicated in the onset of AIG, possibly due to antigen mimicry or cross-reactivity[43]. Amedei *et al*[44] reported that in genetically susceptible individuals, *Helicobacter pylori* infection can activate cross-reactive gastric T cells, thereby inducing gastric autoimmunity through molecular mimicry. Faller *et al*[45] have detected PCA and anti-PCA in *Helicobacter pylori*-infected gastritis, suggesting some similarities between the pathogenic mechanisms of *Helicobacter pylori*-infected gastritis and AIG. However, the ongoing discourse regarding whether *Helicobacter pylori* infection exerts a positive or negative influence on the progression of AIG remains unclear. Kotera *et al*[46] reported a case of AIG in which gastric mucosal

Top 20 references with the strongest citation bursts

References	Year	Strength	Begin	End	2016-2023
Neumann WL, 2013, NAT REV GASTRO HEPAT, V10, P529, DOI 10.1038/nrgastro.2013.101, DOI	2013	6.36	2016	2018	
Toh BH, 2014, AUTOIMMUN REV, V13, P459, DOI 10.1016/j.autrev.2014.01.048, DOI	2014	5.5	2016	2019	
Soykan I, 2012, DIGESTION, V86, P20, DOI 10.1159/000338295, DOI	2012	2.35	2016	2017	
Bizzaro N, 2014, AUTOIMMUN REV, V13, P565, DOI 10.1016/j.autrev.2014.01.042, DOI	2014	2.33	2016	2019	
Du YQ, 2014, J CLIN GASTROENTEROL, V48, P328, DOI 10.1097/MCG.0000000000000033, DOI	2014	2.09	2016	2018	
Vannella L, 2013, ALIMENT PHARM THER, V37, P375, DOI 10.1111/apt.12177, DOI	2013	2.85	2017	2018	
Kalkan C, 2016, DIGEST DIS SCI, V61, P1597, DOI 10.1007/s10620-015-4021-1, DOI	2016	1.9	2017	2018	
Venerito M, 2015, ALIMENT PHARM THER, V41, P686, DOI 10.1111/apt.13097, DOI	2015	1.81	2017	2020	
Miceli E, 2015, J CLIN GASTROENTEROL, V49, PE1, DOI 10.1097/MCG.0000000000000101, DOI	2015	2.62	2018	2020	
Goncalves C, 2014, WORLD J GASTROENTERO, V20, P15780, DOI 10.3748/wjg.v20.i42.15780, DOI	2014	1.99	2018	2019	
Cavalcoli F, 2017, WORLD J GASTROENTERO, V23, P563, DOI 10.3748/wjg.v23.i4.563, DOI	2017	1.92	2018	2020	
Murphy G, 2015, CLIN GASTROENTEROL H, V13, P2282, DOI 10.1016/j.cgh.2015.05.040, DOI	2015	3.56	2019	2020	
Di Sabatino A, 2017, DIGEST LIVER DIS, V49, P947, DOI 10.1016/j.dld.2017.06.010, DOI	2017	2.84	2019	2020	
Hughes JW, 2017, ENDOCR PRACT, V23, P1297, DOI 10.4158/EP-2017-0056, DOI	2017	1.77	2019	2020	
Rusak E, 2016, ADV MED SCI-POLAND, V61, P175, DOI 10.1016/j.advms.2015.12.004, DOI	2016	3.21	2020	2021	
Husebye ES, 2018, NEW ENGL J MED, V378, P1132, DOI 10.1056/NEJMra1713301, DOI	2018	1.64	2020	2021	
Lenti MV, 2020, NAT REV DIS PRIMERS, V6, P0, DOI 10.1038/s41572-020-0187-8, DOI	2020	6.98	2021	2023	
Terao S, 2020, DIGEST ENDOSC, V32, P364, DOI 10.1111/den.13500, DOI	2020	3.29	2021	2023	
Miceli E, 2019, ALIMENT PHARM THER, V50, P1172, DOI 10.1111/apt.15540, DOI	2019	2.66	2021	2023	
Notsu T, 2019, INTERNAL MED, V58, P1817, DOI 10.2169/internalmedicine.2292-18, DOI	2019	2.36	2021	2023	

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Figure 8 Top 20 references with the strongest citation bursts. The red bars represent the starting and ending, and the duration of citation bursts.



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Figure 9 CiteSpace visualization cluster view of co-cited references. In this visual map, each node represents a co-cited reference, and the size of the nodes is proportional to the number of co-citations, and the lines between nodes represent co-citation relationships. The thickness and number of connections between nodes indicate the strength of the links between co-citations.

atrophy showed improvement after *Helicobacter pylori* eradication; however, the underlying reasons for this change remain unexplained. Conversely, the literature has documented cases of AIG progression after *Helicobacter pylori* eradication, suggesting that *Helicobacter pylori* infection may inhibit AIG activity[47]. In summary, the role of *Helicobacter pylori* in AIG remains a subject of ongoing debate and should be a focal theme for future research; (2) Diagnostic criteria for AIG and reference values of serum antibodies (keywords: autoantibody, parietal cell antibody): AIG diagnosis

currently lacks a universally accepted standard. The most recent diagnostic criteria, published in 2023 by Kamada *et al*[8] in "Diagnostic criteria and endoscopic and histological findings of autoimmune gastritis in Japan", have set the diagnostic criteria for AIG as the fulfillment of endoscopic and/or histological manifestations that meet specific criteria, alongside positive findings for PCA and/or IFA[8]. Given that early-stage AIG lacks distinct endoscopic manifestations, its diagnosis requires the fulfillment of histological criteria and the presence of autoantibodies. Autoantibodies such as PCA and IFA, which represent diagnostic markers for AIG, hold significant value. However, the absence of autoantibodies does not definitively exclude the possibility of AIG. This is attributed to the fact that PCA titers tend to gradually decline from early- to late-stage AIG, and late-stage patients may exhibit PCA negativity due to the depletion of antigens resulting from parietal cell loss[48]. Furthermore, evidence suggests an age-related correlation, with PCA negativity being more common among older adult AIG patients[49]. Notably, recent research by Krişe *et al*[50] suggests that PCA and PGI/II constitute the optimal combination for AIG detection, while the diagnostic value of IFA is limited. In summary, the academic community currently lacks consensus regarding AIG diagnostic standards, and the specificity and sensitivity of autoantibodies, such as PCA and IFA, in AIG diagnosis remains a subject of debate. These findings provide avenues for future research and potential breakthroughs; (3) Comorbidity mechanisms between AIG and other autoimmune diseases (keyword: Autoimmune disease): As an autoimmune disease, AIG often coexists with other autoimmune diseases. When AIG is combined with other autoimmune afflictions and results in deficiencies in various endocrine organs, it is termed autoimmune multiglandular syndrome[51]. Thyroid disorders are the most common comorbidities in patients with AIG, affecting approximately 53% of AIG cases[10]. Known autoimmune disorders that commonly coincide with AIG include type 1 diabetes, rheumatoid arthritis, primary Sjögren's syndrome, inflammatory bowel disease, vitiligo, chronic cheilitis, myasthenia gravis, Addison's disease, *etc*[11,52]. Although the current evidence suggests a connection between AIG and other autoimmune disorders, the mechanisms underlying their comorbidity are yet to be elucidated. The shared comorbidity mechanisms of AIG and other autoimmune diseases may be a focal point for future research; (4) Specific risks of AIG complicating gastric and other cancers (keywords: Risk, gastric cancer, cancer): Gastric carcinoma and neuroendocrine tumors represent the most severe known complications and latent risks of AIG [53]. The damaged parietal cells are supplanted by tissues undergoing pseudopyloric and intestinal metaplasia, thus fostering the metamorphosis into gastric cancer[54]. This destruction of parietal cells leads to a reduction in gastric acid secretion, thereby triggering negative feedback regulation in the gastric antrum, resulting in increased levels of gastrin in the bloodstream[55]. Gastrin has the potential to stimulate the proliferation of enterochromaffin-like cells in the intestines, culminating in the development of neuroendocrine tumors[55]. The OLGA/OLGIM systems indicate a heightened risk of gastric cancer in patients with AIG at stages III-IV, which is primarily associated with concomitant *Helicobacter pylori* infection[56]. Notably, a recent long-term clinical observation suggested that AIG did not elevate the risk of gastric cancer when compared to the general population, suggesting that the previously reported increased risk may be attributed to undetected concurrent *Helicobacter pylori* infection[57]. Nevertheless, Waldum[58] contends that the design of this clinical observation lacked rationality and posited that the role of *Helicobacter pylori* infection in augmenting gastric cancer risk among AIG patients remains to be substantiated. In summary, AIG may augment the risk of neuroendocrine tumors, yet its impact on gastric cancer risk remains a subject of contention. The precise risk of AIG complicating gastric cancer and other malignancies warrants further investigation. In summary, AIG leads to an increased risk of neuroendocrine tumors; however, its effect on the risk of gastric cancer remains controversial. The specific risk of AIG being complicated by gastric and other cancers is an interesting area of research; and (5) Potential role of vitamin B12 supplementation in patients with early-stage AIG (keywords: Vitamin B12 deficiency, pernicious anemia): In early investigations, pernicious anemia was regarded as a complex ailment encompassing serology, gastroenterology, and immunology[30]. During AIG, the destruction of parietal cells and the formation of IFA jointly lead to a decline in intrinsic factor levels, consequently hindering the absorption of vitamin B12 and ultimately leading to pernicious anemia. Notably, clinical observations by Hershko and others[59] revealed that iron-deficiency anemia, rather than megaloblastic anemia, was the most common type of anemia in patients with AIG. They suggested that this anemia occurred before the onset of pernicious anemia caused by prolonged vitamin B12 deficiency, with its etiology linked to parietal cell destruction, leading to gastric acid deficiency. Furthermore, multicenter studies have indicated that mild hematological alterations and deficiencies in trace nutrients, such as iron and vitamins, appear before overt anemia[60]. These indications suggest that iron and vitamin B12 supplementation might be advantageous for patients before the diagnosis of iron-deficiency anemia or pernicious anemia, although there is insufficient evidence to support this inference. In summary, because pernicious anemia rarely occurs in the early stages of AIG, the need for early vitamin B12 and iron supplementation is debatable and is a research topic of interest for future studies in the field of AIG.

Limitations

Although we rigorously adhered to bibliometric research methods, our study has limitations. First, to ensure the quality of this study, we exclusively obtained data from the WoSCC database. This decision may have led to an incomplete search for articles. Second, our study was limited to English-language publications, and abstract-only papers were excluded. This may have reduced the comprehensiveness of the research findings. Third, as WoSCC was continuously updated, the citation counts and H-index of the included articles continuously evolves, introducing inevitable temporal limitations to this study.

CONCLUSION

To the best of our knowledge, this is the first comprehensive quantitative bibliometric analysis of AIG. It scrutinized

factors such as publication year, country, institution, author, journal, and keywords while also clarifying key areas for future research: (1) The role of *Helicobacter pylori* in AIG pathogenesis; (2) diagnostic criteria for AIG and the significance of serum antibodies; (3) comorbidity mechanisms of AIG and other autoimmune diseases; (4) specific risks of AIG complicated by gastric cancer and other malignancies; and (5) the potential role of vitamin B12 supplementation in patients with early-stage AIG. This study is expected to provide valuable insight for further AIG research.

ARTICLE HIGHLIGHTS

Research background

Autoimmune gastritis (AIG) is a distinctive type of chronic atrophic gastritis that is considered a potential risk factor for some tumors. Increasing numbers of researchers are focusing on the pathogenesis, diagnosis, and prognosis of AIG.

Research motivation

Bibliometrics is one of the most commonly used methods to evaluate the research status in a field. To date, there are no bibliometric studies related to AIG. Bibliometric analysis provide a more comprehensive overview of the research status and research hotspots in AIG.

Research objectives

This study aims to provide a comprehensive understanding of the knowledge structure and research hotspots of AIG and to offer new ideas and directions for its research.

Research methods

Data for this study were sourced from the Web of Science Core Collection. The proposed search formula was "translational science = Autoimmune gastritis OR Autoimmune atrophic gastritis". The search was conducted until May 2023, the restricted language was English, and the type of studies included were articles and review articles.

Research results

In total, 316 relevant articles were included. From 2015 to 2022, the number of publications increased annually. The countries, institutions, authors, and journals with the highest number of publications in this field were Italy, Monash University, Toh BH, and Internal Medicine. The main keywords usd in this field of research were pathogenesis, *Helicobacter pylori*, autoantibody, parietal cell antibody, atrophic gastritis, classification, diagnosis, autoimmune disease, risk, cancer, gastric cancer, vitamin B12 deficiency, and pernicious anemia.

Research conclusions

This was the first bibliometric analysis related to AIG, highlighting hot topics and emerging trends in the field, and offering new ideas to further scientific research and clinical applications.

Research perspectives

The following directions may be popular for future research: (1) The role of *Helicobacter pylori* in the pathogenesis of AIG; (2) diagnostic criteria for AIG and the reference value of serum antibodies; (3) comorbidity mechanisms between AIG and other autoimmune diseases; (4) specific risks of AIG complicating gastric and other cancers; and (5) the role of vitamin B12 supplementation in patients with early-stage AIG.

FOOTNOTES

Co-first authors: Yun-Feng Yu and Ke-Ke Tong.

Author contributions: Yu YF conceived and designed the study; Yu YF and Tong KK participated in data processing and statistical analysis; Yu YF, Tong KK, Wu JY, and Shangguan XL drafted the manuscript; Shangguan XL, Yang XY, Hu G, Tan CC, and Wu JY contributed to data analysis and interpretation; Yu YF, Tong KK, Yang XY, Yu R, and Tan CC supervised the review of the study; All authors seriously revised and approved the final manuscript. Yu YF and Tong KK contributed equally to this work as co-first authors. The reasons for designating Yu YF and Tong KK as co-first authors are threefold. First, the research was performed as a collaborative effort, and the designation of co-first authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper. This also ensures effective communication and management of post-submission matters, ultimately enhancing the paper's quality and reliability. Second, the overall research team encompassed authors with a variety of expertise and skills from different fields, and the designation of co-first authors best reflects this diversity. Third, Yu YF and Tong KK contributed efforts of equal substance throughout the research process. The choice of these researchers as co-first authors acknowledges and respects this equal contribution, while recognizing the spirit of teamwork and collaboration of this study. In summary, we believe that designating Yu YF and Tong KK as co-first authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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L-Editor: A
P-Editor: Yuan YY

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EDITORIAL

Endoscopic submucosal dissection for early gastric cancer: It is time to consider the quality of its outcomes

Gwang Ha Kim

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Abstract

Endoscopic resection, particularly endoscopic submucosal dissection (ESD), is widely used as a standard treatment modality for early gastric cancer (EGC) when the risk of lymph node metastasis is negligible. Compared with surgical gastrectomy, ESD is a minimally invasive procedure with additional advantages, such as preservation of the entire stomach and maintenance of the patient's quality of life. However, not all patients achieve curative resection after ESD of EGC. Several patients require surgical gastrectomy after ESD to achieve a curative treatment. Additional surgery after ESD, owing to non-curative resection, places considerable emotional and financial burdens on patients. Recently, as the number of endoscopists performing ESD has increased, the rate of non-curative resection after ESD has increased correspondingly. In order to decrease the non-curative resection rate, as well as determine the ideal rate of non-curative resection after ESD, it is time to consider quality indicators for the outcomes of ESD for EGC.

Key Words: Early gastric cancer; Endoscopic resection; Quality indicator

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Core Tip: Endoscopic resection, particularly endoscopic submucosal dissection (ESD), is widely used as a standard treatment modality for early gastric cancer (EGC) when the risk of lymph node metastasis is negligible. Recently, the policy of "diagnostic ESD" has been commonly implemented, especially when accurate prediction of the depth of EGC invasion before ESD is impossible; however, it is neither ideal nor scientific. Therefore, it is time to consider quality indicators for the outcomes of ESD for EGC.

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INTRODUCTION

Gastric cancer (GC) is the fifth most common malignant tumor and the fourth leading cause of cancer-related deaths worldwide[1]. The diagnosis rate of early GC (EGC) has been increasing owing to the widespread use of endoscopy, especially during health checkups, and the development of advanced endoscopy techniques, such as high-definition endoscopy and virtual chromoendoscopy[2-4]. Endoscopic resection, particularly endoscopic submucosal dissection (ESD), is widely used as a standard modality for the curative treatment of EGC when the risk of lymph node metastasis is negligible[5,6]. Compared with surgical gastrectomy, ESD is a minimally invasive procedure with additional advantages, such as preservation of the entire stomach and maintenance of the patient's quality of life. Curative resection after ESD is confirmed based on the following lesion characteristics: (1) Differentiated-type mucosal cancer without ulceration, irrespective of tumor size; (2) Differentiated-type mucosal cancer measuring ≤ 3 cm with ulceration; (3) Differentiated-type cancer measuring ≤ 3 cm with minimal submucosal invasion (depth of invasion into the submucosa < 500 μm); and (4) Undifferentiated-type mucosal cancer measuring ≤ 2 cm without ulceration[5,7]. Although ESD achieves *en bloc*/R0 resection in > 90% of cases, it is not curative in up to 20% of cases. This may be due to previously unsuspected submucosal invasion or horizontal extension, changes in histopathological type (especially to the undifferentiated-predominant mixed type), or lymphovascular invasion on histopathological examination[8]. Therefore, accurate evaluation of the size, invasion depth, horizontal extent, and histopathological type of EGC is essential to improve patient selection for curative ESD.

Advances in ESD techniques and devices, as well as increased opportunities to learn ESD (*e.g.*, visiting ESD training centers, participating in *ex vivo* ESD courses, or watching online or offline videos), have enabled more endoscopists to safely and completely perform ESD in clinical practice, especially in Asian countries. However, not all patients achieve curative resection after ESD of EGC. The main risk factors for non-curative resection are as follows: Tumor location in the upper body, large tumor size (≥ 2 cm), presence of an ulcer, presence of undifferentiated-type component tumor, submucosal invasion, and an inexperienced endoscopist[9-11]. Several patients require surgical gastrectomy after ESD to achieve a curative treatment. Although ESD has merits (*e.g.*, preservation of the stomach and maintenance of quality of life), additional surgery after ESD, owing to non-curative resection, places considerable emotional and financial burdens on patients. Recently, as the number of endoscopists performing ESD has increased, the rate of non-curative resection after ESD has increased correspondingly. In order to decrease the non-curative resection rate, as well as determine the ideal rate of non-curative resection after ESD, we return to the basics.

TIPS FOR SUCCESSFUL ESD OF EGC

Successful ESD of EGC requires accurate prediction of the invasion depth, horizontal extent, and histopathological type of the tumor[2]. To accurately predict the depth of EGC invasion, the macroscopic morphology of the tumor is first considered. Macroscopic findings such as tumor size > 30 mm, remarkable redness, uneven surface, subepithelial tumor-like margin elevation, and mucosal fold convergence are useful for determining the depth of tumor invasion, with a reported accuracy of 83%-97%[12]. However, endoscopic prediction of the invasion depth is subjective and influenced by the endoscopist's level of experience. Endoscopic ultrasonography (EUS) can also be used for determining the depth of EGC invasion, and its overall accuracy with a high-frequency (20 MHz) catheter probe is 81%[13]. EUS is an operator-dependent procedure. In addition, in a retrospective study comparing the accuracy of EUS and conventional endoscopy (based on macroscopic findings) in determining the depth of EGC invasion, EUS was not superior to conventional endoscopy [14]. An integrated diagnostic strategy combining conventional endoscopy and EUS has shown an accuracy of > 85%[15].

The horizontal extent of EGC is mainly determined using conventional endoscopy; however, making accurate prediction becomes challenging when the height and color of the tumor are similar to those of the surrounding normal mucosa. In this situation, chromoendoscopy with indigo carmine alone or indigo carmine and acetic acid, and magnifying endoscopy with narrow-band imaging (ME-NBI) can increase the accuracy of horizontal extent prediction to 90% approximately[16,17]. However, in undifferentiated-type EGC, predicting the horizontal extent using these modalities is challenging. Therefore, during endoscopic examination, biopsy specimens should be obtained from the surrounding tissues and examined histopathologically prior to ESD[12].

The histopathological type of EGC is usually determined based on the results of endoscopic forceps biopsies. However, because these results often do not correctly reflect the final histopathology, histological discrepancies may occur between endoscopic biopsy and ESD-resected specimens. Although the macroscopic morphology and color of lesions have been shown to help predict the histopathological type of EGC, adequate evidence is lacking. Several studies have reported that microsurface and microvascular patterns on ME-NBI can predict the histopathological type of EGC[2,18]. However, systematic ME classification systems, such as those for colorectal polyps and esophageal lesions, have not yet been developed for EGC.

Table 1 Suggested quality indicators for outcomes of endoscopic submucosal dissection for early gastric cancer

Quality indicator	Performance target
En bloc resection rate	> 95%
Complete resection rate	> 90%
Curative resection rate	> 80%
Adverse events	
Post-ESD bleeding	< 10%
Perforation	< 5%

ESD: Endoscopic submucosal dissection.

Hence, other methods to accurately predict the depth of invasion and horizontal extent of EGC are required. Recent studies have reported the use of artificial intelligence (AI) systems for this purpose. Two recent meta-analyses reported that the pooled sensitivity and specificity of AI for predicting deep submucosal invasion were 72%-82% and 79%-90%, respectively[19,20]. In the future, endoscopist-AI cooperation can improve the predictive rates of the depth of invasion, horizontal extent, and histopathological type of EGC before ESD.

CONCLUSION

Recently, the policy of "diagnostic ESD" has been commonly implemented, especially when accurate prediction of the depth of EGC invasion before ESD is impossible. Many young endoscopists have adopted this approach. However, despite its value as diagnostic ESD, it is neither ideal nor scientific. The standard endoscopic process for EGC consists of the following steps: Presence diagnosis (determination of the presence or absence of cancer), qualitative diagnosis (determination of histopathological type), and quantitative diagnosis (determination of depth of invasion and horizontal extent) [2]. Although this process is not perfect, it is recommended for all endoscopists as a basis for every effort to avoid non-curative resection after ESD. Performing ESD without such efforts is likely to increase the non-curative resection rate after ESD for EGC, leading to emotional and economic burdens on patients. Based on the accuracy of endoscopic prediction of the depth of tumor invasion (about 80%) and horizontal tumor extent (about 90%), the ideal non-curative resection rate after ESD is < 15%-20%. According to a recent meta-analysis, *en bloc*, complete, and curative resection rates in Eastern studies were 95% [95% confidence interval (CI): 94%-96%], 89% (95%CI: 88%-91%), and 82% (95%CI: 81%-84%), respectively[21]. EGC cases in which ESD is performed beyond the current ESD indications, considering patient factors such as comorbidities, life expectancy, and the ability to tolerate surgery, should be excluded from this calculation. Furthermore, the main adverse events of ESD, such as post-ESD bleeding and perforation, should be considered when evaluating the quality of ESD outcomes. The overall rates of delayed bleeding and perforation are reported to be 2.6%-8.5% and 2.3%-3.9%, respectively[22-24]. Based on previous reports, I suggest quality indicators for the outcomes of ESD for EGC in Table 1.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study

Development of a machine learning-based model for predicting risk of early postoperative recurrence of hepatocellular carcinoma

Yu-Bo Zhang, Gang Yang, Yang Bu, Peng Lei, Wei Zhang, Dan-Yang Zhang

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Abstract

BACKGROUND

Surgical resection is the primary treatment for hepatocellular carcinoma (HCC). However, studies indicate that nearly 70% of patients experience HCC recurrence within five years following hepatectomy. The earlier the recurrence, the worse the prognosis. Current studies on postoperative recurrence primarily rely on postoperative pathology and patient clinical data, which are lagging. Hence, developing a new pre-operative prediction model for postoperative recurrence is crucial for guiding individualized treatment of HCC patients and enhancing their prognosis.

AIM

To identify key variables in pre-operative clinical and imaging data using machine learning algorithms to construct multiple risk prediction models for early postoperative recurrence of HCC.

METHODS

The demographic and clinical data of 371 HCC patients were collected for this retrospective study. These data were randomly divided into training and test sets at a ratio of 8:2. The training set was analyzed, and key feature variables with predictive value for early HCC recurrence were selected to construct six different machine learning prediction models. Each model was evaluated, and the best-performing model was selected for interpreting the importance of each variable. Finally, an online calculator based on the model was generated for daily clinical practice.

RESULTS

Following machine learning analysis, eight key feature variables (age, intratumoral arteries, alpha-fetoprotein, pre-operative blood glucose, number of tumors, glucose-to-lymphocyte ratio, liver cirrhosis, and pre-operative platelets) were selected to construct six different prediction models. The XGBoost model outperformed other models, with the area under the receiver operating characteristic curve in the training, validation, and test datasets being 0.993 (95% confidence interval: 0.982-1.000), 0.734 (0.601-0.867), and 0.706 (0.585-0.827), respectively. Calibration curve and decision curve analysis indicated that the XGBoost model also had good predictive performance and clinical application value.

CONCLUSION

The XGBoost model exhibits superior performance and is a reliable tool for predicting early postoperative HCC recurrence. This model may guide surgical strategies and postoperative individualized medicine.

Key Words: Machine learning; Hepatocellular carcinoma; Early recurrence; Risk prediction models; Imaging features; Clinical features

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Core Tip: The current study aimed at employing machine learning techniques to select imaging and pre-operative clinical characteristic variables, to which the clinicians were easily accessible, to develop six different risk prediction models for early postoperative recurrence of hepatocellular carcinoma (HCC). We compared the sensitivity and specificity of these models in detecting patients at high risk of early postoperative recurrence of HCC. In addition, to increase the feasibility and applicability of the constructed model, we generated a calculator online based on the predictive model to help clinicians apply it in their daily medical practice.

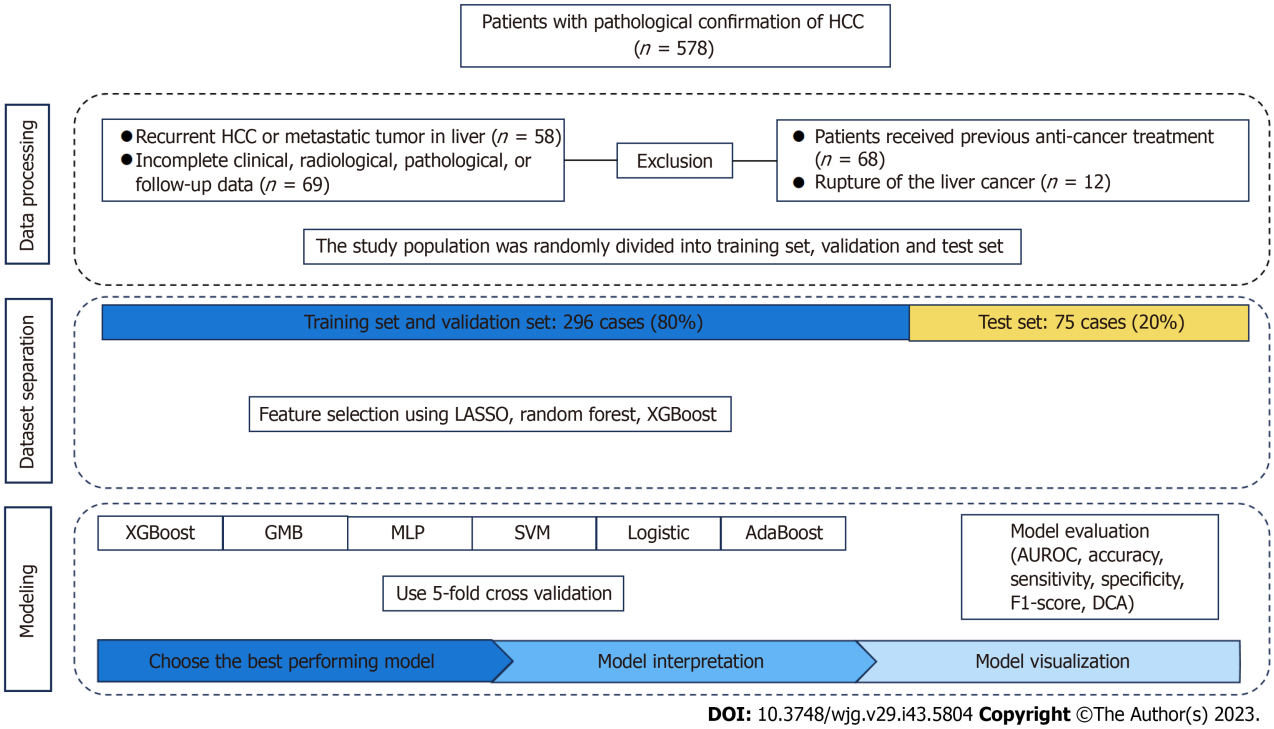
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INTRODUCTION

Liver cancer is one of the most common malignant tumors globally, and its incidence ranks 5th in various malignant tumors. According to statistics, there were about 905700 new cases of liver cancer worldwide in 2020, and about 830200 patients with liver cancer died. The incidence and mortality of liver cancer are expected to increase by > 50% in 2040[1]. Hepatocellular carcinoma (HCC) is the primary type of liver cancer. Currently, surgical resection is still the main treatment for HCC. However, nearly 70% of patients experience HCC recurrence within five years after hepatectomy[2,3], which is the main factor affecting patients' survival rate after surgery. HCC recurrence can be classified into early (≤ 2 years) or late recurrence (> 2 years after HCC hepatectomy). A study has shown that the earlier the recurrence of HCC, the worse the prognosis, and the overall survival rate of patients with early recurrence of HCC tends to be lower than those with late recurrence[4]. For patients with recurrent HCC, radical treatment is more effective than palliative therapies in prolonging their survival[5,6]. For patients at high risk of potentially postoperative recurrence, neoadjuvant therapy, appropriate intraoperative margin expansion, and early postoperative use of targeted therapies and immuno-therapies may improve their prognosis[7]. However, the lack of specific tumor biomarkers and clinical features makes it challenging to identify the patients at high risk of postoperative recurrence before surgery. Therefore, developing pre-operative non-invasive predictive methods will be highly significant in identifying patients at high risk of postoperative recurrence and precise management of those patients by closely monitoring and individualized treatment on time.

Currently, there are many prediction models to predict the early recurrence of HCC in the clinic[8-10]. However, these models are usually constructed based on linear assumptions, which may not model the complex, multidimensional, and nonlinear relationships among different predictor variables that may exist. Their predictive value is greatly limited[11,12]. In recent years, machine learning has great potential for research in the field of disease prediction because machine learning can better capture the relationships between nonlinearities, deeply mine the hidden relationships in massive data through various algorithms, and build treatment models. These models can efficiently and accurately predict the prognosis of diseases and guide clinical treatment. Previous studies have used artificial intelligence techniques and machine learning algorithms to extract imaging features from pre-operative computed tomography (CT) or magnetic resonance imaging (MRI) images and clinical data to predict the risk of liver cancer recurrence[13,14]. Although these studies make superior predictions, these constructed models remain not highly interpretable and could not be widely applied in most medical scenarios, a common problem with machine learning research.

This study aimed to utilize machine learning techniques to select easily accessible imaging and pre-operative clinical characteristic variables. The goal was to develop six different risk prediction models for the early postoperative re-currence of HCC. We compared the sensitivity and specificity of these models in identifying patients at high risk of early



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Figure 1 Flow chart of the study process. HCC: Hepatocellular carcinoma; AUROC: Area under the receiver operating characteristic curve; DCA: Decision curve analysis; GNB: Complement NB; MLP: Multilayer perceptron; SVM: Support vector machine.

postoperative HCC recurrence. Furthermore, to enhance the feasibility and applicability of the constructed model, we created an online calculator based on the predictive model. This tool was designed to assist clinicians in their daily medical practice.

MATERIALS AND METHODS

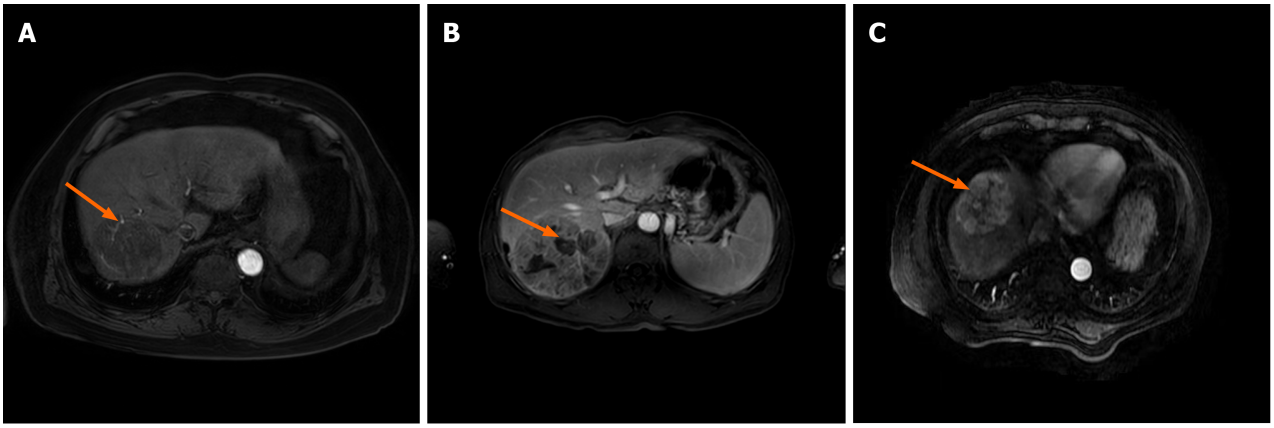
Research subjects

The demographic and clinical data of age, gender, pathology, radiological images, hepatectomy, and postoperative follow-up of 578 patients with primary liver cancer at Ningxia Medical University General Hospital from January 2017 to June 2021 were retrospectively collected according to the inclusion and exclusion criteria. After excluding those with non-primary liver cancer, incomplete data, receiving neoadjuvant therapy, or liver cancer rupture, the remaining cases were selected for this study, randomized by a simple number generated with a computer, and divided into training and test sets at a ratio of 8:2. The training set of data was used to construct the machine learning model, and the test set of data was used to validate the model performance. The study was approved by the Ethics Committee of the General Hospital of Ningxia Medical University and individual patients provided written informed consent. The study was performed per the Declaration of Helsinki, and the study design followed the TRIPOD statement[15] (Multifactorial predictive reporting norms for individual prognosis or diagnosis). The process of this study is shown in Figure 1.

The inclusion criteria included: (1) Postoperatively pathologically confirmed primary HCC (the first treatment option was radical hepatectomy); (2) undergoing pre-operative abdominal contrast-enhanced MRI examinations; and (3) complete clinical, pathological, and imaging data and postoperative follow-up. The exclusion criteria were: (1) Postoperative pathological diagnosis of secondary HCC; (2) patients undergoing emergency surgery for the ruptured bleeding of liver cancer; (3) receiving pre-operative anti-tumor therapy, such as interventional, ablative, immunologic, or targeted therapy; and (4) acute or chronic infection within 2 wk prior to surgery.

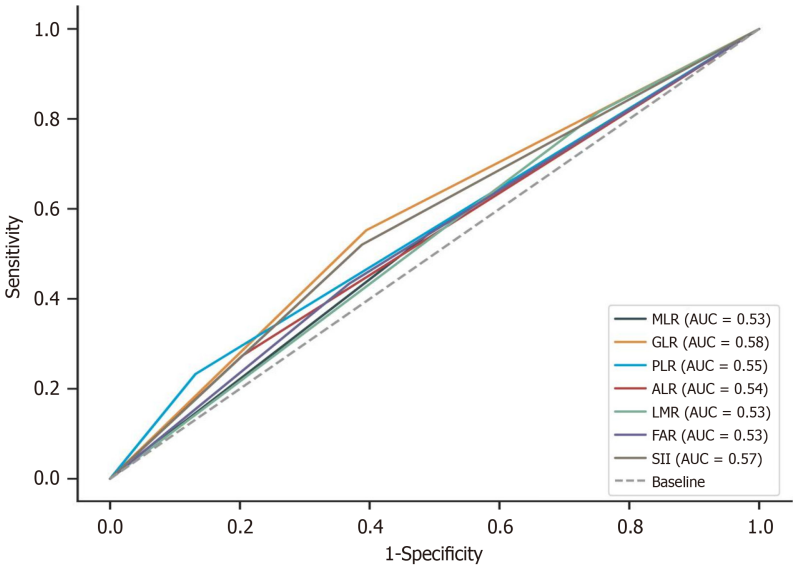
The early liver cancer recurrence was defined according to a previous report[16]. Briefly, a new tumor appeared in the liver within two years after surgical resection of the primary liver cancer. The new tumors were evaluated by radiological imaging or pathological examination. The time to recurrence was defined as the period from the surgical resection of primary liver cancer to the appearance of a new tumor in the liver.

Patients were followed postoperatively in our outpatient service from monthly to every 3 mo in the first-year post-surgical resection of the primary liver cancer. The patients were tested for serum liver functions and alpha-fetoprotein (AFP) levels, chest CT scanning, and abdominal contrast enhanced CT or MRI. Subsequently, the patients who survived were followed every 6 mo starting from the second year after surgery. This follow-up continued until April 2023 or until tumor recurrence, loss of patient follow-up, or death.



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Figure 2 Representative magnetic resonance imaging images. A: Intratumoral arteries; B: Intratumoral necrosis; C: Uniform tumor enhancement.



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Figure 3 Receiver operating characteristic curve analyses of diagnostic performance of monocyte to lymphocyte ratio, γ-glutamyl transferase to lymphocyte ratio, platelet count to lymphocyte count ratio, alkaline phosphatase to lymphocyte count ratio, lymphocyte to monocyte count ratio, fibrinogen to albumin level ratio, or systemic immune-inflammation index. AUC: Area under the curve; MLR: Monocyte to lymphocyte ratio; GLR: γ-glutamyl transferase to lymphocyte ratio; PLR: Platelet count to lymphocyte count ratio; ALR: Alkaline phosphatase to lymphocyte count ratio; LMR: Lymphocyte to monocyte count; FAR: Fibrinogen to albumin level ratio; SII: Systemic immune-inflammation index.

Feature variable filtering

The characteristic variables included: (1) Patient's demographic characteristics and pre-operative laboratory tests, such as gender, age, AFP, blood platelet count (PLT), blood glucose, HBeAg, HBsAg, monocyte to lymphocyte ratio (MLR), γ-glutamyl transferase (GGT) to lymphocyte ratio (GLR), PLT to lymphocyte count ratio (PLR), alkaline phosphatase (ALP) to lymphocyte count ratio (ALR), lymphocyte to monocyte count ratio (LMR), fibrinogen to albumin level ratio (FAR), systemic immune-inflammation index (SII; neutrophil count × PLT/lymphocyte count), ALP, and albumin-bilirubin (ALBI) score; and (2) pre-operative imaging features (abdominal contrast-enhanced MRI): Tumor size, tumor number, homogeneous tumor enhancement, intratumoral necrosis, tumor envelope, whether tumor margin is regular, intratumoral arteries, tumor adjacency to major vessels, ascitic fluid, and liver cirrhosis (Figure 2).

Data pre-processing

The data in the training set were analyzed using the receiver operating characteristic (ROC) curve to determine the best cut-off points for the continuous variable values GLR, PLR, ALR, LMR, FAR, and SII to divide the patients into two groups (as shown in Figure 3), where the best cut-off value was 0.285 [95% confidence interval (95%CI): 0.482-0.601] for MLR; 32.16 (95%CI: 0.526- 0.642) for GLR; 138.18 (95%CI: 0.494-0.61) for PLR; 80.29 (95%CI: 0.465-0.583) for ALR; 5.33 (95%CI: 0.40-0.517) for LMR; 0.07 (95%CI: 0.484-0.6) for FAR; and 312.71 (95%CI: 0.507-0.624) for SII in this population. Z-score normalization of continuous variables was performed. When the proportion of missing values was less than 10%,

the mean value was used for interpolation, and the case was deleted when the proportion of missing values of the variable was greater than 10%.

Statistical analysis

All statistical analyses were performed using the R 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria) and Python language. Categorical variables are expressed as *n* (%) and were analyzed by the chi-square test or Fisher's exact test. Continuous variables are expressed as the mean ± SD or the median and interquartile range and were analyzed by the independent-sample *t*-test or the Mann–Whitney *U* test based on their distribution. A *P* value of < 0.05 was considered statistically significant.

The most significant feature variables were chosen from the intersection of the three methods used for important feature analysis. Six different machine learning models were constructed based on these feature sets. The hyperparameters for each model were determined through a grid search to select the best parameters and perform a five-fold cross-validation on the training data set. Cross-validation provided a more accurate evaluation of model performance through metrics from multiple experiments. The predictive performance of the model was validated using discriminative and calibration methods, and the clinical usefulness of the model was evaluated by clinical decision curve analysis (DCA). After selection, the importance of each characteristic in the best model was interpreted using the SHAP package in Python to provide insight into the relationships among them.

RESULTS

Patient characteristics

According to the inclusion and exclusion criteria, 207 out of 578 cases were excluded, and the remaining 371 patients were finally included for this study. Their demographic and clinical characteristics are shown in Table 1. Among them, 219 (59.02%) patients suffered from early HCC recurrence. Those 371 patients were randomized at a ratio of 8:2 into the training and test sets. As a result, 296 patients were in the training set, including 221 males and 75 females, of whom 172 (58.11%) experienced early HCC recurrence; 75 patients in the test set, including 59 males and 16 females, of whom 47 had early HCC recurrence (62.67%). No statistically significant difference was observed in any measure tested between the training and test groups ($P > 0.05$ for all; Table 1).

Feature filtering

A total of 26 potential characteristic variables were selected for this study. Their importance was analyzed using LASSO regression, XGBoost, and random forest (RF). Ten variables with the highest importance were ranked in descending order. Eight key variables were identified after screening the intersection of the variables using the three models. In no particular order, these variables were age, intratumoral arteries, AFP, blood glucose, number of tumors, GLR, liver cirrhosis, and PLT (Figure 4).

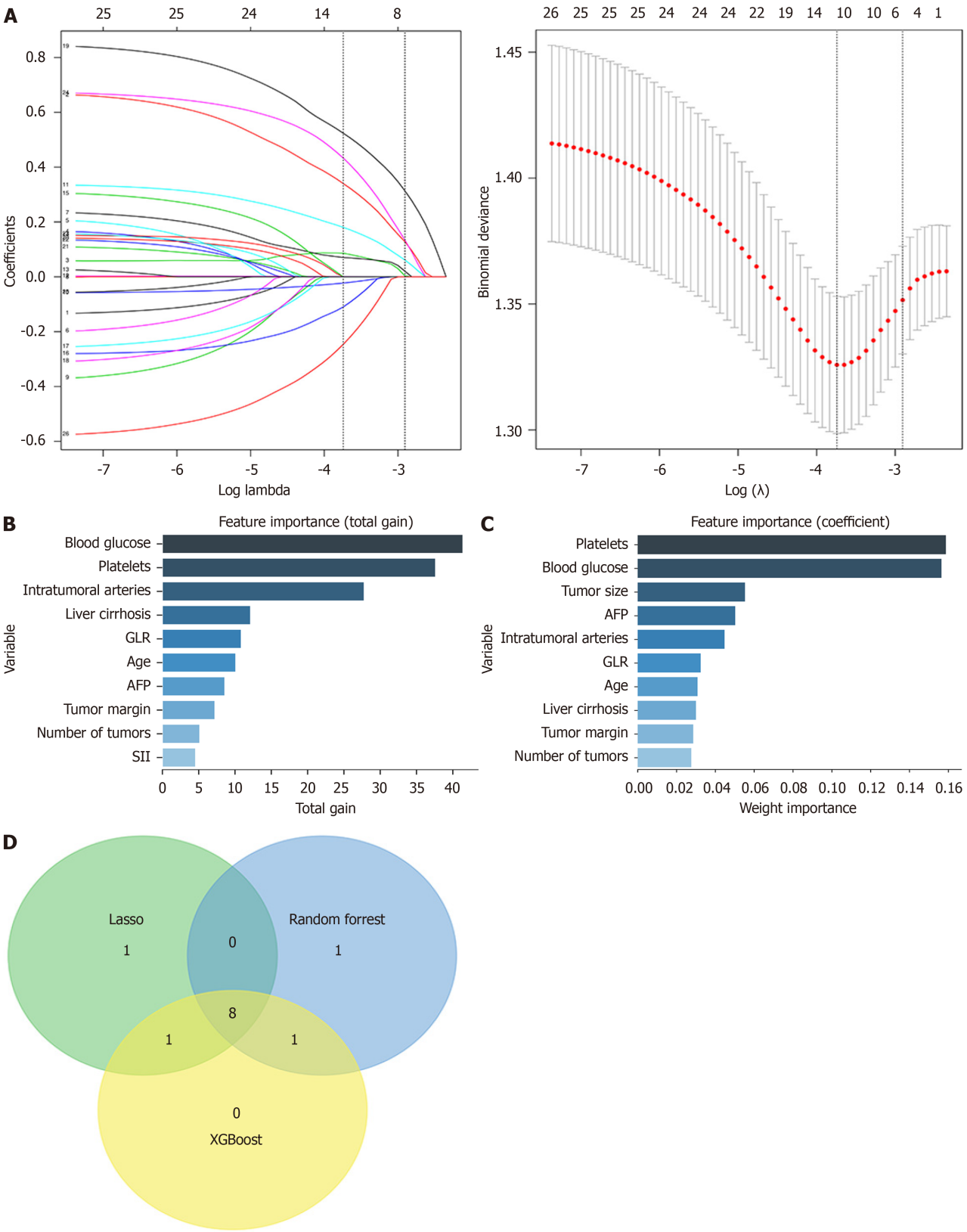
Model construction and performance validation

We eventually constructed six machine learning models, and calculated the area under the ROC curve (AUROC) in both the training set and validation set (Figure 5A and B) and generated the forest plots of the area under the curve (AUC) values of the six predictive models in the training set (Figure 6). The XGBoost analyses indicated larger and better AUROCs in the training, validation, and test sets of 0.993 (95%CI: 0.982-1.000), 0.734 (95%CI: 0.601-0.867), and 0.706 (95%CI: 0.585-0.827), respectively (Figure 7). After the five-fold cross-validation, the AUC had an SD of 0.002 for the XGBoost model, the smallest value among the six models, indicating that the XGBoost model had the most stable performance (Figure 6) and that XGBoost achieved lower and better Brier scores (Figure 5C). The DCA results revealed that the XGBoost model was the best diagnostic tool and had good clinical utility compared to other models (Figure 5D). In comparison with individual models (Tables 2 and 3), although the RF model had better indicators for each evaluation in the training dataset, the XGBoost model was an optimal model after a five-fold cross-validation, considering that the RF model might have overfitting. The XGBoost model achieved an AUC of 0.706 (95%CI: 0.585-0.827), accuracy of 0.64, sensitivity of 0.857, specificity of 0.545, and F1 score of 0.766 in the test dataset. The optimal critical value for the prediction probability of the XGBoost model was 55.0 %, according to the Youden index.

The XGBoost model was further analyzed using the SHAP software package, the results unveiled the importance and impact of each feature in the sample, and the importance from the highest to lowest ranked as blood glucose, PLT, intratumoral arteries, AFP, liver cirrhosis, GLR, age, and number of tumors.

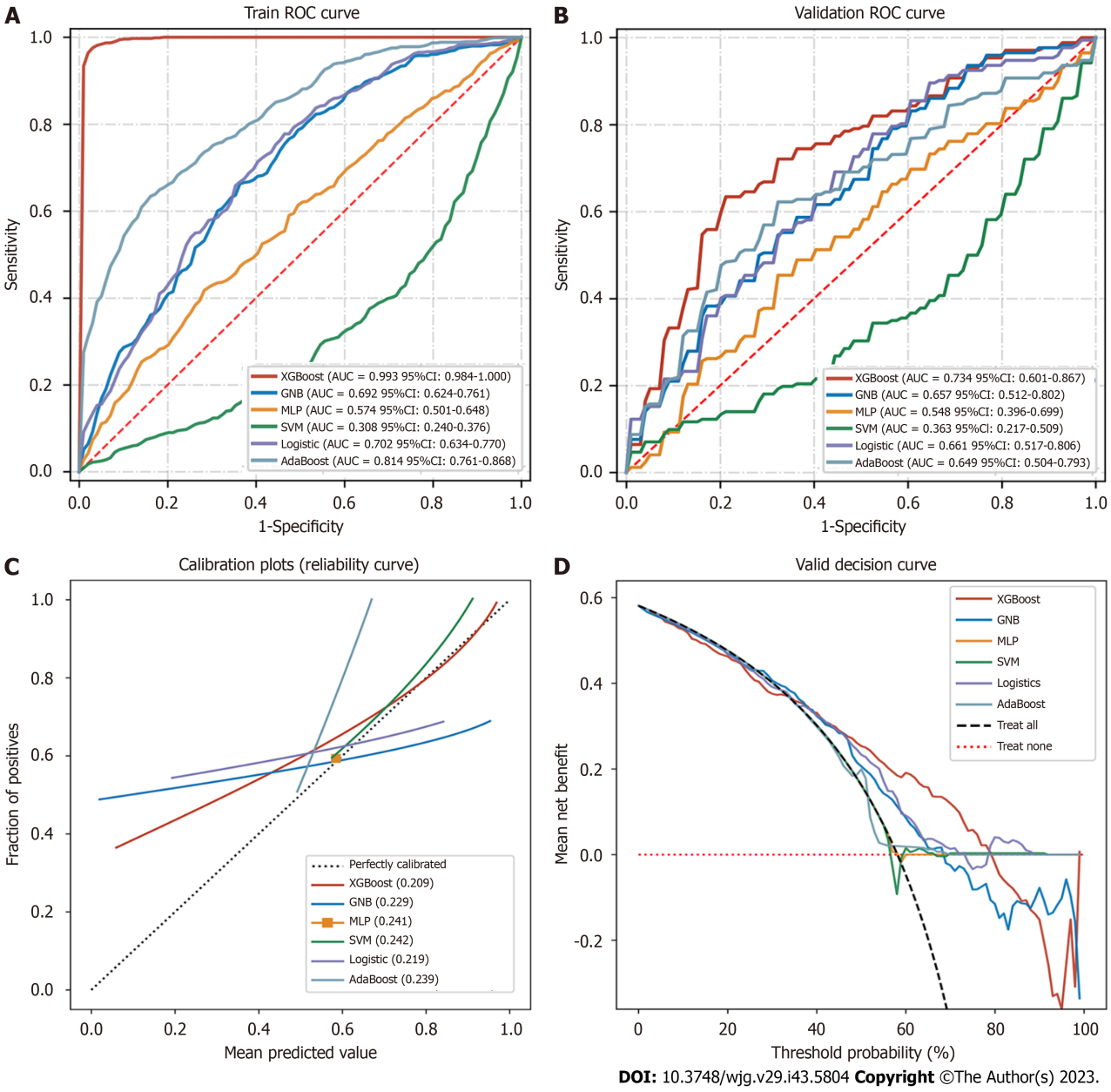
Model demonstrations and applications

Analysis of the XGBoost model using the SHAP package indicated that each feature in the sample had both positive and negative impacts. The relationship between the magnitude of the eigenvalues and the predicted impact is shown in Figure 8. For applying the XGBoost model, the optimal cut-off point for the model's prediction probability was 55%. If the model predicted > 55%, it would indicate that patients undergoing hepatectomy are at higher risk of early postoperative HCC recurrence. In addition, we generated a calculator based on the model online for clinicians to apply this model in their daily practice (available at: http://152.136.184.184:5050/).



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Figure 4 Importance of feature variables analyzed by LASSO regression, XGBoost, and random forest, and top 10 variables ranked in their importance from the highest to lowest and selected. The Venn diagram was drawn by selecting the features common to all three models (taking the intersection). A: LASSO regression with a vertical line was drawn at the value selected using the ten-fold cross-validation, and a minimum mean square error of λ of 0.024 was chosen to obtain the characteristics of the 11 non-zero coefficients; B: The importance of variable features was analyzed using the XGBoost algorithm; C: The importance of variable features was analyzed using the random forest algorithm; D: Venn diagram with eight key characteristic variables at the intersection: Age, intratumoral arteries, alpha-fetoprotein, blood glucose, number of tumors, γ-glutamyl transferase to lymphocyte ratio, liver cirrhosis, and platelets. AFP: alpha-fetoprotein; GLR: γ-glutamyl transferase to lymphocyte ratio; SII: Systemic immune-inflammation index.



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Figure 5 Receiver operating characteristic analyses and validation of six prediction models. A: Receiver operating characteristic (ROC) curves of the six prediction models in the training dataset; B: ROC curves of the six prediction models in the validation dataset after five-fold cross-validation; C: Calibration plots of nine models. The XGBoost achieved lower (better) Brier scores than the other models; D: Decision curve analysis of six machine learning models. The XGBoost model is the best diagnostic tool for early postoperative hepatocellular carcinoma recurrence. ROC: Receiver operating characteristic.

DISCUSSION

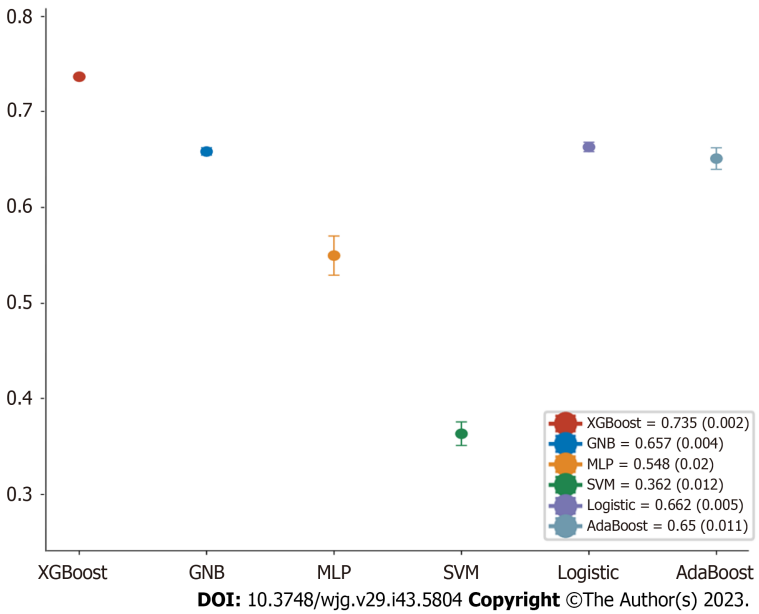
Early HCC recurrence is one of the main factors shortening the survival of postoperative HCC patients. Given that most HCC patients experience HCC recurrence within 5 years post-resection of their primary tumors[2], prevention, early diagnosis, and precise treatment will be critical for limiting postoperative recurrence and improving the survival of HCC patients. This study found an early HCC recurrence rate of 59.02% in this population. While most cases with early HCC recurrence usually come from the hidden micrometastasis of the initial HCC in the body, the late HCC recurrence appears two years after resection. It is commonly from new polyclonal origins of HCC lesions that may completely differ from the initial liver cancer. Actually, early HCC recurrence is usually associated with more aggressive features and a worse prognosis. A previous study has suggested that adjuvant therapy for HCC patients at risk of early recurrence may prolong their survival[17]. However, identifying the patients at high risk of postoperative recurrence before surgery is complicated because of the lack of specific tumor biomarkers and clinical features. Therefore, clinical prediction models will be valuable for predicting early HCC recurrence. However, most available clinical prediction models are often constructed based on the assumption of linear relationships and usually fail to reflect the nature of complex, multidimensional, nonlinear relationships between different predictor variables in the pathogenesis of HCC to affect their predictive performance. Machine learning has shown great potential in analyses of data with nonlinear relationships, and in general, machine learning models outperform traditional regression models in predicting the development of HCC[18].

Table 1 Demographic and clinical characteristics of patients

Variable		Overall (*n* = 371)	No HCC recurrence (*n* = 152)	HCC recurrence (*n* = 219)	*P* value	Training set (*n* = 296)	Test set (*n* = 75)	*P* value
Liver cirrhosis, *n* (%)	No	85 (22.911)	28 (18.421)	57 (26.027)	0.086	85 (22.911)	69 (23.311)	0.716
	Yes	286 (77.089)	124 (81.579)	162 (73.973)		227 (76.689)	59 (78.667)	
Tumor size, *n* (%), cm	≤ 2	42 (11.321)	16 (10.526)	26 (11.872)	0.027	29 (9.797)	13 (17.333)	0.308
	2-5	168 (45.283)	78 (51.316)	90 (41.096)		137 (46.284)	31 (41.333)	
	5-10	109 (29.380)	46 (30.263)	63 (28.767)		87 (29.392)	22 (29.333)	
	≥ 10	52 (14.016)	12 (7.895)	40 (18.265)		43 (14.527)	9 (12.000)	
Tumor number, *n* (%)	1	285 (76.819)	128 (84.211)	157 (71.689)	0.005	229 (77.365)	56 (74.667)	0.621
	≥ 2	86 (23.181)	24 (15.789)	62 (28.311)		67 (22.635)	19 (25.333)	
Homogeneous tumor enhancement, *n* (%)	No	297 (80.054)	119 (78.289)	178 (81.279)	0.479	238 (80.405)	59 (78.667)	0.736
	Yes	74 (19.946)	33 (21.711)	41 (18.721)		58 (19.595)	16 (21.333)	
Intratumoral necrosis, *n* (%)	No	142 (38.275)	69 (45.395)	73 (33.333)	0.019	113 (38.176)	29 (38.667)	0.938
	Yes	229 (61.725)	83 (54.605)	146 (66.667)		183 (61.824)	46 (61.333)	
Tumor envelope intactness, *n* (%)	No	145 (39.084)	60 (39.474)	85 (38.813)	0.898	117 (39.527)	28 (37.333)	0.728
	Yes	226 (60.916)	92 (60.526)	134 (61.187)		179 (60.473)	47 (62.667)	
Regular tumor margin, *n* (%)	No	176 (47.439)	73 (48.026)	103 (47.032)	0.850	139 (46.959)	37 (49.333)	0.713
	Yes	195 (52.561)	79 (51.974)	116 (52.968)		157 (53.041)	38 (50.667)	
Intratumoral arteries, *n* (%)	No	174 (46.900)	89 (58.553)	85 (38.813)	< 0.001	135 (45.608)	39 (52.000)	0.322
	Yes	197 (53.100)	63 (41.447)	134 (61.187)		161 (54.392)	36 (48.000)	
Adjacency to major vessels, *n* (%)	No	216 (58.221)	93 (61.184)	123 (56.164)	0.335	171 (57.770)	45 (60.000)	0.727
	Yes	155 (41.779)	59 (38.816)	96 (43.836)		125 (42.230)	30 (40.000)	
Ascitic fluid, *n* (%)	No	262 (70.620)	106 (69.737)	156 (71.233)	0.756	207 (69.932)	55 (73.333)	0.564
	Yes	109 (29.380)	46 (30.263)	63 (28.767)		89 (30.068)	20 (26.667)	
Age, *n* (%), yr	< 60	227 (61.186)	85 (55.921)	142 (64.840)	0.083	187 (63.176)	40 (53.333)	0.118
	≥ 60	144 (38.814)	67 (44.079)	77 (35.160)		109 (36.824)	35 (46.667)	
Gender, *n* (%)	Male	280 (75.472)	118 (77.632)	162 (73.973)	0.421	221 (74.662)	59 (78.667)	0.472
	Female	91 (24.528)	34 (22.368)	57 (26.027)		75 (25.338)	16 (21.333)	
HBsAg, *n* (%)	Negative	101 (27.224)	41 (26.974)	60 (27.397)	0.928	76 (25.676)	25 (33.333)	0.183
	Positive	270 (72.776)	111 (73.026)	159 (72.603)		220 (74.324)	50 (66.667)	
HBeAg, *n* (%)	Negative	289 (77.898)	123 (80.921)	166 (75.799)	0.242	229 (77.365)	60 (80.000)	0.623
	Positive	82 (22.102)	29 (19.079)	53 (24.201)		67 (22.635)	15 (20.000)	
ALBI, *n* (%), grade	1	67 (18.059)	26 (17.105)	41 (18.721)	0.907	54 (18.243)	13 (17.333)	0.710
	2	288 (77.628)	119 (78.289)	169 (77.169)		228 (77.027)	60 (80.000)	
	3	16 (4.313)	7 (4.605)	9 (4.110)		14 (4.730)	2 (2.667)	
MLR, *n* (%)	< 0.285	171 (46.092)	75 (49.342)	96 (43.836)	0.295	140 (47.297)	31 (41.333)	0.355
	≥ 0.285	200 (53.908)	77 (50.658)	123 (56.164)		156 (52.703)	44 (58.667)	
GLR, *n* (%)	< 32.16	190 (51.213)	92 (60.526)	98 (44.749)	0.003	152 (51.351)	38 (50.667)	0.916

	≥ 32.16	181 (48.787)	60 (39.474)	121 (55.251)		144 (48.649)	37 (49.333)	
PLR, *n* (%)	< 138.18	300 (80.863)	132 (86.842)	168 (76.712)	0.015	241 (81.419)	59 (78.667)	0.588
	≥ 138.18	71 (19.137)	20 (13.158)	51 (23.288)		55 (18.581)	16 (21.333)	
ALP, *n* (%)	< 80.29	280 (75.472)	121 (79.605)	159 (72.603)	0.123	222 (75.000)	58 (77.333)	0.675
	≥ 80.29	91 (24.528)	31 (20.395)	60 (27.397)		74 (25.000)	17 (22.667)	
LMR, *n* (%)	< 5.33	82 (22.102)	28 (18.421)	54 (24.658)	0.155	67 (22.635)	15 (20.000)	0.623
	≥ 5.33	289 (77.898)	124 (81.579)	165 (75.342)		229 (77.365)	60 (80.000)	
FAR, *n* (%)	< 0.07	220 (59.299)	96 (63.158)	124 (56.621)	0.208	174 (58.784)	46 (61.333)	0.688
	≥ 0.07	151 (40.701)	56 (36.842)	95 (43.379)		122 (41.216)	29 (38.667)	
SII, *n* (%)	< 312.71	198 (53.369)	93 (61.184)	105 (47.945)	0.012	157 (53.041)	41 (54.667)	0.801
	≥ 312.71	173 (46.631)	59 (38.816)	114 (52.055)		139 (46.959)	34 (45.333)	
AFP, *n* (%), ng/mL	< 20	171 (46.092)	81 (53.289)	90 (41.096)	0.012	138 (46.622)	33 (44.000)	0.857
	20-400	86 (23.181)	37 (24.342)	49 (22.374)		69 (23.311)	17 (22.667)	
	≥ 400	114 (30.728)	34 (22.368)	80 (36.530)		89 (30.068)	25 (33.333)	
PLT, median (IQR), 109/L		168.000 (113.000, 209.000)	154.000 (112.000, 200.000)	171.000 (118.000, 216.000)	0.110	165.000 (111.000, 208.000)	178.000 (133.000, 220.000)	-1.451
Blood glucose, median (IQR), mmol/L		4.720 (4.340, 5.310)	4.840 (4.390, 5.660)	4.660 (4.300, 5.170)	0.009	4.710 (4.350, 5.310)	4.770 (4.300, 5.310)	-0.066

HCC: Hepatocellular carcinoma; ALBI: Albumin-bilirubin; MLR: Monocyte to lymphocyte ratio; GLR: γ-glutamyl transferase to lymphocyte ratio; PLR: Platelet count to lymphocyte count ratio; ALR: Alkaline phosphatase to lymphocyte count ratio; LMR: Lymphocyte count to monocyte count; FAR: Fibrinogen to albumin ratio; SII: Systemic immune-inflammation index; PLT: Blood platelets; AFP: Alpha-fetoprotein; IQR: Interquartile range.



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Figure 6 Forest plot of the area under the curve scores of the six models. The XGBoost model achieved a smaller (better) SD compared with the other models. GNB: Complement NB; MLP: Multilayer perceptron; SVM: Support vector machine.

In this study, we analyzed pre-operative imaging and clinical indicators that were easily accessible and non-invasive and selected eight important feature variables using machine learning algorithms, including pre-operative blood glucose value, PLT, intratumoral artery mass, methemoglobin, cirrhosis, GLR, age, and number of tumors. Subsequently, we generated six machine learning prediction models and validated their value in predicting the risk of early postoperative HCC recurrence. After randomly dividing the independent test set, we applied hyperparameter tuning in the training dataset with five-fold cross-validation and validated them in the test dataset. The results indicated that the XGBoost model exhibited better discriminative and predictive value than other models constructed. The calibration plots for the

Table 2 Performance metrics of six models in the training dataset

Model	AUC (95%CI)	Accuracy (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)	F1 score (95%CI)
XGBoost	0.993 (0.984-1.000)	0.974 (0.969-0.979)	0.980 (0.971-0.988)	0.976 (0.966-0.986)	0.981 (0.977-0.985)
GNB	0.692 (0.624-0.761)	0.671 (0.660-0.683)	0.754 (0.689-0.820)	0.566 (0.486-0.646)	0.728 (0.705-0.750)
MLP	0.574 (0.501-0.648)	0.563 (0.516-0.611)	0.517 (0.274-0.761)	0.637 (0.410-0.863)	0.549 (0.411-0.686)
SVM	0.308 (0.240-0.376)	0.453 (0.391-0.514)	0.204 (-0.182-0.591)	0.800 (0.413-1.187)	NaN
Logistic	0.702 (0.634-0.770)	0.677 (0.657-0.696)	0.747 (0.654-0.841)	0.589 (0.499-0.679)	0.727 (0.689-0.765)
AdaBoost	0.814 (0.761-0.868)	0.725 (0.701-0.748)	0.661 (0.585-0.737)	0.825 (0.773-0.876)	0.737 (0.700-0.773)

95%CI: 95% confidence interval; AUC: Area under the curve; GNB: Complement NB; MLP: Multilayer perceptron; SVM: Support vector machine; NaN: Not a number.

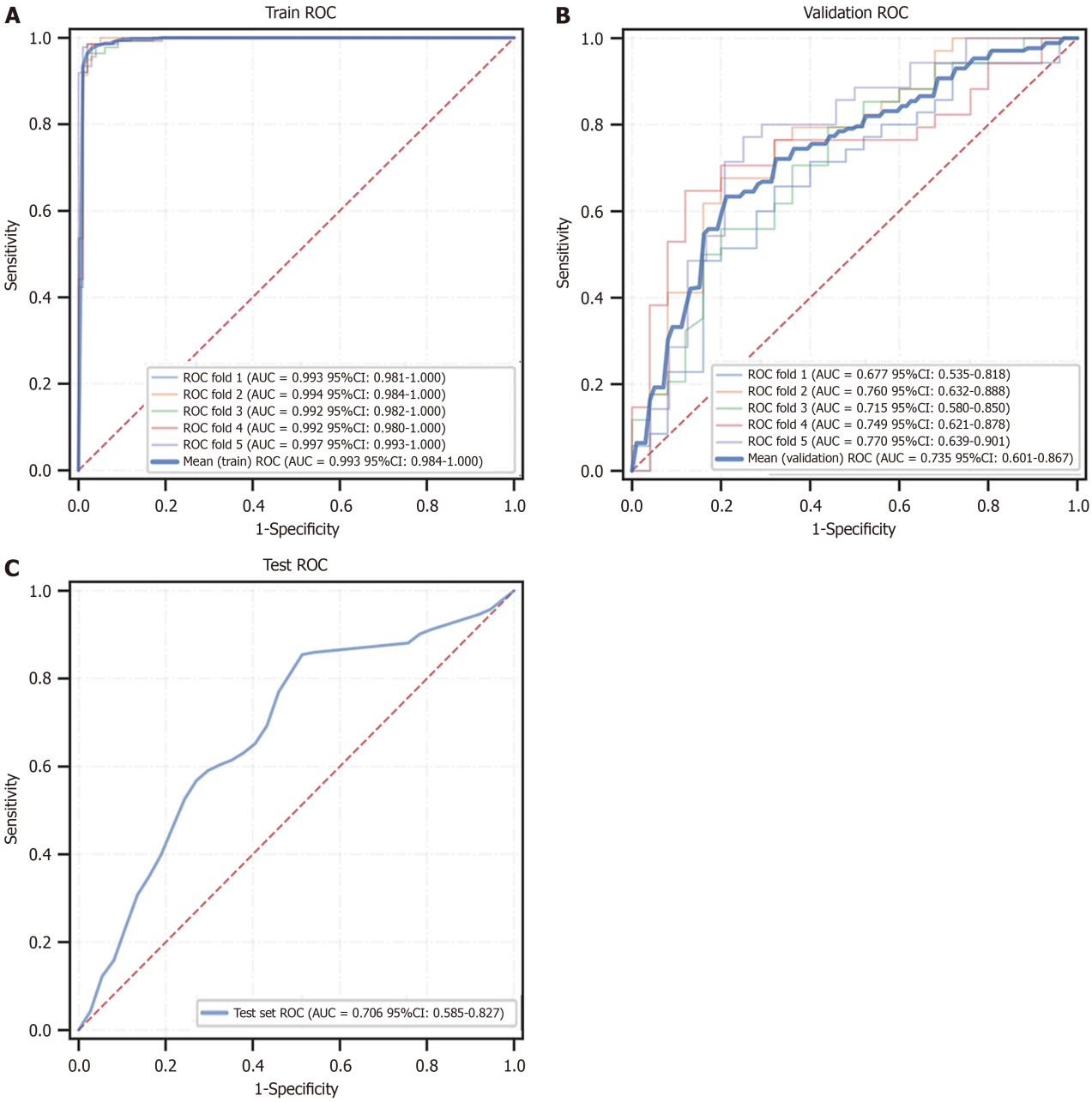
Table 3 Performance metrics of six models in the validation dataset

Model	AUC (95%CI)	Accuracy (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)	F1 score (95%CI)
XGBoost	0.734 (0.601-0.867)	0.683 (0.658-0.708)	0.662 (0.596-0.729)	0.782 (0.717-0.847)	0.694 (0.636-0.752)
GNB	0.657 (0.512-0.802)	0.618 (0.583-0.653)	0.647 (0.443-0.851)	0.662 (0.464-0.861)	0.641 (0.535-0.748)
MLP	0.548 (0.396-0.699)	0.514 (0.452-0.575)	0.575 (0.398-0.752)	0.627 (0.491-0.764)	0.577 (0.471-0.682)
SVM	0.363 (0.217-0.509)	0.446 (0.381-0.511)	0.452 (0.035-0.870)	0.617 (0.188-1.046)	NaN
Logistic	0.661 (0.517-0.806)	0.632 (0.602-0.661)	0.797 (0.709-0.886)	0.525 (0.414-0.637)	0.728 (0.706-0.751)
AdaBoost	0.649 (0.504-0.793)	0.618 (0.569-0.667)	0.591 (0.330-0.852)	0.780 (0.610-0.950)	0.617 (0.403-0.831)

95%CI: 95% confidence interval; AUC: Area under the curve; GNB: Complement NB; MLP: Multilayer perceptron; SVM: Support vector machine; NaN: Not a number.

XGBoost model also displayed an excellent consistency between predictions and actual observations, and the DCA unveiled that the XGBoost model exhibited excellent clinical application. The model was, therefore, selected as the optimal model with an optimal cut-off value of 55% for the prediction probability. If a patient has a predicted probability of greater than 55%, the patient will have an increased risk of early HCC recurrence after primary tumor resection. Therefore, the prediction model may be valuable for predicting HCC patients for their risk of early postoperative HCC recurrence. Accordingly, to make the prediction model more convenient for clinical practice, we generated a model-based calculator online for physicians to use this model to predict early postoperative HCC recurrence. This will significantly improve the efficiency of the practical application of the prediction model.

To ensure better performance and clinical interpretation of the predictive model, we used the shape model to analyze its interpretation after determining the importance of the relationship between each characteristic variable and early postoperative HCC recurrence. The results indicated that the pre-operative blood glucose value was the variable with the highest importance, consistent with previous studies[19-21]. Hyperglycemia has now been recognized as a risk factor for the development and prognosis of HCC. Shi *et al*[19] found that HCC cells employed glycolysis to support their rapid growth in hypoxic and oxidative stress in the body. On the other hand, hyperglycemia can enhance the growth, invasion, and metastasis of HCC cells by promoting the expression of vascular endothelial growth factor[20]. Similarly, PLT also influenced the early HCC recurrence more, possibly by a positive feedback regulation[22]. Elevated pre-operative PLT are independently associated with increased tumor load, extrahepatic recurrence and metastasis, and poor prognoses of HCC [23]. In turn, HCC cells can promote platelet generation by secreting platelet-generating hormones, and the increased PLT further provide favorable conditions for HCC cell proliferation and metastasis[24,25]. Intratumoral arterial features on pre-operative imaging are a significant risk factor for early postoperative HCC recurrence. A previous study has shown that the distribution and number of blood vessels supplying the tumor can impact the prognosis of HCC patients[26]. Multiple small arteries supplying blood to the tumor may reflect high pressure inside the tumor. The high-pressure environment tends to cause the tumor cells to shed and form microvascular invasion in the surrounding vessels, which may even spread to other locations in the liver, increasing the risk of early postoperative HCC recurrence. Moreover, inflammatory factors are essential for the tumor microenvironment, and high levels of GGT will lead to high cysteine levels *in vivo*, which influence tumor development regarding redox regulation and drug resistance[27]. GGT is a new potential prognostic indicator of inflammation in HCC[28], while lymphocytes are crucial for the systemic and local immune responses, including anti-tumor immunity[29]. A decrease in the number of lymphocytes can result in a weakened immune function, which may enhance angiogenesis and extracellular matrix remodeling, creating a mutagenic environment for tumor cells and promoting tumorigenesis and development[30]. Indeed, GLR has been shown to be



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Figure 7 Receiver operating characteristic curves of the XGBoost model in training, validation, and test datasets. ROC: Receiver operating characteristic; 95%CI: 95% confidence interval.

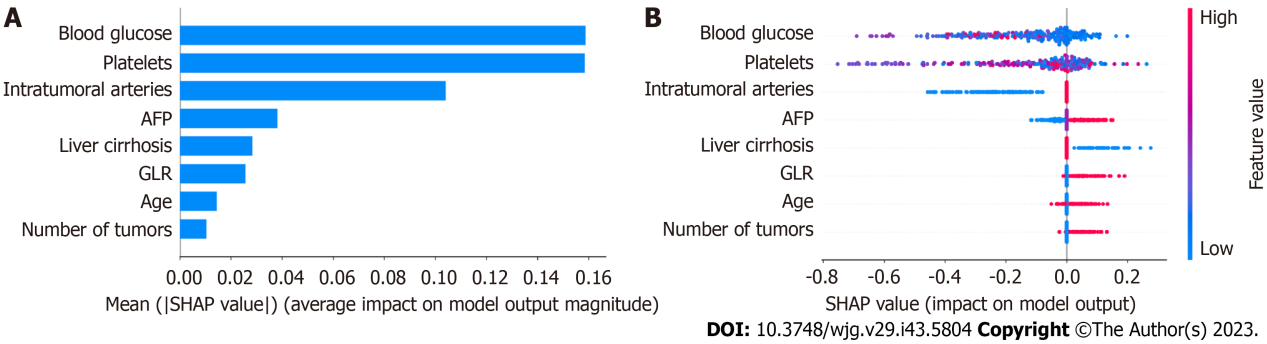
valuable for predicting early postoperative HCC recurrence in a population of 606 patients[31]. Therefore, these vital feature variables included in the best mode are critical for predicting early postoperative HCC recurrence.

In summary, the predictive model constructed in this study may help clinicians predict the risk of early postoperative HCC recurrence in individual HCC patients and better manage them before, during, and after surgery. Our findings may open new avenues for investigating therapeutic strategies for postoperative recurrence of HCC.

We acknowledge that the current study has limitations. First, our study was conducted with a relatively small sample size in a single center and did not include external validation from other centers. Therefore, future studies are needed to validate the value of this model in a larger patient population across multiple centers. Second, the optimal thresholds for the inflammation-related ratio in this study may need to be re-evaluated due to the limited sample size. Considering these limitations, we plan to expand the sample size through a multicenter study in the future and compare our model with other prediction models to further verify its reliability.

CONCLUSION

Compared to other models, the XGBoost model demonstrated superior performance and emerged as the best predictive



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Figure 8 SHAP analysis of the XGBoost model. A: Visual representation of each feature in the XGBoost model and the relationship between the importance of each feature. The color represents the value of the variable, with red representing the larger value and blue representing the smaller value. AFP: Alpha-fetoprotein; GLR: γ-glutamyl transferase to lymphocyte ratio.

measurement model. This predictive model is easily accessible in daily clinical practice and may serve as a crucial tool in guiding postoperative follow-up and individualized medicine for HCC patients.

ARTICLE HIGHLIGHTS

Research background

Surgical resection is still the main treatment for hepatocellular carcinoma (HCC). HCC recurrence is the main factor affecting patients' survival rate after surgery. Developing pre-operative non-invasive predictive methods will be highly significant in identifying patients at high risk of postoperative recurrence and precise management of those patients by closely monitoring and individualized treatment on time.

Research motivation

To develop a new risk prediction model for the early postoperative recurrence of HCC and enhance the feasibility and applicability of the constructed model.

Research objectives

This study aimed to identify key variables in pre-operative clinical and imaging data using machine learning algorithms to construct multiple risk prediction models for early postoperative recurrence of HCC.

Research methods

The demographic and clinical data of 371 HCC patients were collected and analyzed, and the key feature variables were selected to construct six different machine learning prediction models. Each model was evaluated, and the best-performing model was selected for interpreting the importance of each variable. Finally, an online calculator based on the model was generated for daily clinical practice.

Research results

Following machine learning analysis, eight key feature variables were selected to construct six different prediction models. The XGBoost model outperformed other models, with the area under the receiver operating characteristic curve in the training, validation, and test datasets being 0.993 [95% confidence interval (95%CI): 0.982-1.000], 0.734 (95%CI: 0.601-0.867), and 0.706 (95%CI: 0.585-0.827), respectively. Calibration curve and decision curve analysis indicated that the XGBoost model also had good predictive performance and clinical application value.

Research conclusions

The XGBoost model exhibits superior performance and is a reliable tool for predicting early postoperative HCC recurrence. This model may guide surgical strategies and postoperative individualized medicine.

Research perspectives

A multicenter study with large samples should be conducted in the future, and comparing our model with other prediction models is needed to further verify its reliability.

FOOTNOTES

Author contributions: Zhang YB analyzed the data and wrote the manuscript; Yang G analyzed the data and wrote the original draft; Bu

Y contributed the resources; Lei P designed the research; Zhang W analyzed the data; Zhang DY wrote the original draft.

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Institutional review board statement: The study was approved by the Ethics Committee of the General Hospital of Ningxia Medical University (KYLL-2023-0378).

Informed consent statement: Written informed consent was provided by individual patients.

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ORIGINAL ARTICLE

Observational Study

Knowledge, attitude, and practice of patients living with inflammatory bowel disease: A cross-sectional study

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Abstract

BACKGROUND

Patients with inflammatory bowel diseases (IBDs) generally have poor knowledge, attitude, and practice of their disease, while the data from China are lacking.

AIM

To address this knowledge disparity among Chinese patients with IBD.

METHODS

This web-based, cross-sectional study was conducted on a cohort of IBD patients who visited the Second Affiliated Hospital of Wenzhou Medical University between December 2022 and February 2023. Their socio-demographic information and the knowledge, attitude, and practice scores were collected and estimated using a self-designed questionnaire. Pearson's correlation analysis was used to determine the pairwise correlations among knowledge, attitude, and practice scores. A multivariate logistic regression analysis was further performed to determine the independent factors associated with their knowledge, attitude, and practice scores.

RESULTS

A total of 353 patients (224 males) with IBD completed the questionnaires. The mean knowledge, attitude, and practice scores were 10.05 ± 3.46 (possible range: 0-14), 41.58 ± 5.23 (possible range: 0-56), 44.20 ± 7.39 (possible range: 0-56), respectively, indicating good knowledge, positive attitude, and proactive practice toward IBD. Pearson's correlation analysis showed that the knowledge score had significant positive correlations with the attitude score (r = 0.371, P < 0.001) and

practice score (r = 0.100, P < 0.001). The attitude score had a significant positive correlation with the practice score (r = 0.452, P < 0.001). Moreover, multivariate logistic regression analysis showed that aged 30-40 years [odds ratio (OR) = 4.06, 95% confidence interval (CI): 1.04-15.82, P = 0.043], middle school education (OR = 3.98, 95%CI: 1.29-12.33, P = 0.017), high school/technical secondary school education (OR = 14.06, 95%CI: 3.92-50.38, P < 0.001), and junior college/bachelor's degree and above education (OR = 15.20, 95%CI: 4.15-55.650, P < 0.001) were independently associated with good knowledge. The higher knowledge score was independently associated with a positive attitude (OR = 1.23, 95%CI: 1.11-1.36, P < 0.001). The higher attitude score was independently associated with proactive practice (OR = 1.20, 95%CI: 1.11-1.30, P < 0.001).

CONCLUSION

Chinese patients with IBD might have good knowledge, a positive attitude, and proactive practice toward their disease. However, a small number of specific items require education.

Key Words: Attitude; Cross-sectional study; Inflammatory bowel disease; Knowledge; Practice; Questionnaire

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Core Tip: To address this knowledge disparity among Chinese patients with inflammatory bowel disease (IBD), a web-based, cross-sectional study was conducted on 353 IBD patients (224 males). Their mean knowledge, attitude, and practice scores were 10.05 ± 3.46 (range: 0-14), 41.58 ± 5.23 (range: 0-56), and 44.20 ± 7.39 (range: 0-56), respectively. Multivariate logistic regression analysis showed that age and education were independently associated with knowledge. Knowledge was independently associated with attitude. The attitude was independently associated with the practice. In conclusion, patients with IBD in China might have good knowledge, a positive attitude, and proactive practice toward their disease. However, some specific items require education.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, non-specific inflammation of the gastrointestinal tract, including ulcerative colitis and Crohn's disease. The IBD usually develops before age 30[1-3]. Moreover, IBD is associated with a poor quality of life and may increase colorectal cancer risk[2-4]. The individual management strategy of IBD is tailored to each patient according to diagnosis, disease activity grade, disease lesion, and personal prognostic factors[1,3-7]. Despite this, IBD continues to be difficult to manage, as treatment adverse effects and repeated exacerbation/recurrence episodes can eventually necessitate costly second-line therapies or even surgery[8-11].

Maintaining proper lifestyle habits is necessary and complementary to medical treatments in patients with IBD[12,13]. Fundamental to patient self-management is knowing which foods and situations to avoid and what can be done to alleviate symptoms[12,13]. To implement adequate self-management, a thorough understanding of IBD causes, risk factors for exacerbation/recurrence, disease mechanisms, and treatments is essential, and this knowledge needs to be translated into more effective (but not infallible) self-management. In addition, since there is no cure for IBD, self-management is essential to its treatment[1,5,14]. Indeed, since the management of IBD necessitates the adoption of healthy lifestyle habits, IBD patients are the first to be accountable for their health[12,13], which requires proper knowledge, attitudes, and practice (KAP) of the specific lifestyle routines to implement. The appropriate KAP about IBD can reduce medical acceleration in patients with IBD[15]. Since the 1990s, however, some studies have revealed that patients with IBD have misconceptions and limited knowledge of their disease[16-21]. Such studies are important to identify the gaps in knowledge that represent barriers to the proper management of IBD. Identifying these obstacles could also aid in designing interventions to enhance or rectify knowledge[22,23]. Owing to significant differences in culture, economy, health literacy, healthcare systems, and government policies, KAP data are usually very specific to a given population. Of note, data about the KAP toward IBD in Chinese patients with IBD are lacking.

The KAP methodology provides quantitative and qualitative data on the misconceptions that could represent obstacles to a specific task/subject in a specific population[22,23]. Hence, this study aimed to investigate the KAP of patients with IBD toward their disease in Zhejiang Province, China. The results could help healthcare providers to improve the patient's self-management of IBD.

MATERIALS AND METHODS

Study design and participants

It was a cross-sectional study conducted on patients with IBD at the Second Affiliated Hospital of Wenzhou Medical University using convenience sampling. Our study was approved by the ethics committee of the same hospital (approval No. 2022-K-184-02). Each patient provided written informed consent before completing the survey.

Procedures

The questionnaire was designed with reference to the World Gastroenterology Organization Practice Guidelines for the Diagnosis and Management of IBD in 2010[24] and the clinical nutrition guideline for IBD by the European Society for Clinical Nutrition and Metabolism in 2016[25]. Then, the questionnaire was submitted to 5 experts for review. After the modifications based on their comments, a small-scale validation was performed (33 copies), showing a Cronbach's α of 0.854.

The final questionnaire was in Chinese patients with IBD and included four dimensions with 62 items. Among them, the socio-demographic information dimension consisted of 20 items. The knowledge, attitude, and practice dimensions consisted of 14 items each. The items in the knowledge dimension were scored 1 point for a correct answer and 0 points for a wrong or unclear answer (total score of 0-14). The options from positive to negative (*e.g.*, 4 to 0) were assigned for the attitude and practice dimension. The total scores were 0-56 for the attitude dimension and 0-56 for the practice dimension. The threshold for good knowledge, positive attitude, and proactive practice was ≥ 70.0%.

The questionnaires were administered to the participants through WeChat on the SoJump platform (https://www.wjx.cn/app/survey.aspx). A given IP address could be used to submit a questionnaire only once. All items must be answered before the submission of the questionnaire. Questionnaires that took less than 2 min to complete or with obvious filling patterns were excluded.

Statistics analysis

All analyses were performed using Stata 17.0 (Stata Corporation, College Station, TX, United States). The normal distribution of continuous data was checked using the Kolmogorov-Smirnov test. Those continuous data conforming to the normal distribution were presented as means ± SD and analyzed using Student's *t*-test (two groups) or ANOVA (more than two groups). Otherwise, they were presented as medians (ranges) and analyzed using the Wilcoxon-Mann-Whitney *U*-test (two groups) or the Kruskal-Wallis analysis of variance (more than two groups). Categorical data were displayed as numbers (percent). Pearson's correlation analysis was used to determine the pairwise correlations among KAP scores. A multivariate logistic regression analysis was performed to determine the independent factors relevant to the KAP score. Variables with *P*-values less than 0.20 in the univariate analysis were included in the multivariate logistic analysis. Two-sided *P*-values below 0.05 were regarded as statistically significant.

RESULTS

The present study included a total of 353 valid questionnaires. Most of the participants were male (63.5%), aged 20-30 (32.9%) years. The other social-demographic data are presented in Table 1. The mean knowledge, attitude, and practice scores were 10.05 ± 3.46 (possible range: 0-14), 41.58 ± 5.23(possible range: 0-56), and 44.20 ± 7.39 (possible range: 0-56), respectively, indicating good knowledge, positive attitude, and proactive practice toward IBD (Table 1).

The knowledge items with the lowest score were K2 (21.0%, "At present, and many factors such as heredity, immunity, environment, and microorganisms are involved in the pathogenesis of the disease"), K11 (42.2%, "There are no side effects under the therapy of glucocorticoids, *etc.*"), K4 (60.1%, "Extraintestinal manifestations of IBD include oral ulcers, joint injury, skin injury, eye lesions, hepatobiliary diseases, *etc.*"), and K13 (65.7%, "All patients with IBD cannot normally absorb the nutrients they intake") (Table 2). The attitude item with the lowest score was A8 ("I think that treatment can be stopped when the colonoscopy shows mucosal healing *i.e.*, complete healing of colonic erosions and ulcers") (Table 3). The practice item with the lowest score was P11 ("I will use a diet diary to identify foods that may cause discomforts such as abdominal pain or diarrhea and try to avoid them in my future diet"). In addition, 98.0% of the participants were willing to stop smoking and drinking (Table 4).

The knowledge score was found to be related to the attitude score (r = 0.371, P < 0.001) and practice (r = 0.100, P < 0.001) score, respectively. The attitude score was related to the practice score (r = 0.452, P < 0.001) (Table 5). Moreover, multivariate logistic regression analysis suggested that aged 30-40 years [odds ratio (OR) = 4.06, 95% confidence interval (CI): 1.04-15.82, P = 0.043], middle school education (OR = 3.98, 95%CI: 1.29-12.33, P = 0.017), high school/technical secondary school education (OR = 14.06, 95%CI: 3.92-50.38, P < 0.001), and junior college/bachelor's degree and above education (OR = 15.20, 95%CI: 4.15-55.650, P < 0.001) were independently linked with the knowledge score (Table 6). The knowledge score (OR = 1.23, 95%CI: 1.11-1.36, P < 0.001) was independently associated with the attitude score (Table 7). In addition, the attitude score (OR = 1.20, 95%CI: 1.11-1.30, P < 0.001) had an independent effect on the practice score (Table 8).

Table 1 Characteristics of the participants

Variables	*n* (%)	Knowledge		Attitude		Practice	
		mean ± SD	*P* value	mean ± SD	*P* value	mean ± SD	*P* value
Total	353	10.05 ± 3.46		41.58 ± 5.23		44.20 ± 7.39	
Gender			0.468		0.830		0.077
Male	224 (63.5)	9.95 ± 3.57		41.62 ± 5.02		44.72 ± 7.35	
Female	129 (36.5)	10.2 ± 3.28		41.50 ± 5.59		43.28 ± 7.40	
Age, yr (10 cases missing)			< 0.001		0.142		0.886
≤ 20	41 (11.6)	9.46 ± 3.87		40.95 ± 5.13		44.00 ± 8.15	
20-30	116 (32.9)	10.87 ± 2.94		42.41 ± 5.25		44.37 ± 7.40	
30-40	85 (24.1)	10.56 ± 3.03		41.81 ± 5.24		43.89 ± 6.92	
> 40	101 (28.6)	9.02 ± 3.91		40.88 ± 5.21		44.70 ± 7.27	
Ethnicity (1 case missing)			0.011		0.028		0.609
Han	341 (96.6)	10.13 ± 3.39		41.69 ± 5.16		44.25 ± 7.41	
Minorities	11 (3.1)	7.45 ± 4.89		38.18 ± 6.51		43.09 ± 6.85	
Residence			0.006		0.059		0.002
Rural	149 (42.2)	9.38 ± 3.72		44.18 ± 5.19		43.76 ± 7.76	
City	122 (34.6)	10.66 ± 3.05		42.48 ± 5.05		45.97 ± 6.73	
Suburb/urban-rural combination	82 (23.2)	10.35 ± 3.85		40.95 ± 5.45		42.35 ± 7.13	
Education			< 0.001		0.003		0.089
Primary school and below	25 (7.1)	6.92 ± 3.53		40.00 ± 4.97		42.88 ± 7.21	
Middle school	67 (19.0)	7.99 ± 4.17		39.99 ± 5.82		42.40 ± 8.01	
High school/technical secondary school	84 (23.8)	10.25 ± 2.88		41.42 ± 4.83		44.55 ± 6.98	
Junior college/bachelor's degree and above	177 (50.1)	11.18 ± 2.75		42.47 ± 5.05		44.89 ± 7.29	
Work status			< 0.001		0.002		0.012
Employed	185 (52.4)	10.79 ± 2.96		42.39 ± 4.90		45.13 ± 6.83	
Other	168 (47.6)	9.23 ± 3.78		40.68 ± 5.44		43.17 ± 7.85	
Monthly per capita income			< 0.001		0.003		0.074
< 5000	173 (49.0)	9.32 ± 3.87		40.61 ± 5.20		43.29 ± 7.95	
5000-10000	104 (29.5)	10.88 ± 2.52		42.37 ± 4.96		45.21 ± 6.59	
> 10000	76 (21.5)	10.55 ± 3.27		42.68 ± 5.32		44.87 ± 6.93	
Marital status			0.029		0.939		0.201
Unmarried or other	157 (44.5)	10.50 ± 3.21		41.60 ± 5.25		44.76 ± 7.40	
Married	196 (55.5)	9.69 ± 3.62		41.56 ± 5.23		43.74 ± 7.37	
Smoking habit			0.163		0.386		0.202

No (no smoking)	282 (79.9)	10.18 ± 3.33		41.45 ± 5.13		43.94 ± 7.18	
Yes (smoking or used to smoke)	71 (20.1)	9.54 ± 3.92		42.06 ± 5.63		45.20 ± 8.14	
Drinking habit			0.461		0.744		0.372
No (no drinking)	240 (68.0)	10.14 ± 3.40		41.51 ± 5.27		43.95 ± 7.36	
Yes (drinking or used to drink)	113 (32.0)	9.85 ± 3.59		41.71 ± 5.16		44.71 ± 7.47	
Medical insurance type (multiple choices)							
Basic medical insurance for urban employees	187 (53.0)	10.68 ± 3.06	< 0.001	43.62 ± 4.98	< 0.001	44.79 ± 7.14	0.108
New cooperative medical insurance	112 (31.7)	9.16 ± 4.02	0.001	54.18 ± 5.45	0.001	43.34 ± 7.88	0.138
Basic medical insurance for urban residents	62 (17.6)	9.18 ± 3.77	0.029	55.13 ± 4.87	0.460	44.56 ± 6.71	0.666
Commercial insurance	23 (6.5)	10.74 ± 3.37	0.323	56.30 ± 4.76	0.490	45.61 ± 7.24	0.343
No insurance	3 (0.8)	12.33 ± 1.15	0.252	52.00 ± 4.58	0.235	34.67 ± 9.29	0.025
Which IBD			< 0.001		0.005		0.553
Ulcerative colitis	133 (37.7)	9.16 ± 3.69		54.57 ± 5.06		43.89 ± 7.55	
Crohn's disease	220 (62.3)	10.59 ± 3.21		56.18 ± 5.25		44.38 ± 7.30	
Duration of IBD			0.995		0.948		0.248
< 1 yr	239 (67.7)	10.05 ± 3.46		55.58 ± 5.17		44.65 ± 7.36	
1-2 yr	59 (16.7)	10.08 ± 3.43		55.41 ± 5.30		43.17 ± 7.72	
> 2 yr	55 (15.6)	10.02 ± 3.56		55.73 ± 5.59		43.33 ± 7.09	
Ostomy			0.014		0.088		0.621
Yes	27 (7.6)	8.48 ± 4.37		53.93 ± 5.95		43.52 ± 10.05	
No	326 (92.4)	10.18 ± 3.53		55.71 ± 5.15		44.25 ± 7.14	
Comorbidities			0.463		0.064		0.004
Yes	59 (16.7)	9.75 ± 3.72		54.42 ± 5.11		41.71 ± 7.32	
No	294 (83.3)	10.11 ± 3.41		55.81 ± 5.23		44.64 ± 7.28	
Family history of IBD			0.588		0.991		0.392
Yes	9 (2.5)	10.67 ± 4.21		55.56 ± 4.98		42.11 ± 6.43	
No	344 (97.5)	10.03 ± 3.45		55.58 ± 5.24		44.25 ± 7.41	
Surgical history			0.340		0.487		0.894
Yes	165 (46.7)	10.24 ± 3.39		55.78 ± 5.10		44.14 ± 7.47	
No	188 (53.3)	9.88 ± 3.53		55.39 ± 5.35		44.24 ± 7.34	
History of drug allergy			0.110		0.120		0.890
Yes	48 (13.6)	10.79 ± 2.73		56.67 ± 5.15		44.33 ± 7.36	
No	305 (86.4)	9.93 ± 3.55		55.40 ± 5.23		44.17 ± 7.41	
What kind of treatment is being received?			0.040		0.004		0.276
5-aminosalicylic acid drugs (*e.g.*, mesalazine)	19 (5.4)	8.63 ± 3.44		55.00 ± 4.99		45.32 ± 8.87	

Glucocorticoids	1 (0.3)	12.00	57.00	50.00
Immunosuppressants (*e.g.*, azathioprine, tacrolimus, cyclosporine, *etc.*)	6 (1.7)	6.50 ± 3.27	51.33 ± 6.38	37.83 ± 9.99
Biological agents (*e.g.*, infliximab, vedolizumab, ustekinumab)	301 (85.3)	10.16 ± 3.49	55.97 ± 5.13	44.33 ± 7.26
Biological agents + immunosuppressants	14 (4.0)	9.93 ± 3.34	51.36 ± 5.58	43.57 ± 6.73
Biological agents + 5-aminosalicylic acid drugs	12 (3.4)	11.25 ± 1.48	53.42 ± 4.36	42.50 ± 6.99

IBD: Inflammatory bowel disease.

Table 2 Knowledge dimension, *n* (%)

Knowledge	Correct	Wrong	Unclear
IBD is a group of chronic, non-specific recurrent intestinal inflammatory diseases, including UC and CD	316 (89.5)	2 (0.6)	35 (9.9)
At present, many factors, such as heredity, immunity, environment, and microorganisms, are involved in the pathogenesis of the disease	74 (21.0)	185 (52.4)	94 (26.6)
Symptoms of IBD can include abdominal pain, diarrhea, bloody stool, anemia, fever, joint swelling, pain, *etc.*	293 (83.0)	13 (3.7)	47 (13.3)
Extraintestinal manifestations of IBD include oral ulcers, joint injury, skin injury, eye lesions, hepatobiliary diseases, *etc.*	212 (60.1)	32 (9.1)	109 (30.9)
IBD often occurs in young adults and is more common between the ages of 20-50 yr	267 (75.6)	16 (4.5)	70 (19.8)
IBD is a lifelong disease, and the patient's condition is prolonged and repeated. At present, there is no specific and effective medicine or method to cure the disease	293 (83.0)	11 (3.1)	49 (13.9)
Colonoscopy and mucosal biopsy are the best methods to establish the diagnosis and assess the disease's severity in patients with IBD	285 (80.7)	6 (1.7)	62 (17.6)
Generally, medical treatment is the main treatment for IBD, but surgical treatment is needed when intestinal obstruction, intestinal perforation, and canceration occur	275 (77.9)	5 (1.4)	73 (20.7)
The treatment of patients with IBD varies widely among individuals, with different classifications and severity of the disease leading to different treatment outcomes and efficacy	299 (84.7)	1 (0.3)	53 (15.0)
Drugs for treating IBD include hormones, aminosalicylic acid drugs, immunosuppressants (azathioprine, methotrexate, *etc.*), and biological agents	274 (77.6)	5 (1.4)	74 (21.0)
There are no side effects after treatment with glucocorticoids, *etc.*	149 (42.2)	39 (11.0)	165 (46.7)
Currently, the biological agents approved for treating IBD in China include infliximab, vidrizumab, and ustekinumab	292 (82.7)	6 (1.7)	55 (15.6)
All patients with IBD can't normally absorb the nutrients they intake	232 (65.7)	60 (17.0)	61 (17.3)
Emotion, smoking, drinking, and other behaviors will not affect IBD	286 (81.0)	36 (10.2)	31 (8.8)

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease.

DISCUSSION

The findings of our study suggested that Chinese patients with IBD had good knowledge, positive attitudes, and proactive practice toward their disease. Nevertheless, some specific items warranting more education were identified. These outcomes may be useful for the management and self-management of IBD patients in clinical practice.

Several studies revealed misconceptions and relatively poor knowledge in patients with IBD about their disease[16-21]. A study from England published 30 years ago already acknowledged that patients with IBD had poor knowledge regarding their disease but were willing to acquire information[16]. More contemporary data indicated little progress since then, *i.e.*, that the knowledge of patients with IBD toward their disease was poor[17-21], including in New Zealand [17], Canada[18], Israel[19], Poland[20], and South Korea[21]. Surprisingly, in the present study, the patients with IBD showed good KAP toward IBD, but it could be noted that most participants had a junior college/bachelor's degree and above education and were receiving expensive biological agents, thereby suggesting a higher socioeconomic status that could influence the results.

Table 3 Attitude dimension, *n* (%)

Attitude	Strongly agree	Agree	Neutral	Disagree	Strongly disagree
I am confident in the treatment of IBD	158 (44.8)	134 (38.0)	58 (16.4)	3 (0.8)	0
I think patients with IBD need to avoid certain foods	186 (52.7)	144 (40.8)	22 (6.2)	1 (0.3)	0
I think that patients with IBD combined with malnutrition need to use a combination of intestinal and extra-intestinal nutrition support according to the disease situation if necessary	171 (48.4)	151 (42.8)	29 (8.2)	1 (0.3)	1 (0.3)
I think scientific dietary guidance and management are key to managing IBD	193 (54.7)	141 (39.9)	18 (5.1)	1 (0.3)	0
I think developing a specific treatment plan for IBD needs to be tailored to the individual's situation and developed jointly by the IBD medical specialist and the patient	208 (58.9)	129 (36.5)	15 (4.2)	1 (0.3)	0
I think the adjustment of IBD medication needs to be carried out under the guidance of specialists, and patients should not adjust their own medication	228 (64.6)	112 (31.7)	13 (3.7)	0	0
I believe that during IBD medication, patients need to monitor the side effects of their medication and provide timely feedback to their specialists	216 (61.2)	128 (36.3)	9 (2.5)	0	0
I think treatment can be stopped when the colonoscopy shows mucosal healing (*i.e.*, complete healing of colonic erosions and ulcers)	37 (10.5)	47 (13.3)	92 (26.1)	134 (38.0)	43 (12.2)
I think the early application of biologics, in conjunction with specialist advice, will allow early control of disease activity to change the course of the disease and minimize complications and disability in the bowel	171 (48.4)	147 (41.6)	30 (8.5)	3 (0.8)	2 (0.6)
I think patients with IBD should reduce their intake of saturated fatty acids (animal oil, cream, fatty meats, meat soups, *etc.*)	137 (38.8)	156 (44.2)	52 (14.7)	7 (2.0)	1 (0.3)
I think that IBD patients should try to drink plain hot water and freshly squeezed juices and need to avoid strong tea, sugary drinks, coffee, alcohol, *etc.*	177 (50.1)	148 (41.9)	24 (6.8)	2 (0.6)	2 (0.6)
I think the IBD disease has obviously increased the family's financial burden	172 (48.7)	133 (37.7)	42 (11.9)	5 (1.4)	1 (0.3)
I think I can get married, get pregnant, and give birth normally if my IBD disease is controlled	125 (35.4)	166 (47.0)	52 (14.7)	8 (2.3)	2 (0.6)
I think IBD has affected my normal work, study, and social interaction	98 (27.8)	134 (38.0)	86 (24.4)	30 (8.5)	5 (1.4)

IBD: Inflammatory bowel disease.

The present investigation also demonstrated that age and educational attainment were independently associated with knowledge scores. Specific knowledge items that need improvement include the etiology of IBDs, the possible extra-intestinal manifestations of IBDs, the side effects of glucocorticoids, and nutrient absorption. Even though the other knowledge items had high scores, none scored above 90%, indicating they would benefit from additional instruction. Furthermore, knowledge was the only factor independently associated with the attitude score, and attitude was the only factor independently connected with the practice score. Hence, improving the knowledge of patient about IBDs should enhance their KAP. Since a proper KAP of IBDs has been associated with better IBD outcomes[15], improving the KAP can improve patient outcomes, given that self-management is at the core of IBD management[12,13].

Still, patients obtain knowledge primarily from available resources (books, the internet, newspapers, *etc.*), their social network, and healthcare professionals. A study highlighted variable access to high-quality information on IBD-related nutrition[26], and nutrition is a major factor influencing the intestinal microflora and the outcomes of IBDs[27-29]. Furthermore, a study in New Zealand showed that the KAP of IBD in the general population was also low[30,31], suggesting that patient education is deficient or ineffective since the patients with IBD have poor KAP. Healthcare providers are the primary source of reliable patient information, but studies unveiled that the KAP of IBD among healthcare providers was also low[32,33]. Having limited knowledge about a disease can impede the spread of accurate information. Therefore, previous studies suggest that priority should be placed on educating patients, healthcare professionals, and the general public.

There are some limitations in this study. It was conducted at a single institution, limiting its applicability to other hospitals in China. The questionnaire was designed by local investigators and was probably influenced by local policies and guidelines, further restricting the exportability of the questionnaire. The study has local scope, and the results cannot be extrapolated to other populations, which makes similar studies necessary in other locations. The study population shows high education and use of biological products, which suggests a selection bias. KAP surveys represent the situation of a specific population at a precise time point. Therefore, studies in other populations and time might be necessary to examine the actual KAP situation in China and the effect of education. Finally, all KAP surveys were susceptible to

Table 4 Practice dimension, *n* (%)

Practice	Always	Often	Sometimes	Seldom	Never
I will actively cooperate with the medical staff for my treatment and nursing	244 (69.1)	93 (26.3)	15 (4.2)	1 (0.3)	0
I will communicate with specialists regularly and follow up regularly	166 (47.0)	116 (32.9)	62 (17.6)	9 (2.5)	0
I will vent my bad emotions correctly, such as through exercise relaxation, music relaxation, and implied adjustment, to relieve mental stress	106 (30.0)	125 (35.4)	92 (26.1)	25 (7.1)	5 (1.4)
I will communicate with family members, close friends, and patients and gain encouragement and emotional support	94 (26.6)	117 (33.1)	92 (26.1)	41 (11.6)	9 (2.5)
	Yes, *n* (%)		No, *n* (%)		
I will take care to quit smoking and drinking	346 (98.0)		7 (2.0)		
I will take care to avoid staying up late and overworking	120 (34.0)	119 (33.7)	87 (24.6)	23 (6.5)	4 (1.1)
I will take care to choose appropriate physical exercise according to my physical condition	96 (27.2)	95 (26.9)	105 (29.7)	50 (14.2)	7 (2.0)
If there is an ostomy, I will go to an IBD specialist for standard treatment	336 (95.2)	17 (4.8)	0	0	0
If I am treated with biological agents, I will pay attention to monitoring the related side effects	179 (50.7)	107 (30.3)	52 (14.7)	11 (3.1)	4 (1.1)
If a food allergy is identified, I will take care to avoid it in my daily diet	210 (59.5)	105 (29.7)	30 (8.5)	5 (1.4)	3 (0.8)
I will use a "diet diary" to identify foods that may cause discomfort, such as abdominal pain or diarrhea, and try to avoid them in my future diet	106 (30.0)	94 (26.6)	67 (19.0)	42 (11.9)	44 (12.5)
I will improve my understanding of diseases and treatment through WeChat groups, networks, and popular science lectures	100 (28.3)	85 (24.1)	104 (29.5)	52 (14.7)	12 (3.4)
I will insist on taking medicine or receiving infusion treatment of biological agents as prescribed by my physician	247 (70.0)	86 (24.4)	16 (4.5)	3 (0.8)	1 (0.3)
I will encourage and help other people with IBD as much as I can	135 (38.2)	78 (22.1)	93 (26.3)	37 (10.5)	10 (2.8)

IBD: Inflammatory bowel disease.

Table 5 Correlation analysis

	Knowledge dimension	Attitude	Practice
Knowledge dimension	1		
Attitude	0.371 ($P < 0.001$)	1	
Practice	0.100 ($P < 0.001$)	0.452 ($P < 0.001$)	1

Table 6 Univariate and multivariate analysis of knowledge

Variables	Univariate analysis		Multivariate analysis	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Gender				
Male	1.09 (0.62-1.92)	0.751		
Female	Ref			
Age				
≤ 20	Ref		Ref	
20-30	2.80 (1.10-7.09)	0.030	3.01 (0.97-9.38)	0.057
30-40	2.17 (0.84-5.63)	0.111	4.06 (1.04-15.82)	0.043
> 40	0.76 (0.33-1.75)			

Ethnicity				
Han	3.83 (1.13-12.94)	0.031	3.70 (0.80-16.97)	0.093
Minorities	Ref		Ref	
Residence				
Rural	Ref		Ref	
City	3.08 (1.56-6.07)	0.001	1.69 (0.74-3.84)	0.213
Suburb/urban-rural combination	1.95 (0.97-3.90)	0.060	1.33 (0.58-3.03)	0.496
Education				
Primary school and below	Ref			
Middle school	2.99 (1.15-7.76)	0.025	3.98 (1.29-12.33)	0.017
High school/technical secondary school	11.80 (4.20-33.17)	< 0.001	14.06 (3.92-50.38)	< 0.001
Junior college/bachelor's degree and above	20.70 (7.75-55.28)	< 0.001	15.20 (4.15-55.65)	< 0.001
Work status				
Employed	4.07 (2.23-7.41)	< 0.001	1.34 (0.63-2.85)	0.444
Other	Ref		Ref	
Monthly per capita income				
< 5000	Ref		Ref	
5000-10000	3.94 (1.84-8.43)	< 0.001	2.04 (0.81-5.18)	0.133
> 10000	2.46 (1.17-5.18)	0.018	0.90 (0.34-2.36)	0.823
Marital status				
Unmarried or other	Ref		Ref	
Married	0.61 (0.35-1.07)	0.083	0.85 (0.33-2.16)	0.734
Smoking habit				
No (no smoking)	Ref			
Yes (smoking or used to smoke)	0.60 (0.33-1.12)	0.10		
Drinking habit				
No (no drinking)	Ref			
Yes (drinking or used to drink)	0.85 (0.49-1.50)	0.584		
What kind of IBD is being diagnosed				
Ulcerative colitis	0.50 (0.29-0.85)	0.011	0.57 (0.28-1.18)	0.132
Crohn's disease	Ref		Ref	
Duration of IBD				
< 1 yr	1.14 (0.54-2.39)	0.729		
1-2 yr	0.98 (0.39-2.45)	0.964		
> 2 yr	Ref			
Ostomy?				
Yes	0.51 (0.21-1.23)	0.135		
No	Ref			
Comorbidities				
Yes	0.62 (0.32-1.19)	0.149		
None	Ref			
Family history of IBD				
Yes	1.86 (0.23-15.16)	0.560		

No	Ref	
Surgical history		
Yes	1.15 (0.67-1.97)	0.613
No	Ref	
History of drug allergy		
Yes	1.40 (0.60-3.29)	0.433
No	Ref	
What kind of treatment is being received?		
5-aminosalicylic acid drugs (*e.g.*, mesalazine)	Ref	
Glucocorticoids	-	-
Immunosuppressants (*e.g.*, azathioprine, tacrolimus, cyclosporine, *etc.*)	0.46 (0.07-2.99)	0.418
Biological agents (*e.g.*, infliximab, vedolizumab, ustekinumab)	2.11 (0.77-5.80)	0.148
Biological agents + immunosuppressants	1.69 (0.34-8.40)	0.520
Biological agents + 5-aminosalicylic acid drugs	-	-

IBD: Inflammatory bowel disease.

Table 7 Univariate and multivariate analysis of attitude

Variables	Univariate analysis		Multivariate analysis	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Knowledge score (as continuous variables)	1.24 (1.14-1.34)	< 0.001	1.23 (1.11-1.36)	< 0.001
Gender				
Male	1.42 (0.73-2.74)	0.300		
Female	Ref			
Age				
≤ 20	Ref			
20-30	1.49 (0.52-4.25)	0.461		
30-40	1.29 (0.43-3.82)	0.651		
> 40	1.27 (0.44-3.65)	0.656		
Ethnicity				
Han	4.69 (1.31-16.80)	0.017	3.21 (0.66-15.59)	0.149
Minorities	Ref		Ref	
Residence				
Rural	Ref		Ref	
City	2.47 (1.06-5.76)	0.037	1.63 (0.61 4.32)	0.329
Suburb/urban-rural combination	1.12 (0.51-2.44)	0.779	0.98 (0.39-2.47)	0.968
Education				
Primary school and below	Ref		Ref	
Middle school	0.95 (0.30-2.97)	0.925	0.85 (0.23-3.08)	0.803
High school/technical secondary school	2.37 (0.70-8.05)	0.165	0.87 (0.22-3.49)	0.841
Junior college/bachelor's degree and above	2.91 (0.95-8.94)	0.062	0.82 (0.20-3.26)	0.774
Work status				
Employed	2.65 (1.32-5.30)	0.006	1.54 (0.64-3.70)	0.338

Other	Ref		Ref	
Monthly per capita income				
< 5000	Ref		Ref	
5000-10000	2.22 (0.97-5.09)	0.060	1.10 (0.42-2.89)	0.850
> 10000	2.16 (0.85-5.46)	0.105	1.06 (0.35-3.17)	0.924
Marital status				
Unmarried or other	Ref			
Married	0.97 (0.51-1.88)	0.937		
Smoking habit				
No (no smoking)	Ref			
Yes (smoking or used to smoke)	0.88 (0.40-1.94)	0.755		
Drinking habit				
No (no drinking)	Ref			
Yes (drinking or used to drink)	1.16 (0.57-2.36)	0.689		
What kind of IBD is being diagnosed				
Ulcerative colitis	0.75 (0.39-1.44)	0.383		
Crohn's disease	Ref			
Duration of IBD				
< 1 yr	0.92 (0.36-2.35)	0.866		
1-2 yr	0.91 (0.29-2.90)	0.873		
> 2 yr	Ref			
Ostomy?				
Yes	0.55 (0.19-1.53)	0.250		
No	Ref			
Comorbidities				
Yes	0.68 (0.31-1.51)	0.341		
None	Ref			
Family history of IBD				
Yes	1.05 (0.13-8.64)	0.962		
No	Ref			
Surgical history				
Yes	1.14 (0.59-2.19)	0.698		
No	Ref			
History of drug allergy				
Yes	1.52 (0.52-4.47)	0.448		
No	Ref			
What kind of treatment is being received?				
5-aminosalicylic acid drugs (*e.g.*, mesalazine)	Ref		Ref	
Glucocorticoids	-	-	-	-
Immunosuppressants (*e.g.*, azathioprine, tacrolimus, cyclosporine, *etc.*)	0.24 (0.02-2.22)	0.206	0.38 (0.03-4.43)	0.438
Biological agents (*e.g.*, infliximab, vedolizumab, ustekinumab)	1.10 (0.24-5.02)	0.899	0.88 (0.17-4.53)	0.878
Biological agents + immunosuppressants	0.21 (0.03-1.32)	0.096	0.16 (0.02-1.17)	0.070
Biological agents + 5-aminosalicylic acid drugs	0.35 (0.05-2.51)	0.298	0.17 (0.02-1.51)	0.113

IBD: Inflammatory bowel disease.

Table 8 Univariate and multivariate analysis of practice

Variables	Univariate analysis		Multivariate analysis	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Knowledge score (as continuous variables)	1.10 (1.02-1.19)	0.020	0.96 (0.87-1.06)	0.412
Attitude score (as continuous variables)	1.21 (1.13-1.30)	< 0.001	1.20 (1.11-1.30)	< 0.001
Gender				
Male	1.62 (0.86-3.04)	0.134		
Female	Ref			
Age				
≤ 20	Ref			
20-30	1.25 (0.45-3.50)	0.672		
30-40	1.65 (0.53-5.11)	0.386		
> 40	1.16 (0.41-3.30)	0.780		
Ethnicity				
Han	0.65 (0.14-3.11)	0.589		
Minorities	Ref			
Residence				
Rural	Ref		Ref	
City	2.41 (1.08-5.40)	0.033	2.01 (0.80-5.04)	0.139
Suburb/urban-rural combination	1.12 (0.53-2.38)	0.768	1.12 (0.49-2.58)	0.788
Education				
Primary school and below	Ref			
Middle school	0.66 (0.20-2.22)	0.503		
High school/technical secondary school	2.10 (0.56-7.84)	0.272		
Junior college/bachelor's degree and above	1.58 (0.49-5.11)	0.441		
Work status				
Employed	2.76 (1.41-5.40)	0.003	1.93 (0.88-4.21)	0.099
Other	Ref		Ref	
Monthly per capita income				
< 5000	Ref		Ref	
5000-10000	2.42 (1.06-5.51)	0.036	1.31 (0.51-3.33)	0.578
> 10000	1.71 (0.74-3.94)	0.207	0.86 (0.32-2.28)	0.755
Marital status				
Unmarried or other	Ref			
Married	0.65 (0.34-1.25)	0.200		
Smoking habit				
No (no smoking)	Ref			
Yes (smoking or used to smoke)	0.86 (0.41-1.84)	0.706		
Drinking habit				
No (no drinking)	Ref			

Yes (drinking or used to drink)	1.18 (0.60-2.35)	0.631		
What kind of IBD is being diagnosed				
Ulcerative colitis	0.80 (0.43-1.52)	0.501		
Crohn's disease	Ref			
Duration of IBD				
< 1 yr	1.39 (0.59-3.27)	0.445		
1-2 yr	0.74 (0.27-2.01)	0.558		
> 2 yr	Ref			
Ostomy				
Yes	0.62 (0.22-1.72)	0.354		
No	Ref			
Comorbidities				
Yes	0.43 (0.21-0.88)	0.022	0.50 (0.23-1.09)	0.082
None	Ref		Ref	
Family history of IBD				
Yes	-	-		
No	Ref			
Surgical history				
Yes	1.11 (0.59-2.09)	0.741		
No	Ref			
History of drug allergy				
Yes	0.83 (0.35-1.99)	0.682		
No	Ref			
What kind of treatment is being received?				
5-aminosalicylic acid drugs (*e.g.*, mesalazine)	Ref			
Glucocorticoids	-	-		
Immunosuppressants (*e.g.*, azathioprine, tacrolimus, cyclosporine, *etc.*)	0.19 (0.02-1.41)	0.104		
Biological agents (*e.g.*, infliximab, vedolizumab, ustekinumab)	1.38 (0.38-4.97)	0.622		
Biological agents + immunosuppressants	2.44 (0.23-26.30)	0.463		
Biological agents + 5-aminosalicylic acid drugs	0.94 (0.13-6.63)	0.948		

IBD: Inflammatory bowel disease.

social desirability bias, in which participants may have been more likely to provide the expected response than the actual answer[34].

CONCLUSION

In conclusion, this study suggests that Chinese patients with IBD have good knowledge, positive attitudes, and active practice toward their disease. Nevertheless, some specific items warranting more education were identified, especially regarding the etiology and contributing factors to the disease, extraintestinal manifestations, glucocorticoid side effects, and nutrient absorption.

ARTICLE HIGHLIGHTS

Research background

The management of inflammatory bowel disease (IBD) necessitates the adoption of healthy lifestyle habits, which requires proper knowledge, attitudes, and practice of the specific lifestyle routines to implement. However, patients with IBD generally have poor knowledge, attitude, and practice (KAP) of their disease, while the data from China are lacking.

Research motivation

The motivation of this study is to help healthcare providers to improve the patient's self-management of IBD.

Research objectives

The object of this study is to investigate the KAP of patients with IBD toward their disease in Zhejiang Province, China.

Research methods

Self-designed questionnaires were administered to the participants through WeChat on the SoJump platform (https://www.wjx.cn/app/survey.aspx). Pearson's correlation analysis was used to determine the pairwise correlations among KAP scores. A multivariate logistic regression analysis was further performed to determine the independent factors associated with their KAP scores.

Research results

A total of 353 patients (224 males) with IBD completed the questionnaires. Their mean KAP scores were 10.05 ± 3.46 (possible range: 0-14), 41.58 ± 5.23 (possible range: 0-56), 44.20 ± 7.39 (possible range: 0-56), respectively, indicating good knowledge, positive attitude, and proactive practice toward IBD. Age and education were independently associated with their KAP.

Research conclusions

Chinese patients with IBD might have good knowledge, a positive attitude, and proactive practice toward their disease. Nevertheless, some specific items warranting more education were identified, especially regarding the etiology and contributing factors to the disease, extraintestinal manifestations, glucocorticoid side effects, and nutrient absorption.

Research perspectives

The findings of this study may be useful for the management and self-management of IBD patients in clinical practice.

FOOTNOTES

Author contributions: Fang LY and Guo XR carried out the study and participated in collecting data; Shao XX drafted the manuscript; Wang WZ and Shi RX performed the statistical analysis and participated in its design; Lin DP participated in the acquisition, analysis, and interpretation of data; and all authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Observational Study

Helicobacter pylori infection in Xinjiang Uyghur Autonomous Region: Prevalence and analysis of related factors

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Abstract

BACKGROUND

^{14}C urea breath test (^{14}C UBT) and immunohistochemical staining (IHC) are widely used for detection *Helicobacter pylori* (*H. pylori*) infection with different sensitivity, and there is a difference in *H. pylori* infection rate in Uyghur and Han ethnic groups. Both need large cohort studies to evaluate the differences more accurately.

AIM

To analyze the difference between ^{14}C UBT and IHC for *H. pylori* detection in Xinjiang Uyghur Autonomous Region and the difference between Uyghur and Han populations.

METHODS

There were 3944 cases of *H. pylori* infection detected by both IHC and ^{14}C UBT at the same time (interval < 1 wk, with sampling site including gastric antrum, selected from 5747 patients). We compared the sensitivity of ^{14}C UBT and IHC. We

also compared 555 pairs of Han/Uyghur cases (completely matched for gender and age) for their *H. pylori* infection rates. The overall *H. pylori* infection rate of all 5747 cases and the correlation with other clinicopathological data were also further analyzed. SPSS V23.0 software was used for statistical analysis.

RESULTS

The sensitivity was 94.9% for ^{14}C UBT and 65.1% for IHC, which was a significant difference (n = 3944, P < 0.001). However, among those cases negative for *H. pylori* by ^{14}C UBT (detection value ≤ 100), 4.8% were positive by IHC. Combining both methods, the overall *H. pylori* infection rate was 48.6% (n = 5747), and differences in gender, age group, ethnicity and region of residence significantly affected the *H. pylori* positive rates. According to age group (Han/Uyghur), the positive rates were ≤ 30 years (62.2%/100.0%), 31-40 years (45.2%/85.7%), 41-50 years (47.2%/79.2%), 51-60 years (44.6%/76.1%), 61-70 years (40.9%/68.2%), 71-80 years (41.7%/54.1%) and ≥ 81 years (42.9%/NA). The *H. pylori* infection rates of Han/Uyghur paired cases were 41.4% and 73.3%, which was a significant difference (P < 0.001) (555 pairs). *H. pylori* positivity was significantly related to moderate-severe grade 2-3 chronic/active gastritis and intestinal metaplasia (all P < 0.05).

CONCLUSION

The sensitivity of ^{14}C UBT was significantly higher, but combined application can still increase the accuracy. The prevention *H. pylori* should be emphasized for Uygur and young people.

Key Words: *Helicobacter pylori*; Immunohistochemistry; ^{14}C urea breath test; Han; Uyghur; Xinjiang Uyghur Autonomous Region

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Core Tip: The sensitivity of ^{14}C urea breath test (^{14}C UBT) for detecting *Helicobacter pylori* (*H. pylori*) is significantly higher than that of immunohistochemistry (IHC) with endoscopy specimens. Combination of ^{14}C UBT and IHC is necessary to improve detection accuracy, and increasing the number of biopsy specimens (≥ 2) can improve the positive rate significantly. The overall rate of *H. pylori* infection in Xinjiang Uyghur Autonomous Region was higher than in previous studies. *H. pylori* infection was more prevalent in the Uyghur population and the infection rate decreased as age increased. Therefore, the prevention and intervention of *H. pylori* infection in Xinjiang Uyghur Autonomous Region should emphasize Uyghur and young people.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium that mainly parasitizes the oral cavity, stomach, and duodenum after infection[1]. It was first isolated and cultured from patients with gastritis by Warren and Marshall[2] in Australia. It was extensively studied and reported that the vast majority of gastric and duodenal ulcers are associated with *H. pylori* infection, which is also associated with gastric cancer. *H. pylori* has been classified by World Health Organization as a class I carcinogen[3]. Diseases related to *H. pylori* are infectious as emphasized by the Kyoto Consensus [4]. It is known that transmission pathway of *H. pylori* includes fecal-oral and oral-oral transmission[1,5].

The Xinjiang Uyghur Autonomous Region of China has a vast territory and is inhabited by multiple ethnic groups; the main two being Han and Uyghur. Due to the differences in climate, lifestyle, dietary habits, and medical and health conditions, there are differences in *H. pylori* infection status. Although there are some previous reports from Chinese journals, the studies involved small series or their detection methods were not sufficiently sensitive[6-9].

The most important way to more accurately investigate the infection rate of *H. pylori* is to use a more accurate method of detection. Clinical *H. pylori* testing is divided into noninvasive and invasive types[10-12]. The noninvasive tests are mainly carried out in the endoscopy rooms or laboratories of various medical institutions, and the highly recommended method is the ^{14}C urea breath test (^{14}C UBT) test. For the invasive tests, detection of *H. pylori* is through gastroscopic biopsy of pathological tissue. Previous studies have shown that immunohistochemical staining to detect *H. pylori* infection has the highest sensitivity and specificity among all histopathological methods for endoscopic biopsies[13-15]. At present, the consensus is that both immunohistochemistry (IHC) and ^{14}C UBT detection of *H. pylori* infection can be used for diagnosing *H. pylori* infection[16-18]. However, there is controversy in the literature about the sensitivity of IHC and ^{14}C UBT. One study (n = 150) reported that UBT was more sensitive[19] but another study (n = 239) reported that IHC had higher sensitivity[20]. Larger studies are needed to compare the differences between ^{14}C UBT and IHC for detection

of *H. pylori* infection.

Thus, we retrospectively studied a large series of patients who underwent both ^{14}C UBT and IHC and on histopathological diagnosed gastroscopy specimens between January 2019 and February 2021 and through gastroscopy at our hospital in Urumqi. We aimed to investigate the differences between the two detection methods; evaluate *H. pylori* infection rate in Xinjiang Uyghur Autonomous Region; and establish whether there were differences in *H. pylori* infection due to factors such as age, gender, region, and especially, ethnic group (Uyghur and Han). It is hoped that our study might provide more accurate data about the difference in the two widely used *H. pylori* detection methods, and would be useful for the detection, prevention and intervention of *H. pylori* infection in Xinjiang Uyghur Autonomous Region.

MATERIALS AND METHODS

Patients and data collection criteria

A retrospective control study was conducted among patients who went to the Traditional Chinese Medical Hospital of Xinjiang Medical University between March 2019 and February 2021 due to gastrointestinal discomfort for which they underwent pathological examination of gastric biopsy tissue. The screening criteria were as follows: (1) All enrolled patients also underwent ^{14}C UBT; (2) No history of medication (such as antibiotics, proton pump inhibitors, and bismuth-based agents) that affected *H. pylori* activity, or a positive detection rate within 1 mo before the examination; and (3) Complete clinical information. This study met the ethical requirements of the Ethics Committee of Traditional Chinese Medical Hospital of Xinjiang Medical University.

Pathological features

H. pylori infection was detected using hematoxylin and eosin (H&E) staining, IHC staining, and ^{14}C UBT.

H&E staining of paraffin-embedded tissue

All specimens were fixed with 10% neutral formaldehyde solution, routinely dehydrated, embedded, and cut into 4-5 μm sections. We used an automated staining machine (LEICA Company, AUTOSTAINER) for H&E staining.

IHC staining

IHC staining was performed using a BENCHMARK-XT automatic staining machine (Roche) with *H. pylori* antibody working solution (MX104 mouse antihuman monoclonal antibody; Fuzhou Maixin Company), and the positive signal was stained with DAB. Both positive and negative controls were established. The positive control was confirmed to be *H. pylori* positive gastric biopsy tissue with plenty of *H. pylori* (staining > 100 bacterial bodies/*H. pylori*, F). The negative control used a blank antibody solution instead of the first antibody working solution.

Interpretation of pathological results

H&E staining: Pathological diagnosis and gastritis grading (including grading of chronic gastritis, active gastritis, atrophy, and intestinal metaplasia). Classification: Grade 0 (none), grade 1 (mild), grade 2 (moderate), and grade 3 (severe), and statistical analysis was conducted in sequence.

IHC staining sections of H. pylori

Positive/negative results were based on the presence or absence of *H. pylori* bacteria under the microscope. All sections were independently reviewed by two senior pathologists, and for those cases with inconsistency, a third senior pathologist provided the final interpretation.

^{14}C UBT

Patients were tested on an empty stomach or fasting for > 2 h, and the test was positive if ≥ 100 dpm/mmol, and negative if 0-100 dpm/mmol. The ^{14}C urea capsule and related reagents, as well as the *H. pylori* detector, were all provided by Shenzhen Zhonghe Headway Bio-Sci & Tech Co. Ltd.

Criteria for determining positive H. pylori infection

At least one positive result from ^{14}C UBT and IHC is considered as a positive *H. pylori* infection. In the literature, both are the preferred methods for noninvasive and invasive detection; therefore, a positive result with either one can directly diagnose *H. pylori* infection. Both methods have high specificity (default close to 100%). Comparison of the two detection methods in this study focused on the sensitivity aspect. Sensitivity was calculated as: sensitivity = number of true positive cases/(number of true positive cases + number of false negative cases) × 100%.

Grouping and matching and statistical analysis

In order to accurately compare the results of ^{14}C UBT and IHC, it is required that: (1) Gastroscopic pathological examination (H&E and IHC), ^{14}C UBT and the interval between pathological examinations should be < 1 wk; (2) The sampling sites should include the gastric antrum; and (3) The number of samples taken by clinicians should be recorded as it may affect the detection results. A total of 3944 eligible cases were selected from all cases undergoing both ^{14}C UBT and IHC.

We speculated that ethnic differences would be the main factor affecting *H. pylori* infection. We used pathological IHC and ^{14}C UBT, with Uyghur as the case group and Han as the control group, and matched them 1:1. The matching variables included gender and age. A total of 555 fully matched Uyghur and Han cases were obtained, including 249 males and 306 females (male to female ratio of approximately 1:1.23), aged 24-80 years (average 54.1 years, median 48 years), to further investigate the exact impact of ethnic differences on *H. pylori* infection. SPSS version 23.0 was used for statistical analysis and comparison (using χ^2 tests), and $P < 0.05$ represented a statistically significant difference.

RESULTS

Clinical features

A total of 5747 cases were included. Overall, the male to female ratio was 1:1.15 (2672/3075). Age ranged from 19 to 92 years, with a median of 56 years. There were 4925 cases of Han and 822 cases of Uyghur. Divided by the Tianshan Mountains, there were 4919 cases residing in Northern Xinjiang, 508 in Southern Xinjiang, 265 in the Xinjiang Production and Construction Corps (XPCC), and 55 in areas outside Xinjiang Uyghur Autonomous Region. The case information is detailed in Table 1.

Comparative analysis of IHC and ^{14}C UBT for detecting H. pylori infection

For the typical *H. pylori*-positive cases, which were definitively confirmed in the H&E sections (Figure 1A), spiral-shaped or curved bacteria were seen in the superficial mucus secreted from the gastric mucosa, or in the gastric glandular cavity. However, when the number of *H. pylori* was limited or their morphology was atypical (such as coccoid shapes), it was difficult to recognize them (Figures 1B and C). Using *H. pylori* antibodies and IHC can specifically label *H. pylori*. For typical positive cases, *H. pylori* uniformly appeared like small brown rods (Figure 1D). For those cases with low bacterial count, they can also be more easily identified and confirmed, which helps to prevent missed diagnosis or false negative result (Figures 1E and F).

Among the 3944 cases who underwent both IHC and ^{14}C UBT during gastroscopy (interval < 1 wk, including gastric antrum), the overall positive rate of *H. pylori* infection was 49.7% (1962/3944): 29.8% (1176/3944) of the cases were positive by both ^{14}C UBT and IHC; 2.6% (101/3944) were solely positive for IHC; 17.4% (685/3944) were solely positive for ^{14}C UBT; and 50.3% (1982/3944) were negative. According to the previously established standards, the sensitivity of ^{14}C UBT detection was 94.9% (1861/1962), and the sensitivity of IHC detection was 65.1% (1277/1962). Pearson's χ^2 test confirmed that there was a significant difference between the two methods ($P < 0.001$).

When the *H. pylori* positive rates detected by IHC were compared with the value ranges detected by ^{14}C UBT, it would be 4.2%,11.1%, 25.6%, 52.6%, 66.8% and 73.9% in the value ranges 0-49, 50-100, 101-199, 200-499, 500-999 and ≥ 1000, respectively. For cases negative by ^{14}C UBT, the *H. pylori* positive rate by IHC was 4.8%, while for cases defined as positive by ^{14}C UBT, the *H. pylori* positive rate by IHC was 63.2% (Tables 2 and 3).

To clarify the impact of the difference in the number of biopsies taken during gastric endoscopy on the positive rate by IHC, we divided those cases into two groups: Single biopsy (only 1 specimen) and multiple biopsies (≥ 2 specimens). Pearson's test confirmed that the positive rate for immunohistochemical detection in the single biopsy group was 28.4% (987/3476), and the positive rate in the multiple biopsy group was 40.4% (189/468). The two groups were significantly different ($P < 0.001$) by Pearson χ^2 test, and the *H. pylori*-positive rate in the multiple biopsy group was significantly increased (Table 2).

Correlation between overall infection status of H. pylori and clinical factors such as ethnicity, age, gender and region

According to the *H. pylori* positive criteria, the overall positive rate of *H. pylori* infection was 48.6% (2794/5747). The infection rate in male and female patients was 51.0% (1362/2672) and 46.6% (1432/3075), respectively, with a significant difference by χ^2 test ($P = 0.001$). The *H. pylori* infection rates in the Han and Uyghur ethnic groups were 44.2% (2175/4925) and 75.3% (619/822), respectively, with a significant difference ($P < 0.001$). Further statistical analysis showed that the *H. pylori* infection rate was 47.4% (1091/2303) in Han Chinese males and 41.3% (1084/2622) in Han females, which was a significant difference by χ^2 test ($P < 0.001$). The infection rate was 73.4% (271/369) in Uighur men and 76.8% (348/453) in Uighur women, which was not a significant difference by χ^2 test ($P = 0.291$).

When grouped according to age, the *H. pylori* infection rates in the Han ethnic group were 62.2% (23/37) in the ≤ 30 years group, 45.2% (109/241) in the 31-40 years group, 47.2% (523/1108) in the 41-50 years group, 44.6% (814/1825) in the 51-60 years group, 40.9% (488/1193) in the 61-70 years group, 41.7% (194/465) in the 71-80 years group, and 42.9% (24/56) in the ≥ 81 years group. The *H. pylori* infection rates in the Uyghur ethnic group were 100.0% (16/16) in the ≤ 30 years group, 85.7% (48/56) in the 31-40 years group, 79.2% (171/216) in the 41-50 years group, 76.1% (242/318) in the 51-60 years group, 68.2% (122/179) in the 61-70 years group, 54.1% (20/77) in the 71-80 years group, and data for the ≥ 81 years group were missing. The trend in *H. pylori*-positive rate with age group can be seen in Figure 2.

According to region of residence, the *H. pylori* infection rate was 47.4% (2333/4919) in northern Xinjiang, 66.3% (337/508) in southern Xinjiang, 37.0% (98/265) in the XPCC, and 47.3% (26/55) outside Xinjiang Uyghur Autonomous Region. There was a significant difference in *H. pylori* infection rate among the regions, and the rate in southern Xinjiang was the highest ($P < 0.001$). The *H. pylori*-positive rates of the Han and Uyghur ethnic groups in northern Xinjiang region were 44.5% (1959/4405) and 72.8% (374/514), respectively, and 47.8% (99/207) and 79.1% (238/301) in southern Xinjiang. The infection rates in the Han and Uyghur ethnic groups in the XPCC region were 36.5% (96/263) and 100.0% (2/2), and 42.0% (96/263) and 100.0% (5/5), respectively.

Table 1 The essential epidemic features of cases detected *Helicobacter pylori* with immunohistochemical staining and ^{14}C urea breath test methods (*n* = 5747)

Characteristics		Total	Han	Uyghur
Gender (cases)	Male	2672 (46.5%)	2303 (46.8%)	369 (44.9%)
	Female	3075 (35.5%)	2622 (53.2%)	453 (55.1%)
Age (yr)	Range	19-92	19-92	22-80
	Average	56.3	56.7	53.4
	Median	56.0	56.0	54.0
Residing region (cases)	Northern Xinjiang	4919 (85.6%)	4405 (89.4%)	514 (62.5%)
	Southern Xinjiang	508 (8.8%)	207 (4.2%)	301 (36.6%)
	Xinjiang Production and Construction Corps	265 (4.6%)	263 (5.3%)	2 (0.2%)
	Extra Xinjiang Uyghur Autonomous Region	55 (1.0%)	50 (1.0%)	5 (0.6%)
Total (cases)		5747	4925 (85.7%)	822 (14.3%)

Table 2 Comparison of differences between immunohistochemical staining and ^{14}C urea breath test for detecting *Helicobacter pylori* infection (*n* = 3944)

			H. pylori positive rate (%)					
			Positive[1]	Negative[1]	*P* value[1]	Positive[2]	Negative[2]	*P* value (single *vs* multiple biopsy)[2]
^{14}C UBT (total, *n* = 3944)		Positive	1176 (29.8%)	685 (17.4%)	*P* < 0.001	1962 (49.7%)	1982 (50.3%)	-
		Negative	101 (2.6%)	1982 (50.3%)				
Subgroup	Single biopsy (1 piece, *n* = 3476)	Positive	987 (28.4%)	608 (17.5%)	*P* < 0.001	1068 (30.7%)	2408 (69.3%)	*P* < 0.001
		Negative	81 (2.3%)	1800 (51.8%)				
	Multiple biopsies (≥ 2 pieces, *n* = 468)	Positive	189 (40.4%)	77 (16.5%)	*P* < 0.001	209 (44.7%)	259 (55.3%)	
		Negative	20 (4.3%)	182 (38.9%)				

[1]Immunohistochemical staining.
[2]Immunohistochemical staining & ^{14}C urea breath test combined.
^{14}C UBT: ^{14}C urea breath test; *H. pylori*: *Helicobacter pylori*.

Precise matched comparison for H. pylori infection rates between Han and Uyghur groups

For the 555 matched cases of Uyghur and Han groups, the positive rate of *H. pylori* infection in the Uyghur group was 73.3%, while the positive rate in the matched Han cases was 41.4%. The positive rates of *H. pylori* infection in the Uyghur group were higher than those in the Han group by using IHC (53.2% and 27.7%) or ^{14}C UBT (69.5% and 38.2%). These differences were significant by χ^2 test (all $P < 0.001$). In the matched cases, the infection rate of *H. pylori* in Han men was 47.4%, which was higher than that in Han women (36.6%), which was a significant difference by χ^2 test ($P = 0.012$). The infection rate in Uyghur men was 72.3%, and that of Uyghur women was 74.2%, but there was no significant difference by χ^2 test ($P = 0.630$), Table 4.

Correlation between main disease types diagnosed by gastric biopsy and H. pylori infection

The pathological changes in the H&E-stained slides of gastric mucosa were classified as follows: (1) Benign lesions: 4609 cases of gastritis; 4 with ulcer diagnosed definitively; 440 cases of gastric fundic gland polyps; 502 cases of hyperplastic (regenerative) polyps; three cases of xanthoma; and 36 cases of duodenal ectopic gastric mucosa; (2) Precancerous lesions: Nine cases of LGIN; seven cases of gastric HGIN; two cases of esophageal LGIN; two cases of esophageal HGIN; 92 cases of BE; and one case of duodenal LGIN; and (3) Tumors: 34 cases of gastric adenocarcinoma; four cases of lymphoma; one case of neuroendocrine tumor; and one case of stromal tumor.

To ensure the accuracy of *H. pylori* positive rates, we only show disease types with more than 20 samples here, and the other types are included in Table 5. The *H. pylori* positive rates obtained solely by IHC were as follows: (1) Benign lesions: Gastritis 35.8%, duodenal ectopic gastric mucosa 19.4%, gastric fundic gland polyps 17.3%, and hyperplastic

Table 3 Comparison of the differences between immunohistochemical staining and ^{14}C urea breath test and the in detecting *Helicobacter pylori* infection at different value range levels (*n* = 3944)

Qualitative results[1]	Value ranges (dpm/mmol)[1]	Positive[2]	Negative[2]	Positive ratio (%)[2]	IHC positive rate[2]
Negative (*n* = 2083)	0-49	80	1813	4.2	4.8% (101/2083)
	50-100	21	169	11.1	
Positive (*n* = 1861)	101-199	46	134	25.6	63.2% (1176/1861)
	200-499	193	174	52.6	
	500-999	324	161	66.8	
	≥ 1000	613	216	73.9	

[1]*Helicobacter pylori* of ^{14}C urea breath test.
[2]Immunohistochemical staining for *Helicobacter pylori*.
IHC: Immunohistochemistry.

Table 4 Comparison of *Helicobacter pylori* infection rates between Han and Uyghur undergoing gastroscopy examination among accurately matched pairs (*n* = 555)

Method	*H. pylori* positive rate								
	Male (*n* = 249)[1]	Female (*n* = 306)[1]	*P* value (male/female, Pearson)[1]	Subtotal (*n* = 555)[1]	Male (*n* = 249)[2]	Female (*n* = 306)[2]	*P* value (male/female, Pearson)[2]	Subtotal (*n* = 555)[2]	*P* value (total, Han and Uyghur), Pearson
IHC	89 (35.7%)	65 (21.2%)	*P* < 0.001	154 (27.7%)	134 (53.8%)	161 (52.6%)	*P* = 0.778	295 (53.2%)	*P* < 0.001
^{14}C UBT	111 (44.6%)	101 (33.0%)	*P* = 0.005	212 (38.2%)	170 (68.3%)	216 (70.0%)	*P* = 0.556	386 (69.5%)	*P* < 0.001
IHC & ^{14}C UBT	118 (47.4%)	112 (36.6%)	*P* = 0.012	230 (41.4%)	180 (72.3%)	227 (74.2%)	*P* = 0.630	407 (73.3%)	*P* < 0.001

[1]Han.
[2]Uyghur.
^{14}C UBT: ^{14}C urea breath test; *H. pylori*: *Helicobacter pylori*; IHC: Immunohistochemistry; Pearson: Pearson χ^2 test.

(regenerative) polyps 7.4%; (2) Precancerous lesions: BE 41.3%; and (3) Malignant tumors: 41.2% of gastric adenocarcinoma. If the combination of two detection methods was used, the *H. pylori* infection rate in patients with various types of diseases diagnosed by gastroscopy was higher: (1) Benign lesions: Gastritis 53.8%, gastric fundic gland polyps 27.5%, duodenal ectopic gastric mucosa 22.2%, and hyperplastic (regenerative) polyps 17.5%; (2) Precancerous lesions: *H. pylori*-positive rate of BE was 58.7% (based on gastric samples taken simultaneously, Figure 3); and (3) Malignant tumors: 67.6% gastric adenocarcinoma, Table 5.

Correlation between H. pylori infection rate and grading of chronic or active gastritis, atrophy, and intestinal metaplasia

For gastritis, 91.6% (4222/4609) of the cases showed intact structure of the mucosal lamina propria, which was evaluated and graded according to chronic gastritis, active gastritis, atrophy and intestinal metaplasia. The positive rates for *H. pylori* were as follows: (1) Chronic gastritis: *H. pylori* positive rates of grades 1-3 were 29.9%, 85.2% and 95.6%, respectively; (2) Active gastritis: The positive rates of *H. pylori* in grades 0-3 were 27.6%, 74.9%, 95.6% and 100%, respectively; (3) Atrophy: The positive rates of *H. pylori* in grades 0-3 were 54.4%, 57.3%, 65.5% and 80%, respectively; and (4) Intestinal metaplasia: The positive rates of *H. pylori* in grades 0-3 were 55.6%, 48.6%, 63.5% and 66.7%, respectively. The *H. pylori* infection rates in patients with different grades of chronic gastritis and active gastritis differed significantly (both $P < 0.001$). There was a significant difference in the *H. pylori* infection rate among patients with different grades of intestinal metaplasia, by χ^2 test ($P = 0.032$), indicating that *H. pylori* infection was associated with higher grade of chronic/active gastritis and intestinal metaplasia (Figure 4). However, there was no significant difference ($P = 0.084$ by χ^2 test) in the *H. pylori* infection rates between patients with different grades of atrophy (Table 6).

Table 5 *Helicobacter pylori* infection among main disease types diagnosed by biopsies from endoscopy in Xinjiang Uyghur Autonomous Region (*n* = 5747)

Classification of disease	Pathological diagnosis	*H. pylori* positive rate	
		IHC	IHC & ^{14}C UBT combined
Benign lesions	Gastritis	35.8% (1652/4609)	53.8% (2478/4609)
	Duodenal ectopic gastric mucosa	19.4% (7/36)	22.2% (8/36)
	Gastric fundic gland polyps	17.3% (76/440)	27.5% (121/440)
	Hyperplastic (regenerative) polyps	7.4% (37/502)	17.5% (88/502)
	Ulcer	50.0% (2/4)	100% (4/4)
	Xanthoma	66.7% (2/3)	100% (3/3)
Pre-malignant lesions	Barrett's esophagus	41.3% (38/92)	58.7% (54/92)
	Duodenal LGIN	100% (1/1)	100% (1/1)
	Gastric LGIN	55.6% (5/9)	55.6% (5/9)
	Esophageal HGIN	50.0% (1/2)	100% (2/2)
	Gastric HGIN	28.6% (2/7)	57.1% (4/7)
	Esophageal HGIN	0% (0/2)	0% (0/2)
Malignant tumors	Gastric adenocarcinoma	41.2% (14/34)	67.6% (23/34)
	Lymphoma	75.0% (3/4)	75% (3/4)
	Neuroendocrine tumor	0% (0/1)	0% (0/1)
	GIST	0% (0/1)	0% (0/1)

^{14}C UBT: ^{14}C urea breath test; HGIN: High-grade intraepithelial neoplasia; *H. pylori*: *Helicobacter pylori*; IHC: Immunohistochemistry; LGIN: Low-grade intraepithelial neoplasia; GIST: Gastrointestinal stromal tumor.

DISCUSSION

For diagnosis of *H. pylori* infection, both invasive and noninvasive tests are used. Histology with special stains, rapid urease tests (RUTs), bacterial culture, and polymerase chain reaction (PCR) are examples of invasive tests, which require endoscopy and biopsy. Although PCR-based methods were proposed as gold standard tests[10], expensive, complex, and time-consuming methods, they were not widely used for screening, and were preferred for cases when an etiological role of *H. pylori* was clinically suggested but histopathological confirmation was not possible[21]. To increase the specificity of the histological test, special stains such as modified Giemsa stain, Warthin-Starry Silver stain, and immunohistochemical stain can be used, and IHC is considered to be the most reasonable histological method[21-23]. Serology, stool antigen testing, and UBT are examples of noninvasive tests[24]. UBT is often considered as the gold standard diagnostic test for *H. pylori* infection[10], and consistently produces better results in comparison to many other available tests. IHC and ^{14}C UBT are most widely used in our hospital for routine screening of *H. pylori*. It was not clear whether we needed to perform the two methods simultaneously for *H. pylori* detection, and there were controversial reports about the sensitivity of IHC as being either superior[20] or inferior[19] to ^{14}C UBT. The number of cases of the two studies mentioned above is relatively small, thus the conclusion might have coincidence or selection bias.

The novelty of our study was that it strongly proved that the sensitivity of ^{14}C UBT was significantly higher than that of IHC. We also found about 20% inconsistency between the results of ^{14}C UBT and IHC, although most were negative for IHC and positive for ^{14}C UBT (> 17%). It is speculated that there is a significant focal distribution pattern of *H. pylori* infection in these cases[25,26], and the site and size of biopsy specimen obtained by endoscopic sampling are limited, causing false-negative results[24]. Our research showed that increasing the number of biopsy specimens ($n \geq 2$) significantly improved the positive rate of *H. pylori* detection in pathological IHC methods as expected, and the greater the number of biopsy specimens, the fewer false-negative results from sampling errors[24]. However, it might be unacceptable to do multiple biopsies for almost-healthy patients. ^{14}C UBT can overcome this drawback and reflect the overall *H. pylori* infection status in the upper gastrointestinal tract[18].

We should point out that ^{14}C UBT might be unreliable when the reported value is around the cut-off value, and these cases might be infected with a lower density of *H. pylori*[10,27]. That could explain why a small number of cases (< 3%) were negative for ^{14}C UBT but positive for IHC in our study. IHC is superior for tracing *H. pylori* infections in such circumstances, relying on both morphological review and the specificity of the *H. pylori* antibodies used for IHC[15]. The specificity of the primary antibody used in IHC prevents mistaking other resident or contaminating bacteria for *H. pylori* [13,15].

Table 6 Correlation between grading (chronic/active, atrophy, intestinal metaplasia grade) and *Helicobacter pylori* infection rate in gastritis

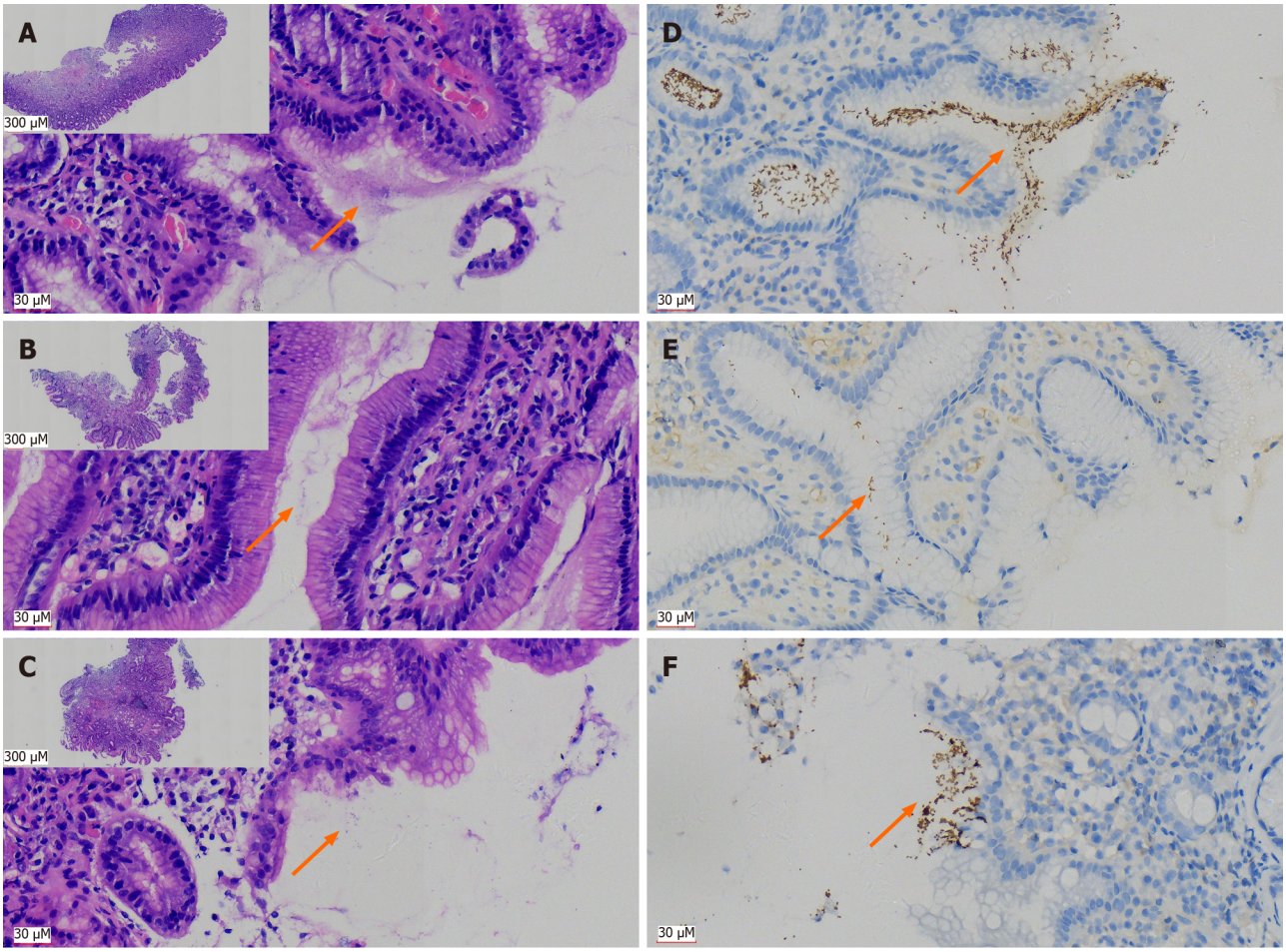
Characteristics	Grading	*H. pylori* positive rate		
		IHC	IHC & ^{14}C UBT combined[1]	*P* value
Chronic	G1	13.3% (312/2348)	29.9% (703/2348)	$P < 0.001$[2]
	G2	65.6% (1110/1692)	85.2% (1441/1692)	
	G3	81.3% (148/182)	95.6% (174/182)	
Active	G0	10.5% (229/2174)	27.6% (599/2174)	$P < 0.001$[2]
	G1	54.1% (626/1157)	74.9% (867/1157)	
	G2	80.3% (705/878)	95.6% (839/878)	
	G3	76.9% (10/13)	100% (13/13)	
Atrophy	G0	37.0% (1367/3697)	54.4% (2010/3697)	$P = 0.084$[1]
	G1	37.2% (170/457)	57.3% (262/457)	
	G2	46.6% (27/58)	65.5% (38/58)	
	G3	60.0% (6/10)	80.0% (8/10)	
Intestinal metaplasia	G0	38.8% (1235/3183)	55.6% (1771/3183)	$P = 0.032$[1]
	G1	30.9% (289/934)	48.6% (480/934)	
	G2	43.8% (42/96)	63.5% (61/96)	
	G3	44.4% (4/9)	66.7% (6/9)	

[1]Fisher's exact probability method.
[2]Pearson χ^2 test.
^{14}C UBT: ^{14}C urea breath test; G0: Grade 0 = none; G1: Grade1 = mild; G2: Grade 2 = moderate; G3: Grade 3 = severe; *H. pylori*: *Helicobacter pylori*; IHC: Immunohistochemistry.

We showed that although IHC gave direct evidence of *H. pylori* infection, its sensitivity was lower and there was a higher risk of false-negative results. ^{14}C UBT is still the preferred method for detecting *H. pylori* infection clinically. However, to improve the positive rate of *H. pylori* detection, we recommend performing ^{14}C UBT and IHC simultaneously, especially for patients unexplained gastritis, previously treated with low organism density, and with results around the cut-off value[15]. Our statistical analysis supports that the *H. pylori* positive rate can increase significantly as multiple endoscopic biopsies are submitted. One limitation of our was is that we did not perform a PCR-based method as the gold standard. However, potential pitfalls still exist with PCR methods, and more studies need to be conducted on diagnostic tests for *H. pylori* to find a reliable gold standard. Positive findings with either IHC or ^{14}C UBT are highly specific[14,28], and are widely recognized to establish diagnosis of *H. pylori* infection[18,29,30]; thus, we focused on their sensitivity.

H. pylori infection is found worldwide, but the rate varies significantly among different countries (11%-91%)[29]. China has a high incidence of *H. pylori* infection, and it has decreased to 40%-60% after economic development and changes in lifestyle[30,31]. Our study showed that the overall infection rate of *H. pylori* in a single center in Xinjiang Uyghur Autonomous Region (Urumqi) was 48.6%, which was close to the national infection rate[30,31]. However, the infection rate was significantly higher than previously reported (36.5%) in Shihezi (located adjacent to Urumqi) in the XPCC[7]. We speculate that *H. pylori* infection was detected by morphological examination without special stains, and was lower in sensitivity. The majority of the population in Shihezi area is of Han nationality. The overall infection rate of *H. pylori* in this center was also higher than the 43.6% overall positive infection rate reported by the First Affiliated Hospital of Xinjiang Medical University in the same region of Urumqi in 2012[6], which used a combination of RUT and ^{14}C UBT. Thus, the higher *H. pylori* positivity rate in our center was likely related to the higher sensitivity of our immunohistochemical assay compared to their RUT assay. Our overall infection rate was lower than that reported in Yili region[9], because the majority of cases in the other study were from multiple ethnic groups, whose *H. pylori* infection rate were all higher than in the Han group.

Xinjiang Uyghur Autonomous Region is a vast region with multiple ethnic groups living together. Previous reports have shown that there are differences in *H. pylori* infection rates between different genders, ages, ethnic groups, and regions[6-9]. Our single center data also showed that the overall infection rate in males was significantly higher than in females, which is consistent with reports in the Shihezi area[7]. However, these were inconsistent with the reports in the Ili area[9], with no significant gender difference. We believe that both our cases and the cases in Shihezi area were randomly enrolled with a large sample size, and the majority were Han ethnicity. The infection rate among Uyghur women in our study was also higher than in men, and the proportion of ethnic minorities in the study from Ili region[9] is



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Figure 1 Immunohistochemical staining using antibodies against *Helicobacter pylori* has significant advantages over hematoxylin and eosin staining in the pathological tissue for gastroscopy biopsy. A: Distribution of curved rod-shaped bacteria [hematoxylin and eosin (H&E, 400 ×)], on the surface of glands and in the mucus of typical *Helicobacter pylori* (*H. pylori*)-positive patients in H&E section; B: A case with low bacterial count of *H. pylori* (H&E, 400 ×); C: A case with atypical *H. pylori* morphology (mainly coccoid rather than rod-shaped), which was difficult to identify (H&E, 400 ×), lower magnification figures of A-C are in the upper left corner (H&E, 40 ×); D: Immunohistochemistry (IHC) with antibodies against *H. pylori* indicated a large number of bacteria, exhibiting brown yellow deep staining (DAB, high magnification, one case the same as in A); E: IHC displayed *H. pylori* bacilli more clearly than H&E due to a lower bacterial count (DAB staining, high magnification, one case the same as in B); F: Atypical coccoid *H. pylori* were clearly present and confirmed by IHC (DAB staining, high magnification, one case the same as in C); orange arrows in A-F indicate distribution of *H. pylori*.

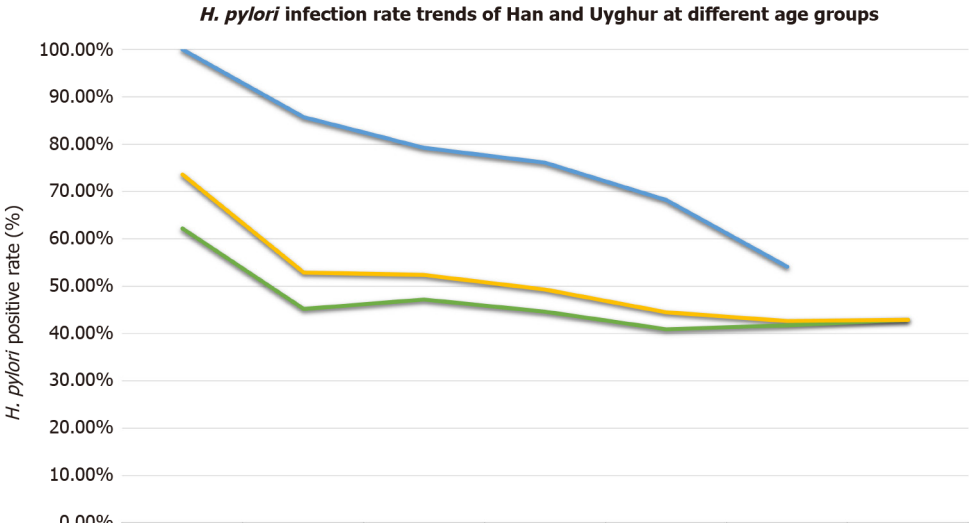
larger than in our cases from Urumqi.

From a perspective of regional difference, patients from southern Xinjiang had the highest positive rate of *H. pylori* infection, followed by patients from northern Xinjiang, and patients from the XPCC had the lowest *H. pylori* infection rate. We speculate that it may be related to the fact that southern Xinjiang is mainly inhabited by Uyghur ethnic groups, while northern Xinjiang is mainly inhabited by Han ethnic groups, as is the XPCC[7].

These results mentioned above suggest that ethnic difference is the most important cause of difference in *H. pylori* infection rate, which was confirmed by our matched comparative analysis. The differences in ethnicity might be one of the most important factors affecting the epidemic characteristics of *H. pylori* infection. The positive rate for *H. pylori* obtained by combining the two most sensitive methods in Uyghur was 75.3%, which was higher than previously reported (59.8%-62.38%)[6,8,9], indicating that the prevention and control for *H. pylori* infection in Uyghur people might be more challenging.

In addition, regardless of the Uyghur or Han ethnic groups, the *H. pylori* infection rate calculated by age group showed a decreasing trend as age increased. In the Han ethnic group, there was an increase after the age of 70 years, which is consistent with previous reports[6,32,33], indicating that we might need to eradicate *H. pylori* infection in young people as early as possible.

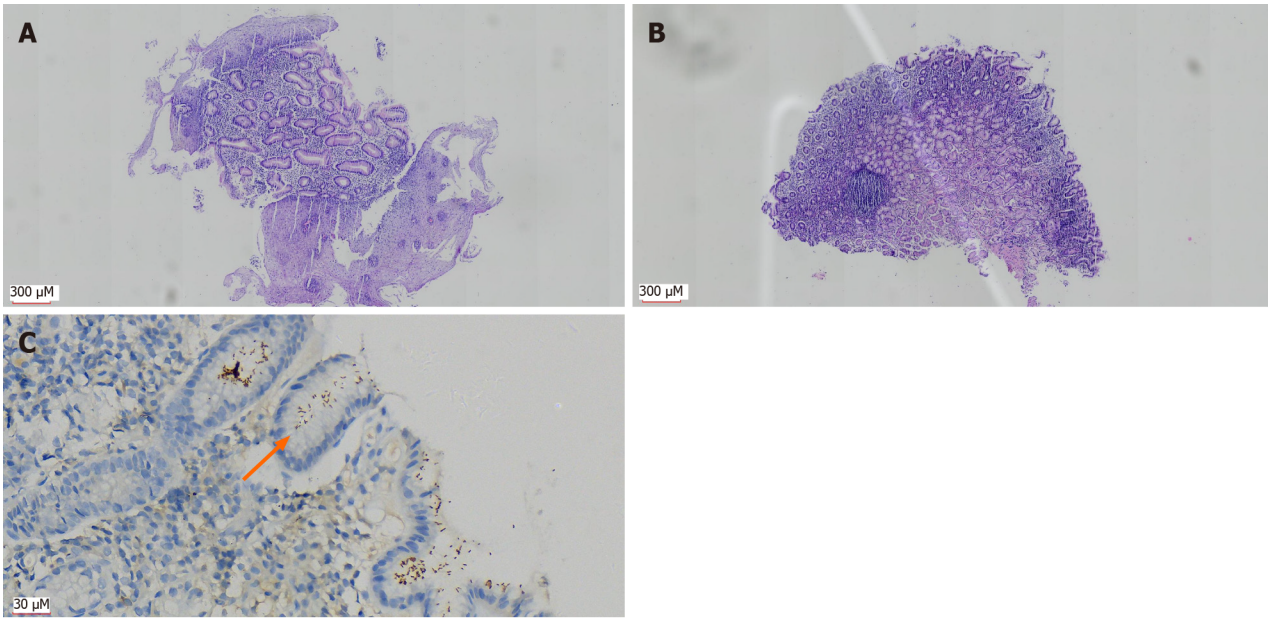
Since our hospital is a general hospital focused on traditional Chinese medicine, the majority of patients have mild stomach discomfort. Overall, benign inflammatory lesions were the main cause (> 90%) in this continuous randomized study. Therefore, there were few cases of malignant lesions such as gastric adenocarcinoma included in this study. Malignant tumors have a closer correlation with *H. pylori* infection, with over two-thirds of gastric adenocarcinoma cases and 75% of lymphoma cases having *H. pylori* infection. According to the literature, eradicating *H. pylori* in the first-degree relatives of gastric cancer patients is an important factor in reducing the incidence of gastric cancer[34]. In addition, *H. pylori*-related gastric lymphoma patients can achieve complete remission by eradicating *H. pylori*[35,36]. BE is a precan-



	≤ 30	31-40	41-50	51-60	61-70	71-80	≥ 81
Han	62.20%	45.20%	47.20%	44.60%	40.90%	41.70%	42.90%
Uyghur	100.00%	85.70%	79.20%	76.10%	68.20%	54.10%	
Total	73.60%	52.90%	52.40%	49.30%	44.50%	42.60%	42.90%

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Figure 2 *Helicobacter pylori* positive rate changed with age in Xinjiang Uyghur Autonomous Region. Regardless of the Uyghur, Han or the overall series, the *Helicobacter pylori* (*H. pylori*) infection rates calculated by age group showed a decreasing trend as age increased. In Han group, the positive rate of *H. pylori* infection increased in two age groups after the age of 70 years. *H. pylori*: *Helicobacter pylori*.

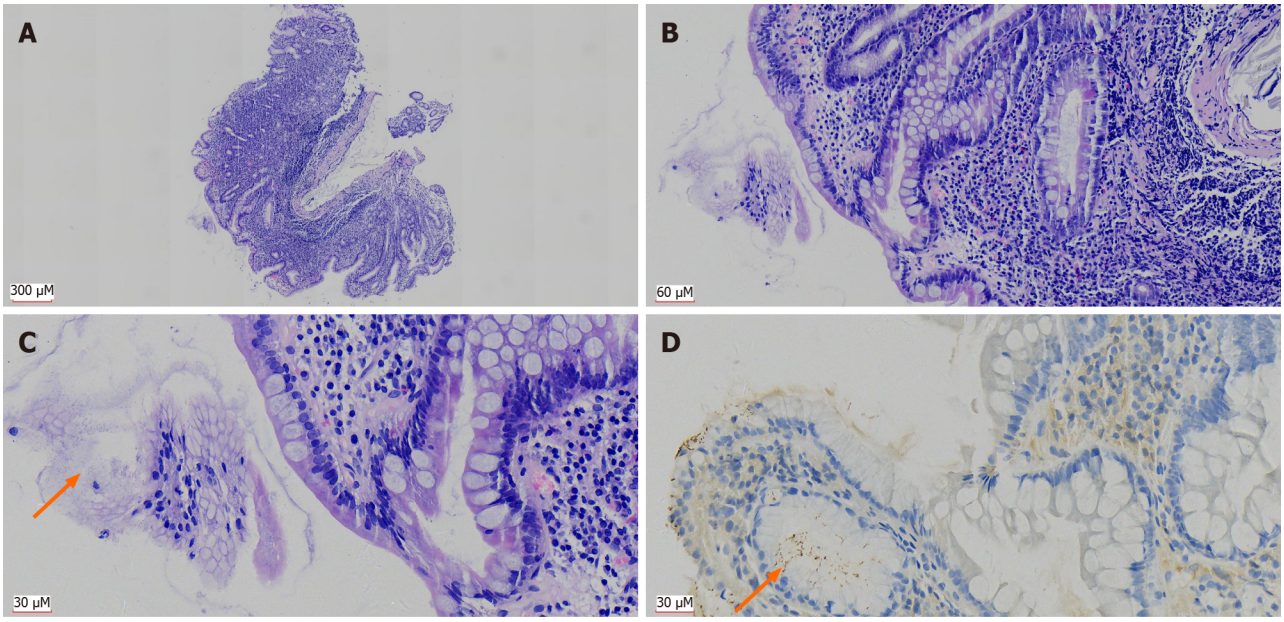


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Figure 3 Patients with Barrett's esophagus concurrent with *Helicobacter pylori* infection. A: Changes with Barrett's esophagus: Many hyperplastic glands in the esophageal wall, partially replacing the squamous epithelial mucosa [hematoxylin and eosin (H&E, 40 ×)]; B: Mild inflammation (H&E, 40 ×) in the gastric wall tissue biopsied simultaneously; C: Immunohistochemistry showing *Helicobacter pylori* (*H. pylori*) infection (DAB, 400 ×); orange arrow in C indicates distribution of *H. pylori*.

cerous lesion and some studies have shown that *H. pylori* infection and BE are inversely related[37,38]. The *H. pylori* infection rate (58.7%) was higher than the overall positive level, and BE may be related to *H. pylori* infection; however, the positive association between *H. pylori* infection and BE seemed to be a paradox. Well-designed prospective cohort studies with a powered sample size are required[39].

For gastritis, the *H. pylori* infection rate significantly increased with grade of chronic/active gastritis, and intestinal metaplasia grading. Infection with *H. pylori* may be an important factor leading to gastritis and intestinal metaplasia[40]. Eradicating *H. pylori* can improve the inflammatory response of the gastric mucosa, prevent or delay development of atrophy and intestinal metaplasia[40,41], block the Correa mode of intestinal gastric cancer evolution, and eliminate the



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Figure 4 *Helicobacter pylori* infection associated with elevated grade of chronic/active gastritis and intestinal metaplasia. A: Patient with severe chronic gastritis (grade 3) in the gastric wall biopsy tissue; inflammatory cell infiltration can be seen throughout the lamina propria of the mucosa [hematoxylin and eosin (H&E, 40 ×)]; B: Infiltrating inflammatory cells were mainly lymphocytes, with scattered neutrophils, indicating chronic gastritis combined with active gastritis (H&E, 200 ×); C: Gastric glands were infected by *Helicobacter pylori* (*H. pylori*) and were accompanied by severe intestinal metaplasia (H&E, 400 ×); D: *H. pylori* infection was confirmed by immunohistochemistry (DAB, 400 ×); all images were from the same patient, orange arrows in C and D indicates distribution of *H. pylori*.

risk of (intestinal) gastric cancer[18,29,33,34]. By combining the IHC and ^{14}C UBT results, we can obtain a better linear correlation between *H. pylori* infection and severity of gastritis, thus it is important to use two diagnostic tests at the same time.

CONCLUSION

Our results show that the sensitivity of ^{14}C UBT for detecting *H. pylori* is significantly higher than that of IHC with pathological specimens obtained by gastroscopy. The combination of ^{14}C UBT and IHC is necessary to improve the detection rate of *H. pylori* infection, and increasing the number of biopsy specimen (≥ 2) can significantly improve the positive rate of *H. pylori* detection in pathological immunohistochemical methods. The overall *H. pylori* infection rate in Xinjiang Uyghur Autonomous Region was higher than previously reported, and ethnicity was the most important factor for detection by combination of the two methods. Uyghur people have a higher *H. pylori* infection rate. The overall *H. pylori* infection rate calculated by age group showed a decreasing trend as age increased. Therefore, the key populations for the prevention and control of *H. pylori* infection in Xinjiang Uyghur Autonomous Region are the Uyghur and young people.

ARTICLE HIGHLIGHTS

Research background

There are differences in *Helicobacter pylori* (*H. pylori*) infection rate in Uyghur and Han ethnic groups. ^{14}C urea breath test (^{14}C UBT) and immunohistochemistry (IHC) with tissue from gastroscopic biopsy are widely used detection methods, but both lack large cohort studies to accurately evaluate their performance.

Research motivation

To compare the difference between ^{14}C UBT and IHC for accurate testing for *H. pylori* infection, and to study the difference in infection positive rate between Uyghur and Han ethnic groups.

Research objectives

We included 5747 cases with *H. pylori* infection detected by both IHC and ^{14}C UBT. We detected 3944 by simultaneous IHC and ^{14}C UBT and 555 pairs of Han/Uyghur were compared for their *H. pylori* infection rate.

Research methods

IHC and ^{14}C UBT were performed at the same time (interval < 1 wk, with sampling site including gastric antrum), and 3944 cases were screened out. The overall *H. pylori* infection positive rate was calculated by combining IHC and ^{14}C UBT results (n = 5747). Correlation between *H. pylori* infection and patients' clinical parameters (gender, age, ethnicity and region) was analyzed. 555 pairs of Han/Uyghur cases (completely matched for gender and age) were compared for their *H. pylori* infection rates. The *H. pylori* infection rate and pathological diagnosis, including gastritis (chronic/active inflammation, atrophy, and intestinal metaplasia), were analyzed.

Research results

Among the 3944 cases for which ^{14}C UBT and IHC were performed at the same time, the sensitivity was 94.9% for ^{14}C UBT and 65.1% for IHC, which was a significant difference (P < 0.001). Among those positive by ^{14}C UBT (detection value > 100), the *H. pylori* positive rate with IHC was 63.2%, and among those negative for ^{14}C UBT (detection value ≤ 100), the IHC positive rate was 4.8%. In combination with both detection methods, the total rate of *H. pylori* infection in all 5747 patients was 48.6%, and there were significant differences for gender, age, ethnicity, and region (P values were 0.001, < 0.001, < 0.001 and < 0.001). The *H. pylori* infection rates for the 555 Chinese/Uyghur paired cases (completely matched for gender and age) were 41.4% and 73.3%, which was a significant difference (P < 0.001). For benign gastric lesions, the combined *H. pylori* infection rate was 53.8% for inflammation, 27.5% for fundus gland polyps, 22.2% for duodenal ectopic gastric mucosa, 17.5% for hyperplastic polyps, 58.7% for BE, and 67.6% for gastric adenocarcinoma. Positivity for *H. pylori* infection was significantly related to moderate-severe (grade 2-3) chronic inflammation, moderate-severe active inflammation and moderate-severe (grade 2-3) intestinal metaplasia (P < 0.001, < 0.001 and 0.032 in order).

Research conclusions

The sensitivity of ^{14}C UBT was significantly higher than that of IHC when detecting *H. pylori* infection, but there were still *H. pylori* positive cases missed that were detected by IHC. Combination of both methods can increase the detection accuracy of *H. pylori* infection, and the overall infection rate of *H. pylori* in our study was higher than previously reported in Xinjiang Uyghur Autonomous Region. Ethnic difference was the most important factor affecting the *H. pylori* infection rate, and the Uyghur people had more *H. pylori* infection. The *H. pylori* infection rate decreased with age, and was more correlated with precancerous lesions and malignant tumors, and increased with severity of inflammation.

Research perspectives

Our study highlights the importance of using IHC and ^{14}C UBT together for *H. pylori* infection, and the prevention and intervention of *H. pylori* infection in Xinjiang Uyghur Autonomous Region and emphasizes that the Uyghur and young people should be examined as early as possible.

FOOTNOTES

Co-first authors: Yu-Hua Peng and Xue Feng.

Author contributions: Peng YH and Feng X collected samples and clinicopathological data, participated in the data analysis and drafted the manuscript; Zhou Z finished the pathological experimental procedures; Yang L performed the statistical analysis; Shi YF designed and supervised the study, revised the manuscript; and all authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Chemical components and protective effects of *Atractylodes japonica* Koidz. ex Kitam against acetic acid-induced gastric ulcer in rats

Bi-Xian Zhen, Qian Cai, Feng Li

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Abstract

BACKGROUND

Atractylodes japonica Koidz. ex Kitam. (*A. japonica*, Chinese name: Guan-Cangzhu, Japanese name: Byaku-jutsu), a perennial herb, which is mainly distributed in northeast area of China, it's often used to treat digestive system diseases such as gastric ulcer (GU). However, the mechanism of its potential protective effects against GU remains unclear.

AIM

To investigate the protective effects of *A. japonica* on acetic acid-induced GU rats.

METHODS

The chemical constituents of *A. japonica* were determined by ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) analysis. The rat model of GU was simulated by acetic acid method. The pathological changes of gastric tissues were evaluated by hematoxylin-eosin stain, the levels of epidermal growth factor (EGF), EGF receptor (EGFR), nuclear factor kappa-B (NF-κB), interleukin-1β (IL-1β), IL-10, Na^+-K^+-ATPase (NKA) in serum and gastric tissues were determined by enzyme-linked immunosorbent assay, and the mRNA expressions of EGFR, NF-κBp65, IkappaBalpha (IκBα) and Zonula Occludens-1 (ZO-1) in gastric tissues were determined by real-time reverse transcription polymerase chain reaction, and the efficacy was observed. Then, plasma metabolomic analysis was performed by UPLC-MS/MS to screen the specific potential biomarkers, metabolic pathways and to explore the possible mechani-sms.

RESULTS

48 chemical constituents were identified. Many of them have strong pharmacological activity, the results also revealed that *A. japonica* significantly improved the pathological damage of gastric tissues, increased the expression levels of IL-10,

IκBα related to anti-inflammatory factors, decreased the expression levels of IL-1β, NF-κB, NF-κBp65, related to proinflammatory factors, restored the levels of factors about EGF, EGFR, ZO-1 associated with ulcer healing and the levels of factors about NKA associated with energy metabolism. Metabolomic analysis identified 10 potential differential metabolites and enriched 7 related metabolic pathways.

CONCLUSION

These findings contribute to the understanding of the potential mechanism of *A. japonica* to improve acetic acid-induced GU, and will be of great importance for the development and clinical application of natural drugs related to *A. japonica.*

Key Words: *Atractylodes japonica* Koidz. ex Kitam.; Ulcer; Acetic acid; Digestive system diseases; Metabolomics; Rats

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Core Tip: *Atractylodes japonica* Koidz. ex Kitam. is a commonly used folk medicine for the treatment of gastric ulcer (GU), but the mechanism of the treatment of GU is still unclear. In this study, chemical composition, pharmacodynamics and metabolomics were studied to explore the potential mechanism of it. The finding was closely related to anti-inflammation, ulcer healing and other factors.

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INTRODUCTION

Gastric ulcer (GU) is a common global gastrointestinal disorder with common symptoms such as loss of appetite, stomach pain, acid reflux, gastric distention, nausea, and in severe cases, gastric bleeding[1]. Due to its long treatment cycle, relapsing easily, and other characteristics, it has serious physiological and psychological effects on patients. Although the mechanism of ulceration is not clear, pathological studies have found that gastric mucosal lesions are significantly contributed to the development of ulcers[2]. With the accelerated pace of life and bad health habits such as excessive alcohol consumption, smoking, use of non-steroidal anti-inflammatory drugs[3], and increased psychological burden from work, study, family and social environment , the incidence of GU has been rising every year. Therefore, the treatment of GU has become one of the hotspots in research[4]. Although current treatments with traditional western drugs such as proton-pump inhibitors, H2-receptor antagonists, M1-receptor antagonists, and antibiotics against *Helicobacter pylori* are effective, their side effects are often unavoidable[5-7]. Therefore, it is imperative to explore new therapeutic agents. However, herbal medicines for GU have a long history and have received considerable attention for their high efficacy, low side effects, and affordability[8].

Atractylodes japonica (*A. japonica*) is a perennial herb in the Asteraceae family that grows on hillsides, bushes, tussah forests, *etc* The herb has irregular masses or irregularly curved cylinders (3-8 cm in length, 2-3cm in diameter)[9] and is mainly distributed in the Siberian region of Russia, northeastern China, Korea, and Japan[10]. The earliest record of *Atractylodes* in ancient books can be found in Shennong's Classic of Materia Medica[11]. In the 2020th edition of the Chinese Pharmacopoeia, *Atractylodes lancea* (Thunb.) DC. and *Atractylodes chinensis* (DC.) Koidz. were included as sources of *Atractylodis rhizoma*, *A. japonica* was not included in it[12], but was included in the Japanese Pharmacopoeia as a source of *Atractylodis rhizoma*. The dried rhizome of *A. japonica* is commonly used in the treatment of various diseases, such as loss of appetite, indigestion, abdominal distension, diarrhea, night blindness, rheumatic arthritis, *etc*[13]. The chemical components of *A. japonica* involve sesquiterpenoids, acetylene, saccharides, *etc*[14]. Modern pharmacological studies have shown that extracts of *A. japonica* have a variety of biological activities, including anti-inflammation, anti-viral, hypoglycemic, diuretic, cardioprotective, and other bioactive effects, along with specific efficacy in the treatment of digestive disorders such as GU[10,15,16]. In recent years, the demand for *A. japonica* at home and abroad has gradually increased.

There are a few studies on *A. japonica's* chemical composition, which forms the basis for its mechanism of action. Although *A. japonica* has a good therapeutic effect, there is little information about its anti-ulcer activity and related mechanisms. In the preliminary study, the research group who adopted the rheumatoid arthritis model established by Freund's complete adjuvant method, which also proved to have a better anti-inflammatory effect[17]. Therefore, this study intends to focus on the potential anti-inflammatory and protective effects of *A. japonica*. In addition, because of its comprehensive and dynamic characteristics, metabolomics is often used in the study of diseases. According to the changes in endogenous metabolites in the body after the intervention of herbal medicines, the mechanism of action is systematically explained. In this study, pharmacodynamic and metabolomic approaches were used to elucidate the protective mechanism of *A. japonica* in a model for acetic acid-induced GU in rats. Thus, our study may provide a

theoretical basis for the application of *A. japonica.*

MATERIALS AND METHODS

Plant materials and reagents

Collected *Atractylodes japonica* Koidz. ex Kitam (Kuandian, Liaoning, China) was identified as the rhizome of *A. japonica* by Professor Feng Li and deposited in Liaoning University of Traditional Chinese Medicine. Omeprazole was purchased from Shiyao Group Ouyi Pharmaceutical Co., Ltd. (Shijiazhuang, Hebei, China) (Drug approval number: H20046430). This drug is commonly used in clinical treatment of GU and as a positive drug for experimental studies[18,19], transformed into powder, and prepared into a 2 mg/mL solution before use. The drug was crushed through a 180-mesh sieve. Low, middle, and high doses of *A. japonica* were prepared into suspensions of 0.047 g/mL, 0.094 g/mL and 0.188 g/mL, respectively, and shaken well before use. Formic acid, methanol, and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, United States). The SweScript RT I First Strand cDNA Synthesis Kit was provided by Servicebio (Wuhan, Hubei, China). Yeasen Biotechnology (Shanghai, China) provided HieffTM qPCR SYBR® Green Master Mix (No Rox Plus). Enzyme-linked immunosorbent assay (ELISA) kits of epidermal growth factor (EGF), EGF receptor (EGFR), nuclear factor kappa-B (NF-κB), interleukin-1β (IL-1β), IL-10, and Na^+-K^+-ATPase (NKA) were purchased from Lianshuo Biotechnology Co., Ltd. (Shanghai, China).

Sample preparation and detection

Approximately 1 g of *A. japonica* (through 60 mesh sieve) was added to 20 mL of 80% methanol (v/v) for 40 min using ultrasonication, centrifuged for 10 min at 13780 × *g* and the supernatants were obtained. Finally, the supernatants were filtered through a 0.22 μm membrane filter. Then, the sample was analyzed using LC-MS (UPLC, Thermo, United States, Q Exactive MS, Thermo, United States) platform. Chromatographic analysis was performed using a ACQUITY UPLC C18 Column (100 mm × 2.1 mm, 1.8 μm, Waters, United States). The mobile phases consisted of acetonitrile (A) and 0.1% aqueous formic acid (v/v) (B). The analysis was carried out with an elution gradient using the following steps: 0-5 min, 95%-46% B; 5-11 min, 46% B; 11-20 min, 46%-23% B; 20-30 min, 23%-5% B, with the flow rate at 0.3 mL/min. The column temperature was kept at 30 °C, and the injection volume was 2 μL. The ESI source conditions were set as follows: Sheath gas flow rate as 35 Arb, aux gas flow rate as 15 Arb, capillary temperature as 320 °C, full MS resolution as 70000, MS/MS resolution as 17500, collision energy as 40 in NCE mode, and spray voltage as 4.0 kV (positive) or -3.0 kV (negative), respectively. The mass range scanned was 100-1500 m/z. Furthermore, MS data were collected with Xcalibur software (version 3.0).

Animals and treatment

Male Sprague-Dawley rats (specific pathogen-free grade), weighing 180-220 g, were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (SCXK 2020-0001, Liaoning, China). Prior to the experiment, the rats were raised under normal laboratory conditions: Housed for 7 d at 24 °C and 45%-55% relative humidity. After acclimatization for 7 d, all rats were randomly divided into the normal group (NG), the acetic acid-induced model group (MG), the omeprazole group (OG), the low dose group of *A. japonica* (LA), the middle dose group of *A. japonica* (MA), and the high dose group of *A. japonica* (HA), n = 8 for each group. GU was induced by acetic acid treatment in rats according to the method of Okabe *et al*[20] and Okabe *et al*[21] with partial modifications[19]. All rats were fasted for 24 h, and then a laparotomy was performed through a left subcostal incision after anesthesia with isoflurane. The stomach was gently exteriorized and clamped 3 mm away from the pylorus with special tweezers, a double-ring tweezer with a 9-mm diameter that can clamp the injection site[19]. Furthermore, a 0.2 mL mixture of mineral oil and 60% acetic acid (v/v) in the same syringe was injected into the subserosal layer in the glandular portion of the anterior wall in the clamping region, and the solution was aspirated off after 45 s. In the NG group, saline was used instead of acetic acid. The opened abdomen was then cautiously placed back, cleaned with penicillin, and sutured. Then the rats were fed normally. Except for the NG and MG groups, they were given the same amount of normal saline for 10 d (20 mL/kg/d), twice a day. Other groups were treated with intragastric administration for 10 d (20 mL/kg/d), twice a day. 12 h after the last administration, all rats were anesthetized[22]. Blood was collected from rats, then centrifuged at 3910 × *g* for 10 min. The clear serum and plasma for ELISA assays and metabolomics were then stored at -80 °C until the measurement. The stomach was removed and washed with ice-cold saline. The gastric mucosal injury was observed and evaluated by calculating ulcer score[23]. Then, the ulcer tissue was cut into three parts, and one part was immediately fixed with a 4% paraformaldehyde solution for pathological analysis. Other parts were used for ELISA and mRNA level assays; they were also stored at -80 °C until the measurement.

Gastric histopathology examination

Gastric tissues were immediately fixed with a 4% paraformaldehyde solution for pathological analysis; gradient alcohol and xylene were used for dehydration and transparency separately. The tissues were impregnated with wax and embedded. The sections were made to a thickness of 5 μm, stained with hematoxylin for 30 min, counterstained with eosin, dehydrated with gradient alcohol, thoroughly permeabilized with xylene, sealed with neutral resin, observed under a light microscope (BX53, Olympus, Tokyo, Japan), and blind analysis was performed by an experienced histopathologist.

ELISA analysis

Serum and gastric tissues were thawed at 4 °C before use. Gastric tissues, weighing 100 mg, were homogenized into a 10% tissue homogenate prepared by adding 900 μL of ice-cold saline, centrifuging for 10 min at 3910 × *g*, and obtaining the supernatants. The levels of EGF, EGFR, NF-κB, IL-1β, IL-10, and NKA were analyzed by ELISA (Infinite M200, TECAN, Switzerland) at 450 nm.

Gene expression analysis

According to the supplier's instructions, the trizol method was used to extract total RNA[24], 2.5 μL of the solution to be tested was aspirated, and RNA concentration and purity were detected using an ultra-micro spectrophotometer (Nanodrop 2000, Thermo, America). The SweScript RT I First Strand cDNA Synthesis Kit was used to reverse transcribe to cDNA; the specific reaction system was 20 μL. The HieffTM qPCR SYBR® Green Master Mix was used to perform DNA amplification. Primers were provided by Tianyi Huiyuan Biotechnology Co., Ltd. (Wuhan, China), and data were analyzed using the comparative cycle threshold (CT) method. The primers for the gene sequences are shown in Table 1.

Metabolomics analysis

Plasma samples were thawed on ice. 100 μL sample was taken and placed into an EP tube, extracted with 300 μL methanol, vortexed for 30 s, and then ultrasound treated for 30 min (incubated in ice water) and incubated for 1 h at -40 °C to precipitate proteins, followed by vortexing for 30 s, and incubated for 0.5 h at 4 °C. Then the sample was centrifuged at 13780 × *g* for 15 min at 4 °C. The entire supernatant was collected and placed into an EP tube and incubated for 1 h at -40 °C after centrifugation at 13780 × *g* for 15 min at 4 °C. 200 μL of supernatants, including 5 μL internal standard (2-chloro-DL-phenylalanine, 0.5 mg/mL), were transferred to LC-MS vials by mixing. The quality control (QC) samples were prepared by mixing an equal aliquot of the supernatants from all samples. Plasma samples were analyzed by LC-MS (UPLC, Waters, United States; Q Exactive MS, Thermo, United States). Chromatographic analysis was performed using an UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm, Waters, United States). The mobile phase consisted of 0.05% formic acid in water (A) and acetonitrile (B). The analysis was carried out with an elution gradient using the following steps: 0-1 min, 5% B; 1-12 min, 5%-95% B; 12-13.5 min, 95% B; 13.5-13.6 min, 95%-5% B; 13.6-16 min, 5% B, with the flow rate of 0.3 mL/min. The column temperature was kept at 40 °C. The autosampler temperature was 4 °C, and the injection volume was 5 μL. The ESI source conditions were set as follows: Sheath gas flow rate as 45 Arb, aux gas flow rate as 15 Arb, capillary temperature as 350 °C, full MS resolution as 70000, MS/MS resolution as 17500, collision energy as 15/30/45 in NCE mode, and spray voltage as 3.0 kV (positive) or -3.2 kV (negative), respectively.

Data process and multivariate analysis

First of all, the sample data were obtained with feature extraction and preprocessed with Compound Discoverer software (version 2.1, Thermo, United States). Then, the normalized data were imported into SIMCA-P software (version 14.1, Umetrics, Umea, Sweden) for performing multivariate statistical analysis (MVA), including principal component analysis (PCA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA)[25]. The validity of the OPLS-DA model was evaluated based on the results of the permutation test. Meanwhile, METLIN (http://metlin.scripps.edu/), HMDB (http://www.hmdb.ca/), and KEGG (http://www.kegg.com/) were used to identify potential biomarkers by comparing the mass spectrometry fragmentation information. Furthermore, potential markers were visualized in the enrichment pathway using MetaboAnalyst (http://www.metaboanalyst.ca/).

Statistics analysis

The results were expressed as the mean ± SD. Student's *t*-test, one-way analysis of variance (ANOVA) by Dunnett's post-hoc test and Kruskal-Wallis test were used for statistical analysis. $P < 0.05$ was statistically significant, and $P < 0.01$ was highly significant. GraphPad Prism software (version 9.5, San Diego, United States) and TBtools (version 1.108, Guangzhou, China) were used for visualization.

RESULTS

Identification of the constituents by UPLC Q-Exactive Orbitrap MS

UPLC Q-Exactive Orbitrap was employed for comprehensive analysis in positive and negative modes to identify the chemical constituents using related databases and literature. Therefore, a total of 48 components (Table 2) were identified as terpenoids, flavonoids, organic acids, *etc.* A typical total ion chromatogram of *A. japonica.* in positive and negative ion modes are shown in Figure 1.

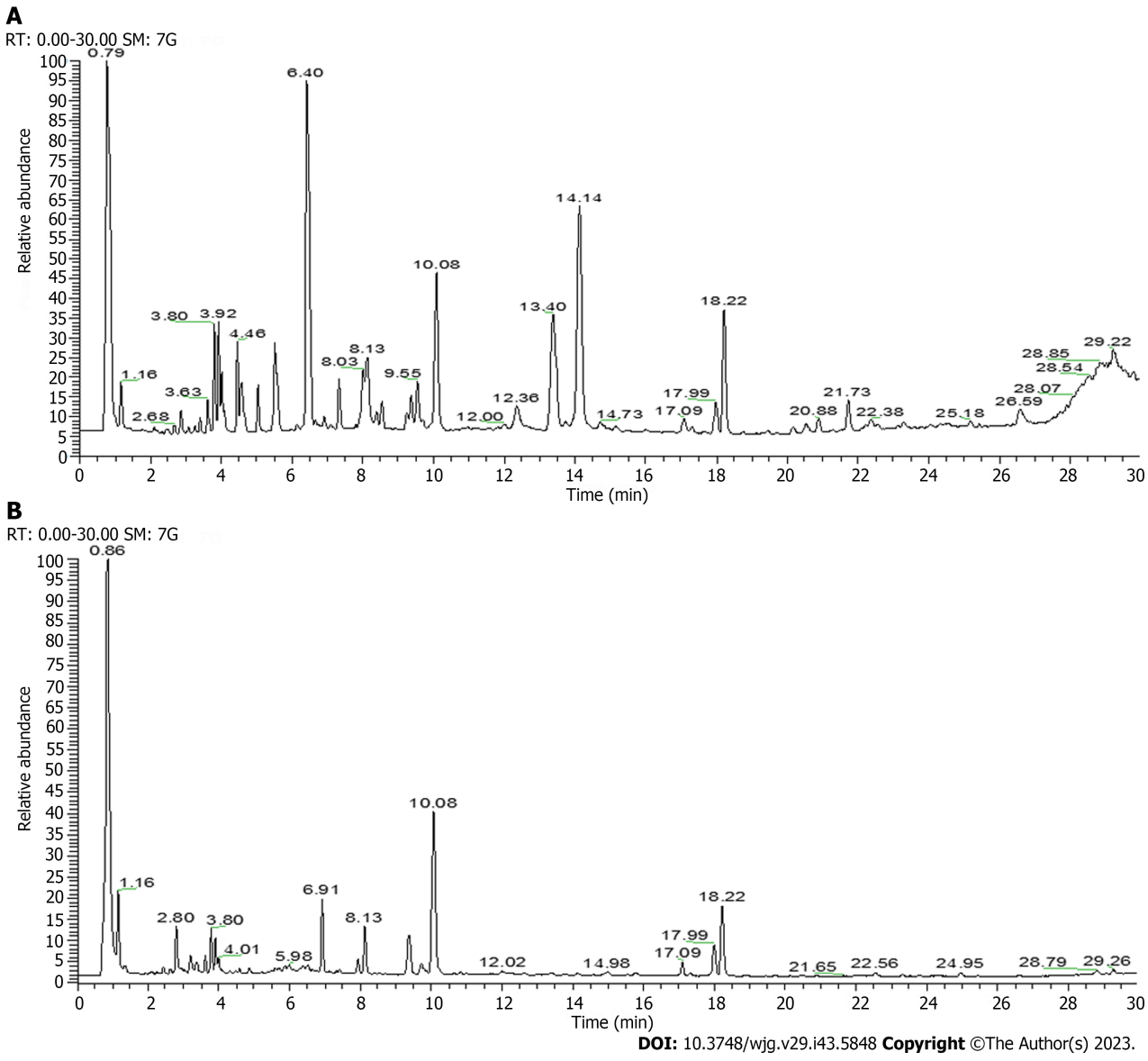
Effects of A. japonica on GU by histological analysis

Macroscopic analyses showed the gastric mucosa in NG group was smooth and complete with continuous mucosal plica and no edema. The gastric mucosa of MG group showed swelling and congestion. The treatment group and the positive drug group had different degrees of improvement, the surface of gastric mucosa in OG group was more flat, with no obvious ulcer surface. The ulcer was round or nearly round in the treated group, with slight bulge around the ulcer, and the ulcer area was smaller than that in MG group, especially in MA and HA groups, Specific information was given in Supplementary Figure 1 and Supplementary Table 1. Histopathological results showed that the gastric glands were closely arranged and regular, and the epithelial cells remained intact without congestion or edema in the NG group.

Table 1 Sequences of the primers for real-time reverse transcription polymerase chain reaction

Gene	Forward primer	Reverse primer	Length
EGFR	CCTATGGGCCAAAGATCCCA	GAGGTTCCACGAGCTCTCTC	162 bp
ZO-1	CACCACAGACATCCAACCAG	CACCAACCACTCTCCCTTGT	230 bp
NF-κBp65	AGGCCATTGAAGTGATCCAG	CAGTGAGGGACTCCGAGAAG	204 bp
IκBα	CACGGAAGATGAGTTGCCCT	CAAGTCCACGTTCCTTTGGC	91 bp
GAPDH	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT	207 bp

EGFR: Epidermal growth factor receptor; ZO-1: Zonula Occludens-1; NF-κB: Nuclear factor kappa-B; IκBα: IkappaBalpha.



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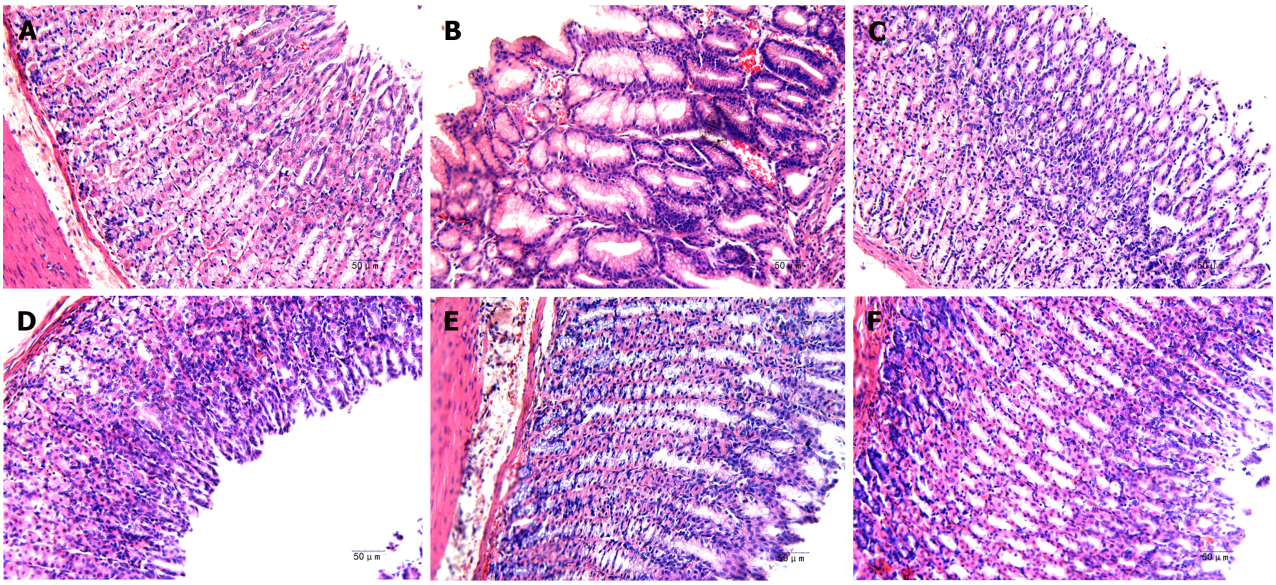
Figure 1 Total ion current chromatogram of *Atractylodes japonica* by liquid chromatography-tandem mass spectrometry. A: Positive ion mode; B: Negative ion mode.

Compared with the NG group, the epithelial cells of the gastric mucosa were disorganized with edema and inflammatory cell infiltration in the MG group, showing pathological changes. The histopathology associated with GU was improved, and inflammatory cell infiltration was reduced by the intervention of positive control drug and *A. japonica*, especially in the HA group (Figure 2).

Table 2 Identification of chemical constituents of *Atractylodes japonica* by UPLC Q-Exactive Orbitrap MS

No.	tR/min	Compound	Molecular formula	Adduct ion	Theoretical (m/z)	Measured (m/z)	Error (ppm)
1	0.83	Asparagine	$C_4H_8N_2O_3$	$[M+H]^+$	133.06076	133.06033	-3.297
2	0.85	Sucrose	$C_{12}H_{22}O_{11}$	[M−H+HCOOH]	387.11441	387.11374	-1.741
3	0.86	Manninotriose	$C_{18}H_{32}O_{16}$	$[M-H]^-$	503.16092	503.16175	-1.665
4	0.85	Valine	$C_5H_{11}NO_2$	$[M+H]^+$	118.08625	118.08591	-2.923
5	0.87	5-hydroxymethylfurfural	$C_6H_6O_3$	$[M+H]^+$	127.03897	127.03864	-2.602
6	0.91	Niacinamide	$C_6H_6N_2O$	$[M+H]^+$	123.05529	123.05509	-1.620
7	1.19	Pyroglutamic acid	$C_5H_7NO_3$	$[M+H]^+$	130.04986	130.04953	-2.612
8	1.25	Tyrosine	$C_9H_{11}NO_3$	$[M+H]^+$	182.08116	182.08063	-2.964
9	1.27	Citric acid	$C_6H_8O_7$	$[M-H]^-$	191.01972	191.01880	-4.847
10	1.35	Isoleucine	$C_6H_{13}NO_2$	$[M+H]^+$	132.10190	132.10153	-2.840
11	1.46	Leucine	$C_6H_{13}NO_2$	$[M+H]^+$	132.10190	132.10153	-2.840
12	2.10	Phenylalanine	$C_9H_{11}NO_2$	$[M+H]^+$	166.08625	166.08577	-2.921
13	2.25	Neochlorogenic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	353.08781	353.08783	0.050
14	2.44	Chlorogenic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	353.08781	353.08752	-0.808
15	2.44	Atractyloside A	$C_{21}H_{36}O_{10}$	[M−H+HCOOH]	493.22905	493.22864	-0.831
16	2.80	Cryptochlorogenic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	353.08781	353.08728	-1.488
17	2.86	Caffeic acid	$C_9H_8O_4$	$[M+H]^+$	181.04953	181.04890	-3.509
18	2.86	7-hydroxycoumarin	$C_9H_6O_3$	$[M+H]^+$	163.03897	163.03841	-3.439
19	3.17	Catechin	$C_{15}H_{14}O_6$	$[M-H]^-$	289.07176	289.07172	-0.143
20	3.19	Isochlorogenic acid B	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.11950	515.11890	-1.163
21	3.27	Isoorientin	$C_{21}H_{20}O_{11}$	$[M+H]^+$	449.10784	449.10669	-2.556
22	3.51	Rutin	$C_{27}H_{30}O_{16}$	$[M-H]^-$	609.14610	609.14630	0.315
23	3.52	Hyperoside	$C_{21}H_{20}O_{12}$	$[M-H]^-$	463.08819	463.08826	0.131
24	3.52	Quercetin	$C_{15}H_{10}O_7$	$[M+H]^+$	303.04992	303.04895	-3.528
25	3.68	Kaempferol-3-O- rutinoside	$C_{27}H_{30}O_{15}$	$[M-H]^-$	593.15119	593.15125	0.096
26	3.77	Isorhamnetin 3-O-neohesperidin	$C_{28}H_{32}O_{16}$	$[M-H]^-$	623.16176	623.16107	-1.104
27	3.78	Narcissoside	$C_{28}H_{32}O_{16}$	$[M+H]^+$	625.17631	625.17371	-4.161
28	3.78	Isorhamnetin	$C_{16}H_{12}O_7$	$[M+H]^+$	317.06558	317.06421	-4.318
29	3.83	Vanillin	$C_8H_8O_3$	$[M+H]^+$	153.05462	153.05406	-3.663
30	3.86	Isochlorogenic acid A	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.11950	515.11914	-0.697
31	3.96	Isochlorogenic acid C	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.11950	515.11896	-1.047
32	6.40	Palmitic acid	$C_{16}H_{32}O_2$	$[M+NH_4]^+$	274.27406	274.27292	-4.141
33	6.47	Cinnamyl alcohol	$C_9H_{10}O$	$[M+H]^+$	135.08044	135.07999	-3.343
34	6.51	Curcumenol	$C_{15}H_{22}O_2$	$[M+H]^+$	235.16925	235.16826	-4.237
35	7.34	Atractylenolide III	$C_{15}H_{20}O_3$	$[M+H]^+$	249.14852	249.14752	-4.018
36	7.62	Atractylenolactam	$C_{15}H_{19}NO$	$[M+H]^+$	230.15394	230.15340	-2.350
37	9.24	3β-hydroxyatractylone	$C_{15}H_{20}O_2$	$[M+H]^+$	233.15361	233.15279	-3.502
38	9.55	Atractylenolide II	$C_{15}H_{20}O_2$	$[M+H]^+$	233.15361	233.15274	-3.716
39	12.00	Atractylenolide VI	$C_{15}H_{22}$	$[M+H]^+$	203.17943	203.17889	-2.644

40	12.01	α-linolenic acid	$C_{18}H_{30}O_2$	$[M+H]^+$	279.23185	279.23108	-2.782
41	12.14	3β-acetoxyatracty	$C_{17}H_{22}O_3$	$[M+H]^+$	275.16417	275.16333	-3.057
42	12.38	Atractylenolide I	$C_{15}H_{18}O_2$	$[M+H]^+$	231.13796	231.13721	-3.229
43	12.95	Atractylenolide V	$C_{15}H_{20}O_4$	$[M+H]^+$	265.14343	265.14258	-3.227
44	13.42	(6E,12E)-tetradecadiene-8,10-diyne-1,3-diol diacetate	$C_{18}H_{22}O_4$	$[M+H]^+$	303.15908	303.15805	-3.416
45	13.97	β-elemene	$C_{15}H_{24}$	$[M+H]^+$	205.19508	205.19443	-3.155
46	14.14	Eudesma-4(15),7(11)-dien-8-one	$C_{15}H_{22}O$	$[M+H]^+$	219.17434	219.17346	-4.024
47	20.55	Atractylon	$C_{15}H_{20}O$	$[M+H]^+$	217.15869	217.15800	-3.186
48	21.83	Biatractylolide	$C_{30}H_{38}O_4$	$[M+H]^+$	463.28428	463.28290	-2.992



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Figure 2 Effect of *Atractylodes japonica* on pathological changes of gastric tissues in gastric ulcer rats after 10 d of treatment (*n* = 8). A-F: Histological analysis was performed by hematoxylin and eosin staining (200 × original magnification, scale bar 50 μm). Normal group (A); acetic acid-induced model group (B); omeprazole group (C); low dose group of *Atractylodes japonica* (*A. japonical*) (D); middle dose group of *A. japonica* (E); high dose group of *A. japonica* (F).

Effects of A. japonica on the levels of EGF, EGFR, NF-κB, IL-1β, IL-10 and NKA in serum and gastric tissues

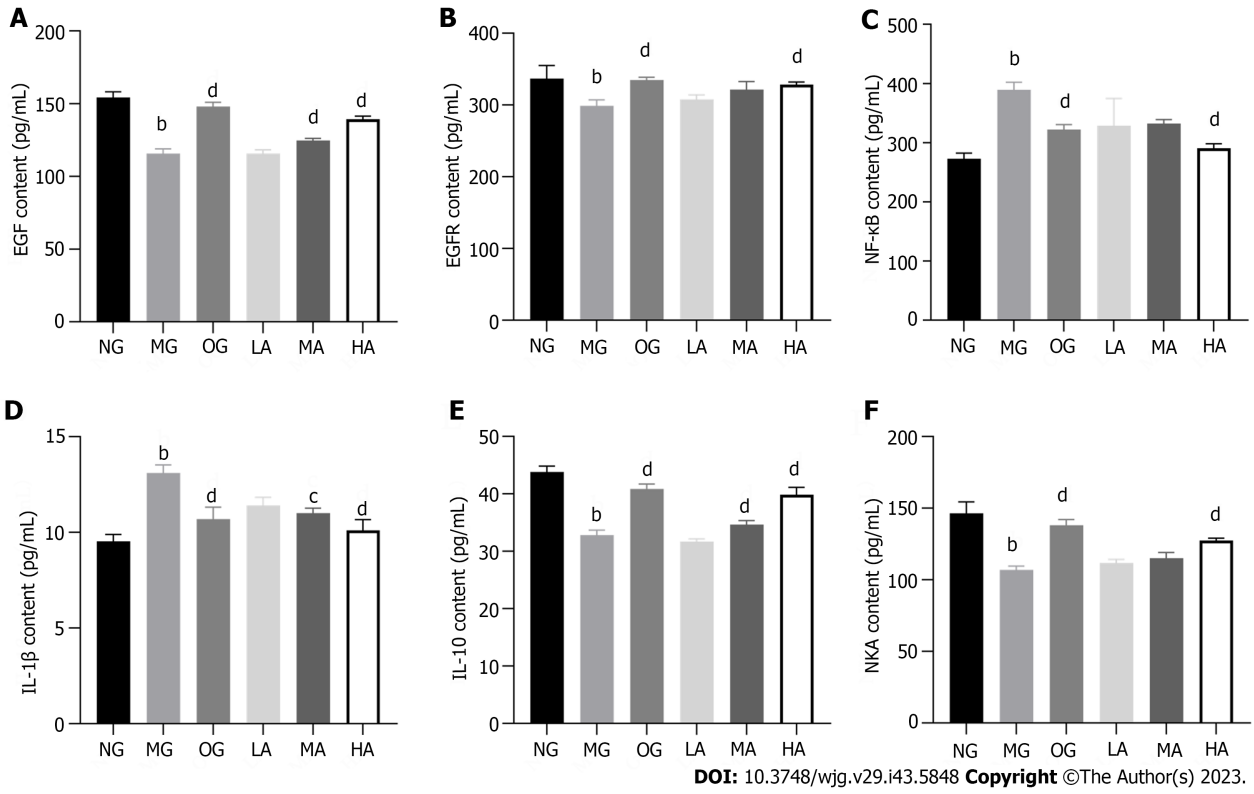
The levels of EGF, EGFR, NF-κB, IL-1β, IL-10, and NKA in the serum and gastric tissues of rats were detected by ELISA. The results showed that the levels of EGF, EGFR, IL-10, and NKA in the MG group were significantly lower ($P < 0.01$), while the levels of NF-κB and IL-1β were significantly higher than in the NG group ($P < 0.01$). Compared with the MG group, the HA group could significantly increase EGF, EGFR, IL-10, and NKA levels and decrease the levels of NF-κB and IL-1β in the serum and gastric tissues ($P < 0.05$ or $P < 0.01$). In addition, compared with the MG group, the levels of EGF and EGFR increased and IL-1β decreased in the MA group's serum ($P < 0.05$ or $P < 0.01$). The levels of EGFR increased in the MA and LA groups ($P < 0.01$) and IL-1β decreased in the MA group of gastric tissues ($P < 0.05$) (Figures 3 and 4).

Effects of A. japonica on the mRNA expressions of EGFR, Zonula Occludens-1, NF-κBp65 and IkappaBalpha in gastric tissues

The mRNA expressions of EGFR, Zonula Occludens-1 (ZO-1), and IkappaBalpha (IκBα) in the MG group were significantly lower ($P < 0.01$), while the expression of NF-κBp65 was significantly higher than in the NG group ($P < 0.01$). Compared with the MG group, the mRNA expressions of EGFR, ZO-1, and IκBα significantly decreased, while the expression of NF-κBp65 significantly increased in the HA group ($P < 0.05$). In addition, the expressions of EGFR and IκBα could also increase in MA and LA groups ($P < 0.01$) (Figure 5).

MVA

The multivariate pattern recognition analysis was first analyzed by PCA, an unsupervised learning method. The results of the PCA score plot including the QC samples showed a certain trend of separation among the different groups (Figure 6), where tightly aggregated QC samples indicated MS platform was stable. Next, the plasma metabolism



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Figure 3 Effect of *Atractylodes japonica* on gastric ulcer-associated factors level in serum of gastric ulcer rats (*n* = 8). A-F: The epidermal growth factor (EGF), EGF receptor, nuclear factor kappa-B, interleukin-1β (IL-1β), IL-10, and Na^+-K^+-ATPase levels were detected by enzyme-linked immunosorbent assay in serum of gastric ulcer rats. $^bP < 0.01$ *vs* normal group; $^cP < 0.05$ and $^dP < 0.01$ *vs* model group. EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; NF-κB: Nuclear factor kappa-B; IL: Interleukin; NKA: Na^+-K^+-ATPase; NG: Normal group; MG: Model group; OG: Omeprazole group; LA: Low dose group of *Atractylodes japonica*; MA: Middle dose group of *Atractylodes japonica*; HA: High dose group of *Atractylodes japonica*.

between NG and MG groups and between MG and HA groups were analyzed by OPLS-DA, a supervised discriminant analysis statistical method. It was observed that there was a clear trend of separation between different groups (Figures 7A and C). The permutation test results (values of R2 and Q2) also showed that the model was stable and reliable (Figures 7B and D).

Biomarker screenings and pathway enrichment analysis

A total of 10 endogenous metabolites were identified as potential biomarkers by database analysis (Table 3). The heat map directly reflected the differences in the expression of the relative abundance of metabolites among groups (Figure 8A). MetaboAnalyst 5.0 was used for enrichment analysis of potential biomarkers, and the main metabolic pathways associated with pathway enrichment analysis included arginine biosynthesis, primary bile acid biosynthesis, taurine and hypotaurine metabolism, glycerophospholipid metabolism, arginine and proline metabolism, purine metabolism, steroid hormone biosynthesis (Table 4 and Figure 8B).

DISCUSSION

The acetic acid-induced ulcer model is one of the most commonly used models. Since its development in 1969, it has been widely recognized in the scientific community. Its pathological morphology and repair process are similar to human GUs, with the advantages of easy induction process, good model repeatability, and a high survival rate[19,26]. Consequently, the findings of this study demonstrated that *A. japonica* had a therapeutic impact on gastrointestinal ulcers, with improved recovery outcomes observed across multiple related indexes. According to pathological sections, the ulcer status of rats was significantly improved.

Since metabolites are downstream products of gene and protein expression, they can respond more quickly and provide effective information when the organism is affected[27]. MVA is characterized by the analysis of the statistical rules of multiple interrelated research objects, monitoring variables, and focusing on the internal changes of variables. Therefore, it is widely used in metabolomics research, such as biomarker selection through the construction of models. The research fields cover animals, plants, medicine, the environment, *etc*[28-31]. Typically, the MVA is divided into supervisory and non-supervisory methods. At present, the most commonly used unsupervised recognition mode is PCA [32], which maximizes the extraction of the original information while reducing the dimension of the data. If PCA is not successful in distinguishing subtle differences between sample groups, the supervised models PLS-DA and OPLS-DA can

Table 3 Identified metabolites of plasma

No.	Compound name	R.T. (min)	Formula	Exact mass (m/z)	HMDB ID	MG *vs* NG	*P* value	HA *vs* MG	*P* value
1	L-arginine	0.77	$C_6H_{14}N_4O_2$	174.1116	HMDB0000517	Decreased	< 0.01	Increased	< 0.05
2	Citrulline	0.79	$C_6H_{13}N_3O_3$	175.0956	HMDB0000904	Decreased	< 0.01	Increased	< 0.05
3	Taurine	0.79	$C_2H_7NO_3S$	125.0146	HMDB0000251	Decreased	< 0.05	Increased	< 0.05
4	Adenine	1.42	$C_5H_5N_5$	135.0545	HMDB0000034	Increased	< 0.05	Decreased	< 0.05
5	Glycocholic acid	6.31	$C_{26}H_{43}NO_6$	465.3090	HMDB0000138	Decreased	< 0.05	Increased	< 0.05
6	Aldosterone	7.59	$C_{21}H_{28}O_5$	360.1936	HMDB0000037	Decreased	< 0.05	Increased	< 0.05
7	Glycochenodeoxycholic acid	8.09	$C_{26}H_{43}NO_5$	449.3138	HMDB0000637	Decreased	< 0.05	Increased	< 0.05
8	LysoPC (16:0/0:0)	10.17	$C_{24}H_{50}NO_7P$	495.3324	HMDB0010382	Increased	< 0.01	Decreased	< 0.01
9	LysoPC (18:0/0:0)	11.20	$C_{26}H_{54}NO_7P$	523.3637	HMDB0010384	Increased	< 0.05	Decreased	< 0.05
10	Eicosadienoic acid	13.76	$C_{20}H_{36}O_2$	308.2715	HMDB0005060	Increased	< 0.01	Decreased	< 0.05

MG: Model group; NG: Normal group; HA: High dose group of the crude *Atractylodes japonica*.

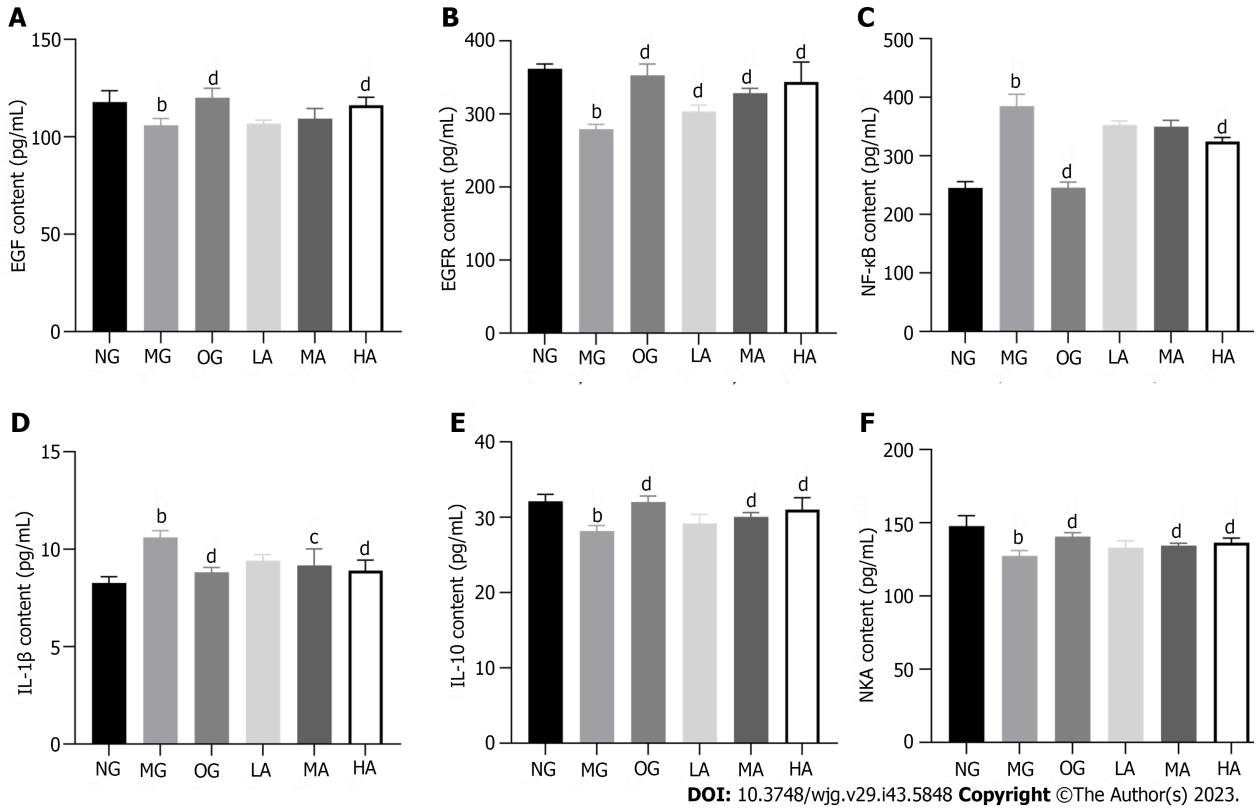
Table 4 Results of enrichment analysis of biomarkers

No.	Pathway name	Match status	*P* value	-Log (*P*)	Impact
1	Primary bile acid biosynthesis	3/46	0.0012354	2.9082	0.0254
2	Arginine biosynthesis	2/14	0.0020576	2.6866	0.30457
3	Taurine and hypotaurine metabolism	1/8	0.040642	1.391	0.42857
4	Glycerophospholipid metabolism	1/36	0.17174	0.76513	0.01736
5	Arginine and proline metabolism	1/38	0.18047	0.74359	0.05786
6	Purine metabolism	1/65	0.29073	0.53651	0.00528
7	Steroid hormone biosynthesis	1/85	0.3638	0.43913	0.01032

be used to maximize the degree of separation between sample groups. The drawback is that data can be overfitted. Therefore, strict cross-validation must be carried out to ensure the reliability of the model. The differentiated metabolites obtained by screening often have functional similarities or complementarity in biology or participate in positive or negative regulation of the same metabolic pathway, which is manifested as similar or opposite expression characteristics between different experimental groups. Therefore, cluster analysis of these characteristics is helpful to speculate on the function of metabolites and explore the mechanism of disease treatment. This study made full use of MVA for metabonomic data analysis in order to make the experimental results more accurate and reliable[33].

Liquid chromatography-mass spectrometry technology has been widely used for the identification and analysis of traditional Chinese medicine or complex unknown substances. In this study, technology was used to analyze the extract of *A. japonica*. Among the identified components, multiple of them have potential biological activity. Terpenoids are the most common active components, of which atractylon and atractylenolide I, II, and III have anti-inflammatory effects and regulate gastrointestinal function[34,35]. The organic acid component chlorogenic acid[36] has obvious antioxidant, antibacterial, anti-inflammatory, antiviral, and other effects. Isoorientin[37], a flavonoid component, has anti-oxidative and anti-inflammatory effects, regulates the intestinal flora, *etc*. These components may be closely related to the treatment of GU and the potential key pharmacological bases of *A. japonica*.

GU is the most common cause of gastric mucosal inflammation and injury. NF-κB is a primary regulator of the inflammatory response, and one of its subunits, NF-κBp65 is the main functional protein. IκB, as a suppressor protein of the NF-κB signaling pathway, when stimulated by injury factors such as ulcers, will activate NF-κB, leading to the degradation of IκB, and subsequently promoting the expression of multiple inflammatory factors[38-40]. Cytokines IL-1β and IL-10 are common indicators of acute inflammation and are closely related to the severity of GUs[41,42]. IL-1β, as a pro-inflammatory factor, regulates the function of various gastric epithelial cells and interacts with NF-κB to cause the release of inflammatory mediators and stimulate the secretion of other cytokines, exacerbating the inflammatory response[43]. IL-10 can inhibit the pro-inflammatory response and limit unnecessary tissue destruction caused by inflammation[44]. The results showed that the level of IL-1β and expression of NF-κBp65 were significantly increased, while the level of IL-10 and expression of IκBα were significantly decreased in GU rats, suggesting that the progression of GU is closely related to inflammation. After the intervention of *A. japonica*, the expression levels of inflammatory factors were significantly re-regulated, which is in accordance with the research results of Hu *et al*[45] and Zhou *et al*[46]. Simultaneously, pathological



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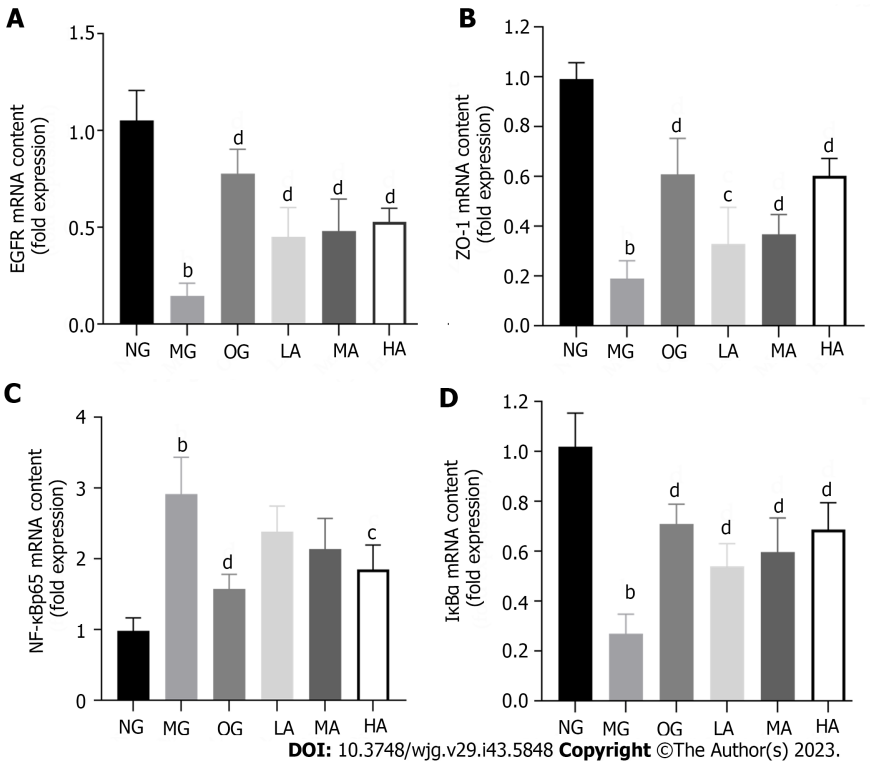
Figure 4 Effect of *Atractylodes japonica* on gastric ulcer-associated factors level in gastric tissues of gastric ulcer rats (*n* = 8). A-F: The epidermal growth factor (EGF), EGF receptor, nuclear factor kappa-B, interleukin-1β (IL-1β), IL-10, and Na^+-K^+-ATPase levels were detected by enzyme-linked immunosorbent assay in gastric tissues of gastric ulcer rats. $^bP < 0.01$ *vs* normal group; $^cP < 0.05$ and $^dP < 0.01$ *vs* model group. EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; NF-κB: Nuclear factor kappa-B; IL: Interleukin; NKA: Na^+-K^+-ATPase; NG: Normal group; MG: Model group; OG: Omeprazole group; LA: Low dose group of *Atractylodes japonica*; MA: Middle dose group of *Atractylodes japonica*; HA: High dose group of *Atractylodes japonica*.

results showed *A. japonica* had a significant protective effect on GU tissue and reduced inflammatory damage to the gastric mucosa. All these results suggest that the gastroprotective effect of *A. japonica* is related to the inhibition of the inflammatory response.

On the other hand, GU causes mucosal damage and destroys the structure and function of the gastric mucosa. EGF is an endogenous substance that can inhibit gastric acid secretion, promote epithelial cell proliferation, tissue repair, and cytoprotection, and is an important factor in promoting wound healing. Its receptor EGFR also plays an important role in cell proliferation and other effects, both of which generally combine and activate downstream effectors to promote the repair and healing of injured mucosa[47,48]. ZO-1 is a bridging protein, which is an important component of tight junctions; most of them are located at the junctions between cells. It mainly interacts with extracellular signal transduction pathways and the cytoskeleton, and the normal expression of ZO-1 is closely related to mucosal integrity[49, 50]. It plays an important role not only in regulating the transport of intracellular substances and maintaining epithelial polarity but also in cell proliferation and differentiation. According to the results, the ulcer may destroy the integrity and permeability of the gastric mucosa, leading to the degradation of the protein structure[51]. NKA is a ubiquitous transmembrane protein that maintains the normal function of mucosal cells and membrane permeability and maintains Na^+ and K^+ gradients across the cell membrane through energy from adenosine triphosphate (ATP). External stimulation will cause abnormal function of this enzyme and mucosal damage[52]. According to the results, the expression levels of EGF, EGFR, and ZO-1 were significantly decreased in GU rats, suggesting that GU leads to impaired mucosal barrier function and disrupted structure. After intervention, the expression levels of all factors were significantly increased, indicating that *A. japonica* could promote ulcer healing and improve gastric mucosal function. Based on the above pharmacodynamic results, it was found that the HA group had the best effect among the treatment groups, so the HA group was selected for further metabolic analysis.

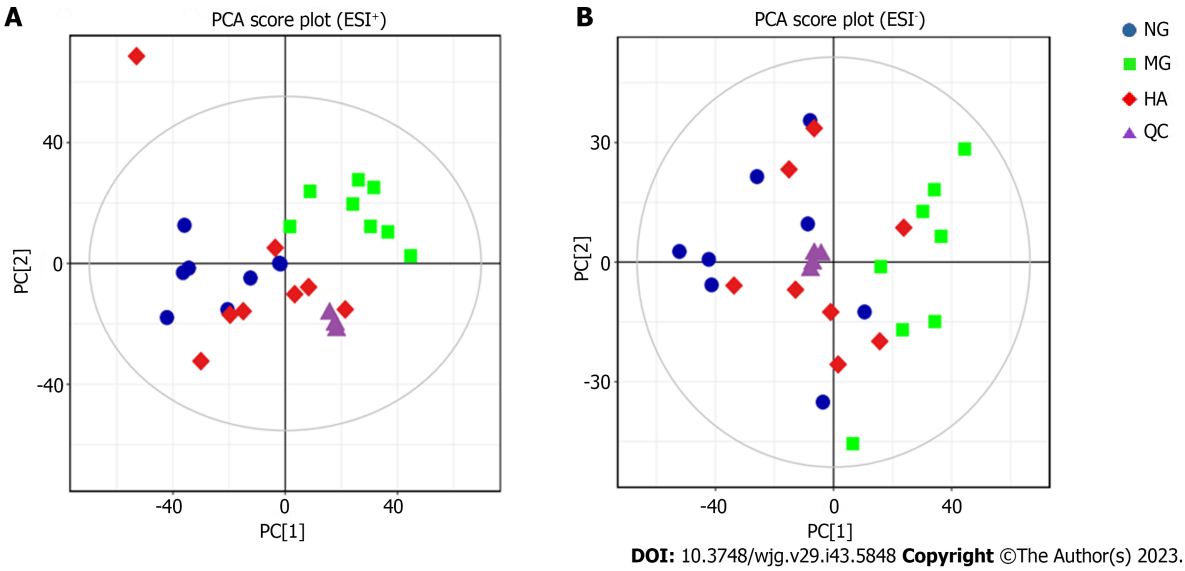
The metabolomic study showed that GUs are involved in multiple metabolic pathways. Amino acid metabolism: L-arginine has a variety of biological effects, including gastric protection and promoting ulcer healing properties. Its metabolite, citrulline, has been confirmed to protect gastric mucosa from ulcer-induced mucosal damage and regulate mucosal integrity[53,54]. Taurine is also a cytoprotective factor, maintaining the storage of an important antioxidant and free radical scavenger glutathione in the body, increasing membrane stability, and preventing inflammation and gastric mucosal damage[55,56]. According to the results, the levels of amino acids decreased in the MG group, which indicates an amino acid metabolic disorder; however, *A. japonica* can significantly recover the levels of amino acids.

Lipid metabolism: Multiple experimental studies have shown that lysophosphatidylcholines are a class of metabolites associated with inflammatory damage caused by GU, which can induce impairment of gastric mucosal barrier function,



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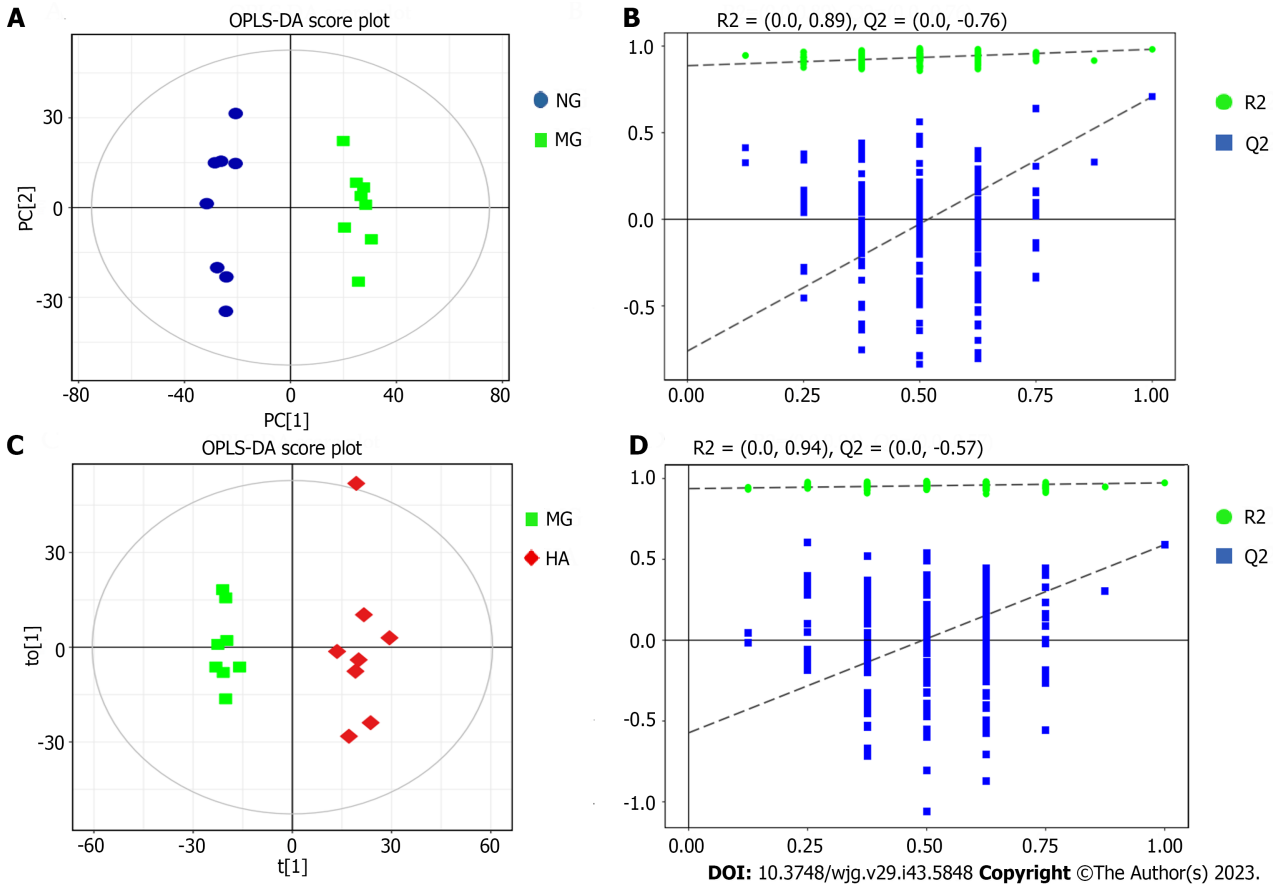
Figure 5 Effect of *Atractylodes japonica* on gastric ulcer-associated genes in acid-induced gastric tissues (*n* = 8). A-D: The mRNA levels of epidermal growth factor receptor, Zonula Occludens-1, nuclear factor kappa-B p65, IkappaBalpha in gastric tissues were analysed by real-time reverse transcription polymerase chain reaction. $^{b}P < 0.01$ *vs* normal group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* model group. EGFR: Epidermal growth factor receptor; ZO-1: Zonula Occludens-1; NF-κB: Nuclear factor kappa-B; IκBα: IkappaBalpha; NG: Normal group; MG: Model group; OG: Omeprazole group; LA: Low dose group of *Atractylodes japonica*; MA: Middle dose group of *Atractylodes japonica*; HA: High dose group of *Atractylodes japonica*.



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Figure 6 Principal component analysis score plots of plasma metabolomics analysis (*n* = 8). Principal component analysis score plots among normal group, model group, high dose group of *Atractylodes japonica* and quality control groups. A: Positive ion mode(ESI^{+}); B: Negative ion mode(ESI^{-}). PCA: Principal component analysis; NG: Normal group; MG: Model group; HA: High dose group of *Atractylodes japonica*; QC: Quality control.

leading to gastric mucosal damage[57,58]. Eicosadienoic acid is an n-6 polyunsaturated fatty acid with certain pro-inflammatory activity, which can not only metabolize to the eicosanoid precursor compound arachidonic acid to promote an inflammatory response but also promote the expression of inflammatory mediators such as NF-κBp65 to cause mucosal inflammatory damage[59,60]. Another study has found that eicosadienoic acid can be positively associated with ulcerative colitis[61]. Furthermore, since reactive oxygen species (ROS) is an important factor in the early stage of inflammation and mediates the onset of inflammation, eicosadienoic acid can act by reducing ROS level induced by lipopolysaccharide and was presumed to have potential anti-inflammatory activity to some extent[62]. The dualism effect of this



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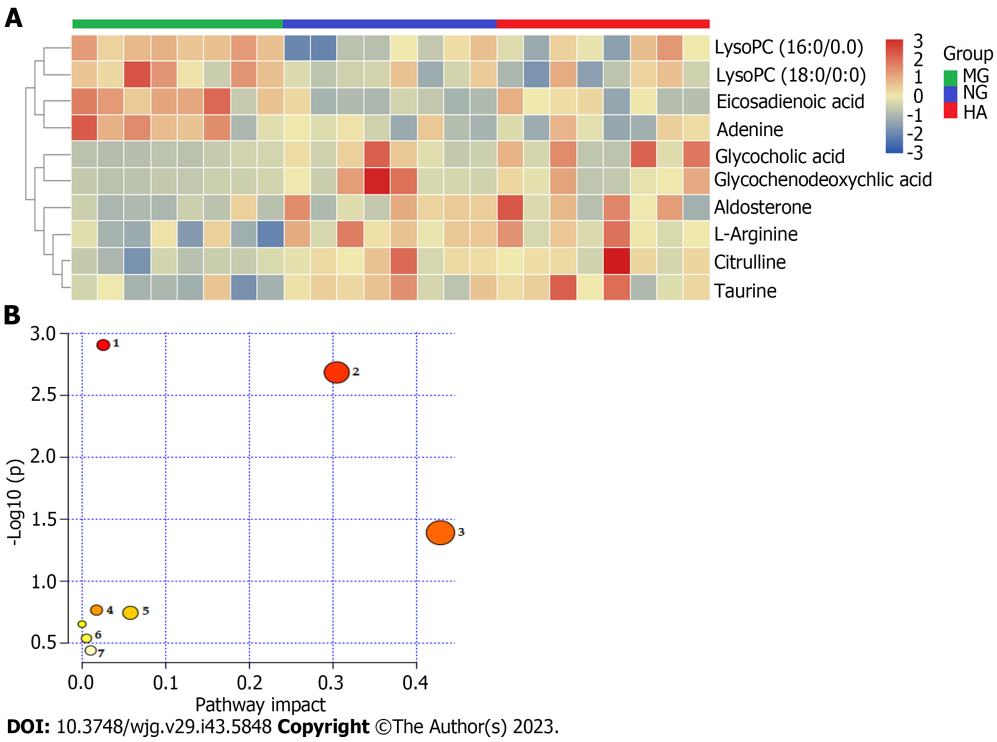
Figure 7 Orthogonal projections to latent structures-discriminant analysis score plots and 200-permutation test of plasma metabolomics analysis (*n* = 8). A: Orthogonal projections to latent structures-discriminant analysis (OPLS-DA) score plots between normal group (NG) and model group (MG) groups; B: 200-permutation test between NG and MG groups; C: OPLS-DA score plots between MG and high dose group of *Atractylodes japonica* (HA) groups; D: 200-permutation test between MG and HA groups. OPLS-DA: Orthogonal projections to latent structures-discriminant analysis; MG: Model group; HA: High dose group of *Atractylodes japonica*.

metabolite may be related to the disease and the course of disease. It is worth further exploring. According to the results, the levels were significantly increased in the MG group, suggesting that lipid metabolism disorder is one of the metabolic pathways exacerbating ulcers, and the levels recovered after intervention.

Bile acids are a class of cholesterol derivatives that regulate a variety of metabolic and inflammatory pathways[63]. We found that the levels of glycochenodeoxycholic acid and glycocholic acid in the MG decreased significantly in this study. According to existing reports, glycochenodeoxycholic acid promotes cell proliferation in a concentration-dependent manner through activation of Gαi, reduction of cyclic AMP, and an increase in H2AX phosphorylation[64]. The Farnesoid X receptor (FXR), also known as the bile acid receptor, regulates lipid metabolism, mitigates the inflammatory response, and enhances barrier function. Some studies showed that glycocholic acid can improve tissue growth performance, reduce tissue damage, and play a protective role in mucous membranes by activating FXR[65]. In our study, the levels of two kinds of cholic acid were significantly reduced in GU rats. It is hypothesized that ulcers may disrupt barrier function and activate the inflammatory response by interfering with the bile acid metabolic pathway. Treatment with *A. japonica* significantly alleviates the inflammatory response and improves metabolite levels. Stepien *et al*[66] have shown that these two cholic acids increased in a variety of liver diseases, such as hepatocellular carcinoma. Aragonès *et al*[67] found that the level of glycocholic acid was significantly elevated in patients with non-alcoholic fatty liver disease. It is suggested that the same metabolite may have opposite tendency for different diseases.

The adrenal cortex produces the steroid hormone aldosterone, which regulates electrolyte and water balance by increasing sodium renal retention and potassium excretion. There are a few studies on aldosterone-related GU. Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) are closely related to ulcers. MMPs belong to a class of endogenous proteolytic enzymes that generally aggravate the inflammatory response and prolong healing time. TIMPs are endogenous specific suppressors of MMPs, which play a role in the process of ulcer healing by inhibiting and regulating MMPs[68]. Studies have shown that aldosterone can induce TIMP-1 secretion and promote collagen accumulation[69]. It is speculated that ulcers interfered with aldosterone production and that downstream factors could not play a healing role. However, treatment with *A. japonica* alleviated inflammation and promoted ulcer healing.

Adenine is a component of adenosine. When adenosine reacts with three phosphate groups, it forms the nucleotide ATP, the body's most direct source of energy. Adenine increased in the MG group, suggesting that ulcers damaged the body's energy metabolism, resulting in blocked ATP synthesis and adenine accumulation. When *A. japonica* intervened to restore the process of energy metabolism, the levels of adenine decreased.



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Figure 8 Enrichment analysis of metabolic markers (*n* = 8). A: Heatmap of potential biomarkers and the degree of the changes are marked in red (up-regulation) and blue (down-regulation); B: Metabolic pathways involved in the therapeutic effects of *Atractylodes japonica* on gastric ulcer. NG: Normal group; MG: Model group; HA: High dose group of *Atractylodes japonica*.

CONCLUSION

In this study, 48 potential bioactive compounds were identified by UPLC–MS/MS that may be active components of GU. Additionally, the pathogenesis of acetic acid-induced GU in rats and the therapeutic effect of *A. japonica* were explored from the perspective of metabonomics for the first time. The results showed that *A. japonica* could correct the metabolic disorder of GU with its gastroprotective effect and effectively relieve mucosal inflammatory injury. The specific anti-ulcer effect is closely related to the anti-inflammatory activity produced by down-regulating NF-κB and IL-1β and up-regulating IL-10 and IκBα, as well as the gastric protective effect produced by up-regulating EGF, EGFR, ZO-1, and NKA. Combined with omics enrichment of metabolic pathways, we found that lipid metabolism, amino acid metabolism, and other metabolic pathways play a protective role in the stomach through anti-inflammatory, antioxidative, ulcer healing, and other functions. This study provides a research direction for the potential mechanism of *A. japonica* in the treatment of GU, which is of great significance for its drug development and clinical application.

ARTICLE HIGHLIGHTS

Research background

Gastric ulcer (GU) is a common digestive system disease. In addition to western medicine treatment, more and more Chinese herbs have become the first choice for alternative treatment due to their long history of use, high efficacy, low side effects and low price.

Research motivation

Although the herbal medicine *Atractylodes japonica* Koidz. ex Kitam. (*A. japonica*) has an obvious therapeutic effect on GU, there are few relevant mechanism studies at present.

Research objectives

The object of this study is to investigate the protective effects of *A. japonica* on acetic acid-induced GU rats.

Research methods

We used ultra performance liquid chromatography tandem mass spectrometry, hematoxylin-eosin stain, enzyme-linked immunosorbent assay, real-time reverse transcription polymerase chain reaction and acetic acid-induced GU model to evaluate the therapeutic effect of *A. japonica*.

Research results

48 chemical constituents of *A. japonica* were identified, the herb significantly improved the pathological damage of gastric tissues, increased the expression levels of anti-inflammatory factors, decreased the expression levels of proinflammatory factors, restored the levels of factors about ulcer healing and energy metabolism. and identified 10 potential differential metabolites and enriched 7 related metabolic pathways of metabolomic analysis.

Research conclusions

The therapeutic effect of *A. japonica* on GU rats is closely related to anti-inflammation and repair of gastric injury, and is regulated and treated through a combination of multiple pathways.

Research perspectives

These findings contribute to the understanding of the potential mechanism of *A. japonica* to improve acetic acid-induced GU, and will provide great importance for the development and clinical application of *A. japonica.*

FOOTNOTES

Author contributions: Li F and Cai Q designed the experiment; Zhen BX completed the whole experiment and wrote the manuscript; and all authors approved the final version of the article.

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CASE REPORT

Pediatric-type follicular lymphoma in a Crohn's disease patient receiving anti-α4β7-integrin therapy: A case report

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Abstract

BACKGROUND

Patients with autoimmune conditions receiving immunosuppressants are at risk of non-Hodgkin lymphomas (NHL). Vedolizumab (anti-α4β7-integrin antibody), a treatment-of-choice for Crohn's disease (CD), reduces inflammatory lymphocyte trafficking into the intestinal mucosa. This effect is believed to be confined to the colon.

CASE SUMMARY

We report the case of a CD patient on vedolizumab for five years who developed pediatric-type follicular lymphoma. Work-up prior to therapy revealed a reduction in circulating T-lymphocytes and their suppressed response to mitogens. Rituximab, cyclophosphamide, vincristine, and prednisone chemo-immunotherapy resulted in durable lymphoma remission, and vedolizumab treatment was continued. While the patient's T-lymphocyte population and immunoglobulin production recovered, the T-lymphocyte mitogen response remained suppressed.

CONCLUSION

This patient's NHL may be linked to receiving anti-α4β7 therapy. Further research could be beneficial to determine if proactive surveillance for NHL and other systemic diseases is indicated in patients on vedolizumab.

Key Words: Pediatric-type follicular lymphoma; Crohn's disease; Vedolizumab; Immunosuppression; Non-Hodgkin lymphoma; Case report

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Core Tip: The literature is inconclusive on the association between anti-α4β7-integrin therapy and oncogenesis. This case report highlights a young adult on chronic vedolizumab, a monoclonal antibody targeting α4β7-integrin, who develops pediatric-type follicular lymphoma. The patient recovered with rituximab, cyclophosphamide, vincristine, prednisone immunotherapy, but T-lymphocyte mitogen response remained suppressed.

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INTRODUCTION

Follicular lymphoma (FL) is one of the most common non-Hodgkin lymphomas (NHL) worldwide[1]. Pediatric-type FL (PTFL) was first identified as a distinct clinicopathological condition in the 4th edition of the World Health Organization classification of lymphoid neoplasms[2]. PTFL lacks the t(14;18) translocation characteristic of adult-type FL that leads to overexpression of the B-cell leukemia/lymphoma 2 (*BCL2*) oncogene. Instead, the most commonly affected gene in PTFL is *MAP2K1* on chromosome 15 encoding the serine/threonine kinase MEK1[3]. MEK1 plays a role in the RAS-MAPK signaling pathway and directs cell growth, differentiation, and apoptosis. PTFL commonly presents with localized disease (stage I or II) involving lymph nodes of the head and neck region. Hilar and mediastinal lymph nodes may also be involved. The adenopathy is typically painless and without mass effect on adjacent anatomical structures[4]. The disease course of PTFL is indolent and cure rate is high. Standard-of-care treatment approaches are surgical resection *versus* rituximab, cyclophosphamide, vincristine, and prednisone-like regimens for patients with advanced or unresectable conditions[5].

Patients with autoimmune conditions such as inflammatory bowel disease (IBD) and those treated with systemic immunosuppressants are at risk of developing lymphoid malignancies[6,7]. The incidence of such comorbidities remains low, and reports are limited to adult patient cohorts. Crohn's disease (CD) is a chronic IBD with increasing incidence in the United States over the past several decades. The pathogenesis of Crohn's is mediated by auto-reactive T-cells migrating into intestinal tissue, perpetuating inflammation and tissue necrosis. Targeted therapies have been developed to block inflammatory cell activation and migration in an organ-specific manner[8,9]. Vedolizumab is an anti-α4β7 integrin monoclonal antibody that inhibits interaction of T cells, monocytes, and dendritic cells with the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed on vascular endothelium. Paradoxically, ligation of α4β7 results in downregulation of genes controlling expression of innate immune receptors, chemokines, and their cognate receptors. Previous clinical studies suggest that vedolizumab does not have a systemic immunosuppressive effect and does not increase risk for malignancy[10,11].

We present a case of a 27-year-old male with CD who was diagnosed with PTFL five years into treatment with vedolizumab. Surprisingly, at the time of diagnosis, the patient had reduced numbers and suppressed function of circulating T cells, potentially contributing to a permissive immune environment for PTFL development. This case provides a useful addition to the existing knowledge of PTFL putative risk factors and vedolizumab systemic effects on the immune system.

CASE PRESENTATION

Chief complaints

A 27-year-old male presented with a painless mass in his right mandibular fossa.

History of present illness

Review of systems was negative. The patient denied constitutional B symptoms (fever, night sweats, weight loss), signs of gastrointestinal distress (*e.g.*, pain, nausea, vomiting, diarrhea), and oral pain or dysphagia.

History of past illness

Past medical history was pertinent for CD on long-term vedolizumab therapy.

Personal and family history

All other personal and family medical history was noncontributory.

Physical examination

Physical exam appreciated submandibular and upper cervical lymphadenopathy; there were no other concerning findings. Abdomen was soft, non-tender, non-distended, and without palpable masses; bowel sounds were present.

Laboratory examinations

Core-needle biopsy of the parotid mass demonstrated neoplastic infiltrate with a follicular pattern. The follicles did not have polarized germinal centers and immunophenotype. The neoplastic cells were intermediate-sized with scant cytoplasm and irregular nuclei. The neoplastic cells expressed CD10, CD20, BCL6, PAX5, and CD23; they did not express CD3, CD5, CD21, cyclin D1, BCL2, MUM1, or BCL2. Ki-67 stain was positive in approximately 70% of cells (Figure 1). Molecular studies identified a MAP2K1p.g128D mutation.

Imaging examinations

Computed tomography confirmed the mass within the parotid gland encasing the right-sided facial nerve.

FINAL DIAGNOSIS

The above pathomorphological and molecular features were consistent with FL, pediatric-type.

TREATMENT

Pre-therapy baseline immunologic testing revealed reduced absolute and relative levels of circulating $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells (Table 1). *In vitro* mitogen stimulation with phytohemagglutinin (PHA) and concanavalin A (ConA) also revealed suppressed proliferative response (Table 2). Of interest, while the peripheral blood B-cell population had a polyclonal pattern, the T-lymphocyte pool demonstrated expansion of two distinct T cell receptor (TCR)-rearranged monoclonal populations (Table 1), a phenomenon characteristic for autoimmune conditions such as IBD[12,13]. The patient was also positive for Epstein-Barr virus (EBV) viral capsid antigen (VCA) immunoglobulin (Ig)G [antibody index (AI) 7.1]; negative for EBV VCA IgM (0.2 AI); positive for EBV early antigen Ab (1.5 AI); and positive for EBV nuclear antigen Ab (7.8 AI), indicating chronic EBV viremia/reactivation. The parotid mass tissue was not stained for EBV, and EBV DNA quantification in blood was not performed.

The parotid gland was not amenable to surgical resection or local radiation therapy; therefore, 6 cycles of rituximab, cyclophosphamide, vincristine, and prednisone chemo-immunotherapy were administered concurrently with monthly vedolizumab. Complete remission for the lymphoma was successfully achieved with good control of CD symptoms (no recurrence or flares).

OUTCOME AND FOLLOW-UP

Repeat assessment at 12 mo off-therapy revealed ongoing complete remission of both PTFL and CD. Of note, one of the TCR-rearranged T-lymphocyte clones became undetectable (Table 1). Surprisingly, functional suppression of peripheral blood T-cells persisted. It remains unclear whether recurrent immune suppression at this clinical stage may only be attributed to continued vedolizumab therapy.

DISCUSSION

Lymphoproliferative disorders (including NHL) have long been recognized as complications for autoimmune inflammatory conditions[14]. Latent infection with EBV (as in this patient) or human herpesvirus 8 may also contribute to cancer genesis[15]. Vedolizumab, the monoclonal antibody against α4β7-integrin, uniquely inhibits interaction of T cells, monocytes, and dendritic cells with the vascular endothelium to reduce local inflammation for disease control[10]. While serious adverse events are reported in 41% of Crohn's patients, benign and malignant neoplasms are noted in only 6.8% of treated patients at an incidence rate of 20.8 per 1000 person-years[16]. Several other studies suggested that treatment with vedolizumab is rather safe and does not carry an excessive burden of new or recurrent malignancies[17].

However, recent insights into the mechanisms of vedolizumab's biologic activity suggest it may extend beyond inhibition of the α4β7-integrin interaction with MAdCAM-1. It appears that several important genes regulating immune effectors and mechanisms of their cross-talk *via* chemokines and cytokines [*e.g.*, CXC chemokine ligand (CXCL)9, CXCL10, FCGR3B, interleukin (IL)23A, IL17, interferon-γ] were down-regulated in IBD patients who achieved clinical remission with vedolizumab[18,19]. Additional findings suggest that the interaction of α4β7-integrin with MAdCAM-1 may serve as an alternative co-stimulatory pathway for T cells, triggering expression of genes encoding multiple cytokines (*e.g.*, IL-2, IL-3, IL-4, IL-8, IL-13, IL-17A, IL-17F, IL-22) in a similar fashion to CD28-mediated signaling. This potentially implies that inhibition of α4β7-integrin-MAdCAM-1 interaction results in down-regulation of T-cell function, although the argument requires further research[20].

Our patient was found to have decreased numbers of both $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells as well as a diminished response to ConA and PHA at the time of NHL diagnosis, *i.e.*, while receiving ongoing vedolizumab therapy. At the end of lymphoma chemo-immunotherapy and at 12 mo post-therapy, peripheral blood lymphocyte subset quantification demonstrated improvement of the absolute $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cell counts; however, the proliferative response

Table 1 Peripheral blood lymphocyte subsets, and B- and T-lymphocyte clonality

Component	Ref range & units	Testing time point		
		Pre-Tx	EOTx	12 mo off Tx
$CD3^+$ T cell	60%-89%	43	72	52
$CD3^+$ T cell	958-2388 cells/uL	473	792	869
$CD3^+CD4^+$ T cell	34%-61%	27	39	32
$CD3^+CD4^+$ T cell	533-1674 cells/uL	304	425	526
$CD3^+CD8^+$ T cell	10%-41%	14	28	18
$CD3^+CD8^+$ T cell	175-958 cells/uL	151	303	293
$CD19^+$ B cell	5%-22%	20	0	23
$CD19^+$ B cell	75-660 cells/uL	224	0	385
NK cell	5%-25%	37	27	24
NK cell	102-565 cells/uL	406	297	394
CD4/CD8 ratio	1.10-3.25	2.02	1.40	1.79
B cell clonality				
IGH FR1		Nonclonal		Nonclonal
IGH FR2		Nonclonal		Nonclonal
IGH FR3		Nonclonal		Nonclonal
IGH DH1-6-J		Nonclonal		Nonclonal
IGK V-J		Nonclonal		Nonclonal
IGK V-Kde		Nonclonal		Nonclonal
TCR clonality				
TCRB A (V-J)		Nonclonal		Nonclonal
TCRB B (V-J)		Nonclonal		Nonclonal
TCRB C (V-J)		Clonal peak (180 bp)		Clonal peak (180 bp)
TCRG D		Clonal peak (189 bp)		Nonclonal

pre-Tx: Pre-treatment; EOTx: End of therapy; 12 mo off Tx: 12 mo off therapy anti-lymphoma therapy; NK: Natural killer; TCR: T cell receptor.

Table 2 Response of peripheral blood lymphocytes to *in vitro* mitogen stimulation

Component	Reference range & units	Testing time point	
		Pre-Tx	12 mo off Tx
Mitogen control	> 50 CPM	434	2297
Phytohemagglutinin	≥ 188800 CPM	173643	125008
Pokeweed mitogen	> 68549 CPM	77631	76811
Concanavalin A	> 81283 CPM	49198	92755

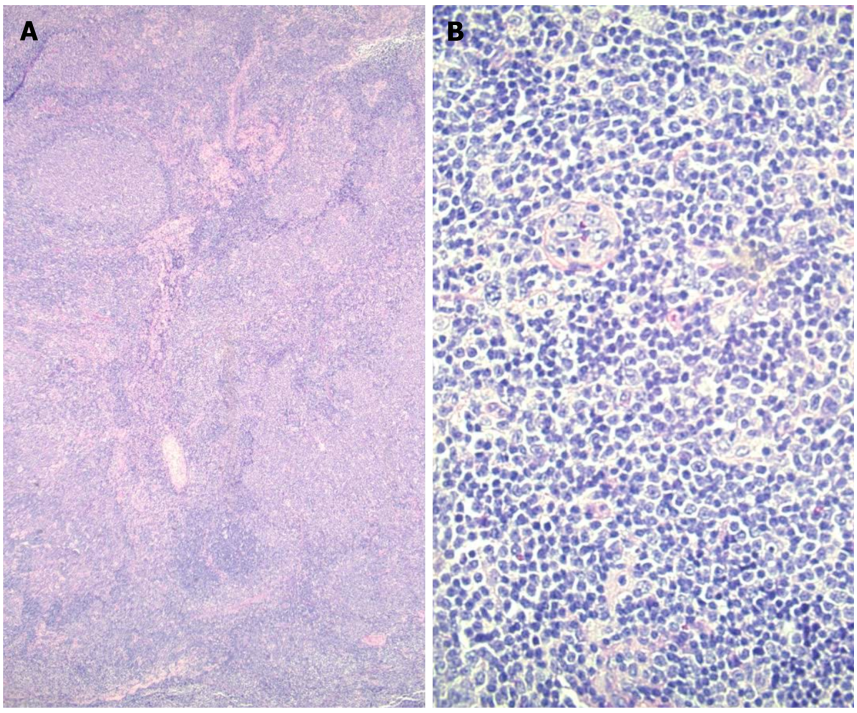
CPM: Counts per million; pre-TX: Pre-treatment; 12 mo off Tx: 12 mo off therapy anti-lymphoma therapy.

to ConA remained suppressed. At this phase of the clinical course, reduced response to the *in vitro* mitogen stimulation may be attributable to vedolizumab, although the suppressive effect of an auto-inflammatory state cannot be ruled out. Further research is necessary to discern if vedolizumab has responsible mechanisms. Regardless, the patient was able to recover his $CD19^+$ B-cell population, which was completely depleted by rituximab at the end of treatment, as well as maintain immunoglobulin production (Table 3). Surprisingly, one of the two clonally expanded populations of T-cells were not detectable following therapy completion, suggesting eradication by the anti-lymphoma chemotherapy.

Table 3 Immune globulin production before treatment, at the end of therapy and at 12 mo off therapy follow up

Component	Ref range & units	Testing time point		
		Pre-Tx	EOTx	12 mo off Tx
IgA	68-408 mg/dL	250	177	170
IgM	35-263 mg/dL	55	30	31
IgG	768-1632 mg/dL	1376	921	927
IgG Subclass 1	240-1118 mg/dL	763	-	503
IgG Subclass 2	124-549 mg/dL	371	-	335
IgG Subclass 3	21-134 mg/dL	162 (H)	-	64
IgG Subclass 4	1-123 mg/dL	64	-	40

Ig: Immunoglobulin; pre-Tx: Pre-treatment; EOTx: End of therapy; 12 mo off Tx: 12 mo off therapy anti-lymphoma therapy.



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Figure 1 Histopathological appearance and immunophenotype of pediatric-type follicular lymphoma. A and B: Typical appearance of pediatric-type follicular lymphoma at low- (A, 4 ×) and high-power (B, 40 ×) resolution of hematoxylin and eosin staining lymphoma tissue. Pattern of expression of CD10, CD20, BCL6 and Ki67 at 20 × resolution is also shown.

CONCLUSION

PTFL is a rare type of B-cell NHL. The clinical course is indolent despite a high proliferative index and blastoid histopathological features. Due to its rarity, predisposing factors have not been clearly postulated. The role of underlying immune suppression in PTFL pathogenesis has not been established. Nodal PTFL is believed to arise from B-lymphocytes in follicle germinal centers. Immune system workup and cytogenetic analysis in PTFL patients may help reveal an etiological correlation between the malignancy and immunomodulatory processes. Attention must also be paid to possible systemic consequences of vedolizumab therapy hitherto unreported in the literature.

FOOTNOTES

Author contributions: Buhtoiarov I was the patient's primary oncology attending, he provided the patient charts with informed consent, prepared images and tables for submission, and edited the manuscript; Yerigeri K prepared initial manuscript drafts and managed the submission and revisions.

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ORIGINAL ARTICLE

Clinical and Translational Research

Causal associations between inflammatory bowel disease and anxiety: A bidirectional Mendelian randomization study

Ying He, Chun-Lan Chen, Jian He, Si-De Liu

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Abstract

BACKGROUND

Anxiety is common in patients with inflammatory bowel disease (IBD), including those with ulcerative colitis (UC) and Crohn's disease (CD); however, the causal relationship between IBD and anxiety remains unknown.

AIM

To investigate the causal relationship between IBD and anxiety by using bidirectional Mendelian randomization analysis.

METHODS

Single nucleotide polymorphisms retrieved from genome-wide association studies (GWAS) of the European population were identified as genetic instrument variants. GWAS statistics for individuals with UC (6968 patients and 20464 controls; adults) and CD (5956 patients and 14927 controls; adults) were obtained from the International IBD Genetics Consortium. GWAS statistics for individuals with anxiety were obtained from the Psychiatric Genomics Consortium (2565 patients and 14745 controls; adults) and FinnGen project (20992 patients and 197800 controls; adults), respectively. Inverse-variance weighted was applied to assess the causal relationship, and the results were strengthened by heterogeneity, pleiotropy and leave-one-out analyses.

RESULTS

Genetic susceptibility to UC was associated with an increased risk of anxiety

[odds ratio: 1.071 (95% confidence interval: 1.009-1.135), P = 0.023], while genetic susceptibility to CD was not associated with anxiety. Genetic susceptibility to anxiety was not associated with UC or CD. No heterogeneity or pleiotropy was observed, and the leave-one-out analysis excluded the potential influence of a particular variant.

CONCLUSION

This study revealed that genetic susceptibility to UC was significantly associated with anxiety and highlighted the importance of early screening for anxiety in patients with UC.

Key Words: Inflammatory bowel disease; Anxiety; Causal effect; Mendelian randomization; Genome-wide association studies

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Core Tip: Our study provides evidence that genetic susceptibility to ulcerative colitis (UC) is associated with an increased risk of anxiety [odds ratio: 1.071 (95% confidence interval: 1.009, 1.135), P = 0.023], while genetic susceptibility to Crohn's disease (CD) is not associated with an increased risk of anxiety. No causal effects of anxiety on UC and CD were observed in this study. In conclusion, our study demonstrates the causal effect of UC on anxiety. These findings may be helpful to increase physicians' awareness of the need to recognize anxiety in UC patients and influence the management of anxiety in clinical practice.

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INTRODUCTION

Inflammatory bowel disease (IBD), mainly composed of ulcerative colitis (UC) and Crohn's disease (CD), is a debilitating chronic inflammatory disease with varying degrees of severity[1,2]. UC and CD influence not only the gastrointestinal tract but also other systems[3]. Emerging evidence has reported the role of the gut-brain axis in the interactions between gastrointestinal diseases and neuropsychiatric disorders[4,5]. Anxiety is a common comorbidity in patients with IBD (prevalence varies from 19.1% to 35.1%) compared with the general population (3.4%)[4,6]. The association between IBD and anxiety, or vice versa, has received considerable attention due to the putative pathophysiological mechanisms regulated by the gut-brain axis.

Some observational studies have investigated the temporal relationship between IBD and anxiety and have suggested that the relationship between IBD and anxiety may be bidirectional[7]. Patients with IBD might be at higher risk for anxiety than control individuals. Specifically, newly diagnosed patients with IBD had a rising prevalence (incidence rate ratio: 1.39) of anxiety when compared with matched control individuals during 10 years of follow-up[7]. However, some observational studies have shown that patients with anxiety are prone to suffering from IBD[8,9]. A cohort study suggested a higher prevalence of IBD in patients with newly diagnosed anxiety compared with control individuals during 6.7 years of follow-up[9]. Collectively, the existing findings from observational studies demonstrated the bidirectional relationships between IBD and anxiety, which is partly influenced by residual confounders. Therefore, more evidence is needed to clarify the causal relationships between IBD and anxiety. A recent Mendelian randomization (MR) study inferred the causal relationships between IBD and depression and demonstrated a causal effect of depression on IBD but no causal effect of IBD on depression[10]. Depression and anxiety are common co-occurrence in IBD, while the causality between IBD and anxiety has not been investigated.

MR is a genetic approach to estimate causality between the exposure and the outcome by using genetic instrument variants (IVs) identified through genome-wide association studies (GWAS), usually using single-nucleotide polymorphisms (SNPs) as IVs. Since genetic makeup is assigned at conception and is unlikely to be affected by disease later in life, unidirectional causality can be deduced by MR analysis. Potential confounders that could affect the outcomes were eliminated from the analysis, effectively forming naturally blinded randomized controlled trials[11]. Therefore, this study aimed to evaluate the causal associations between IBD and anxiety by performing bidirectional two-sample MR analysis.

MATERIALS AND METHODS

GWAS statistics sources for UC, CD and anxiety

The International IBD Genetics Consortium (IIBDGC), a large-scale consortium consisting of hundreds of researchers from more than 20 countries worldwide, collects GWAS data from over 75000 patients with IBD. The IIBDGC is an

authoritative organization aimed at identifying genetic risk factors for IBD. GWAS summary statistics for UC and CD were obtained from the IIBDGC[12], which contains adult individuals of European descent with UC (6968 patients, 20464 control individuals) and CD (5956 patients, 14927 control individuals) (Supplementary Table 1). UC and CD were diagnosed by physicians based on comprehensive evidence of clinical symptoms, endoscopic findings, and histopathological and imaging results.

GWAS summary statistics for anxiety were obtained from two separate databases (Supplementary Table 1): (1): The Psychiatric Genomics Consortium (PGC) (https://pgc.unc.edu/) from Otowa *et al*[13]; and (2) the FinnGen project (https://www.finngen.fi/en). The PGC[14], the largest international psychiatric consortium consisting of more than 800 investigators from 38 countries, is dedicated to finding the genetic variants of psychiatric disorders. The FinnGen project is an academic-industrial collaboration aimed at deciphering genotype-phenotype relationships from more than 500000 Finnish participants. The participants in the GWAS databases for anxiety were adults of European descent. The number of patients and control individuals for anxiety were 2565/14745 in PGC and 20992/197800 in FinnGen, respectively.

We used published GWAS statistics and did not collect initial data. Patient informed consent and ethics approval were not needed for this study, as these materials had already been obtained in each of the preliminary studies.

Genetic IV selection

To select eligible SNPs as IVs from the GWAS statistics, a series of quality control steps were applied. The three following assumptions must be satisfied[11]: (1) Correlation assumption: The IV is strongly correlated with the exposure; (2) independence assumption: The IV does not influence the outcome through the confounding factors; and (3) exclusion assumption: the IV does not directly influence the outcome, but only influences the outcome *via* indirect exposure (Figure 1).

To satisfy the correlation assumption, the following criteria were set for identifying instrumental SNPs: (1): Genome-wide strongly significant ($F > 10$, $P < 5 \times 10^{-8}$) association with the exposure. For a single variant, the F statistic, which should be over 10 to avoid weak instrument bias, was calculated by the following equation[15]: $F = [\beta/se]^2$, where β means estimated effect size and se means standard error of β; and (2) independent SNPs are selected by linkage disequilibrium (LD) clumping ($r^2 < 0.001$, window size = 1 Mb). To satisfy the independence and exclusion assumption, we checked each SNP associated with the exposure at PhenoScanner (http://www.phenoscanner.medschl.cam.ac.uk/) and eliminated SNPs significantly related to the potential confounders and the outcome. The potential confounders that may influence anxiety include smoking, body mass index, neuropsychiatric disease, drinking, and hypertension[16,17]. The potential confounders that may influence UC or CD include smoking, body mass index, and intestinal malabsorption[18,19]. The subsequent harmonization process was used to unify the effect direction and effect allele, ensure SNPs with a minor allele frequency (> 0.01), and remove the palindromic and incompatible SNPs.

For MR estimation from UC or CD to anxiety, the selection criteria of SNPs associated with UC or CD satisfy the abovementioned three assumptions. For MR estimation from anxiety to UC or CD, no eligible SNPs associated with anxiety could be obtained from GWAS statistics in PGC after LD clumping, so we used GWAS statistics from the FinnGen project to extract anxiety-related SNPs. Because anxiety-related SNPs could not be obtained by the statistical P value of $< 5 \times 10^{-8}$, we used a suggested P value of $< 5 \times 10^{-6}$ to extract SNPs, which had been applied in a previous study to decipher bidirectional relationships between prescription opioid use and anxiety risk by MR analysis[20].

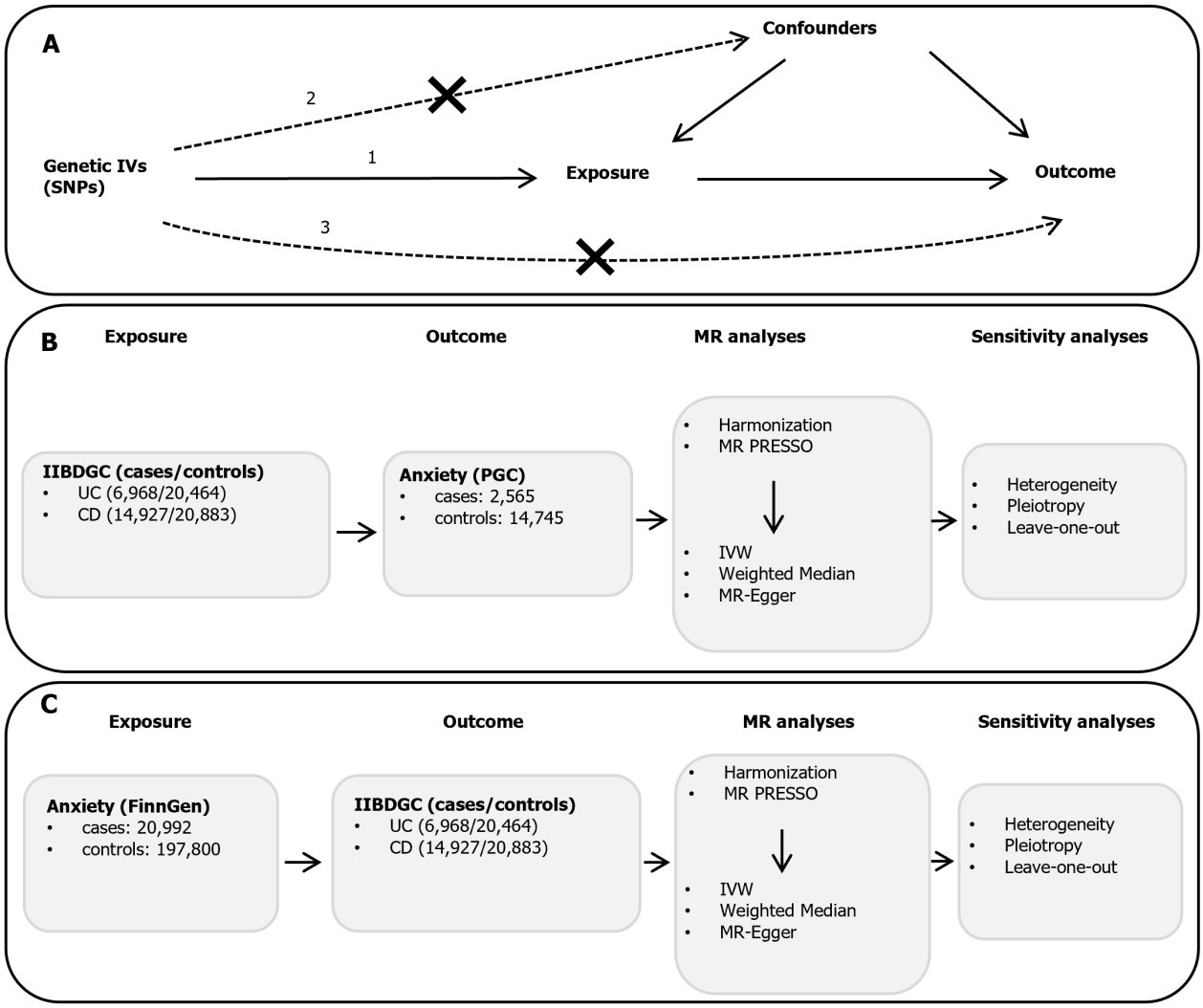
The MR-Pleiotropy Residual Sum and Outlier (MR-PRESSO) test was used to detect the outlier SNPs in MR analysis [21]. The MR-PRESSO test consists of three main parts: (1) Detecting horizontal pleiotropy; (2) correcting horizontal pleiotropy by removing the outlier; and (3) testing significant distortion in the causal estimates before and after removing the outlier. The MR-PRESSO test requires that more than 50% of the SNPs are efficient IVs with balanced pleiotropy. The number of distributions was set to 3000 in the MR-PRESSO test.

MR analysis

Three different methods [inverse-variance weighted (IVW), MR Egger, and weighted median] were used in the MR analysis[15]. IVW, the most efficient causal estimation method allowing balanced pleiotropy in MR analysis, was used as the main method[22]. The assessments by the IVW method are efficient, consistent and close to the true effect when the sample size of IVs is large enough and the pleiotropy of IVs is not significant[23]. If no significant heterogeneity (IVW-derived Cochran Q statistic $P \geq 0.05$) was detected, the fixed-effects IVW model was adopted; otherwise, the random-effects IVW model was applied. Since unbalanced pleiotropy may result in bias to the causal estimates by IVW, supplementary MR and sensitivity analyses are usually needed to verify the robustness of causal estimation in MR analysis[24]. MR Egger and weighted median were applied as supplementary MR methods to verify the causal estimates obtained by the IVW method[15,25]. Although they have less statistical power [wider confidence intervals (CIs)], they can provide more robust and reliable causal estimations across a wider range of scenarios. All statistical analyses were conducted using R (version 4.2.1), the Two-Sample MR package (version 0.5.6), and the MR-PRESSO package (version 1). $P < 0.05$ was considered indicative of statistical significance.

Sensitivity analysis

The MR Egger method was applied to assess the potential horizontal pleiotropy of SNPs. If the intercept P value was < 0.05, there was significant pleiotropy. Heterogeneity was tested by the IVW-derived Cochran Q statistic. A Cochran Q statistic P value of < 0.05 was considered to indicate the presence of significant heterogeneity. Scatter plots were used to visualize the results from MR analysis to show efficiency and reliability. The leave-one-out analysis aimed to verify that a single SNP does not affect the results by eliminating a single SNP one by one and performing MR analysis on the remaining SNPs.



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Figure 1 Schematic overview of the study design. A: Mendelian randomization (MR) analysis illustration. There are three principal assumptions in MR design: (1) Relevance assumption: the genetic instrument variants (IVs) are strongly associated with the exposure; (2) independence assumption: the genetic IVs do not affect the outcome through the confounders; and (3) exclusion assumption: The genetic IVs do not affect the outcome directly but only *via* indirect exposure; B: MR analysis from ulcerative colitis (UC) or Crohn's disease (CD) to anxiety. UC- or CD-related single-nucleotide polymorphisms (SNPs) obtained from genome-wide association studies (GWAS) statistics in the International Inflammatory Bowel Disease Genetics Consortium (IIBDGC) consortium were identified as IVs, and GWAS summary statistics of anxiety were retrieved from Psychiatric Genomics Consortium; C: MR analysis from anxiety to UC or CD. Anxiety-related SNPs retrieved from FinnGen were identified as IVs, and GWAS statistics for UC and CD were obtained from the IIBDGC consortium. MR analysis was performed after the harmonization process and MR Pleiotropy Residual Sum and Outlier test and subsequently sensitivity analyses to strengthen the MR estimates. MR: Mendelian randomization; IVs: Instrumental variables; UC: Ulcerative colitis; CD: Crohn's disease; PGC: Psychiatric Genomics Consortium; IVW: Inverse-variance weighted; PRESSO: Pleiotropy Residual Sum and Outlier; IIBDGC: International Inflammatory Bowel Disease Genetics Consortium.

MR analysis procedure

First, the abovementioned three assumptions were used to identify the genetic IVs (SNPs) of the exposure. Second, after the harmonization process, MR-PRESSO analysis was applied to detect and remove the outlier SNPs. Third, after the outlier SNPs were removed, MR analysis was performed, and subsequently, sensitivity analyses were conducted to determine whether pleiotropy existed and which IVW model was adopted according to heterogeneity. A schematic overview of the procedure is detailed in Figure 1.

RESULTS

Description of selected genetic IVs

Data for 34652 individuals of European descent who participated in the GWAS cohorts were obtained from seven CD and eight UC collections with combined genome-wide SNP data. After LD clumping and harmonization processes were completed, the MR-PRESSO test did not detect any CD- or UC-related outlier SNPs. Finally, 46-51 confounder-independent CD-related SNPs and 34-36 UC-related SNPs were identified to evaluate the causal effects on anxiety

(Table 1). Twenty-one anxiety-related SNPs from the FinnGen database were obtained to evaluate the causal effects on UC and CD (Table 1). The single-variant F statistic was calculated, and the F statistic values were all over 10, indicating that these selected SNPs were strongly associated with the exposures. Independent UC-related SNPs are listed in Supplementary Table 2, CD-related SNPs are listed in Supplementary Table 3, and anxiety-related SNPs are listed in Supplementary Tables 4 and 5.

Causal estimations from UC or CD to anxiety

We used IVW as the primary method to assess the causal estimates. We only observed a causal effect of UC on anxiety [odds ratio (OR): 1.071, 95%CI: 1.009-1.135, P = 0.0226] (Table 1). However, CD had no causal effect on anxiety. Additionally, MR Egger and weighted median methods verified the reliability of the causality estimated by IVW. Even though the MR Egger-derived P value and weighted median-derived P value were over 0.05, the ORs from MR Egger (OR = 1.138; 95%CI: 0.945-1.371) and weighted median (OR = 1.064; 95%CI: 0.981-1.154) methods were in the same direction as IVW estimation from UC to anxiety, indicating the robustness of causality (Table 1). Moreover, the supplementary MR analysis also did not provide evidence for the causal effect of CD on anxiety.

Heterogeneity was tested by the IVW-derived Cochran's Q statistic. The results showed that no statistically significant heterogeneity (all P > 0.05) was observed for the causal effects of UC and CD on anxiety (Table 1). Thus, we used the fixed-effects IVW model in the MR analysis. In addition, the effects of individual UC- or CD-related SNPs on anxiety are presented in scatter plots (Figure 2). The horizontal pleiotropic effect was performed by the MR Egger method to determine whether genetic IVs associated with UC or CD could affect anxiety through other potential pathways. Significant horizontal pleiotropy was not observed in our MR analysis (Table 1), which verified the robustness and credibility of the IVW-derived causal estimates. After one-by-one removal of each individual SNP, the following causal estimates of the remaining SNPs on anxiety were tested by leave-one-out analysis, which was consistent with the results of MR analysis and demonstrated that the causal effects were unlikely to be caused by any individual SNP (Supplementary Figure 1). Overall, our MR analysis showed that genetic susceptibility to UC, but not CD, was associated with an increased risk of anxiety.

Causal estimations from anxiety to UC or CD

Both IVW and the supplementary methods showed no causal effects (all P > 0.05) of anxiety on UC or CD (Table 1). In addition, no heterogeneity or horizontal pleiotropy (all P > 0.05) was observed in the sensitivity analyses (Table 1), which indicated that these MR estimations were reliable and robust (Figure 3). The leave-one-out analysis also suggested that the MR analysis was reliable (Supplementary Figure 2). Taken together, our MR analysis suggested that genetic susceptibility to anxiety was not associated with UC or CD.

DISCUSSION

In this study, we estimated the bidirectional causal relationships between IBD and anxiety by MR analysis. Our results suggested that genetic susceptibility to UC, but not CD, was associated with anxiety; however, genetic susceptibility to anxiety was not associated with UC or CD.

Previous observational studies showed that IBD was a risk factor for anxiety. A recent meta-analysis showed that the pooled prevalence of anxiety in IBD patients was 12% (95%CI, 8%-18%)[26]. Another population-based cohort study in the United Kingdom demonstrated that young IBD patients had a significantly higher incidence and risk of anxiety (adjusted hazard ratio, 1.25; 95%CI, 1.06-1.48)[27]. In addition, two large nationwide cohort studies showed that anxiety was more commonly seen in both patients with adult-onset IBD and those with childhood-onset IBD[28,29]. However, the abovementioned results could not be used to clarify the causality and directionality of the relationship between IBD and anxiety. Our study is the first to estimate the causal associations between IBD and anxiety using MR analysis and provides evidence that UC has a causal effect on anxiety.

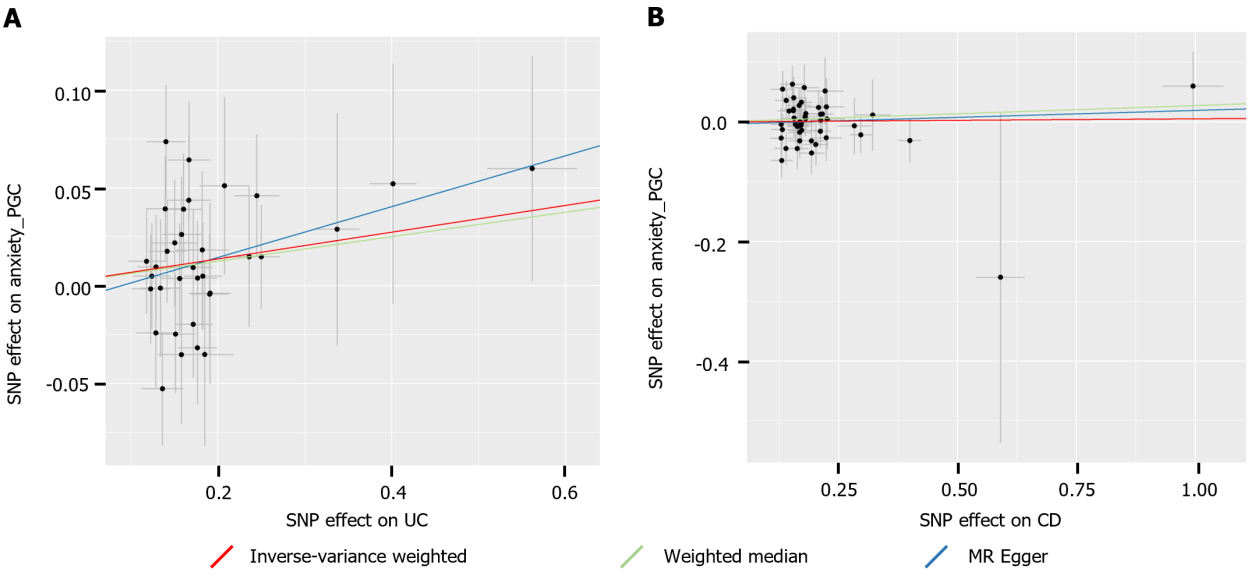
Some studies observed that CD patients had a higher OR value of anxiety symptoms than UC patients[30-32]. However, in our study, genetic susceptibility to UC but not CD was associated with an increased risk of anxiety. This result may be explained as follows: (1) UC and CD are two different diseases of the gut, with UC mainly restricted to the colonic mucosa, whereas CD involves the immune response of the entire gastrointestinal tract. Differences in the distribution of the enteric nervous system in the gastrointestinal tract may affect the function of different brain regions *via* the gut-brain axis[33,34]; and (2) discrepancies in gut microbiota and immune cell populations between UC and CD may have different influences on the brain through the microbiota-gut-brain axis[35-37]. These results reminded us that many factors other than genetic predisposition to IBD can also increase the risk of developing anxiety during the progression of IBD.

The biological route from UC or CD to anxiety has not yet been fully clarified. Increased evidence indicates that the gut-brain axis regulated by inflammation can affect neuronal development and subsequent behavioral phenotypes[5,38,39]. Circulating leukocytes and cytokines can reach the brain by crossing the blood-brain barrier, even leading to neuropsychiatric disorders[40]. For example, induced colitis in mice can result in increased levels of circulating cytokines, which influence certain brain regions, especially the hippocampus[41,42]. The hippocampus is related to memory and emotions, and damage to the hippocampus is closely associated with anxiety and depression[18,43-45]. Additionally, the gut microbiota plays a critical role in the interconnections between the gut and the brain[46,47]. The key routes or mediators between the gut microbiota and the brain are the enteric vagus nervous system, tryptophan metabolites, and microbial products[38,48]. Therefore, dysregulated inflammation and gut microbiota in patients with UC may lead to the

Table 1 Causal estimates between ulcerative colitis or Crohn's disease and anxiety by Mendelian randomization analysis

Exposure	Outcome	SNPs (*n*)	IVW			Weighted median		MR Egger		
			OR [95%CI]	*P* value	Cochran's Q *P* value	OR [95% CI]	*P* value	OR [95%CI]	*P* value	Intercept *P* value
UC	Anxiety	34	1.071 (1.009-1.135)	**0.023**	0.709	1.064 (0.981-1.154)	0.131	1.138 (0.945-1.371)	0.182	0.501
CD	Anxiety	46	1.005 (0.960-1.052)	0.825	0.713	1.027 (0.960-1.100)	0.435	1.023 (0.914-1.145)	0.689	0.734
Anxiety	UC	21	0.920 (0.779-1.086)	0.325	0.858	0.820 (0.654-1.027)	0.085	0.807 (0.594-1.096)	0.187	0.333
Anxiety	CD	21	1.012 (0.851-1.204)	0.892	0.808	0.998 (0.771-1.291)	0.989	1.016 (0.744-1.388)	0.919	0.974

MR: Mendelian randomization; UC: Ulcerative colitis; CD: Crohn's disease; IVW: Inverse-variance weighted; SNPs: Single-nucleotide polymorphisms; OR: Odds ratio; CI: Confidence intervals.



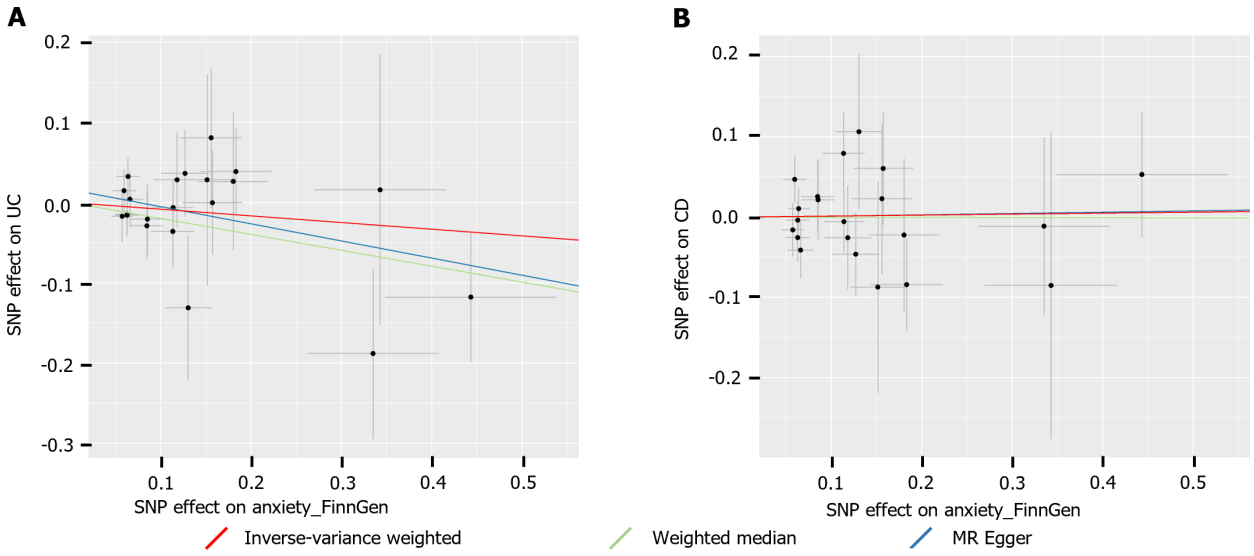
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Figure 2 Scatter plots of Mendelian randomization analysis showing the effect of ulcerative colitis and Crohn's disease on anxiety. A: Analysis of ulcerative colitis (UC) and anxiety; B: Analysis of Crohn's disease (CD) and anxiety. The x-axes represent the genetic instrument-UC or instrument-CD associations, and the y-axes represent genetic instrument-anxiety associations. Black dots denote the genetic instruments included in the primary Mendelian randomization (MR) analysis. Red: Inverse-variance weighted; Green: Weighted median; blue: MR Egger. CD: Crohn's disease; UC: Ulcerative colitis; PGC: Psychiatric Genomics Consortium; SNP: Single-nucleotide polymorphism.

progression of neuropsychiatric conditions, such as anxiety.

There are two main strengths in this study. First, this is the first study to assess the causal associations between IBD and anxiety using rigorous MR analysis. Second, the large-scale GWAS summary statistics for UC, CD and anxiety were all obtained from individuals of European descent, which would avoid the bias caused by a sample of individuals with different ethnicities. Meanwhile, some limitations should be considered. First, patients with CD or UC came from different medical units, and discrepancies in diagnostic approaches, data collection, and data processing may generate bias. Second, stratification analyses, especially those for sex, diverse severity, age, and drug use, are not viable due to using summary statistics for MR analysis. Third, all GWAS statistics included in the MR analysis came from European individuals; thus, the results of this study might not be generalizable to other ethnic groups.

Although this study investigated the causal relationship between IBD and anxiety, the precise biological mechanisms by which UC affects the development of anxiety remain unclear, such as whether and how the gut-brain axis plays a role. Hence, more basic and clinical studies regarding the identification of key regulators and pathways are needed to further uncover the biological mechanisms.



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Figure 3 Scatter plots of Mendelian randomization analysis showing the effect of anxiety on ulcerative colitis and Crohn's disease. A: Analysis of anxiety and ulcerative colitis (UC); B: Analysis of anxiety and Crohn's disease (CD). The x-axes represent the genetic instrument-anxiety associations, and the y-axes represent genetic instrument-UC or instrument-CD associations. Black dots denote the genetic instruments included in the primary Mendelian randomization (MR) analysis. Red: inverse-variance weighted; green: weighted median; blue: MR Egger. Due to the same estimate from the weighted median and MR Egger methods in some analyses, those figures only contain two lines. However, the color of the overlapped lines is darker than that of the MR Egger. CD: Crohn's disease; UC: Ulcerative colitis; SNP: Single-nucleotide polymorphism.

CONCLUSION

This study revealed that genetic susceptibility to UC was significantly associated with anxiety and highlighted the importance of early screening for anxiety in patients with UC, which may be helpful to strengthen physicians' awareness of recognizing anxiety.

ARTICLE HIGHLIGHTS

Research background

Inflammatory bowel disease (IBD), mainly consisted of Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammatory disease. Depression and anxiety are common co-occurrence in IBD. A recent Mendelian randomization (MR) study has inferred the causal effect of depression on IBD, while the causality between IBD and anxiety has not been investigated.

Research motivation

Previous observational studies showed that IBD patients had a significantly higher incidence and risk of anxiety. Despite the existing findings demonstrated the bidirectional relationship between IBD and anxiety, the causal association between them remain unclear. This study seeks to find out causal association between IBD and anxiety from the genetic perspective by using MR analysis, potentially offering new insights into the pathogenesis and clinical significance of anxiety in IBD.

Research objectives

The study aims to investigate the causal relationship between IBD and anxiety by performing bidirectional MR analysis, to better understand the gene susceptibility of anxiety in IBD.

Research methods

Single nucleotide polymorphisms retrieved from genome-wide association studies (GWAS) were identified as instrument variants. GWAS statistics for UC and CD were obtained from the International IBD Genetics Consortium. GWAS statistics for anxiety were obtained from the Psychiatric Genomics Consortium and FinnGen project. Inverse-variance weighted was applied to assess the causal relationship, and the results were strengthened by sensitivity analyses.

Research results

This study found that the genetic susceptibility to UC was associated with the increased risk of anxiety [odds ratio: 1.071 (95% confidence interval: 1.009, 1.135), P = 0.023]; while genetic susceptibility to CD was not associated with anxiety.

However, genetic susceptibility to anxiety was not associated with UC or CD. No heterogeneity and pleiotropy were found and leave-one-out analysis excluded the potential influence of a particular variant.

Research conclusions

This study identified that the genetic susceptibility to UC was significantly associated with anxiety, and provided the insight that early screening for the trait of anxiety is important for patients with UC.

Research perspectives

Although this study investigated the causal relationship between IBD and anxiety, the precise biological mechanisms by which UC affects the development of anxiety remain unclear, such as whether and how the gut-brain axis plays a role in this process. Hence, more basic and clinical studies are needed for the identification of key regulators and pathways to further uncover the biological mechanisms.

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FOOTNOTES

Co-first authors: Ying He and Chun-Lan Chen.

Author contributions: He Y, Chen CL and Liu SD proposed the study conception and designed the study methods; He Y, Chen CL and He J contributed to the data acquisition/analysis; He Y and He J contributed to the statistical analysis/interpretation; Liu SD supervised and managed the whole research process; He Y, Chen CL drafted the original manuscript; Liu SD reviewed and revised the original manuscript; all authors have read and approve the final manuscript. He Y and Chen CL contributed equally to this work; they were designated as co-first authors because they made equal and substantial contributions to the study conception, design, data analysis, and manuscript preparation and editing, each playing key roles in ensuring the integrity and quality of the manuscript.

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Institutional review board statement: The study used public GWAS statistics and did not collect new human data. Hence, ethical approval was not required by the ethics committee of Nanfang Hospital of Southern Medical University.

Clinical trial registration statement: As this study was not a clinical trial and did not involve any human studies, the clinical trial registration was not required for our research.

Informed consent statement: The study used public GWAS statistics and did not collect new human data. Hence, ethical approval was not required by the ethics committee of Nanfang Hospital of Southern Medical University.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Data sharing statement: All GWAS summary data that support the findings of this study are openly available in the IIBDGC (https://www.ibdgenetics.org/), PGC (https://pgc.unc.edu/), and FinnGen project (https://www.finngen.fi/en).

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ORIGINAL ARTICLE

Retrospective Study

Changing trends and characteristics of peptic ulcer disease: A multicenter study from 2010 to 2019 in Korea

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Abstract

BACKGROUND

The clinical trend and characteristics of peptic ulcer disease (PUD) have not fully been investigated in the past decade.

AIM

To evaluate the changing trends and characteristics of PUD according to age and

etiology.

METHODS

We analyzed seven hospital databases converted into the Observational Medical Outcomes Partnership-Common Data Model between 2010 and 2019. We classified patients with PUD who underwent rapid urease tests or *Helicobacter pylori* (*H. pylori*) serology into three groups: *H. pylori*-related, drug [nonsteroidal anti-inflammatory drugs (NSAIDs) or aspirin]-related, and idiopathic (*H. pylori*/NSAID/aspirin-negative) PUD and compared the yearly trends and characteristics among the three groups.

RESULTS

We included 26785 patients in 7 databases, and the proportion of old age (≥ 65 years) was 38.8%. The overall number of PUD exhibited no decrease, whereas PUD in old age revealed an increasing trend (P = 0.01 for trend). Of the 19601 patients, 41.8% had *H. pylori*-related, 36.1% had drug-related, and 22.1% had idiopathic PUD. *H. pylori*-related PUD exhibited a decreasing trend after 2014 (P = 0.01), drug-related PUD demonstrated an increasing trend (P = 0.04), and idiopathic PUD showed an increasing trend in the old-age group (P = 0.01) during 10 years. Patients with drug-related PUD had significantly more comorbidities and concomitant ulcerogenic drugs. The idiopathic PUD group had a significantly higher number of patients with chronic liver disease.

CONCLUSION

With the aging population increase, the effects of concomitant ulcerogenic drugs and preventive strategies should be investigated in drug-induced PUD. Further studies are required to clarify the relationship between idiopathic PUD and chronic liver disease.

Key Words: Peptic ulcer disease; Drug; Idiopathic; Trend; Characteristics

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Core Tip: In the multicenter study including 26785 peptic ulcer disease (PUD) patients from 7 databases, the overall number of PUD exhibited no decrease, whereas PUD in old age revealed an increasing trend from 2010 to 2019 in Korea. According to etiology, decreasing trend of *Helicobacter pylori*-related PUD after year 2014, and increasing trend of drug-related PUD were observed in the past decade. Drug-related PUD showed significantly more comorbidities and exposure to concomitant ulcerogenic drugs, and the idiopathic PUD group had a significantly higher proportion in the chronic liver disease. Further studies are required to clarify the relationship between idiopathic PUD and chronic liver disease.

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INTRODUCTION

Peptic ulcer disease (PUD) remains a critical cause of hospitalization, particularly when complicated by hemorrhage, perforation, or obstruction[1]. The main etiologies of PUD include *Helicobacter pylori* (*H. pylori*) infection and the use of aspirin or nonsteroidal anti-inflammatory drugs (NSAIDs)[1]. The overall decline of *H. pylori* infection in the general population and advances in the management of *H. pylori* infection have led to a decline in the PUD incidence over the past two decades, however, prescriptions of aspirin and NSAIDs have increased over the same period owing to the rising number of older patients and patients with comorbidities[2]. Furthermore, direct oral anticoagulants (DOAC), newer NSAIDs, and antiplatelet agents continue to be used in chronic disease, and public awareness of *H. pylori* eradication has improved. Consequently, trends of PUD have demonstrated inconsistent results among regions[2-7]. A recent population-based study reported that morbidity and mortality due to PUD decreased significantly from 1990 to 2019, while a gradual upward trend has been observed in the recent 15 years, which might be associated with changes in risk factors [2]. Meanwhile, the incidence of non-*H. pylori*, non-NSAIDs/aspirin PUD, also termed idiopathic PUD, has increased in recent years, particularly in Asian countries[8]. The clinical outcomes of idiopathic PUD revealed recurrent ulcer bleeding and higher mortality in previous studies[9-14]; however, characteristics of idiopathic PUD remain poorly understood and warrant further investigation.

To date, few large-scale studies have comprehensively investigated the recent changing trends and clinical characteristics of PUD, including the multiple risk factors. Therefore, we investigated the trends and characteristics of PUD according to age and etiology in Korea between 2010 and 2019.

MATERIALS AND METHODS

Data source and study design

We analyzed seven hospital databases converted into the Observational Medical Outcomes Partnership-Common Data Model (OMOP-CDM) using the FEEDER-NET platform, which is a coordinating platform that enables data user and supplier connections and multi-institution analyses, with the anonymity of patients' personal information, as applied in previous studies[15-17]. The included institutions were Ajou University Medical Center (AUMC), Daegu Catholic Medical Center (DCMC), Ewha Womans University Medical Center (EUMC), Gyeongsang National University Hospital (GNUH), Kangdong Sacred Heart Hospital (KDH), Kangwon National University Hospital (KNUH), and Wonkwang University Hospital (WKUH). The detailed characteristics of each database are presented in Supplementary Table 1.

The characteristics of PUD were analyzed using the characterization tab on ATLAS, which is a web-based open platform developed by the Observational Health Data Sciences and Informatics community[18]. We analyzed basic demographic information, prescriptions, and comorbidities during the year before entering the cohort. We evaluated for exposure to aspirin and other antiplatelet agents, NSAIDs, anticoagulants, and potential ulcerogenic drugs (steroids, antidepressants, bisphosphonates, and immunosuppressive agents). Further, the mean Charlson Comorbidity Index (CCI) was calculated for each database. The same analytic codes were applied to seven hospital databases, and the results of each database were combined as proportions without extracting the raw data. The combined results were analyzed yearly for trend analysis and compared according to age group and etiology of PUD. This study was approved by the Institutional Review Board (IRB) of Kangdong Sacred Hospital (IRB number: 2021-11-001). The other six hospitals were affiliated with the Research Border Free Zone, which recognizes IRB approval of the research organizing center and waives individual IRB approval.

Definition of PUD

We defined a newly diagnosed PUD as having a 1-year observation period between January 1, 2010, and December 31, 2019, combining diagnostic codes for PUD, esophagogastroduodenoscopy, and exposure to proton pump inhibitors (PPIs). This operational definition was based on previous reports[3,19]. The index date was defined as the initial diagnosis of PUD. Exclusion criteria were as follows: (1) Age < 20 years; (2) Observation period < 1 year before the index date; (3) No exposure to PPIs 30 d before or after the index date; (4) No exposure to esophagogastroduodenoscopy 30 d before or after the index date; (5) Gastric cancer before the index date; and (6) Benign gastric neoplasm before the index date. Each concept name and identifier included in the OMOP-CDM databases matched to diagnostic codes or drugs are listed in Supplementary Table 2.

PUD classification according to etiology

We classified PUD patients who underwent rapid urease tests or *H. pylori* serology tests according to etiology into the following three groups: (1) *H. pylori*-related; (2) Drug-related (*H. pylori*-negative and NSAIDs/aspirin-related); and (3) Idiopathic (*H. pylori*/NSAID/aspirin-negative) PUD. *H. pylori*-related PUD included patients who were concomitantly prescribed *H. pylori* eradication therapy (PPI, amoxicillin, clarithromycin, bismuth, tetracycline, and metronidazole) for 7-14 d. Drug-related PUD included patients who were not exposed to *H. pylori* eradication therapy and were exposed to aspirin or NSAIDs before the index date. Idiopathic PUD included patients without exposure to aspirin, NSAIDs, or *H. pylori* eradication therapy. We further performed a subgroup analysis according to age and sex. We defined the old age group as ≥ 65 years, and the young age group as < 65 years.

Statistical analysis

Categorical variables were compared using the χ^2 or Fisher's exact test based on the results from the ATLAS version 2.7.6. Continuous variables were expressed as means with standard deviations. The yearly trend was analyzed with simple linear regression, and the yearly trend for each group was compared using the Cochran Armitage test for trends. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using R version 4.2.1 (R Foundation for Statistical Computing).

RESULTS

Characteristics of PUD in total patients

The flowchart of the study is presented in Figure 1. A total of 26785 patients (AUMC, *n* = 4629; DCMC, *n* = 4690; EUMC, *n* = 3118; GNUH, *n* = 2998; KDH, *n* = 6320; KNUH, *n* = 1854; and WKUH, *n* = 3176) were included between 2010 and 2019. Finally, 15544 (58%) were men, and 11241 (42%) were women. The yearly trend of PUD exhibited no declining pattern in the total patients ($P = 0.69$ for trend) (Figure 2A). The yearly PUD trend in each database is presented in Supplementary Figure 1.

The baseline demographics are presented in Table 1. The proportion of gastric ulcers (69.2%) was over twofold that of duodenal ulcers (31.3%). The most prevalent comorbidity was hypertensive disorder, followed by diabetes mellitus. The proportions of exposure to NSAIDs, aspirin, and antiplatelet agents were 19.9%, 15.2%, and 11.4%, respectively. Regarding anticoagulants, warfarin and DOAC were prescribed in 2.2% and 1.1% of total patients, respectively. The proportion of exposure to steroids was 12.7%. Detailed information for each database is presented in Supplementary Table 3.

Table 1 Baseline characteristics of all patients with peptic ulcer disease, *n* (%)

	Total (*n* = 26785)
Age ≥ 65 yr	10392 (38.8)
Men	15544 (58.0)
RUT or serology	19601 (73.2)
Gastric ulcer	18527 (69.2)
Duodenal ulcer	8377 (31.3)
Diagnosis	
Hypertensive disorder	4773 (17.8)
Diabetes mellitus	2239 (8.4)
Hyperlipidemia	2038 (7.6)
Chronic kidney disease	1282 (4.8)
Ischemic heart disease	2221 (8.3)
Cerebrovascular disease	812 (3.0)
Chronic liver disease	1436 (5.4)
Osteoarthritis	425 (1.6)
Chronic obstructive lung disease	515 (1.9)
Medication	
Aspirin	4068 (15.2)
Other antiplatelet agent[1]	3065 (11.4)
Clopidogrel	2386 (8.9)
Cilostazole	691 (2.6)
NSAID	5342 (19.9)
Warfarin	579 (2.2)
DOAC	297 (1.1)
Steroid	3404 (12.7)
Antidepressant	6308 (23.6)
Bisphosphonate	488 (1.8)
Immunosuppressant	578 (2.2)

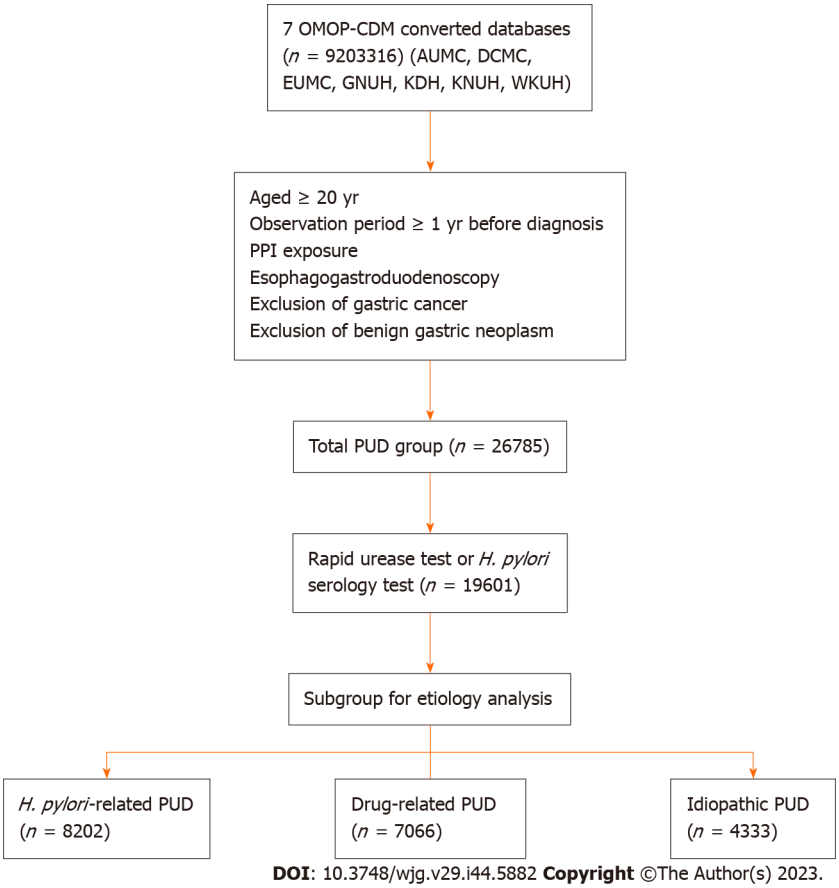
[1]Antiplatelet agents other than aspirin include clopidogrel, cilostazole, ticlopidine, triflusal, ticagrelor, and prasugrel.
RUT: Rapid urease test; NSAIDs: Nonsteroidal anti-inflammatory drugs; DOAC: Direct oral anticoagulant.

Characteristics of PUD in old age

The proportion of patients with old age (≥ 65 years) was 38.8% (10392/26785). The PUD with the old-age group demonstrated an increasing annual trend (P = 0.01 for trend), whereas the young age group (< 65 years) showed no specific trend overall (P = 0.47 for trend) (Figure 2A). The mean CCI in old age was higher than that in total patients (old-age group *vs* total group; 3.18 ± 2.6 *vs* 2.58 ± 2.4 in AUMC; 2.94 ± 2.2 *vs* 2.30 ± 1.9 in DCMC; 2.37 ± 1.9 *vs* 1.77 ± 1.5 in EUMC; 2.61 ± 2.0 *vs* 2.19 ± 1.8 in GNUH; 2.58 ± 2.0 *vs* 2.05 ± 1.7 in KDH; 2.78 ± 2.1 *vs* 2.36 ± 1.9 in KNUH; and 3.21 ± 2.4 *vs* 2.66 ± 2.2 in WKUH).

Characteristics of PUD according to etiology

Of the total patients, 19601 underwent rapid urease or *H. pylori* serology tests. The number of *H. pylori*-related, drug-related, and idiopathic PUD patients was 8202 (41.8%); 7066 (36.1%); and 4333 (22.1%), respectively. The proportions according to etiology in each hospital are presented in Supplementary Table 3. *H. pylori*-related PUD exhibited a decreasing trend after 2014 (P = 0.01 for trend), and drug-related PUD showed a slightly increasing trend in the past 10 years (P = 0.04 for trend). In contrast, idiopathic PUD revealed no statistically increasing trend in the past 10 years (P = 0.08 for trend) (Figure 2B).



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Figure 1 Flow chart of the study. OMOP-CDM: Observational Medical Outcomes Partnership-Common Data Model; AUMC: Ajou University Medical Center; DCMC: Daegu Catholic Medical Center; EUMC: Ewha Womans University Medical Center; GNUH: Gyeongsang National University Hospital; KDH: Kangdong Sacred Heart Hospital; KNUH: Kangwon National University Hospital; WKUH: Wonkwang University Hospital; PPI: Proton pump inhibitor; PUD: Peptic ulcer disease; *H. pylori* : *Helicobacter pylori*.

The comparison of characteristics among the three groups is presented in Table 2. The *H. pylori*-related PUD group showed the lowest proportion of old age among the three groups. The proportion of duodenal ulcers was significantly higher in the *H. pylori*-related PUD than in the other groups. The drug-related PUD group had significantly more elderly patients, less predominance of men, more gastric ulcers, comorbidities except for chronic liver disease, exposure to concomitant potential ulcerogenic drugs than the other groups (Table 2). Notably, there were more patients with chronic liver disease in the idiopathic PUD group than in the other groups. In addition, there were significantly more patients with alcoholic liver damage and cirrhosis in the idiopathic PUD group (Table 2).

Subgroup analysis according to age and sex

Of 19601 patients, we conducted a subgroup analysis according to age group and sex. In the old-age (≥ 65 years) group (*n* = 7486), *H. pylori*-related PUD was 2043 (27.3%); drug-related was 3842 (51.3%); and idiopathic was 1601 (21.4%). In the young age (< 65 years) group (*n* = 12115), *H. pylori*-related PUD was 6159 (50.8%); drug-related was 3224 (26.6%); and idiopathic was 2732 (22.6%). In the old-age group, *H. pylori*-related PUD exhibited a decreasing trend after 2014 ($P < 0.01$ for trend), whereas drug-related and idiopathic PUD showed an overall increasing trend (drug-related, $P = 0.001$ for trend; idiopathic, $P = 0.01$ for trend) (Figure 2C). In the young age group, there was only a decreasing trend for *H. pylori*-related PUD after 2014 ($P = 0.01$ for trend) (Figure 2D). The yearly trend did not differ according to sex.

In the old-age group, more women had drug-related PUD than total patients (Table 3). The proportion of gastric ulcers was highest in drug-related PUD, regardless of age, whereas the proportion of duodenal ulcers showed different patterns according to age. In the old-age group, the proportion of duodenal ulcer was highest in the idiopathic PUD, and it was highest in *H. pylori*-related PUD in the young age group (Table 3). Other comorbidities or exposure to concomitant drugs did not differ according to age (Table 3). Supplementary Table 4 lists the subgroup analysis according to sex. No significant difference was found according to sex.

DISCUSSION

The findings of this multicenter OMOP-CDM-based study revealed that the total number of PUD patients demonstrated no decreasing trend, whereas newly diagnosed PUD in the old-age group showed an increasing trend in the past 10

Table 2 Comparison of characteristics according to the etiology of peptic ulcer disease

	H. pylori-related PUD (*n* = 8202)	Drug-related PUD (*n* = 7066)	Idiopathic PUD (*n* = 4333)	*P* value	All (*n* = 19601)
Age ≥ 65	2043 (24.9)	3842 (54.4)	1601 (36.9)	< 0.001	7486 (38.2)
Men, *n* (%)	5268 (64.2)	3774 (53.4)	2747 (63.4)	< 0.001	11789 (60.1)
Gastric ulcer	5062 (61.7)	5052 (71.5)	2758 (63.7)	< 0.001	12872 (65.7)
Duodenal ulcer	3272 (39.9)	2171 (30.7)	1574 (36.3)	< 0.001	7017 (35.8)
Diagnosis, *n* (%)					
Hypertensive disorder	928 (11.3)	2036 (28.8)	529 (12.2)	< 0.001	3493 (17.8)
Diabetes mellitus	438 (5.3)	954 (13.5)	256 (5.9)	< 0.001	1648 (8.4)
Hyperlipidemia	587 (7.2)	829 (11.7)	129 (3.0)	< 0.001	1545 (7.9)
Chronic kidney disease	173 (2.1)	604 (8.5)	123 (2.8)	< 0.001	900 (4.6)
Ischemic heart disease	483 (5.9)	1098 (15.5)	49 (1.1)	< 0.001	1630 (8.3)
Cerebrovascular disease	161 (2.0)	372 (5.3)	35 (0.8)	< 0.001	568 (2.9)
Chronic liver disease	224 (2.7)	447 (6.3)	349 (8.1)	< 0.001	1020 (5.2)
Alcoholic liver damage	164 (2.0)	253 (3.6)	234 (5.4)	< 0.001	651 (3.3)
Liver cirrhosis	175 (2.1)	381 (5.4)	325 (7.5)	< 0.001	881 (4.5)
Osteoarthritis	84 (1.0)	191 (2.7)	13 (0.4)	< 0.001	288 (1.5)
Chronic obstructive lung disease	76 (0.9)	209 (3.0)	74 (1.7)	< 0.001	359 (1.8)
Medication, *n* (%)					
Antiplatelet agent[1]	634 (7.7)	1514 (21.4)	77 (1.8)	< 0.001	2225 (11.4)
Warfarin	84 (1.0)	258 (3.7)	34 (0.8)	< 0.001	376 (1.9)
DOAC	33 (0.4)	129 (1.8)	20 (0.5)	< 0.001	182 (0.9)
Steroid	690 (8.4)	1423 (20.1)	227 (5.2)	< 0.001	2340 (11.9)
Antidepressant	1188 (14.5)	2408 (34.1)	868 (20.0)	< 0.001	4464 (22.8)
Bisphosphonate	94 (1.1)	223 (3.2)	18 (0.4)	< 0.001	335 (1.7)
Immunosuppressant	125 (1.5)	275 (3.9)	35 (0.8)	< 0.001	435 (2.2)

[1]Antiplatelet agents other than aspirin include clopidogrel, cilostazole, ticlopidine, triflusal, ticagrelor, and prasugrel.
PUD: Peptic ulcer disease; DOAC: Direct oral anticoagulant; *H. pylori*: *Helicobacter pylori*.

years. We classified PUD patients who underwent *H. pylori* serology or rapid urease tests into *H. pylori*-related, drug-related, and idiopathic PUD to clarify the characteristics and changing trends of PUD according to etiology. *H. pylori*-related PUD showed a decreasing trend after 2014; drug-related PUD, an increasing trend; and idiopathic PUD, an increasing trend only in the old-age group. Drug-related PUD revealed significantly more comorbidities and exposure to concomitant ulcerogenic drugs. Notably, the proportion of patients with chronic liver disease was significantly higher in idiopathic PUD.

Several studies have investigated the trends of PUD, and the overall prevalence has exhibited a decreasing trend in the past few decades; however, the trend differed according to region or sex owing to changes in the distribution of the etiologies of PUD[2,3]. A recent global study has reported that the age-standardized incidence rate exhibited an increasing annual trend with increasing age[2], and our results showed an increasing trend of PUD in the old-age group. A recent Korean nationwide cohort study conducted in 2006-2015 showed a decreasing trend in the *H. pylori* infection rate and no change in drug exposure that increases the risk of peptic ulcer bleeding (PUB)[3]. Furthermore, the *H. pylori* infection rate was 34.4% in that study when it was defined by including patients who received *H. pylori* eradication therapy out of the patients who underwent rapid urease tests, *H. pylori* cultures, urea breath tests, Warthin-Starry silver stains, and *H. pylori* stool antigen tests[3]. The lower *H. pylori* infection rate than that in our study may be attributed to the lack of *H. pylori* serology testing and false-negative results in the PUB setting in that study. *H. pylori*-related PUD was 41.8% in our study, which was consistent with a recent Korean nationwide multicenter study that reported 43.9% *H. pylori* seropositivity from 2016 to 2017[20]. Our proportion of *H. pylori*-related PUD also included patients who had *H. pylori* eradication therapy, which suggests that the knowledge and awareness of the public was improved in the past decade. A

Table 3 Subgroup analysis of characteristics of peptic ulcer disease according to age, *n* (%)

	Age (yr)	*H. pylori*-related PUD (*n* = 8202)	Drug-related PUD (*n* = 7066)	Idiopathic PUD (*n* = 4333)	*P* value
Men	≥ 65	1161/2043 (56.8)	1869/3842 (48.6)	934/1601 (58.3)	< 0.001
	< 65	4107/6159 (66.7)	1905/3224 (59.1)	1813/2732 (66.4)	< 0.001
Gastric ulcer	≥ 65	1481/2043 (72.5)	2855/3842 (74.3)	1093/1601 (68.3)	< 0.001
	< 65	3581/6159 (58.1)	2197/3224 (68.1)	1665/2732 (60.9)	< 0.001
Duodenal ulcer	≥ 65	612/2043 (30.0)	1117/3842 (29.1)	561/1601 (35.0)	< 0.001
	< 65	2660/6159 (43.2)	1054/3224 (32.7)	1013/2732 (37.1)	< 0.001
Diagnosis					
Hypertensive disorder	≥ 65	452/2043 (22.1)	1431/3842 (37.2)	321/1601 (20.0)	< 0.001
	< 65	476/6159 (7.7)	605/3224 (18.8)	208/2732 (7.6)	< 0.001
Diabetes mellitus	≥ 65	194/2043 (9.5)	610/3842 (15.9)	148/1601 (9.2)	< 0.001
	< 65	244/6159 (4.0)	344/3224 (10.7)	108/2732 (4.0)	< 0.001
Hyperlipidemia	≥ 65	201/2043 (9.8)	533/3842 (13.9)	53/1601 (3.3)	< 0.001
	< 65	386/6159 (6.3)	296/3224 (9.2)	76/2732 (2.8)	< 0.001
Chronic kidney disease	≥ 65	76/2043 (3.7)	382/3842 (9.9)	64/1601 (4.0)	< 0.001
	< 65	97/6159 (1.6)	222/3224 (6.9)	59/2732 (2.3)	< 0.001
Ischemic heart disease	≥ 65	247/2043 (12.1)	794/3842 (20.7)	35/1601 (2.6)	< 0.001
	< 65	236/6159 (3.8)	304/3224 (9.4)	14/2732 (0.6)	< 0.001
Cerebrovascular disease	≥ 65	92/2043 (4.5)	288/3842 (7.5)	25/1601 (1.6)	< 0.001
	< 65	69/6159 (1.1)	84/3224 (2.7)	10/2732 (0.5)	< 0.001
Chronic liver disease	≥ 65	42/2043 (2.3)	202/3842 (5.3)	120/1601 (7.5)	< 0.001
	< 65	182/6159 (3.0)	245/3224 (7.6)	229/2732 (8.4)	< 0.001
Alcoholic liver damage	≥ 65	20/2043 (1.1)	85/3842 (2.2)	64/1601 (4.0)	< 0.001
	< 65	144/6159 (2.3)	168/3224 (5.2)	170/2732 (6.2)	< 0.001
Liver cirrhosis	≥ 65	29/2043 (1.6)	161/3842 (4.2)	113/1601 (7.1)	< 0.001
	< 65	146/6159 (2.4)	220/3224 (6.8)	212/2732 (7.8)	< 0.001
Osteoarthritis	≥ 65	56/2043 (2.7)	145/3842 (3.8)	10/1601 (1.1)	< 0.001
	< 65	28/6159 (0.5)	46/3224 (1.6)	3/2732 (0.2)	< 0.001
Chronic obstructive lung disease	≥ 65	53/2043 (2.6)	176/3842 (4.6)	57/1601 (3.6)	0.001
	< 65	23/6159 (0.4)	33/3224 (1.0)	17/2732 (0.6)	0.001
Medication					
Antiplatelet agent	≥ 65	324/2043 (15.9)	1058/3842 (27.5)	51/1601 (3.2)	< 0.001
	< 65	310/6159 (5.0)	456/3224 (14.1)	26/2732 (1.0)	< 0.001
Warfarin	≥ 65	49/2043 (2.4)	190/3842 (4.9)	28/1601 (1.9)	< 0.001
	< 65	35/6159 (0.6)	68/3224 (2.1)	6/2732 (0.3)	< 0.001
DOAC	≥ 65	23/2043 (1.2)	112/3842 (2.9)	19/1601 (1.2)	< 0.001
	< 65	10/6159 (0.2)	17/3224 (0.6)	1/2732 (0.1)	0.015
Steroid	≥ 65	237/2043 (11.6)	807/3842 (21.0)	111/1601 (6.9)	< 0.001
	< 65	453/6159 (7.4)	616/3224 (19.1)	116/2732 (4.2)	< 0.001
Antidepressant	≥ 65	456/2043 (22.3)	1568/3842 (40.8)	435/1601 (27.2)	< 0.001
	< 65	732/6159 (11.9)	840/3224 (26.1)	433/2732 (15.8)	< 0.001

Bisphosphonate	≥ 65	54/2043 (2.6)	174/3842 (4.5)	11/1601 (0.7)	< 0.001
	< 65	40/6159 (0.6)	49/3224 (1.5)	7/2732 (0.4)	< 0.001
Immunosuppressant	≥ 65	49/2043 (2.4)	108/3842 (2.8)	13/1601 (0.9)	< 0.001
	< 65	76/6159 (1.2)	167/3224 (5.2)	22/2732 (1.0)	< 0.001

PUD: Peptic ulcer disease; DOAC: Direct oral anticoagulant; *H. pylori*: *Helicobacter pylori*.

recent meta-analysis also revealed that technology-enhanced communication initiatives effectively improve compliance to the *H. pylori* eradication regimen and increase the eradication rate[21].

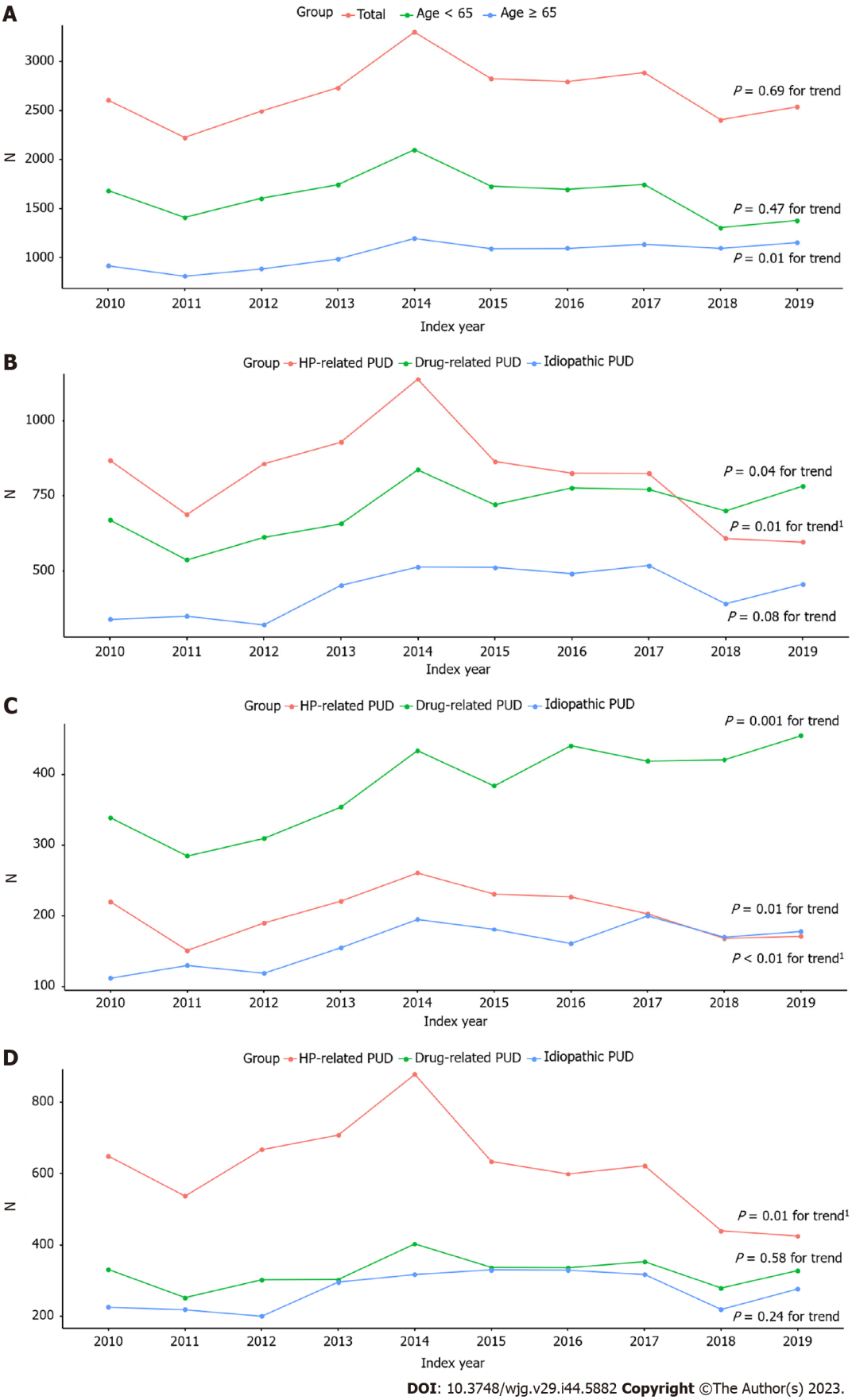
The use of NSAIDs or aspirin has increased dramatically in recent decades, and the prevalence of NSAID use in patients aged ≥ 65 years is reported to be as high as 96%[22,23]. The proportion of drug-related PUD revealed an increasing trend, and it was prominent in the old-age group. The drug-related PUD group included more elderly patients and showed comorbidities and exposure to concomitant ulcerogenic drugs compared with the other groups, consistent with previous reports[24]. Drug-related PUD may cause serious complications, including bleeding or perforation[25]. Clinical practice guidelines for the appropriate treatment and prevention of drug-related PUD have been recently developed[26,27]. The guidelines recommend high-risk patients who are on long-term NSAID medications receive low-dose PPIs to prevent PUD and its complications; however, evidence in patients who take multiple ulcerogenic drugs remains lacking[26]. In a previous case series analysis from seven population-based healthcare databases, concomitant use of NSAIDs or aspirin with selective serotonin reuptake inhibitors, aldosterone antagonists, corticosteroids, or anticoagulants significantly increased the risk of upper gastrointestinal bleeding[28]. Our results suggest that drug-induced PUD may have more severe clinical outcomes; therefore, further strategies should be investigated to prevent complications in elderly patients.

The proportion of idiopathic PUD was 22.1% in our study, which is consistent with the proportion in the previous multicenter prospective study in 2008[9] and higher than that in the study by Chung *et al*[8] (8.6%) of patients with PUB in Korea[12]. Idiopathic PUD can be defined after excluding the missed diagnosis of *H. pylori* infection and undocumented use of NSAIDs or aspirin; therefore, the definition and diagnosis of *H. pylori* infection differed among the previous studies. Epidemiological studies have consistently reported an increasing proportion of *H. pylori*-negative PUD, particularly in Asian countries[8]; however, most of these studies were conducted before 2014, and few large-scale studies have investigated the changing trends or characteristics of idiopathic PUD in recent years. Our idiopathic PUD group included patients who had no exposure to aspirin, NSAIDs, or *H. pylori* eradication therapy before the index date. In our study, the exact results of the *H. pylori* tests could not be obtained, and patients with idiopathic PUD may overlap with those with drug-induced PUD. Despite these limitations, our study was the first to demonstrate the increasing trend of idiopathic PUD in old age in the past decade. In addition, we confirmed that the proportion of idiopathic PUD may differ regionally depending on the regional *H. pylori* prevalence.

The proportions of most of the comorbidities and drug exposures were higher in drug-induced PUD in our study, and only chronic liver disease was significantly higher in patients with idiopathic PUD among the three groups. Previous studies have compared the characteristics of idiopathic PUD with those of *H. pylori*-related PUD. The risks for idiopathic PUD included older age, smoking, alcohol, comorbid diseases, and higher psychological stress[8]. In our study, among three PUD groups, only the proportion of chronic liver disease was significantly higher in the idiopathic PUD; therefore, it may be a distinct characteristic compared with drug-induced PUD. The relationship between idiopathic PUD and liver disease has been suggested in several studies. Kim *et al*[29] have reported that despite the decreased *H. pylori* infection in patients with severe liver cirrhosis, PUD was increased in 288 patients with liver cirrhosis. Therefore, factors other than *H. pylori* infection may be involved in the pathogenesis of PUD in patients with cirrhosis[29,30]. A proposed pathophysiological mechanism is that portal hypertension induces splanchnic vascular congestion followed by gastrointestinal mucosal changes involving impaired mucosal secretion and microvascular flow, leading to peptic ulcer formation[31]. Moreover, we demonstrated that alcoholic liver disease was more prevalent in idiopathic PUD, suggesting that alcohol may be another risk factor for idiopathic PUD, although the causal relationship is uncertain.

Our study had several limitations. First, the proportion of idiopathic PUD may have been overestimated since we could not identify the exact *H. pylori* infection or confirmation after *H. pylori* eradication using an administrative database. However, this limitation may be overcome by converting text data to CDM in future studies. Second, our study was not conducted nationwide; therefore, we could not demonstrate the PUD trend as a proportion of the total patients. However, we observed that the pattern or severity of PUD may vary according to the scale of each hospital. Third, patients may overlap across hospital databases. Patients may be diagnosed with PUD in one hospital and treated in another. Fourth, the claims data-based research design may include misclassification bias or inaccurate data. Lastly, we could not include data on smoking or alcohol consumption; therefore, it could not be evaluated as a cause of idiopathic PUD.

Despite these limitations, our study had the following strengths. The main strength is that the analysis was performed using the OMOP-CDM database, which can be applied to other databases worldwide with the same analytic code. Second, our study used the operational definition of PUD based on a previous validation study, which showed high sensitivity and specificity[19]. Lastly, our study confirmed the changing trends and characteristics of PUD according to etiology and age group in the past 10 years using a large-scale multicenter design.



DOI: 10.3748/wjg.v29.i44.5882 **Copyright** ©The Author(s) 2023.

Figure 2 Yearly trend of peptic ulcer disease according to age group, etiology in total, in the old age (≥ 65) group, and in the young age (< 65) group. A: Age group; B: Etiology in total group; C: Etiology in the old age (≥ 65) group; D: Etiology in the young age (< 65) group. PUD: Peptic ulcer disease; HP: *Helicobacter pylori*. [1]*P* for trend after 2014.

CONCLUSION

In conclusion, PUD exhibited an increasing trend in the old-age group in the past decade. Regarding etiology, *H. pylori*-related PUD decreased, whereas drug-related and idiopathic PUD increased, particularly in the old-age group. With the rising number of older patients, the effects of concomitant ulcerogenic drugs on PUD should be investigated, and preventive strategies for drug-induced PUD should be developed. Further studies are required to clarify the relationship between idiopathic PUD and chronic liver disease.

ARTICLE HIGHLIGHTS

Research background

To date, few large-scale studies have comprehensively investigated the recent changing trends and clinical characteristics of peptic ulcer disease (PUD), including the multiple risk factors.

Research motivation

The incidence of idiopathic PUD, has increased in recent years, particularly in Asian countries. The clinical outcomes of idiopathic PUD revealed recurrent ulcer bleeding and higher mortality in previous studies; however, characteristics of idiopathic PUD remain poorly understood and warrant further investigation.

Research objectives

We aimed to evaluate the changing trends and characteristics of PUD according to age and etiology.

Research methods

We analyzed seven hospital databases that were converted to a common data model between 2010 and 2019. We classified PUD patients who underwent rapid urease testing or *Helicobacter pylori* (*H. pylori*) serology testing into the following three groups according to etiology: (1) *H. pylori*-related; (2) drug-related [*H. pylori*-negative and nonsteroidal anti-inflammatory drugs (NSAIDs)/aspirin-related]; and (3) Idiopathic (*H. pylori*/NSAID/aspirin-negative) PUD.

Research results

The overall number of PUD exhibited no decrease, whereas PUD in old age revealed an increasing trend. *H. pylori*-related PUD exhibited a decreasing trend after 2014, drug-related PUD demonstrated an increasing trend, and idiopathic PUD showed an increasing trend in the old-age group during 10 years. The idiopathic PUD group had a significantly higher number of patients with chronic liver disease.

Research conclusions

There was an increase in the incidence of PUD in the older age group during the last decade. There was a decrease in *H. pylori*-related PUD and an increase in drug-related and idiopathic PUD, especially in the elderly group.

Research perspectives

Further preventive strategies for drug-induced PUD should be developed. Further studies are required to clarify the relationship between idiopathic PUD and chronic liver disease.

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FOOTNOTES

Author contributions: Choi YJ and Seo SI contributed to the acquisition of data, analysis and interpretation of data, drafting of the manuscript; Kim TJ, Bang CS, Lee YK, and Lee MW participated in the technical or material support of this study; Nam SY and Shin WG contributed to the critical revision of the manuscript for important intellectual content; Seo SI involved in the study concept and design, and study supervision.

Institutional review board statement: This study was approved by the Institutional Review Board (IRB) of Kangdong Sacred Hospital (IRB number: 2021-11-001). The other six hospitals were affiliated with the Research Border Free Zone, which recognizes IRB approval of the research organizing center and waives individual IRB approval.

Informed consent statement: As the study used anonymous and pre-existing data, the requirement for the informed consent from patients was waived.

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Data sharing statement: Dataset available from the corresponding author's e-mail doctorssi@kdh.or.kr.

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ORIGINAL ARTICLE

Retrospective Study

Role of intelligent/interactive qualitative and quantitative analysis-three-dimensional estimated model in donor-recipient size mismatch following deceased donor liver transplantation

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Abstract

BACKGROUND

Donor-recipient size mismatch (DRSM) is considered a crucial factor for poor outcomes in liver transplantation (LT) because of complications, such as massive intraoperative blood loss (IBL) and early allograft dysfunction (EAD). Liver volumetry is performed routinely in living donor LT, but rarely in deceased donor LT (DDLT), which amplifies the adverse effects of DRSM in DDLT. Due to the various shortcomings of traditional manual liver volumetry and formula methods, a feasible model based on intelligent/interactive qualitative and quantitative analysis-three-dimensional (IQQA-3D) for estimating the degree of DRSM is needed.

AIM
To identify benefits of IQQA-3D liver volumetry in DDLT and establish an estimation model to guide perioperative management.

METHODS
We retrospectively determined the accuracy of IQQA-3D liver volumetry for standard total liver volume (TLV) (sTLV) and established an estimation TLV (eTLV) index (eTLVi) model. Receiver operating characteristic (ROC) curves were drawn to detect the optimal cut-off values for predicting massive IBL and EAD in DDLT using donor sTLV to recipient sTLV (called sTLVi). The factors influencing the occurrence of massive IBL and EAD were explored through logistic regression analysis. Finally, the eTLVi model was compared with the sTLVi model through the ROC curve for verification.

RESULTS
A total of 133 patients were included in the analysis. The Changzheng formula was accurate for calculating donor sTLV (P = 0.083) but not for recipient sTLV (P = 0.036). Recipient eTLV calculated using IQQA-3D highly matched with recipient sTLV (P = 0.221). Alcoholic liver disease, gastrointestinal bleeding, and sTLVi > 1.24 were independent risk factors for massive IBL, and drug-induced liver failure was an independent protective factor for massive IBL. Male donor-female recipient combination, model for end-stage liver disease score, sTLVi ≤ 0.85, and sTLVi ≥ 1.32 were independent risk factors for EAD, and viral hepatitis was an independent protective factor for EAD. The overall survival of patients in the 0.85 < sTLVi < 1.32 group was better compared to the sTLVi ≤ 0.85 group and sTLVi ≥ 1.32 group (P < 0.001). There was no statistically significant difference in the area under the curve of the sTLVi model and IQQA-3D eTLVi model in the detection of massive IBL and EAD (all P > 0.05).

CONCLUSION
IQQA-3D eTLVi model has high accuracy in predicting massive IBL and EAD in DDLT. We should follow the guidance of the IQQA-3D eTLVi model in perioperative management.

Key Words: Intelligent/interactive qualitative and quantitative analysis-three-dimensional; Donor-recipient size mismatch; Intraoperative blood loss; Early allograft dysfunction

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Core Tip: This is a retrospective study to identify benefits of intelligent/interactive qualitative and quantitative analysis-three-dimensional (IQQA-3D) liver volumetry in deceased donor liver transplantation and establish an estimation model to guide perioperative management. Patients with estimation total liver volume index (eTLVi) ≥ 1.24 have an increased risk of massive intraoperative blood loss and patients with eTLVi ≤ 0.85 or eTLVi ≥ 1.32 have an increased risk of early allograft dysfunction. To improve the overall survival of patients, we should follow the guidance of the IQQA-3D eTLVi model either for organ allocation or perioperative management.

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INTRODUCTION

An understanding of the interaction between donors and recipients is crucial to ensure optimum outcomes in liver transplantation (LT)[1]. Studies have investigated the effects of mismatch in age, gender[2,3] and ethnicity[4] on the outcomes of LT, with donor-recipient size mismatch (DRSM) being the most important factor[5,6]. Nowadays, with most organ allocation systems worldwide relying on the 'sickest first' policy. However, they do not consider the mismatch of the mismatch of the morphological parameters in the abdominal cavity between donors and recipients[7]. Liver volumetry is rarely undertaken in deceased donor LT (DDLT), which amplifies the adverse effects of DRSM. Massive intraoperative blood loss (IBL), early allograft dysfunction (EAD)[8-10], and other complications caused by small-for-size syndrome (SFSS) or large-for-size syndrome (LFSS) have been found to lead to lower allograft survival and higher patient mortality[11,12]. Therefore, experienced centers have focused more on liver volumetry for total liver volume (TLV) and are committed to establishing a model to estimate the degree of DRSM.

The use of the Archimedes drainage method, the gold standard for liver volumetry, is restricted due to the disadvantage of measuring only for liver *in vitro*[13]. Whether TLV is calculated by height/weight or by body surface

area (BSA), the results are subject to differences in race, gender, and various clinical factors[14]. Estimations by simple empirical formulas are handy and suitable for donor TLV but not for recipient TLV (mostly accompanied by ascites, hepatic carcinomas, cirrhosis, or post-hepatectomy status). Over the years, imaging equipment and visualization techniques have improved significantly and become increasingly refined. Consequently, liver volumetry, based on Doppler ultrasound (DUS), contrast-enhanced computed tomography (CT), or magnetic resonance imaging (MRI) scans, has shown a close correlation with TLV when highly trained operators spend considerable time in postprocessing analysis[15-17]. However, the expensive and time-consuming post process may impede the widespread application of non-automatic liver volumetry, particularly in some emerging transplantation centers.

With advanced technology, the trend towards automated interactive volumetry-assist software replacing manual liver volumetry in the future is anecdotally known. Intelligent/interactive qualitative and quantitative analysis-three-dimensional (IQQA-3D), one of the automated computerized liver volumetry calculators, is characterized by real accuracy, high intelligence, and robust applicability. Scattered reports[18] indicated that the advantages of high repeatability, stability, and reliability of IQQA-3D in measuring standard liver volume can reduce IBL, operative duration, and postoperative complications in precise hepatectomy and living donor LT (LDLT). However, few results have been reported on the role of IQQA-3D in liver volumetry in DDLT. Thus, we conducted this study to identify the benefits of IQQA-3D-based liver volumetry in DDLT and establish a convenient, feasible, and accurate estimation model to guide perioperative management, especially for DRSM-induced massive IBL and EAD.

MATERIALS AND METHODS

Patient selection

We retrospectively analyzed patients who underwent DDLT by a single experienced surgeon between November 2017 and February 2022 in our center and ensured that all surviving patients had been followed up for more than 1 year. All liver allografts were allocated by China Organ Transplant Response System which follows the sickest first policy. Recipients with extreme marginal allografts were excluded according to the following criteria: (1) Donors over 70 years old; (2) Severe allografts steatosis with more than 60% macrosteatosis confirmed by wedge biopsy 1 h after reperfusion; (3) The warm ischemia time of allografts of > 20 min or cold ischemia time of allografts of > 12 h; (4) Prolonged hypotension of the donors (diastolic blood pressure < 60 mmHg, maintenance time > 2 h); (5) Continuous serum total bilirubin and transaminase higher than normal by more than 3 times (maintenance time > 7 d); and (6) ABO-incompatible LT. The recipients of multiorgan transplants or liver re-transplants and recipients with missing demographic characteristics or LT-related information were also excluded.

The study was conducted in accordance with the Helsinki Declaration, and the protocol was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (No. XHEC-D-2023-076).

Procedures

IQQA-3D was applied to calculate estimated TLV (eTLV) for all recipients by analyzing the imported contrast-enhanced CT/MRI scan and automatically outlining the liver parenchyma contour in each layer according to the liver anatomy and density. To improve the accuracy of eTLV, further amendments were carried out by experienced surgeons if necessary.

Donors included in this study were free of liver disease and hence the donor eTLV was similar to that of the normal population. Therefore, due to the extensive practicability for the Chinese population among existing studies, we chose the formula[19] derived by the Shanghai Changzheng Hospital to calculate donor eTLV: TLV (cm^3) = 758.259 × BSA (m^2) - 124.272. Standard TLV (sTLV) was preferably determined using the Archimedes method and secondly deduced from liver weight and density[20] (1.00 ± 0.06 kg/L). To estimate the degree of DRSM, the sTLV index (sTLVi), which was calculated as the ratio of donor TLV to recipient TLV, became a crucial parameter and a gold standard model in this study.

All enrolled patients underwent classic orthotopic LT without the piggyback technique, portocaval shunt, or veno-venous bypass. Anesthesiologists ensured that hypotension and hypothermia did not occur during the operation. An autotransfusion machine was used for all patients to not only maintain the concentration of blood hemoglobin but also to determine IBL. Liver allografts were weighed or volume was measured using the Archimedes method immediately after back-table procedures. Native diseased livers were subjected to the same measurement after removing the gallbladder and accessory ligaments. The donor risk index (DRI) for liver allografts was calculated using the formula described formula[21].

Outcomes

The primary outcomes were massive IBL and EAD caused by DRSM during the perioperative period. Massive IBL was defined as 2000 mL as the baseline. EAD was determined based on the presence of 1 or more of the following most widely accepted criteria[22]: (1) Bilirubin ≥ 10 mg/dL on post operative day 7; (2) International normalized ratio ≥ 1.6 on post operative day 7; and (3) Alanine or aspartate aminotransferase ≥ 2000 IU/L within the first 7 d.

Secondary outcomes included: (1) Procedure-related outcomes including tracheal extubation time, intensive care unit (ICU) stay, postoperative hospital stay, and complications, such as infection and incision nonunion; and (2) perioperative mortality and overall survival (OS) at the end of follow-up.

Statistical analysis

Continuous variables were expressed as mean ± SD or median (interquartile range) and categorical variables as numbers and percentages. Statistical methods used in this study included the Student's *t*-test (or Mann-Whitney U test), Fisher's exact test (or Pearson's chi-square test), Kaplan-Meier method for survival analysis, and Cox regression analyses. All tests were two-sided and differences were considered statistically significant when the *P*-value was < 0.05. Receiver operating characteristic (ROC) curves were drawn to not only detect the optimal cut-off values for the index but also confirm the equivalence of the sTLVi model and the eTLV index (eTLVi) model in predicting massive IBL and EAD. Univariate and multivariable logistic regression analyses were performed successively to predict how sTLVi affected the development of massive IBL and EAD.

RESULTS

Demographics of recipients and donors

A total of 133 patients were enrolled in this study. Patients with chronic hepatitis B virus/hepatitis C virus (HBV/HCV) infection accounted for the majority (63.9%) of the patients, followed by those with chronic alcoholic liver disease (ALD) (18.0%) and drug-induced liver failure (DILF) (9.0%; Table 1). Gastrointestinal bleeding (64.7%), ascites (51.9%), and hepatic encephalopathy (23.3%) were the most common clinical symptoms in the included patients. Pathologically confirmed hepatic carcinomas were found in 42 removed livers (31.6%). Twenty-eight patients (21.1%) used to undergo open upper abdominal surgery and 29 patients (21.8%) had a history of one or more artificial liver support system (ALSS) use.

Donors were younger (mean age, 48.1 ± 13.1 years *vs* 49.4 ± 12.8 years, *P* = 0.388) than recipients and had a higher proportion of males (83.5% *vs* 76.7%, *P* = 0.002). Although the body shape of recipients was generally smaller than that of donors, which was reflected in mean height (168.1 ± 10.2 cm *vs* 170.6 ± 11.2 cm, *P* = 0.032) and mean weight (67.5 ± 18.0 kg *vs* 73.7 ± 17.5 kg, *P* = 0.003), there was no statistical difference in sTLV of recipients and donors (1299 ± 482 mL *vs* 1311 ± 267 mL, *P* = 0.799). Notably, considering the shortage of donor pool in China, 23 liver allografts with HBV or HCV infection (17.3%) and 43 allografts with mild or moderate steatosis (32.3%) were transplanted after exclusion of extreme marginal allografts through a rapid biopsy. The mean DRI of liver allografts was 2.28 ± 0.42. Male donor-female recipient (MD-FR) combination and female donor-male recipient (FD-MR) combination were observed in 26 cases (19.5%) and 17 cases (12.8%), respectively, which were also considered for our analyses.

Operational parameters and outcomes

The mean cold ischemia time was 5.9 ± 1.8 h, and the mean anhepatic phase time was 50.7 ± 9.0 min. Massive IBL developed in 71.4% of recipients with a mean IBL of 3117 ± 1725 mL and a mean intraoperative blood transfusion (IBT) of 1949 ± 1749 mL. The median tracheal extubation time, ICU stay, and postoperative hospital stay were 1 (1-3) d, 2 (1-6) d, and 15 (11-23) d, respectively. EAD developed in 42.1% of recipients, of which SFSS was the cause in 36 recipients (27.1%) and LFSS was the cause in 20 recipients (15.0%). Infection occurred in 36.8% of recipients, and incision nonunion occurred in 21.8%. In terms of perioperative adverse events, there were 29 perioperative deaths (21.8%), including 7 blood loss-specific deaths (5.3%), and 17 EAD-specific deaths (12.8%).

Accuracy of IQQA-3D and the formula method for calculating donor and recipient eTLV

Compared with donor sTLV, there was no statistical difference in donor eTLV calculated using the Changzheng formula (1287 ± 207 mL *vs* 1311 ± 267 mL, *P* = 0.083). However, compared with recipient sTLV, the Changzheng formula for calculating recipient eTLV was not accurate (1213 ± 212 mL *vs* 1299 ± 482 mL, *P* = 0.036), while recipient eTLV calculated using IQQA-3D was highly matched up with recipient sTLV (1311 ± 522 mL *vs* 1299 ± 482 mL, *P* = 0.221). Therefore, the IQQA-3D eTLVi model was defined as the ratio of donor eTLV to IQQA-3D recipient eTLV and selected as an estimation model in the study.

Association between sTLVi and massive IBL

Univariate logistic regression revealed that sTLVi was a risk factor for massive IBL [odds ratio (OR) = 2.968, *P* = 0.037]. The optimal cut-off value of sTLVi in predicting massive IBL calculated using the ROC curve was 1.24, with a sensitivity of 46.3% and a specificity of 94.7% (Figure 1A). Except for a higher proportion of males (82.8% *vs* 65.2%, *P* = 0.023) in the sTLVi < 1.24 group, there was no significant statistical difference in other recipient characteristics (all *P* > 0.05). The distribution of donor characteristics was similar, except for the MD-FR combination (11.5% *vs* 34.8%, *P* = 0.001) and allograft steatosis (25.3% *vs* 45.7%, *P* = 0.017). In addition, the sTLVi < 1.24 group showed advantages in terms of the anhepatic phase time (49.3 ± 7.2 min *vs* 53.6 ± 11.4 min, *P* = 0.024), IBL (2430 ± 1203 mL *vs* 4417 ± 1821 mL, *P* < 0.001), and IBT (1366 ± 1235 mL *vs* 3052 ± 2039 mL, *P* < 0.001). In terms of recovery course, the sTLVi < 1.24 group had a longer survival time (45.6 ± 2.8 mo *vs* 37.0 ± 4.1 mo, *P* = 0.132), and shorter tracheal extubation time (median, 1 d *vs* 2 d, *P* = 0.089), ICU stay (median, 2 d *vs* 3 d, *P* = 0.082) and postoperative hospital stay (median, 15 d *vs* 16 d, *P* = 0.239) than the sTLVi ≥ 1.24 group. These data were not statistically significant except for blood loss-specific mortality (1.1% *vs* 13.0%, *P* = 0.007).

The results of multivariate logistic regression analysis revealed that sTLVi ≥ 1.24 (OR = 18.43, *P* < 0.001), ALD (OR = 9.371, *P* = 0.040) and gastrointestinal bleeding (OR = 3.954, *P* = 0.005) were associated with massive IBL, while DILF (OR = 0.226, *P* = 0.047) was protective against massive IBL (Table 2).

Table 1 Demographics, operational parameters and outcomes of recipients and donors

	Recipient	Donor
Demographics		
Age (yr)	49.4 ± 12.8	48.1 ± 13.1
Male	102 (76.7)	111 (83.5)
Height (cm)	168.1 ± 10.2	170.6 ± 11.2
Weight (kg)	67.5 ± 18.0	73.7 ± 17.5
sTLV (mL)	1299 ± 482	1311 ± 267
eTLV by formula (mL)	1213 ± 212	1287 ± 207
eTLV by IQQA-3D (mL)	1311 ± 522	-
DRI	-	2.28 ± 0.42
Cold ischemia time (h)	-	5.9 ± 1.8
Liver disease		
HBV/HCV	85 (63.9)	23 (17.3)
ALD	24 (18.0)	0
DILF	12 (9.0)	0
Steatosis	8 (6.0)	43 (32.3)
Hepatic carcinoma	42 (31.6)	0
Signs and symptoms		
Moderate or severe ascites	69 (51.9)	-
Gastrointestinal bleeding	86 (64.7)	-
Hepatic encephalopathy	31 (23.3)	-
Operational parameters		
Anhepatic phase time (min)	50.7 ± 9.0	-
IBL (mL)	3117 ± 1725	-
IBT (mL)	1949 ± 1749	-
Hospitalization Information		
Tracheal extubation time (d)	1 (1-3)	-
ICU stay (d)	2 (1-6)	-
Postoperative hospital stay (d)	15 (11-23)	-
Complications		
Massive intraoperative blood loss	95 (71.4)	-
EAD caused by SFSS	36 (27.1)	-
EAD caused by LFSS	20 (15.0)	-
Infection	49 (36.8)	-
Incision nonunion	29 (21.8)	-
Outcomes and follow-up		
Perioperative mortality	29 (21.8)	-
Blood loss-specific mortality	7 (5.3)	-
EAD-specific mortality	17 (12.8)	-
All-cause mortality	46 (34.6)	-
Follow-up time (mo)	27 (9-44)	-

Values are mean ± SD, *n* (%), or median (interquartile range). sTLV: Standard total liver volume; eTLV: estimated total liver volume; DRI: Donor risk index; HBV: Hepatitis B virus; HCV: Hepatitis C virus; ALD: Alcoholic liver disease; DILF: Drug-induced liver failure; IBL: Intraoperative blood loss; IBT: Intraoperative blood transfusion; ICU: Intensive care unit; EAD: Early allograft dysfunction; SFSS: Small-for-size syndrome; LFSS: Large-for-size syndrome; IQQA-3D: Intelligent/interactive qualitative and quantitative analysis-three-dimensional.

Table 2 Univariate and multivariate logistic regression to predict massive intraoperative blood loss

	Univariate			Multivariate		
	OR	95% CI	*P* value	OR	95% CI	*P* value
Recipient age	1.038	1.007-1.070	0.017			0.106
Male	0.835	0.336-2.074	0.697			0.815
sTLVi ≥ 1.24	22.00	4.804-100.8	< 0.001	18.43	3.809-89.15	< 0.001
Donor age	1.014	0.986-1.042	0.340			
MD-FR combination	1.873	0.650-5.396	0.245			
FD-MR combination	0.698	0.238-2.046	0.513			
Graft steatosis (< 60%)	2.187	0.903-5.297	0.083			
Cold ischemia time	1.005	0.815-1.239	0.963			
DRI	1.074	0.437-2.644	0.876			
HBV/HCV	1.225	0.564-2.661	0.608			
ALD	11.82	1.535-90.99	0.018	9.371	1.112-78.98	0.040
DILF	0.165	0.406-0.586	0.005	0.226	0.052-0.983	0.047
Hepatic carcinoma	1.422	0.615-3.288	0.410			
Gastrointestinal bleeding	2.393	1.104-5.188	0.027	3.954	1.502-10.41	0.005
Moderate or severe ascites	1.065	0.502-2.261	0.869			
History of open upper abdominal surgery	2.108	0.737-6.032	0.164			
Platelet count	1.000	0.955-1.005	0.914			
PT	1.011	0.976-1.045	0.577			
Child-Pugh grade C	2.347	0.961-5.731	0.061			
MELD score	1.012	0.975-1.052	0.526			
Anhepatic phase time	1.020	0.975-1.067	0.396			

OR: Odds ratio; 95%CI: 95% confidence interval; sTLVi: Standard total liver volume index; MD-FR: Male donor-female recipient; FD-MR: Female donor-male recipient; DRI: Donor risk index; HBV: Hepatitis B virus; HCV: Hepatitis C virus; ALD: Alcoholic liver disease; DILF: Drug-induced liver failure; PT: Prothrombin time; MELD: Model for end-stage liver disease.

Association between sTLVi with EAD

Unconventional sTLVi was found to be related to an increased risk of EAD ($P < 0.001$). The optimal cut-off values of sTLVi calculated using the ROC curve for predicting EAD caused by SFSS and LFSS were 0.85 (sensitivity 96.0%, specificity 88.0%) and 1.32 (sensitivity 95.0%, specificity 85.0%), respectively (Figure 1B and C). In the sTLVi ≥ 1.32 group, there were significant differences in the proportion of males (61.1% *vs* 83.8% and 81.7%, $P = 0.034$), MD-FR combination (41.7% *vs* 8.1% and 13.3%, $P < 0.001$), and allograft steatosis (50.0% *vs* 13.5% and 33.3%, $P = 0.004$) compared to the 0.85< sTLVi <1.32 group and the sTLVi ≤ 0.85 group. The mean Child-Pugh score for patients in the 0.85 < sTLVi < 1.32 group was the lowest among the 3 groups (8.2 ± 2.8 *vs* 9.6 ± 2.8 and 9.4 ± 2.7, $P = 0.033$). The distribution of other characteristics was similar between the 3 groups, except for the prolonged anhepatic phase time with the increase of sTLVi ($P = 0.002$).

An sTLVi of ≤ 0.85 (OR = 21234, $P < 0.001$) and model for end-stage liver disease (MELD) score (OR = 1.333, $P = 0.002$) were associated with EAD caused by SFSS, whereas HBV/HCV (OR = 0.095, $P = 0.042$) infection was protective against EAD on multivariate logistic regression (Table 3). An sTLVi of ≥ 1.32 (OR = 78.56, $P < 0.001$) and MD-FR combination (OR = 6.540, $P = 0.008$) were associated with EAD caused by LFSS on multivariate logistic regression.

In general, patients with sTLVi between 0.85 and 1.32 had a longer survival time (52.5 ± 2.6 mo *vs* 37.3 ± 4.4 mo and 25.0 ± 4.3 mo, $P < 0.001$), shorter tracheal extubation time (median, 1 d *vs* 1 d and 2 d, $P = 0.002$), lower EAD-associated

Table 3 Univariate and multivariate logistic regression to predict early allograft dysfunction

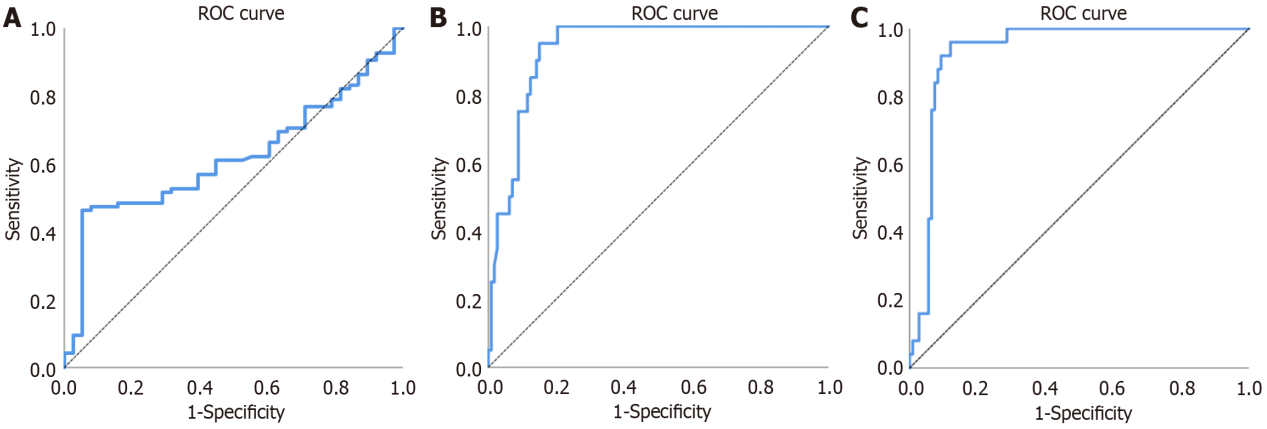
	EAD caused by SFSS						EAD caused by LFSS					
	Univariate			Multivariate			Univariate			Multivariate		
	OR	95%CI	*P* value	OR	95%CI	*P* value	OR	95%CI	*P* value	OR	95%CI	*P* value
Recipient age	0.980	0.948-1.013	0.235			0.992	1.062	1.014-1.113	0.012			0.069
Male	1.268	0.433-3.715	0.665			0.357	0.295	0.109-0.800	0.016			0.134
sTLVi ≤ 0.85/≥ 1.32	175.4	21.85-1407	< 0.001	21234	126-3585713	< 0.001	100.3	16.06-970.4	< 0.001	78.56	9.529-648.0	< 0.001
Donor age	0.992	0.961-1.025	0.645				1.003	0.967-1.040	0.887			
MD-FR combination	0.504	0.139-1.833	0.298				10.61	3.693-30.47	< 0.001	6.540	1.617-26.45	0.008
FD-MR combination	0.916	0.242-3.464	0.897				1.923	0.557-6.637	0.301			
Graft steatosis (< 60%)	0.065	0.009-0.502	0.009			0.444	2.424	0.923-6.368	0.072			
Cold ischemia time	0.923	0.725-1.174	0.512				1.006	0.772-1.312	0.962			
DRI	2.376	0.859-6.573	0.095			0.086	1.069	0.346-3.305	0.908			
HBV/HCV positive graft	0.163	0.021-1.271	0.083				2.420	0.817-7.174	0.111			
HBV/HCV	0.361	0.149-0.877	0.025	0.095	0.010-0.919	0.042	1.058	0.391-2.862	0.912			
ALD	0.838	0.259-2.712	0.768				1.649	0.535-5.084	0.384			
DILF	3.607	1.040-12.51	0.043			0.979	0.488	0.059-4.005	0.504			
Hepatic carcinoma	0.480	0.167-1.381	0.173				0.493	0.154-1.579	0.234			
Hepatic encephalopathy	1.739	0.667-4.534	0.258				3.384	1.249-9.167	0.016			0.186
History of ALSS	2.475	0.957-6.400	0.062				1.677	0.581-4.842	0.339			
Child-Pugh grade C	18.85	2.408-147.5	0.005			0.319	2.200	0.563-8.598	0.257			
MELD score	1.082	1.035-1.133	0.001	1.333	1.109-1.602	0.002	1.024	0.978-1.073	0.305			
Anhepatic phase time	0.949	0.893-1.009	0.097				1.049	1.000-1.100	0.050			0.808

OR: Odds ratio; 95%CI: 95% confidence interval; sTLVi: Standard total liver volume index; MD-FR: Male donor-female recipient; FD-MR: Female donor-male recipient; DRI: Donor risk index; HBV: Hepatitis B virus; HCV: Hepatitis C virus; ALD: Alcoholic liver disease; DILF: Drug-induced liver failure; PT: Prothrombin time; MELD: Model for end-stage liver disease.

morbidity (3.4% *vs* 64.9% and 52.8%, $P < 0.001$) and lower EAD-specific mortality (1.7% *vs* 13.5% and 30.6%, $P < 0.001$) compared to the sTLVi ≥ 1.32 group and the sTLVi ≤ 0.85 group. However, the sTLVi did not influence ICU stay ($P = 0.383$) and postoperative hospital stay ($P = 0.101$). There were significant differences in terms of median survival time between the 3 groups (median, 57 mo *vs* 60 mo *vs* 24 mo, $P < 0.001$). Figure 2 shows that the OS of patients with sTLVi between 0.85 and 1.32 was significantly superior to those with sTLVi ≤ 0.85 ($P = 0.006$) and sTLVi ≥ 1.32 ($P < 0.001$).

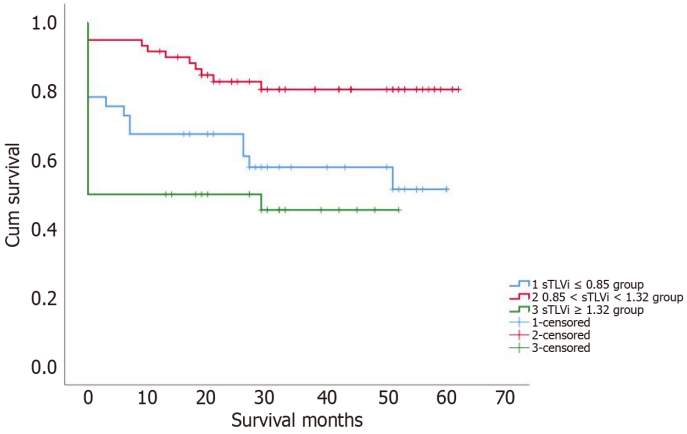
Equivalence between the sTLVi model and IQQA-3D eTLVi model

The area under the curve (AUC) of the sTLVi and IQQA-3D eTLVi models for the detection of massive IBL were 0.618 and 0.598 ($Z = 0.889$, $P = 0.374$; Figure 3A), respectively. The AUC of the sTLVi model and IQQA-3D eTLVi model for predicting EAD caused by SFSS and LFSS were 0.932 and 0.889 ($Z = 1.501$, $P = 0.133$), 0.933 and 0.922 ($Z = 0.710$, $P =$



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Figure 1 Optimal cut-off values of the index in predicting massive intraoperative blood loss and early allograft dysfunction. A: The standard total liver volume index (sTLVi) value of 1.24 was the optimal cutoff value for predicting massive intraoperative blood loss, with a sensitivity of 46.3% and a specificity of 94.7%; B: The sTLVi value of 0.85 was the optimal cutoff value for predicting early allograft dysfunction (EAD) caused by small-for-size syndrome, with a sensitivity of 96.0% and a specificity of 88.0%; C: The sTLVi value of 1.32 was the optimal cutoff value for predicting EAD caused by large-for-size syndrome, with a sensitivity of 95.0% and a specificity of 85.0%. ROC: Receiver operating characteristic.



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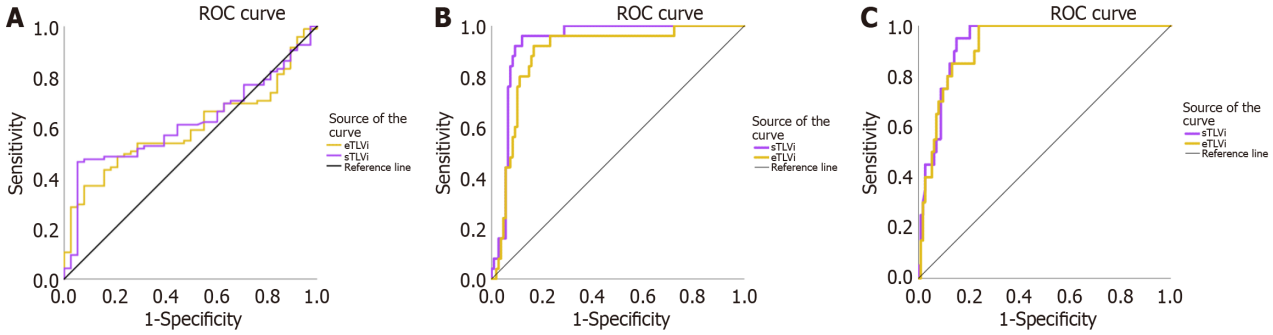
Figure 2 Overall survival of patients in 0.85 < standard total liver volume index < 1.32 group was significantly superior to those in standard total liver volume index ≤ 0.85 group (P = 0.006) and sTLVi ≥ 1.32 group (P < 0.001). sTLVi: Standard total liver volume index.

0.478), respectively (Figure 3B and C). There were no statistically significant differences in the AUC of the sTLVi model and IQQA-3D eTLVi model for predicting massive IBL and EAD. Finally, we found that the IQQA-3D eTLVi model was also applicable to all optimal cut-off values and was equivalent to the sTLVi model in predicting massive IBL and EAD after verification.

DISCUSSION

Postoperative complications associated with DRSM have been reported in an increasing number of DDLT studies[8-12, 23]. Conclusions drawn from LDLT-related studies are that sTLVi < 0.5 is associated with poor outcomes[24] and cannot be used for DRSM in DDLT because additional risk factors, such as brain death and longer preservation injury, result in the need for a larger allograft in DDLT. Therefore, this study aimed to establish an easy, feasible, and accurate estimation model using IQQA-3D to predict massive IBL and EAD associated with DRSM and guide perioperative management in DDLT.

Massive IBT has been reported to possibly increase the risk of acute renal failure, surgical site infections, and recurrence of hepatic carcinomas in patients[25-27]. McCluskey *et al*[28] and Cywinski *et al*[29] attempt to create a model to predict the demand for IBT based on preoperative variables was not successful. The influence of preoperative risk factors and surgical factors on massive IBL have been widely studied[30], but donor factors were rarely mentioned. We have concluded that sTLVi ≥ 1.24 is an independent risk factor for massive IBL, which has not been reported in existing literatures. This may be attributed to the greater surgical difficulty and longer anhepatic phase time. In addition, the



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Figure 3 The equivalence of the standard total liver volume index model and the estimation total liver volume index model in predicting massive intraoperative blood loss and early allograft dysfunction. A: The area under the curve (AUC) of standard total liver volume index (sTLVi) model and intelligent/interactive qualitative and quantitative analysis-three-dimensional (IQQA-3D) estimation total liver volume index (eTLVi) model in detection of massive IBL were 0.618 and 0.598 (Z = 0.889, P = 0.374), demonstrating equivalence; B: The AUC of sTLVi model and IQQA-3D eTLVi model in detection of early allograft dysfunction (EAD) caused by small-for-size syndrome were 0.932 and 0.889 (Z = 1.501, P = 0.133), demonstrating equivalence; C: The AUC of sTLVi model and IQQA-3D eTLVi model in detection of EAD caused by large-for-size syndrome were 0.933 and 0.922 (Z = 0.710, P = 0.478), demonstrating equivalence. eTLVi: estimation total liver volume index; sTLVi: Standard total liver volume index; ROC: Receiver operating characteristic.

probability of large allografts accompanied by steatosis is higher than that of small allografts, which was seen in our analysis. Theoretically, a large allograft can fill the abdominal space to tamponade of bleeding, but allograft steatosis can have a greater effect on massive IBL due to the delayed recovery of coagulation function after reperfusion.

Besides DRSM, we observed that ALD and gastrointestinal bleeding were independent risk factors for massive IBL. Patients with ALD always experience a long course of liver disease and usually experience portal hypertension, plentiful collateral circulation, and cavernous transformation of the portal vein. Gastrointestinal bleeding can serve as a significant marker of portal hypertension, indicating a greater likelihood of multiple thin and varicose blood vessels being transected during surgical dissection. Animal experiments have shown that replacing the exact IBL volume results in an increase in portal pressure by 20%[31], higher rates of massive IBL, and worse outcome[32] in portal hypertensive rats subjected to a period of gastrointestinal bleeding. This has subsequently been demonstrated in our study, as adequate IBT was usually given to recipients with gastrointestinal bleeding before transplantation to correct hypovolemia. Surprisingly, we observed that DILF, a severe liver disease characterized by rapid onset and progression, has become an independent protective factor for massive IBL. Since patients with DILF rarely show anatomic changes in the native liver, the incidence of massive IBL can be reduced with a shorter hepatectomy duration, which is consistent with other reports[33].

Studies have reported factors related to EAD, with the most important being ischemia-reperfusion injury (IRI), which is difficult to regulate. We found that improper sTLVi is associated with an increased risk of EAD and this effect is 'U' shaped. Due to the unavailability of an index to quantify the magnitude of the DRSM effect and of a statistical methodology to formulate the model for describing its nonlinear effect, scattered studies[5,10] only reported the impact of SFSS or LFSS on EAD separately. To overcome the statistical difficulty, we divided recipients into 3 groups and found that the effect on EAD becomes stronger toward both ends of sTLVi values.

MELD score and sTLVi ≤ 0.85 were independent risk factors for EAD caused by SFSS. SFSS occurs when the functional liver volume is too small to provide enough activated hepatocytes for hepatic metabolism. Moreover, the small allograft will withstand the entire blood flow of the original liver, leading to severe congestion of the hepatic sinuses and portal hypertension after reperfusion. MELD score has been reported by various authors as an independent risk factor for the occurrence of EAD in DDLT recipients[34,35], which is consistent with our conclusion.

The MD-FR combination and sTLVi ≥ 1.32 were independent risk factors for EAD caused by LFSS. Patients with sTLVi ≥ 1.32 underwent a higher number of challenging surgeries, had longer anhepatic phase time, and had a higher probability of allograft steatosis, which aggravated IRI. Other anatomical causes of LFSS-related EAD included external compression affecting allograft perfusion and outflow tract obstruction, which were also potential factors with the lowest median survival time in the sTLVi ≥ 1.32 group. In terms of secondary outcomes, the tracheal extubation time in the sTLVi ≥ 1.32 group was longer than that in the other 2 groups, indicating the potential impact of large volume allograft on respiratory complications, consistent with Levesque *et al* report[6]. Gender mismatch, especially the FD-MR combination, has been reported in most studies as an independent risk factor for EAD[2,36,37]. Hormonal factors and differences in IRI and SFSS are the main hypotheses currently[38]. It is speculated that female allografts face a greater risk of IRI due to the sudden loss of protection from estrogen during implantation[2]. However, in our study, the MD-FR combination rather than the FD-MR combination played a significant role in EAD, size which could be related to the differences among males and females.

Because of time constraints with organ allocation and limited services at the donor hospital, donor eTLV measured using cross-sectional imaging is usually not feasible. We confirmed the accuracy of the Changzheng formula method in calculating donor eTLV rather than recipient eTLV. Therefore, we focused on IQQA-3D because of its high accuracy, shorter time, and not having to rely on experienced operators[39,40]. Foreign studies[41] have reported the application of IQQA-3D in precise hepatectomy and LDLT, which not only shortens the operation time but also reduces IBL and other complications. The application of IQQA-3D was rarely reported in DDLT. However, our study has innovatively used

IQQA-3D into the eTLVi model after demonstrating its accuracy in measuring recipient eTLV and confirmed its equivalence with the sTLVi model in predicting massive IBL and EAD. For patients with stable liver diseases, the IQQA-3D eTLVi model can be used to exclude extremely sized mismatched allografts. For patients with critical diseases having minimal choice but to receive unsuitable allografts, timely and sufficient perioperative management can suppress adverse outcomes. For patients with eTLVi ≥ 1.24, sufficient blood products, antifibrinolytics, terlipressin, autotransfusion machine, and liver resection time should be prepared for and reserved before DDLT to reduce IBL. For patients with eTLVi ≤ 0.85, the intraoperative ligation of the splenic artery or resection of the spleen should be considered to control the velocity of the portal vein. Also, if necessary, ALSS therapy should be performed promptly to reduce the burden of the allografts. For patients with eTLVi ≥ 1.32, in addition to sufficient preoperative treatments to reduce the Child-Pugh score and MELD score, strict respiratory management and frequent DUS monitoring to prevent vascular complications, reduced-size LT should be considered.

To improve the accuracy of the results of this impact, we first used sTLVi calculated using the Archimedes method as the gold standard instead of the formula method based on BSA[9,42,43] or graft-to-recipient weight ratio[6,44]. If a simpler or more accurate liver volumetry tool other than IQQA-3D is developed in the future, researchers can still create a new eTLVi model to compare with the sTLVi model in our study. However, like the shortcomings of other studies on DRSM, we only compared TLV between donors and recipients, but failed to compare the abdominal parameters in detail. Due to the limitations of a single-center retrospective study without numerous patients and sufficient follow-up time, future prospective studies are warranted to carried out.

CONCLUSION

We established the IQQA-3D eTLVi model to estimate the degree of DRSM and predict massive IBL and EAD in DDLT. Patients with eTLVi ≥ 1.24 have an increased risk of massive IBL and patients with eTLVi ≤ 0.85 or eTLVi ≥ 1.32 have an increased risk of EAD. To improve the OS of patients, we should follow the guidance of the IQQA-3D eTLVi model either for organ allocation or perioperative management.

ARTICLE HIGHLIGHTS

Research background

Donor-recipient size mismatch (DRSM) is considered a crucial factor for poor outcomes in deceased donor liver transplantation (DDLT).

Research motivation

A feasible model for estimating the degree of DRSM is needed.

Research objectives

To identify benefits of intelligent/interactive qualitative and quantitative analysis-three-dimensional (IQQA-3D) liver volumetry in DDLT and establish an estimation model to guide perioperative management.

Research methods

A retrospective study was conducted to determine the accuracy of IQQA-3D liver volumetry and to establish an estimation total liver volume (TLV) index (eTLVi) model. Receiver operating characteristic curves and logistic regression analysis were used to detect the influencing factors for the occurrence of massive intraoperative blood loss (IBL) and early allograft dysfunction (EAD) in DDLT.

Research results

Recipient estimation TLV calculated using IQQA-3D highly matched with recipient standard TLV (P = 0.221). Alcoholic liver disease, gastrointestinal bleeding, and standard TLV index (sTLVi) > 1.24 were independent risk factors for massive IBL, and drug-induced liver failure was an independent protective factor for massive IBL. Male donor-female recipient combination, model for end-stage liver disease score, sTLVi ≤ 0.85, and sTLVi ≥ 1.32 were independent risk factors for EAD, and viral hepatitis was an independent protective factor for EAD. The overall survival of patients in the 0.85 < sTLVi < 1.32 group was better compared to the sTLVi ≤ 0.85 group and sTLVi ≥ 1.32 group (P < 0.001). There was no statistically significant difference in the area under the curve of the sTLVi model and IQQA-3D eTLVi model in the detection of massive IBL and EAD (all P > 0.05).

Research conclusions

IQQA-3D eTLVi model has high accuracy in predicting massive IBL and EAD in DDLT. We should follow the guidance of the IQQA-3D eTLVi model in perioperative management.

Research perspectives

By establishing the eTLVi model, the degree of DRSM was estimated and IQQA-3D proved to be of guiding value in

perioperative management in DDLT.

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FOOTNOTES

Co-first authors: Zhi-Guo Ding and Wen-Jing Xiao.

Co-corresponding authors: Hao Cai and Yi-Chi Zhang.

Author contributions: Gong W, Cai H and Zhang YC contributed to the study conception and design; Material preparation and data collection were performed by Ding H, Ding ZG and Wang Q; Data analysis was performed by Xiao WJ and Mao XN; The first draft of the manuscript was written by Ding H and all authors commented on previous versions of the manuscript; All authors read and approved the final manuscript. Ding ZG and Xiao WJ contributed equally to this work as co-first authors. The follow-up and data collection of 133 patients included in this study were all completed by Ding ZG, which is a time-consuming and difficult task. Therefore, it is reasonable to list him as a co-first author. The data analysis and figure drawing involved in this article were mostly completed by Xiao WJ. She maintained the rigorous principle and ensured data authenticity in the process of data analysis, which justifies her as a qualified co-first author. Cai H and Zhang YC contributed equally to this work as co-corresponding authors. Cai H consulted a large amount of relevant literature and designed this study together with Gong Wei and Zhang YC. The details of the study (such as primary outcomes, secondary outcomes, influencing factors, *etc*.) were determined by Cai H, which makes it reasonable for him to become a co-corresponding author. Zhang YC also participated in the design of the study, and as an experienced surgeon, he was mainly responsible for calculating eTLV using IQQA-3D and carrying out further amendments if necessary. In addition, he was mainly committed to revising the manuscript, which makes it reasonable for him to become a co-corresponding author.

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ORIGINAL ARTICLE

Retrospective Study

Tenofovir amibufenamide *vs* tenofovir alafenamide for treating chronic hepatitis B: A real-world study

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Abstract

BACKGROUND

The efficacy and safety profile of tenofovir amibufenamide (TMF) in chronic hepatitis B (CHB) patients is not well-established.

AIM

To compare the efficacy and safety of TMF and tenofovir alafenamide (TAF) over a 48-wk period in patients with CHB.

METHODS

A total of 215 subjects meeting the inclusion criteria were enrolled and divided into two groups: TMF group (n = 106) and the TAF group (n = 109). The study included a comparison of virological response (VR): Undetectable hepatitis B virus DNA levels, alanine transaminase (ALT) normalization rates, renal function parameters, and blood lipid profiles.

RESULTS

At 24 and 48 wk, VR rates for the TMF group were 53.57% and 78.57%, respectively, compared with 48.31% and 78.65% for the TAF group (P > 0.05). The VR rates were also similar in both groups among patients with low-level viremia, both hepatitis B e antigen (HBeAg)-positive and HBeAg-negative subgroups. The TMF cohort showed ALT normalization and renal safety profiles similar to the TAF group. There was a notable increase in total cholesterol levels in the TAF group (P = 0.045), which was not observed in the TMF group (P > 0.05). In patients with liver cirrhosis, both groups exhibited comparable VR and ALT normalization rates and renal safety profiles. However, the fibrosis 4 score at 48 wk showed a significant reduction in the TAF group as compared to the TMF group within the liver cirrhosis subgroup.

CONCLUSION

Our study found TMF is as effective as TAF in treating CHB and has a comparable safety profile. However, TAF may be associated with worsening lipid profiles.

Key Words: Alanine transaminase normalization; Chronic hepatitis B; Renal safety; Virological response; Blood lipid; Tenofovir

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Core Tip: This is a retrospective study to compare the efficacy and safety of tenofovir amibufenamide (TMF) and tenofovir alafenamide (TAF) for 48 wk in patients with chronic hepatitis B (CHB). Our study found that TMF is as effective as TAF in treating CHB and has comparable safety profiles. In addition, TAF may cause deterioration of lipid profiles. These results suggest that TMF may be a viable alternative to TAF for CHB treatment.

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INTRODUCTION

Hepatitis B virus (HBV) infection represents a significant economic and health burden worldwide. As of 2019, over 1.5 million preventable new infections continue to occur annually, and there are approximately 296 million people living with chronic HBV infection, resulting in over 820,000 deaths annually due to liver cirrhosis and hepatocellular carcinoma (HCC)[1]. Achieving complete suppression of HBV in a safe and effective manner is crucial for preventing HBV-related adverse health events[2]. Consequently, efforts in this regard have primarily focused on antiviral treatment over the past decades. Current international guidelines recommend as first-line treatments newer antiviral agents with a high genetic barrier to HBV mutation, such as entecavir, tenofovir (TFV) disoproxil fumarate (TDF) and tenofovir alafenamide (TAF) [3-5]. Previous studies have shown that these drugs are safe and effective in treating chronic hepatitis B (CHB). However, long-term use of TDF leads to high levels of circulating TFV, resulting in kidney and bone toxicity, particularly in aging populations[6]. TAF, a TFV prodrug, is converted into the active form of TFV diphosphate *in vivo*, similar to TDF. At a dose of ≤ 25 mg, TAF reduces the total body exposure of TFV by more than 90%[7]. The correlativity study demonstrated that TAF, with its low concentration of TFV in the circulation, reduces the drug load on the kidneys and bones, thereby improving their safety[8].

Currently, tenofovir amibufenamide (TMF) is recommended as the fourth nucleoside analog for first-line treatment of CHB in mainland China[9]. TMF, another prodrug of TFV, is produced by ProTide technology and features an additional methyl group compared to TAF. This extra methyl group may enhance TMF's stability in peripheral blood and facilitate intracellular conversion[10]. *In vitro* studies have shown that TMF has a lower EC_{50} in HepG2.2.15 cells than TAF and TDF [11]. In randomized clinical trials and prospective clinical studies with treatment durations of 48 and 96 wk, TMF was found to be similarly effective in viral suppression to TDF, but with significantly less bone and renal toxicity[12,13]. TMF was approved in June 2021 and was included in the 2021 China National Reimbursement Drug List for CHB treatment.

Due to the recent introduction of TMF in the Chinese market and the limited real-world research data for the Chinese population, there is currently a knowledge gap regarding the drug's safety and efficacy. Therefore, we conducted this clinical study to assess the safety and effectiveness of TMF in treating patients with CHB in China.

MATERIALS AND METHODS

Study design and patient selection

In this retrospective study, we enrolled a total of 587 patients aged 18 and above who had been HBsAg positive for more than 6 mo. These patients were treated at Xiangya Hospital of Central South University between July 2021 and April 2022. Patients were excluded if they met any of the following criteria: (1) Concomitant with other liver diseases, such as alcoholic liver disease, nonalcoholic fatty liver disease, autoimmune liver disease, drug-induced liver injury, hepatolenticular degeneration, or other viral infections [hepatitis A, C, and E virus or human immunodeficiency virus (HIV)]; (2) pregnant or lactating women; (3) concomitant with malignant tumors or other serious diseases affecting survival time; (4) added or changed to other antiviral drugs during treatment; and (5) patients with missing data. Of the enrolled patients, 215 were included in the final analysis and were divided into two groups based on their drug selection: The TMF group and the TAF group.

The study protocol was approved by the Medical Ethics Committee of Xiangya Hospital Central South University (approval No. 202303047).

Treatment and follow-up

During the study period, all patients received anti-HBV treatment with 25 mg of TMF (Hansoh Pharmaceuticals Co., Ltd, Jiangsu, China) or 25 mg of TAF (Gilead Sciences, Inc.) once daily immediately after diagnosis of CHB. Additionally, liver protection drugs were used according to the needs of the disease as prescribed by clinicians. Clinical results and related indicators were collected for each participant during the 48-week follow-up period. These parameters were recorded at baseline, approximately at week 24, and again at week 48.

The efficacy endpoint at week 48 was defined as the proportion of patients achieving a virological response (VR), which is characterized by a reduction in serum HBV DNA levels to less than 10 IU/mL, as measured by the real-time polymerase chain reaction method. Additionally, a pre-specified safety outcome included the percentage change in renal function markers and lipid profiles at weeks 24 and 48 in comparison with the baseline values.

Data collection

Clinical and laboratory data were collected during hospitalization, including clinical characteristics, routine blood test results [including white blood cells (WBC) and platelets (PLT)], liver function tests [including albumin, globulin, total bilirubin (TBIL), direct bilirubin (DBIL), alanine aminotransferase (ALT), and aspartate aminotransferase (AST)], renal function tests [including serum creatinine (Cr), blood urea nitrogen (BUN), and estimated glomerular filtration rate (eGFR)], HBV DNA quantification, serological biomarkers, blood lipids [including triglycerides, total cholesterol (TC), high-density lipoprotein (HDL), and low-density lipoprotein (LDL)], serum phosphorus, alpha-fetoprotein (AFP), and liver stiffness measurement (LSM). The model for fibrosis 4 (FIB-4) score was calculated using the following formula[14]: FIB-4 = age [(year) × AST (U/L)] /[(PLT (10(9)/L) × [ALT (U/L) (1/2)]. In our study, ultrasound examinations were employed to diagnose liver cirrhosis in patients. Low-level viremia (LLV) was characterized as either persistent or intermittent detection of HBV DNA at levels below 2000 IU/mL, with a detection threshold of 10 IU/mL, following 48 wk of antiviral therapy.

Statistical analyses

The sample size for this study was calculated using G Power version 3.1.9.2 (Heinrich-Heine-Universität Düsseldorf). We predetermined the effect size f to range between 0.1 (small) and 0.4 (large), with a type I error rate (alpha) of 0.05 and a power of 0.8, considering two independent groups: TMF and TAF. Employing a one-way ANOVA model, the estimated sample size necessary varied from 84 for a large effect size to 788 for a small effect size. We ultimately recruited 215 participants for the study.

Statistical analyses were performed using SPSS for Windows, version 25.0. Continuous variables were reported as mean ± SD or median (interquartile range), while categorical variables were reported as percentages. The Student *t*-test and rank sum test were used to compare continuous variables, while the chi-squared test was used for categorical variables. All statistical tests were two-sided, and a P-value < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics of the study population

A total of 587 patients with CHB were identified at our hospital between April 2022 and December 2021, of which 372 patients were excluded for various reasons (Figure 1). The final study population consisted of 215 patients, with 106 patients receiving TMF treatment and 109 patients receiving TAF treatment. The mean age of the study population was 40.57 ± 10.54 years, with 145 (67.74%) male patients. The mean LSM using FibroScan was 10.43 ± 3.99, and 42 (19.53%) of patients were diagnosed with cirrhosis. As shown in Table 1, there were no significant differences in baseline characteristics between the two treatment groups, including age, gender proportion, underlying disease, serum Cr, eGFR, BUN, albumin, globulin, AST, ALT, TBIL, DBIL, AFP, blood phosphorus, WBC, PLT, LSM, and FIB-4 score (all P > 0.05). These findings suggest that the two treatment groups were comparable.

Safety

During the 48-wk follow-up period, no significant drug-related adverse reactions were observed with either oral antiviral drug.

VRs

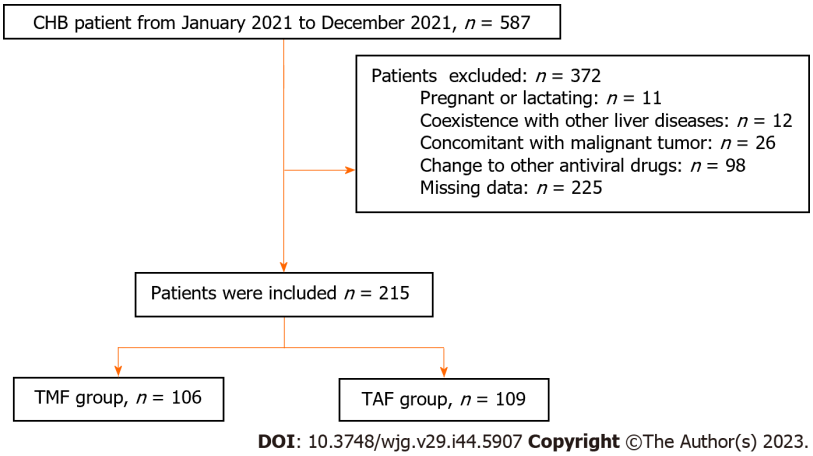
At week 48, the rate of undetectable HBV DNA (HBV DNA < 10 IU/mL) was slightly higher in the TMF treatment group (78.57%), compared to the TAF group (78.65%), although the difference was not statistically significant (Figure 2A). Similarly, the VR rates were similar in both treatment groups for patients with LLV (P > 0.05) (Figure 2B). Among the hepatitis B e antigen (HBeAg)-positive population, 74.36% of patients receiving TMF and 76.09% receiving TAF achieved HBV DNA less than 10 IU/mL (Figure 2C). In the HBeAg-negative population, 82.22% and 81.40% of patients in the TMF and TAF groups, respectively, achieved HBV DNA less than 10 IU/mL (Figure 2D).

The carrying capacity of HBV DNA decreased from 3.96 ± 2.18 to 2.13 ± 0.84 Log10 (IU/mL) in the TMF group and from 4.55 ± 2.31 to 2.35 ± 1.33 Log10 (IU/mL) in the TAF group (Figure 3A). While the TMF group showed a similar VR

Table 1 Baseline characteristics of the study population, *n* (%)

Variable	TMF group 25 mg (*n* = 106)	TAF group 25 mg (*n* = 109)	*P* value
Male	71 (66.98)	74 (67.89)	0.887
Age (yr)	40.96 ± 11.25	40.19 ± 9.84	0.707
Routine blood test			
WBC (× 10^9/L)	5.59 ± 1.64	5.59 ± 1.49	0.792
PLT (× 10^9/L)	193.97 ± 67.44	181.98 ± 68.63	0.218
Liver function			
Albumin (g/L)	45.57 ± 3.64	45.46 ± 3.59	0.746
Globulin (g/L)	29.32 ± 3.66	29.49 ± 3.70	0.617
TBIL (μmol/L)	4.55 (3.70, 5.90)	4.75 (2.20, 18.78)	0.070
DBIL (μmol/L)	2.50 (1.50, 3.90)	5.85 (4.10, 8.33)	0.152
ALT (U/L)	28.30 (20.10, 46.70)	32.40 (22.35, 49.60)	0.203
AST (U/L)	30.50 (24.90, 39.00)	30.00 (24.83, 40.80)	0.740
Kidney function			
BUN (mmol/L)	4.75 (3.99, 6.11)	4.88 (4.22, 5.70)	0.856
Creatinine (μmol/L)	79.50 (66.05, 91.00)	83.30 (73.00, 93.90)	0.177
eGFR (ml/min/1.73 m^2)	90.58 (79.84, 103.80)	97.19 (87.335, 106.38)	0.180
Viral load			
HBV DNA < 10 IU/mL	34 (32.08)	44 (40.37)	0.206
HBeAg positive	39 (36.79)	46 (42.20)	0.417
Blood lipid			
Triglycerides (mmol/L)	1.57 ± 0.82	1.65 ± 1.19	0.719
Total cholesterol (mg/dl)	4.83 ± 1.09	4.30 ± 1.54	0.173
HDL	1.18 ± 0.21	1.10 ± 0.14	0.341
LDL	3.19 ± 0.91	3.20 ± 0.94	0.877
Phosphorus (mmol/L)	1.64 ± 3.84	1.05 ± 0.44	0.958
AFP (ng/mL)	5.19 ± 8.90	5.24 ± 7.89	0.167
LSM (Kpa)	10.17 ± 4.41	10.94 ± 3.37	0.108
FIB-4 score	1.15 (0.75, 1.77)	1.27 (0.87, 2.03)	0.552
Underlying diseases			
Diabetes	5 (4.72)	6 (5.50)	0.793
Cirrhosis	23 (21.70)	19 (17.43)	0.430
Decompensated cirrhosis	4 (3.77)	4 (3.67)	0.968
Hepatocellular carcinoma	2 (1.89)	3 (2.75)	0.674
NAFLD	26 (24.53)	28 (25.69)	0.845
Treatment naïve	63 (59.43)	61 (55.96)	0.607

Data are frequency (%), median M (P25, P75), or mean ± SD deviation. TMF: Tenofovir amibufenamide; TAF: Tenofovir alafenamide; WBCs: White blood cells; PLTs: Platelets; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; DBIL: Direct bilirubin; BUN: Blood urea nitrogen; eGFR: Estimated glomerular filtration rate; P: Phosphorus; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; AFP: Alpha fetoprotein; LSM: Liver stiffness measurement; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; NAFLD: Nonalcoholic fatty liver disease; FIB-4: Fibrosis-4.



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Figure 1 Flow chart of the patient inclusion process. CHB: Chronic hepatitis B; TMF: Tenofovir amibufenamide; TAF: Tenofovir alafenamide.

within 48 wk compared with the TAF group, there was no statistical difference between the two groups (Figure 3B).

On-treatment ALT normalization

The ALT normalization rate in the TMF group was 66.04% and 78.30% at 24 and 48 wk, respectively. In the TAF group, the ALT normalization rate was 55.96% and 74.31% at 24 and 48 wk, respectively. Although the ALT normalization rate in the TAF group showed a higher trend compared to the TMF group from baseline to 48 wk, this difference did not reach statistical significance (Figure 3C). As shown in Figure 3D, both TMF and TAF groups had similar trends in ALT changes during the 48-wk period.

Changes in renal function in TMF and TAF groups

After 48 wk of treatment, Cr levels in the TMF group decreased from 79.50 (66.05, 91.00) μmol/L to 72.00 (63.00, 83.00) μmol/L, and that in the TAF group decreased from 83.30 (73.00, 93.90) μmol/L to 78.10 (61.00, 90.70) μmol/L. Meanwhile, eGFR in both groups increased slightly. However, there was no significant difference in the changes of Cr and eGFR between the two groups within 48 wk (Table 2).

Changes in blood lipids

In this study, plasma lipids consisted primarily included triglycerides, TC, LDL, and HDL. There was no significant change observed in the triglycerides, HDL, and LDL levels at 48 wk. Specifically, in the TMF group, the TC levels demonstrated a mean change of -0.23 ± 0.71 mg/dL at the 48-wk mark (P = 0.822) (Table 3). Conversely, in the TAF group, TC values exhibited a continuous rise from 4.30 ± 1.54 mg/dL at baseline to 5.2 ± 0.99 mg/dL at week 48 (P = 0.045) (Table 3).

Antiviral therapy in patients with liver cirrhosis

In our study, 23 patients in the TMF group and 19 patients in the TAF group had liver cirrhosis. The complete VR rates after 48 wk of treatment were 78.26% in the TMF group and 73.68% in the TAF group, with no significant difference between the two groups (Figure 4A). The normalization rate of ALT was similar in the two groups after 48 wk of treatment (Figure 4B). Renal safety profiles for TMF were similar to those observed for TAF at the 48-wk mark (Supplementary Table 1).

The regression of liver fibrosis was evaluated using FIB-4 scores and LSM in this study (Supplementary Table 2). Liver stiffness was measured using FibroScan. The LSM of cirrhotic patients in the TAF group decreased from baseline to the 48th week (P > 0.05). For the TMF cohort, both LSM and FIB-4 scores demonstrated a marginal increase after 48 wk of treatment; however, these differences did not reach statistical significance. In patients with liver cirrhosis, there was no significant difference between the two groups in the reduction of LSM from baseline level at the 48th week. However, the decrease in FIB-4 in patients with TAF was significantly greater than in patients with TMF [0.29 (0.06, 0.77) *vs*. -0.43 (1.16, -0.08); P = 0.001].

DISCUSSION

TFV ester prodrugs, a class of nucleotide analogs (NAs), are the first-line clinical anti-HBV drugs with potent antiviral efficacy, low resistance rates, and high safety. Various types of ester prodrugs of TFV have been designed in recent decades to improve its antiviral activity and reduce its adverse reactions[15,16].

TMF, the third commercially available TFV ester prodrug, was developed by modifying TAF through the addition of a single methyl group. It received approval from China's National Medical Products Administration for the treatment of HBV infection in 2021. Clinical trials have demonstrated that TMF possesses superior plasma stability compared to TDF

Table 2 Comparison of changes in serum creatinine and estimated glomerular filtration rate between the tenofovir amibufenamide and tenofovir alafenamide groups.

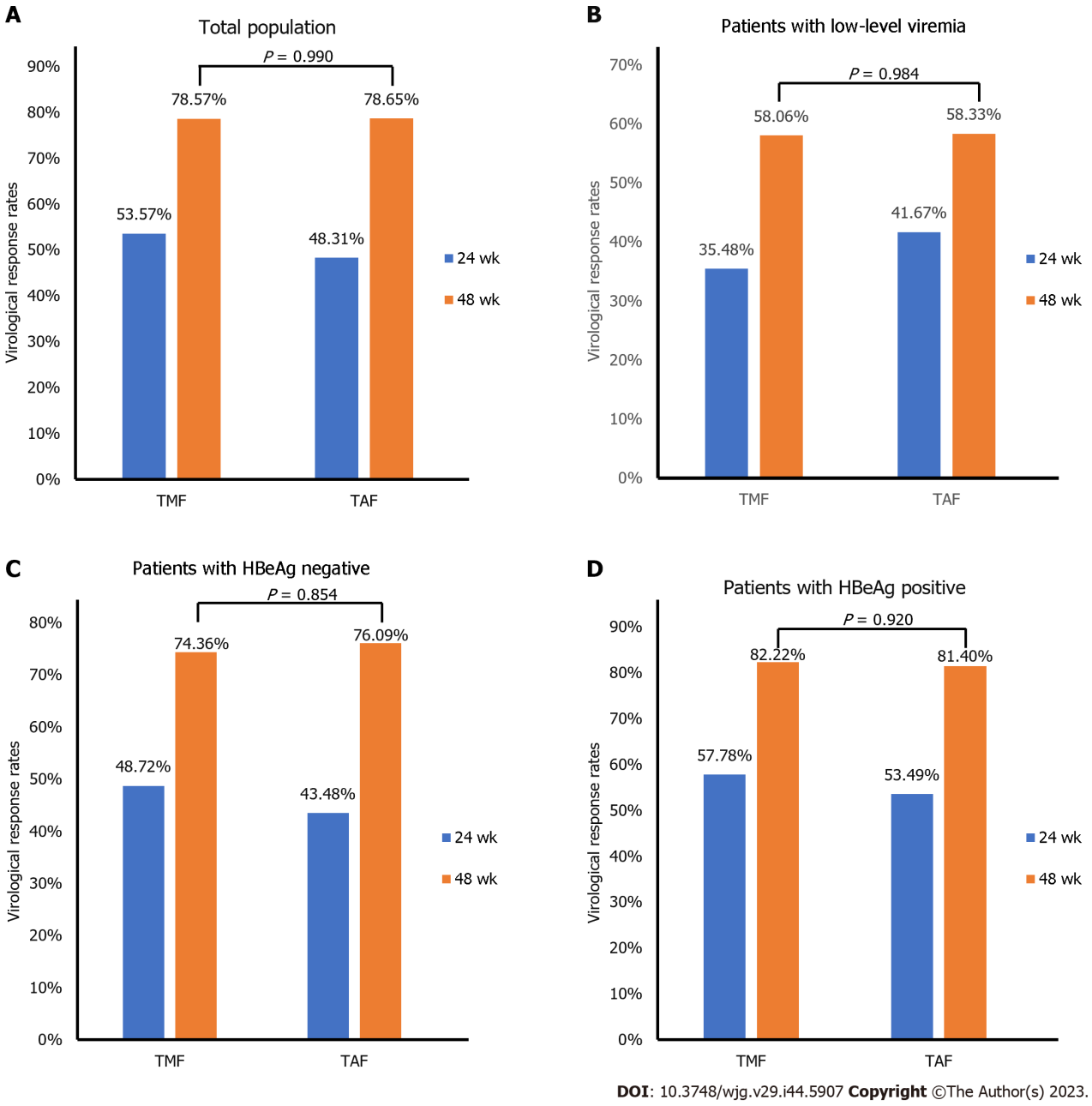
	TMF group (*n* = 106)	TAF group (*n* = 109)	*P* value
Creatinine (μmol/L)			
Before treatment	79.50 (66.05, 91.00)	83.30 (73.00, 93.90)	0.856
After 48 wk	72.00 (63.00, 83.00)	78.10 (61.00, 90.70)	0.194
Reduction	4.00 (-19.65, 19.50)	3.37 (-7.96, 26.13)	0.728
P (baseline *vs*. 48 wk)	0.053	0.105	
eGFR (mL/min/1.73 m^2)			
Before treatment	90.58 (79.84, 103.80)	97.19 (87.35, 106.38)	0.180
After 48 wk	106.37 (94.58, 113.15)	105.17 (88.15,129.56)	0.617
Reduction	-2.22 (-9.72, 16.75)	-4.17 (-227.89, 7.67)	0.093
P (baseline *vs*. 48 wk)	0.301	0.108	

Data are median M (P25, P75). TMF: Tenofovir amibufenamide; TAF: Tenofovir alafenamide; eGFR: Estimated glomerular filtration rate.

Table 3 Changes in blood lipid profiles between the tenofovir amibufenamide and tenofovir alafenamide groups

	TMF group (*n* = 106)	TAF group (*n* = 109)	*P* value
Triglycerides (mmol/L)			
Before treatment	1.57 ± 0.82	1.65 ± 1.19	0.719
After 48 wk	2.16 ± 1.34	1.81 ± 0.87	0.931
Reduction	-0.64 ± 1.02	0.19 ± 0.31	0.103
P (baseline *vs*. 48 wk)	0.099	0.359	
Total cholesterol (mg/dl)			
Before treatment	4.83 ± 1.09	4.30 ± 1.54	0.173
After 48 wk	4.82 ± 1.52	5.20 ± 0.99	0.581
Reduction	-0.23 ± 0.95	-1.02 ± 1.18	0.182
P (baseline *vs*. 48 wk)	0.822	0.045	
HDL (mmol/L)			
Before treatment	1.18 ± 0.21	1.10 ± 0.14	0.341
After 48 wk	1.43 ± 0.74	1.23 ± 0.31	0.977
Reduction	-0.23 ± 0.71	-0.09 ± 0.16	0.672
P (baseline *vs*. 48 wk)	0.430	0.225	
LDL (mmol/L)			
Before treatment	3.19 ± 0.91	3.20 ± 0.94	0.877
After 48 wk	3.15 ± 1.18	3.40 ± 0.71	0.428
Reduction	0.10 ± 0.94	-0.04 ± 0.9	0.791
P (baseline *vs*. 48 wk)	0.807	0.332	

Data are median M (P25, P75). TMF: Tenofovir amibufenamide; TAF: Tenofovir alafenamide; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.



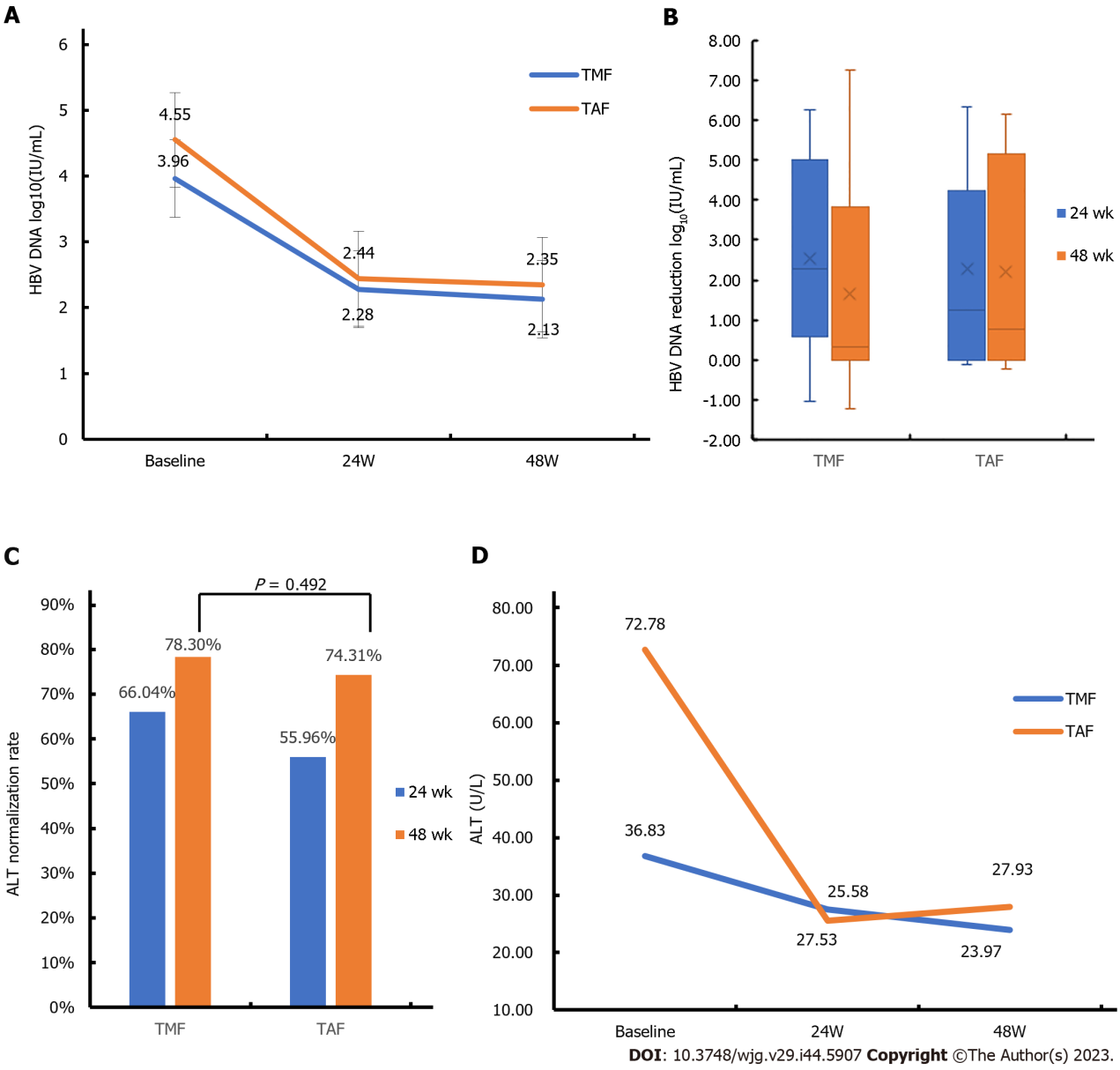
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Figure 2 Comparison of virological response rates between tenofovir amibufenamide and tenofovir alafenamide. A: Virological response (VR) rates of tenofovir amibufenamide (TMF) and tenofovir alafenamide (TAF) groups at 24 and 48 wk; B: VR rates of TMF and TAF groups at 24 and 48 wk with low-level viremia; C: VR rates of TMF and TAF groups at 24 and 48 wk with hepatitis B e antigen (HBeAg) negative; D: VR rates of TMF and TAF groups at 24 and 48 wk with HBeAg positive. TMF: Tenofovir amibufenamide; TAF: Tenofovir alafenamide; VR: Virological response; HBeAg: Hepatitis B e antigen.

and exhibits a similar potency in inhibiting HBV, even when administered at a mere 1/30 of TDF's dosage[17]. Another study, however, revealed that both TMF and TAF displayed enhanced anti-HBV activity and corrective effects on liver biochemical metabolism disturbances relative to TDF *in vitro* and *in vivo*, with TMF exhibiting marginally superior performance to TAF[11]. Given the relatively recent introduction of TMF to the Chinese market and the scarcity of real-world research data for the Chinese population, limited information exists regarding its safety and efficacy.

Consequently, we conducted a real-world investigation to assess the safety and effectiveness of TMF in treating patients with CHB in Southern China. Our findings indicated that, apart from a few mild side effects, TMF did not induce any serious reactions, thus establishing its safety for the treatment of CHB.

In our present study, we observed that the antiviral effectiveness of the TMF and TAF treatment groups was comparable across various patient subpopulations, including the general population, those with LLV, and HBeAg-positive and HBeAg-negative individuals. Throughout the 48-wk TMF treatment duration, no instances of virological breakthrough were encountered. In the 48th week, prior research demonstrated the sustained non-inferiority of VR rates between TMF and TDF treatments, regardless of HBeAg status[13]. Another study corroborated that TAF maintained its efficacy in inhibiting HBV replication relative to TDF, with no emergence of virologic resistance[18]. These findings align with our results, substantiating the equivalent antiviral potency of TMF and TAF in patients with CHB following 48 wk of



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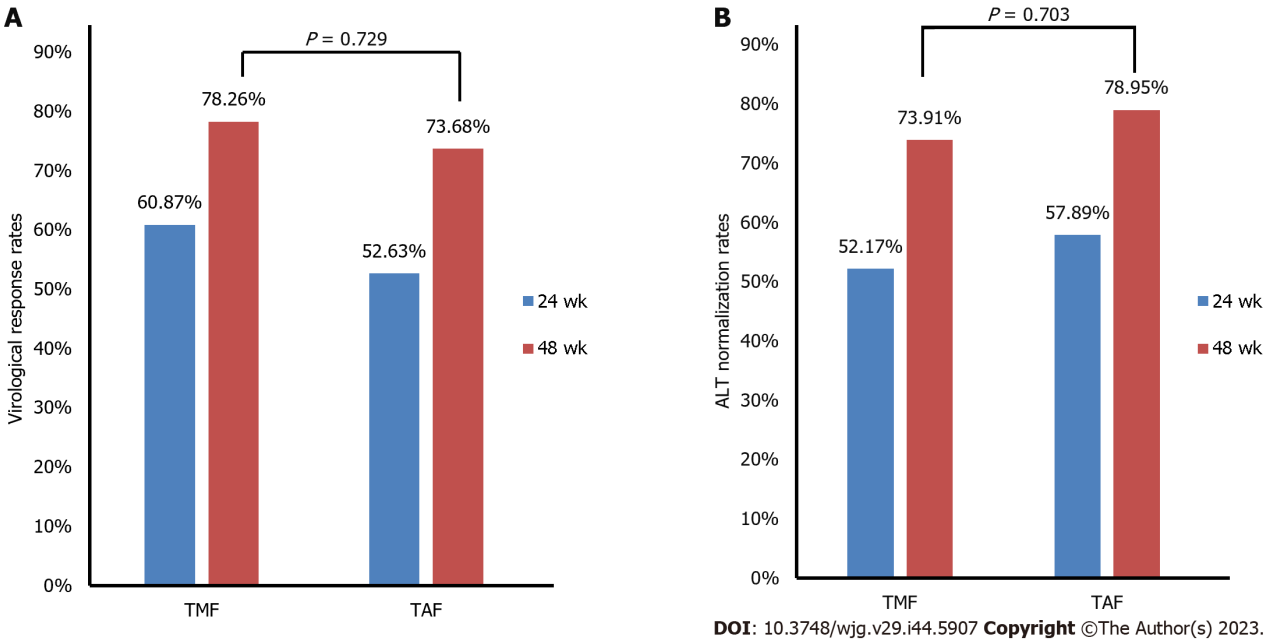
Figure 3 Comparison of changes in hepatitis B virus DNA level, the ratios of alanine aminotransferase normalization and alanine aminotransferase level between the tenofovir amibufenamide and tenofovir alafenamide groups. A: Hepatitis B virus (HBV) DNA levels at baseline week, 24 wk and 48 wk in the tenofovir amibufenamide (TMF) and tenofovir alafenamide (TAF) groups; B: HBV-DNA reduction from 24 wk to 48 wk in the TMF and TAF groups; C: Alanine aminotransferase (ALT) normalization rate of TMF and TAF groups at 24 and 48 wk; D: ALT levels at baseline week, 24 wk and 48 wk in the TMF and TAF groups. TMF: Tenofovir amibufenamide; TAF: Tenofovir alafenamide; ALT: Alanine aminotransferase; HBV: Hepatitis B virus.

therapy.

The significance of achieving on-treatment ALT normalization in CHB patients has been emphasized in recent literature. A large-scale observational study revealed that patients who attained normal on-treatment ALT in the first 48 wk of antiviral treatment exhibited a reduced risk of hepatic events[19]. Liu *et al*[13] reported a notably higher ALT normalization rate for TMF-treated patients compared to those receiving TDF. Concurrently, Agarwal *et al*[18] observed a significantly greater ALT normalization rate among CHB patients treated with TAF relative to TDF recipients. In contrast, our study established that, at week 48, the rate of ALT normalization in the TMF group was comparable to that in the TAF group. These findings, taken together with the virological inhibition rate and biochemical response, confirm the equivalent efficacy of TMF and TAF in the treatment of CHB patients over a 48-wk period.

Prior research has demonstrated the nephrotoxic and osteotoxic effects of TFV[20], emphasizing the need to consider nephrotoxicity when developing TFV prodrugs. Renal impairment associated with TDF primarily arises from proximal tubulopathy[21], with the ensuing tubular dysfunction evidenced by increased serum Cr and reduced serum phosphate levels. The superior renal safety profile of TAF, compared to TDF, is attributable to the primary elimination of TAF through fecal excretion, with less than 1% excreted renally[22].

Since all NAs are eliminated *via* the kidneys, it is crucial for clinicians to monitor for progression of renal dysfunction [23]. The ability of TAF to reduce the risk of renal damage renders it a favorable option for CHB patients who have potential or associated risk factors for renal damage. Studies have confirmed that TAF can continuously enhance renal



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Figure 4 Comparison of the virological response rate and the alanine aminotransferase normalization rate between tenofovir amibufenamide and tenofovir alafenamide groups in patients with cirrhosis. A: Virological response rates of tenofovir amibufenamide (TMF) and tenofovir alafenamide (TAF) groups at 24 and 48 wk; B: Alanine aminotransferase normalization rate of TMF and TAF groups at 24 and 48 wk. TMF: Tenofovir amibufenamide; TAF: Tenofovir alafenamide; ALT: Alanine aminotransferase.

function and maintain bone safety in patients with CHB[7,24]. Our findings indicate that the renal safety profile of the TMF group is comparable to that of the TAF group, suggesting that TMF could emerge as a novel therapeutic option for CHB patients, particularly those with an elevated risk of renal damage.

TDF and TAF are both efficacious nucleoside analogs, with TAF being preferred over TDF due to its lower incidence of renal and bone toxicities. However, there is evidence indicating a worsening of the lipid profile following the transition from TDF- to TAF-containing antiretroviral regimens in patients with HIV, as documented in clinical trials and observational studies[25,26]. Given the association between dyslipidemia, cardiovascular disease, and metabolic/non-alcoholic fatty liver disease-which may elevate the risk of HCC-it becomes imperative to determine whether TAF monotherapy alone adversely affects lipid profiles in CHB patients. This study compared lipid profile alterations in a cohort of CHB patients managed with either TMF or TAF over a 48-wk observation period. The findings indicated a significant increase in serum TC levels in the TAF group (4.3 ± 1.54 *vs*. 5.2 ± 0.99, mg/dL, $P < 0.05$) compared to the TMF cohort (4.83 ± 1.09 *vs* . 4.82 ± 1.52 mg/dL, $P > 0.05$). Therefore, this study suggests that TAF might contribute to the worsening of lipid profiles, whereas TMF appears to have a negligible impact on serum lipids. These conclusions are in contrast with the findings presented by Li *et al*[27] Despite these insights, the underlying mechanism by which TFV affects serum lipids remains to be elucidated. Further research is essential to fully understand this aspect. Nevertheless, physicians should monitor lipid levels vigilantly in patients at the higher end of the normal range when prescribing TAF.

Chronic HBV infection constitutes the primary cause of liver cirrhosis in China and may progress to decompensated liver cirrhosis and primary liver cancer, severely impacting the quality of life of patients. An increasing body of evidence indicates that sustained and effective antiviral therapy can reverse liver fibrosis and cirrhosis[4,28]. Therefore, our study evaluated the efficacy and safety of treatment in patients with cirrhosis.

In cirrhotic patients, the FIB-4 score reduction observed in the TAF cohort was significantly more pronounced than that in the TMF cohort. This could be partly attributed to the marginally higher ALT normalization rate associated with TAF treatment and the limited sample size of both groups. In contrast, no significant difference was discerned in the LSM values between the TMF and TAF groups. However, implications of these findings are not entirely clear, as it remains uncertain if the changes reflect true fibrosis regression or merely a biochemical variation. The observed decline in FIB-4 scores is noteworthy, warranting further research to ascertain if such biochemical alterations correspond to actual histological improvements. Where appropriate, liver tissue biopsies should be considered for conclusive evidence.

Our study is not without limitations. Firstly, the follow-up period of 48 wk may be insufficient to fully capture the antiviral effect, and a more extended timeframe would provide a clearer representation. Secondly, serum Cr and eGFR were employed as markers of renal function in this study, but incorporating indicators reflecting renal tubular function could bolster the study's reliability based on established clinical pharmacological research. Thirdly, the applicability of our findings is restricted, as TMF is not available worldwide. Fourthly, our study did not include data on bone health, such as that obtained *via* DEXA scans, and relied on serum Cr as a surrogate marker for renal function. Lastly, as a single-center retrospective study, future multi-center investigations with larger cohort and longer follow-up durations for CHB patients are essential to corroborate our findings.

CONCLUSION

In summary, our results indicate that TMF demonstrates comparable efficacy to TAF in terms of VR, ALT normalization rate, and renal safety among CHB patients in China. Nevertheless, TMF has an advantage over TAF in patients with hyperlipidemia. Additionally, TMF exhibits effectiveness and safety in cirrhotic patients. Collectively, these results suggest that TMF presents a viable therapeutic alternative for patients with CHB.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B virus (HBV) infection may lead to cirrhosis and hepatocellular carcinoma, and the exploration of optimal antiviral drugs can improve patient prognosis.

Research motivation

Tenofovir amibufenamide (TMF) is a new antiviral drug with limited research on its safety and efficacy. Our research may provide new evidence for the treatment of patients with HBV infection.

Research objectives

To compare the efficacy and safety of TMF and tenofovir alafenamide (TAF) for 48 wk in patients with chronic hepatitis B (CHB). The primary outcome was the proportion of virological responses (VR) at 48 wk. Additional outcomes included the changes of renal function and lipid characteristic markers at weeks 24 and 48 compared to baseline.

Research methods

In this retrospective study, we enrolled a total of 587 patients who had been HBsAg positive for more than 6 mo. Of the enrolled patients, 215 were included in the final analysis and were divided into two groups based on their drug selection: The TMF group and the TAF group.

Research results

The VR rates of the TMF group and TAF group were comparable at 24 and 48 wk of treatment ($P > 0.05$). In patients with low-level viremia, hepatitis B e antigen (HBeAg) positive, and HBeAg negative, their VR rates are also similar. The alanine transaminase (ALT) normalization rate and renal safety of TMF are also comparable to those of TAF. However, total cholesterol levels increased in the TAF group ($P = 0.045$). In patients with liver cirrhosis, the renal safety, VR, and ALT normalization rate were comparable between the TMF group and the TAF group.

Research conclusions

TMF is as effective as TAF in treating CHB and has considerable safety. Moreover, TMF may have more advantages in lipid profile compared to TAF.

Research perspectives

The design and research of new nucleotide analogs should continue in the hope of achieving clinical cure of hepatitis B infection as soon as possible.

FOOTNOTES

Author contributions: Fu L designed the research and supervised the study; Peng WT, Chen KY, Zhou NQ, and Yang FL collected the clinical data; Peng WT, Liu JQ, and Jiang C performed the experiments and wrote the manuscript; Peng SF assisted in experiments; all authors critically reviewed the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Tousled-like kinase 1 promotes gastric cancer progression by regulating the tumor growth factor-beta signaling pathway

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Abstract

BACKGROUND

The role of Tousled-like kinase 1 (TLK1) in in gastric cancer (GC) remains unclear.

AIM

To investigate the expression, biological function, and underlying mechanisms of TLK1 in GC.

METHODS

We measured TLK1 protein expression levels and localized TLK1 in GC cells and tissues by western blot and immunofluorescence, respectively. We transfected various GC cells with lentiviruses to create TLK1 overexpression and knockdown lines and established the functional roles of TLK1 through *in vitro* colony formation, 5-ethynyl-2`-deoxyuridine, and Transwell assays as well as flow cytometry. We applied bioinformatics to elucidate the signaling pathways associated with TLK1. We performed *in vivo* validation of TLK1 functions by inducing subcutaneous xenograft tumors in nude mice.

RESULTS

TLK1 was significantly upregulated in GC cells and tissues compared to their normal counterparts and was localized mainly to the nucleus. TLK1 knockdown significantly decreased colony formation, proliferation, invasion, and migration but increased apoptosis in GC cells. TLK1 overexpression had the opposite effects. Bioinformatics revealed, and subsequent experiments verified, that the tumor growth factor-beta signaling pathway was implicated in TLK1-mediated GC progression. The *in vivo* assays confirmed that TLK1 promotes tumorigenesis in GC.

CONCLUSION
The findings of the present study indicated that TLK1 plays a crucial role in GC progression and is, therefore, promising as a therapeutic target against this disease.

Key Words: Gastric cancer; Tousled-like kinase 1; Tumor growth factor-beta; Tumour progression; Targeted therapy

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Core Tip: We demonstrated that Tousled-like kinase 1 (TLK1) is highly expressed in gastric cancer (GC), localized mainly to the nucleus, significantly promotes GC cell proliferation, invasion, and migration, and inhibits apoptosis. TLK1 may facilitate GC progression by modulating tumor growth factor-beta expression. We believe that TLK1 could be a crucial therapeutic target for GC, and propose that future investigations evaluate the feasibility and practicality of targeting TLK1 in GC treatment.

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INTRODUCTION

Gastric cancer (GC) is the fifth most prevalent cancer and the third leading cause of cancer-related mortality worldwide [1]. Endoscopy is the mainstay of early-stage GC treatment whereas advanced GC must be managed through surgery and other interventions including chemotherapy and targeted therapy[2]. Despite significant progress in GC control, however, its overall survival remains unsatisfactory. Hence, potential therapeutic targets against this disease are urgently required.

Tousled kinase and Tousled-like kinase (TLK) are serine-threonine enzymes implicated in DNA replication[3], transcription[4,5], and chromatin assembly[6]. TLK promotes glioma progression[7] and modulates latent viral activation [8]. Thus, it plays a critical role in various cellular processes. The two known TLK genes are TLK1 and TLK2. However, the former has received more research attention than the latter, and prior investigations focused primarily on the mechanisms by which TLK1 regulates DNA replication and repair. It interacts with Aurora kinase and chromatin assembly factors, and together they precisely control spindle assembly and S-phase progression[9]. The ataxia-telangiectasia-mutated-checkpoint kinase (Chk1)-TLK pathway uses TLK1 as a target to direct chromatin assembly[10, 11]. TLK1 collaborates with Chk1 to regulate RAD9 checkpoint clamp component A (Rad9A) phosphorylation and, by extension, modulate the DNA damage response[12,13]. It also confers resistance to ultraviolet irradiation and, therefore, helps maintain cellular integrity and survival[14-17].

Recent research efforts have aimed to clarify the roles of TLK1 in cancers. TLK1 mediates prostate cancer progression *via* the TLK1-MAPK-activated protein kinase 5 and TLK1-NIMA-related kinase 1 axes[18-21]. The phenothiazine analog J54 is a potent TLK1 inhibitor and a possible therapeutic agent against prostate cancer[22]. TLK1 may promote the progression of glioma[23-25] and oral cancer[26]. However, the expression patterns and functional relevance of TLK1 in GC remain to be determined. Hence, the present study aimed to elucidate the functional significance of TLK1 in GC cells and potentially identify a novel therapeutic target against this disease.

MATERIALS AND METHODS

Bioinformatics analysis

The RNA-sequencing data obtained from The Cancer Genome Atlas (TCGA) website (https://portal.gdc.cancer.gov/) was subjected to a transcriptome analysis in R v. 4.1.2 (R Core Team, Vienna, Austria) to determine the functional relevance of TLK1 to GC. Differential gene expression between the high- and low-TLK1 expression groups was evaluated. The differentially expressed genes were then subjected to a Kyoto Encyclopedia of Genes and Genomes analysis (http://www.kegg.jp/) to identify and characterize the enrichment pathways with which they were associated. A gene set enrichment analysis was then used to analyze specific pathways and identify the interactions between them and TLK1 in GC. This multifaceted approach established novel molecular mechanisms and regulatory pathways underlying TLK1-mediated GC progression and elucidated GC biology.

Cell culture

The normal gastric epithelial cell line GES-1 as well as the AGS, SGC7901, MGC803, BGC823, and HGC27 GC cell lines were sourced from GeneChem (Shanghai, China) in December 2021. Short tandem repeat analyses verified the authenticity of each cell line and the final test was conducted on March 30, 2022. The cells were cultured in PMIS-1640 medium (Corning Inc., Corning, NY, United States) supplemented with 10% fetal bovine serum (FBS; Clark Bioscience, Richmond,

VA, United States), 1% penicillin, and 1% streptomycin (HyClone Laboratories, Logan, UT, United States) and incubated in a Thermo Fisher incubator (Thermo Fisher Scientific, Waltham, MA, United States) under a 5% CO_2 atmosphere at 37°C. They were then subcultured after they reached 80% confluence and maintained in the log phase. Strict quality control measures including regular mycoplasma testing were implemented to mitigate the risk of contamination.

Western blotting

Total protein was extracted with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with phosphatase and protease inhibitors (BBI Life Sciences Corporation, Shanghai, China). Denatured sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate the proteins and they were then blotted onto polyvinylidene fluoride (PVDF) membranes. The latter were blocked with 5% skim milk for 1 h and incubated at 4°C overnight with primary antibodies including anti-GAPDH (1:2,500; No. 7074T; Cell Signaling Technology, Danvers, MA, United States), anti-TLK1 (1:1,000; No. 13564-1-AP; Proteintech Group, Rosemont, IL, United States), and anti-tumor growth factor-beta (TGF-β) (1:1,000; No. 346599; ZenBio, Chengdu, China) to detect the protein expression levels. The PVDF membranes were then washed with Tris-buffered saline with Tween-20 (TBST), incubated with secondary antibody at room temperature for 1 h, rinsed again with TBST, and visualized by enhanced chemiluminescence (ECL; Bridgen, Beijing, China). The ECL signals were captured on a Tanon-5200 multi-platform (Tanon Science & Technology Co. Ltd., Shanghai, China). The protein band gray values were then quantified with ImageJ software (National Institutes of Health (NIH), Bethesda, MD, United States).

Immunofluorescence staining

Sterile slides were placed in a 24-well plate, drops of cell suspension were dispensed onto them, and they were incubated overnight. The following day, the cells were fixed with 4% formaldehyde, blocked with 5% bovine serum albumin (BSA) for 1 h, and incubated at 4°C overnight with anti-TLK1 antibody (1:1,000; No. 13564-1-AP; Proteintech Group). The next day, the slides were subjected to fluorescent secondary antibody (1:250; No. A11012; Thermo Fisher Scientific) at room temperature for 1 h. Then 4`,6-diamidino-2-phenylindole (DAPI; 1:100; No. D9542; Sigma-Aldrich Corp., Roedermark, Germany) nuclear stain was applied to them for 15 min and they were observed and photographed under a confocal laser scanning microscope (CLSM; No. LSM800; Carl Zeiss AG, Jena, Germany).

Lentivirus infection

Three distinct small hairpin RNAs (shRNAs) targeting human TLK1 and a lentiviral TLK1 overexpression construct were procured from GeneChem (Shanghai, China). Cells were seeded in 12-well plates and the lentiviral particles were added to them at a multiplicity of infection = 10 in the presence of a transfection aid per the manufacturer's instructions. The medium was replaced and the cells were passaged after 24 h and 48 h, respectively. Successful lentiviral infection was confirmed by screening the cells with a medium containing puromycin. Transfection efficiency was assessed *via* protein extraction. The shRNA sequences used in the experiment were as follows: sh1: 5`-GAUACAGAUACGUUUUGUACA-dTdT-3`; sh2: 5`-CUCGUAGGGUAGAAACCAAUAdTdT-3`; and sh3: 5`-GCAGGCACUUACUGGUAUUUAdTdT-3`.

Colony formation assay

Cells were seeded in six-well plates at a density of 800/well and incubated with gentle agitation under optimal conditions. The culture medium was renewed every 3 d and the experiment was terminated when cell colonies emerged. The cells were then fixed with 4% formaldehyde for 20 min, washed thrice with phosphate-buffered saline (PBS), stained with 0.1% crystal violet for 15 min, air-dried, and imaged.

5-ethynyl-2`-deoxyuridine assay

An 5-ethynyl-2`-deoxyuridine (EdU) Kit (No. C0078S; Beyotime Biotechnology, Shanghai, China) was utilized for this assay. Sterile slides were aseptically placed in 12-well plates. Cells were seeded onto them and incubated overnight until the optimal cell density was attained. The next day, a 2 EdU solution was prepared according to the manufacturer's instructions and mixed in equal proportions with the culture medium. The mixture was then added to the 12-well plates and incubated at 37°C for 2 h. The culture medium was removed and the cells were fixed with 4% paraformaldehyde for 15 min and washed with PBS containing 3% BSA (Beyotime Biotechnology). Then 0.3% Triton X-100 was added and the cells were incubated at room temperature for 15 min. The cells were rinsed and a pre-configured Click reaction solution was added to the 12-well plates. The cells were incubated for 30 min and their nuclei were stained with Hoechst33342 for 10 min. An anti-quenching agent was added and the cells were viewed under a microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Transwell assays

Cells in the log phase were seeded at optimal density and subjected to serum deprivation for 24 h. Matrigel Basement Membrane Matrix (BD Biosciences, Shanghai, China) was diluted to a working concentration and uniformly spread onto the upper layer of a Transwell chamber (Corning Inc.). The latter was incubated at 37°C for 5 h. The cell concentration was adjusted and maintained at 8 10^5/mL. One hundred microliters cell suspension was added to the upper chamber, 650 μL high-serum medium was added to the lower chamber, and the Transwell was incubated in a suitable environment. After a predetermined incubation period, the Transwell chambers were extracted. Their contents were then fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and observed and photographed under a microscope (Leica Microsystems GmbH).

Flow cytometry

An Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Kit (Yeasen Biotechnology, Shanghai, China) was used for this assay. Cell supernatant and digested cells were collected in a centrifuge tube, washed with PBS, and resuspended in 100 μL of 1 × binding buffer. Then 5 μL Annexin V-FITC and 10 μL PI staining solution were added and the suspension was incubated in the dark at room temperature for 15 min. Then a CytoFLEX Flow Cytometry Platform (Beckman Coulter, Brea, CA, United States) was used to detect apoptosis.

Xenograft mouse model

Four-Week-old male BALB/C nude mice were obtained from GemPharmatech, Jiangsu, China, and maintained under specific pathogen-free conditions. The control and experimental groups each included six mice. SGC7901 cells were digested and resuspended in PBS and 5 x 10^6 were subcutaneously injected into the left underarm of each mouse. During the experiment, the mice were provided with sufficient food and water, and their body weight was periodically measured. The volumes of any subcutaneous tumors that had formed were determined every 4 d. Humane euthanasia was performed when the body weight decreased by ≥ 20% and/or the tumor diameter was > 1.5 cm.

Immunohistochemical staining

Immunohistochemical (IHC) staining was conducted per established methods[27]. Protein expression levels were independently evaluated by two pathologists blinded to the clinical information of the patients. The IHC score was calculated based on the staining intensity and area to assess the protein expression levels.

Statistical analysis

All data were analyzed with SPSS v. 22.0 (SPSS Inc., Chicago, IL, United States), GraphPad Prism v. 7.0 (GraphPad Software, La Jolla, CA, United States), and R v. 4.1.2 (R Core Team, Vienna, Austria). Student's *t*-test or one-way ANOVA was used to detect significant differences between treatments. $P < 0.05$ was considered statistically significant ($^aP < 0.05$).

Ethics statement

Studies involving human participants were reviewed and approved (No. 20180323) by the Ethics Committee of Anhui Medical University, Anhui, China. All patients and participants provided written informed consent. All animal experiments were approved (No. 20180345) by the Institutional Animal Care and Use Committee of Anhui Medical University.

RESULTS

TLK1 was significantly upregulated in GC cells and tissues and localized mainly to the nucleus

We performed a bioinformatics analysis using TCGA to determine TLK1 expression and localization in GC. We found that TLK1 was upregulated in GC and other tumors of the digestive tract (Figure 1A). We then conducted a western blot analysis to determine the TLK1 expression levels in the GC cells and tissues. We measured TLK1 protein expression in normal gastric epithelial cells (GES-1) and the GC cells AGS, SGC7901, MGC803, BGC823, and HGC27 to clarify the association between the expression and localization of TLK1 in GC. TLK1 expression was significantly higher in GC than in GES-1 cells (Figure 1B and C). We then measured TLK1 expression in GC and their adjacent normal tissues and found that it was significantly higher in the former than in the latter (Figure 1D and E). We then subjected HGC27 and MGC803 GC cells to immunofluorescence staining and observed that TLK1 was localized mainly to their nuclei (Figure 1F). The foregoing findings suggest that TLK1 is upregulated in GC cells and tissues and is localized to the nucleus.

TLK1 significantly enhanced GC cell clonogenesis, proliferation, invasion, and migration in vitro

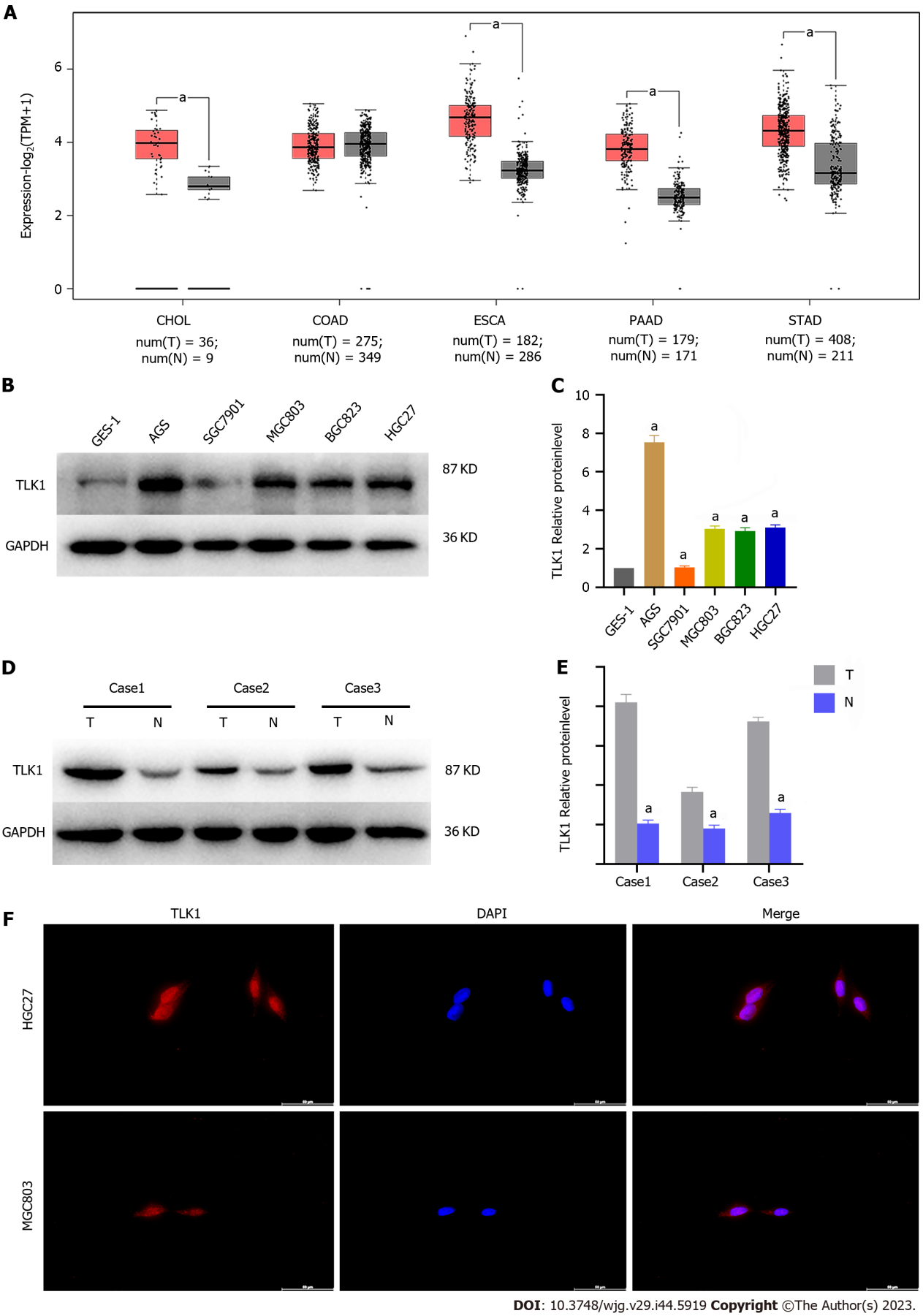
We increased TLK1 expression in SGC7901 cells *via* lentiviral transfection and verified its upregulation *via* western blot to elucidate its role in GC cells (Figure 2A). We then used a colony formation assay to assess the impact of TLK1 overexpression on GC cell proliferation. TLK overexpression substantially increased the number of SGC7901 cell colonies compared to the control (Figure 2B and C). The EdU assay demonstrated a dramatic increase in the proportion of proliferative SGC7901 cells in response to TLK1 overexpression (Figure 2D and E). The preceding experiments showed that TLK1 overexpression augments SGC7901 cell proliferation.

A Transwell assay showed that TLK1 overexpression significantly increased SGC7901 GC cell migration and invasion relative to the control (Figure 2F and G). Overall, TLK1 overexpression promoted clonal expansion, proliferation, invasion, and migration in SGC7901 GC cells.

TLK1 knockdown inhibited GC cell clonal formation, invasion, and migration

We transfected AGS and HGC27 cells with three lentiviral sequences designed to knock down/silence TLK1 and used western blot analysis to assess transfection efficiency. We selected sh2 and sh3 for the subsequent experiments as they exhibited superior knockdown efficacy (Figure 3A). A colony formation assay demonstrated that TLK1 knockdown markedly reduced the number of AGS and HGC27 cell colonies compared with the control (Figure 3B and C).

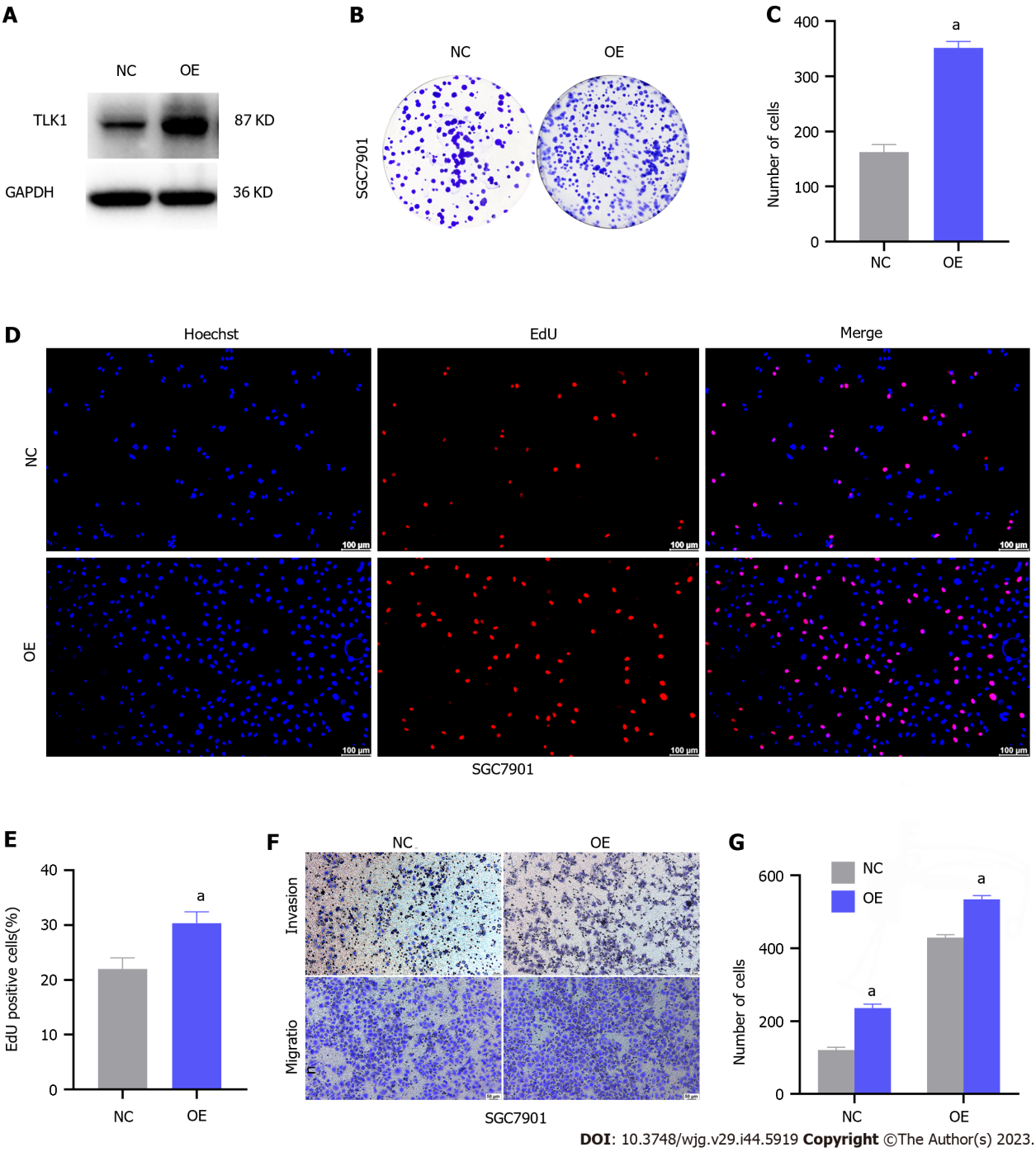
A Transwell assay revealed that TLK1 knockdown significantly diminished AGS GC cell invasion and migration relative to the control (Figure 3D and E). Similar results were obtained for HGC27 cells subjected to TLK1 knockdown (Figure 3F and G). The foregoing findings suggest that TLK1 knockdown effectively suppresses clonal formation,



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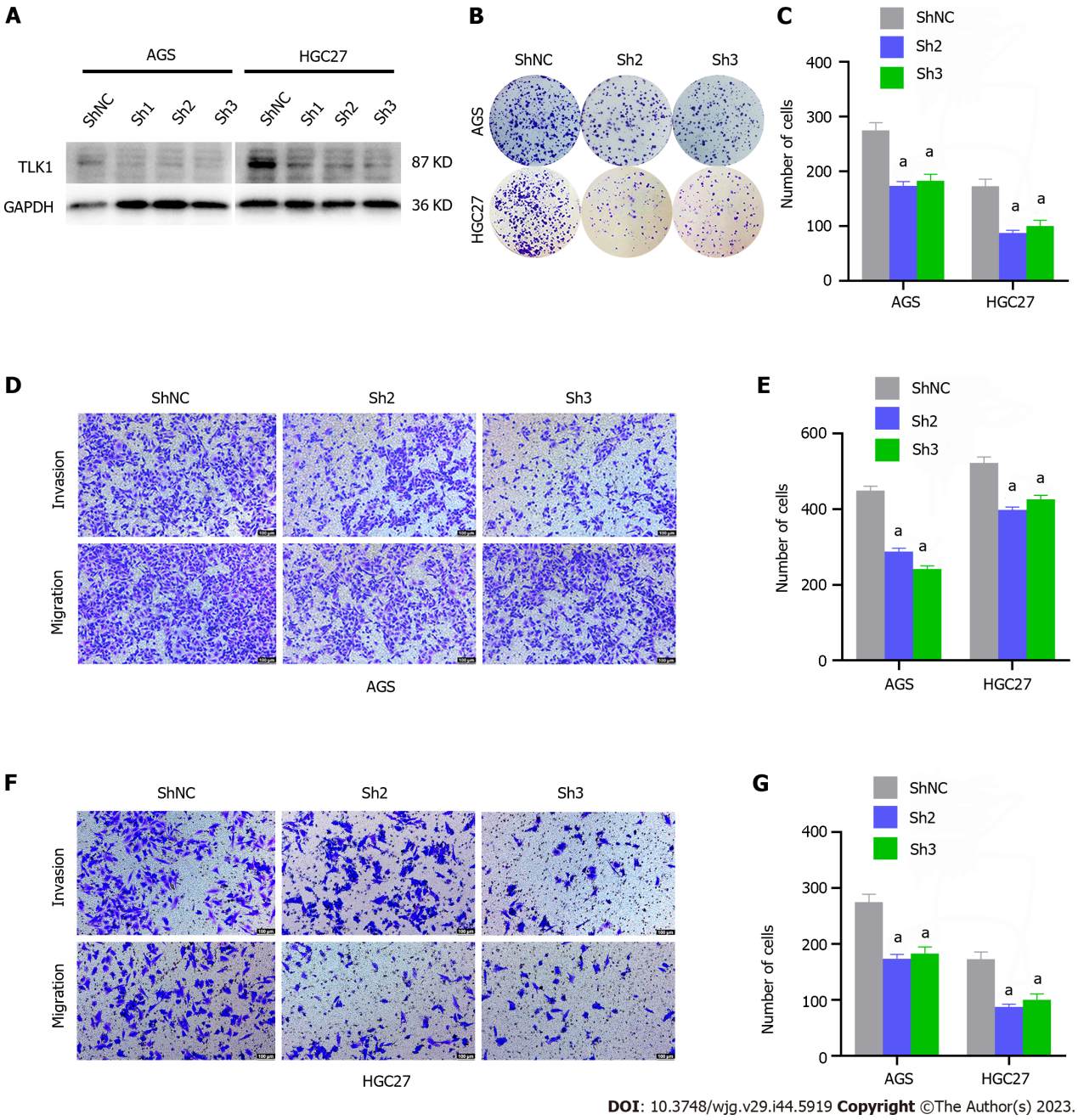
Figure 1 Tousled-like kinase 1 was upregulated and localized mainly to the nucleus in gastric cancers. A: Tousled-like kinase 1 (TLK1) expression in various tumors was explored using The Cancer Genome Atlas database; B and C: Western blot measuring TLK1 expression in normal gastric epithelial

cells GES-1 and in gastric cancer (GC) cell lines; D and E: Western blot detecting differential TLK1 expression between GC and adjacent non-neoplastic tissues; F: Immunofluorescence detecting subcellular TLK1 compartmentalization in HGC27 and MGC803 cell lines. [a]*P* < 0.05. TLK1: Tousled-like kinase 1; CHOL: Cholangiocarcinoma; COAD: Colon adenocarcinoma; ESCA: Esophageal carcinoma; PAAD: Pancreatic adenocarcinoma; STAD: Stomach adenocarcinoma.



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Figure 2 Tousled-like kinase 1 upregulation potentiated tumorigenesis in gastric cancer cell lines. A: Western blot demonstrated the effects of Tousled-like kinase 1 (TLK1) overexpression in SGC7901 cells; B and C: Impact of TLK1 overexpression on clonogenesis in SGC7901 cells; D and E: 5-ethynyl-2`-deoxyuridine assay evaluating the influence of TLK1 overexpression on SGC7901 cell proliferation; F and G: Transwell assay showing the effects of TLK1 overexpression on SGC7901 cell invasion and migration. [a]*P* < 0.05. TLK1: Tousled-like kinase 1; NC: Negative control; OE: Overexpression; EdU: 5-ethynyl-2`-deoxyuridine.



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Figure 3 Tousled-like kinase 1 knockdown attenuated clonal formation, invasion, and migration in gastric cancer cell lines. A: Western blot revealing the impact of Tousled-like kinase 1 (TLK1) knockdown on AGS and HGC27 cell lines; B and C: Effects of TLK1 suppression on clonogenesis in AGS and HGC27 cell lines; D and E: Transwell assay disclosing the effects of TLK1 knockdown on AGS cell invasion and migration; F and G: Transwell assay demonstrating the influences of TLK1 suppression on HGC27 cell invasion and migration. [a]$P < 0.05$. TLK1: Tousled-like kinase 1; shNC: Short hairpin RNA of negative control.

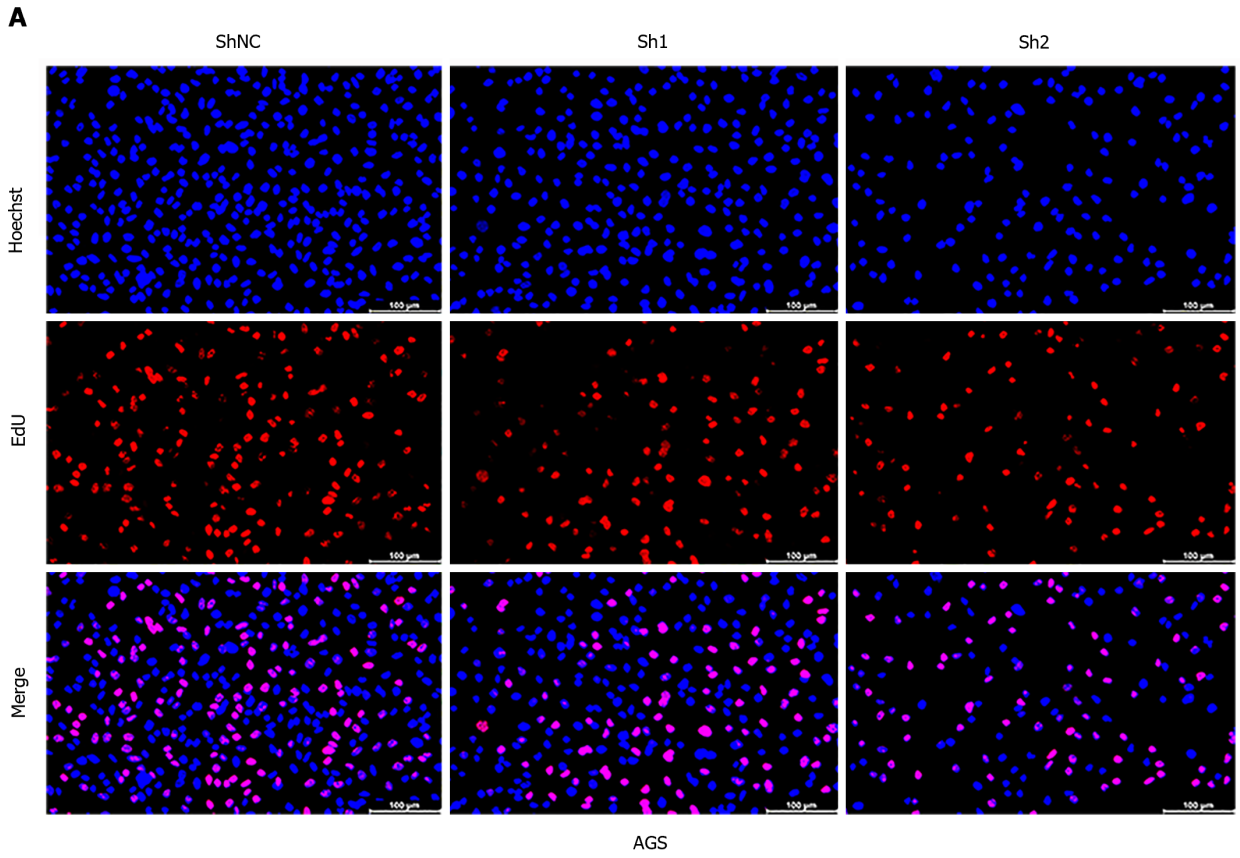
invasion, and migration in GC cells.

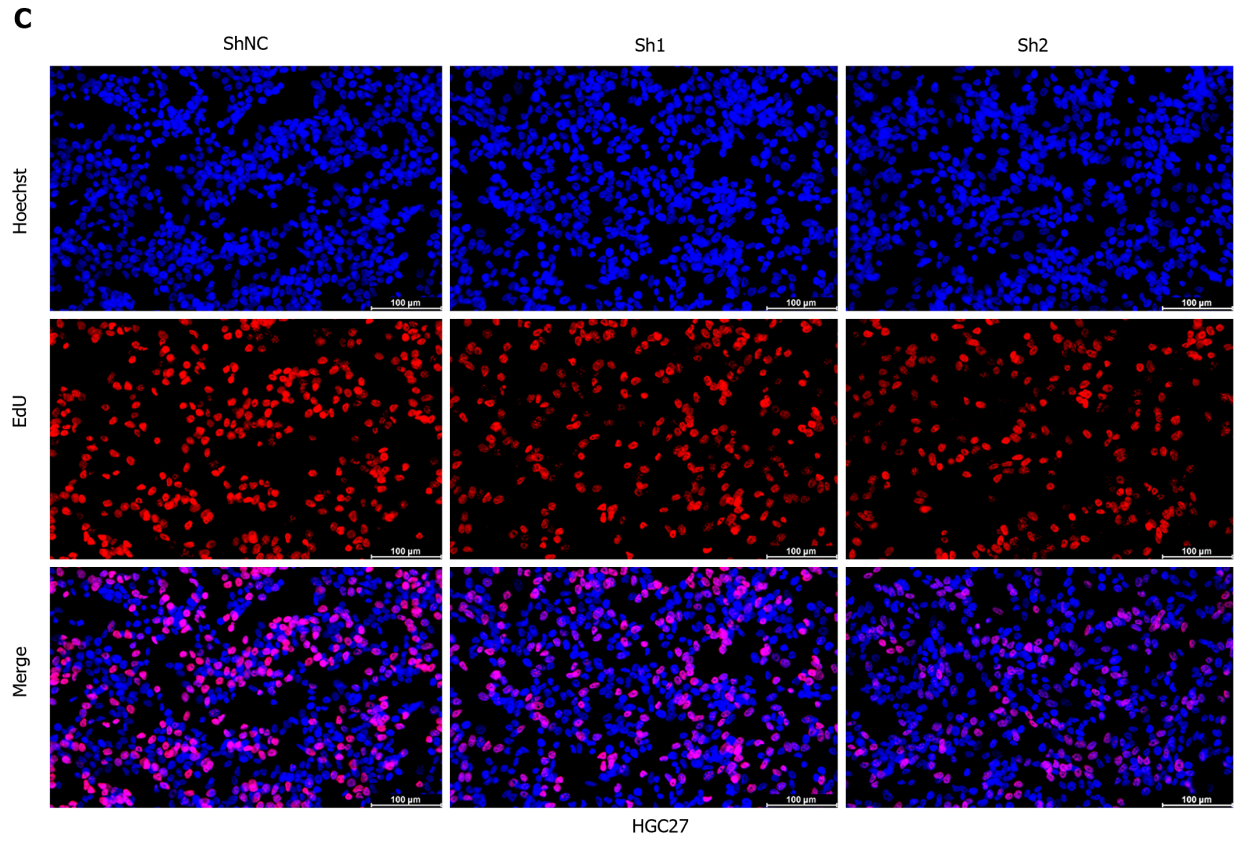
TLK1 knockdown diminished proliferation and augmented apoptosis in GC cells

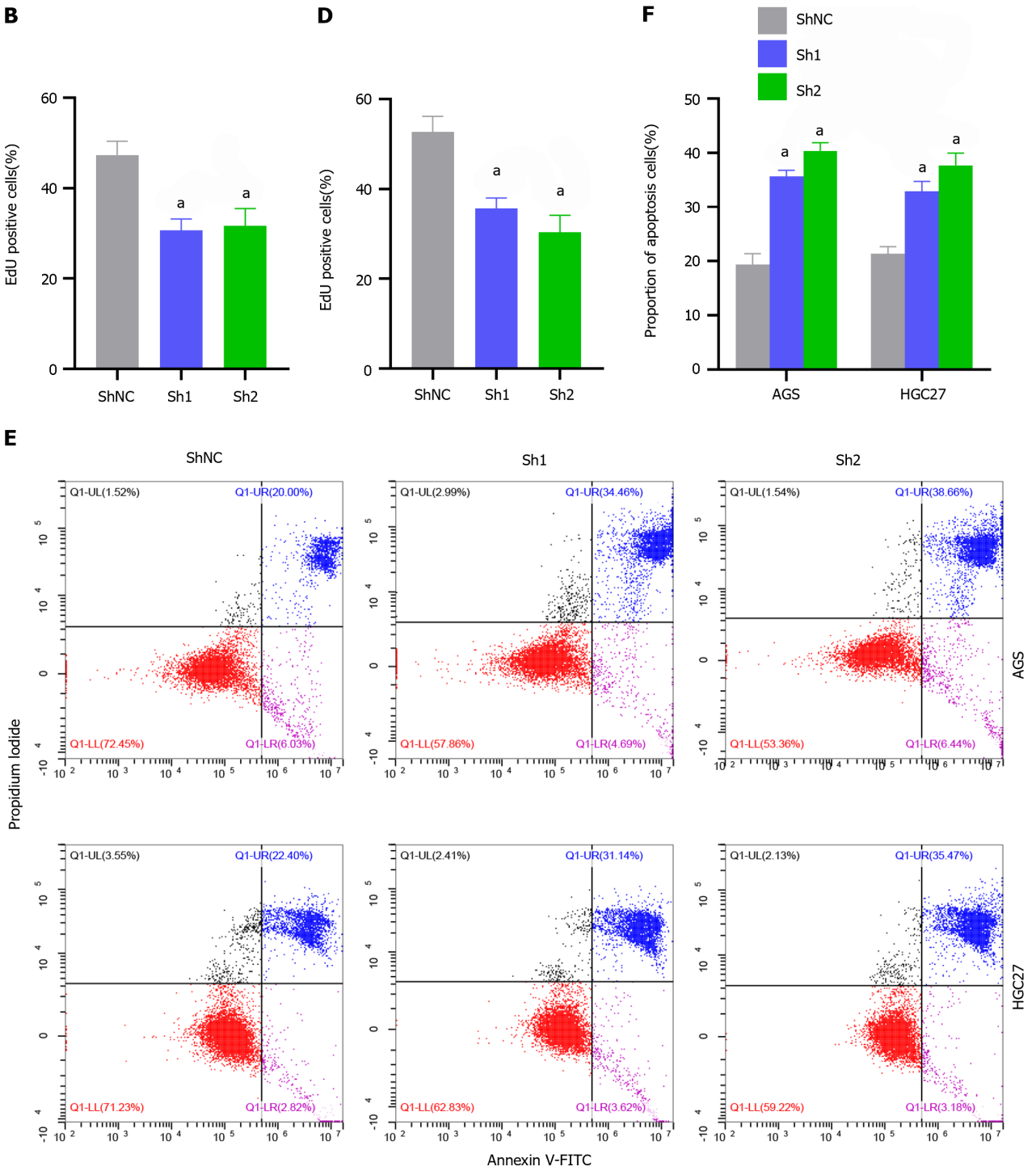
An EdU assay disclosed that TLK1 knockdown substantially inhibited AGS cell proliferation compared to the control (Figure 4A and B). Similar results were obtained for HGC27 cells (Figure 4C and D). Flow cytometry also showed that TLK1 knockdown considerably increased the apoptosis ratios in AGS and HGC27 relative to the control (Figure 4E and F).

TLK1 promotes GC progression by regulating the TGF-β signaling pathway

We applied bioinformatics to identify the genes associated with TLK1 and elucidate the mechanisms by which TLK1 affects GC cell clonal formation, proliferation, invasion, and migration. The expression levels of DDB1- and CUL4-associated factor 17 (DCAF17), enhancer of polycomb homolog 2 (EPC2), and membrane-associated ring-CH-type finger 7 (MARCH7) were positively correlated with that of TLK1 (Figure 5A) whereas those of COPI coat complex subunit epsilon (COPE), RNA pseudouridine synthase domain containing 1 (RPUSD1), and protein phosphatase 4, catalytic subunit


A
ShNC
Sh1
Sh2
Hoechst
EdU
Merge
AGS


C
ShNC
Sh1
Sh2
Hoechst
EdU
Merge
HGC27

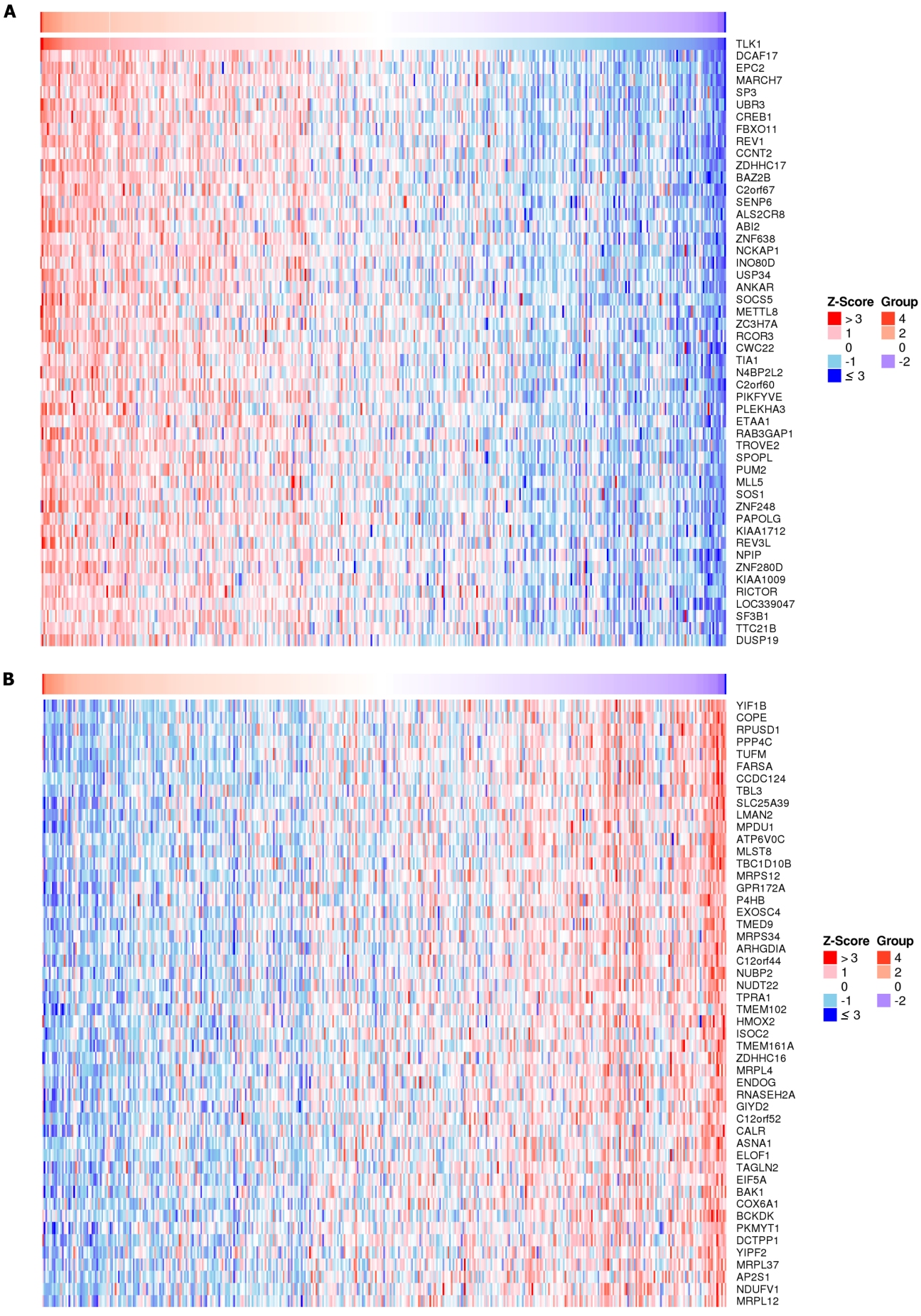


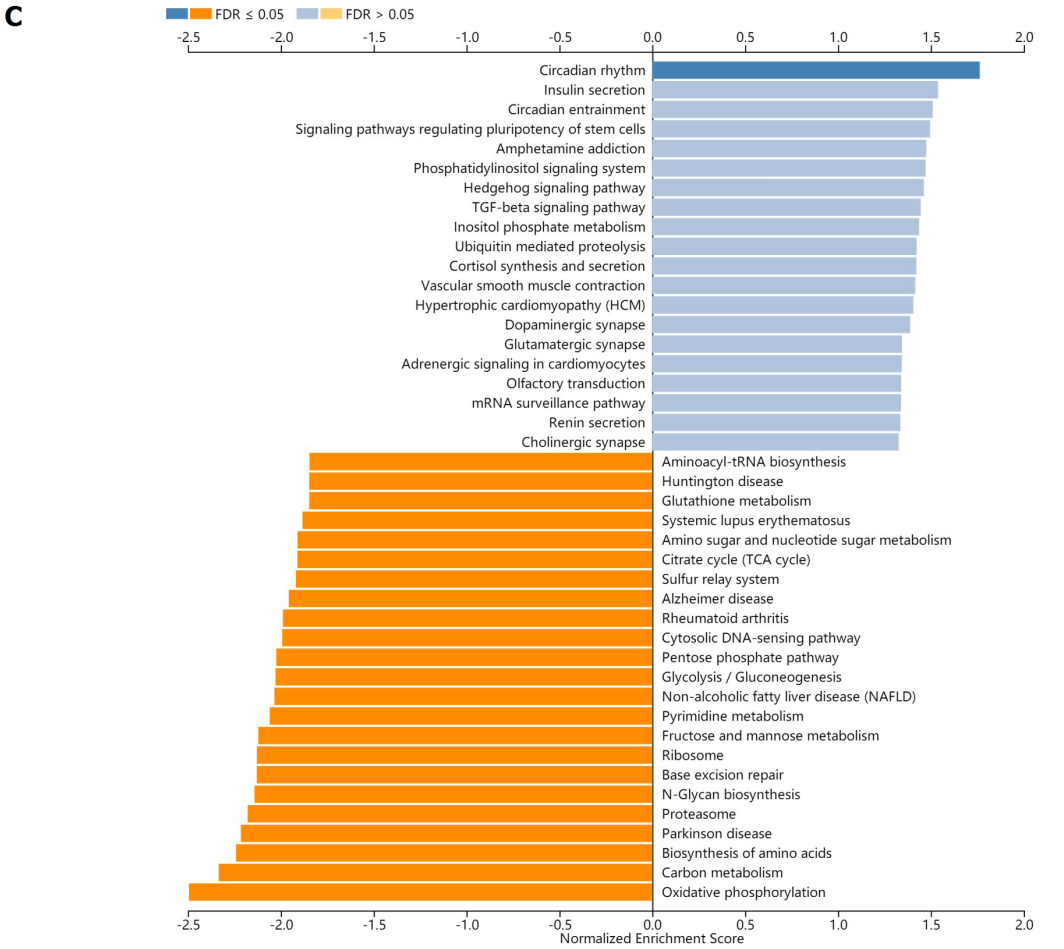
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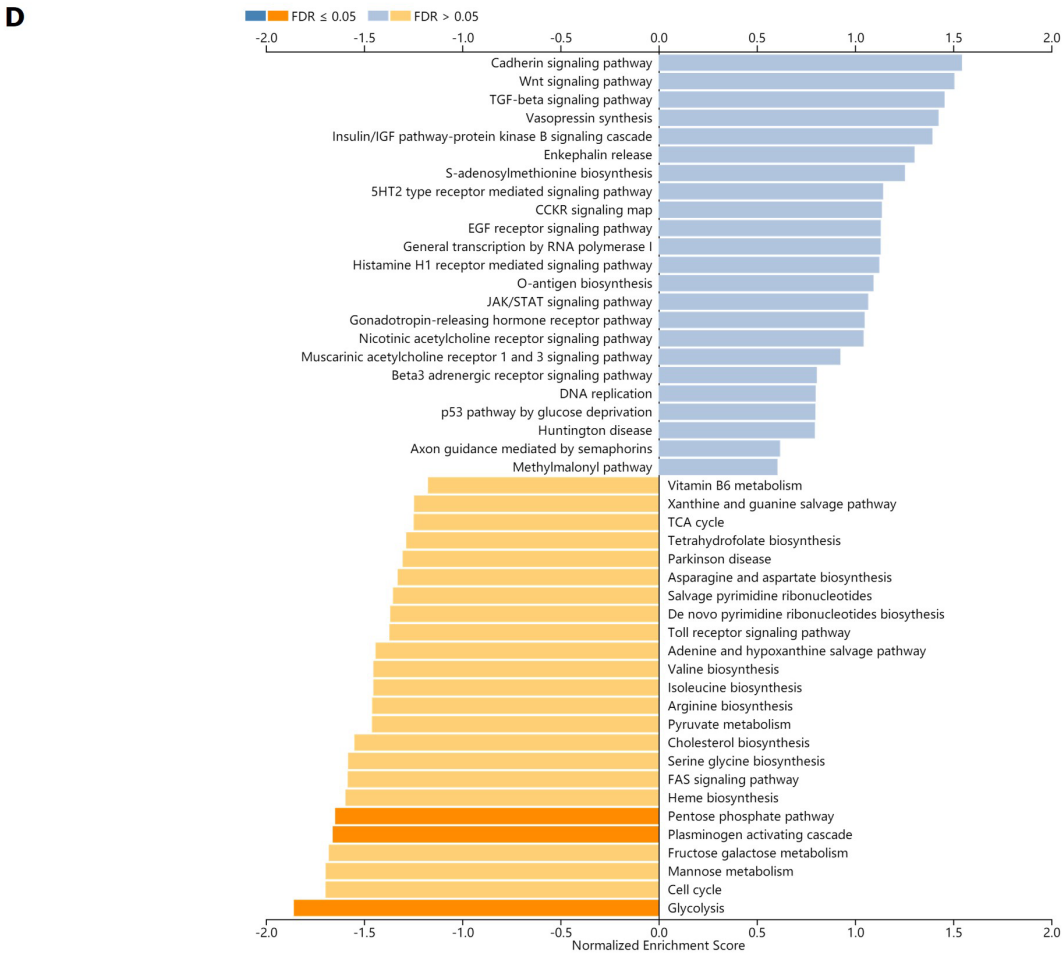
Figure 4 Tousled-like kinase 1 inhibition diminished proliferation and augmented apoptosis in gastric cancer cell lines. A and B: 5-ethynyl-2`-deoxyuridine (EdU) assay showing the effects of Tousled-like kinase 1 (TLK1) suppression on AGS cell proliferation; C and D: EdU assay revealing the impact of TLK1 knockdown on HGC27 cell proliferation; E and F: Flow cytometry evaluating the effects of TLK1 inhibition on apoptosis in AGS and HGC27 cell lines. $^{a}P < 0.05$. EdU: 5-ethynyl-2`-deoxyuridine; shNC: Short hairpin RNA of negative control.

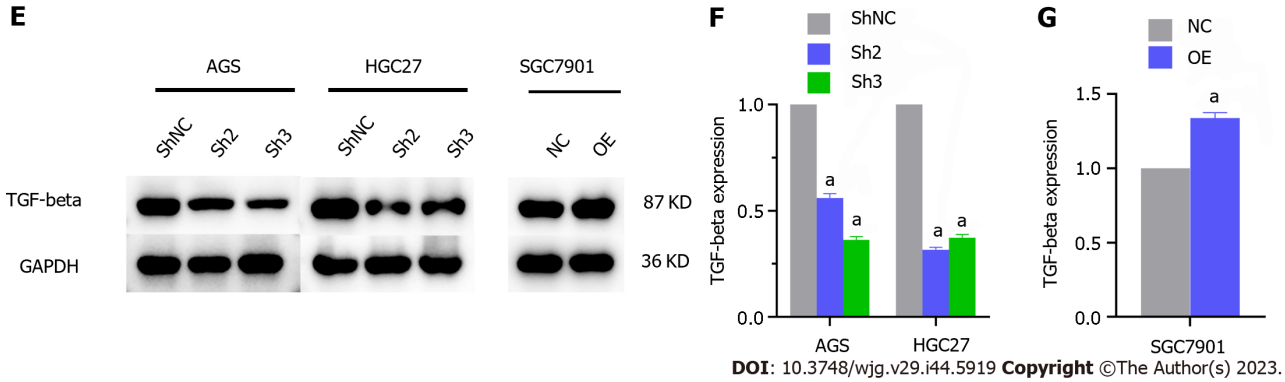
(PPP4C) were negatively correlated with it (Figure 5B).

We then discovered that the TGF-β signaling pathway might mediate the impact of TLK1 on the clonal formation, proliferation, invasion, and migration of GC cells (Figure 5C and D). We then used western blot to measure TGF-β protein expression in GC cells subjected to TLK1 knockdown and overexpression. We observed that the former downregulated TGF-β in AGS and HGC27 cells whereas the latter upregulated TGF-β in SGC7901 cells. Hence, TGF-β signaling determines the influence of TLK1 on GC progression (Figure 5E-G).


A
TLK1
DCAF17
EPC2
MARCH7
SP3
UBR3
CREB1
FBXO11
REV1
CCNT2
ZDHHC17
BAZ2B
C2orf67
SENP6
ALS2CR8
ABI2
ZNF638
NCKAP1
INO80D
USP34
ANKAR
SOCS5
METTL8
ZC3H7A
RCOR3
CWC22
TIA1
N4BP2L2
C2orf60
PIKFYVE
PLEKHA3
ETAA1
RAB3GAP1
TROVE2
SPOPL
PUM2
MLL5
SOS1
ZNF248
PAPOLG
KIAA1712
REV3L
NPIP
ZNF280D
KIAA1009
RICTOR
LOC339047
SF3B1
TTC21B
DUSP19
Z-Score
> 3
1
0
-1
≤ 3
Group
4
2
0
-2
B
YIF1B
COPE
RPUSD1
PPP4C
TUFM
FARSA
CCDC124
TBL3
SLC25A39
LMAN2
MPDU1
ATP6V0C
MLST8
TBC1D10B
MRPS12
GPR172A
P4HB
EXOSC4
TMED9
MRPS34
ARHGDIA
C12orf44
NUBP2
NUDT22
TPRA1
TMEM102
HMOX2
ISOC2
TMEM161A
ZDHHC16
MRPL4
ENDOG
RNASEH2A
GIYD2
C12orf52
CALR
ASNA1
ELOF1
TAGLN2
EIF5A
BAK1
COX6A1
BCKDK
PKMYT1
DCTPP1
YIPF2
MRPL37
AP2S1
NDUFV1
MRPL12
Z-Score
> 3
1
0
-1
≤ 3
Group
4
2
0
-2


C
FDR ≤ 0.05
FDR > 0.05
-2.5 -2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0
Circadian rhythm
Insulin secretion
Circadian entrainment
Signaling pathways regulating pluripotency of stem cells
Amphetamine addiction
Phosphatidylinositol signaling system
Hedgehog signaling pathway
TGF-beta signaling pathway
Inositol phosphate metabolism
Ubiquitin mediated proteolysis
Cortisol synthesis and secretion
Vascular smooth muscle contraction
Hypertrophic cardiomyopathy (HCM)
Dopaminergic synapse
Glutamatergic synapse
Adrenergic signaling in cardiomyocytes
Olfactory transduction
mRNA surveillance pathway
Renin secretion
Cholinergic synapse
Aminoacyl-tRNA biosynthesis
Huntington disease
Glutathione metabolism
Systemic lupus erythematosus
Amino sugar and nucleotide sugar metabolism
Citrate cycle (TCA cycle)
Sulfur relay system
Alzheimer disease
Rheumatoid arthritis
Cytosolic DNA-sensing pathway
Pentose phosphate pathway
Glycolysis / Gluconeogenesis
Non-alcoholic fatty liver disease (NAFLD)
Pyrimidine metabolism
Fructose and mannose metabolism
Ribosome
Base excision repair
N-Glycan biosynthesis
Proteasome
Parkinson disease
Biosynthesis of amino acids
Carbon metabolism
Oxidative phosphorylation
Normalized Enrichment Score


D
FDR ≤ 0.05
FDR > 0.05
-2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0
Cadherin signaling pathway
Wnt signaling pathway
TGF-beta signaling pathway
Vasopressin synthesis
Insulin/IGF pathway-protein kinase B signaling cascade
Enkephalin release
S-adenosylmethionine biosynthesis
5HT2 type receptor mediated signaling pathway
CCKR signaling map
EGF receptor signaling pathway
General transcription by RNA polymerase I
Histamine H1 receptor mediated signaling pathway
O-antigen biosynthesis
JAK/STAT signaling pathway
Gonadotropin-releasing hormone receptor pathway
Nicotinic acetylcholine receptor signaling pathway
Muscarinic acetylcholine receptor 1 and 3 signaling pathway
Beta3 adrenergic receptor signaling pathway
DNA replication
p53 pathway by glucose deprivation
Huntington disease
Axon guidance mediated by semaphorins
Methylmalonyl pathway
Vitamin B6 metabolism
Xanthine and guanine salvage pathway
TCA cycle
Tetrahydrofolate biosynthesis
Parkinson disease
Asparagine and aspartate biosynthesis
Salvage pyrimidine ribonucleotides
De novo pyrimidine ribonucleotides biosythesis
Toll receptor signaling pathway
Adenine and hypoxanthine salvage pathway
Valine biosynthesis
Isoleucine biosynthesis
Arginine biosynthesis
Pyruvate metabolism
Cholesterol biosynthesis
Serine glycine biosynthesis
FAS signaling pathway
Heme biosynthesis
Pentose phosphate pathway
Plasminogen activating cascade
Fructose galactose metabolism
Mannose metabolism
Cell cycle
Glycolysis
Normalized Enrichment Score



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Figure 5 Tousled-like kinase 1 drives gastric cancer progression by modulating the tumor growth factor-beta signaling pathway. A and B: Genes positively and negatively correlated with Tousled-like kinase 1 (TLK1) were identified from The Cancer Genome Atlas database; C: Kyoto Encyclopedia of Genes and Genomes analysis identifying the differentially expressed genes and their associated enriched pathways; D: Gene set enrichment analysis of pathway enrichment; E–G: Western blot evaluating tumor growth factor-beta expression in gastric cancer cell lines in response to TLK1 knockdown or overexpression. $^{a}P < 0.05$. TGF-beta: Tumor growth factor-beta; shNC: Short hairpin RNA of negative control; NC: Negative control; OE: Overexpression.

In vivo assays confirmed that TLK1 drives GC progression

We then validated the impact of TLK1 on GC progression through subcutaneous xenograft tumor induction in nude mice. The study included control and TLK1 overexpression groups (Figure 6A). Compared to the former, the latter presented significantly larger tumor size, volume, and mass (Figure 6B–D). Western blot and IHC verified that the TLK1 and TGF-β expression levels were considerably higher in the treatment group presenting large tumors than in the control group exhibiting small tumors (Figure 6E-G).

Antigen Kiel 67 staining of the tumors revealed that cancer proliferation was markedly higher in the TLK1 overexpression group than in the control group (Figure 6H and I). Hematoxylin and eosin and terminal deoxynucleotidyl transferase dUTP nick end labeling staining exposed substantially greater apoptotic necrosis in the control group than in the TLK1 overexpression group (Figure 6J and K).

DISCUSSION

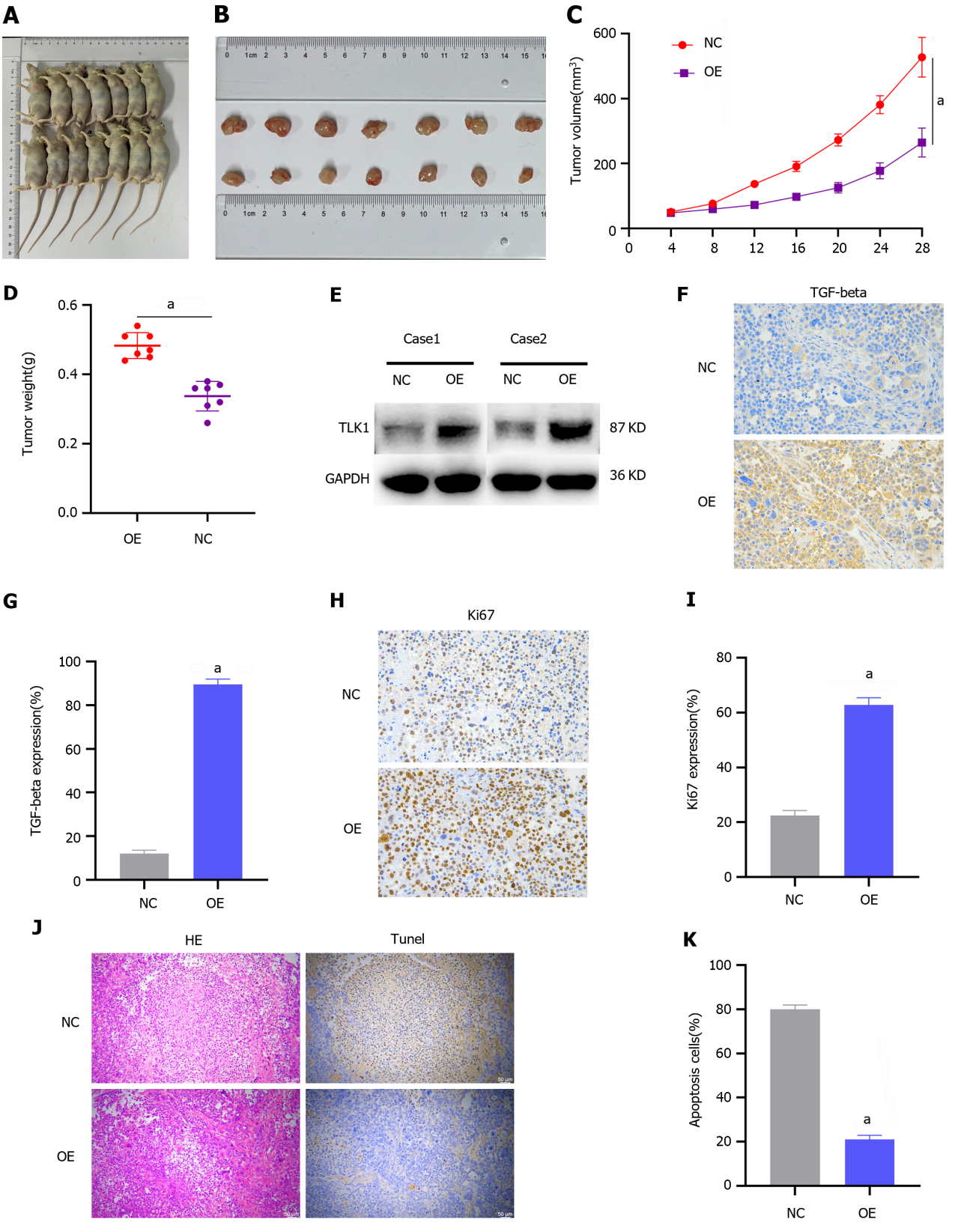
The mortality rate of advanced GC remains high despite the progress that has been made in the therapeutic approaches used against it[28]. Hence, novel treatments for GC are urgently needed. TLK promotes the progression of various malignancies. Therefore, research on TLK in the context of cancer therapy should be prioritized[18-21,23-26]. Here, we examined TLK1 expression in GC cells, investigated its effects on their functions, and used *in vivo* experiments to clarify how it modulates GC progression.

Previous studies reported that TLK1 was upregulated in gliomas[23,25]. In the present work, we discovered that TLK1 was significantly overexpressed in GC cells and tissues (Figure 1B–E). Immunofluorescence staining also revealed that TLK1 was localized mainly to GC cell nuclei (Figure 1F).

We then assessed the impact of TLK1 on GC cell function. TLK1 was overexpressed in the SGC7901 cell line (Figure 2A). Colony formation, EdU, and Transwell assays disclosed that TLK1 overexpression promoted SGC7901 cell proliferation, invasion, and migration (Figure 2B–G). TLK1 knockdown had the opposite effects on AGS and HGC27 cell lines (Figures 3 and 4). An earlier study reported similar findings for the roles of TLK1 in other cancer types[25]. Taken together, these results suggest that TLK1 contributes to GC progression. Our bioinformatics analysis revealed that the mechanism of TLK1 was associated with the TGF-β signaling pathway in GC (Figure 5C and D). The TGF-β signaling pathway comprises TGF-β itself, activins, nodal, bone morphogenetic proteins, growth and differentiation factors, and other factors[29,30] and plays vital roles in human embryonic development and homeostasis[31]. A recent study showed that alterations in TGF-β signaling may result in immunocompromise, fibrosis, and carcinogenesis[32]. The TGF-β signaling pathway may either inhibit or promote tumorigenesis depending upon the tumor microenvironment or cancer stage[29,33,34]. We used western blot to measure TGF-β expression in response to TLK1 overexpression or knockdown and found a positive correlation between TLK1 and TGF-β (Figure 5E and F). Thus, TLK1 may promote GC progression by upregulating TGF-β. We validated this mechanism *in vivo* by inducing subcutaneous xenograft tumor formation in nude mice (Figure 6) and substantiated the critical role of TLK1 in GC progression. To the best of our knowledge, the present work is one of the first to delineate the expression, localization, and functional impact of TLK1 in GC.

CONCLUSION

We demonstrated that TLK1 is highly expressed in GC, localized mainly to the nucleus, significantly promotes GC cell proliferation, invasion, and migration, and inhibits apoptosis. TLK1 may facilitate GC progression by modulating TGF-β



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Figure 6 *In vivo* confirmation of the role of Tousled-like kinase 1 in gastric cancer progression. A and B: Relative tumorigenesis between the control and Tousled-like kinase 1 (TLK1) overexpression groups; C and D: Differences in tumor volume and mass between the control and TLK1 overexpression groups; E: Western blot verifying differential TLK1 expression in tumor tissues; F and G: Immunohistochemical (IHC) staining detecting the differences in tumor growth factor-beta expression between the control and TLK1 overexpression groups; H and I: IHC staining showing the differences in Ki-67 expression between the control and TLK1 overexpression groups; J and K: IHC and HE staining disclosing the differences in apoptosis between the control and TLK1 overexpression groups. $^{a}P < 0.05$. TGF-beta: Tumor growth factor-beta; NC: Negative control; OE: Overexpression.

expression. We believe that TLK1 could be a crucial therapeutic target for GC, and propose that future investigations evaluate the feasibility and practicality of targeting TLK1 in GC treatment.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is the fifth most prevalent cancer and the third leading cause of cancer-related mortality worldwide. Endoscopy is the mainstay of early-stage GC treatment whereas advanced GC must be managed through surgery and other interventions including chemotherapy and targeted therapy. Despite significant progress in GC control, however, its overall survival remains unsatisfactory.

Research motivation

Potential therapeutic targets against GC are urgently required.

Research objectives

The present study aimed to elucidate the functional significance of Tousled-like kinase 1 (TLK1) in GC cells and potentially identify a novel therapeutic target against this disease.

Research methods

We measured TLK1 protein expression levels and localized TLK1 in GC cells and tissues by western blot and immunofluorescence, respectively. We transfected various GC cells with lentiviruses to create TLK1 overexpression and knockdown lines and established the functional roles of TLK1 through *in vitro* colony formation, 5-ethynyl-2`-deoxyuridine, and Transwell assays as well as flow cytometry. We applied bioinformatics to elucidate the signaling pathways associated with TLK1. We performed *in vivo* validation of TLK1 functions by inducing subcutaneous xenograft tumors in nude mice.

Research results

TLK1 was significantly upregulated in GC cells and tissues compared to their normal counterparts and was localized mainly to the nucleus. TLK1 knockdown significantly decreased colony formation, proliferation, invasion, and migration but increased apoptosis in GC cells. TLK1 overexpression had the opposite effects. Bioinformatics revealed, and subsequent experiments verified, that the tumor growth factor-beta (TGF-β) signaling pathway was implicated in TLK1-mediated GC progression. The *in vivo* assays confirmed that TLK1 promotes tumorigenesis in GC.

Research conclusions

We demonstrated that TLK1 is highly expressed in GC, localized mainly to the nucleus, significantly promotes GC cell proliferation, invasion, and migration, and inhibits apoptosis. TLK1 may facilitate GC progression by modulating TGF-β expression. We believe that TLK1 could be a crucial therapeutic target for GC.

Research perspectives

Future investigations evaluate the feasibility and practicality of targeting TLK1 in GC treatment.

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FOOTNOTES

Co-first authors: Ruo-Chuan Sun and Jing Li.

Co-corresponding authors: Ming-Liang Wang and Yong-Xiang Li.

Author contributions: Li YX and Wang ML conceived the study design; Wang HZ and Dal E wrote the manuscript. Sun RC and Li J performed the experiments; Li YX participated in the cell culture and *in vitro* experiments; All authors contributed to the article and approved the final submitted version. Li YX and Wang ML contributed equally to this work as co-corresponding authors. The reasons for designating Li YX and Wang ML as co-corresponding authors are twofold. First, the main design of this project is completed by Li YX and Wang ML, which makes our project more rigorous. Second, the choice of these researchers as co-corresponding authors acknowledges and respects this equal contribution, while recognizing the spirit of teamwork and collaboration of this study. Sun RC and Li J contributed equally to this work as co-first authors. The reasons for designating Sun RC and Li J as co-first authors are also twofold. Sun RC and Li J completed all *in vitro* and *in vivo* experiments of this study, and made great contributions to this study. They contributed efforts of equal substance throughout the research process. Second, the research was performed as a collaborative effort, and

the designation of co-first authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper. This also ensures effective communication and management of post-submission matters, ultimately enhancing the paper's quality and reliability. summary, we believe that designating Li YX and Wang ML as co-corresponding authors and Sun RC and Li J as co-first authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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CASE REPORT

Mucosal esophageal carcinoma following endoscopic submucosal dissection with giant gastric metastasis: A case report and review of literature

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Abstract

BACKGROUND

Esophageal carcinoma is a highly aggressive digestive cancer responsible for a notable proportion of cancer-related deaths worldwide. Its elevated metastatic rate contributes to a poor prognosis in affected patients. In this case review, we aim to summarize the metastatic characteristics of intramural gastric metastasis (IGM) in mucosal esophageal squamous carcinoma.

CASE SUMMARY

A 56-year-old man was admitted to our hospital because of a dry cough with an esophageal sensation for one year. Endoscopic examination revealed a 2.0 cm 1.0 cm, superficial esophageal squamous cell carcinoma, and the patient underwent endoscopic submucosal dissection (ESD). Fifteen months after ESD, positron emission tomography/computed tomography revealed that the metabolism of the stomach cardia wall had increased slightly. However, the mucosa of the gastric cardia was smooth under gastroendoscopy. Two years after ESD, endoscopic examination revealed a giant gastric cardia carcinoma, while the esophageal mucosa was smooth, and no advanced cancer was found. A biopsy of the gastric cardia indicated squamous-cell carcinoma. The patient received immunochemotherapy and radiotherapy for esophageal cancer for 8 mo and is currently under follow-up.

CONCLUSION

Early-stage esophageal carcinoma with IGM is rare. Despite the ESD of the primary lesion, IGM may still occur and should be closely monitored after ESD.

Key Words: Esophageal squamous cell carcinoma; Intramural metastasis; Endoscopic submucosal dissection; Case report

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Core Tip: This study presents a rare case of mucosal esophageal squamous cell carcinoma (ESCC) post endoscopic submucosal dissection with giant gastric cardia metastasis. intramural gastric metastasis has also been reported to plays a role in distant metastasis, particularly in the liver. Preoperative and postoperative endoscopic follow-up of patients with ESCC of any stage, although the depth of an esophageal cancer may be T1.

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INTRODUCTION

Globally, esophageal squamous cell carcinoma (SCC) (ESCC) is the sixth leading cause of cancer-related deaths. In Asian countries, SCC is the most common pathological type of esophageal cancer. The prognosis of ESCC is poor owing to its increased tendencyfor direct spread and metastasis. The 5-year survival rate of ESCC patients with metastatic disease is less than 5%[1]. ESCC often metastasizes to the lymph nodes and distant organs, and intramural metastasis (IM) occasionally occurs, which is seen in approximately 10% of esophageal carcinomas[2,3]. IM is a form that tumor can occur as a skip lesion in the other parts of esophagus or to the stomach. IM incidence in ESCC in the head and neck is 25%, compared with 11% in stage I ESCC[4]. However, the reported intramural gastric metastasis (IGM) is seldom, accounting for only 1%–4.58% of ESCC through surgical treatment[5-9]. A higher incidence of IGM has been reported in the advanced ESCC, whereas only a few cases of IGM have been reported in the early-stage ESCC[10-13]. Herein, we report a case of mucosal esophageal carcinoma with multi-pathway and multi-organ metastases (including IGM, lymphatic metastasis of celiac lymph nodes, and hematogenous metastasis of the liver and right adrenal gland) that occurred in a metachronous manner, while the IGM progressed rapidly over 1 year. We hope that this case will guideclinicians in identifying unique metastasis of ESCC in some cases when making a treatment plan.

CASE PRESENTATION

Chief complaints

In August 2020, a 56-year-old man was admitted to our hospital for a dry cough with an esophageal sensation.

History of present illness

The symptoms started 1 year before presentation.

History of past illness

The patient had no relevant past illness.

Personal and family history

The patient had a smoking history for 20 years, with 20 cigarettes daily and drinking approximately 4000 mL of beer 3–4 times a week for 20 years.

Physical examination

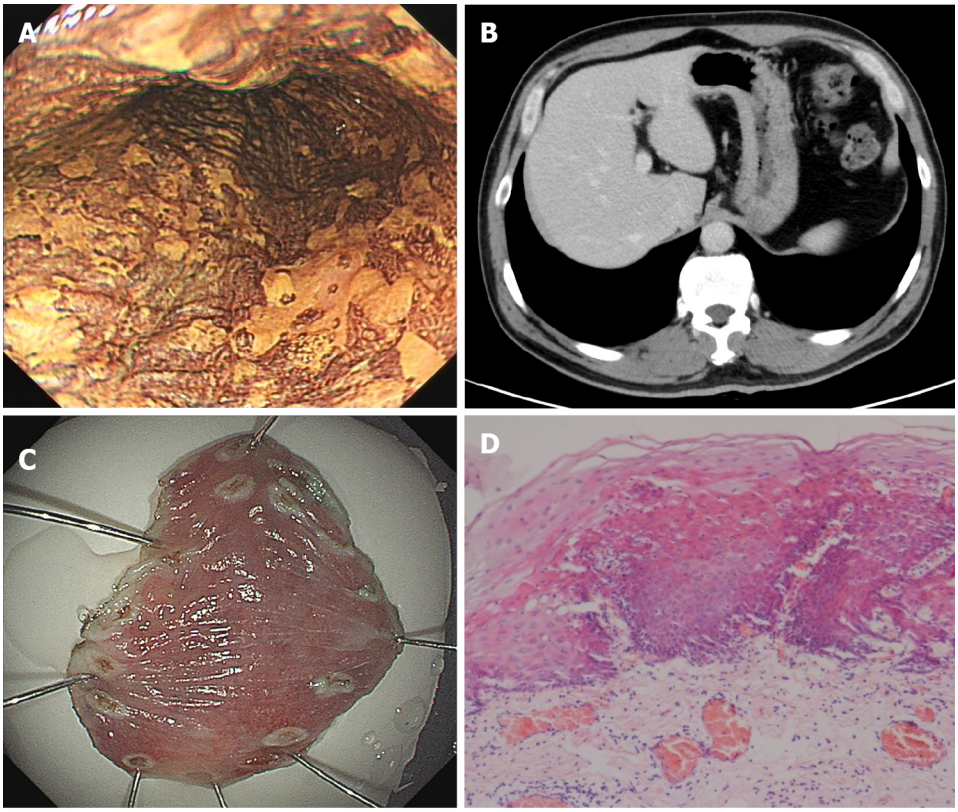
There was no abnormal in physical examination.

Laboratory examinations

All blood test results, including tumor markers, were within normal limits.

Imaging examinations

Gastroendoscopy showed a 2.0 cm × 1.0 cm, superficial, flat, slightly rough, and red esophageal lesion 30 cm from the incisor teeth. Magnifying endoscopy with narrow-band imaging observations showed that the background mucus was brown, and the intrapapillary capillary loop was Type B1 (JES). Iodine staining showed no stain but showed its apparent margins more, and the pink sign was positive in the central part. The remaining esophageal mucosa was scattered in patchy light-stained areas, ranging from 0.2–0.4 cm (Figure 1A). Computed tomography (CT) revealed a small cyst in the



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Figure 1 The first gastroendoscopy examination in August 2020. A: After iodine staining, the esophageal mucosa was scattered in patchy light-stained areas, ranging from 0.2 cm to 0.4 cm, and a 2.0 cm × 1.0 cm, superficial, flat lesion (30 cm from the incisor teeth) did not stain, whereas the pink areas were positive; B: Computed tomography showed no abnormal thickening in the gastrointestinal wall; C: Endoscopic submucosal dissection was performed 1 wk after gastroendoscopy; D: Postoperative pathology revealed moderate to severe dysplasia of squamous epithelium.

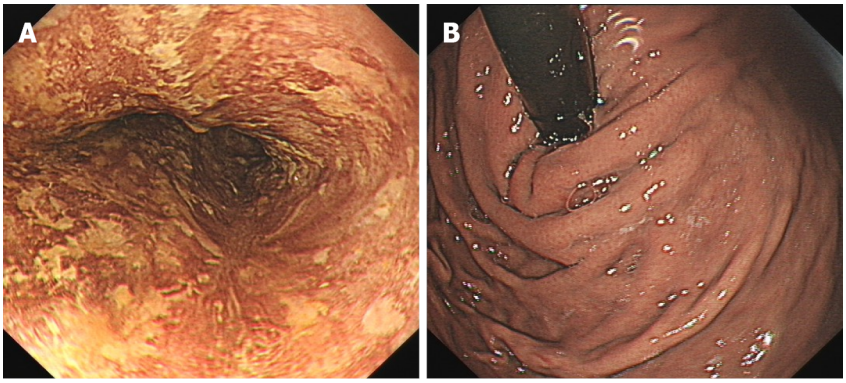
liver; however, no abnormal thickening of the gastrointestinal wall was observed (Figure 1B). Endoscopic submucosal dissection (ESD) was performed 1 wk after the endoscopy (Figure 1C), and postoperative pathology revealed mucosal ESCC (Figure 1D).

In March 2021, the patient was re-examined 7 mo after ESD. Endoscopic examination showed a 1.0 cm × 2.0 cm scar on the right posterior wall of the esophagus 30 cm from the incisor teeth. Iodine staining showed that the esophageal mucosa was scattered in a patchy light-stained area, with a pink sign (-) (Figure 2A). The mucosa of the gastric fundus and cardia were smooth without any abnormalities (Figure 2B).

In July 2021, the patient was examined again 1 year after ESD. The esophageal mucosa was the same as that at the final endoscopic examination. While gastroendoscopy also showed a 0.6 cm × 0.8 cm IIa + IIc lesion near the anterior wall of the gastric fundus near the cardia, biopsy of this lesion revealed erosion with regeneration and moderate dysplasia locally. The other mucosa of the gastric fundus and cardia were smooth without any abnormalities (Figure 3A). CT also revealed a small cyst in the liver; however, no abnormal thickening was observed in the gastrointestinal wall (Figure 3B). The patient underwent ESD of the fundus lesion (Figure 3C), and postoperative pathology revealed moderate-to-severe dysplasia of the gastric mucosa (Figure 3D).

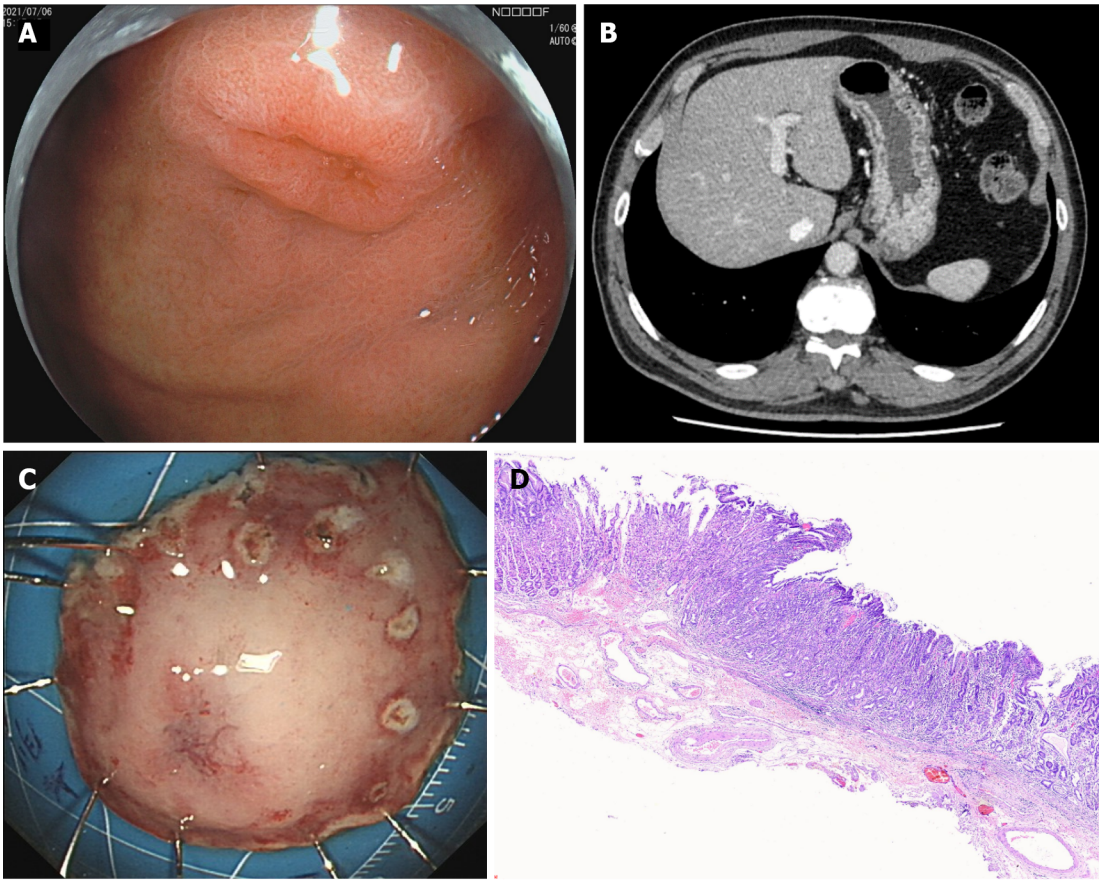
In November 2021, the patient received a 3rd examination 15 mo after the 1st ESD. Gastroendoscopy revealed patchy light-stained areas throughout the esophagus after iodine staining (Figure 4A). The mucosa of the gastric fundus and cardia was smooth, except for the scar after ESD, and no abnormalities were found on a gastroendoscopy (Figure 4B). CT showed no abnormal thickening of the gastrointestinal wall. However, positron emission tomography (PET)-CT revealed that the metabolism of the lower esophagus increased slightly, the metabolism of the stomach cardia wall increased slightly, and lymph nodes in the retroperitoneal area had high-density shadows and increased metabolism (Figure 4C).

In November 2022, the patient received a 4th endoscopy examination. The esophageal mucosa was smooth, and no advanced cancer was found (Figure 5A). Circumferential submucosal tumor (SMT)-like swelling was observed in the cardia and stomach body; local ulceration was observed, and multiple ulcers were formed. The mucosa surrounding the ulcer was regular and tough, the mucosa surrounding the lesion was clustered, and the lower edge of the lesion involved a minor curvature in the middle of the stomach body (Figure 5B). A biopsy of the cardia revealed SCC (Figure 5C). CT revealed a cardia tumor (Figure 5D), perigastric lymph node enlargement, and multiple metastatic tumors of the liver (Figure 5E) and right adrenal gland (Figure 5F).



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Figure 2 Re-examination of the patient 7 mo after endoscopic submucosal dissection in March 2021. A: Gastroendoscopy revealed a 1.0 cm × 2.0 cm scar on the right posterior wall of the esophagus, 30 cm from the incisor teeth. Iodine staining revealed that the esophageal mucosa was scattered in the patchy, lightly stained area, indicated by the pink sign (-); B: The mucosa of gastric fundus and cardia was smooth without abnormality.

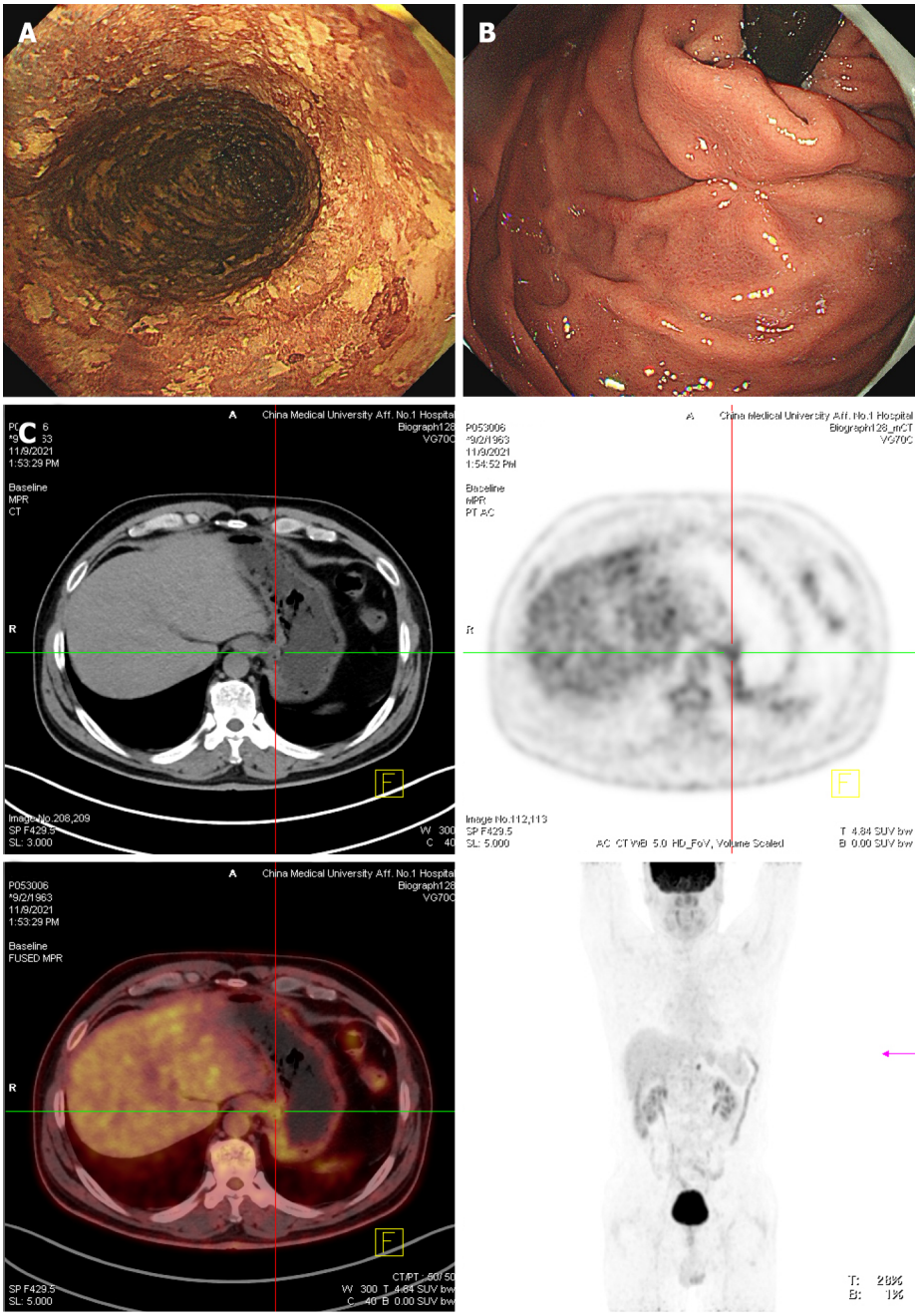


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Figure 3 The patient was re-examined for the second time 1 year after endoscopic submucosal dissection in July 2021. A: Gastroendoscopy revealed a 0.6 cm × 0.8 cm IIa + IIc lesion near the anterior wall of the gastric fundus near the cardia. B: Computed tomography showed no abnormal thickening in the gastrointestinal wall; C: The patient underwent Endoscopic submucosal dissection of the fundus lesion; D: Postoperative pathology revealed moderate to severe dysplasia of gastric mucous.

FINAL DIAGNOSIS

The patient was diagnosed as early-stage esophageal carcinoma with giant gastric cardia metastasis.



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Figure 4 In November 2021, the patient underwent the third re-examination 15 mo after the first endoscopic submucosal dissection. A: The entire esophagus showed patchy, lightly stained areas after iodine staining in gastroendoscopy. B: The mucosa of the gastric fundus and cardia was smooth except for the scar after Endoscopic submucosal dissection operation, and no abnormality was noted on gastroendoscopy. C: Computed tomography (CT) revealed no abnormal thickening in the gastrointestinal wall. However, positron emission tomography-CT revealed that the metabolism of the lower esophagus and stomach cardia wall slightly increased. A lymph node was noted in the retroperitoneal area with high-density shadow and increased metabolism.

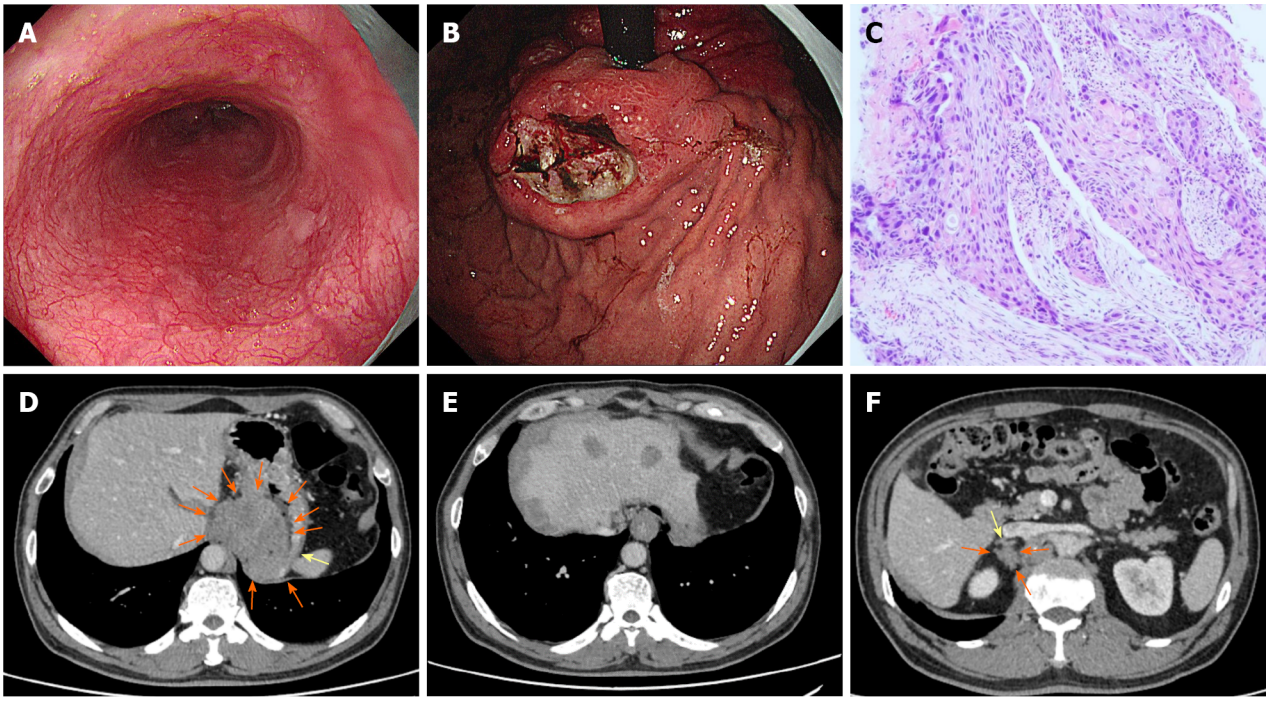
TREATMENT

To alleviate the observed multi-organ metastasis, the patient was administered immunochemotherapy (carrelizumab 200 mg D1, albumin paclitaxel 400 mg D1, and carboplatin 450 mg D1) and radiotherapy (30 courses) for esophageal cancer. Six immunochemotherapy and 30 radiotherapy courses were completed.

The development and treatment of the disease in the present case are summarized in Figure 6.

OUTCOME AND FOLLOW-UP

The immunochemotherapy and radiotherapy was completed and the patient was still alive. The follow-up is ongoing.



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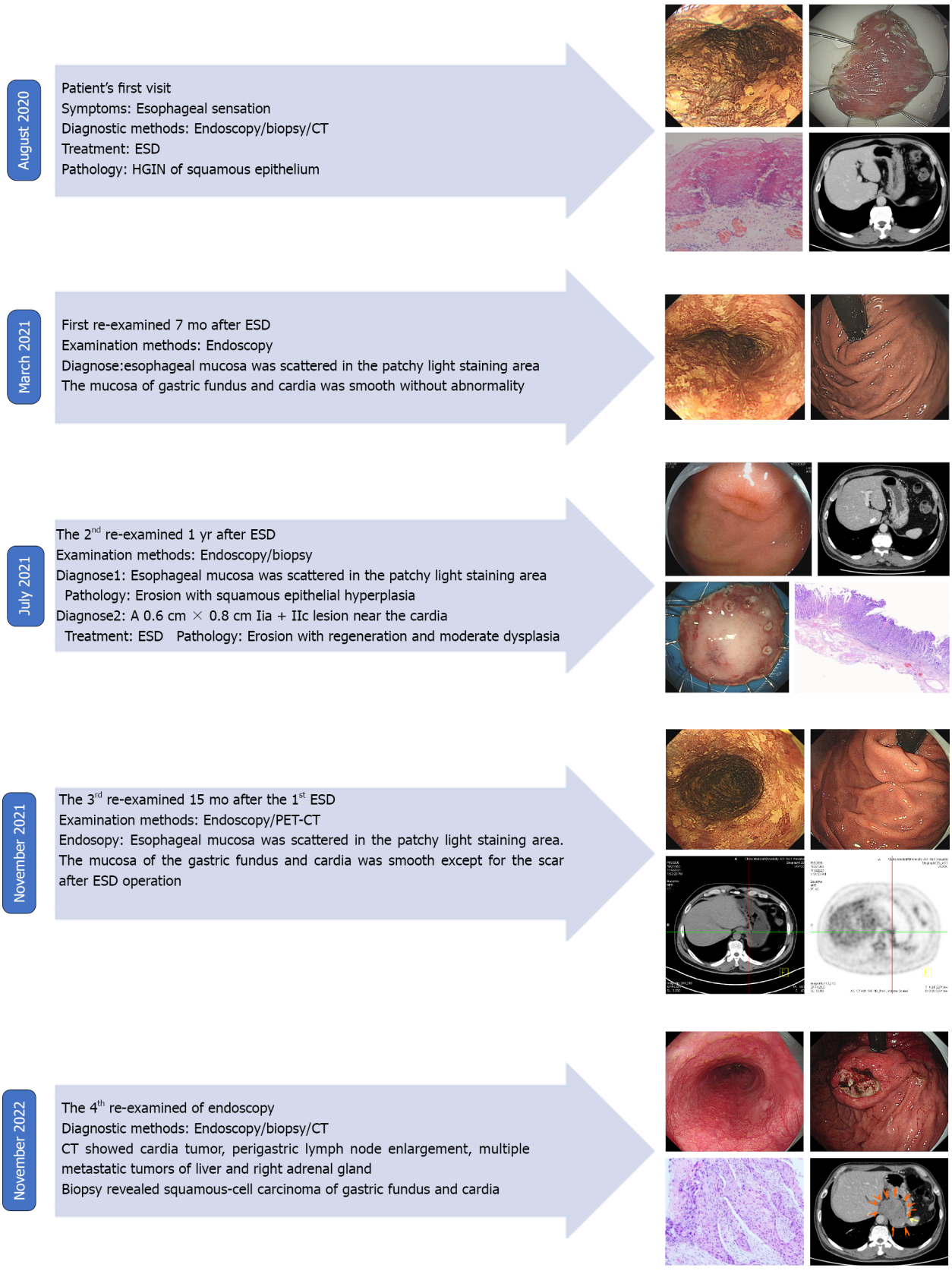
Figure 5 In November 2022, the patient underwent the fourth endoscopy re-examination. A: Esophageal mucosa was smooth, and no advanced cancer was noted; B: Circumferential submucosal tumor-like swelling was observed in the cardia and stomach body, along with local ulceration and the formation of multiple ulcers; C: Biopsy of cardia revealed squamous-cell carcinoma; D: Computed tomography (CT) showed cardia tumor; E: CT revealed multiple metastatic tumors in the liver; F: CT showed metastatic tumors in the right adrenal gland.

DISCUSSION

ESCC is an extremely aggressive cancer and has a poor prognosis owing to its higher propensity for lymph nodes and distant metastases. Historically, cancer-distal metastases are considered as the most important barrier to achieving significant advances in cancer management; moreover, it is responsible for 90% of cancer-related deaths[14,15]. The incidence of distant metastases in newly diagnosed ESCC is approximately 20%–30%. However, this proportion raised to approximately 40%–50% in recurrent cases after radical esophagectomy[16] or concurrent radiochemotherapy[17] in ESCC. IM is another metastasis pathway in esophageal cancer. Based on previous endoscopic findings, clinical IM, including gastric metastasis from ESCC, had mainly three characters: (1) Metastatic tumor often separated distantly from the primary tumor; (2) The metastatic lesion presented in the esophagus or stomach wall; and (3) the SMT lacked of an intraepithelial component, occasionally with an erosive change[6]. Accompanied by these findings, the ESCC strongly suggests an IM tumor[18].

Longitudinal lymphatic vessels contained in the mucosa lamina propria and submucosa of the esophagus are the anatomic basis for IM[19]. The mucosal lymphatic channels in esophagus are not directly connected to those in stomach; however, submucosal channels may have a direct connection with the submucosal gastric lymphatics[20]. Therefore, tumor cells go through the submucosal lymphatic system to the stomach wall and form metastatic loci[20]. Watson and Goodner[21] first reported there existed an interflow of lymphatic channels between the esophagus and stomach through virtue of neo-lymphangiogenesis in the carcinomatous lesion and explained IM as an extension of submucous lymphatics. Kato *et al*[6] documented that the location of the IM toward the primary tumor did not indicate a predilection in the direction of the lymphogenic spread of carcinoma of the esophagus. However, another study[5] showed that among 1259 patients, although the depth of esophageal cancer may be T1, the possibility of IGM still exists. The incidence of IGM is higher in advanced-stage ESCC[5,22], as only a few cases of IGM in the early-stage ESCC have been reported. From mucosal ESCC, to our best knowledge, only 6 cases (including the present one) have been documented[10-13,23]. Therefore, although the probability of lymphatic metastasis is very low, it can still occur even if the tumor is confined to the mucosa.

IGM from esophageal cancer is rare[5,9]; nonetheless, it is considered to be one of the most significant poor prognostic factors due to its higher rate of lymphatic invasion[24,25] and distant metastasis. The correlation between metastatic tumors and lymph node metastasis can be attributed to the fact that gastric metastases occur *via* the lymphatic system. In the middle and lower parts of the esophagus, submucosal lymphatic drainage is regarded to be connected to gastric from the cardia and fundus. At times, the primary esophageal lesion could appear to be small, compared with the gastric metastatic lesion, which is exophytic in morphology because of the submucosal site of implantation[22]. In this report, the primary tumor was in the middle-thoracic esophagus, and the IGM was found in the gastric cardia.



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Figure 6 Summary of disease development and treatment of the present case.

In addition, IGM plays a role in distant metastasis, particularly in the liver[6]. IGM of ESCC is categorized as distant metastasis, described as M1b stage IVB, according to the Union for International Cancer Control for International Cancer Control Classification[26]. However, other studies have reported metastases in the reconstructed stomach. IGM develops principally in the upper third of the stomach wall; however, it can also appear in the gastric body[5,9]. These mean IGM happen without direct involvement of the lymphatic system[27]. In this report, the tumor cells giving rise to IGM could not have spread *via* the lymphatic system because the primary tumor was confined to the mucosa with no lymphatic connection to the submucosa. Thus, IGM sometimes develops *via* routes other than the lymphatic system, including vascular routes. In addition, we found multiple distant metastases to the liver and right adrenal gland, which indicated that there were hematogenous pathways of metastasis in our case.

In our case, what really surprised us was that from November 2021 (PET-CT suggested abnormal cardiac metabolism) to November 2022 (gastroendoscopy revealed a Borrmann type II lesion in the cardiac area), metastases to the cardia grew rapidly and were so large that no significant primary lesions were found in the esophagus. The cardiac area is rich in blood supply, which is conducive to the root proliferation of squamous cells, and the cardiac area is significantly large [23,24]. Primary gastric adenocarcinoma sometimes has a similar gross appearance but a higher incidence in the lower part of the stomach. Gastrointestinal stromal tumors also appear as exophytic lesions. The detection rate of IM of ESCC ranges 5.5% between 16.6%[6,28]. General endoscopy has certain limitations when the metastases are confined below the mucosa without breaking through the surface. The radiological imaging, especially fluorodeoxyglucose-PET, can detect out early metastatic lesions in time[29-31]. Therefore, preoperative and postoperative radiographic follow-up is conducive to indicating the presence of IM[20,32]. The five-year disease-free survival rate of patients with ESCC with IM is significantly lower than that of patients without IM[6,33]. Therefore, multimodal treatments with strong antitumor effects should be administered.

CONCLUSION

In conclusion, preoperative and postoperative endoscopic follow-up of patients with ESCC of any stage, although the depth of an esophageal cancer may be T1, in addition to paying attention to esophageal lesions, the stomach should also be carefully examined, especially for the submucosal eminence in the upper part of the stomach, to consider the possibility of IGM, and the nature of the submucosal eminence can be clarified by combining endoscopic ultrasonography and ultrasonic puncture biopsy. Therefore, enhancing the preoperative and postoperative imaging and endoscopic ultrasonography are beneficial for indicating the existence of IM.

FOOTNOTES

Co-corresponding authors: Sun MJ and Zhang HJ.

Author contributions: Zhang HJ and Sun MJ had full access to all the content in the study and takes responsibility for the integrity and the accuracy of the data analysis; Yang MQ and Sun MJ performed information collection; Yang MQ and Zhang HJ wrote the paper and performed editing. Sun MJ and Zhang HJ contributed equally to this work as co-corresponding authors. The reasons for designating Sun MJ and Zhang HJ as co-corresponding authors are threefold. Firstly, our research project is a collaborative effort. Throughout the entire research process, we worked closely together, sharing responsibilities in experimental design, data analysis, and result interpretation. Each of us made significant contributions in different aspects, whether it be in experimental operations, data processing, or writing and revising the manuscript. Therefore, we believe that designating two corresponding authors accurately reflects our collective efforts and academic contributions. Secondly, the selection of two corresponding authors is also driven by our commitment to academic fairness and transparency. This also promotes the most comprehensive and in-depth examination of the research topic, ultimately enriching readers' understanding by offering various expert perspectives. Thirdly, Sun MJ and Zhang HJ contributed efforts of equal substance throughout the research process. Sun MJ made a significant contribution to the diagnostic process of this case. The choice of these researchers as co-corresponding authors acknowledges and respects this equal contribution, while recognizing the spirit of teamwork and collaboration of this study. In summary, we believe that designating Sun MJ and Zhang HJ as co-corresponding authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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EDITORIAL

Gut microbiota in women: The secret of psychological and physical well-being

Giuseppe Marano, Gianandrea Traversi, Eleonora Gaetani, Antonio Gasbarrini, Marianna Mazza

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Abstract

The gut microbiota works in unison with the host, promoting its health. In particular, it has been shown to exert protective, metabolic and structural functions. Recent evidence has revealed the influence of the gut microbiota on other organs such as the central nervous system, cardiovascular and the endocrine-metabolic systems and the digestive system. The study of the gut microbiota is outlining new and broader frontiers every day and holds enormous innovation potential for the medical and pharmaceutical fields. Prevention and treatment of specific women's diseases involves the need to deepen the function of the gut as a junction organ where certain positive bacteria can be very beneficial to health. The gut microbiota is unique and dynamic at the same time, subject to external factors that can change it, and is capable of modulating itself at different stages of a woman's life, playing an important role that arises from the intertwining of biological mechanisms between the microbiota and the female genital system. The gut microbiota could play a key role in personalized medicine.

Key Words: Gut microbiota; Women; Metabolites; Health promotion; Immune system; Gut-brain axis

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Core Tip: The function of the gut microbiota on health is of primary importance, as it educates and controls the immune system, allows to metabolize and absorb nutrients correctly and protects from pathogens invasion. This paper focuses on the importance of the microbiota for women's physical and psychological well-being. The gut microbiota has a strategic role in crucial moments at every stage of a woman's life: From childhood to adolescence, from fertile age to pregnancy-partum, up to menopause. In the future, the study of the gut microbiota could be useful in the treatment of autoimmune and metabolic diseases and even in the fight against tumors, allowing the latest generation of oncological treatments, including immunotherapy, to be more effective.

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INTRODUCTION

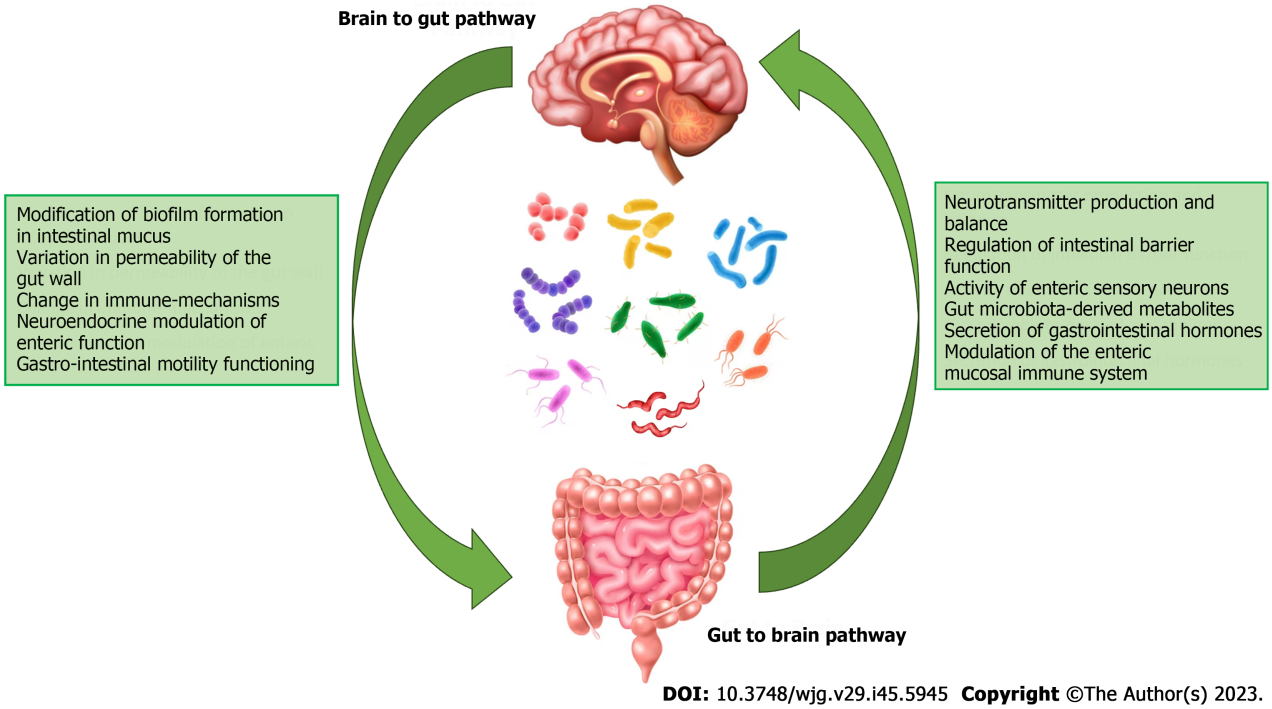
Symbiotic microbes are present in several sites across the human body and contribute to the healthy physiology of our organism. The microbiota consists of all these microorganisms (bacteria, viruses, fungi and parasites) in a multicellular living organism that live in symbiosis with it without harming it. The assemblage of microbes and their respective genomes constitute our "microbiome" and contribute to shape and regulate many aspects of healthy bodily function. Gastrointestinal, skin, vaginal, and respiratory microbiomes are featured across those respective anatomical and functional sites[1].

In particular, the gastrointestinal tract is composed of different anatomical structures and is the theatre of complex biochemical processes, as well as parallel interactions with sensory, neurological, and endocrinological networks. Not surprisingly, the gut environment is characterized by a heterogeneous collection of distinct habitats along the rostral-caudal axis, which host the most abundant and diverse microbiota in the human body[2].

In recent years, a large body of studies on the human gut microbiota has increased impressively, deepening the awareness that the composition of the gut microbiota can greatly influence health status. Taking part in the digestive process, the gut microbiota plays a fundamental role in the synthesis of short-chain fatty acids, certain vitamins and essential amino acids, which contribute to the health of the body and the gut[3]. The gut microbiota has a strong influence on the immunoregulation, and on metabolic and cardiovascular health[4,5]. Furthermore, it can contribute to the correct functioning of the central nervous system and can even condition the response to drugs[3,6]. States of dysbiosis can adversely affect host health by promoting enrichment of pathogenic species, compromising the permeability of the intestinal barrier, and contributing to localized or generalized inflammatory states[7]. These conditions can lead to the onset of diseases such as cancer, inflammatory bowel disease, metabolic diseases, or even affect the health of other body districts *e.g.*, through gynaecological and dermatological diseases.

The gut microbiota is directly involved not only in the genesis of gastroenterological diseases, such as chronic inflammatory bowel disease, but also in the neurological and psychiatric fields due to the role of the so-called gut-brain axis[8]. The microbiota-gut-brain axis is a bidirectional communication system that connects the central nervous system with the gut microbiota. This axis describes a bidirectional interaction between the inside of the enteric environment (the intestinal epithelium, microbiota, enteric nervous system) and the outside (the central nervous system), connecting centers of the cognitive and emotional spheres, endocrine, and immunological activity. Growing scientific evidence indicates that the gut microbiota can modulate the functions of the central nervous system and *vice versa*[9]. The marked synergy and continuous exchange of information along this axis is possible because of the vast neurochemical pool available to the enteric nervous system, which innervates the gastrointestinal tract, comparable only to that of the central nervous system. Cells in both systems use the same chemical mediators[3]. Under stressful conditions, the autonomic nervous system can alter intestinal motility and blood flow, and lead to an excessive secretion of hormones and neurotransmitters such as adrenaline and cortisol[10]: All this translates into an alteration in the composition and functional activity of the gut microbiota (Figure 1).

Due to dysbiosis, injurious molecules released from the gut and mediators of the immune response (especially cytokines and interleukins) released during the subsequent chronic inflammatory process can damage and overcome the blood-brain barrier. As a result, brain areas that are critical for the control of emotions and behaviour, such as the limbic and frontal lobes, can be damaged[11]. The study conducted by Carloni *et al*[12] highlighted that alteration of the blood-brain barrier, induced by chronic intestinal inflammation, can be a cause of severe depressive and anxiety disorders.



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Figure 1 Gut-brain axis interaction.

The emerging role of the gut microbiota in women

There is a complex controlled exchange of bacteria and other microorganisms from mother to child, even before birth. This dialogue prepares the newborn's immune system to face the outside world. Each individual acquires at birth his or her own personal gut microbiota, which will be an integral part of his or her defence mechanisms: An extremely heterogeneous set of bacteria, protozoa, fungi, and viruses that form the barrier between the external environment and the internal part of the organism (Figure 2). Its strength lies in diversity: A microbiota with high diversity can maintain certain functions and is usually a guarantee of a healthy immune system, while a low diversity microbiota can more easily undergo deficiencies and can cause impaired immune defences[13]. At birth, the digestive tract of newborns is completely sterile and is colonized immediately, starting from birth, by the microorganisms with which it comes into contact[13]. The gut microbiota of newborns delivered by caesarean section and/or those artificially breastfed appears profoundly different and takes longer to stabilize. In the first 4-36 mo of life, as a result of contact with parents, the external environment and food, the gut microbiota develops and changes rapidly.

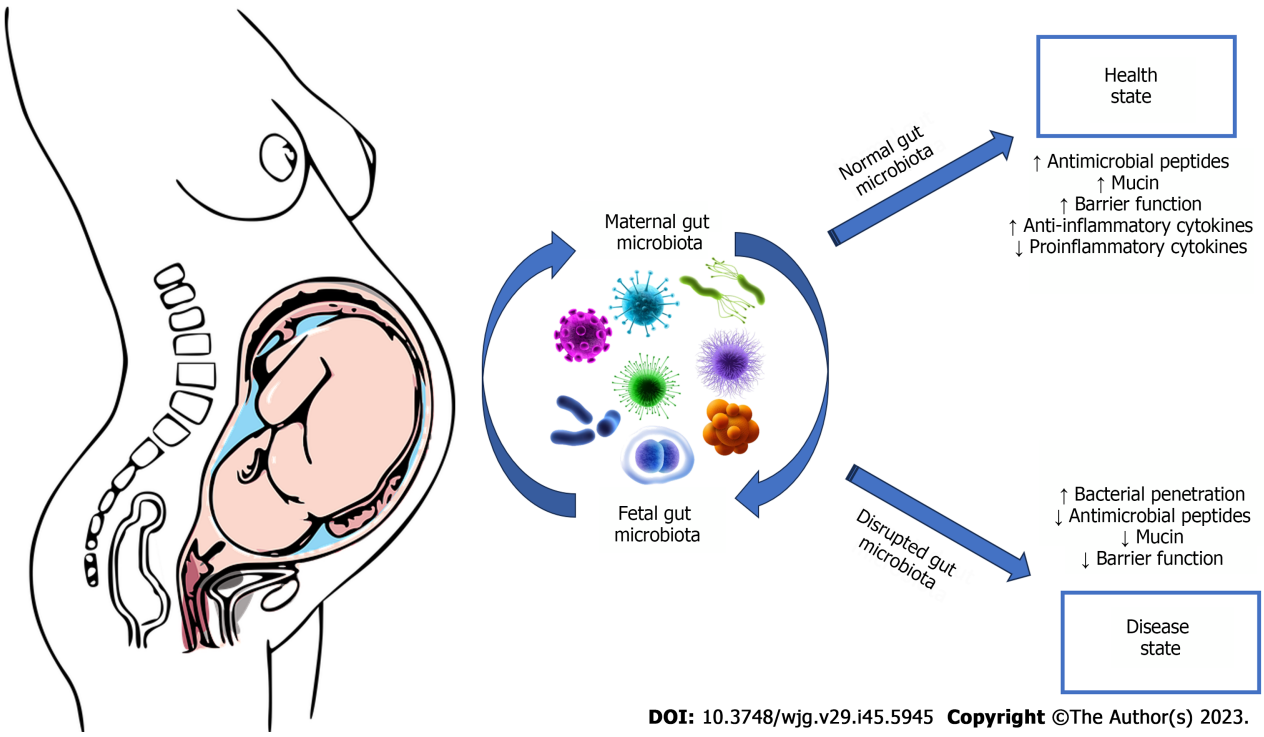
In the first years of life, a real genetic "imprinting" of the gut microbiota takes place, which then determines the state of health in the rest of existence: There it is written whether or not there is predisposition toward certain diseases[14]. This is a crucial time in an individual's life, it occurs within the age of 4-6 years, and for this reason all factors that can alter it should be avoided[15].

At this age, any intervention on the bacterial flora takes on a very important meaning as it will leave an indelible mark on what will be the adult gut microbiota. From this individual basic nucleus, various changes continue to be observed at different stages of life or if particular pathological conditions are established.

Differences in gut microbiota between men and women

The factors that can influence the composition and balance of the gut microbiota in adulthood are the most diverse: Environmental conditions, stress levels, genetic predisposition, hormonal structure (such as pregnancy, menopause, premenstrual period), pharmacological therapies, eating habits, styles of life[16]. There are diversities between male and female gut microbiota[17] related to hormonal[18], autoimmune[19], ethnic[20] or purely physiological differences such as body mass index body mass index[21] and age[22]. Since microbes of the same species can produce different metabolites depending on the gender of the host and interact differently with sex hormones producing different effects [23] for better characterization it is important to take into account gender differences. These differences appear after puberty, suggesting that sexual hormones have a key role in influencing composition of gut microbiota[24]. The gut microbiota progressively develops along with the maturation of the immune and nervous system throughout the lifespan in men and women with resultant different microbial communities as well as immune and neuro-inflammatory pathways in adult males and females[24].

The embryo differentiates in a male direction due to the Y chromosome and the genes contained therein, which condition circulating testosterone levels. In mice, the microbiota also seems to follow the same paradigm. There is a basic female-type microbiota, which progressively differentiates into a male-type microbiota in parallel with the increase in circulating testosterone[25,26]. Boys and girls during childhood show significant differences in *Actinomycetota*, *Bacillota*, and *Bacteroidota* phyla amount with higher *Bacteroidota/Bacillota* ratio in boys compared to girls. In adult population men



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Figure 2 Interplay between maternal and fetal microbiota.

and women maintain different amount of gut microbiota phyla (*Bacillota, Verrucomicrobia, Bacteroidota, Fusobacteria* and *Actinomycetota*)[27].

Gut microbiota during different stages of a woman's life

Puberty: In women, estrogens are important for differentiating the microbiota with health-promoting characteristics. The vaginal microbiota, whose population of microorganisms changes radically after puberty, is essential for the defence of the vagina throughout the fertile age, with a particular value for the pregnancy protection. After menopause, the impoverishment of the microbiota by sexual hormones deficiency leads to a depletion of many of its local, loco-regional and systemic beneficial health functions[24-26].

The estrobolome is capable of producing and metabolizing estrogen, which plays a key physiological role in maintaining women's health therefore modulating estrogen levels can have effects on weight, libido, and even mood, important dimensions for well-being of every person[28]. As regards estrogens in particular, intestinal microorganisms can modify their levels mainly through the activity of certain enzymes with which some bacteria are equipped, thus transforming estrogens into their active form capable of triggering physiological effects. An altered gut microbiota may play a leading role in the onset and development of reproductive system disorders with hormonal imbalances, such as polycystic ovary syndrome, endometriosis, and infertility[29]. Similarly, it has been suggested that microbiota imbalances with impairment of the gut's physiological "barrier" function and consequent translocation of microbes and/or microbial components have an active role in the development of vaginal and urinary tract infections[30,31].

Pregnancy: Pregnancy represents the period most clearly exemplifying the existing relationships between microbiota and hormone levels, where the modification of gut bacteria produces relevant and evident alterations ranging from a "low-grade" inflammatory condition typical of the pregnant state, to a reduced glucose tolerance, increased adiposity, among other transmissible as has been demonstrated in some experimental observations conducted on laboratory mice transplanted with the microbiota of women in the third trimester of pregnancy[30,31]. During pregnancy, the gut microbiota primarily defends the health of the mother, for example, by helping her not to develop gestational diabetes, and at the same time, it also preserves that of the child who will be exposed to the first "transfer" of microorganisms at the time of birth[32]. The vertical transmission of the microbiota from mother to fetus, which appears to begin during intrauterine life, contributes to the development of the child's gastrointestinal microbiota[33]. Going toward the third trimester, the relative abundance of some bacteria that are then transferred to the newborn in the peripartum (in the case of vaginal birth) increases: In fact, the child's gut microbiota consists of 22% bacteria derived from the mother's gut microbiota, particularly *Bifidobacteria*, *Bacteroides*, and *Escherichia coli*[31]. A more recent study showed important differences in the gut microbiota related to the sex of the individual and allowed the identification of microorganisms that remain in the female gut until fertile age and are then transmitted from mother to child during birth[34]. These strains are part of the genus *Bifidobacterium*, a group of microorganisms that has been much studied because they are associated with a positive impact on host health. Thus, knowing the composition of the microbiota during pregnancy can be a most useful tool both for preserving a woman's health status and for "working" to ensure that a healthy stock of microorganisms is inherited at the time of birth.

Menopause: In menopausal women it has been observed a depletion of *Bacillota* and a progressive reorganization of the gut microbiome (post-menopausal women tend to be more similar to age-matched men as to pre-menopausal women regarding *e.g.*, *Bacillota* to *Bacteroidota* ratio). This could suggest that testosterone may play a major role in shaping the gut microbiota, but more studies focusing on different ages with data comparing both male and female subjects are warranted in order to enlighten the distinct role of sexual hormones as direct or indirect choirmasters of human gut microbiota[27].

Gut microbiota and diseases in women

The fact that the gut microbiota differs between males and females can cause some sex-specific changes in immunity and it has been demonstrated that differences in male and female microbiota can drive chronic diseases, ranging from gastrointestinal inflammatory and metabolic conditions to neurological, cardiovascular, and respiratory illnesses[35]. Gut microbiota has a bidirectional relationship with inflammation and depending on its composition, it can inhibit or stimulate inflammatory pathways. Altered gut microbiota can contribute to subacute systemic inflammation reinforcing the disease state[36].

Potential role of the gut microbiota and its dysbiosis has been described in many diseases affecting women, such as polycystic ovarian syndrome, female cancers (breast, cervical, and ovarian cancer) and postmenopausal period illnesses such as menopausal obesity, Alzheimer's disease, and bone diseases[37]. The International Cancer Microbiome Consortium has suggested a key role of the gut microbiota in disease development, tumour protection, response to therapies, and control of side effects of cancer treatments[38]. Wang *et al*[39] found that the feces composition of women with benign pathology differed from that of women suffering from ovarian carcinoma where *Akkermansia* were low. The researchers showed that transferring gut microbes from ovarian carcinoma patients to mice with the same tumour increased their growth. Subsequent addition of *Akkermansia* bacteria to the microbiota transplant slowed tumour growth, probably due to increased T-cell activation. *Akkermansia* supplementation increased levels of short-chain fatty acids, which are associated with an improved ability of specific T lymphocytes to kill tumour cells.

The profile of the gut microbiota and its metabolites can serve as biomarkers for breast cancer. Some potentially pathogenic bacteria such as *Clostridium*, *Citrobacter*, and *Escherichia* may negatively influence the development and progression of breast cancer cells[40,41]. However, some bacterial species have shown a protective effect against this disease. *Faecalibacterium prausnitzii* and *Roseburia intestinalis* are producers of butyrate, this molecule has powerful anti-inflammatory and intestinal permeability-reducing activities, demonstrating a protective effect against the development of tumour cells[42,43].

The gut appears to be directly connected to the breast through an "axis", which may underlie a correlation between gut and breast microbiota dysfunction: The gut microbiota-mammary axis[44]. Altered composition of the various bacterial populations, no longer harmoniously represented within the microbiota, could affect not only local intestinal inflammation, but also systemic inflammation through "hyperactivation" of the immune system. In fact, several studies have shown the presence of dysbiosis in the breast microbiota of women with cancer, but not of healthy women[45]. Costantini *et al*[46] pointed out that the microbiota may predispose to neoplastic transformation. The clinical importance of this observation suggests that "working" on the microbiota to promote its rebalancing could result in a subsequent reduction in the risk of tumour transformation (Table 1).

The gut microbiota also actively and importantly influences the effectiveness of the response to immunotherapy. Several studies have observed that patients in whom immunotherapy is effective have a gut microbiome very rich in different species, whereas in patients who are resistant to this treatment the microbiota repertoire is more limited. Thus, the gut microbiota represents a first important modulation tool in regulating the anti-tumour immune response[47]. For example, *Bacteroidota* are biomarkers of response in melanoma patients: Their presence is associated with a possible reduction in response rates. In melanoma patients, the presence of three types of bacteria (*Bifidobacterium pseudocatenulatum*, *Roseburia* spp. and *Akkermansia muciniphila*) appears to be associated with better response to immunotherapy, but the link between microbiota and response to therapy involves different species in different patient groups [48]. On the other hand, *Faecalibacterium*, *Bifidobacterium* and *Oscillospiraceae* can improve the response to immune checkpoint inhibitors[49]. In addition, the gut microbiota may also play a role in the response to chemotherapy and in reducing the impact of treatment side effects, such as oral mucositis and inflammation[50,51].

CONCLUSION

Although there is a growing interest on influence of the gut microbiota on other organs, our knowledge on the role of gut microbiota in diseases is currently still limited. Some researchers have focused on the variation of the gut microbiome *via* diet and through supplementation with pre/pro/postbiotics in various female health issues. Interdisciplinary studies on the microbiota are achieving progress toward a better understanding of the molecular basis responsible for microorganism-host interaction and possible positive or negative implications on women's health. The modulation of the gut microbiome as a both preventative and therapeutic strategy needs to be accomplished. Theoretically, enriching the gut microbiota with "good" bacteria at the expense of "bad" bacteria, good health is promoted. However, there cannot be an ideal microbiota that is the same for everyone: Genes and individual characteristics play a determining role. Increasingly advanced techniques for analyzing the gut microbiota and its functions may also contribute to the development of precision medicine.

Table 1 Summary of gut microbiota in women

	Summary of gut microbiota
Healthy female[27]	Decreased: *Bacteroides* abundance with ↑diversity than in men
	Increased: *Lactobacillus*, *Bifidobacterium* and *Parabacteroides* than in men
Menstruation[29]	Decreased: *Bacteroidota*, *Butyricicoccus*, *Extibacter*, *Megasphaera*, *Parabacteroides*
Pregnancy[31-33]	Increased: *Actionbacteria*, *Proteobacteria*, *Akkermansia*, *Bifidobacterium*, *Bacillota*
	Decreased: Short chain fatty acids producers
Polycistic ovarian syndrome[36]	Increased: *Phocaeicola vulgatus*, *Bacillota*, *Streptococcus*, *Escherichia/Shigella*
	Decreased: *Tenericutes*, *Akkermansia*, *Oscillospiraceae*
Menopause[22]	Increased: *Bacillota*, *Roseburia*, *Lachnospira*, *Bacteroidota*
	Decreased: *Bilophila*, *Prevotella*, *Parabacteroides*
Breast cancer[40,41]	Increased: *Eubacteriales*, *Bacillus*, *Enterobacteriaceae*, *Staphylococcus*
Cervical cancer[37]	Increased: *Proteobacteria*, *Prevotella*, *Porphyromonas*, *Dialister*
	Decreased: *Bacteroides*, *Alistipes*, *Lachnospiraceae*
Ovarian cancer[39]	Increased: *Prevotella*, *Coriobacteriaceae*, *Bifidobacterium*

FOOTNOTES

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ORIGINAL ARTICLE

Observational Study

Enduring association between irritable bowel syndrome and war trauma during the Nicaragua civil war period: A population-based study

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Abstract

BACKGROUND

Psychosocial and physical trauma are known risk factors for irritable bowel syndrome (IBS), including in war veterans, whereas war exposure in civilians is unclear. Nicaragua experienced two wars, 1970-1990: The Sandinistas Revolution (1970s) and The Contra War (1980s). Our aim was to investigate the role of exposure to war trauma in the subsequent development of IBS in the context of an established health surveillance system (11000 households).

AIM

To investigate in a civilian population the relationship between exposure to war trauma and events and the subsequent development of IBS in the context of an

established public health and demographic surveillance system in western Nicaragua.

METHODS
We conducted a nested population-based, cross-sectional study focused on functional gastrointestinal disorders based on Rome II criteria. 1617 adults were randomly selected. The Spanish Rome II Modular Questionnaire and Harvard Trauma Questionnaire were validated in Nicaragua. War exposure was assessed with 10 measures of direct and indirect war trauma and post-war effects. Multiple exposures were defined by ≥ 3 measures.

RESULTS
The prevalence of IBS was 15.2% [Female (F) 17.1%, Male (M) 12.0%], war exposure 19.3% (F 9.3%, M 36.7%), and post-traumatic stress disorder (PTSD) 5.6% (F 6.4%, M 4.3%). Significant associations with IBS in the civilian population were observed (adjusted by gender, age, socioeconomic status, education): physical and psychological abuse [adjusted odds ratio (aOR): 2.25; 95% confidence interval: 1.1-4.5], witnessed execution (aOR: 2.4; 1.1-5.2), family member death (aOR: 2.2; 1.2-4.2), and multiple exposures (aOR: 2.7; 1.4-5.1). PTSD was independently associated with IBS (aOR: 2.6; 1.2-5.7).

CONCLUSION
An enduring association was observed in the Nicaragua civilian population between specific civil war-related events and subsequent IBS. Civilian populations in regions with extended armed conflict may warrant provider education and targeted interventions for patients.

Key Words: Irritable bowel syndrome; Functional gastrointestinal disorders; War trauma; Civil war; Post-traumatic stress disorder; Central America

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Core Tip: What is known: Psychosocial and physical trauma are known risk factors for irritable bowel syndrome (IBS), including in war veterans. What is new: An enduring association was observed in the Nicaragua civilian population between specific civil war-related events and subsequent IBS. Civilian populations in regions with extended armed conflict may warrant provider education and targeted interventions for patients.

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INTRODUCTION

Irritable bowel syndrome (IBS) is an important functional gastrointestinal disorder (FGID) affecting 1.1% to 35.5% of the general population. In North America, Europe, Australia and New Zealand the prevalence is estimated to be 8.1% [95% confidence interval (95%CI): 7.0-8.3][1]. Recent data developed in North America and the United Kingdom estimated an IBS prevalence with Rome IV criteria in 4.6% (95%CI: 4.1%-5.2%)[2]. In Latin America, the prevalence may be 2-3 times higher (17.5%, 95%CI: 16.9%- 18.2%)[1]. Furthermore, in central America the IBS studies are scarce, and not reported prevalence of IBS according to subtype[3]. The pathophysiology of IBS is a complex bio-psychosocial disease model. Variation in disease epidemiology may be explained by the heterogeneity of the biology, cultural and socioeconomic context, evolution of diagnostic criteria, and the microbiome, including parasite and viral infections[1,4-6] IBS is associated with multiple comorbidities, including somatic pain syndromes, other gastrointestinal disorders and psychiatric disorders, which affect patient quality of life (QOL) and health care utilization[1,7]. IBS and war veterans most studies of FGIDs and war trauma have focused on professional soldiers. Studies in deployed United States veterans identified a positive association between IBS, life stressors and gastroenteritis[8-10]. An analysis from the millennium cohort study, antecedent infectious gastroenteritis, female gender, life stressors, and anxiety are significantly associated with IBS[8]. This suggests that FGIDs may develop following war exposure, and may exert significant long term adverse effects. In addition, the risk of IBS in women veterans is nearly three times higher [odds ratio (OR): 2.82, 95%CI: 2.06-3.85) in those with posttraumatic stress disorder (PTSD)[11]. A recent meta-analysis based on 8 studies found PSTD to be a significant risk factor for IBS (OR: 2.80, 95%CI: 2.06-3.54)[12]. In Gulf War veterans, a case-control study found a positive association with IBS with chronic multi-symptom illness condition (OR: 11.57, 95%CI: 3.66-36.58)[9,10]. Similarly, Tuteja *et al*[10], founded a long-term persistence of FGIDs up to 16 years post-deployment, and a positive association with psychological disorders, extra-intestinal symptoms, and lower of QOL. The Central America Four region (CA-4), Nicaragua, Honduras, El Salvador, and Guatemala, are interconnected by geography, history, language, and development indices

[13]. In 2006, the CA-4 opened borders, similar to the European Union, and has been in transition toward a union of many aspects of their infrastructure, which has had implications for the health systems. The development indices in the CA-4 rank among the lowest in the Western Hemisphere: Multidimensional poverty approaches 20%, and nearly half (48%) of the CA-4 population lives in rural areas[13]. Civil wars dominated the region from 1970-1996, centered in Nicaragua, El Salvador and Guatemala. Importantly, the CA-4 countries account for a large number of the recent immigrant population to the United States, which makes the region unique among global low/middle income countries (LMICs) from a United States perspective[13]. A number equivalent to one quarter of the population of El Salvador lives in the United States, and most are foreign born. From the United States viewpoint, research and prevention initiatives in the CA-4 may be informative for United States. Hispanic populations, particularly for those from the region (> 5 million)[13]. The Nicaragua Civil War period, 1970-1990 Nicaragua suffered through two civil wars in 1970s and 1980s: The Popular Sandinista Revolution in the 1970s, with peak combat intensity in 1975-1979, and the counter-revolution "Contra War" in the 1980s. The Sandinista Revolution was an intense conflict throughout the country, with the greatest intensity in the western region in the cities of León and Chinandega. The civilian population of Leon lived with armed confrontations and aerial bombing[14]. The Contra War developed after the Sandinista victory, in the context of regional conflicts in Central America and the Cold War. It is estimated that 50000 people were killed in each of the two conflicts, and tens of thousands more were exposed to war events and trauma[14,15]. Based upon the enduring oral history of the civil wars in the Leon region, and the emerging bio-psychosocial model of IBS, we hypothesized a potential association between civil conflict and IBS. Our aim was to investigate in a civilian population the relationship between exposure to war trauma and events and the subsequent development of IBS in the context of an established public health and demographic surveillance system in western Nicaragua.

MATERIALS AND METHODS

Study design

We conducted a nested population-based, cross-sectional study of IBS and the FGIDs with the Rome II criteria in western Nicaragua, utilizing the León Health and Demographic Surveillance System (León-HDSS). The Centre of Epidemiology and Health Research, within the National Autonomous University of Nicaragua-León, oversees the León-HDSS, established in 1993. The municipality of León has an area of 820 km^2, including rural and urban populations[16]. The Leó n-HDSS encompasses 10994 households with 54647 individuals, distributed in 208 randomly-distributed geographical clusters, which encompass approximately 22% of the population of the municipality of León[16]. The current study is nested within a comprehensive study of the population prevalence of the FGIDs, based upon Rome 2 criteria, with final enrollment of 1617 persons randomly selected from urban and rural areas (the sample size was based upon an estimated IBS prevalence of 11%, alpha 5%, and 5% losses to follow-up within the León-HDSS). Thereafter, individuals born before 1975 were selected to meet the criteria of having been at least 5 years of age during the peak war period of 1975-1979 in the Leon region (n = 1012).

Variables and instruments

Socioeconomic status was measured by a validated poverty index based on the United Nations unsatisfied basic needs index (UBN)[16,17]. This is a scale from 0 to 4 (0-1 non-poor, 2-3 poor, and 4 extremely poor) that is based on four indicators: Household condition, water and sanitation services, school enrolment, and number of dependents in the household. This scale has been adapted and validated to specific conditions in Nicaragua[16]. Educational level was categorized into four levels: No schooling or illiterate, primary school, secondary school and university/professional formation. The Rome II Modular Questionnaire (R2MQ) was used to identify IBS cases, which were confirmed by physician interview[18,19]. To validate the R2MQ instrument in Nicaragua, we performed an independent selection of 400 individuals (not included in the study sample) for dual R2MQ assessments by trained field coordinators with an interval of 15 d (± 1 d) between the first and second interview. Alternate field coordinators conducted the second R2MQ assessment. The study subjects were randomly selected per the León-HDSS standing protocol. Three hundred and eighty subjects completed the two R2MQ assessments. Case status was confirmed thereafter *via* interviews by trained physicians, who were blinded to the survey results. We documented an R2MQ accuracy of 88% and repeatability of 84% (data not shown). The assessment of war trauma included direct exposures, indirect exposures, and post-war effects. The direct exposures refer to those experienced by the individual and included forced conscription, service in combat, personal trauma and disability, witnessed executions, and physical or psychological abuse. Indirect exposures included experiences of family members, and death of a family member. The post-war effects were focused on unemployment, loss of personal property, and rejection by family members. Unemployment includes difficulty finding employment in the immediate post-war period, and extended unemployment. The Harvard Trauma Questionnaire (HTQ) was used as a proxy to measure PTSD. The war trauma instrument was developed by Mollica *et al*[20], and includes a section covering 30 symptoms likely to be present after traumatic experiences, scored from 1 (not at all) to 4 (extremely). The trauma symptoms section of the HTQ is derived from the Diagnostic and Statistical Manual IV criteria for PTSD, with some additional questions specific to refugee trauma. The HTQ was validated in victims after the 1974 earthquake in Nicaragua, achieved a sensitivity of 95% and specificity of 77% with a cut-off 50/51 out of a maximum score 120[21].

Statstical methods and ethics

The variable distribution by groups [gender and strata age (29-39, 40-49 and ≥ 50)] was analysed with Mantel-Haenszel chi square test for categorical variables. The prevalence of IBS across the exposure variables (gender, age, socioeconomic

Table 1 Demographic, irritable bowel syndrome and war exposure summary

Variable	Total	Gender		*P* value[1]	Age group			*P* value[1]
	N (%)	Men, *n* (%)	Women, *n* (%)		29-39, *n* (%)	40-49, *n* (%)	50-65, *n* (%)	
Poverty				0.175				0.010[2]
Non-poor	657 (64.9)	229 (62.2)	428 (66.5)		227 (59.6)	224 (65.9)	206 (70.8)	
Poor/extremely poor	355 (35.1)	139 (37.8)	216 (33.5)		154 (40.4)	116 (34.1)	85 (29.1)	
Educational level				0.933				< 0.001[2]
Primary school	489 (48.3)	175	314 (48.8)		117 (30.7)	163 (47.8)	209 (71.8)	
High school	372 (36.8)	(47.6) 137	235 (36.5)		183 (48.0)	124 (36.5)	65 (22.3)	
Professional	151 (14.9)	(37.2) 56 (15.2)	95 (14.7)		81 (21.2)	53 (15.6)	17 (5.8)	
IBS				0.029[2]				0.593
No	858 (84.8)	324 (88.0)	534 (82.9)		320 (84.0)	286 (84.1)	252 (86.6)	
Yes	154 (15.2)	44 (12.0)	110 (17.1)		61 (16.0)	54 (15.9)	39 (13.4)	
PTSD				0.180				0.537
No	955 (94.4)	352 (95.7)	603 (93.6)		362 (95.0)	317 (93.2)	276 (94.8)	
Yes	57 (5.6)	16 (4.3)	41 (6.4)		19 (5.0)	23 (6.8)	15 (5.2)	
War				< 0.001[2]				0.022[2]
No	817 (80.7)	233 (63.3)	584 (90.7)		321 (84.3)	259 (76.2)	237 (81.4)	
Yes	195 (19.3)	135 (36.7)	60 (9.3)		60 (15.7)	81 (23.8)	54 (18.6)	
PTSD + War				0.422				0.095
No	987 (97.0)	357 (97.0)	630 (97.8)		376 (98.7)	327 (96.2)	284 (97.6)	
Yes	25 (3.0)	11 (3.0)	14 (2.2)		5 (1.3)	13 (3.8)	7 (2.4)	
Dose Effect				< 0.001[2]				0.199
0-2 exposures	945 (93.4)	308 (83.7)	637 (98.9)		358 (94.0)	311 (91.5)	276 (94.8)	
≥ 3 exposures	67 (6.6)	60 (16.3)	7 (1.1)		23 (6.0)	29 (8.5)	15 (5.2)	
Total	1012 (100.0)	368 (100.0)	644 (100.0)		381 (100.0)	340 (100.0)	291 (100.0)	

[1]Mantel-Haenszel chi square test.
[2]*P* value < 0.05.
IBS: Irritable bowel syndrome; PTSD: Post-traumatic stress disorder.

status, educational level, war exposure, and PTSD). The multiple logistic regression model was adjusted for age, gender, education, and poverty level. In the analysis, age [age of ≥ 45 years old (29-44 or 45-65)], socioeconomic status [non-poor (0 to 1) *vs* poor (2 to 4)], and educational level (elementary primary school or less educational level *vs* secondary school or more) were dichotomized. The model was used to examine the relationship between IBS and war events and exposures (direct and indirect exposures, and postwar effects). A distinct analysis was conducted for PTSD with and without war exposure and the association with IBS. The 95%CI and *P* value < 0.05 is used for the reported values. All analyses were done using IBM SPSS Statistics for Mac, Version 20.0 (IBM Corp., Armonk, NY, United States). This study was approved by the Institutional Review Boards of the University of North Carolina, Chapel Hill (#02-MED-461) and UNAN-Leon. All Good Clinical Practice standards were followed, including informed consent with each subject, participant confidentiality and anonymized data. There were no conflicts of interest with respect to industry or government.

RESULTS

A total of 1617 individuals were randomly selected and consented from the León-HDSS for household interviews, of whom 1012 met age inclusion criteria (see Methods). Nearly two-thirds (n = 644), were women in Table 1. The mean age was 43.8 years old (range 29-65). The distribution of poverty and educational level by gender was similar, with p values of 0.175 and 0.933, respectively. Men were exposed to a greater number of war events than women (36.7% *vs* 9.3%, *P* <

Table 2 Irritable bowel syndrome prevalence by Rome II criteria among 1012 study participants

Variables	IBS		*P* value[1]
	N (%)	95%CI	
Gender			0.029[2]
Male	44 (12.0)	9.0–15.7	
Female	110 (17.1)	14.4–20.2	
Age group			0.593
29-39	61 (16.0)	12.7-20.0	
40-49	54 (15.9)	12.4-20.1	
≥ 50	39 (13.4)	9.9-17.8	
Socioeconomic status[3]			0.298
Non-poor	97 (14.8)	12.3-17.7	
Poor/Extremely poor	57 (16.1)	12.6-20.24	
Educational level			0.701
Primary school/Lower	70 (14.3)	11.5-17.7	
High school	61 (16.4)	13.0-20.5	
University/Professional	23 (15.2)	10.4-21.8	
War			0.237
No	119 (14.6)	12.3-17.1	
Yes	35 (17.9)	13.2-23.9	

[1]Mantel-Haenszel chi square test.
[2]*P* value < 0.05.
[3]Based on United Nations Poverty Index, adapted to Nicaraguan population.
95%CI: 95% confidence interval; IBS: Irritable bowel syndrome.

0.001), and men were more likely than women to have three or more war exposures (16.3% and 1.1%, $P < 0.001$). Participants ages 40-49 were more likely to have war experiences (23.8%, $P = 0.022$) compared with the 29-39 age group (15.7%) and 50-65 years old group (18.6%).

IBS prevalence and associations

The overall prevalence of IBS was 15.2% (95%CI: 13.1-17.6), and significantly higher among women (17.1% *vs* 12.0%, $P = 0.029$). No significant differences in IBS prevalence were noted by age, education, or poverty level. The IBS prevalence was greater among individuals with war exposure, but not statistically significant (17.9% and 14.6%, respectively, $P = 0.237$) in Table 2. Three specific war exposures were significantly associated with over two times the future risk of IBS in the logistic regression model adjusted for age, gender, education, and poverty level in Table 3. These included physical or psychological abuse [adjusted OR (aOR): 2.25, 1.1-4.5], a witnessed execution (aOR: 2.4, 1.1-5.2), and the death of a family member (aOR: 2.2, 1.2-4.2). In addition, a dose effect was noted, as participants with ≥3 war exposures also had over two times the IBS risk (aOR: 2.7, 1.4-5.1), with a prevalence of 23.9%. The remaining war event exposures were not associated with IBS, including direct combat involvement, physical injury or disability, a wounded family member, and post-war events (loss of personal property, rejection by family and unemployment in table.

PTSD relationchip to war exposure and IBS

The overall prevalence of PTSD was 5.6%, and without differences observed by gender or age (Table 1). PTSD specifically caused by war exposure was 3.0% overall, and similar in men and women (3.0 *vs* 2.2, $P = 0.422$) and among the age strata (1.3% *vs* 3.8% *vs* 2.4%, $P = 0.095$) in Table 1. Notably, 42.1% (95%CI: 30.2-55.0) and 56% (95%CI: 37.1-73.3) of subjects who fulfilled the criteria for IBS had PTSD and war-associated PTSD, respectively, in Table 2. Adjusted for age, gender, education, and poverty level, a strong relationship was observed between IBS and war-associated PTSD (aOR: 3.3, 1.1-10.0) in Table 3.

Table 3 Association between Irritable bowel syndrome and specific war exposures

Condition	IBS, *n* (%)	Non-IBS, *n* (%)	Unadjusted[1], OR (95%CI)	Adjusted[2], OR (95%CI)
Direct Exposures				
War experience	35 (22.7)	160 (18.6)	1.28 (0.84-1.94)	1.30 (0.81-2.09)
Direct combat involvement	13 (8.4)	77 (9.0)	0.93 (0.50-1.72)	0.61 (0.27-1.38)
Abuse, physical or psychological[3]	17 (11.0)	47 (5.5)	2.19 (1.19-3.83)	2.25 (1.12-4.49)
Witnessed execution[3]	17 (11.0)	51 (5.9)	1.96 (1.10-3.50)	2.41 (1.11-5.23)
Physical injury or disability	3 (1.9)	8 (0.9)	2.11 (0.55-8.04)	1.29 (0.27-6.02)
Indirect Exposures				
Death of a family member[3]	20 (13.0)	68 (7.9)	1.73 (1.01-2.94)	2.21 (1.17-4.17)
Family member wounded	9 (5.8)	42 (4.9)	1.20 (0.57-2.53)	0.80 (0.33-1.93)
Post-war effects				
Loss of personal property	3 (1.9)	19 (2.2)	0.87 (0.25-3.00)	0.86 (0.24-3.08)
Rejection by family	5 (3.2)	16 (1.9)	2.58 (0.88-7.54)	2.57 (0.82-8.07)
Unemployment	9 (5.8)	36 (4.2)	1.41 (0.66-3.00)	1.58 (0.69-3.65)
Dose effect				
≥ 3 exposures[3]	16 (10.4)	67 (7.8)	1.83 (1.01-3.30)	2.67 (1.39-5.13)
PTSD				
PTSD[3]	24 (15.6)	33 (3.8)	4.61 (2.64-8.05)	2.63 (1.21-5.73)
PTSD, war associated[3]	14 (9.1)	11 (1.3)	7.70 (3.42-17.3)	3.31 (1.09-10.0)

[1]Crude odds ratio.
[2]Multiple logistic regression analysis adjusted by gender, age, poverty and educational level.
[3]Significant association with IBS.
95%CI: 95% confidence interval; IBS: Irritable bowel syndrome; PTSD: Post-traumatic stress disorder.

DISCUSSION

Our population-based study in the civilian population of Nicaragua demonstrates an association of direct and indirect exposure to war events with the subsequent development of IBS. IBS was associated with specific war exposures, including physical or psychological abuse, a witnessed execution, and the death of a family member. A 'dose effect' is also observed, with an increased risk of IBS among those with ≥ 3 types of war trauma. Riddle *et al*[8] describe an increase association in relation with the numbers of life stressors and female gender. Notably, the majority of studies to date have evaluated the association between deployed veterans and risk of gastrointestinal symptoms or IBS, and from the first Gulf War in particular[9,22-24]. The prevalence of IBS was 14.2% (95%CI: 12.1-16.5) in the general population (without war trauma exposures), which is similar to estimations in Latin America 17.5% (95%CI: 16.9-18.2), some of which hvw used different Rome instruments[1]. The IBS prevalence in females (17.1%; 95%CI: 14.4-20.2) was greater than males (12.0%; 95%CI: 9.0-15.7), also consistent with Latin America data[1,25]. We observed a lower IBS prevalence in subjects ≥ 50 years old, similar to the findings by Lovell and Ford[26] in a review of 14 studies (OR: 0.75; 95%CI: 0.62-0.92). The IBS prevalence was not significantly different among levels of poverty. In this regard, Lovell and Ford[26] noted an insufficient number of studies (n = 4) of the IBS association with socioeconomic status, and insufficient for assessment of heterogeneity. We found that the abuse of civilians in a civil armed conflict setting, physical or psychological, was independently associated with IBS. The association of abuse and IBS is a consistent finding in patients[27-29]. Koloski *et al*[29] reported likelihood abuse (physical, emotional and/or verbal) during adulthood in subjects with symptoms of IBS in contrast with controls. Kanuri *et al*[28] demonstrated a higher prevalence of abuse in the IBS population in comparison with the non-FGID population. Specific forms of abuse, including physical, emotional and sexual, were higher in IBS groups. Abuse experiences have lasting effects on the mental and gastrointestinal health of individuals, thereby establishing a relationship between these experiences and IBS during the adulthood[27-29]. In the Nicaraguan civilian population, IBS was associated with PTSD, likely linked to prior war events exposure (42.1%, 95%CI: 30.2%-55.0%, P value < 0.001). The increased risk of gastrointestinal syndromes among men and women is a consistent finding with warzone exposure[23,30]. Increased rates of IBS among deployed United States veterans[9,22,24]. In a population-based military survey in China, appreciable differences in the prevalence of IBS were noted between aircrew and ground personnel (5.72% *vs* 3.70%), P < 0.05)[31]. In one of the major cohort studies among United States military personnel from all service branch, Riddle *et al*[8], identified a strong association between IBS and PTSD. Maguen *et al*[32] conducted a

retrospective, cross-sectional analysis of > 600000 Iraq and Afghanistan War veterans, finding that IBS was more likely among both males and females among those with PTSD. A meta-analysis with 648375 subjects reported a pooled IBS risk OR of 2.80 (95%CI: 2.06-3.54; $P < 0.001$) with PTSD[12]. Our finding demonstrated similar association in the logistic regression model. In 2016, the Institute of Medicine confirmed that there was sufficient evidence demonstrating an association between deployment to the Gulf War and gastrointestinal symptoms consistent with FGIDs such as IBS or functional dyspepsia[22]. Conversely, a United Kingdom study on the Iraq War estimated that the prevalence of IBS was relatively low on return from Iraq, although it was significant higher during deployment[33]. Tuteja *et al*[10], demonstrated the long-term persistence of IBS with higher scores for all psychological disorders under study (depression, anxiety, somatization, and global symptoms index) in deployed Gulf War veterans. Our results in the Nicaragua civil conflict setting suggest a long-term risk of IBS and its association with PTSD and specific war exposures. Likewise, Riddle *et al*[8] describe an increase association in relation with the numbers of life stressors and female gender. Our study has several strengths including the random selection of a civilian population from an established health and demographic surveillance system, thereby minimizing selection bias. The variables under study were well defined and used instruments validated in the Nicaraguan population. Our study evaluated a general civilian population and therefore may generalize to civilians in prior or current war zones. The study focused on the period over 15 years after the ending of Contra-Revolution war, and consistent with studies suggesting longstanding risk for the development of clinical IBS [10,34]. Several study limitations are noted. The cross-sectional design does not confirm causality between war exposure and IBS. Rome II criteria were used, which have different IBS sensitivity and specificity in comparison with Rome III and Rome IV criteria, but notably, each IBS case was verified by a physician interview in our protocol. Our study was conducted several years after war exposure and potentially subject to recall bias, although arguably these recollections are vivid. Lastly, the impact of gastrointestinal infections in this study population in the tropical environment was not evaluated[6].

CONCLUSION

The prevalence of IBS in the LMIC setting of in Central America is significant and consistent with studies in Latin America. An enduring association in the civilian population of IBS with prior exposure to specific war-related events is observed, in a region of extended civil conflict. Our findings have important implications for healthcare programs for providers and civilian populations in the post-war period[35].

ARTICLE HIGHLIGHTS

Research background

Post-traumatic stress disorder (PTSD) in veterans, and the association with irritable bowel syndrome (IBS) is well described, but the impact on civilian population is poor described.

Research motivation

According to United Nations Refugee Agency 108.4 million of people were forced to flee their homes to escape conflicts in 2023. During this exodus the people are exposed to suffer different PTSD. The Nicaraguan population between 1970 and 1988 was part of this worldwide phenomena.

Research objectives

To determinate the association between PTSD and IBS in civilians exposed to war.

Research methods

A nested cross-sectional study was design. A population data set was used to develop a random selection. Different instruments were validated to collect data. The instruments were focused on IBS, PTSD and poverty. Logistic regression model was developed to respond to our aim.

Research results

Positive association between IBS and PTSD by war exposure in civilians was obtained.

Research conclusions

PTSD and IBS symptoms are persistent over the time. The association between then is positive.

Research perspectives

Other populations in the world could be affected by IBS as a result of PTSD originated in different stressful conflicts. Interventions in primary health care could be implemented to improve the gut and mental health.

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FOOTNOTES

Author contributions: Morgan D and Peña R designed the research; Peña-Galo EM, Peña R, and Cortes L contributed with data acquisition; Peña-Galo EM, Wurzelmann D, Peña R, Alcedo J, and Morgan D analyzed and interpreted of data; Morgan D, and Peña EM wrote manuscript; Peña-Galo EM, Peña R, Alcedo J, Wurzelmann D, Cortes L, and Morgan D performed a critical review.

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Institutional review board statement: This study was approved by the Institutional Review Boards of the University of North Carolina, Chapel Hill (#02-MED-461) and UNAN-Leon. All Good Clinical Practice (GCP) standards were followed, including informed consent with each subject, participant confidentiality and anonymized data.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: There were no conflicts of interest with respect to industry or government.

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ORIGINAL ARTICLE

Observational Study

Association of low muscle strength with metabolic dysfunction-associated fatty liver disease: A nationwide study

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Abstract

BACKGROUND

There is limited evidence regarding the association between muscle strength and metabolic dysfunction-associated fatty liver disease (MAFLD).

AIM

To investigate the association between muscle strength and MAFLD in the general population in Korea.

METHODS

This nationwide representative cross-sectional study included 31649 individuals aged ≥ 19 years who participated in the Korea National Health and Nutrition Examination Survey between 2015 and 2018. Odds ratios (ORs) and 95% confidence intervals (95%CIs) for MAFLD according to sex-specific quartiles of muscle strength, defined by relative handgrip strength, were calculated using multivariable logistic regression analysis. Additionally, multivariable logistic regression analysis was used to assess the association between muscle strength and probable liver fibrosis in patients with MAFLD.

RESULTS

Of all the participants, 29.3% had MAFLD. The prevalence of MAFLD was significantly higher in the lower muscle strength quartile groups for all participants, sexes, and age groups ($P < 0.001$). A 1.92-fold (OR = 1.92, 95%CI: 1.70–2.16) and 3.12-fold (OR = 3.12, 95%CI: 2.64–3.69) higher risk of MAFLD was

observed in the lowest quartile (Q1) group than in the other groups (Q2–Q4) and the highest quartile (Q4) group, respectively. The ORs of MAFLD were significantly increased in the lower muscle strength quartile groups in a dose-dependent manner (*P* for trend < 0.001). These associations persisted in both sexes. An inverse association between muscle strength and the risk of MAFLD was observed in all subgroups according to age, obesity, and diabetes mellitus. In patients with MAFLD, the odds of severe liver fibrosis were higher in Q1 (OR = 1.83, 95%CI: 1.25–2.69) than in other groups (Q2–Q4).

CONCLUSION

Among Korean adults, low muscle strength was associated with an increased risk of MAFLD and liver fibrosis in patients with MAFLD.

Key Words: Muscle strength; Handgrip strength; Metabolic dysfunction-associated fatty liver disease; Liver fibrosis; Korea National Health and Nutrition Examination Survey

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Core Tip: Limited evidence exists regarding the association between muscle strength and metabolic dysfunction-associated fatty liver disease (MAFLD). This nationwide cross-sectional study analyzed 17349 individuals in the general community who participated in the Korea National Health and Nutrition Examination Survey and measured their grip strength between 2015 and 2018. Among the participants, 29.3% had MAFLD. The prevalence of MAFLD was significantly higher in the lower muscle strength quartiles. The odds ratios of MAFLD were significantly increased in the lower muscle strength quartile groups in a dose-response manner. Among Korean adults, low muscle strength was associated with an increased risk of MAFLD and liver fibrosis in patients with MAFLD.

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INTRODUCTION

The prevalence of non-alcoholic fatty liver disease (NAFLD) has surged alongside the obesity pandemic, making it a significant global public health issue, particularly within the Asian population. Over the last three decades, the overall burden of NAFLD has grown exponentially[1-3]. NAFLD has significant clinical implications because it causes liver cirrhosis and liver cancer, which are major causes of liver-related mortality[1]. In response to the broader multi-system nature of NAFLD and the rising prevalence of metabolic disorders, a recent introduction has been made regarding a new definition: metabolic dysfunction-associated fatty liver disease (MAFLD)[2]. While reports suggest that the prevalence of MAFLD in Asian countries ranges from 10%–30%, it exhibits a discernible upward trajectory[2]. Notably, a 23-year follow-up United States study revealed that MAFLD was associated with increased all-cause mortality; furthermore, advanced fibrosis in MAFLD had a higher all-cause mortality risk than that in NAFLD[4]. Given the clinical significance and the imperative to alleviate the disease burden associated with MAFLD, a thorough analysis of risk factors is essential.

Previous studies have suggested an association between sarcopenia and NAFLD. Specifically, muscle mass or strength has shown an inverse relationship with NAFLD[5-7]. Furthermore, it is worth noting that reduced muscle strength has shown a significant association with liver fibrosis in individuals afflicted with NAFLD[8]. Muscle strength, a marker for cardiometabolic fitness, is inversely associated with morbidity[9,10], encompassing conditions such as metabolic syndrome and mortality, particularly within the context of NAFLD[11-14]. Thus, we hypothesized that sarcopenia, particularly muscle strength, is associated with the risk of MAFLD and advanced fibrosis in MAFLD.

Although the body of evidence is evolving, it is worth noting that only a few cross-sectional studies have shown that sarcopenia, measured using dual-energy X-ray absorptiometry and mid-upper arm circumference, was associated with liver fibrosis in patients with MAFLD[15,16]. Limited evidence exists on the association between muscle strength and the prevalence of MAFLD and liver fibrosis in MAFLD in the general population[17]. In light of the public health burden of MAFLD in Asia, we investigated the association between muscle strength and MAFLD using a Korean nationally representative database.

MATERIALS AND METHODS

Survey description and study participants

This study used data from the Korea National Health and Nutrition Examination Survey (KNHANES). The Ministry of Health and Welfare and the Korea Disease Control and Prevention Agency jointly conduct the KNHANES to calculate national health statistics, which is the fundamental database for healthcare policymaking in South Korea. This annual examination recruits an average of 10000 participants in South Korea and consists of 11 different variables, including blood tests and physical examinations, such as measuring body weight, height, handgrip strength, and blood pressure (BP). The KNHANES ensures the quality of the data entered into the database through data collection by well-trained staff and quality control of procedures by internal and external professionals. Details of the KNHANES database have been covered in depth in the past[18].

Because the KNHANES database includes pediatric and adolescent participants, only Korean citizens aged ≥ 19 years who participated in the KNHANES during 2015-2018 were initially included in the analysis. Among the 31649 individuals who participated in the KNHANES between 2015 and 2018, we excluded individuals aged < 19 years (n = 6315), heavy drinkers who consumed ≥ 210 g of alcohol per week for men and ≥ 140 g per week for women (n = 2712) based on self-reported questionnaires, those who tested positive serological markers for hepatitis B or C virus (n = 976), those diagnosed with liver cirrhosis or hepatocellular carcinoma (n = 84), and those with missing data (n = 4213). Ultimately, the data from 17349 individuals were included in the analysis. All participants provided written informed consent for data collection.

Assessment of muscle strength

Muscle strength was assessed using relative handgrip strength, which has been utilized in prior studies as an indicator of muscle strength[8,19]. To measure handgrip strength, the participants were instructed to squeeze a handgrip dynamometer (Digital grip strength dynamometer, T.K.K 5401, Takei Scientific Instruments Co., Ltd., Tokyo, Japan) for at least 3 S using their dominant arm with the elbow extended and the participant in a standing position. Handgrip strength was measured thrice with a 1-min interval for rest between each measurement. Muscle strength was defined as the mean handgrip strength (kg) divided by the body mass index (BMI, kg/m^2). As the present study aimed to understand the association between muscle strength and MAFLD, the study participants were divided into sex-specific quartile groups of muscle strength, with Q1 and Q4 being the lowest and highest quartiles, respectively. The cutoff values for the quartiles were 1.30, 1.53, and 1.77 in men, and 0.76, 0.94, and 1.11 in women, respectively.

Definition of MAFLD and liver fibrosis

NAFLD was defined using a validated fatty liver prediction model called the hepatic steatosis index (HSI)[20]. HSI was defined as 8 × alanine aminotransferase (ALT)/aspartate aminotransferase (AST) + body mass index (BMI, + 2, if diabetic; +2, if female). HSI > 36 was defined as NAFLD[20]. Previous studies have reported that HSI could predict NAFLD with high sensitivity and specificity in the Korean population[20,21].

MAFLD was defined as NAFLD (HSI > 36) with the presence of at least one of the following metabolic risk factors[2]: (1) Overweight or obesity (BMI ≥ 23 kg/m^2) based on Asian standards[22]; (2) Type 2 diabetes [physician diagnosis, fasting serum glucose ≥ 126 mg/dL, or glycated hemoglobin (HbA1c) ≥ 6.5%]; and (3) Normal BMI (< 23 kg/m^2) with two or more of the following metabolic risk factors[23]: (1) Waist circumference (WC) ≥ 90 cm and ≥ 80 cm for men and women; (2) BP ≥130/85 mmHg or being administered anti-hypertensive medication(s); (3) Triglyceride level ≥ 150 mg/dL or being administered lipid-lowering medication(s); (4) High-density lipoprotein cholesterol (HDL-C) < 40 mg/dL for men and < 50 mg/dL for women, or being administered lipid-lowering medication(s); (5) Diagnosis of prediabetes state, defined as fasting serum glucose of 100-125 mg/dL or HbA1c of 5.7%–6.4%; and (6) Serum high-sensitivity C-reactive protein (hs-CRP) level > 2 mg/L.

To evaluate advanced liver fibrosis in patients with MAFLD, we used the following prediction equation: Fibrosis-4 (FIB-4) score = age (years) × AST (IU/L)/[platelet (10^9/L)] × [ALT (IU/L)]$^{1/2}$. The risk of advanced fibrosis in MAFLD was classified as either 1.3 ≤ FIB-4 score < 2.67 (intermediate risk) or FIB-4 ≥ 2.67 (high risk)[24].

Measurements and covariates

Participants' sociodemographic information and data on health behaviors were assessed using a self-report questionnaire. Smoking status was classified based on whether the participant was a current smoker. Based on the modified version of the International Physical Activity Questionnaire[25,26], regular physical activity was defined as: (1) Moderate-intensity physical activity for ≥ 150 min/wk; (2) High-intensity physical activity for ≥ 75 min/week; or (3) A combination of moderate- and high-intensity physical activity per week, where 1 min of high-intensity physical activity is equivalent to 2 min of moderate-intensity physical activity, with the collective minutes satisfying either one of the above criteria. Household income was segmented into quartiles, and educational attainment was assessed based on whether participants had completed more than 12 years of education (or high school graduate).

The physical examination was conducted by certified staff. Height, body weight, and WC were measured, and BMI was defined as the weight in kilograms divided by the square of height in meters. Using a standard sphygmomanometer, three BP measurements were conducted at 5-min intervals, and the mean values of the second and third BP measurements were recorded. Blood samples were drawn after fasting for ≥ 8 h, and the serum concentrations of AST, ALT, total cholesterol, HDL-C, low-density lipoprotein cholesterol (LDL-C), triglycerides, fasting glucose, HbA1c, hs-CRP, hepatitis B surface antigen, hepatitis C virus antibody, and platelet count were assessed.

Hypertension was defined as either a medical diagnosis by a physician or a systolic/diastolic BP reading of ≥ 140/90 mmHg[27]. Diabetes mellitus (DM) was defined as having a physician's diagnosis, a fasting glucose level of ≥ 126 mg/dL, or an HbA1c level of ≥ 6.5%[28]. Dyslipidemia was characterized by either a physician's diagnosis or a total cholesterol level of ≥ 240 mg/dL[29]. Obesity was defined as BMI ≥ 25 kg/m^2[30].

Statistical analyses

Continuous variables were summarized as mean ± SE and categorical variables as percentages and were compared using analysis of variance and the Rao–Scott chi-square test, respectively. We performed multivariable logistic regression analysis to evaluate the association between muscle strength and the risk of MAFLD and calculated the odds ratios (ORs) and 95% confidence intervals (95%CIs). Model 1 was not adjusted, and Model 2 was adjusted for age, sex, income, education, smoking status, and physical activity. In Model 3, adjustments were made for ALT, obesity, hypertension, DM, dyslipidemia, and hs-CRP levels, in addition to the confounders in Model 2. The adjusted variables were selected from the statistically significant variables in Table 1, the clinical factors that were expected to be associated with muscle strength and MAFLD, and based on the results of the preliminary logistic regression analysis between baseline variables and MAFLD (Supplementary Table 1) and a literature search. The association between muscle strength and the risk of MAFLD was also evaluated in subgroups stratified by sex, age, obesity, and DM. Multivariable logistic regression analysis was used to assess the association between muscle strength and probable liver fibrosis in patients with MAFLD. Statistical analyses, including pairwise comparison, were performed using IBM SPSS Statistics ver. 22.0 (IBM Corp., Armonk, NY, United States). Complex sample procedures were performed based on the survey design. Statistical significance was set at $P < 0.05$. The statistical methods used in this study were reviewed by Dr. Youn Huh from Uijeongbu Eulji Medical Center.

RESULTS

Characteristics of study participants

Among a total of 31649 potentially eligible individuals, 17349 individuals were included and analyzed in the study. The characteristics of the participants according to the muscle strength quartiles are presented in Table 1. Individuals with weaker muscle strength were older and had lower socioeconomic status. Those with weaker muscle strength were less likely to be current smokers and engaged in regular physical activities (P for trend < 0.001). The mean values of cardiometabolic parameters, such as BMI, WC, BPs, AST, ALT, total cholesterol, LDL-C, triglycerides, fasting glucose, HbA1c, and hs-CRP, tended to be higher in the lower muscle strength quartile groups (P for trend < 0.001). The proportion of those with obesity, hypertension, DM, and dyslipidemia also increased as muscle strength decreased (P for trend < 0.001).

Prevalence of MAFLD according to muscle strength

Among all participants, including within various sex and age groups, the prevalence of MAFLD was significantly higher in the lower muscle strength quartile groups ($P < 0.001$ in Figure 1). Additionally, regardless of sex and age group, the prevalence was higher in the lowest quartile (Q1) group of muscle strength than in the remaining quartile (Q2–Q4) groups ($P < 0.001$ in Figure 1B and C).

Association between muscle strength and the risk of MAFLD

Among all participants, the Q1 group had higher odds of MAFLD than the other groups (Q2–Q4) (OR = 1.92, 95%CI: 1.70–2.16) (Model 3, Table 2). Compared with the Q4 group, the ORs of MAFLD significantly increased in the Q3 (OR = 1.41, 95%CI: 1.22–1.65), Q2 (OR = 2.19, 95%CI: 1.85–2.58), and Q1 (OR = 3.12, 95%CI: 2.64–3.69) groups. The ORs were higher in the lower muscle strength quartile groups in a dose-dependent manner (P for trend < 0.001). Among both men and women, higher odds of MAFLD were observed in the Q1 group than in the Q2–Q4 (OR = 2.05, 95%CI: 1.70–2.47 in men and OR = 1.76, 95%CI: 1.47–2.10 in women) and Q4 groups (OR = 2.88, 95%CI: 2.20–3.78 in men and OR = 3.09, 95%CI: 2.43–3.92 in women). The ORs of MAFLD tended to be higher in the lower muscle strength quartile groups for both sexes (P for trend < 0.001). Furthermore, the receiver operating characteristic analysis to assess the relationship between muscle strength and prevalence of MAFLD revealed that the area under the curve for the entire participant group, as well as for men and women separately, were 0.764, 0.701, and 0.740, respectively (all $P < 0.001$, Supplementary Figure 1).

Subgroup analyses on the association between muscle strength and MAFLD

As indicated in Table 3, a noteworthy inverse relationship between muscle strength and the risk of MAFLD remained consistent across all subgroups. Importantly, no significant interactions were observed based on sex, age, obesity, or DM status in the association between muscle strength and MAFLD.

Association between muscle strength and probable liver fibrosis assessed by FIB-4 in MAFLD

After adjusting for all confounding factors, muscle strength was not significantly associated with the intermediate risk for advanced liver fibrosis (defined as 1.3 ≤ FIB-4 < 2.67) in patients with MAFLD (Table 4). However, the lowest muscle strength group (Q1) had a higher odds of a high risk of advanced fibrosis (defined by FIB-4 ≥ 2.67) than the Q2–Q4 group (OR = 1.83, 95%CI: 1.25–2.69).

Table 1 Characteristics of study participants according to the quartiles of muscle strength

	Q1	Q2	Q3	Q4	*P* for trend
N (unweighted)	4305	4347	4397	4300	
Age (yr)	55.9 ± 0.4	49.2 ± 0.4	44.6 ± 0.3	39.9 ± 0.2	< 0.001
Sex (men)	45.2 (1.0)	45.9 (0.9)	46.2 (0.9)	48.0 (0.9)	0.172
Current smoker	13.6 (0.7)	16.6 (0.7)	17.7 (0.7)	20.3 (0.8)	< 0.001
Physical activity	38.5 (1.0)	46.4 (1.0)	51.1 (0.9)	53.1 (0.9)	< 0.001
Income (lowest quartile)	28.8 (1.0)	16.5 (0.7)	10.7 (0.6)	8.0 (0.5)	< 0.001
Education (≤ 12 yr)	45.4 (1.2)	28.1 (0.9)	16.9 (0.7)	8.5 (0.5)	< 0.001
BMI (kg/m^2)	26.0 ± 0.1	24.7 ± 0.1	23.5 ± 0.1	21.8 ± 0.1	< 0.001
Waist circumference (cm)	88.0 ± 0.2	84.0 ± 0.2	80.5 ± 0.2	76.3 ± 0.2	< 0.001
Handgrip strength (kg)	21.8 ± 0.2	27.8 ± 0.2	31.2 ± 0.2	35.6 ± 0.2	< 0.001
Muscle strength (handgrip strength/body mass index)	0.8 ± 0.01	1.1 ± 0.01	1.3 ± 0.01	1.6 ± 0.01	< 0.001
Systolic BP (mmHg)	122.7 ± 0.4	118.2 ± 0.3	115.2 ± 0.3	112.1 ± 0.3	< 0.001
Diastolic BP (mmHg)	75.3 ± 0.2	75.8 ± 0.2	75.2 ± 0.2	74.2 ± 0.2	< 0.001
AST (IU/L)	24.5 ± 0.2	23.1 ± 0.2	21.5 ± 0.2	20.2 ± 0.1	< 0.001
ALT (IU/L)	26.0 ± 0.5	24.2 ± 0.4	21.0 ± 0.3	18.4 ± 0.2	< 0.001
Total cholesterol (mg/dL)	191.4 ± 0.7	194.3 ± 0.7	194.1 ± 0.6	188.8 ± 0.6	< 0.001
HDL-C (mg/dL)	47.4 ± 0.2	49.9 ± 0.2	51.6 ± 0.2	54.0 ± 0.2	< 0.001
LDL-C (mg/dL)	115.0 ± 0.7	116.9 ± 0.6	116.5 ± 0.6	112.3 ± 0.5	< 0.001
Triglycerides (mg/dL)	145.6 ± 2.0	137.5 ± 2.0	130.1 ± 1.9	112.5 ± 1.6	< 0.001
Fasting glucose (mg/dL)	106.1 ± 0.5	100.8 ± 0.4	97.6 ± 0.4	93.8 ± 0.3	< 0.001
HbA1c (%)	5.9 ± 0.02	5.7 ± 0.01	5.6 ± 0.01	5.4 ± 0.01	< 0.001
hs-CRP (mg/L)	1.7 ± 0.04	1.2 ± 0.04	1.1 ± 0.03	0.8 ± 0.03	< 0.001
Obesity	56.7 (0.9)	44.0 (1.0)	28.7 (0.8)	11.6 (0.6)	< 0.001
Hypertension	45.4 (1.0)	30.5 (0.8)	21.5 (0.7)	12.4 (0.6)	< 0.001
Diabetes mellitus	22.5 (0.7)	13.1 (0.6)	7.8 (0.4)	3.9 (0.3)	< 0.001
Dyslipidemia	31.8 (0.9)	28.1 (0.9)	21.2 (0.7)	14.1 (0.6)	< 0.001

Data are presented as mean ± SE or percentage (SE). Q: Quartile; BMI: Body mass index; BP: Blood pressure; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; HbA1c: Glycated hemoglobin; hs-CRP: High-sensitivity C-reactive protein.

DISCUSSION

In this large-scale nationwide study, we found that the prevalence of MAFLD was greater among individuals with lower muscle strength, which was associated with a higher risk of MAFLD after adjusting for potential confounding variables. The lowest muscle strength quartile group had 3.12-fold, 2.88-fold, and 3.09-fold higher odds of MAFLD than the highest quartile group in all participants, men, and women, respectively. These associations persisted in the subgroups stratified by age, obesity, and DM. Furthermore, in patients with MAFLD, the lowest muscle strength quartile group had 1.83-fold increased odds of high risk of advanced liver fibrosis compared to the other groups.

To the best of our knowledge, no prior studies have examined the association between muscle strength and the risk of MAFLD. However, few studies have examined the association between muscle strength and NAFLD[31]. One cross-sectional study, utilizing data from the KNHANES database, found an association between low muscle strength and NAFLD[8]. In our study, we have effectively demonstrated a significant association between muscle strength and MAFLD. Importantly, our findings suggest that low muscle strength may be a modifiable risk factor for MAFLD. This study also suggests an association between low muscle strength and a high probability of advanced liver fibrosis in patients with MAFLD. Our findings are in line with previous studies reporting an association between low muscle strength and advanced liver fibrosis[8,32]. Although the association did not persist when each muscle strength group was analyzed, the current study demonstrated that low muscle strength might be associated with advanced liver fibrosis in

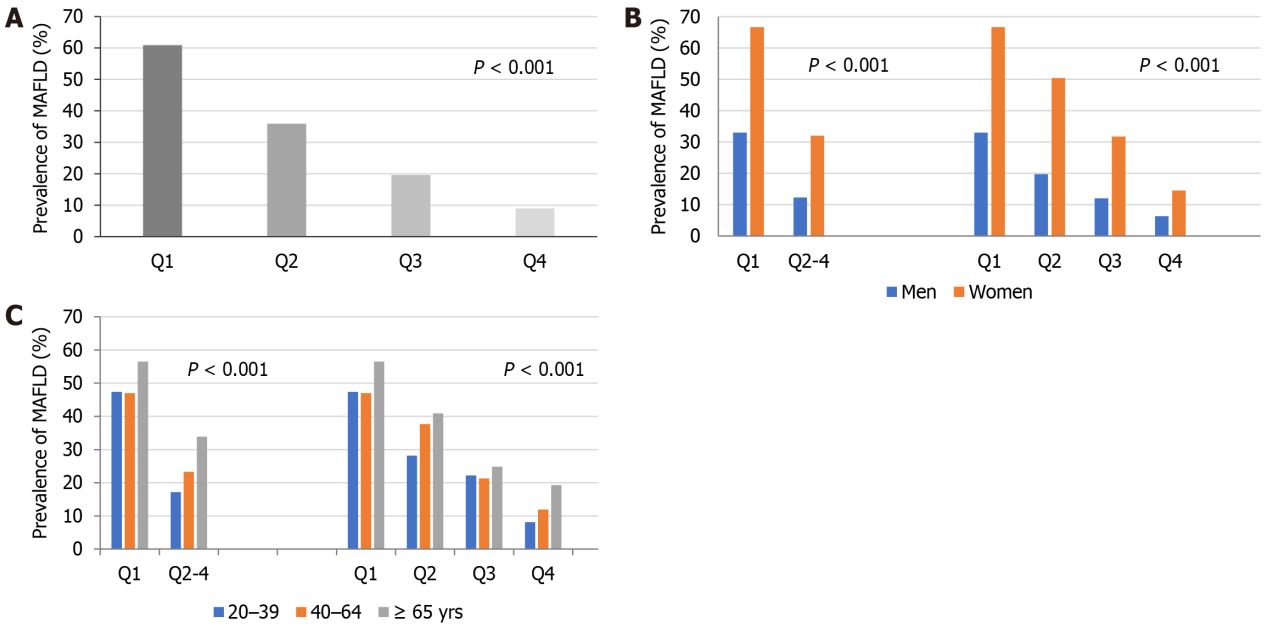
Table 2 Odds ratios (95% confidence intervals) of metabolic dysfunction-associated fatty liver disease according to the categories of muscle strength

Muscle strength	Model 1	Model 2	Model 3
Total			
Q1	3.63 (3.34–3.95)	3.24 (2.94–3.58)	1.92 (1.70–2.16)
Q2–Q4	1 (reference)	1 (reference)	1 (reference)
P value			
Q1	9.12 (7.98–10.41)	8.61 (7.41–10.01)	3.12 (2.64–3.69)
Q2	4.92 (4.29–5.64)	4.75 (4.10–5.50)	2.19 (1.85–2.58)
Q3	2.48 (2.17–2.84)	2.42 (2.10–2.78)	1.41 (1.22–1.65)
Q4	1 (reference)	1 (reference)	1 (reference)
P for trend	< 0.001	< 0.001	< 0.001
Men			
Q1	3.51 (3.02–4.09)	3.31 (2.80–3.90)	2.05 (1.70–2.47)
Q2–Q4	1 (reference)	1 (reference)	1 (reference)
P value	< 0.001	< 0.001	
Q1	7.30 (5.67–9.39)	7.20 (5.52–9.38)	2.88 (2.20–3.78)
Q2	3.68 (2.83–4.78)	3.68 (2.82–4.80)	1.75 (1.32–2.31)
Q3	2.02 (1.54–2.66)	2.03 (1.55–2.68)	1.23 (0.93–1.63)
Q4	1 (reference)	1 (reference)	1 (reference)
P for trend	< 0.001	< 0.001	< 0.001
Women			
Q1	4.20 (3.74–4.71)	3.17 (2.79–3.60)	1.76 (1.47–2.10)
Q2–Q4	1 (reference)	1 (reference)	1 (reference)
P value	< 0.001	< 0.001	< 0.001
Q1	11.76 (9.87–14.02)	9.14 (7.56–11.05)	3.09 (2.43–3.92)
Q2	6.09 (5.15–7.20)	5.30 (4.47–6.30)	2.52 (2.05–3.10)
Q3	2.76 (2.34–3.26)	2.59 (2.20–3.06)	1.52 (1.25–1.85)
Q4	1 (reference)	1 (reference)	1 (reference)
P for trend	< 0.001	< 0.001	< 0.001

Odds ratios (95% confidence intervals) were calculated using multivariable logistic regression analysis; Model 1 = not adjusted; Model 2 = adjusted for age, sex, income, education, smoking status, and physical activity; Model 3 = Model 2 + alanine aminotransferase, obesity, hypertension, diabetes mellitus, dyslipidemia, and high-sensitivity C-reactive protein. Q: Quartile.

patients with MAFLD.

The mechanisms underlying the association between low muscle strength and MAFLD have not yet been fully elucidated. However, potential pathways may include insulin resistance due to hepatic steatosis[33] and physical inactivity and disuse, leading to decreased muscle function[34]. Metabolically inactive muscles undergo disuse atrophy, in which sarcomeres are catabolized and capillaries decrease in number[35]. This risk is especially pronounced in individuals with metabolic syndrome and a sedentary lifestyle. Weight gain leads to adipocyte dysfunction and increased local inflammation, eventually resulting in insulin resistance. Disturbances in fat storage in adipocytes increase the release of fatty acids. Excess fatty acids produce strain in the hepatic mitochondria and subsequently result in the production of reactive oxygen species and mitochondrial damage. Excess fatty acids also increase stress on the endoplasmic reticulum, contributing to mitochondrial dysfunction and cellular death[33]. In particular, reduced muscle strength may be linked to mitochondrial dysfunction in both skeletal muscles and the liver, as suggested by previous studies[36-41]. Data accumulated thus far have indicated that mitochondrial dysfunction may play a role in the development of insulin resistance, and MAFLD and mitochondrial dysfunction may additionally contribute to low muscle strength[42-46]. In relation to hepatic fibrosis, stellate cells are thought to be involved in inflammatory and fibrotic changes in fatty liver disease in response to damage-associated molecular patterns from dying hepatocytes, free



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Figure 1 Prevalence of metabolic dysfunction-associated fatty liver disease by muscle strength. A: The prevalence of metabolic dysfunction-associated fatty liver disease (MAFLD) in the total participants; B: the prevalence in men and women; C: the prevalence in different age groups. The prevalence of MAFLD was higher in the lower muscle strength quartile groups in total participants and age and sex groups. The prevalence was higher in the lowest muscle strength quartile (Q1) group than in the remaining groups (Q2–Q4) in both sexes and all age groups. A dose-response relationship between lower muscle strength quartile and MAFLD was also observed. Q: Quartile; MAFLD: Metabolic dysfunction-associated fatty liver disease.

cholesterol, toll-like receptors, and oxidative stress[33,47].

Sarcopenia is a muscle disease of both young and old and increases the risks of falls, fractures, and mortality[34]. Studies have revealed that muscle strength, rather than muscle mass, is a predictor of mortality[48-50]; thus, the guideline from the European Working Group on Sarcopenia in Older People highlighted low muscle strength as the main characteristic of sarcopenia[34]. Low muscle strength alone is adequate for making a clinical diagnosis of probable sarcopenia. Subsequently, diagnostic assessments for potential underlying causes and appropriate interventions can be initiated[34,51]. Additionally, the guideline recommended handgrip strength as a proxy for whole-body strength[34]. Overall, these findings suggest that muscle strength is a crucial parameter in detecting sarcopenia[52]. Accordingly, we used muscle strength, defined by handgrip strength, as the primary independent variable in this study.

MAFLD increases the risk of liver fibrosis, hepatocellular carcinoma, and cardiovascular diseases; hence, controlling metabolic disorders is essential in the management of MAFLD[2]. Treatment objectives encompass the reversal of hepatic steatosis, steatohepatitis, and hepatic fibrosis, as well as the reduction of cardiovascular risk associated with MAFLD. The mainstay of treatment is the modification of metabolic risk factors and lifestyle[2]. Weight loss can reduce liver steatosis and reverse steatohepatitis or fibrosis[53]. Generally, low-carbohydrate, low-fat, and Mediterranean-type dietary plans with moderate-intensity exercise for 30 min/d for ≥ 5 d/wk or ≥ 150 min/wk or vigorous-intensity exercise for ≥ 20 min/d for ≥ 3 d/wk are recommended[2,54]. The guidelines endorse the inclusion of both aerobic and resistance exercises[2]. In light of the observed association between low muscle strength and a higher prevalence of MAFLD in this study, it is imperative to underscore the importance of resistance training during patient education.

While this study has provided valuable insights, it is important to acknowledge that it also had several limitations. First, the findings of this study are not generalizable to other ethnic groups since only Koreans were included in the analysis. Second, although liver biopsy is the gold standard for diagnosis, the present study used HSI to diagnose NAFLD and MAFLD. However, the diagnostic reliability of the HSI has been validated in previous studies[20,21]. Third, causal relationships could not be fully determined owing to the cross-sectional design of the study. Future prospective longitudinal studies are needed to confirm the role of muscle strength in MAFLD. Fourth, the lack of data on medications for chronic diseases and those potentially affecting liver steatosis is a limitation. Thus, we defined hypertension, DM, and dyslipidemia using a combination of prior physician diagnoses and laboratory blood tests. Fifth, handgrip strength was used to diagnose probable sarcopenia in the present study. The prevalence of probable sarcopenia can vary depending on the diagnostic methods, especially in liver steatosis[55]. Nonetheless, handgrip strength is easy to incorporate into clinical settings and is also a well-studied parameter of sarcopenia in many studies. Despite these shortcomings, the use of KNHANES enabled us to study important exposures and muscle strength in a large representative Korean population and make adjustments for a variety of potential confounders. Furthermore, we were able to successfully assess the new definition of MAFLD and study its prevalence and association with muscle strength, which extends beyond previous studies where only NAFLD was considered.

Table 3 Subgroup analysis for the association between muscle strength and metabolic dysfunction-associated fatty liver disease

	Muscle strength	OR (95%CI)	*P* for interaction
Sex			0.126
Men	Q2–Q4	1 (reference)	
	Q1	2.05 (1.70–2.47)	
Women	Q2–Q4	1 (reference)	
	Q1	1.76 (1.47–2.10)	
Age			0.057
< 65 yr	Q2–Q4	1 (reference)	
	Q1	1.97 (1.70–2.29)	
≥ 65 yr	Q2–Q4	1 (reference)	
	Q1	1.74 (1.44–2.09)	
Obesity			0.264
No	Q2–Q4	1 (reference)	
	Q1	1.63 (1.36–1.96)	
Yes	Q2–Q4	1 (reference)	
	Q1	2.09 (1.75–2.48)	
Diabetes mellitus			0.622
No	Q2–Q4	1 (reference)	
	Q1	1.97 (1.72–2.26)	
Yes	Q2–Q4	1 (reference)	
	Q1	1.71 (1.33–2.22)	

Odds ratios (95% confidence intervals) were calculated using multivariable logistic regression analysis after adjusting for age, sex, income, education, smoking status, physical activity, alanine aminotransferase, obesity, hypertension, diabetes mellitus, dyslipidemia, and high-sensitivity C-reactive protein; Stratified variables (sex, age, obesity, and diabetes mellitus) were omitted from the adjusted variables during the respective subgroup analyses. OR: Odds ratio; 95%CI: 95% confidence interval; Q: Quartile.

Table 4 Association between muscle strength and probable liver fibrosis assessed using fibrosis-4 among patients with metabolic dysfunction-associated fatty liver disease

Muscle strength	OR (95%CI)		
	Model 1	Model 2	Model 3
1.3 ≤ FIB-4 < 2.67			
Q1	2.67 (2.35–3.02)	0.96 (0.82–1.12)	1.03 (0.87–1.21)
Q2–Q4	1 (reference)	1 (reference)	1 (reference)
P value	< 0.001	0.588	0.773
Q1	4.09 (3.50–4.79)	0.89 (0.72–1.09)	1.01 (0.81–1.26)
Q2	2.31 (2.00–2.66)	0.85 (0.71–1.01)	0.94 (0.78–1.13)
Q3	1.67 (1.43–1.94)	0.97 (0.81–1.16)	1.04 (0.86–1.25)
Q4	1 (reference)	1 (reference)	1 (reference)
P for trend	< 0.001	0.117	0.809
FIB-4 ≥ 2.67			
Q1	4.71 (3.38–6.56)	1.72 (1.19–2.50)	1.83 (1.25–2.69)
Q2–Q4	1 (reference)	1 (reference)	1 (reference)

P value	< 0.001	0.004	0.002
Q1	8.13 (4.74–13.95)	1.68 (0.89–3.17)	1.85 (0.92–3.74)
Q2	2.25 (1.22–4.15)	0.78 (0.41–1.47)	0.82 (0.41–1.66)
Q3	2.20 (1.18–4.13)	1.26 (0.65–2.46)	1.31 (0.65–2.65)
Q4	1 (reference)	1 (reference)	1 (reference)
P for trend	< 0.001	0.113	0.073

Odds ratios (95% confidence intervals) were calculated using multivariable logistic regression analysis; Model 1 = not adjusted; Model 2 = adjusted for age, sex, income, education, smoking status, and physical activity; Model 3 = Model 2 + alanine aminotransferase, obesity, hypertension, diabetes mellitus, dyslipidemia, and high-sensitivity C-reactive protein. OR: Odds ratio; 95%CI: 95% confidence interval; FIB-4: Fibrosis-4; Q: Quartile.

CONCLUSION

In this nationwide study of the Korean adult population, low muscle strength was associated with a dose-dependent higher risk of MAFLD in all participants and subgroups. Low muscle strength is associated with a high probability of liver fibrosis in patients with MAFLD. The identification and management of low muscle strength may play a crucial role in preventing MAFLD and liver fibrosis. Nonetheless, additional research is necessary to validate this association.

ARTICLE HIGHLIGHTS

Research background

More evidence is needed regarding the association between muscle strength and metabolic dysfunction-associated fatty liver disease (MAFLD) and only a few cross-sectional studies have shown that sarcopenia was associated with liver fibrosis in patients with MAFLD. In response to the increasing public health burden of MAFLD in Asia, we investigated the association between muscle strength and MAFLD using a Korean nationally representative database.

Research motivation

A recent introduction has been made regarding a new definition: MAFLD. Importantly, MAFLD is associated with increased all-cause mortality and advanced fibrosis in MAFLD had a higher all-cause mortality risk than that in non-alcoholic fatty liver disease. However, the link between muscle strength and MAFLD is not well studied.

Research objectives

We aimed to investigate the association between muscle strength and MAFLD in the general population in Korea. Additionally, we sought to study the risk of liver fibrosis in patients with MAFLD according to muscle strength.

Research methods

This study used data from the Korea National Health and Nutrition Examination Survey. Muscle strength was assessed using relative handgrip strength and the participants were categorized into muscle strength quartiles. We performed multivariable logistic regression analysis to evaluate the association between muscle strength and the risk of MAFLD and calculated the odds ratios and 95% confidence intervals.

Research results

Twenty-nine point three per cent of the participants had MAFLD. The lowest quartile was significantly associated with higher prevalence of MAFLD for all participants, sexes, and age groups. In patients with MAFLD, the odds of severe liver fibrosis were higher in Q1 than in other groups (Q2–Q4). However, causality should be investigated in future studies.

Research conclusions

The nationwide study of the Korean adult population revealed that low muscle strength was associated with a dose-dependent higher risk of MAFLD in all participants and subgroups. Additionally, low muscle strength is associated with a high probability of liver fibrosis in patients with MAFLD. The identification and management of low muscle strength may play a crucial role in preventing MAFLD and liver fibrosis.

Research perspectives

Prospective cohort or randomized controlled trials are needed to confirm the relationship between muscle strength and MAFLD. Future studies should focus on whether physical activity can prevent or reverse MAFLD and liver fibrosis in patients with MAFLD.

FOOTNOTES

Author contributions: Nam GE designed the study; Nam GE and Huh Y were responsible for developing the methodology; Nam GE, Huh Y, Lee GB, and Lee SH participated in the formal analysis and investigation; Lee GB wrote the original draft; Lee GB, Han B, Kim YH, Kim DH, Kim SM, Choi YS, Cho KH, and Nam GE participated in the review and editing; Nam GE acquired funding.

Institutional review board statement: The institutional review board of the Korea University Guro Hospital in Seoul, Republic of Korea approved the study protocol (No. 2022GR0322).

Informed consent statement: Signed informed consent was obtained from all participants.

Conflict-of-interest statement: The authors disclose no conflicts.

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ORIGINAL ARTICLE

Basic Study

Thioridazine reverses trastuzumab resistance in gastric cancer by inhibiting S-phase kinase associated protein 2-mediated aerobic glycolysis

Zheng-Yan Yang, Yi-Wei Zhao, Jing-Rui Xue, Ran Guo, Zhi Zhao, Han-Di Liu, Zhi-Guang Ren, Ming Shi

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Abstract

BACKGROUND

Trastuzumab constitutes the fundamental component of initial therapy for patients with advanced human epidermal growth factor receptor 2 (HER-2)-positive gastric cancer (GC). However, the efficacy of this treatment is hindered by substantial challenges associated with both primary and acquired drug resistance. While S-phase kinase associated protein 2 (Skp2) overexpression has been implicated in the malignant progression of GC, its role in regulating trastuzumab resistance in this context remains uncertain. Despite the numerous studies investigating Skp2 inhibitors among small molecule compounds and natural products, there has been a lack of successful commercialization of drugs specifically targeting Skp2.

AIM

To discover a Skp2 blocker among currently available medications and develop a therapeutic strategy for HER2-positive GC patients who have experienced progression following trastuzumab-based treatment.

METHODS

Skp2 exogenous overexpression plasmids and small interfering RNA vectors were utilized to investigate the correlation between Skp2 expression and trastuzumab resistance in GC cells. Q-PCR, western blot, and immunohistochemical analyses were conducted to evaluate the regulatory effect of thioridazine on Skp2 expression. A cell counting kit-8 assay, flow cytometry, a amplex red glucose/glucose oxidase assay kit, and a lactate assay kit were utilized to measure the proliferation, apoptosis, and glycolytic activity of GC cells in vitro. A xenograft model established with human GC in nude mice was used to assess thioridazine's effectiveness *in vivo*.

RESULTS

The expression of Skp2 exhibited a negative correlation with the sensitivity of HER2-positive GC cells to trastuzumab. Thioridazine demonstrated the ability to directly bind to Skp2, resulting in a reduction in Skp2 expression at both the transcriptional and translational levels. Moreover, thioridazine effectively inhibited cell proliferation, exhibited antiapoptotic properties, and decreased the glucose uptake rate and lactate production by suppressing Skp2/protein kinase B/mammalian target of rapamycin/glucose transporter type 1 signaling pathways. The combination of thioridazine with either trastuzumab or lapatinib exhibited a more pronounced anticancer effect in vivo, surpassing the efficacy of either monotherapy.

CONCLUSION

Thioridazine demonstrates promising outcomes in preclinical GC models and offers a novel therapeutic approach for addressing trastuzumab resistance, particularly when used in conjunction with lapatinib. This compound has potential benefits for patients with Skp2-proficient tumors.

Key Words: Gastric cancer; Trastuzumab resistance; Thioridazine; S-phase kinase associated protein 2; Glycolysis

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Core Tip: S-phase kinase-interacting protein 2 (Skp2) has been shown to be a reliable prognostic indicator of unfavorable outcomes for gastric cancer (GC). However, no agents specifically targeting Skp2 have been successfully developed. In this study, we proved that thioridazine restores the sensitivity of GC cells to trastuzumab both *in vivo* and *in vitro* by inhibiting Skp2-mediated glycolysis. Furthermore, the combination of thioridazine and lapatinib exhibits enhanced inhibitory effects compared with either monotherapy on the growth and survival of trastuzumab-resistant GC cells. Overall, this study suggests the potential of a thioridazine-based therapy to overcome trastuzumab resistance in human epidermal growth factor receptor 2-positive GC by targeting Skp2.

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INTRODUCTION

Gastric cancer (GC) is the fifth most common cancer worldwide, with more than 1 million new cases diagnosed in 2020, and it is the fourth leading cause of cancer-related death[1]. Approximately 7.3%-20.2% of GCs are positive for human epidermal growth factor (EGF) receptor 2 (HER2) /neu, c-ERBB2[2]. Positive expression of HER2 was proven to be associated with many tumorigenic processes and poor prognosis in patients with GC[3,4]. Trastuzumab is an effective anti-HER2 therapeutic agent that showed a survival benefit in the ToGA trial[5]. Trastuzumab in combination with chemotherapy was previously the first-line treatment for HER2-positive metastatic GC. However, due to primary or acquired drug resistance, only 12.8% of patients with HER2-positive GC respond to trastuzumab[6,7]. To overcome trastuzumab resistance, many new agents and combination therapies, such as pertuzumab, margetuximab, lapatinib, tucatinib, trastuzumab emtansine, and pembrolizumab, have emerged. However, the application of most of these drugs in the treatment of trastuzumab-resistant HER-2-positive GC is still in the investigative stage. Thus, the development of new drugs or combination therapies to increase trastuzumab sensitivity is a critical need.

Cancer cells exhibit high levels of glucose uptake and glycolysis, which allow the production of high levels of ATP to facilitate cell proliferation and survival, a phenomenon called the "Warburg effect"[8]. It has been reported that the GATA6 binding protein 6 protein contributes to resistance to trastuzumab in GC by regulating metabolic reprogramming, including reprogramming toward glycolysis[9]. According to Liu *et al*[10], MACC1 activates the phosphatidylin-

ositol-3-kinase (PI3K)/protein kinase B (AKT) signaling pathway to promote the Warburg effect, and downregulation of MACC1 reverses trastuzumab resistance in GC cells. Wang *et al*[11] and colleagues found that combination treatment with metformin and trastuzumab in a circadian pattern resensitized GC cells to trastuzumab, partially by disrupting the BMAL1–CLOCK–PER1–HK2 axis, thus controlling fluctuations in glycolysis. Therefore, glycolysis inhibitors could be used appropriately to overcome trastuzumab resistance in GC. However, historically, glycolysis inhibitors have not been widely applied clinically due to their obvious side effects[12].

S-phase kinase associated protein 2 (Skp2) is a constituent of the F-box protein family and functions as a substrate recognition component within the Skp2-SCF complex, which plays a crucial role in the regulation of ubiquitination, cell cycle progression, cell proliferation, and apoptosis[13]. Extensive evidence has demonstrated that Skp2 acts as an oncogene[14], exhibiting elevated expression levels in breast cancer[15], GC[16], prostate cancer[17], and various other malignant tumors, thereby exhibiting a strong association with poor outcomes in affected individuals. Recent studies have shown that Skp2 regulates glycolysis, trastuzumab sensitivity, and tumorigenesis in breast cancer[18]. However, whether Skp2 regulates trastuzumab sensitivity in GC is unknown.

Several investigations have been conducted on small structure-based inhibitors of Skp2. For instance, the compounds SZL-P1-41[19], SKPin C1[20,21], and DT204[22] were identified as Skp2 inhibitors that could suppress tumor growth. However, treatment with these chemical inhibitors is accompanied by adverse effects. Several natural compounds, such as diosmetin[23], safranal[24], dioscin[25], gartanin[26], betulinic acid[27], linichlorin A[28], and gentian violet[29], have been identified to function as potential antitumor agents through Skp2 inhibition. However, these studies are still in the preliminary stages of preclinical development.

This study proposes that the antipsychotic drug thioridazine can specifically decrease the expression of Skp2, thereby increasing the responsiveness of HER-2-positive GC cells to trastuzumab through the attenuation of glycolysis.

MATERIALS AND METHODS

Cell lines and reagents

The human GC cell lines HGC-27, SGC-7901, MGC-803, MKN-45, and NCI-N87 were purchased from the American Type Culture Collection (Manassas, United States). HGC-27, NCI-N87, and MGC-803 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS); Biological Industries, Israel). SGC-7901 and MKN-45 cells were grown in RPMI 1640 medium containing 10% FBS. Trastuzumab-resistant HGC-27 and SGC-7901 cells were established by culturing cells with increasing concentrations of trastuzumab (Roche, Switzerland) over half a year and were designated HGC-27-R and SGC-7901-R cells, respectively. All cells were cultured in incubators at 37 ℃ with 5% CO_2. Thioridazine and lapatinib were obtained from Selleck Chemicals (United States).

RT-PCR analysis

Quantitative real-time reverse transcription polymerase chain reaction analysis using a Real-Time PCR Detection System (Agilent Technologies) was performed to validate the effect of thioridazine on SKP2 gene expression. The sequences of the primers used were as follows: *5′-ATGCCCCAATCTTGTCCATCT-3′* and *5′-CACCGACTGAGTGATAGGTGT-3′* for SKP2; *5′-GTGGGGCGCCCCAGGCACCA-3′* and *5′-CTTCCTTAATGTCACGCACGATTTC-3′* for β-actin.

Construction of the exogenous overexpression plasmids and SKP2 RNA interference vectors

The pcDNA3.1-3 × Flag-C plasmid carrying the Skp2 coding sequence was constructed. The sense primer sequence was *5'-CCGGAATTCCGGAGGATGCACAGGAAGCACCTCCAGGAG-3'*, and the antisense primer sequence was *5'-CCGCTC-GAGTAGACAACTGGGCTTTTGCAGTGT-3'*. The two recombinant plasmids confirmed to contain the correct sequence were named OX-SKP2-1 and OX-SKP2-2. Small interfering RNA (siRNA) for SKP2 and the negative control (NC) oligonucleotide sequence were synthesized by Sangon Biotech (Guangdong, China). The siRNA duplexes were transfected into HGC27-R cells using Lipofectamine 2000 (Invitrogen, United States).

CCK-8 assay

A cell counting kit-8 (CCK-8), Japan, was used to assess cell proliferation. In brief, cell suspensions (5 × 10^3 cells/well) were seeded in 96-well plates in triplicate, and the plates were incubated for 48 h. Each well was filled with 10 μL of CCK-8 assay solution and incubated for 4 h. A microplate reader was used to measure the optical density at 450 nm.

Western blot

Immunoblotting was performed using antibodies against the following proteins: Poly ADP-ribose polymerase (PARP); 9352, glucose transporter type 1 (Glut1); 73015, Skp2 (2652), p-signal transducer and activator of transcription 3 (9134p), p-AKT (4060), p-mammalian target of rapamycin (mTOR) (5336), and GAPDH (5174) (all obtained from Cell Signaling Technology, United States).

Molecular docking

The 3D structure of thioridazine was obtained from the PubChem Substance database (https://www.ncbi.nlm.nih.gov/) by minimizing structural energy using the ChemBioDraw 3D module. The crystal structure of Skp2 was retrieved from the RCSB Protein Data Bank (PDB ID: 1fs2) and subsequently modified (dehydration and hydrogenation) using AutoDockTools 1.5.6 21 before being exported in pdbqt format. Following definition of the grid on the active site of the

receptor protein, the docking procedure was executed using AutoDock Vina 1.1.2, and the output score was displayed in kcal/mol. PyMOL 2.3.0 and BIOVIA Discovery Studio were utilized in this process.

Apoptosis assays

For apoptosis assays, the following steps were performed according to the instructions of the annexin V-FITC Apoptosis Detection Kit (Solarbio, China). Cells in each sample were washed, suspended in 100 μL of 1 × binding buffer and stained with 5 μL of FITC-labeled annexin V and 5 μL of PI for 5 min. Apoptotic cells were detected by a flow cytometer (BD, United States) at wavelengths of 488 nm and 630 nm.

Cellular thermal shift assay

HGC-27-R cells were exposed to dimethyl sulfoxide or thioridazine for 24 h, collected, washed with PBS containing protease inhibitors, aliquoted into PCR tubes, and heated in a thermal cycler (Bio-Rad, T100) at the indicated temperature for 3 min to denature proteins. The cells were then resuspended in NP40 buffer, subjected to three freeze-thaw cycles with liquid nitrogen, and centrifuged at 20000 × g for 20 min at 4 ℃. The supernatant was boiled in loading buffer for western blotting.

Glycolysis assay

Cells were seeded in 12-well plates at 5 × 10^5 cells/well. After the cells were treated with different reagents for 48 h, the supernatant was collected. An amplex red glucose/glucose oxidase assay kit (Molecular Probes, Carlsbad, CA, United States) was used for glucose uptake measurements. A lactate assay kit (BioVision, Mountain View, CA, United States) was used to detect the production of lactate in the medium.

Animal studies

The animal procedures were approved by the Henan University Institutional Experimental Animal Care and Use Committee (ID: HUSOM2022-439). All experiments were designed and conducted in accordance with the Animal Research: Reporting of In Vivo Experiments guidelines; the United Kingdom Animals (Scientific Procedures) Act 1986 and associated guidelines; and the European Union (EU) Directive 2010/63/EU for animal experiments. Five-week-old male BALB/c athymic nude mice (weighing 16-18 g, SPF grade) were purchased from Peking Vital River Laboratory Animal Technology Company. Prior to use, all cages, bedding, and drinking water were sterilized. The cages, feed, and drinking water were replaced biweekly. The breeding environment adhered to the following specifications: temperature range from 20 to 26 ℃, humidity range from 40% to 70%, and light cycle consisting of 12 h of illumination followed by 12 h of darkness (lights activated from 8 am to 8 pm). A total of 3 × 10^6 HGC27-R or 1 × 10^6 SGC7901-R cells were injected subcutaneously (s.c.) into each mouse. The mice were randomly grouped into four groups with five mice in each group. The mice received either vehicle control, thioridazine (25 mg/kg), trastuzumab (5 mg/kg), lapatinib (70 mg/kg), and thioridazine (25 mg/kg) plus trastuzumab (5 mg/kg) or lapatinib (70 mg/kg) by intraperitoneal injection daily. After two weeks of drug administration, the mice were sacrificed, and tumor weights were determined.

Statistical analysis

Data were expressed as mean ± SD. Comparisons between two groups were performed using a *t* test. One-way ANOVA with the Bonferroni correction was used to analyze differences among three or more groups. Statistical significance was defined as a value of $P < 0.05$.

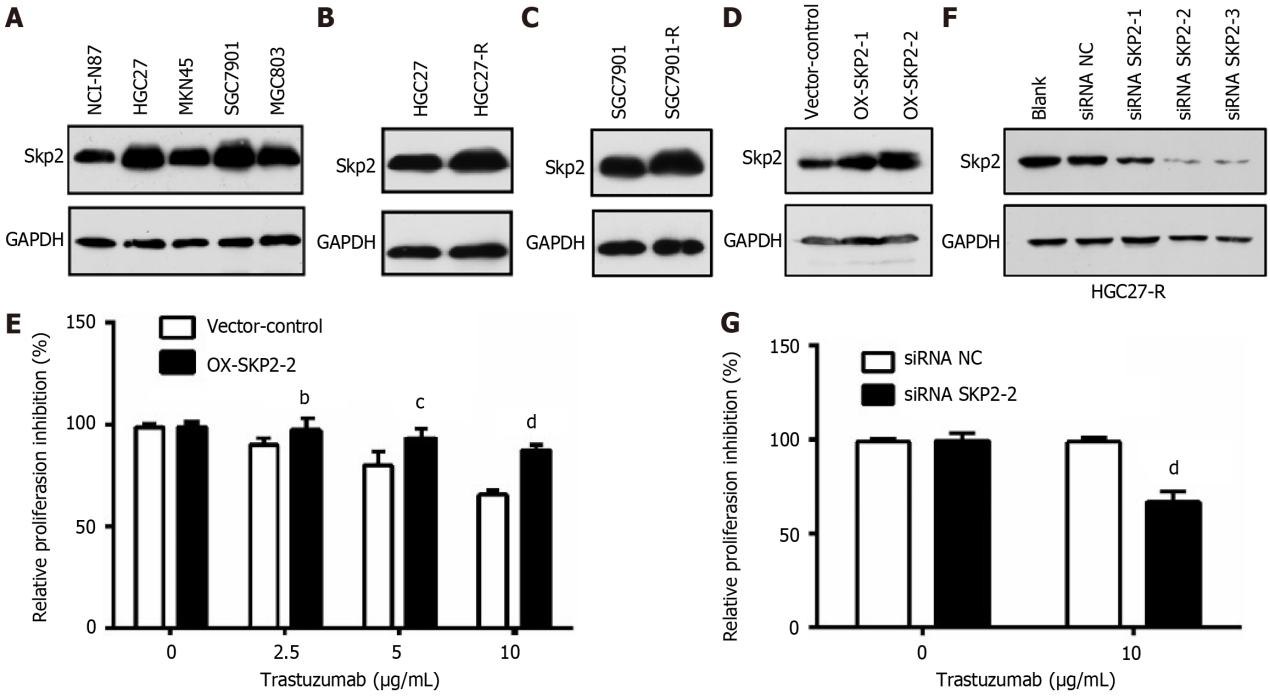
RESULTS

Upregulation of Skp2 expression promotes trastuzumab resistance in HER2-positive GC cells

To ascertain the correlation between Skp2 expression and trastuzumab sensitivity in GC, Skp2 expression was analyzed by immunoblotting in a panel of human GC cell lines with HER2-positive status. Subsequently, the HGC27 and SGC7901 cell lines, exhibiting Skp2 overexpression, were selected for further examination (Figure 1A). Following chronic treatment with 10 μg/mL trastuzumab, two cell lines, HGC27-R and SGC7901-R, were identified as being more resistant to trastuzumab than their parental counterparts. Notably, the Skp2 level was higher in HGC27-R and SGC7901-R cells than in the corresponding parental HGC27 and SGC7901 cells (Figure 1B and C). HGC27 cells with exogenous Skp2 overexpression were employed to investigate the potential decrease in antiproliferative activity associated with upregulated Skp2 expression. The results indicated a decline in trastuzumab activity in isogenic stable Skp2 transfectants (HGC27-OX-SKP2-2 cells) (Figure 1D and E). Additionally, transfection of the SKP2-targeted siRNA effectively downregulated Skp2 expression and significantly enhanced trastuzumab activity in HGC27-R cells (Figure 1F and G). These findings suggest a significant relationship between Skp2 expression and trastuzumab insensitivity, highlighting the importance of Skp2 as a potential therapeutic target in GC.

Thioridazine decreases Skp2 expression in GC

A docking analysis conducted with the drug repurposing compound library revealed that thioridazine exhibits favorable binding potential with Skp2. Thioridazine exhibits a high binding potential (affinity score: -7.0 kcal/mol) for the active pocket of Skp2, as shown in Figure 2A. Specifically, thioridazine engages in van der Waals, pi-alkyl and alkyl interactions with the branched-chain amino acids Leu130, Lys131, Pro132 and Ile205 in Skp2, resulting in a robust interaction between



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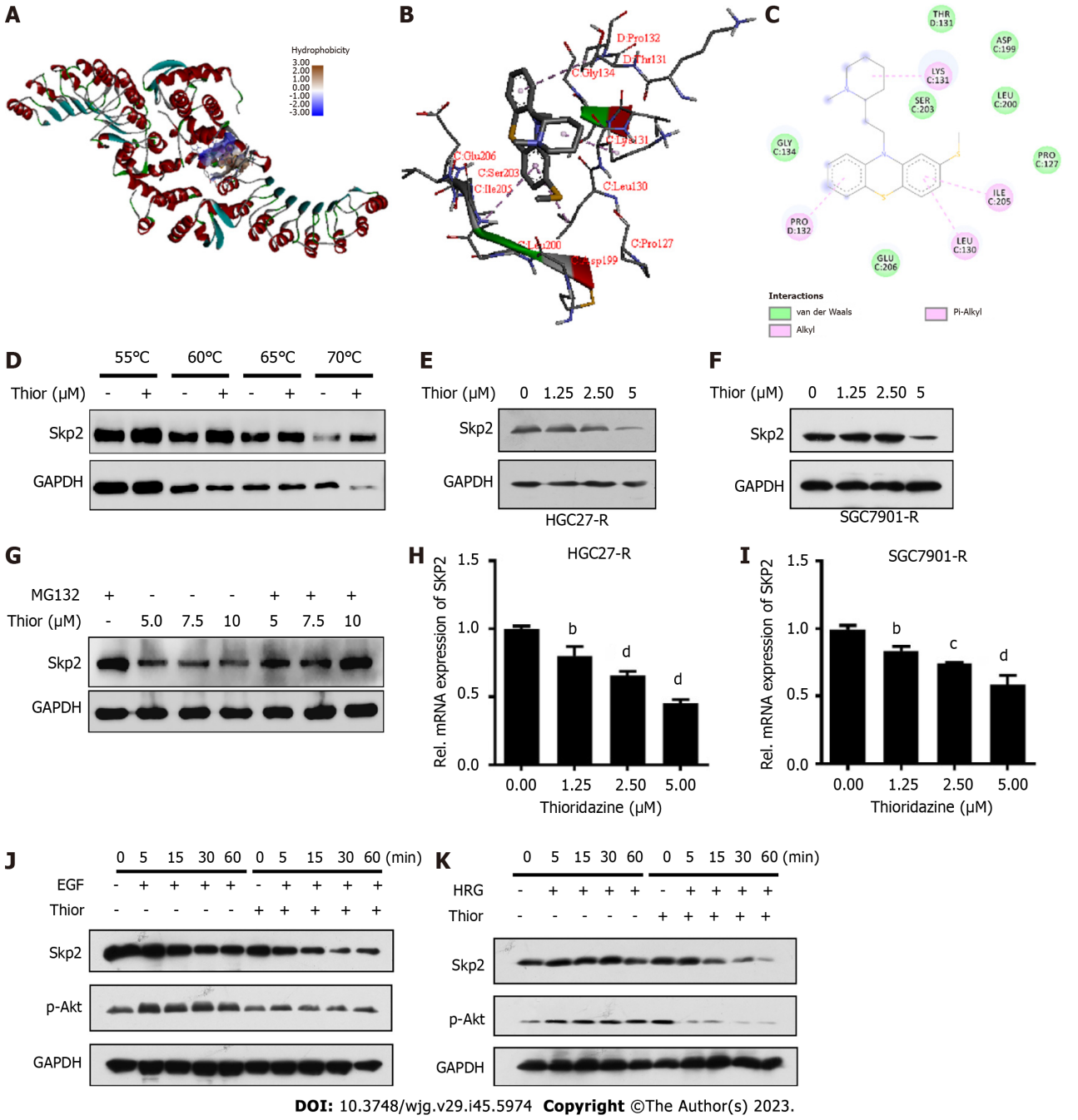
Figure 1 Elevated S-phase kinase associated protein 2 expression contributes to trastuzumab resistance in human epidermal growth factor receptor 2-positive gastric cancer cells. A: Western blotting was used to measure the S-phase kinase associated protein 2 (Skp2) expression level in human gastric cancer (GC) cell lines; B and C: Skp2 expression in HGC27 and HGC27-R cells and SGC7901 and SGC7901-R cells was analyzed by Western blotting; D: Skp2 expression levels in HGC27 cells transfected with empty vector (pcDNA3.1), OX-SKP2-1 or OX-SKP2-2 (pcDNA3.1/SKP2) were determined by western blotting; E: HGC27 cells stably transfected with empty vector or OX-SKP2-2 were cultured with or without trastuzumab (0, 2.5, 5, and 10 μg/mL) for 48 h. Proliferation activity was analyzed by cell counting kit-8 (CCK-8) assays; F: HGC27-R cells were transfected with or without small interfering RNA-negative control (siRNA-NC) or siRNA-SKP2 for 72 h. Skp2 expression was measured by western blotting; G: HGC27 cells transfected with siRNA-NC or siRNA-SKP2-2 were cultured with or without 10 μg/mL trastuzumab. CCK-8 assays were used to analyze proliferation activity. The experiments were performed in triplicate. $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$. Skp2: S-phase kinase associated protein 2; siRNA: Small interfering RNA; NC: Negative control.

the ligand and the Skp2 protein (Figure 2B and C). Cellular thermal shift assay was performed to evaluate the binding affinity of thioridazine for the Skp2 protein. Administration of thioridazine increased the thermal stability of Skp2 but not of GAPDH (Figure 2D), implying a direct binding interaction between thioridazine and the Skp2 protein.

To explore the effect of thioridazine on Skp2 expression, HGC27-R and SGC7901-R cells were treated with varying concentrations of thioridazine (0, 1.25, 2.5, and 5 μM). Western blot analysis revealed a notable decrease in the expression level of Skp2 protein after treatment with thioridazine (Figure 2E and F). MG132 reversed the decrease in Skp2 protein expression induced by thioridazine, thereby indicating that thioridazine may increase the degradation of Skp2 *via* the ubiquitin–proteasome pathway (Figure 2G). Additionally, the mRNA expression level of SKP2 exhibited a dose-dependent decline as the concentration of thioridazine increased, as shown in Figure 2H and I. It has been established that Skp2 plays a role in governing the phosphorylation and activation of Akt in response to ErbB receptor signaling[18], and we found that the phosphorylation of Akt, which is stimulated by EGF and facilitated by Skp2, was abolished after thioridazine treatment (Figure 2J). In a similar vein, our observations also revealed that Skp2 expression and Akt phosphorylation induced by heregulin was effectively inhibited through the administration of thioridazine (Figure 2K). These results suggest that thioridazine can impede the Skp2-mediated Akt phosphorylation and activation induced by ligands of the ErbB2 family.

Thioridazine increases GC cell sensitivity to trastuzumab in vitro and in vivo

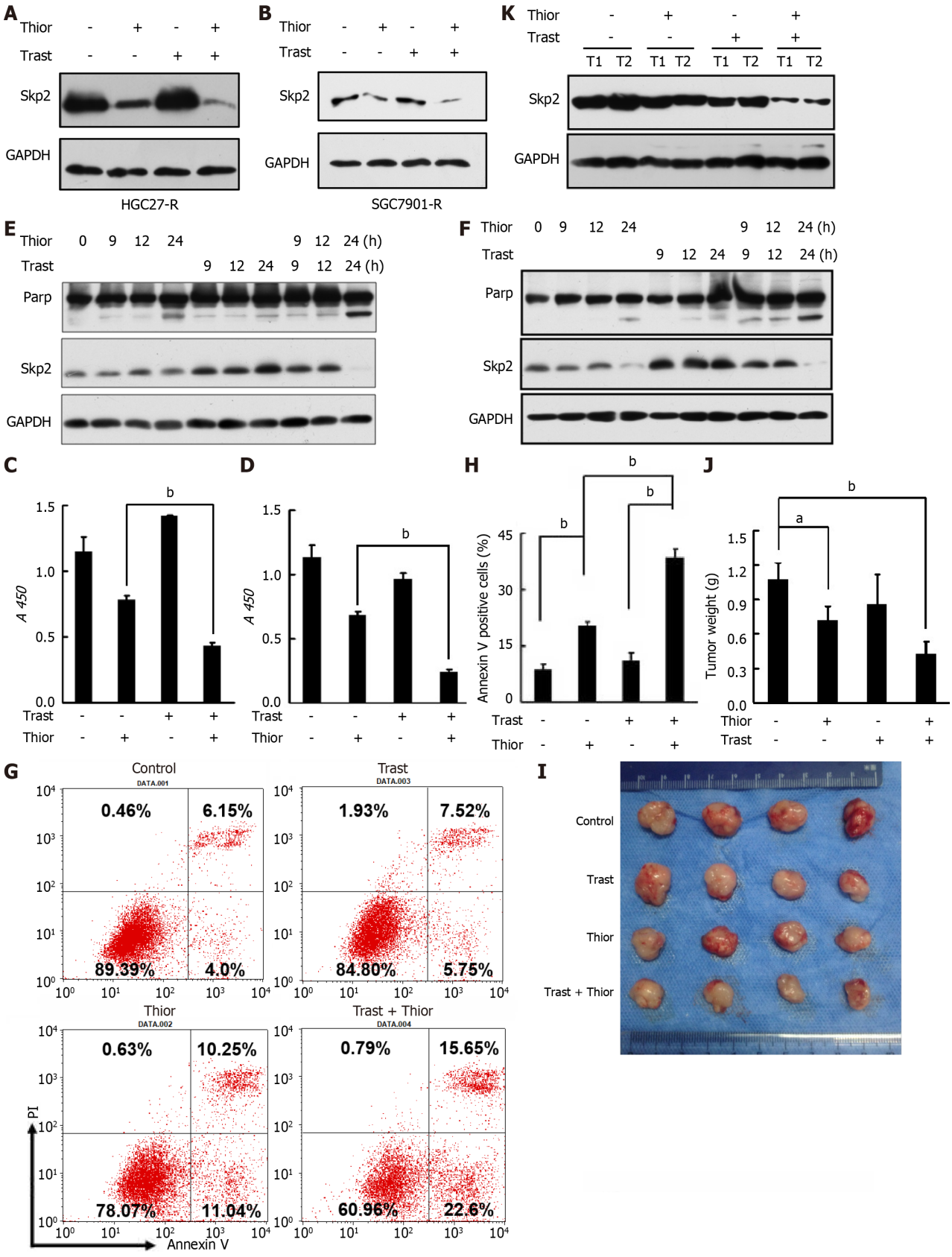
Furthermore, we verified that cotreatment with thioridazine and trastuzumab resulted in a further decrease in Skp2 expression (Figure 3A and B). We next assessed the effect of thioridazine on the proliferation and survival of HGC27-R and SGC7901-R cells. As shown in Figure 3C and D, thioridazine significantly decreased the viability and restored the trastuzumab sensitivity of HGC27-R and SGC7901-R cells. To determine whether thioridazine promotes apoptosis in trastuzumab-resistant cells, we treated HGC27-R and SGC7901-R cells with thioridazine and trastuzumab alone and in combination. PARP is a substrate of caspases. PARP splicing is a key indicator of apoptosis. As shown in Figure 3E and F, trastuzumab induced increased expression of Skp2 but not splicing of PARP. In contrast, combined treatment with thioridazine and trastuzumab increased PARP splicing. The rate of early apoptotic HGC27-R cells treated with thioridazine was 2-fold that of HGC27-R cells treated with trastuzumab, and the difference even increased to 4-fold thioridazine was combined with trastuzumab (Figure 3G and H). These data suggest that thioridazine and trastuzumab synergistically suppress the proliferation and decrease the survival of GC cells.



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Figure 2 Thioridazine can inhibit S-phase kinase associated protein 2 expression and protein kinase B activation triggered by ErbB signaling. A: The interaction between the ligand thioridazine and the active pocket of S-phase kinase associated protein 2 (Skp2) (1FS2) was examined; B: 3D structural schematic showing the binding of thioridazine to Skp2; C: Dihedral angle diagram showing the mode of interaction between thioridazine and Skp2; D: HGC-27-R cells were exposed for 24 h to dimethyl sulfoxide or 30 μM thioridazine and subjected to the Cellular Thermal Shift Assay; E and F: HGC27-R and SGC7901-R cells were treated with different concentrations of thioridazine for 24 h. Western blotting was used to analyze Skp2 expression; G: HGC27 cells were treated with the indicated concentrations for 42 h and were then treated with 5 μM MG132 for 6 h. The protein expression level of Skp2 was analyzed by western blotting; H and I: HGC27-R and SGC7901-R cells were treated with various concentrations of thioridazine for 24 h. The mRNA expression level of SKP2 was analyzed by Q-PCR; J and K: HGC27-R cells were pretreated with or without thioridazine (5 μM) for 2 h, followed by stimulation with epidermal growth factor (100 μg/mL) or heregulin (50 μg/mL). At the indicated times, Skp2 and p-protein kinase B protein levels were assessed. $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$. Thior: Thioridazine; Skp2: S-phase kinase associated protein 2; EGF: Epidermal growth factor; HRG: Heregulin.

To investigate the efficacy of trastuzumab and thioridazine in vivo, we treated mice bearing HGC27-R xenografts with trastuzumab and thioridazine alone or in combination. As monotherapies, trastuzumab and thioridazine showed a limited effect on tumor growth, whereas combined administration of trastuzumab and thioridazine resulted in greater reductions in tumor volume and tumor weight (Figure 3I and J). However, lower expression of Skp2 was found in xenograft tissues of the combined administration group (Figure 3K). These data demonstrate that thioridazine enhances the antitumor activity of trastuzumab *in vitro* as well as *in vivo*.



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Figure 3 Thioridazine increases the primary susceptibility of gastric cancer cells to trastuzumab *in vitro* and *in vivo*. A and B: HGC27-R and SGC7901-R cells were treated with phosphate-buffered saline (PBS), thioridazine (5 μM), trastuzumab (5 μg/mL), or thioridazine (5 μM) plus trastuzumab (5 μg/mL) for 24 h. S-phase kinase associated protein 2 (Skp2) expression was determined by western blotting; C and D: HGC27 and SGC7901 cells were treated with trastuzumab (10 μg/mL) or thioridazine (2.5 μM) alone or in combination for 48 h. Proliferation activity was evaluated by cell counting kit-8 assays; E and F: HGC27 and SGC7901 cells were treated with trastuzumab (10 μg/mL) or thioridazine (2.5 μM) alone or in combination for various times. The expression of Skp2 and Parp

was analyzed by western blotting; G and H: HGC27 cells were treated with trastuzumab (10 μg/mL) or thioridazine (2.5 μM) alone or in combination for 24 h, followed by an apoptosis assay, quantitative results; I-K: Nude mice were injected s.c. with 0.1 mL of an HGC27 cell suspension (3×10^7 cells/mL) in the right upper flank. The mice were treated with PBS, trastuzumab (0.5 mg/kg), thioridazine (25 mg/kg), or trastuzumab (0.5 mg/kg) plus thioridazine (25 mg/kg) daily by intraperitoneal (i.p.) injection beginning 7 d after cell implantation. After two weeks of drug administration, the mice were sacrificed. Primary tumors were excised, photographed, and weighed. Western blot analysis was used to assess Skp2 expression in primary tumors. $^aP < 0.05$; $^bP < 0.01$. Thior: Thioridazine; Trast: Trastuzumab.

Thioridazine combined with lapatinib more effectively suppressed glycolysis mediated by Skp2

Trastuzumab-resistant advanced breast cancer responds to lapatinib, a dual tyrosine kinase inhibitor (TKI), according to the results of the phase III trial EGF104900 and the HER2CLIMB Randomized Clinical Trial[30,31]. However, the addition of lapatinib to a regimen of capecitabine and oxaliplatin did not improve overall survival (OS) in patients with HER2-amplified gastroesophageal adenocarcinoma[32]. In addition, lapatinib plus paclitaxel demonstrated activity in the second-line treatment of patients with HER2 FISH-positive IHC3+ advanced GC but did not significantly improve OS in the intent-to-treat population[33]. Here, we explored the effect of combination treatment with lapatinib and thioridazine on the glycolytic phenotype in GC cells. The combination of thioridazine and lapatinib completely abolished Skp2 expression (Figure 4A-C). It has been reported that Skp2 regulates glycolysis by inducing Glut1 expression and Akt/mTOR pathway activation[34,35]. The decreased protein levels of p-Akt, p-mTOR, and Glut1 were consistent with the downregulation of Skp2 (Figure 4A-C). Furthermore, both glucose uptake and lactate production were decreased more apparently in HGC27-R and SGC7901-R cells treated with thioridazine and lapatinib together than in those treated with either alone (Figure 4D-G). Compared to siRNA-NC-transfected cells, HGC27 cells transfected with SKP2-2 siRNA did not show inhibitory effects of thioridazine on glucose uptake or lactate production (Figure 4H and I). These results indicate that in combination, thioridazine and lapatinib can markedly suppress glycolysis by downregulating Skp2 in trastuzumab-resistant GC cells.

Thioridazine improves the inhibitory effect of lapatinib on GC cells

We further examined the anticancer effect of thioridazine combined with lapatinib. As shown in Figure 5A-C, thioridazine significantly enhanced the anti-proliferative activity of lapatinib in GC cells (HGC27-R, SGC-7901, and NCI-N87-R). Thioridazine (5 μM) in combination with lapatinib (1 or 2 μM) markedly increased apoptosis in HGC27-R and SGC7901-R cells (Figure 5D-F).

Moreover, Figure 6A-C shows that combined administration of thioridazine and lapatinib strongly decreased the growth, volume, and weight of tumors in mice bearing SGC7901-R xenografts. No toxic or side effects were observed during the administration period, and the weight of the mice did not significantly decrease (Figure 6D). Lower expression of Skp2 was found in the combined thioridazine and lapatinib treatment group than in the other groups (Figure 6E). The protein levels of Skp2, p-Akt, and Glut1 were greatly decreased in xenograft tissues from mice treated with both thioridazine and lapatinib (Figure 6F). These data demonstrate that thioridazine enhances the antitumor activity of lapatinib by inhibiting Skp2 expression *in vitro* and *in vivo*.

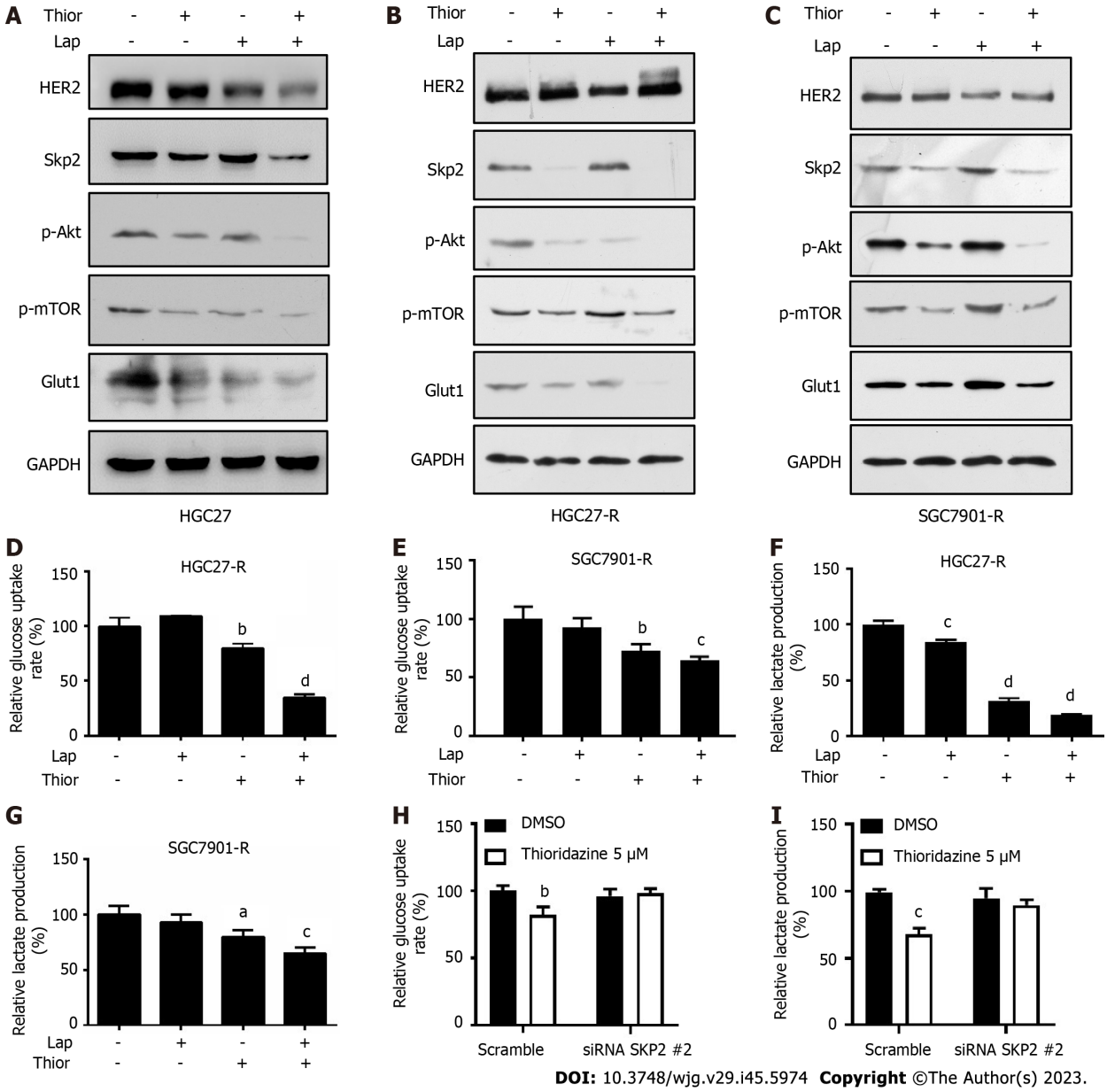
Our study demonstrates that thioridazine can overcome trastuzumab resistance by blocking glycolysis, growth, and apoptosis resistance by downregulating the expression of Skp2 in GC cells.

DISCUSSION

It has been confirmed that HER-2 has the most outstanding clinical significance in advanced GC. However, targeting HER-2 in advanced GC remains challenging due to the high heterogeneity and subsequent resistance caused by prolonged therapy. Despite the development of numerous HER2-targeted drugs, including antibody-drug conjugates, TKIs, bispecific antibodies, vaccines, and immune checkpoint inhibitors, to combat trastuzumab resistance in HER2-positive breast cancer, the efficacy of these treatments in HER2-positive GC remains uncertain.

Trastuzumab combined with palbociclib, a CDK4/6 inhibitor, was demonstrated to yield favorable survival outcomes in patients with advanced breast cancer[36,37]. Multiple studies have demonstrated that the simultaneous administration of supplementary inhibitors, including figitumumab (an insulin-like growth factor 1 receptor inhibitor), ipatasertib (an AKT inhibitor), and MK2206 (another AKT inhibitor), in individuals with HER2-overexpressing tumors increases the efficacy of trastuzumab[38-40]. However, additional extensive research is needed for clinical incorporation of these agents. In contrast to the aforementioned drugs currently under development, the expedited introduction of approved drugs for new therapeutic applications could be facilitated in the clinical market. For example, the potential of combining trastuzumab with metformin as an innovative adjuvant therapy for HER-2-positive breast cancer is being investigated in an ongoing phase II clinical trial[41]. Our findings in the current study highlight the ability of thioridazine to augment the effect of trastuzumab in GC.

The antipsychotic drug thioridazine was first discovered as a phenothiazine-type piperidine drug. In 2013, Sachlos *et al*[42] first discovered that thioridazine can induce the differentiation of acute myeloid leukemia cells and breast cancer stem cells and increase sensitivity to doxorubicin without affecting the function of normal hematopoietic stem cells. It has been shown that thioridazine can inhibit the expression of a multidrug resistance protein (P-gp) and increase sensitivity to chemotherapy drugs in glioblastoma[43]. In addition to inducing reactive oxygen species accumulation and DNA damage, thioridazine can increase autophagy and apoptosis in ovarian cancer cells[44]. It reduces ovarian cancer angiogenesis by inhibiting vascular endothelial growth factor receptor-2, PI3K, and mTOR signaling[45]. Recent studies

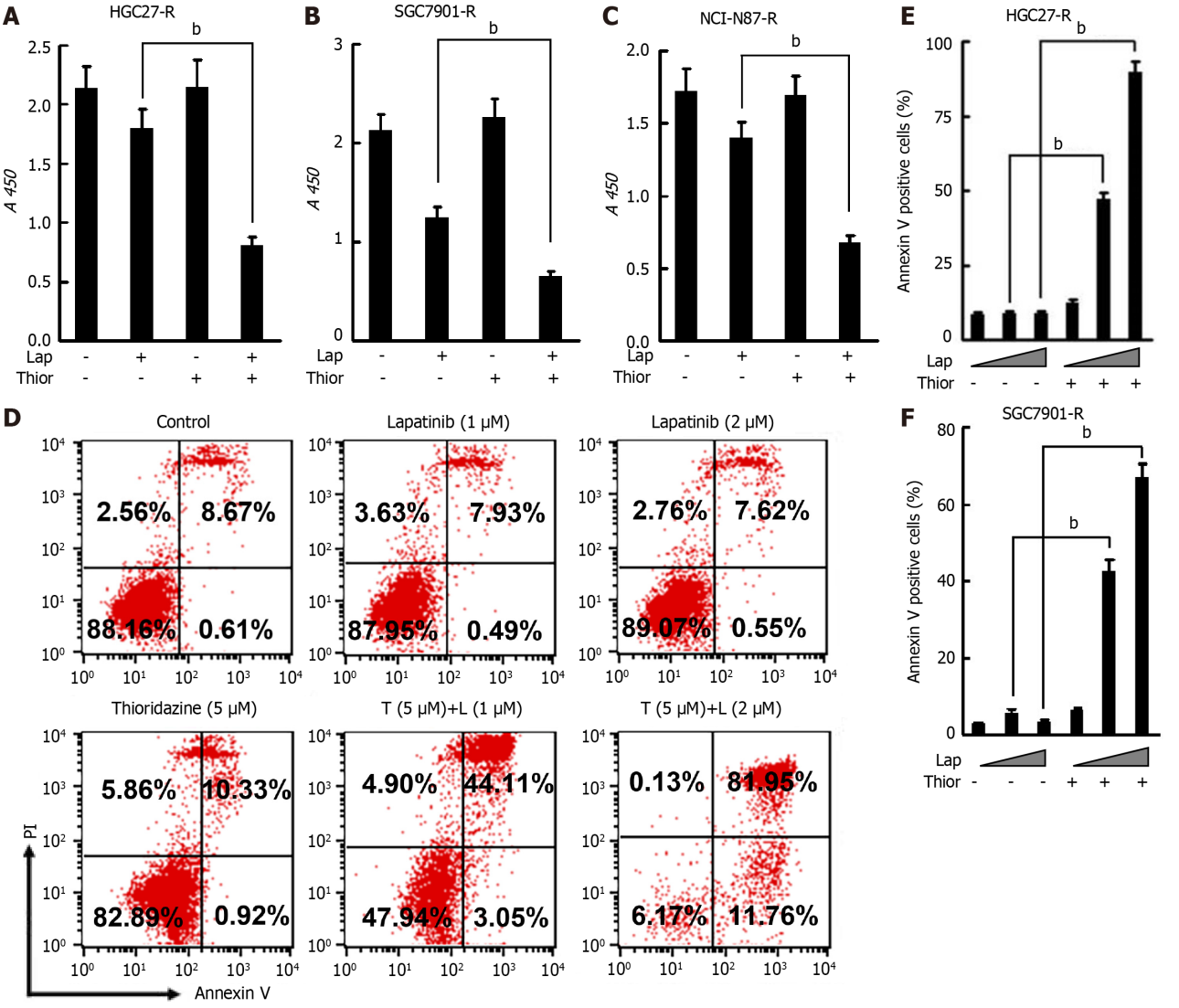


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Figure 4 Thioridazine combined with lapatinib decreases S-phase kinase associated protein 2/p-protein kinase B/p-mammalian target of rapamycin/glucose transporter type 1 protein levels and glycolysis in gastric cancer cells. A-C: HGC27, HGC27-R and SGC7901-R cells were treated with thioridazine (5 μM) alone or in combination with lapatinib (5 μM) for 24 h. S-phase kinase associated protein 2 (Skp2), p-protein kinase B (Akt), Akt, p-mammalian target of rapamycin, and glucose transporter type 1 protein levels were analyzed by western blotting; D-G: HGC27-R cells were treated with thioridazine (5 μM) and lapatinib (5 μM) alone or in combination for 24 h. The glucose uptake rate and lactate production rate were measured following the manufacturer's instructions; H and I: HGC27-R cells were divided into two groups that were transfected with small interfering RNA (siRNA) negative control or siRNA SKP2 2 for 48 h. Each group was treated without or with thioridazine (5 μM) for another 24 h. The glucose uptake rate and lactate production rate in all groups were measured. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$. Thior: Thioridazine; Lap: Lapatinib.

have demonstrated that thiolidazine exhibits the potential to augment the susceptibility of glioblastoma cells towards temozolomide through the inhibition of autophagy[46]. Furthermore, the concurrent administration of thiazidine and oxaliplatin has been found to stimulate immunogenic cell death in colon cancer by inducing endoplasmic reticulum stress [47]. In this study, we first revealed that thioridazine can inhibit glycolysis in GC by downregulating Skp2 expression at both the transcriptional and translational levels. However, further evidence is needed to determine whether the main regulatory mechanism by which thioridazine regulates the expression and function of Skp2 is mediated through transcriptional inhibition, posttranslational inhibition, or blockade by protein interactions. The effectiveness of combining thioridazine with other HER-2- or non-HER-2-targeted drugs, such as neratinib, tucatinib, and apatinib, in treating trastuzumab-resistant GC remains uncertain.

Based on our findings, Skp2 may serve as a predictor of the trastuzumab response in patients with HER-2-positive GC, thereby aiding in identifying the patient subgroups most likely to benefit from HER-2-targeted therapies. Additionally, our study underscores the advantageous impacts of thioridazine when used in combination with synergistic drugs for the management of GC. These findings offer valuable support for future clinical investigations aimed at exploring the



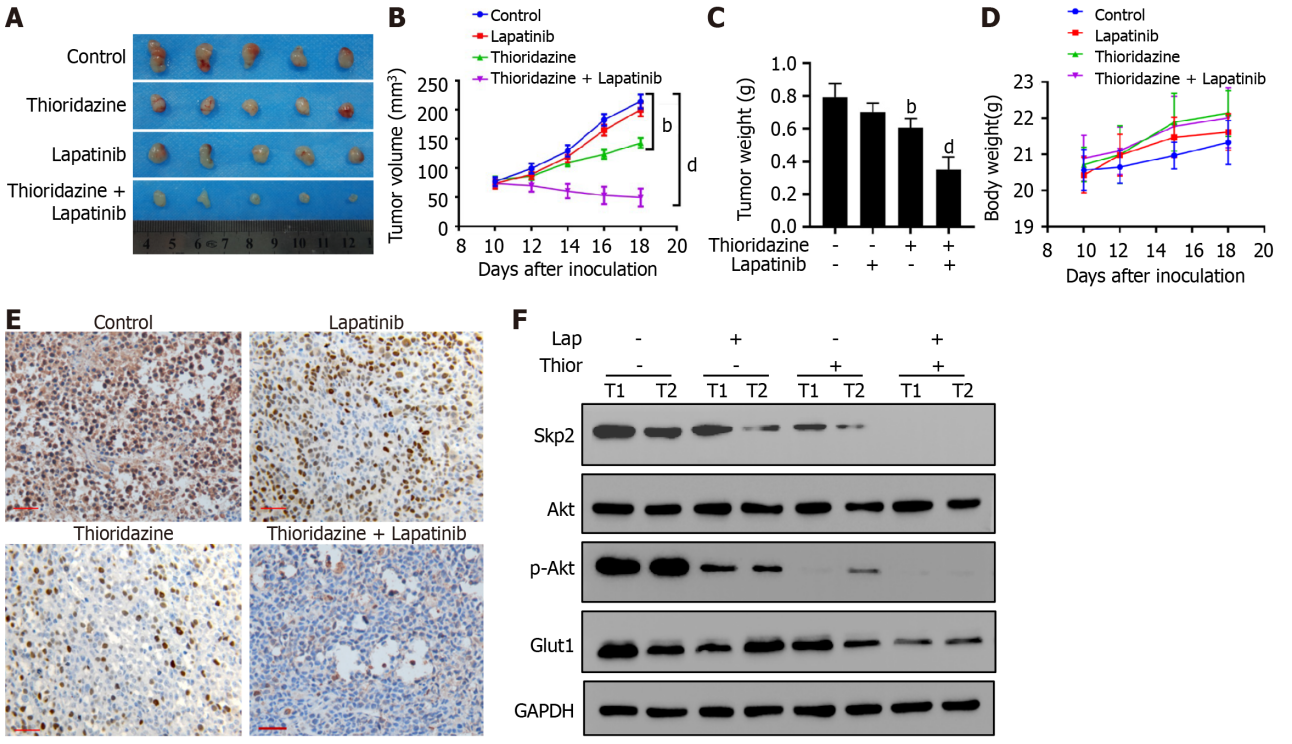
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Figure 5 The combination of thioridazine and lapatinib exhibits more pronounced antitumor activity than either alone in vitro against trastuzumab-resistant gastric cancercells. A-C: To HGC27-R, SGC7901-R, and NCI-N87-R cells were treated with trastuzumab (10 μg/mL) or thioridazine (5 μM) alone or in combination for 48 h. Proliferation activity was evaluated by cell counting kit-8 assays; D-F: HGC27-R cells were treated with lapatinib (1 μM or 2 μM) in the presence or absence of thioridazine (5 μM) for 24 h, followed by an apoptosis assay. Similar experiments were performed and results were quantified in SGC7901-R cells. The experiments were repeated three times. $^{b}P < 0.01$. Thior: Thioridazine; Lap: Lapatinib.

potential of thioridazine as a viable treatment option for GC. Notably, HER-2-targeted drugs possess dosage-independent potential for cardiac toxicity. In addition, thioridazine has been reported to be associated with arrhythmias in a minority of schizophrenia patients. Consequently, when considering the administration of these drugs individually or in combination, it becomes imperative to exclude individuals with preexisting heart conditions and implement vigilant cardiac surveillance.

CONCLUSION

In conclusion, our data demonstrate that thioridazine combined with lapatinib exhibits synergistic effects in impeding cell proliferation, inducing apoptosis, and suppressing tumor growth in GC. This study provides an experimental foundation for the potential utilization of thioridazine in overcoming trastuzumab resistance in GC. The objective of this study is to offer potential drug selection and administration strategies for patients with advanced GC who exhibit resistance to trastuzumab. Furthermore, these findings offer novel insights for the future investigation of Skp2 inhibitors.



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Figure 6 Coadministration of thioridazine and lapatinib demonstrates potent anticancer activity in mice harboring gastric cancer xenografts. A: SGC7901-R cells (1 × 10^6) were injected s.c. into the flanks of nude mice. Ten days post-implantation, the mice were randomized into four groups of five mice each. Then, the mice were treated by i.p. injection with phosphate-buffered saline, lapatinib (70 mg/kg), thioridazine (25 mg/kg), or lapatinib (70 mg/kg) plus thioridazine (25 mg/kg) for 14 d. Gross morphology of the final excised xenograft tumor masses; B: Tumor volumes following treatments were recorded; C: Wet tumor weights; D: Body weights of mice; E: S-phase kinase associated protein 2 (Skp2) expression was evaluated by immunohistochemistry in primary tumors (scale bar = 100 μm); F: The protein levels of Skp2, protein kinase B (Akt), p-Akt, glucose transporter type 1, and GAPDH in two primary tumors from each group were measured by western blotting. $^bP < 0.01$, $^dP < 0.0001$. Thior: Thioridazine; Lap: Lapatinib.

ARTICLE HIGHLIGHTS

Research background

The drug resistance observed in patients with human epidermal growth factor receptor 2 (HER-2)-positive advanced gastric cancer (GC) treated with trastuzumab is a significant concern, as no established targeted therapy regimen for use after the development of drug resistance is available. S-phase kinase associated protein 2 (Skp2) has been identified as a crucial target for GC treatment; however, the development of new drugs targeting Skp2 remains a considerable challenge.

Research motivation

To investigate potential pharmacological interventions targeting Skp2 to increase the efficacy of subsequent therapies for patients with HER-2-positive GC who have developed resistance to trastuzumab.

Research objectives

This study aims to elucidate the inhibitory effect of thioridazine on Skp2 expression and to preliminarily assess the potential of thioridazine in reversing the resistance of HER2-positive GC cells to trastuzumab through both *in vivo* and *in vitro* experiments.

Research methods

The impact of altering the Skp2 protein expression level through overexpression or knockdown on the sensitivity of HER2-positive GC cells to trastuzumab was assessed using a cell counting kit-8 assay. The influence of thioridazine on Skp2 protein expression was demonstrated through computational docking analysis and Cellular Thermal Shift Assay. Flow cytometry, a glucose uptake assay, a lactate production assay, and xenograft experiments in nude mice were employed to evaluate the effects of thioridazine alone or in combination with trastuzumab and lapatinib on the cell cycle, apoptosis, glucose metabolism, and tumor growth.

Research results

Trastuzumab sensitivity can be increased in HER-2-positive GC cells through negative modulation of Skp2 expression. Thioridazine can selectively inhibit Skp2 expression and the protein kinase B/mammalian target of rapamycin signaling

pathway. Thioridazine combined with lapatinib effectively reverses trastuzumab resistance in GC cells by diminishing glycolysis.

Research conclusions

Combining thioridazine with lapatinib is a potential strategy to reverse trastuzumab resistance in GC by suppressing Skp2 expression.

Research perspectives

Further investigation into the optimal combination ratio, initial dosage, and dose-response correlation between thioridazine and lapatinib in GC xenograft models will contribute to the development of more precise drug reference protocols for subsequent clinical trials. A new therapeutic strategy for the management of GC by simultaneous targeting of Skp2 and HER-2 could be introduced.

FOOTNOTES

Co-first authors: Zheng-Yan Yang and Yi-Wei Zhao.

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Author contributions: Yang ZY drafted the manuscript and conducted the experiments of immunoblotting, qPCR, and apoptosis analysis in this study; Zhao YW completed the glucose metabolism analysis and animal experiments; Xue JR contributed to the CCK-8 assay and animal experiments; Guo R assisted in the construction and amplification of recombinant plasmid vectors; Liu HD participated in the data collection of docking; Zhao Z assisted in immunohistochemical analysis of xenograft tumor tissue samples; Ren ZG supervised the experiments, corrected the data, and revised the manuscript; Shi M conceived and designed the main content of this study and was responsible for analyzing the research results; all authors contributed to the article and approved the submitted version.

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ORIGINAL ARTICLE

Basic Study

Pien Tze Huang alleviates Concanavalin A-induced autoimmune hepatitis by regulating intestinal microbiota and memory regulatory T cells

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Abstract

BACKGROUND

Traditional Chinese medicine has used the drug Pien Tze Huang (PTH), a classic prescription, to treat autoimmune hepatitis (AIH). However, the precise mode of action is still unknown.

AIM

To investigate the mechanism of PTH in an AIH mouse model by determining the changes in gut microbiota structure and memory regulatory T (mTreg) cells functional levels.

METHODS

Following induction of the AIH mouse model induced by Concanavalin A (Con A), prophylactic administration of PTH was given for 10 d. The levels of mTreg cells were measured by flow cytometry, and intestinal microbiota was analyzed by 16S rRNA analysis, while western blotting was used to identify activation of

the toll-like receptor (TLR)2, TLR4/nuclear factor-κB (NF-κB), and CXCL16/CXCR6 signaling pathways.

RESULTS

In the liver of mice with AIH, PTH relieved the pathological damage and reduced the numbers of T helper type 17 cells and interferon-γ, tumor necrosis factor-alpha, interleukin (IL)-1β, IL-2, IL-6, and IL-21 expression. Simultaneously, PTH stimulated the abundance of helpful bacteria, promoted activation of the TLR2 signal, which may enhance Treg/mTreg cells quantity to produce IL-10, and suppressed activation of the TLR4/NF-κB and CXCL16/CXCR6 signaling pathways.

CONCLUSION

PTH regulates intestinal microbiota balance and restores mTreg cells to alleviate experimental AIH, which is closely related to the TLR/CXCL16/CXCR6/NF-κB signaling pathway.

Key Words: Pien Tze Huang; Autoimmune hepatitis; Intestinal microbiota; Memory regulatory T cell; Toll-like receptor signaling

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Core Tip: Intestinal microbiota disorder plays an important role in the pathogenesis of autoimmune hepatitis (AIH), in which one of the main characteristics is the hypofunction of memory regulatory T (mTreg) cells to maintain immune tolerance. The interaction between Treg cells and intestinal microbiota may provide a feasible therapeutic strategy for AIH. As a well-known traditional Chinese medicine, Pien Tze Huang (PTH) can effectively treat AIH in the clinic. However, its mechanism is still unclear. In the present study, PTH regulated intestinal microbiota balance and restored mTreg cells to alleviate experimental AIH, which was closely related to the toll-like receptor/CXCL16/CXCR6/nuclear factor-κB signaling pathway.

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INTRODUCTION

Autoimmune hepatitis (AIH) is an interfacial hepatitis characterized by lymphocyte infiltration and the production of autoantibodies; however, its etiology is unknown. Some studies have suggested that AIH is associated with abnormal intestinal permeability[1]. In the case of increased intestinal permeability or intestinal leakage, abnormal intestinal microbiota can enter the submucosa to activate immune cells and produce inflammatory factors or metabolites, which can invade the liver through the portal vein and cause liver injury. Among the many mechanisms that the immune system uses to recognize microbiota are the toll-like receptors (TLRs), which directly promote regulatory T (Treg) cell proliferation and interleukin (IL)-10 production, thereby preventing imiquimod's ability to induce psoriasis-like symptoms on the skin[2]. Moreover, in the peripheral blood of patients with AIH, some studies have discovered a reduced frequency of Treg cells, essential agents which maintain immune homeostasis, indicating that a deficiency in Treg cell quantity and abnormal Treg cell function may play a vital role in the pathogenesis of AIH[3].

The treatment for AIH is usually high doses of a steroid, which can suppress the immune system. Recent studies have suggested using intestinal microbiota as a potential strategy to treat AIH[4,5]. In their study it was shown that compound probiotics may regulate intestinal microbiota and intestinal permeability, reduce the level of T helper type 1 (Th1) and Th17 cells, improve the level of Treg cells, suppress activation of the TLR4/nuclear factor-κB (NF-κB) pathway, and promote the emergence of AIH[6]. Therefore, for patients with AIH, the interaction between Treg cells and intestinal microbiota may provide a feasible therapeutic strategy. However, the activation degree of effector Treg cells is closely related to memory Treg (mTreg) cells[7], suggesting that mTreg cells could quickly transform into effector Treg cells, playing a crucial part in preserving immunological tolerance.

As a well-known traditional Chinese medicine, Pien Tze Huang (PTH) contains Moschus, Bovis calculus, Snake bile, Notoginseng radix et rhizoma, and other ingredients[8,9] and is regularly used to treat liver conditions, including AIH [10-14]. PTH controls the NF-κB signaling pathway and the NLRP3 inflammasome to reduce IL-1, IL-6, and IL-17 production in blood and to reduce joint inflammation in mice with collagen-induced arthritis[10]. However, the definite mechanism of PTH is not fully understood. PTH has a physiological basis for controlling Treg cells and intestinal microbiota. Bile acid, the active component of Bovis calculus and Snake bile, and notoginsenoside R1, is a potent saponin that is extracted from Panax notoginseng. The immune system's signals to the microbiota may be mediated by bile acids [15]. Song *et al*[16] reported that dietary adjustment and flora transplantation could improve a significant population of colonic Foxp3$^+$ Treg cells with highly-expressed RORγ, and optimize the constituents of the gut bile acids pool. Moreover,

Gong *et al*[17] found that notoginsenoside R1 modulates NF-κB and MAPK signaling to exert anti-inflammatory effects to reduce carbon tetrachloride-induced liver fibrosis. Based on the above evidence, we hypothesized that PTH could treat intestinal microbiota imbalance and a lack of mTreg cells. Thus, this research aimed to determine the mechanism of PTH in the AIH mouse model by observing the changes in intestinal microbiota structure and mTreg functional levels.

MATERIALS AND METHODS

Mice

Forty male specific pathogen-free C57BL/6J mice (aged 8-9 wk; 20-22 g) were bought from GemPharmatech Co., Ltd. (Nanjing, China) (Animal Certificate Number: SCXK (Su)2018-0008). The mice were housed at a temperature of 22 ± 1 °C, relative humidity of 50% ± 10%, and a light/dark cycle of 12/12 h. All animal experiments (including euthanasia of mice) were performed in accordance with the Institutional Animal Care and Use Committee's guidelines (Nanchang, China). The experimental protocols (Permit Number: JZLLSC20210090) were approved by The Jiangxi University of Chinese Medicine's Animal Care and Use Committee.

Materials and reagents

Concanavalin A was bought from Sigma-Aldrich Trading Co., Ltd. (CA, United States, No. C2010-250MG). Dexamethasone (DXM) was obtained from Henan Runhong Pharmaceutical Co., Ltd. (Henan, China, No. 2103111). PTH was acquired from Zhangzhou PTH Pharmaceutical Co., Ltd. (Zhangzhou, China, No. 2108123). As mentioned in previous studies by other researchers, the online pressurized liquid extraction-ultra-high-performance liquid chromatography-ion trap-time-of-flight mass spectrometry (online PLE-UHPLC-IT-TOF-MS) method was used for rapid and comprehensive analysis of the components of PTH. PTH produced a total of 73 signals, of which 71 components could only be inferred from the data. Bovis calculus and Snake bile worked together in a synergistic way to contribute to the occurrence of the remaining 11 components, which included 36 from Notoginseng radix et rhizoma, 15 from Snake bile, and 9 from Bovis calculus (Supplementary Table 1)[18,19].

Induction and treatment of experimental AIH

In order to determine the optimal dose of PTH in treating AIH, an animal experiment was carried out on the treatment of AIH with high, middle, and low doses of PTH.

According to earlier research[20,21], the mice were randomly allocated into 6 groups (10 mice in each group): Control group (control), Concanavalin A (Con A) group (model), Con A + low-dose PTH group (PTH-L), Con A + middle-dose PTH group (PTH-M), Con A + high-dose PTH group (PTH-H), and Con A + DXM group (DXM). Mice in the Con A + PTH-L, Con A + PTH-M, and Con A + PTH-H groups received PTH (117 mg/kg, 234 mg/kg, and 468 mg/kg) every day for 10 d by gavage, respectively, while mice in the normal group and Con A group received the same amount of normal drinking water. The DXM group was intraperitoneally injected with DXM (3 mg/kg) every day. On the 10th d, the AIH mouse model was established, three hours after the last administration of PTH, the mice in the normal control group were injected with 0.2 mL of 0.9% sodium chloride solution through the tail vein, and mice in the other 5 groups were injected with 15 mg/kg Con A through the tail vein. At 8 h after Con A injection, fresh blood was collected from the mice after deep anesthesia with 2% sodium pentobarbital (20 mg/kg i.p.). The abdominal cavity was then quickly opened to collect mouse liver, spleen and colon tissues.

Mice in the Con A + PTH-M group received PTH (234 mg/kg) every day for 10 days by gavage, while mice in the control group and Con A group received the same amount of normal drinking water. The DXM group was intraperitoneally injected with DXM (3 mg/kg) every day. On the 10th day, the AIH mouse model was established as originally stated; three hours after the last administration of PTH, 0.2 mL of a 0.9% sodium chloride solution was injected into the tail vein of the mice in the normal control group, and the mice in the other three groups received an intravenous injection of 15 mg/kg Con A into the tail vein. At 8 h after Con A injection, fresh blood was collected from the mice after deep anesthesia with 2% sodium pentobarbital (20 mg/kg i.p.). The abdominal cavity was swiftly opened to collect mouse colon, spleen, and liver tissues.

Serum measurements

Mouse peripheral blood was centrifuged at 1123 g for 15 min to extract the serum. An automated chemistry analyzer was used to measure the concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin (ALB), globulin (GLB), total protein (TP), indirect bilirubin (IBIL), triglyceride (TG), and high-density lipoprotein (HDL) IBIL in mice (AU480, Beckman Coulter, Pasadena, CA, United States).

Pathological observation

After one week of fixing in 4% buffered paraformaldehyde, the sample was dehydrated in a 50% to 100% alcohol gradient, made completely transparent in dimethyl benzene, and embedded in paraffin. Following deparaffinization and rehydration, the samples were cut into sections 4 μm thick and stained with hematoxylin-eosin (Solarbio, Beijing, China). Thereafter, the slices were randomly and double-blindly observed, and scored by pathologists using a biomicroscope (Lecia, Wetzlar, Germany). Using the Ishak scoring system[22] shown in Supplementary Table 2, liver damage was graded with regard to portal and intralobular inflammation, degeneration, and necrosis. The degree of pathology was graded from 0 (no pathology) to 6 (severe pathology).

Immunofluorescence staining of colon tissue

Immunofluorescence of intestinal mucosae was carried out to evaluate occludin expression and distribution. The tissues were quickly deparaffinized, rehydrated, and cleaned in 1% phosphate buffered saline. The samples were then blocked with 5% bovine serum albumin, and subsequently incubated with occludin (1:100, Abcam, MA, United States) at 4 °C overnight. After three washes, at room temperature, the samples were incubated with anti-FITC IgG secondary antibody for two hours. Finally, the slides were then counterstained with DAPI for 5 min. Images were captured using a fluorescent microscope (Leika, Wetzlar, Germany).

Enzyme-linked immunosorbent assay

Mouse liver tissues were removed and cut into pieces in lysis buffer at the proportion of 1:10, homogenized in ice water using an electric homogenizer, incubated at 4 °C for 30 min, and the supernatants were then removed by centrifugation at 15493 *g* for 15 min. The expression levels of IL-4 (No. MM-0165M1), IL-17A (No. MM-0170M1), interferon (IFN)-γ (No. MM-0182M1), IL-1β (No. MM-0040M1), IL-2 (No. MM-0701M1), IL-6 (No. MM-0163M1), IL-10 (No. MM-0176M1, MM-0176M1), IL-21 (No. MM-0688M1) and tumor necrosis factor-alpha (TNF-α) (No. MM-0132M1) were detected by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Jiangsu Meimian Industry Co., Ltd., Jiangsu, China). According to the instructions the absorbance was measured at 450 nm by a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, United States).

Flow cytometry

Mouse spleen was ground in a sterile culture dish and filtered through nylon mesh to prepare a single cell suspension. RPMI 1640 tissue medium was used to suspend the splenocytes, 10% heat-inactivated fetal bovine serum, 50 units per milliliter of penicillin, and 50 nanograms per milliliter of streptomycin were added, and cultivated for three hours at 37 °C with Cell Stimulation Cocktail after erythrocyte lysis (BD Biosciences, Franklin Lakes, NJ, United States). Extracellular antigens were stained for 20 min at 4 °C in RPMI 1640 medium following FcγR blockage with anti-CD16/32 antibodies (BD Biosciences, Franklin Lakes, NJ, United States). Foxp3/Transcription Factor Buffer Set (BD Biosciences, Franklin Lakes, NJ, United States) was used to fix and permeabilize the cells before staining for intracellular cytokines. CellQuest (BD Biosciences, CA, United States), and a BD FACSCalibur flow cytometer were used to collect the samples, and FlowJo software (BD Biosciences) was used to analyze the data. Cell populations were identified by staining with APC-H7 rat anti-mouse CD4 (1:200, BD Biosciences, NJ, United States), PE rat anti-mouse IL-17A (1:100, BD Biosciences, NJ, United States) FITC-CD25 (1:100, BD Biosciences, NJ, United States), BV421 rat anti-mouse Foxp3 (1:100, BD Biosciences, NJ, United States), APC rat anti-mouse programmed cell death ligand 1 (PD-L1)+(1:100, BD Biosciences, NJ, United States), Alexa Fluor 647 rat anti-mouse CCR7 (1:100, BD Biosciences, NJ, United States), and BV510 rat anti-mouse CD45RA (1:100, BD Biosciences, NJ, United States).

Western blot

According to the instructions above, the liver tissue supernatant utilized for western blotting was produced (see the ELISA section). The traditional BCA protein determination method was used to measure the protein concentration (Cwbiotech, Beijing, China). Sodium-dodecyl sulfate gel electrophoresis was used to separate the same amount of protein by 10%-15% before being transferred to polyvinylidene fluoride membranes. After being sealed with 5% skimmed milk, the membranes were incubated with primary antibodies at 4 °C overnight. The antibodies were anti-GAPDH (1:10000, Proteintech, Wuhan, China), Tubulin (1:1000, Abclonal, Wuhan, China), TLR2 (1:1000, Abcam, MA, United States), TLR4 (1:1000, Abcam, MA, United States), CXCR6 (1:1000, Boster, Wuhan, China), NF-κB p65 (1:1000, Cell Signaling Technology, Boston, United States), CXCL16 (1:1000, Bioss, Beijing, China),and Occludin (1:1000, Abcam, MA, United States). The membranes were then rinsed with Tris buffered saline and Tween (TBST) and incubated at 37 °C for 1 h with the secondary antibody (1:10000, Proteintech, Wuhan, China). The tagged protein bands were scanned using the ChemiDoc XRS+Gel Imaging System (Hercules, CA, United States). after a second TBST wash.

Isolation of fecal bacterial microbiota and 16S rRNA gene sequencing analysis

According to the directions on the DNA extraction kit (Omega Bio-tek, Norcross, GA, United States), the DNA of the microbial community was collected. DNA concentration and purity were assessed using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, United States), and the quality of the DNA extracted was determined using 1% agarose gel electrophoresis. The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, United States). The 16S rRNA gene was amplified using PCR in the following manner: A first denaturation was performed at 95 °C for three minutes, followed by 27 cycles of denaturation at 95 °C for thirty seconds, annealing at 55 °C for thirty seconds, and extension at 72 °C for forty-five seconds, followed by a third extension at 72 °C for ten minutes, before finishing at 4 °C. Purified PCR products were quantified after removal from 2% agarose gels using a Quantus TM Fluorometer (Promega, United States) after purification using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States).

Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) used an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, United States) to sequence the purified amplicons in a paired-end manner using equimolar pooling. The NCBI Sequence Read Archive database received the raw reads (Accession Number: SRP392420). In addition, I-sanger was used to conduct co-occurrence network analysis, correlation analysis, and microbiological difference analysis (Majorbio Bio-Pharm Technology Co., Ltd.; www.i-sanger.com).

Statistical analysis

Data were expressed as the mean ± SEM. The Student *t*-test or one-way analysis of variance (ANOVA), followed by the Wilcoxon rank-sum test and Kruskal-Wallis's test were used for multiple comparisons. $P < 0.05$ or $P < 0.01$ was considered significantly different. GraphPad Prism 8.0 and SPSS 21.0 were used for statistical analysis.

RESULTS

Dose screening of PTH relieved liver injury induced by Con A in mice

Con A-induced AIH is a confirmable model for studying human AIH, as it simulates many aspects of this disease, including liver damage, abnormal elevation of serum transaminase level and excessive generation of pro-inflammatory cytokines[23]. The liver weight (Figure 1A) and liver weight index (Figure 1B), spleen weight (Figure 1C), colonic weight (Figure 1D), and colonic weight index (Figure 1E), serum ALT (Figure 1F) and AST (Figure 1G), and the levels of IL-17A (Figure 1J) were raised in the Con A group compared with mice not treated with Con A ($P < 0.01$), while following PTH-L, PTH-M, PTH-H or DXM treatment, these results were the opposite ($P < 0.05$ or $P < 0.01$). The expression of IL-4 (Figure 1H) and IL-10 (Figure 1I) in Con A-induced AIH mice without treatment was lower than in control mice and AIH mice treated with PTH-L, PTH-M, PTH-H or DXM ($P < 0.01$). Compared with the normal control group, mice in the Con A group showed interfacial hepatitis, death of a large number of liver cells, structural disorder of liver lobules, increased infiltration of inflammatory cells, focal (or spotted) lytic necrosis or apoptosis (Figure 1K), and higher Ishak scores (Figure 1L) ($P < 0.01$). After 10 d of PTH treatment, compared with mice in the Con A group, mice in the PTH-L, PTH-M, PTH-H groups showed less interfacial hepatitis and mild focal (or spotted) lytic necrosis or apoptosis, more complete liver lobular structure, reduced inflammatory cell infiltration (Figure 1K), and lower Ishak scores (Figure 1L) ($P < 0.01$); thus, relieving AIH. However, pathological damage of the liver and the expression level of inflammatory factors were further reduced and liver function was further improved following the middle dose of PTH compared with the high dose of PTH and the low dose of PTH in AIH mice. The middle dose of PTH was the best dose to relieve AIH, and this dose was selected for the follow-up study.

PTH relieves liver injury induced by Con A in mice

Compared with the control group, the livers in the Con A group were dark red and swollen. Compared with the Con A group, the livers in the Con A + PTH-M and Con A + DXM groups were bright red with smooth surfaces (Figure 2B). Moreover, the liver weight (Figure 2C) and liver weight index (Figure 2D) were increased in the Con A group compared with mice not treated with Con A ($P < 0.01$), while PTH or DXM treatment decreased the increase in liver weight (Figure 2C) and liver weight index (Figure 2D) ($P < 0.05$). Compared with the normal control group, mice in the Con A group showed interfacial hepatitis, death of a large number of liver cells, structural disorder of liver lobules, increased infiltration of inflammatory cells, focal (or spotted) lytic necrosis or apoptosis (Figure 2F), and higher Ishak scores (Figure 2E) ($P < 0.01$). After 10 d of PTH treatment, compared with mice in the Con A group, mice in the Con A + PTH-M group and Con A + DXM groups showed less interfacial hepatitis and mild focal (or spotted) lytic necrosis or apoptosis, more complete liver lobular structure, reduced inflammatory cell infiltration (Figure 2F), and lower Ishak scores (Figure 2E) ($P < 0.01$). These findings demonstrate that PTH efficaciously relieves the pathological liver injury in Con A-induced AIH mice.

PTH improves liver function in AIH mice

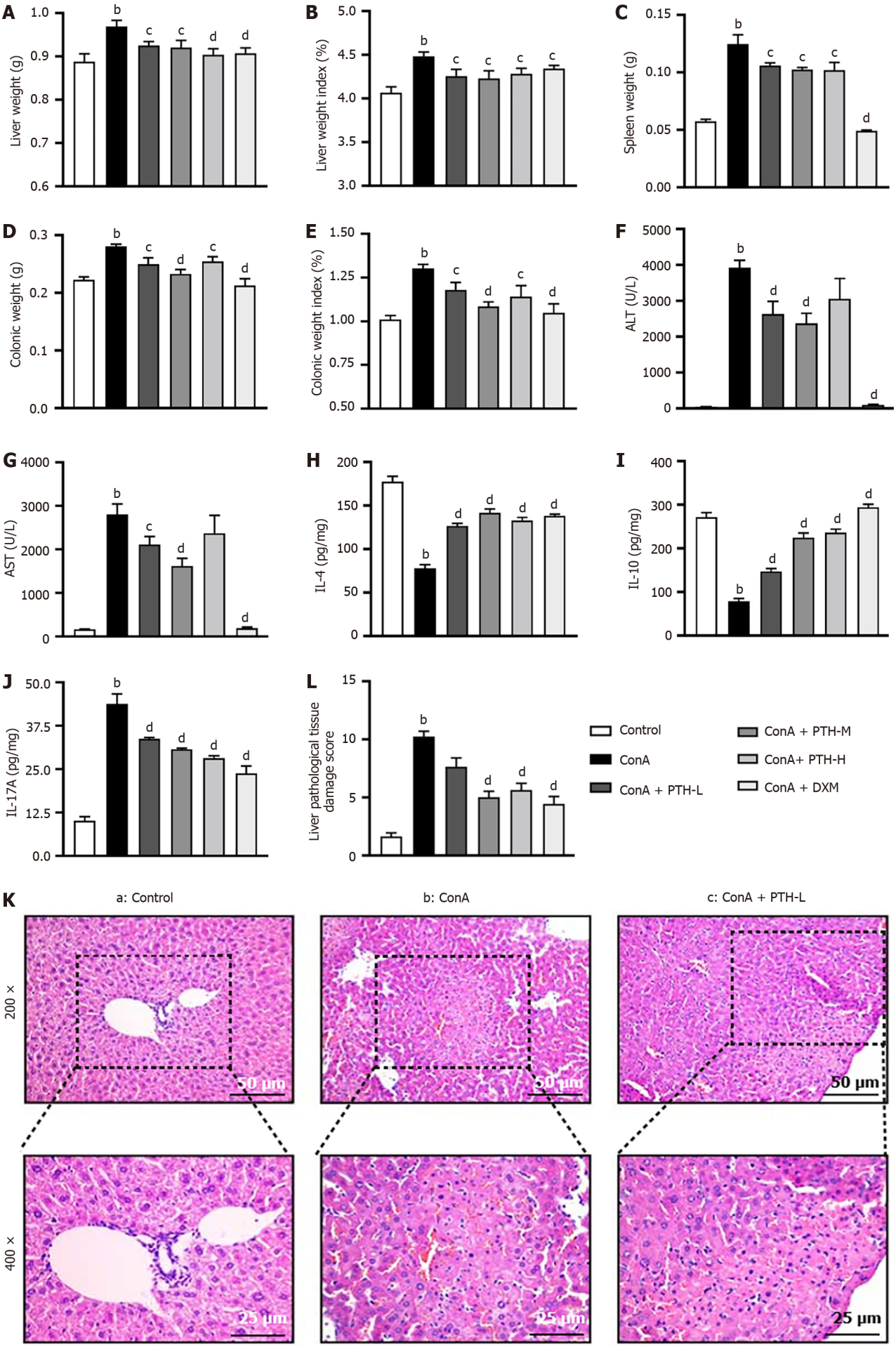
A biochemical liver test is an important method to judge whether there is liver damage, evaluate the severity of liver illness, and judge the treatment effect and prognosis[24-26]. The levels of serum enzymes, including ALT (Figure 3A), AST (Figure 3B), ALP (Figure 3C), IBIL (Figure 3D), and TG (Figure 3E) were raised in mice who received Con A compared with control mice ($P < 0.01$), and mice with Con A-induced AIH treated with PTH and DXM. In addition, decreased levels of TP (Figure 3F), ALB (Figure 3G), GLB (Figure 3H), and HDL (Figure 3I) were detected in serum from mice with Con A-induced AIH treatment ($P < 0.01$), and these levels were increased after treatment with PTH and DXM ($P < 0.05$ or $P < 0.01$). The above results show that PTH can improve liver function in mice with Con A-induced AIH.

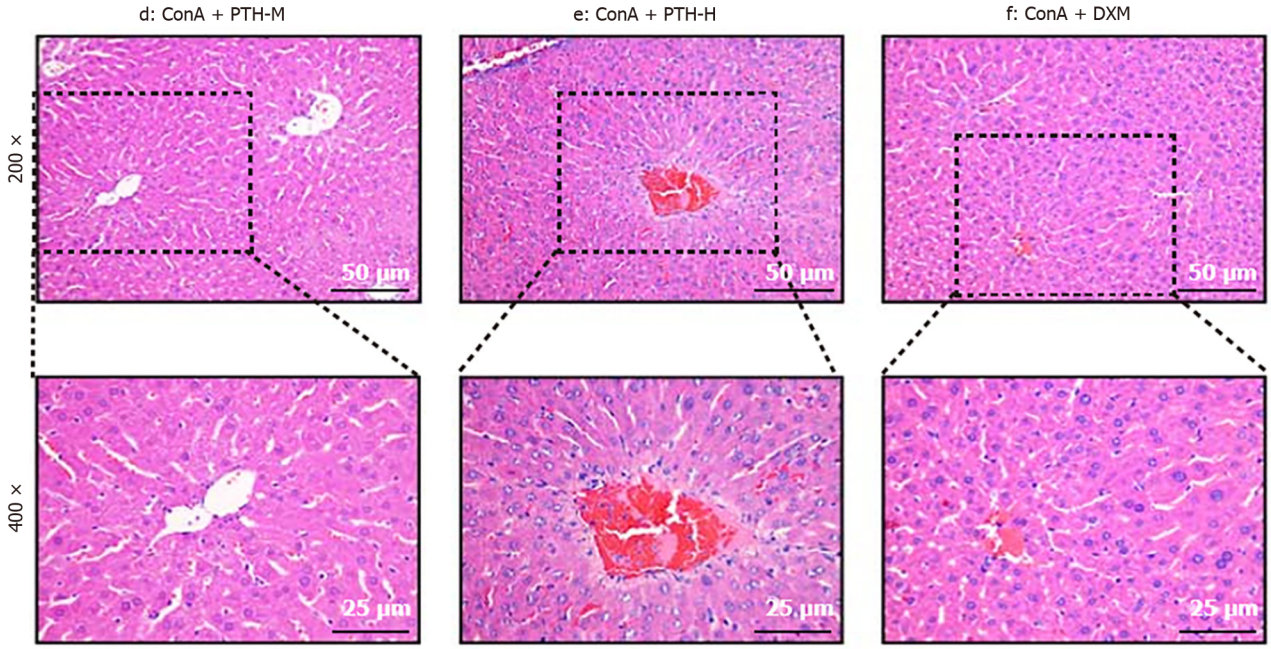
PTH regulates the expression of cytokines in liver tissue

An essential aspect of immunological damage in AIH is abnormal expression of inflammatory cytokines, such as IFN-γ, IL-1β, IL-2, IL-6, IL-21, TNF-α, and IL-10. Th17, Treg, and mTreg cells thereafter undergo transformation and migration into the liver. The secretion of IFN-γ (Figure 4A), IL-1β (Figure 4B), IL-2 (Figure 4C), IL-6 (Figure 4D), IL-21 (Figure 4E), TNF-α (Figure 4F) in liver tissues from AIH mice in the Con A group was higher than that in the control, Con A + PTH-M and Con A + DXM groups ($P < 0.01$). In contrast, the expression of IL-10 (Figure 4G) in Con A-induced AIH mice without treatment was lower than in control mice and AIH mice treated with PTH or DXM ($P < 0.01$). According to these findings, PTH modulates the equilibrium of pro- and anti-inflammatory cytokines in AIH mice.

PTH regulates Th17 and Treg differentiation in AIH mice

Production of the pro-inflammatory cytokine IL-17A is a characteristic of Th17 cells, the phenotype of Treg cells is $CD4^+$ $CD25^+Foxp3^+$, and they play a significant role in autoimmune disease such as AIH and so on[27,28]. Programmed death-1 (PD-1) is a member of the CD28/B7 superfamily of costimulatory molecules that plays an inhibitory effect in the periphery. PD-L1 (also known as B7-H1) and PD-L2 (also known as B7-DC) are ligands for PD-1[29]. In the present study,


A
Liver weight (g)
B
Liver weight index (%)
C
Spleen weight (g)
D
Colonic weight (g)
E
Colonic weight index (%)
F
ALT (U/L)
G
AST (U/L)
H
IL-4 (pg/mg)
I
IL-10 (pg/mg)
J
IL-17A (pg/mg)
L
Liver pathological tissue damage score
Control
ConA
ConA + PTH-L
ConA + PTH-M
ConA+ PTH-H
ConA + DXM
K
a: Control
b: ConA
c: ConA + PTH-L
200 ×
400 ×
50 μm
25 μm



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Figure 1 Dose screening of Pien Tze Huang relieved liver injury induced by Concanavalin A in mice. A: Liver weigh; B: Liver weigh index; C: Spleen weigh; D: Colonic weigh; E: Colonic weigh index; F: Serum alanine aminotransferase; G: Serum aspartate aminotransferase; H-J: The levels of cytokines: Interleukin (IL)-4 (H), IL-10 (I), IL-17A (J) were measured by enzyme-linked immunosorbent assay; K: Representative photographs of hematoxylin and eosin-stained liver sections; L: Scores for liver injury in different groups. According to the Ishak scoring system, liver injury was scored with respect to portal and intralobular inflammation, fragmented necrosis, and bridging necrosis. The extent of the pathology was scored from 0 (no pathology) to 4 (severe pathology). Data are representative images or the mean ± SEM (n = 8). $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* the control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* the Concanavalin A group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IL: Interleukin; ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

the mononuclear cells in peripheral blood and $CD4^+$ lymphocytes were measured by flow cytometry (Figures 5A and B). The percentage of $CD4^+IL\text{-}17A^+$ (Th17) cells (Figure 5C) in the peripheral blood of AIH mice was markedly raised ($P <$ 0.01), while the levels of $CD4^+IL\text{-}17A^+$ (Th17) cells (Figure 5C) were considerably lower in PTH-treated AIH mice than in untreated AIH mice ($P < 0.01$). However, the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells (Figure 5D) and $CD4^+CD25^+Foxp3^+PD\text{-}L1^+$ cells (Figure 5E) in the peripheral blood of mice with Con A-induced AIH were significantly decreased ($P <$ 0.05 or $P < 0.01$), while PTH treatment significantly increased the percentages of $CD4^+CD25^+Foxp3^+$ Treg cells (Figure 5D) and $CD4^+CD25^+Foxp3^+PD\text{-}L1^+$ cells (Figure 5E) in AIH mice ($P < 0.01$). The above results demonstrate that PTH controls the balance of Th17 cells and Treg cells in mice with Con A-induced AIH.

PTH regulates the number of central memory T cells and their transformation into effector T cells in AIH mice

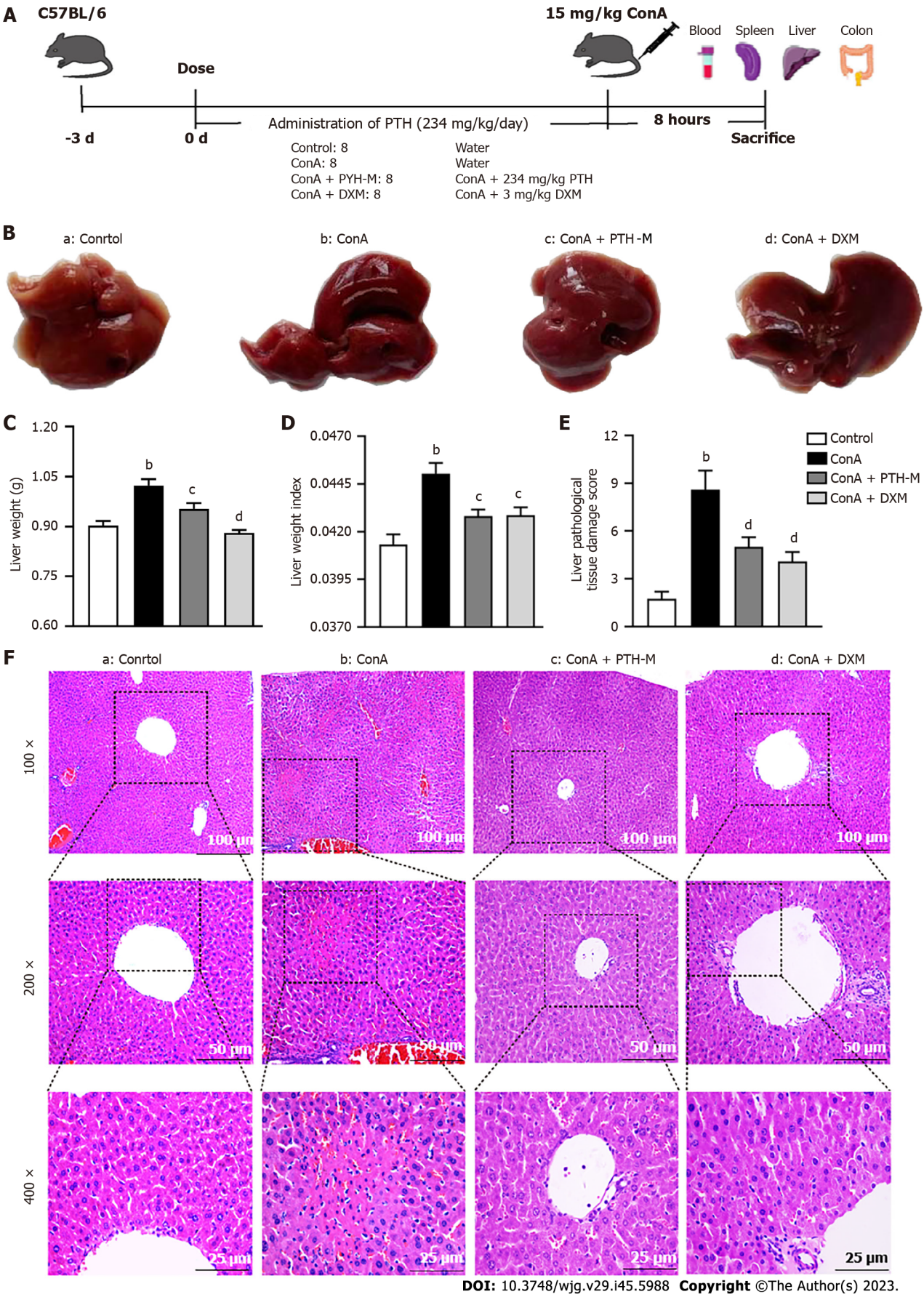
Central memory T (TCM) cells, whose phenotype is $CD4^+CD45RA^-CCR7^+$, mainly exist in lymphoid organs. Following antigen stimulation, TCM cells can rapidly transform into effector T (TEM) cells. The TEM phenotype is $CD4^+CD45RA^-CCR7^-$ and these cells exist in the periphery for a long time to produce IFN-γ, IL-17, and other inflammatory factors. This study used lymph node homing receptors $CD45RA^-$, $CD45RA^+$, and chemokine CC receptor (CCR)7 (Figures 6A-D) to recognize and count memory T cells. The levels of $CD4^+CD45RA^+CCR7^+$ (Figure 6E), $CD4^+CD45RA^+CCR7^-$ (Figure 6F), $CD4^+CD45RA^-CCR7^+$ (TCM) (Figure 6G), and $CD4^+CD45RA^-CCR7^-$ (TEM) (Figure 6H) cells were markedly decreased in untreated AIH mice in contrast to control mice ($P < 0.05$ or $P < 0.01$). After PTH treatment, the levels of $CD4^+CD45RA^+$ $CCR7^+$ (Figure 6E), $CD4^+CD45RA^+CCR7^-$ (Figure 6F), $CD4^+CD45RA^-CCR7^+$ (TCM) (Figure 6G) and $CD4^+CD45RA^-CCR7^-$ (TEM) (Figure 6H) cells were significantly increased ($P < 0.05$ or $P < 0.01$), which suggests that PTH controlled the quantity of TCM cells and TEM cells in AIH mice.

PTH regulates the subgroups of mTreg cells in AIH mice

$CD45RA^+Foxp3^{low}$ cells are static mTreg cells, which become $CD45RA^-Foxp3^{high}$ cells after activation. This study used lymph node homing receptors $CD45RA^-$ and $CD45RA^+$ (Figures 7A-D) to recognize and count memory Treg cells. In contrast to control mice, the number of $CD4^+CD45RA^-Foxp3^{high}$ mTreg (Figure 7E) and $CD4^+CD45RA^+Foxp3^{high}$ (Figure 7F) cells were significantly decreased in untreated AIH mice ($P < 0.05$), which implies that this is one of the primary characteristics in mice with Con A-induced AIH. After PTH treatment, the numbers of $CD4^+CD45RA^-Foxp3^{high}$ mTreg (Figure 7E), and $CD4^+CD45RA^+Foxp3^{high}$ (Figure 7F) significantly increased ($P < 0.05$). These results show that PTH improves mTreg cells level in AIH mice.

PTH improves the composition of gut microbiota in AIH mice

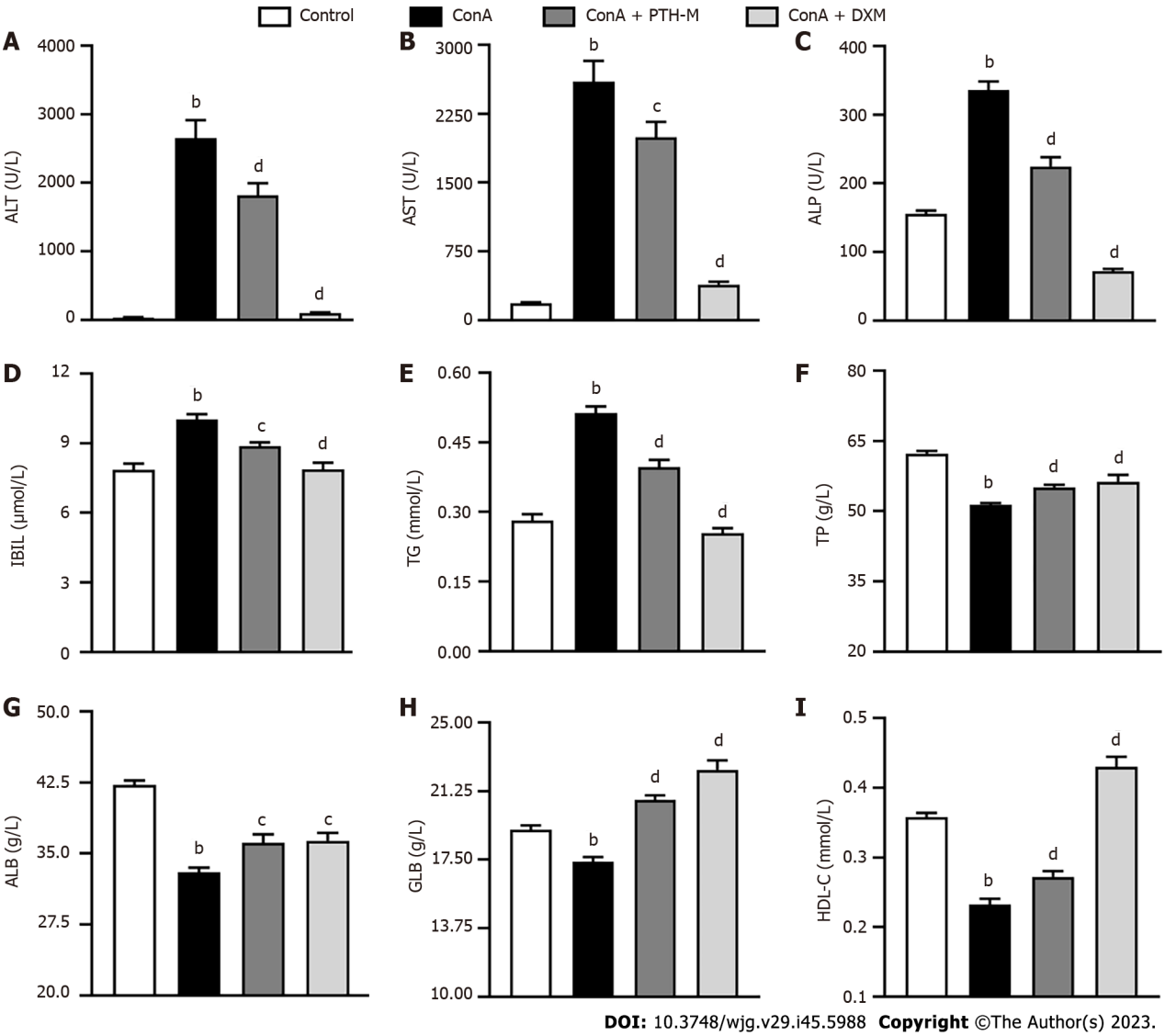
Increasing evidence has suggested that disordered intestinal microbiota is a significant characteristic in mice with Con A-



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Figure 2 Pien Tze Huang relieves liver injury induced by Concanavalin A in mice. A: Timeline of the experimental process of treatment and autoimmune hepatitis induction in mice; B-E: Images of mouse livers (B), liver weigh (C), liver weigh index (D), and scores for liver injury (E) in different groups. According to the Ishak scoring system, liver injury was scored with respect to portal and intralobular inflammation, fragmented necrosis, and bridging necrosis. The

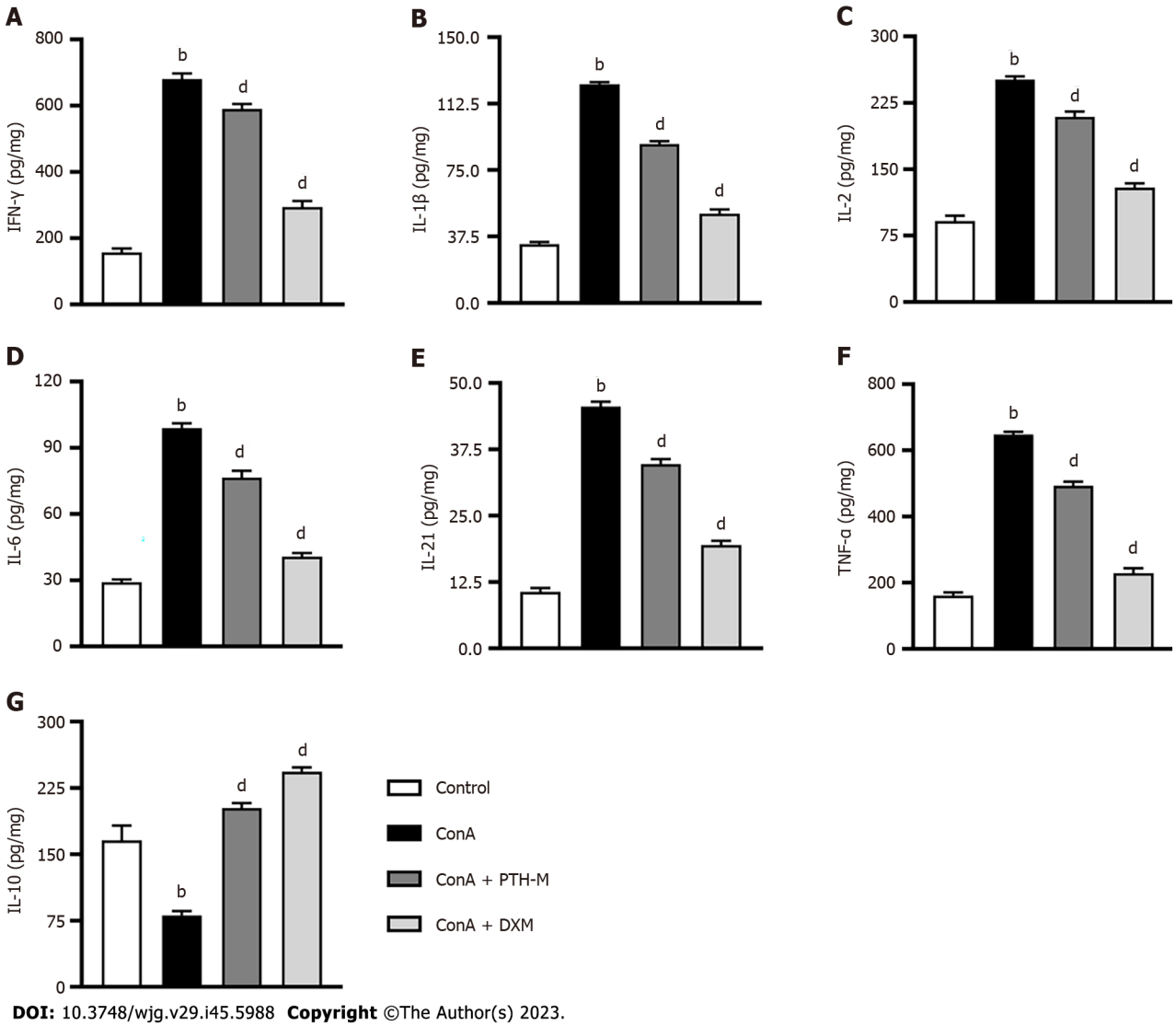
extent of the pathology was scored from 0 (no pathology) to 4 (severe pathology); F: Representative photographs of hematoxylin and eosin-stained liver sections. Data are representative images or the mean ± SEM (n = 8). [a]P < 0.05 and [b]P < 0.01 *vs* the control group; [c]P < 0.05 and [d]P < 0.01 *vs* the Concanavalin A group. ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.



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Figure 3 Pien Tze Huang improves liver function in mice with autoimmune hepatitis. A: Serum alanine aminotransferase; B: Serum aspartate aminotransferase; C: Serum alkaline phosphatase; D: Serum indirect bilirubin; E: Serum triglyceride; F: Serum total protein; G: Serum albumin; H: Serum globulin; I: Serum high-density lipoprotein. Data are representative images or the mean ± SEM (n = 8). [a]P < 0.05 and [b]P < 0.01 *vs* the control group; [c]P < 0.05 and [d]P < 0.01 *vs* the Concanavalin A group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; IBIL: Indirect bilirubin; TG: Triglyceride; TP: Total protein; ALB: Albumin; GLB: Globulin; HDL: High-density lipoprotein; ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

induced AIH[30]. The operational taxonomic unit (OTU) level (Figure 8A) rarefaction curve of the Shannon index at the OTU level levelled off when the sequencing data reached saturation, covering the vast majority of species in the gut microbiome community of AIH mice. The abundance grade curve at the OTU level (Figure 8B) showed the species richness and evenness of species diversity in the sample. When the abscissa position of the extension end point of the sample curve is more backward, this implies that the species richness of the sample is higher, the curve is smoother, and the species distribution of the sample is uniform. The abundance and diversity of the microbial population were expressed by single sample diversity (Alpha diversity) analysis, including the microbial richness indices Shannon and Simpson (Figures 8C-D). Figure 8C shows a substantial variation in the Shannon index at the OTU level between the Con A group and Con A + PTH-M group. Although there was no substantial variation in the Shannon index among the control, Con A, and Con A + DXM groups, the Shannon index at the OTU level was higher in the Con A group than in the control mice and Con A + DXM groups. Interestingly, the result of the Simpson index (Figure 8D), which also represented species richness, showed the opposite trend. The Simpson index at the OTU level was lower in the Con A group than in the control, Con A + PTH-M, and Con A + DXM groups.



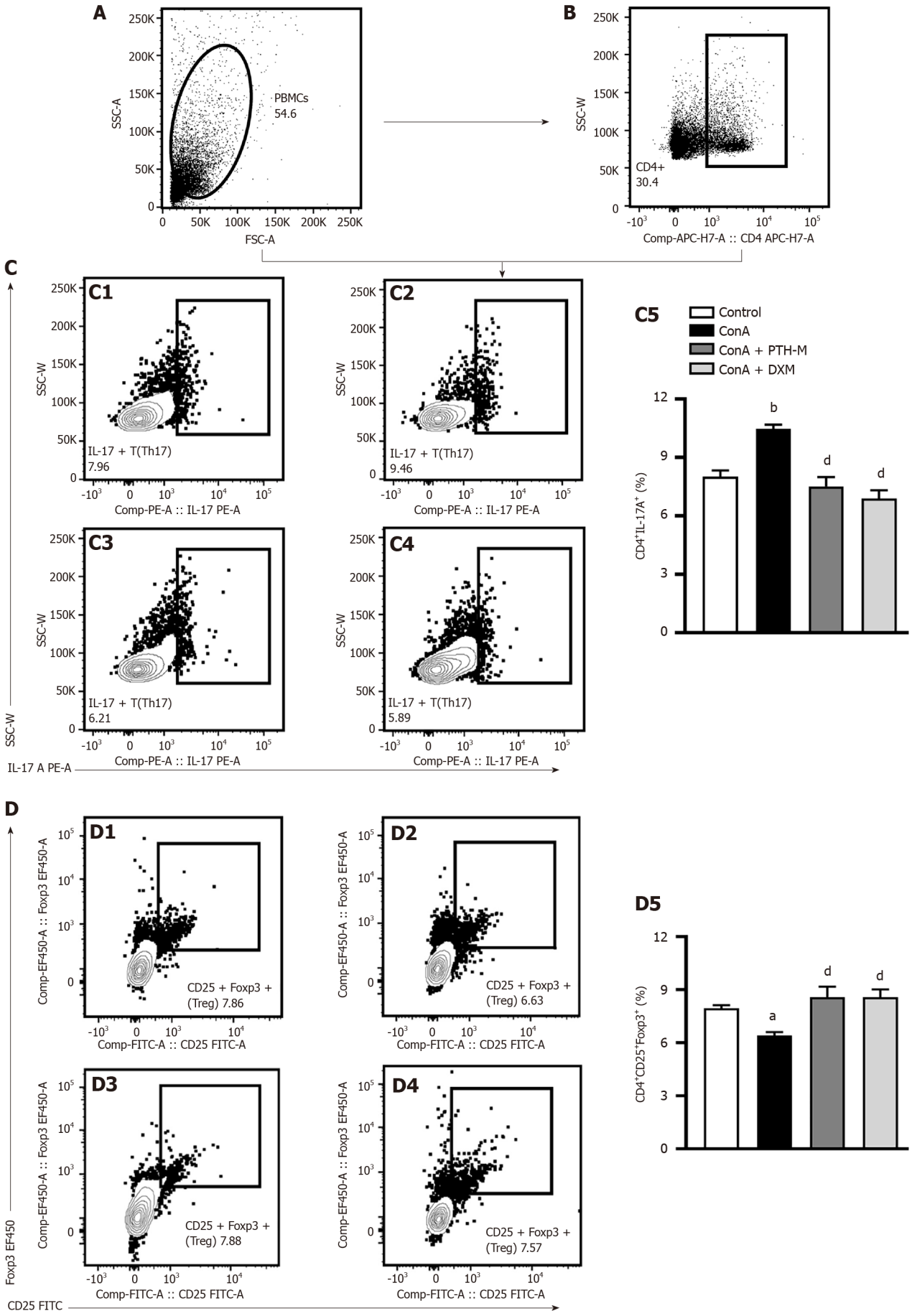
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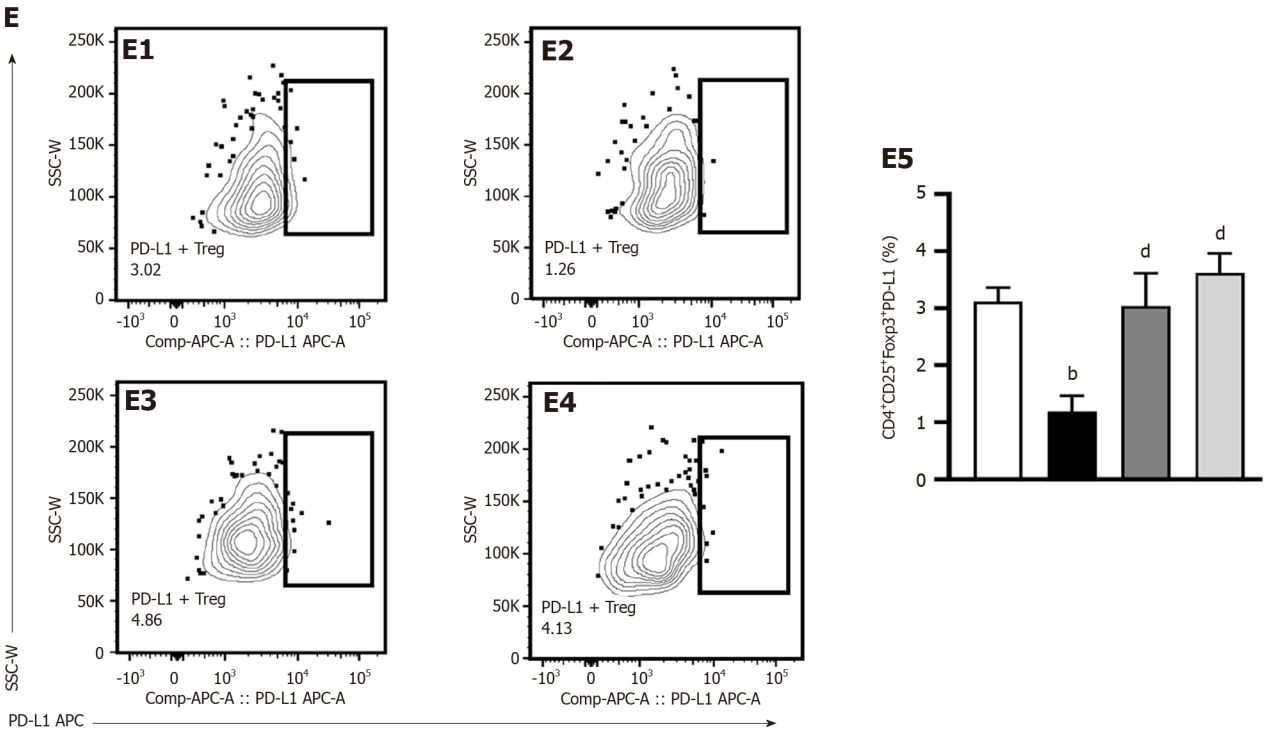
Figure 4 Pien Tze Huang regulates the expression of inflammatory cytokines in mice with autoimmune hepatitis. A-G: The levels of cytokines: Interferon-γ (A), interleukin (IL)-1β (B), IL-2 (C), IL-6 (D), IL-21 (E), tumor necrosis factor-alpha (F), and IL-10 (G), were measured by enzyme-linked immunosorbent assay. Data are representative images or the mean ± SEM (n = 8). $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* the control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* the Concanavalin A group. IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor; ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

Subsequently, in order to compare the diversity of the intestinal microbiota among these four groups, the diversity analyses were used, including principal co-ordinates analysis (PCoA) (Figure 8E). The PCoA analyses proved that the distribution of species in the Con A and Con A + PTH-M groups was distinct from the control group, the Con A + PTH-M group and the control groups were separated from one another by a smaller distance than the Con A and control groups (Figure 8E). Venn diagram analysis at the OTU level revealed additional variations in the intestinal microbiota composition across the four groups (630, 634, 627, and 620 OTUs were seen in the control, Con A, Con A + PTH-M, and Con A + DXM groups, respectively) (Figure 8F).

The percent of community abundance at the genus level (Figure 8G) was used to analyze and compare the species abundance of each group of samples and observe their biological composition and structure. *Lachnospiraceae-NK4A136*, *norank-f-norank-o-Clostridia-UCG-014*, and *Desulfovibrio* were lower in the Con A group than in the control and Con A + PTH-M groups. *Alloprevotella*, *Prevotellaceae-UCG-001*, *Dubosiella*, and *Odoribacter* were superior in the Con A group than in the control and Con A + PTH-M groups.

Figure 8H shows the phylogenetic tree at the genus level. Each branch in the evolutionary tree represented a species. According to the higher taxonomic rank that the species belong to, the branches are colored differently. The bar graph on the right displays the percentage of reads of species in various groups, and the lengths of the branches represent the evolutionary distance between the two species, or the level of species difference. Picrust was used to predict the function of the 16S amplicon sequencing results (Figure 8I). The functions of enrichment mainly include the transport and metabolism of hydrates; transcription; amino acid transport and metabolism; signal transduction mechanism; copy, recombine, repair; translation, ribosome structure, and biogenesis; inorganic ion transport and metabolism; energy production and transformation.


A
PBMCs
54.6
SSC-A
FSC-A
B
SSC-W
CD4+
30.4
Comp-APC-H7-A :: CD4 APC-H7-A
C
C1
IL-17 + T(Th17)
7.96
C2
IL-17 + T(Th17)
9.46
C3
IL-17 + T(Th17)
6.21
C4
IL-17 + T(Th17)
5.89
Comp-PE-A :: IL-17 PE-A
SSC-W
IL-17 A PE-A
C5
Control
ConA
ConA + PTH-M
ConA + DXM
CD4+IL-17A+ (%)
D
D1
CD25 + Foxp3 +
(Treg) 7.86
D2
CD25 + Foxp3 +
(Treg) 6.63
D3
CD25 + Foxp3 +
(Treg) 7.88
D4
CD25 + Foxp3 +
(Treg) 7.57
Comp-EF450-A :: Foxp3 EF450-A
Comp-FITC-A :: CD25 FITC-A
Foxp3 EF450
CD25 FITC
D5
CD4+CD25+Foxp3+ (%)



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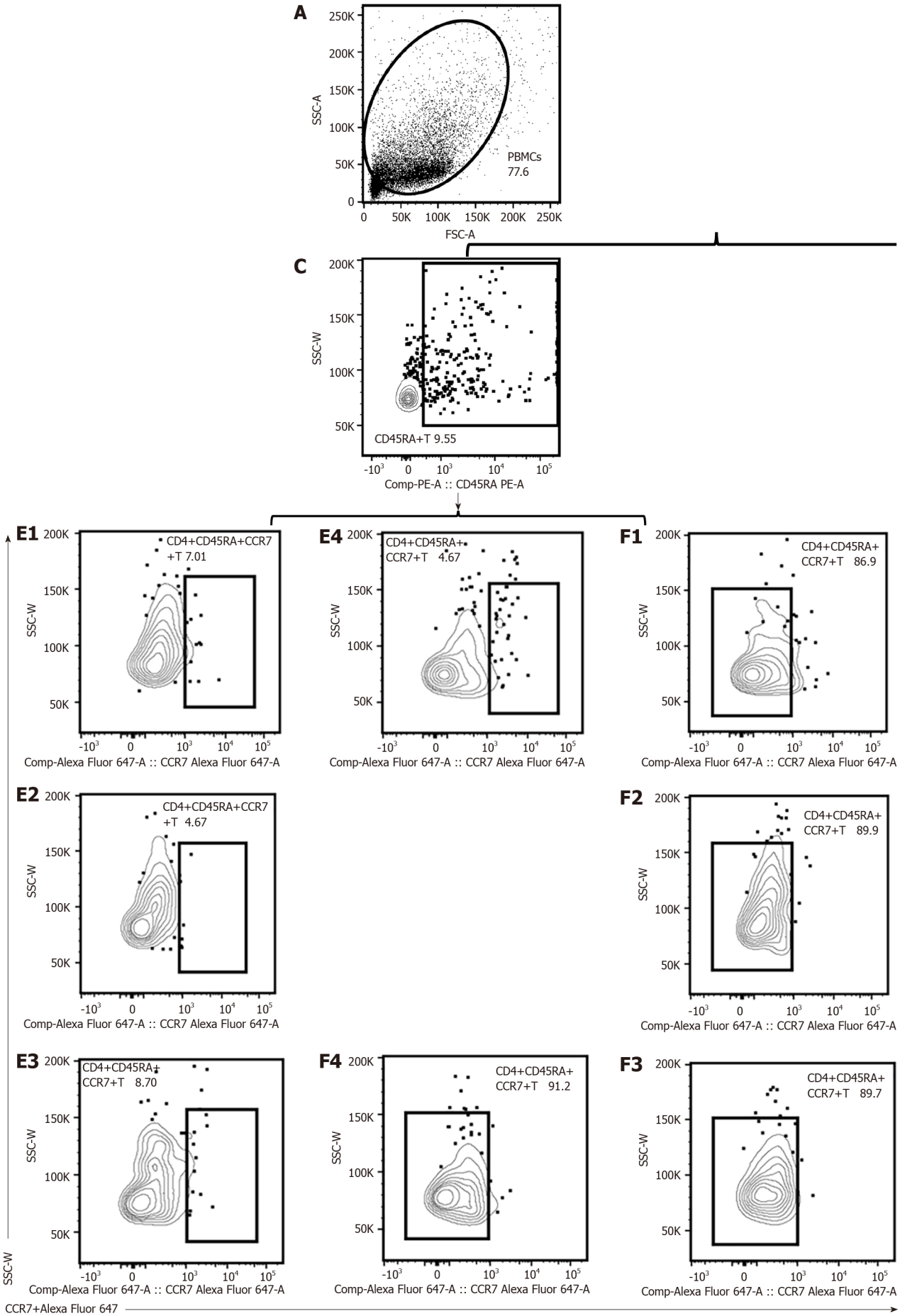
Figure 5 Pien Tze Huang regulates the number of T helper type 17 and regulatory T cells in mice with autoimmune hepatitis. A: Mononuclear cells in peripheral blood; B: $CD4^+$ lymphocytes measured by flow cytometry; C: $CD4^+IL\text{-}17A^+$(T helper type 17) cells. C1-C4: Control, Concanavalin A (Con A), Con A + middle-dose Pien Tze Huang (PTH-M), and Con A + dexamethasone (DXM) group, respectively; C5: Statistical analysis of $CD4^+IL17A^+$ cell frequencies in these four groups; D: $CD4^+CD25^+Foxp3^+$ cells. D1–D4: Control, Con A, Con A + PTH-M, and Con A + DXM group, respectively; D5: Statistical analysis of $CD4^+CD25^+Foxp3^+$ cell frequencies in these four groups; E: $CD4^+CD25^+Foxp3^+PD\text{-}L1^+$ cells. E1–E4: Control, Con A, Con A + PTH-M, and Con A + DXM group, respectively; E5: Statistical analysis of $CD4^+CD25^+Foxp3^+PD\text{-}L1^+$ cell frequencies in these four groups. Data are representative images or the mean ± SEM (n = 8). $^aP < 0.05$ and $^bP < 0.01$ *vs* the control group; $^cP < 0.05$ and $^dP < 0.01$ *vs* the Concanavalin A group. IL: Interleukin; ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

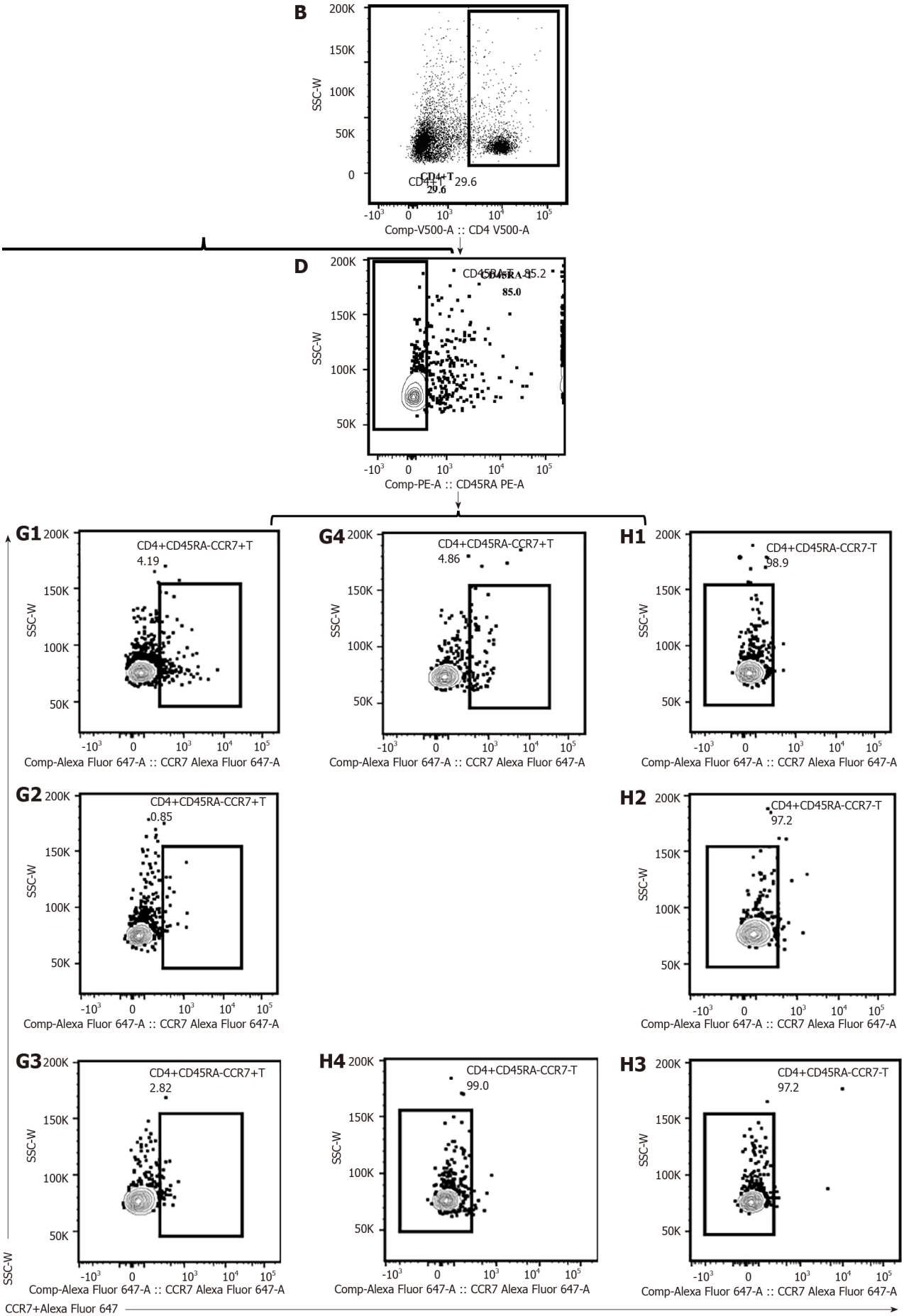
To identify the species with different abundances in various groups (samples) of microbial communities, hypothesis tests were conducted, and the significance of the observed differences were assessed, and rigorous statistical methods of the Kruskal-Wallis *H* test (Figure 9A) and Wilcoxon rank-sum test (Figures 9B and C) were used. As shown in Figure 9A, *Dubosiella* and *Rikenellaceae-RC9*-gut-group were higher in the Con A group than in the control, Con A + PTH-M, and Con A + DXM groups, while *Desulfovibrio* were lower. As shown in Figures 9B and C, *Alloprevotella*, *Rikenellaceae-RC9*-gut-group and *Prevotellaceae-NK3B31*-group were superior in the Con A group than in the control and Con A + PTH-M groups. The above results show that PTH can improve Con A-induced intestinal microbial composition in AIH mice.

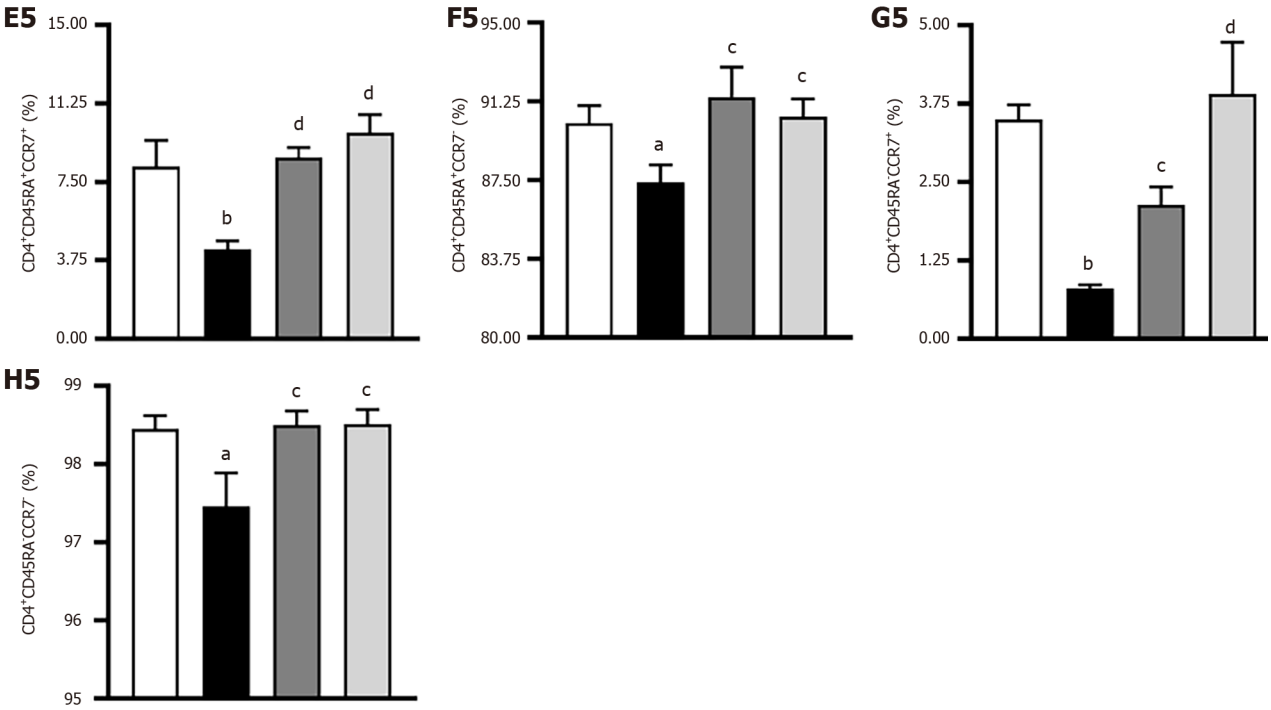
PIH regulates gut microbiota-associated important environmental factors in AIH mice

Based on the actual effects of PTH on Treg/mTreg and intestinal microbiota in AIH mice, the correlation between AST, ALT, HAI, TLR4, $CD4^+CD25^+Foxp3^+$ Treg cells, $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells, and intestinal microbiota was further investigated using a Spearman correlation heat map, redundancy analysis (RDA), and distance-based RDA (db-RDA). In addition, environmental factors such as AST, ALT, HAI, TLR4, $CD4^+CD25^+Foxp3^+$ Treg, and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells were used to examine their relationship with the intestinal microbiota. On the basis of RDA analysis (Figure 9D), the abundance of AST, ALT, HAI, and TLR4 were accordant with the Con A group's intestinal microbiota, while the abundance of $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells were accordant with the gut microbiota of mice in the control and Con A + PTH-M groups. On the basis of db-RDA analysis (Figure 9E), the abundance of AST, ALT, HAI and TLR4 were compatible with the gut microbiota of the Con A group, while the abundance of $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells were similar to the gut microbiota of mice in the Con A + PTH-M group. According to the Spearman correlation heat map at the genus level (Figure 9F), *Desulfovibrio* was negatively correlated with HAI, AST, ALT, TLR4 and positively connected with $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells; *Prevotellaceae-NK3B31*-group was positively connected with HAI, AST, ALT, TLR4, and negatively connected with $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells; *Rikenellaceae-RC9*-gut-group was positively connected with HAI, AST, ALT, and negatively connected with $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells; *Dubosiella* was negatively connected with $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells.

According to Western blot analysis, AIH mice had higher levels of TLR4 protein expression (Figure 9G) than the control group ($P < 0.01$), and the Con A + PTH-M group had lower levels of TLR4 protein expression (Figure 9G) than AIH mice ($P < 0.01$) (Figure 9G). To sum up, PIH regulates gut microbiota-associated important environmental factors in AIH mice.


A
SSC-A
FSC-A
PBMCs
77.6
C
SSC-W
CD45RA+T 9.55
Comp-PE-A :: CD45RA PE-A
E1
CD4+CD45RA+CCR7
+T 7.01
E4
CD4+CD45RA+
CCR7+T 4.67
F1
CD4+CD45RA+
CCR7+T 86.9
E2
CD4+CD45RA+CCR7
+T 4.67
F2
CD4+CD45RA+
CCR7+T 89.9
E3
CD4+CD45RA+
CCR7+T 8.70
F4
CD4+CD45RA+
CCR7+T 91.2
F3
CD4+CD45RA+
CCR7+T 89.7
Comp-Alexa Fluor 647-A :: CCR7 Alexa Fluor 647-A
SSC-W
CCR7+Alexa Fluor 647


B
SSC-W
CD4+T 29.6
Comp-V500-A :: CD4 V500-A
D
CD45RA-T 85.2
Comp-PE-A :: CD45RA PE-A
G1
CD4+CD45RA-CCR7+T 4.19
G4
CD4+CD45RA-CCR7+T 4.86
H1
CD4+CD45RA-CCR7-T 98.9
G2
CD4+CD45RA-CCR7+T 0.85
H2
CD4+CD45RA-CCR7-T 97.2
G3
CD4+CD45RA-CCR7+T 2.82
H4
CD4+CD45RA-CCR7-T 99.0
H3
CD4+CD45RA-CCR7-T 97.2
Comp-Alexa Fluor 647-A :: CCR7 Alexa Fluor 647-A
CCR7+Alexa Fluor 647



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Figure 6 Pien Tze Huang regulates the number of memory T cells in mice with autoimmune hepatitis. A: Peripheral blood mononuclear cells; B: CD4+ lymphocytes measured by flow cytometry; C: CD45RA$^+$ lymphocytes measured by flow cytometry; D: CD45RA$^-$ lymphocytes measured by flow cytometry; E: CD4$^+$CD45RA$^+$CCR7$^+$ cells. E1-E4: Control, Concanavalin A (Con A), Con A + middle-dose Pien Tze Huang (PTH-M), and Con A + dexamethasone (DXM) group in order; E5: Statistical analysis of CD4$^+$CD45RA$^+$CCR7$^+$ cell frequencies in these four groups; F: CD4$^+$CD45RA$^+$CCR7$^-$ cells. F1-F4: Control, Con A, Con A + PTH-M, and Con A + DXM group, respectively; F5: Statistical analysis of CD4$^+$CD45RA$^+$CCR7$^-$ cell frequencies in these four groups; G: CD4$^+$CD45RA$^-$CCR7$^+$ cells. G1-G4: Control, Con A, Con A + PTH-M, and Con A + DXM group, respectively; G5: Statistical analysis of CD4$^+$CD45RA$^-$CCR7$^+$ cell frequencies in these four groups; H: CD4$^+$CD45RA$^-$CCR7$^-$cells. H1-H4: Control, Con A, Con A + PTH-M, and Con A + DXM group, respectively; H5: Statistical analysis of CD4$^+$CD45RA$^-$CCR7$^-$ cell frequencies in these four groups. Data are representative images or the mean ± SEM (n = 8). $^a P < 0.05$ and $^b P < 0.01$ *vs* the control group; $^c P < 0.05$ and $^d P < 0.01$ *vs* the Concanavalin A group. ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

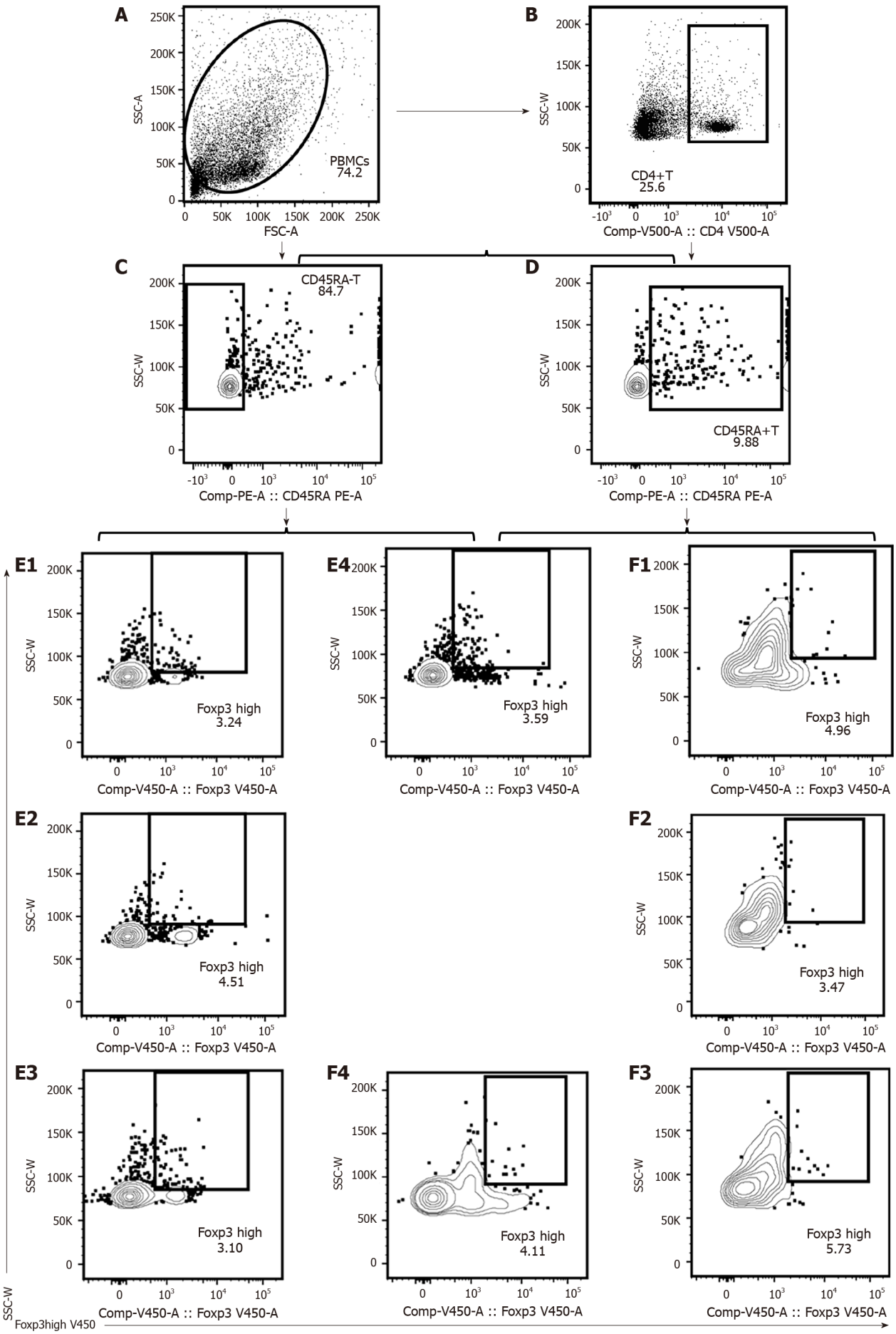
PTH maintains the intestinal barrier in AIH mice

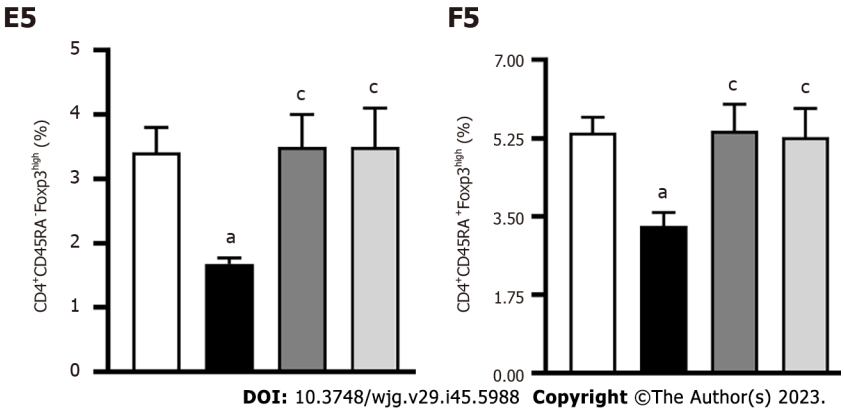
In order to further study whether intestinal microflora and/or other pathogens (or immunocomplexes) can enter the liver through the injured intestinal microbiota barrier and whether PTH can treat AIH by protecting the intestinal microbiota barrier and preventing bacterial translocation, the intestinal microbiota permeability test was performed. In the present study, the colonic weight (Figure 10A), colonic weight index (Figure 10B), and colonic weight/colonic length (Figure 10C) of AIH mice in the Con A group were higher than in the control group ($P < 0.01$), while colonic weight (Figure 10A), colonic weight index (Figure 10B), and colonic weight/colonic length (Figure 10C) of AIH mice in the Con A + PTH-M group were lower than in the Con A group ($P < 0.05$). Also, colonic inflammation was more pronounced in the colonic mucosa and submucosa of AIH model mice than in the control group (Figure 10D). Compared with the untreated Con A group, histological colonic damage was improved following 10-d of PTH and DXM therapy, with fewer inflammatory cells in the colonic mucosa and submucosa (Figure 10D).

Occludin content in tight junction proteins may be an indicator of the intestinal barrier's permeability. The expression of occludin was reduced in the colon tissues of mice in the Con A group than in the control group (Figures 10E and F), while the expression of occludin was elevated in the colon tissues of mice in the Con A + PTH-M group than in the Con A group (Figures 10E and F). The above data suggest that PTH has a protective effect on the intestinal barrier.

PTH regulates TLR/CXCL16/CXCR6 /NF-κB signal activation in AIH mice

Emerging evidence has identified that TLRs are interrelated with autoimmune diseases by recognizing the change in gut microbiota and/or other pathogens (or immunocomplexes). Figures 9D-G show that in the treatment groups, there was a sizable association between the distribution of sample microflora and mTreg cells. The small intestine's epithelial cells are stimulated by *Lactiplantibacillus plantarum 22A-3* to secrete transforming growth factor-beta, and retinoic acid is likely produced through TLR2, resulting in an increase in CD103+ DCs and the Foxp3$^+$ Treg population[31], and the activation of TLR4 by lipopolysaccharide (LPS) leads to the induction of pro-inflammatory Th17 cells and reduces the immunosuppressive function of Treg cells[3,32]. Differentiation of Th17 and Treg cells is adjusted by NF-κB[33,34]. CXCL16 promotes the migration of immune cells to secondary lymphoid organs and inflammatory sites, and CXCL16 is also an effective direct activator of NF-κB. The findings of western blot analysis revealed that the proteins TLR4 (Figure 9G), NF-κB p65 (Figure 11C), CXCL16 (Figure 11D), and CXCR6 (Figure 11E) in the liver were highly expressed in AIH model mice *vs* the control group ($P < 0.01$), while the expression of TLR4 (Figure 9G), NF-κB p65 (Figure 11C), CXCL16 (Figure 11D), and


A
SSC-A
FSC-A
PBMCs
74.2
B
SSC-W
CD4+T
25.6
Comp-V500-A :: CD4 V500-A
C
CD45RA-T
84.7
SSC-W
Comp-PE-A :: CD45RA PE-A
D
CD45RA+T
9.88
SSC-W
Comp-PE-A :: CD45RA PE-A
E1
Foxp3 high
3.24
SSC-W
Comp-V450-A :: Foxp3 V450-A
E4
Foxp3 high
3.59
SSC-W
Comp-V450-A :: Foxp3 V450-A
F1
Foxp3 high
4.96
SSC-W
Comp-V450-A :: Foxp3 V450-A
E2
Foxp3 high
4.51
SSC-W
Comp-V450-A :: Foxp3 V450-A
F2
Foxp3 high
3.47
SSC-W
Comp-V450-A :: Foxp3 V450-A
E3
Foxp3 high
3.10
SSC-W
Comp-V450-A :: Foxp3 V450-A
F4
Foxp3 high
4.11
SSC-W
Comp-V450-A :: Foxp3 V450-A
F3
Foxp3 high
5.73
SSC-W
Comp-V450-A :: Foxp3 V450-A
SSC-W
Foxp3high V450



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Figure 7 Pien Tze Huang regulates the number of special memory regulatory T cells in mice with autoimmune hepatitis. A: Peripheral blood mononuclear cells; B: $CD4^+$ lymphocytes measured by flow cytometry; C: $CD45RA^-$ lymphocytes measured by flow cytometry; D: $CD45RA^+$ lymphocytes measured by flow cytometry; E: $CD4^+CD45RA^-Foxp3^{high}$ cells. E1-E4: Control, Concanavalin A (Con A), Con A + middle-dose Pien Tze Huang (PTH-M), and Con A + dexamethasone (DXM) group, respectively; E5: Statistical analysis of $CD4^+CD45RA^-Foxp3^{high}$ cell frequencies in these four groups; F: $CD4^+CD45RA^+Foxp3^{high}$ cells. F1-F4: Control, Con A, Con A + PTH-M, and Con A + DXM group, respectively; F5: Statistical analysis of $CD4^+CD45RA^+Foxp3^{high}$ cell frequencies in these four groups. Data are representative images or the mean ± SEM (n = 8). $^aP < 0.05$ and $^bP < 0.01$ *vs* the control group; $^cP < 0.05$ and $^dP < 0.01$ *vs* the Concanavalin A group. ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

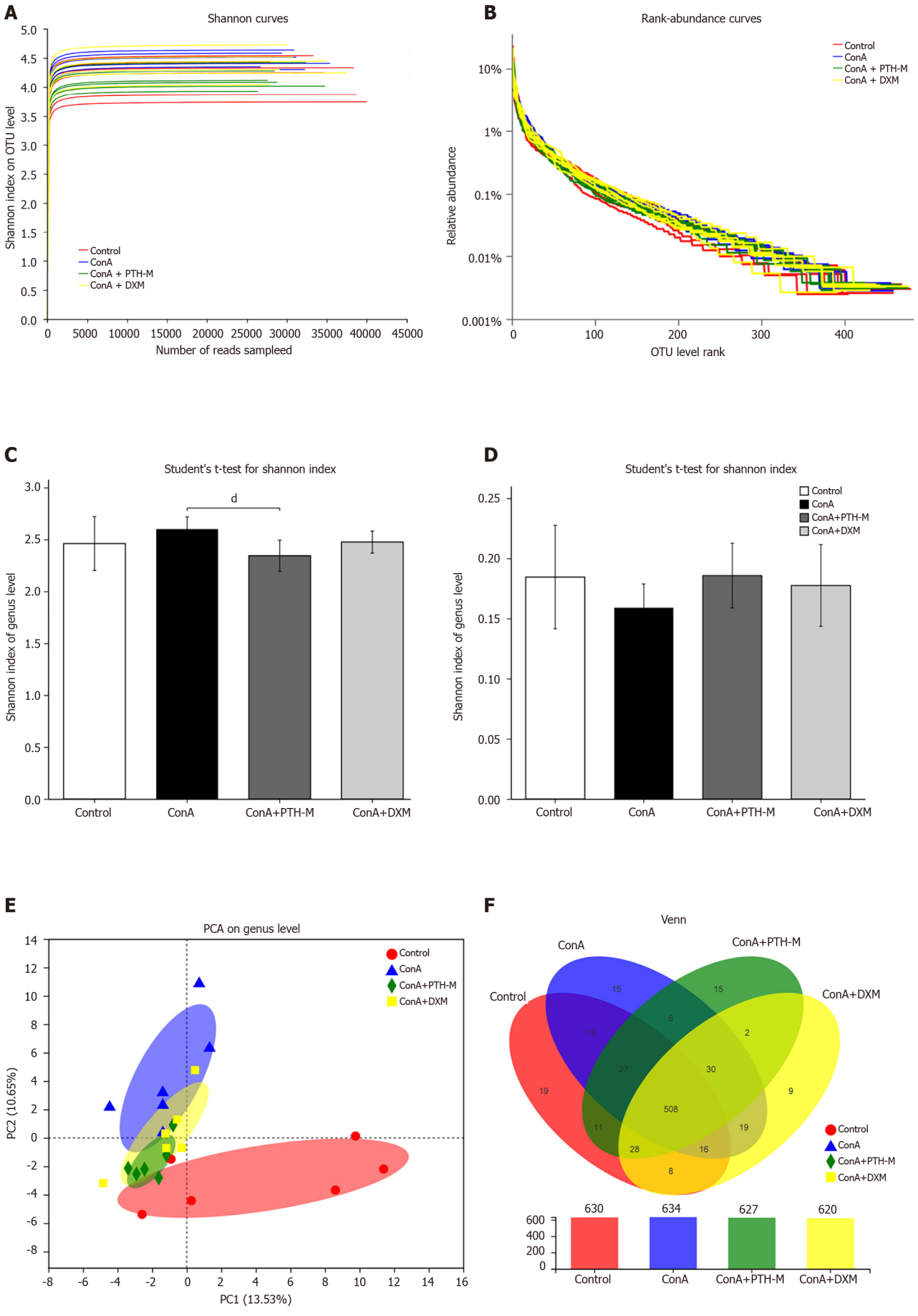
CXCR6 (Figure 11E) proteins were markedly decreased in mice in the Con A + PTH-M group than in mice in the Con A group ($P < 0.01$). However, the protein TLR2 (Figure 11B) in the liver was decreased in AIH model mice compared with the control group ($P < 0.01$). After PTH treatment, the protein TLR2 (Figure 11B) in the liver was lower in AIH model mice than in the Con A group ($P < 0.01$). Hence, these data suggest that PTH regulates Treg cell expression by inhibiting the NF-kB pathway and promoting TLR2 expression.

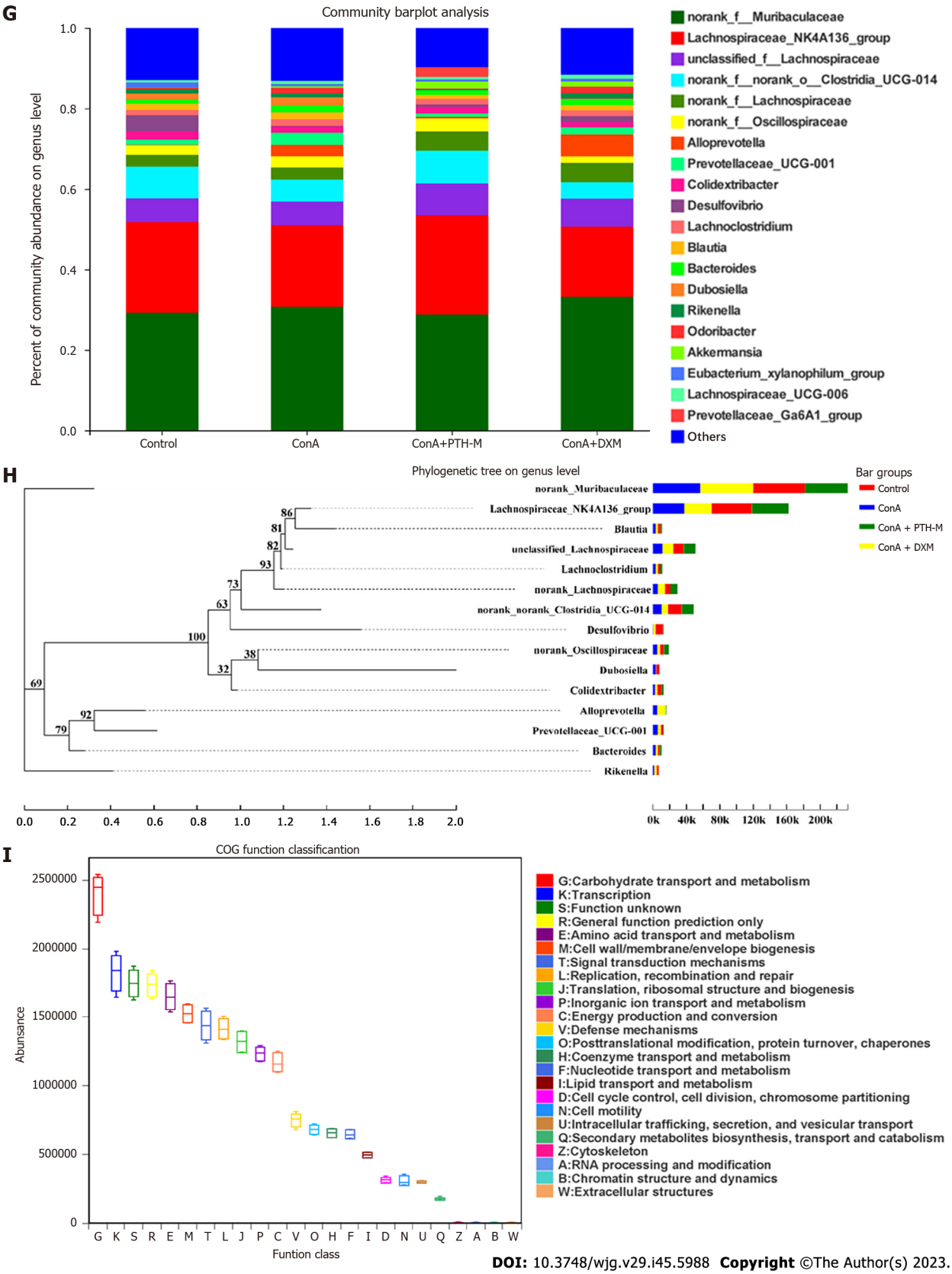
DISCUSSION

PTH has anti-inflammatory, neuroprotective, and immunoregulatory properties and is used in traditional Chinese medicine[10,35], which can reduce the serum homocysteine level and inhibit endoplasmic reticulum stress and the PERK/eIF2α pathway to reduce the severity of alcohol and high-fat diet in rats[36]. PTH can also inhibit apoptosis of intestinal crypt cells induced by 5-fluorouracil by inhibiting the ratio of Bax/Bcl-2, which can effectively reduce 5-fluorouracil-induced intestinal mucositis[37]. The above results suggest that PTH has the potential to treat immune liver injury and sustain the integrity of the gut mucosal barrier.

In order to further investigate the mechanism of PTH in the treatment of AIH, we analyzed the expression of various cytokines in AIH mice, and found that PTH treatment downregulated the expression of pro-inflammatory cytokines, such as IFN-γ, IL-1β, IL-2, IL-6, IL-21, and TNF-α, and upregulated the expression of anti-inflammatory factors, such as IL-10 in mice with Con A-induced HIA. IFN-γ[38]. TNF-α[39], IL-1β[40], IL-2[41], IL-21[42], and IL-6[43] promote autoimmune hepatic damage, whereas IL-10[44] protects the liver from damage. Thus, these data suggest that PTH could control the proportion of pro- and anti-inflammatory factors to improve Con A-induced AIH.

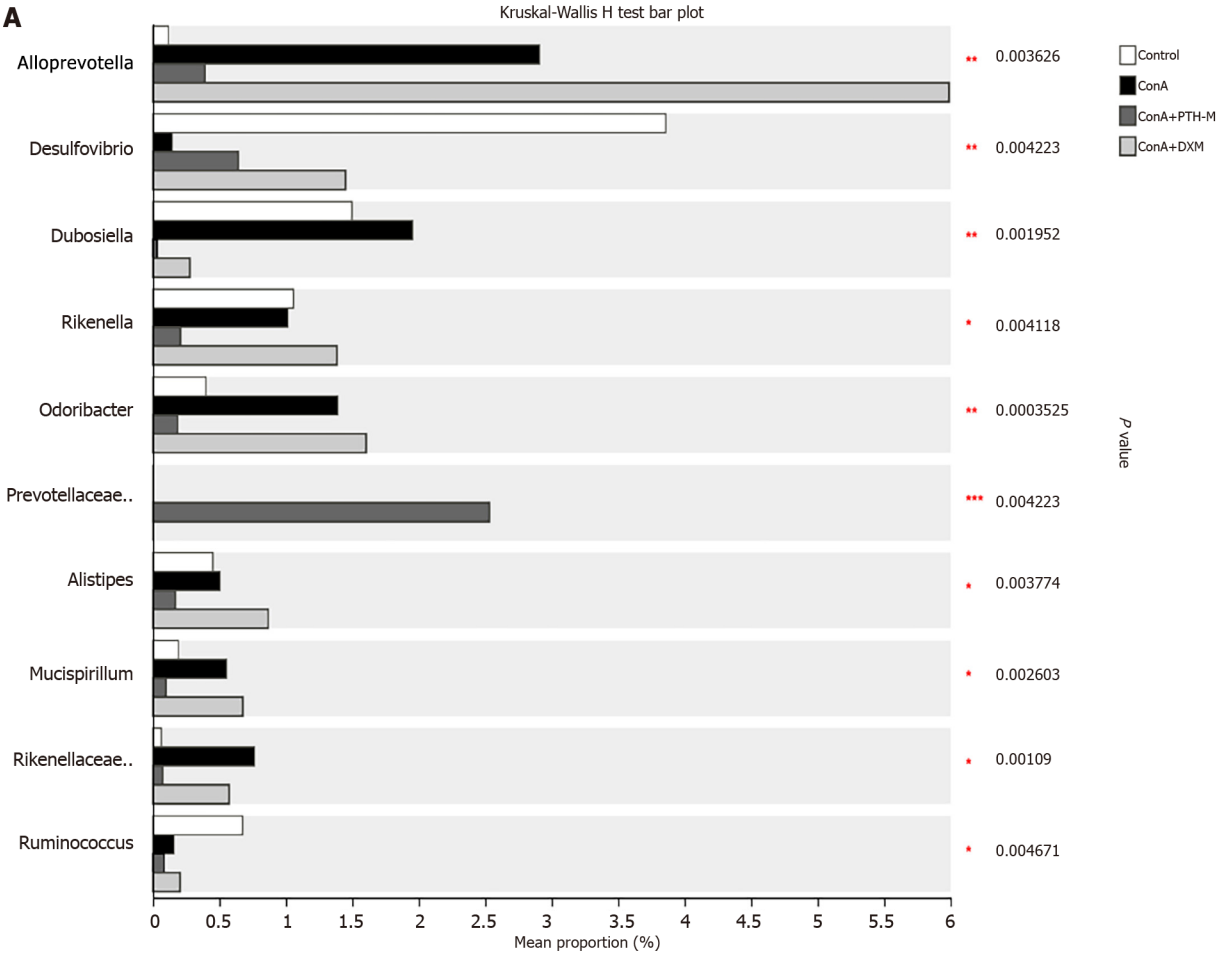
Previous research demonstrated a link between the poor prognosis of AIH and the low expression and functioning of Treg cells in the liver's inflammatory microenvironment[45-47]. It is known that mTreg cells can control memory effector reactions to induce differentiation and proliferation of Treg cells, and subsequently reduce collateral damage to tissues [48,49]. Treg cells have a significant role in adjusting the diversity of intestinal microbiota. They are also key cells that can inhibit intestinal inflammation, inhibit the abnormally activated immune response, and maintain intestinal immune tolerance[50]. When exposed to a foreign antigen again, TCM cells, which are long-lived T cells with the capacity for self-renewal and proliferation, can quickly develop into effector cells (TEM)[51]. Moreover, in mice lacking Foxp3 enhancer CNS1, *Firmicutes* were less abundant in the gut relative to the control group[52]. Additionally, Zhou *et al*[53] found that $Foxp3^+$ expression could be increased by PD-1/$PD\text{-}L1^+$ to improve its inhibitory effect. In the present research, the expression of $CD4^+CD25^+Foxp3^+PD\text{-}L1^+$, $CD4^+CD25^+Foxp3^+$ Treg, $CD4^+CD45RA^-Foxp3^{high}$, TCM, and TEM cells were lower in the Con A group than in control mice, while the expression of $CD4^+IL\text{-}17A^+$ Th17 cells was higher. These findings imply that the decreased PD-L1 expression in $CD4^+CD25^+Foxp3^+$ Treg cells downregulates the number of $CD4^+CD25^+Foxp3^+$ Treg cells. The decreased immune memory function leads to reduced differentiation of Treg cells and more activation of Th17 cell differentiation. The total number of Treg cells was insufficient to preserve immunological tolerance and stop pathogenic elements-such as heteroantigen and pathogenic bacteria-from continuing to have a milder immunosuppressive effect on $CD4^+CD25^+Foxp3^+$ Treg cells. Following PTH treatment for 10 d, the number of $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells was raised to maintain immune tolerance. In order to activate the immunosuppressive function and limit the severity of excessive immunological damage, PTH additionally enhanced the expression of PD-L1 in $CD4^+CD25^+Foxp3^+$ Treg cells and decreased the number of $CD4^+IL17A^+$ Th17 cells. Also, PTH effectively reduced Con A-induced AIH in mice. These findings imply that the equilibrium between Th17, mTreg, and

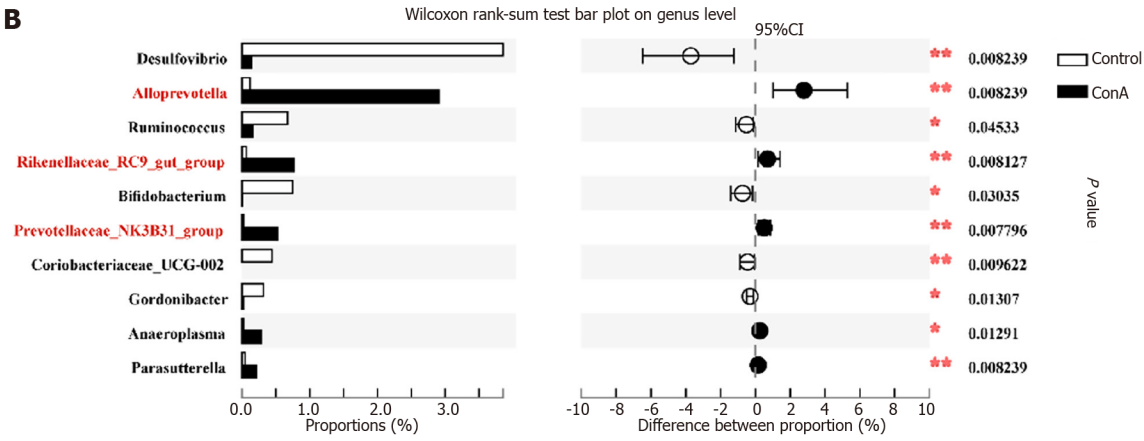

A
Shannon curves
Shannon index on OTU level
Number of reads sampleed
Control
ConA
ConA + PTH-M
ConA + DXM
B
Rank-abundance curves
Relative abundance
OTU level rank
C
Student's t-test for shannon index
Shannon index of genus level
d
Control
ConA
ConA+PTH-M
ConA+DXM
D
Student's t-test for shannon index
E
PCA on genus level
PC2 (10.65%)
PC1 (13.53%)
F
Venn
630
634
627
620

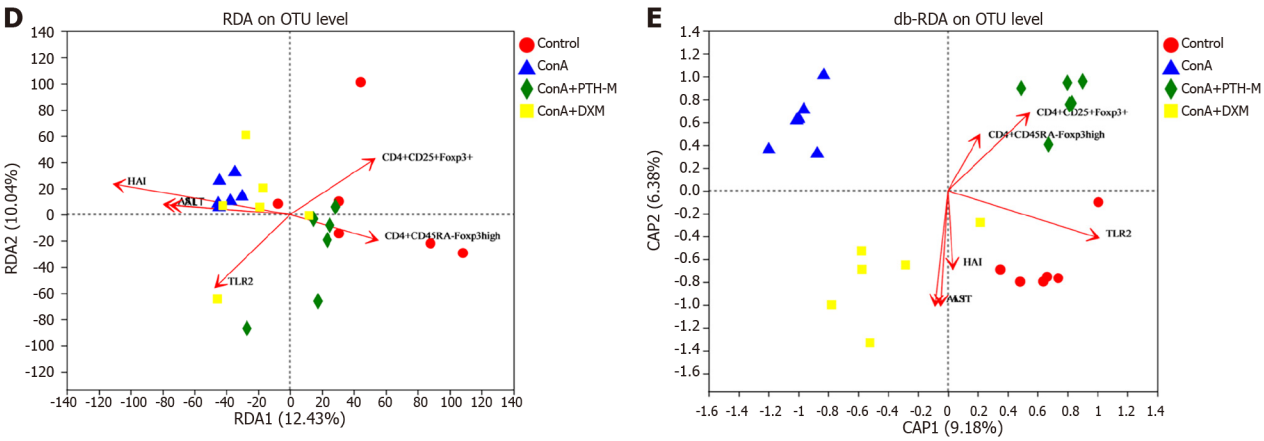


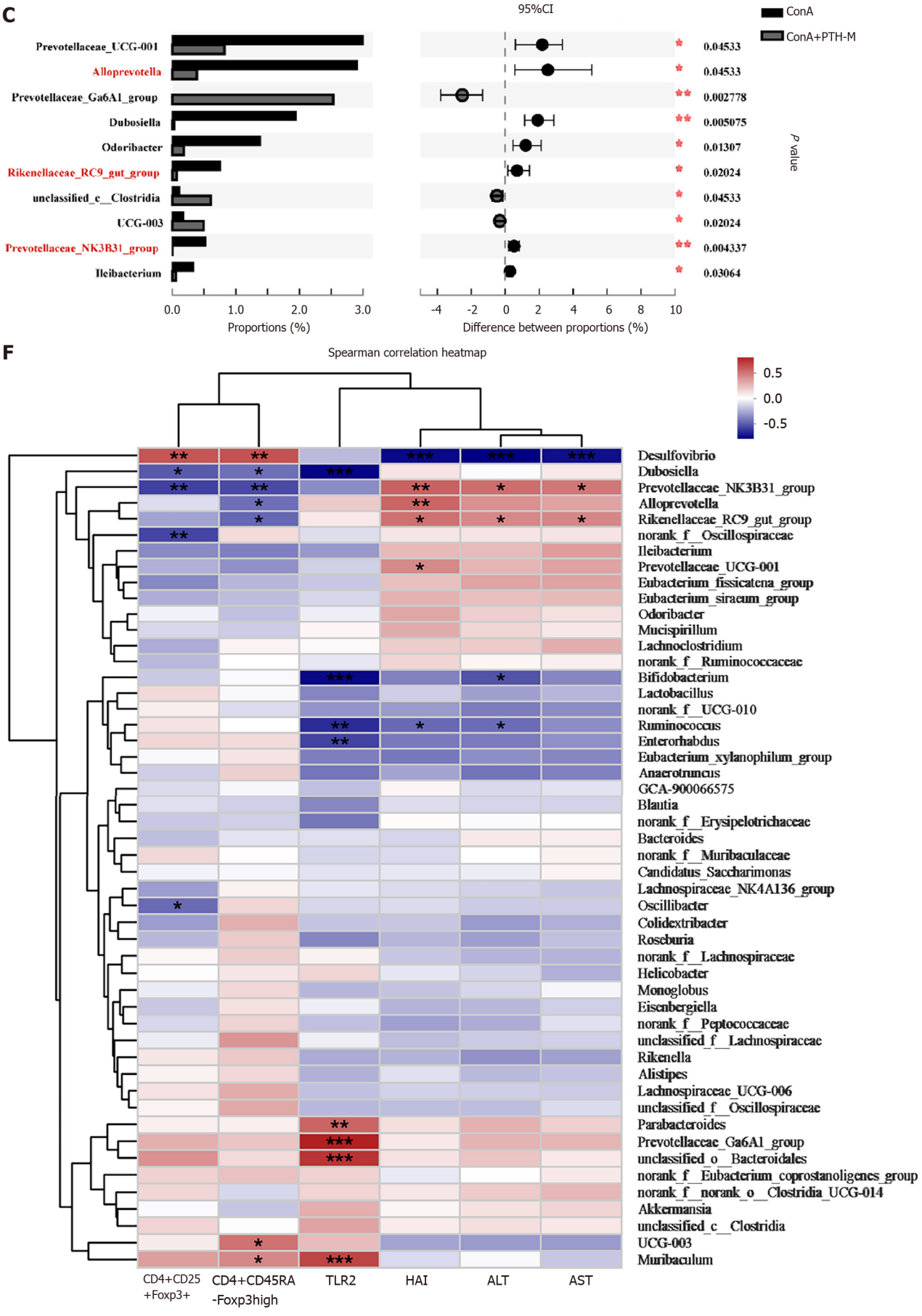
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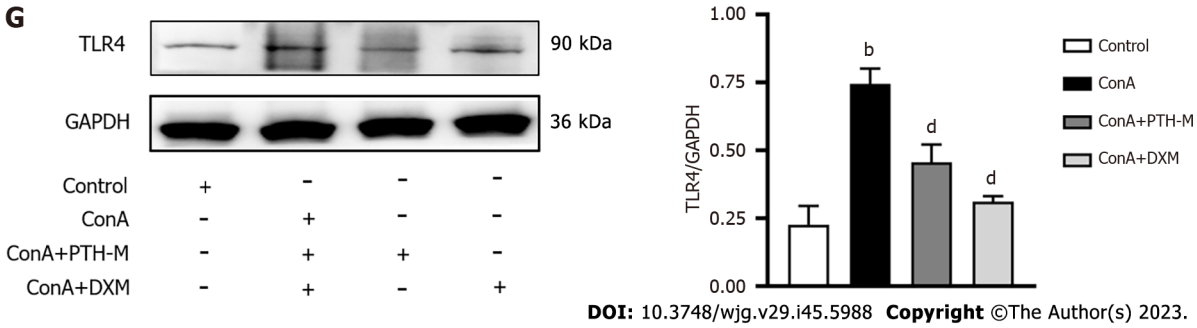
Figure 8 Pien Tze Huang improves the composition of gut microbiota in mice with autoimmune hepatitis. A: The rarefaction curve of the Shannon index at the operational taxonomic unit (OTU) level; B: Rank-Abundance curves at the OTU level; C: Shannon index at the genus level; D: Simpson index at the genus level; E: Partial least squares discriminant analysis score at the genus level; F: The Venn diagram depicts OTUs that differed in each group; G: Community composition bar chart at the genus level; H: Phylogenetic evolutionary tree at the genus level; I: COG function classification. Data are representative images or the mean ± SEM (n = 6). $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* the control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* the Concanavalin A group. ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.


A
Kruskal-Wallis H test bar plot
Alloprevotella
Desulfovibrio
Dubosiella
Rikenella
Odoribacter
Prevotellaceae..
Alistipes
Mucispirillum
Rikenellaceae..
Ruminococcus
0.003626
0.004223
0.001952
0.004118
0.0003525
0.004223
0.003774
0.002603
0.00109
0.004671
P value
Control
ConA
ConA+PTH-M
ConA+DXM
Mean proportion (%)


B
Wilcoxon rank-sum test bar plot on genus level
95%CI
Desulfovibrio
Alloprevotella
Ruminococcus
Rikenellaceae_RC9_gut_group
Bifidobacterium
Prevotellaceae_NK3B31_group
Coriobacteriaceae_UCG-002
Gordonibacter
Anaeroplasma
Parasutterella
0.008239
0.008239
0.04533
0.008127
0.03035
0.007796
0.009622
0.01307
0.01291
0.008239
P value
Control
ConA
Proportions (%)
Difference between proportion (%)


D
RDA on OTU level
Control
ConA
ConA+PTH-M
ConA+DXM
CD4+CD25+Foxp3+
CD4+CD45RA-Foxp3high
HAI
ALT
TLR2
RDA2 (10.04%)
RDA1 (12.43%)
E
db-RDA on OTU level
CD4+CD25+Foxp3+
CD4+CD45RA-Foxp3high
TLR2
HAI
AST
CAP2 (6.38%)
CAP1 (9.18%)


C
95%CI
ConA
ConA+PTH-M
Prevotellaceae_UCG-001
Alloprevotella
Prevotellaceae_Ga6A1_group
Dubosiella
Odoribacter
Rikenellaceae_RC9_gut_group
unclassified_c__Clostridia
UCG-003
Prevotellaceae_NK3B31_group
Ileibacterium
0.04533
0.04533
0.002778
0.005075
0.01307
0.02024
0.04533
0.02024
0.004337
0.03064
P value
0.0
1.0
2.0
3.0
Proportions (%)
-4
-2
0
2
4
6
8
10
Difference between proportions (%)
F
Spearman correlation heatmap
0.5
0.0
-0.5
Desulfovibrio
Dubosiella
Prevotellaceae_NK3B31_group
Alloprevotella
Rikenellaceae_RC9_gut_group
norank_f__Oscillospiraceae
Ileibacterium
Prevotellaceae_UCG-001
Eubacterium_fissicatena_group
Eubacterium_siraeum_group
Odoribacter
Mucispirillum
Lachnoclostridium
norank_f__Ruminococcaceae
Bifidobacterium
Lactobacillus
norank_f__UCG-010
Ruminococcus
Enterorhabdus
Eubacterium_xylanophilum_group
Anaerotruncus
GCA-900066575
Blautia
norank_f__Erysipelotrichaceae
Bacteroides
norank_f__Muribaculaceae
Candidatus_Saccharimonas
Lachnospiraceae_NK4A136_group
Oscillibacter
Colidextribacter
Roseburia
norank_f__Lachnospiraceae
Helicobacter
Monoglobus
Eisenbergiella
norank_f__Peptococcaceae
unclassified_f__Lachnospiraceae
Rikenella
Alistipes
Lachnospiraceae_UCG-006
unclassified_f__Oscillospiraceae
Parabacteroides
Prevotellaceae_Ga6A1_group
unclassified_o__Bacteroidales
norank_f__Eubacterium_coprostanoligenes_group
norank_f__norank_o__Clostridia_UCG-014
Akkermansia
unclassified_c__Clostridia
UCG-003
Muribaculum
CD4+CD25+Foxp3+
CD4+CD45RA-Foxp3high
TLR2
HAI
ALT
AST



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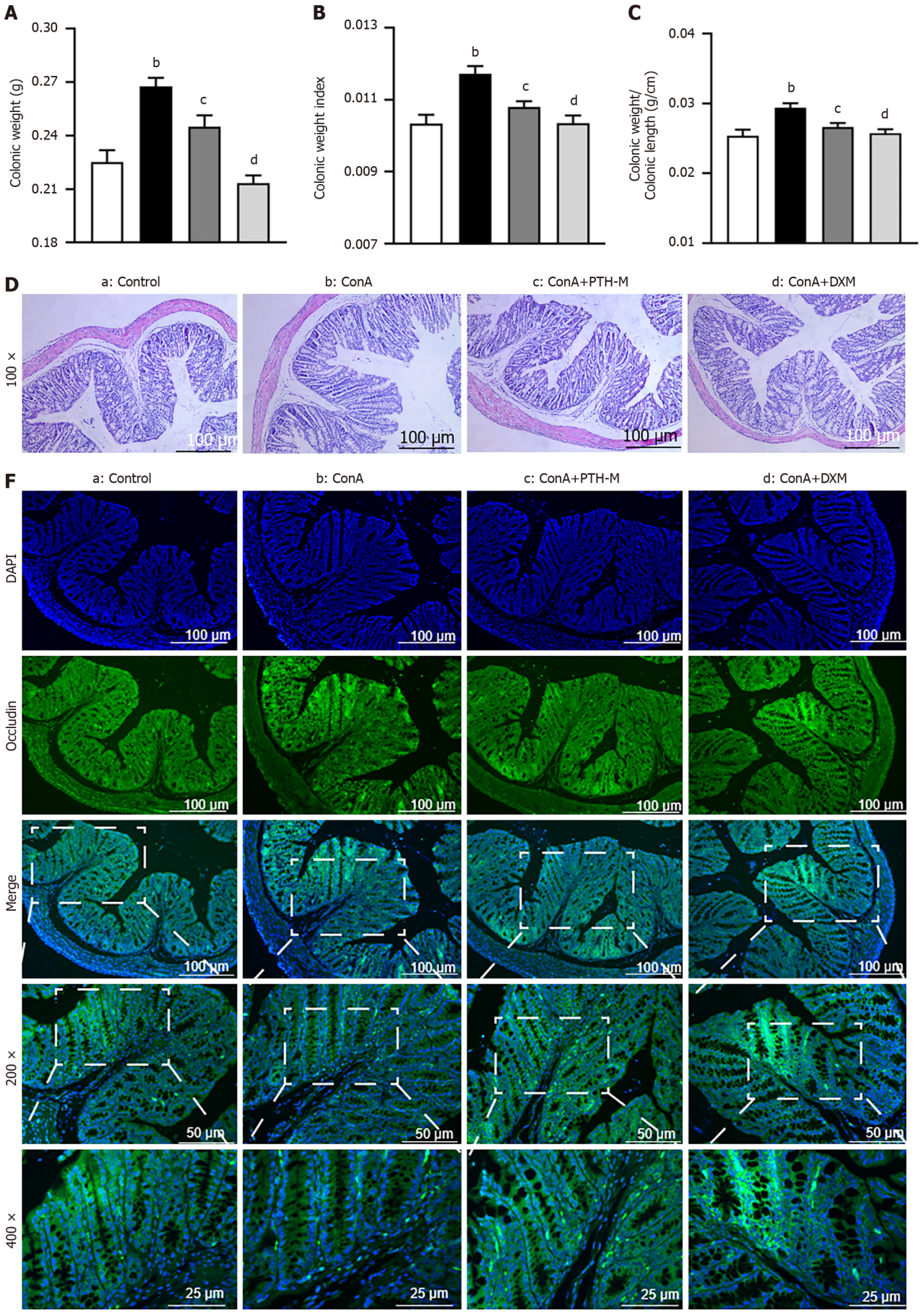
Figure 9 Species differences in intestinal microbiota and correlation analysis. A: Differential analysis among the four groups at the genus level; B: Phylotypes significantly different between the control and Concanavalin A (Con A) groups at the genus level; C: Phylotypes significantly different between Con A and Con A + middle-dose Pien Tze Huang groups at the genus level; D: Redundancy analysis of relationship between toll-like receptor (TLR)4, aspartate aminotransferase (AST), alanine aminotransferase (ALT), HAI, $CD4^+CD25^+Foxp3^+$, $CD4^+CD45RA^-Foxp3^{high}$ and intestinal microbiota; E: Distance-based redundancy analysis of relationship between TLR4, AST, ALT, HAI, $CD4^+CD25^+Foxp3^+$(regulatory T cells) cells, $CD4^+CD45RA^-Foxp3^{high}$ (memory regulatory T cells) cells, and intestinal microbiota; F: Spearman's correlation heatmap of TLR4, AST, ALT, HAI, $CD4^+CD25^+Foxp3^+$, $CD4^+CD45RA^-Foxp3^{high}$ and intestinal microbiota; G: The quantitative analysis of TLR4 protein expression. Data are representative images or the mean ± SEM (n = 6). $^aP < 0.05$ and $^bP < 0.01$ *vs* the control group; $^cP < 0.05$ and $^dP < 0.01$ *vs* the Concanavalin A group. ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

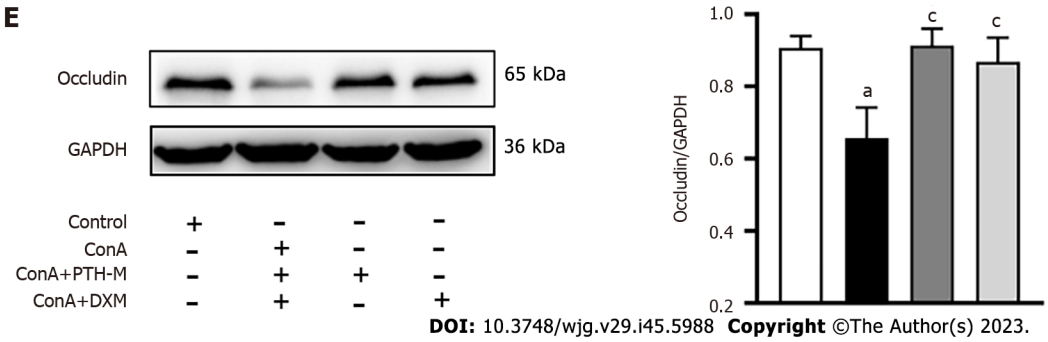
Treg cells may be controlled by PTH to provide its therapeutic impact in mice with Con A-induced AIH.

It is widely recognized that an imbalance in intestinal microbiota is a key trait and pathogenic component of AIH. Intestinal bacteria and/or other pathogens (or immunocomplexes) can enter the liver through the damaged gut mucosal barrier. PTH has a protective role in the liver of mice with Con A-induced AIH by encouraging the growth of particular helpful bacteria and repressing the growth of some potentially harmful bacteria. In this study, lower abundances of *Alloprevotella*, *Prevotellaceae-UCG-001*, *Dubosiella,* and *Odoribacter*, and higher abundances of *Lachnospiraceae-NK4A136*, *norank-f-norank-o-Clostridia-UCG-014,* and *Desulfovibrio* were seen in the PTH group. Moreover, psyllium elicits a broader microbial shift, increasing the abundance of *Parasutterella* and *Akkermansia*, and decreasing *Enterorhabdus* and *Odoribacter* [54]. Chen *et al*[55] found that chondroitin sulfate can ameliorate diet-induced insulin resistance, improve non-alcoholic fatty liver disease *via* the AKT pathway, and modulate gut microbiota composition, increasing the abundance of *Desulfovibrio* and the levels of hydrogen sulfide (H2S). *Lachnospiraceae_NK4A136*, *Parabacteroides,* and *Clostridium* likely strengthened the availability of *Bifidobacterium pseudocatenulatum LI09* against D-GalN-induced rat liver injury[56]. In this study, species differences and correlation analysis of intestinal microbiota showed that PTH could reduce the abundance of *Bacteroides-caecimuris* and *uncultured-bacterium-g-Prevotellaceae-NK3B31*-group and increase the abundance of *unclassified-g-norank-f-Muribaculaceae*. Simultaneously, correlation analysis showed that *Desulfovibrio* was negatively related to HAI, AST, ALT, TLR4, and positively related to $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells; *Prevotellaceae-NK3B31*-group was positively related to HAI, AST, ALT, TLR4, and negatively related to $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells; *Rikenellaceae-RC9*-gut-group was positively related to HAI, AST, ALT, and negatively related to $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells; *Dubosiella* was negatively related to $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells.

The destruction of intestinal mucosa was the main reason for induced bacterial translocation. Tight junction proteins have a prominent role in maintaining the intestinal barrier. Consequently, it is particularly important to reverse the deterioration of the intestinal barrier, bacterial translocation, and/or other pathogens (or immunocomplexes) to the liver. The present results showed that Con A increases intestinal permeability in mice by decreasing occludin protein expression; intestinal microbiota and/or other pathogens (or immunocomplexes) arrive at the liver through the portal vein to induce AIH. In order to treat mice with Con A-induced AIH, PTH controlled the interreaction between the intestinal microbiota and the number of mTreg cells.

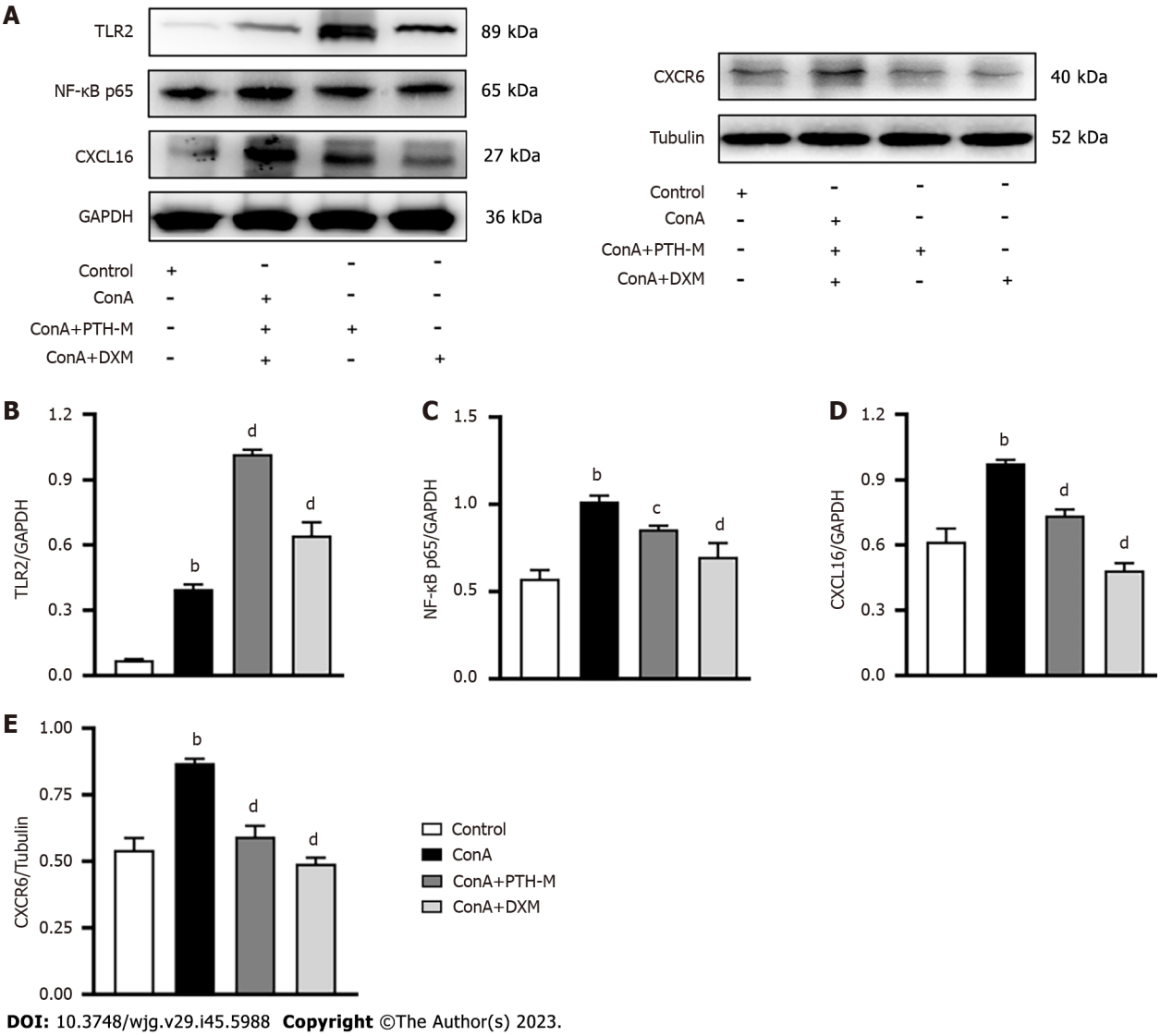
Correlation analysis of environmental factors showed that Treg/mTreg cells were positively correlated with intestinal microbiota, and TLR4 was negatively correlated with intestinal microbiota. Treg/mTreg cells could affect the intestinal microbiota, which in turn could stimulate pattern recognition receptors on the surface of immune cells or metabolites of flora to affect Treg cells. The immune system can sense the intestinal microbiota through the TLRs mechanism[57]. Treg cells mainly express TLR1, TLR2, TLR4, TLR5, and TLR8. It has been demonstrated that the stimulation of TLR2 by the intestinal microbiota or polysaccharide A from *Bacteroides fragilis* plays a defensive role in the gut, lowering inflammation by raising IL-10 production by dendritic and B cells and encouraging Treg proliferation[58]. Moreover, the activation of TLR4 by LPS causes the emergence of pro-inflammatory Th17 cells and reduces the immunosuppressive function of Treg cells[3]. Lai *et al*[59] found that indirubin downregulates LPS-induced inflammation by blocking TLR4 and NF-κB and MAPK activation, which is used to treat mastitis. Studies have also suggested that CXCL16/CXCR6 are elevated in some autoimmune diseases. Treg cells could be inhibited by CXCL16[60]; LPS was discovered to enhance the transcription of CXCL16 *via* the NF-κB signaling pathway, thus promoting the pathogenesis of acute lung injury[61]. In the current study, TLR4, NF-κB p65, CXCR6, and CXCL16 proteins in the liver were markedly expressed in AIH mice, and the protein TLR2 in the liver showed low expression. However, after PTH treatment, the expression of TLR4, NF-κB p65, CXCR6, and CXCL16 was significantly reduced, and TLR2 protein was significantly increased. However, it is still unclear which precise PTH constituents collectively control the interaction between the gut microbiota, TLR/CXCL16/CXCR6/NF-κB signal pathway, and mTreg cells, and which specific bacterium and their metabolites would be significantly enhanced or


A
Colonic weight (g)
0.30
0.27
0.24
0.21
0.18
b
c
d
B
Colonic weight index
0.013
0.011
0.009
0.007
b
c
d
C
Colonic weight/ Colonic length (g/cm)
0.04
0.03
0.02
0.01
b
c
d
D
a: Control
b: ConA
c: ConA+PTH-M
d: ConA+DXM
100 ×
100 μm
F
a: Control
b: ConA
c: ConA+PTH-M
d: ConA+DXM
DAPI
Occludin
Merge
200 ×
400 ×
100 μm
50 μm
25 μm



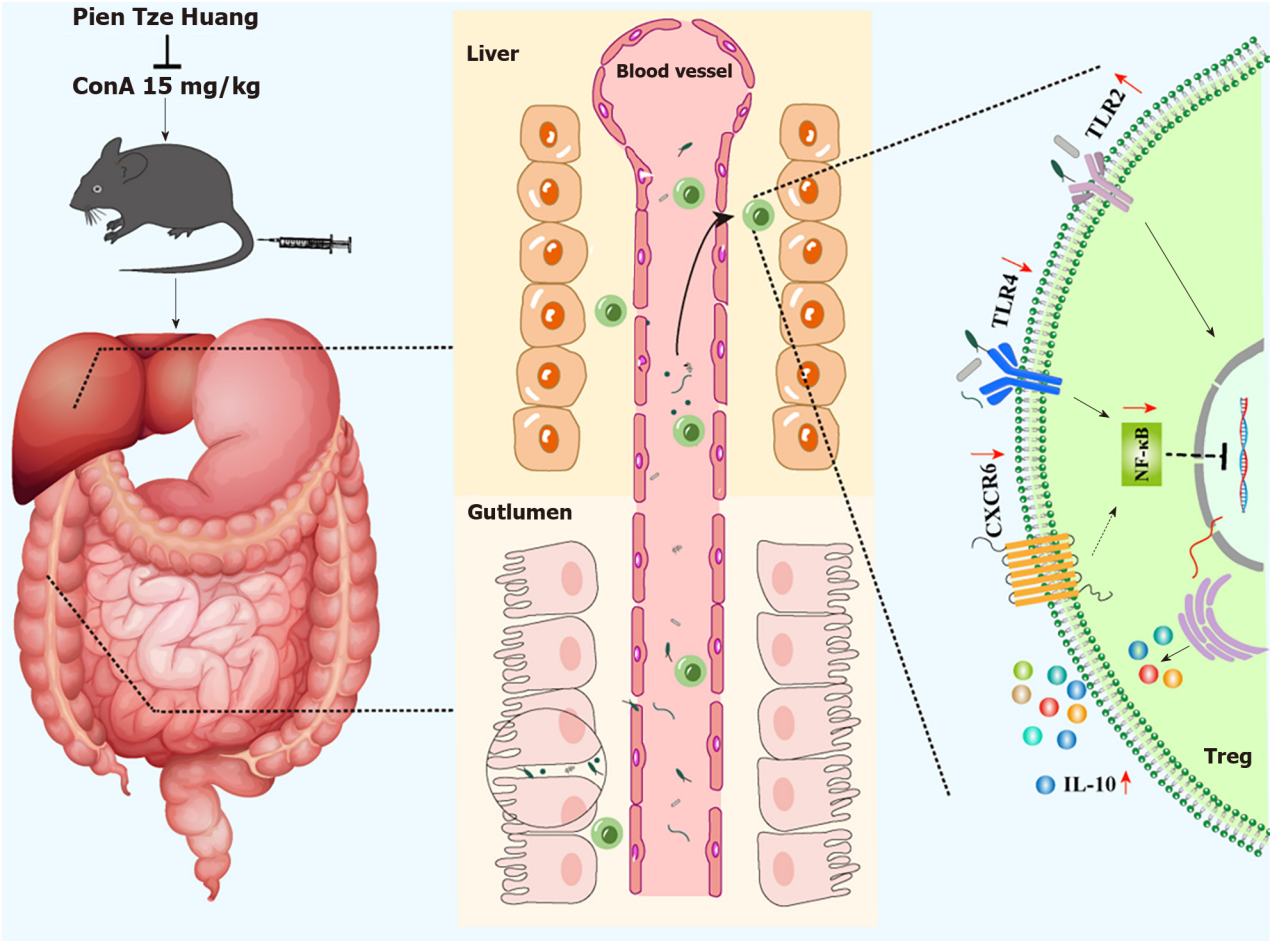
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Figure 10 Pien Tze Huang restores the function of the intestinal barrier in mice with autoimmune hepatitis. A: Colonic weight; B: Colonic weight index; C: Colonic weight/Colonic length; D: Colonic hematoxylin and eosin staining; E: The quantitative analysis of occludin protein expression; F: Representative images of occludin in the colon (IF, 200 ×, 400 ×). Data are representative images or the mean ± SEM (n = 8). [a]P < 0.05 and [b]P < 0.01 *vs* the control group; [c]P < 0.05 and [d]P < 0.01 *vs* the Concanavalin A group. ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.



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Figure 11 Pien Tze Huang regulates the expression of toll-like receptor 2, nuclear factor-κB p65, CXCR6, and CXCL16 in mice with autoimmune hepatitis. A: The protein expression of toll-like receptor (TLR)2, nuclear factor-κB (NF-κB) p65, CXCR6, and CXCL16 analyzed by western blot; B: Quantitative analysis of TLR2; C: Quantitative analysis of NF-κB p65; D: Quantitative analysis of CXCL16; E: Quantitative analysis of CXCR6. Data are representative images or the mean ± SEM (n = 8). [a]P < 0.05 and [b]P < 0.01 *vs* the control group; [c]P < 0.05 and [d]P < 0.01 *vs* the Concanavalin A group. TLR: Toll-like receptor; NF-κB: Nuclear factor-κB; ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.



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Figure 12 Protective mechanisms of Pien Tze Huang against autoimmune hepatitis. TLR: Toll-like receptor; NF-κB: Nuclear factor-κB; ConA: Concanavalin A; IL: Interleukin.

have a significant role in PTH treatment of AIH mice.

CONCLUSION

In conclusion, these findings suggest that PTH controls interactions between mTreg cells and intestinal microbiota to reduce experimental AIH. The effect of PTH was similar to that of DXM; yet, in the PTH group, there was obvious immunoregulation. PTH may enhance the colon barrier effect and the variety of intestinal microbiota, in order to prevent the movement of potentially hazardous intestinal substances to the liver, suppression of the TLR4/NF-κB, CXCL16/CXCR6 pathway, and activation of the TLR2 pathway could increase the expression of mTreg cells to secrete more Treg cells, thus limiting the expression of Th17 cells, reducing the creation of inflammatory substances, and achieving the purpose of treating AIH. These results suggest that PTH may balance intestinal microbiota and interfere with mTreg cells to regulate differentiated Treg cells, which is a natural alternative therapy for AIH (as shown in Figure 12).

Although our studies hoped to determine the mechanism of PTH treatment in mice with AIH, as a compound preparation with multiple and complex components, the definite effective ingredients of PTH in the treatment of AIH are ambiguous. Although specific bacteria had interactions with mTreg cells, these interactions were not defined. Crosstalk between the gut microbiota, mTreg cells, the TLR/CXCL16/CXCR6/NF-κB signaling pathway and PTH requires further investigation in future studies. It is very fortunate that PTH regulates intestinal microbiota balance and restores mTreg cells to alleviate experimental AIH, which is closely related to the TLR/CXCL16/CXCR6/NF-κB signaling pathway. These results will have directive significance and help to determine more immunomodulatory effects of PTH, to widen the scope of the clinical application of PTH treatment in AIH, and to develop new drugs related to PTH. In the future, we will plan to separate and co-cultured mTreg cells with PTH, or culture medium from PTH and the specific bacteria using a transwell microfluidic control chip, and to compare the effect of the inhibitor or agonist of the TLR/CXCL16/CXCR6/NF-κB signaling pathway on mTreg cells, and ultimately discover the definite action target and effective ingredients of PTH in the treatment of AIH.

ARTICLE HIGHLIGHTS

Research background

Autoimmune hepatitis (AIH) is a common and frequently-occurring interfacial hepatitis, and its etiology remains unknown. The interaction between regulatory T (Treg) cells and intestinal microbiota may provide a feasible therapeutic strategy for AIH.

Research motivation

As a well-known traditional Chinese medicine, Pien Tze Huang (PTH) is regularly used to treat liver conditions, including AIH. PTH has a physiological basis for controlling Treg cells and intestinal microbiota. However, it is unknown whether PTH can regulate intestinal microbiota and memory Treg (mTreg) cells to treat AIH.

Research objectives

To explore the mechanism of PTH treated AIH by determining the changes in the structure of intestinal microbiota and the level of mTreg cells.

Research methods

Following establishment of the mouse model of Concanavalin A (Con A)-induced AIH, prophylactic administration of PTH was given for 10 d. The levels of mTreg cells were measured by flow cytometry, and intestinal microbiota was analyzed by 16S rDNA analysis, while western blotting was used to identify the activation of the Toll-like receptor (TLR)2, TLR4/nuclear factor-κB (NF-κB), and CXCL16/CXCR6 signaling pathways.

Research results

In the present study, after PTH administration in mice with AIH, the expression of pro-inflammatory factors [such as tumor necrosis factor-alpha, interferon-γ, interleukin (IL)-1β, IL-2, IL-6, and IL-21] and Th17 cells was decreased, followed by improved pathological liver injury. In addition, the number of Treg/mTreg cells and IL-10 level were markedly increased, and the diversity of intestinal microbiota was regulated. Furthermore, activation of the TLR4/NF-κB, CXCL16/CXCR6 signaling pathway was suppressed in hepatic tissue.

Research conclusions

PTH effectively relieved pathological liver injury in mice with Con A-induced AIH, which was realized by improving intestinal microbiota balance and mTreg levels, and inhibiting the TLR/CXCL16/CXCR6/NF-κB signaling pathway.

Research perspectives

This research indicated that the mechanism of PTH treated mice with AIH was closely related to the regulation of intestinal microbiota balance and improving mTreg cell levels. These results provide scientific evidence for the application of PTH in AIH treatment and for the development of new drugs for AIH.

FOOTNOTES

Author contributions: Zeng X and Liu MH contributed to methodology, visualization, and validation of this study; Zeng X, Liu MH, Xiong Y, Zheng LX, and Guo KE were involved in the investigation of this manuscript; Xiong Y, Yin YT, Liu DY, and Zhou BG participated in the supervision of this manuscript; Xiong Y, Zhao HM, Yin YT, Liu DY, and Zhou BG contributed to the project administration and funding acquisition; Zhou BG and Yin YT contributed equally to this work as co-corresponding authors to collectively design, perform, analyze and complete the study and the paper, and to contribute efforts of equal substance throughout the research process.

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Institutional animal care and use committee statement: All experiments on animals (including the euthanasia of mice) were performed in accordance with the Institutional Animal Care and Use Committee's guidelines (Nanchang, China). The experimental protocols (Permit Number: JZLLSC20210090) were approved by The Jiangxi University of Chinese Medicine's Animal Care and Use Committee.

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

Data sharing statement: No additional data are available.

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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LETTER TO THE EDITOR

Intestinal barrier in inflammatory bowel disease: A bibliometric analysis

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Abstract

The primary objective of this investigation was to examine the evolving trajectories and pivotal focal points within the domain of research on intestinal barriers with regard to inflammatory bowel disease (IBD). Publications germane to the intestinal barrier in the context of IBD were procured from the Science Citation Index Expanded within the Web of Science Core Collection database. Bibliometric scrutiny and visualization were executed employing the R package "bibliometrix" through the R software platform (version: 4.3.0). A comprehensive compilation of 7344 English-language articles spanning from January 1, 2001 to December 31, 2021 was meticulously identified and included in the analysis. Remarkably, China emerged as the preeminent force in the realm of intestinal barrier research in relation to IBD. The significance of the intestinal barrier in the context of IBD has been progressively and comprehensively acknowledged. This recognition has ushered in a fresh therapeutic perspective that offers the promise of enhancing the management of inflammation and prognostication.

Key Words: Intestinal barrier; Inflammatory bowel disease; Bibliometric analysis; Bibliometrix; Gastroenterology

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Core Tip: The intricate architecture and operation of the intestinal mucosal barrier assume a pivotal role in maintaining the delicate equilibrium of both physiological and immune processes within the confines of the intestinal milieu. This study employed advanced bibliometric methodologies to investigate evolving paradigms and conspicuous research domains within the ambit of research on intestinal barriers with a specific focus on inflammatory bowel disease. The results reveal swift progress within the realm of intestinal barrier research characterized by a proliferation of expansive collaborative endeavors.

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TO THE EDITOR

We have recently examined the scholarly work authored by Zhou *et al*[1] titled "Intestinal barrier in inflammatory bowel disease (IBD): A bibliometric and knowledge-map analysis". The primary objective of this study was to consider the evolving trends and focal points within the realm of intestinal barrier research in the context of IBD. Given that barrier surfaces, which are composed of specialized epithelial cells, serve as the critical demarcation between the host's internal milieu and the external environment and are indispensable for the preservation of proper physiological and immunological equilibrium, we acknowledge the profound significance of this investigation[2-4]. Nevertheless, we wish to proffer a series of thoughtful recommendations pertaining to the information retrieval methodologies employed in this study.

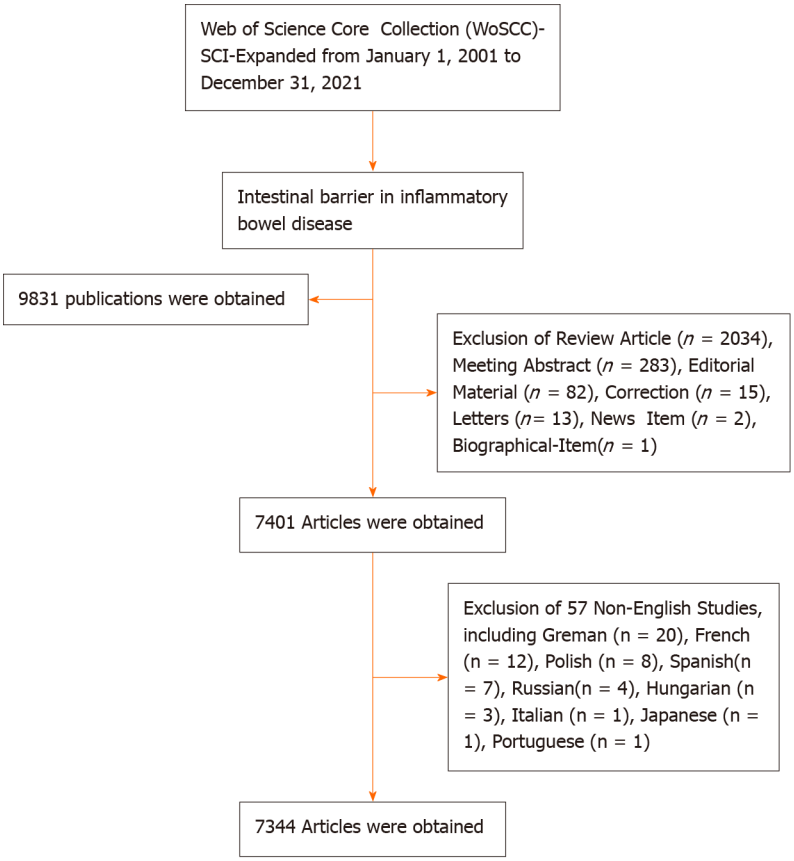
First and foremost, within the domain of bibliometric inquiries, the meticulous formulation of search strategies assumes paramount importance. The authors have duly noted their utilization of the Web of Science Core Collection (WoSCC) as the primary source of original data, a judicious choice in our estimation. However, it is worth noting that WoSCC encompasses at least 10 subdatabases, including the Science Citation Index Expanded (SCI-Expanded), Social Sciences Citation Index, Emerging Sources Citation Index, Arts and Humanities Citation Index, Conference Proceedings Citation Index-Science, Current Chemical Reactions, Conference Proceedings Citation Index-Social Science and Humanities, Book Citation Index-Science, Index Chemicus, and Book Citation Index-Social Sciences and Humanities. In our view, it may not be appropriate to incorporate all of these subdatabases for the retrieval of eligible articles. For instance, utilizing the retrieval formula employed by the authors, no relevant studies could be located in AHCI, CCR-EXPANDED, and IC[5,6]. Consistent with this perspective, some scholars also contend that it is inappropriate to employ such a diverse array of databases that vary in type and level within a singular bibliometric analysis[7,8]. Among these, SCI-Expanded stands out as the most fitting and widely accepted choice for conducting bibliometric analyses[9,10].

Another pivotal aspect that warrants careful consideration pertains to the appropriateness of topic search (TS) for bibliometric endeavors. The "TS" approach, by design, designates a publication as relevant when the search term appears in the "Title (TI)," "Abstract (AB)," "Author keywords (AK)," or "Keywords plus (KP)." It is imperative to determine that "KP" is generated through WoSCC's automated computational algorithms independent of author input. Consequently, the inclusion of "KP" during the search process may inadvertently include a multitude of extraneous publications[11]. In our experience, the prudent course of action involves relying on "TI," "AB," and "AK" as qualifiers to ensure a more precise and semantically meaningful dataset for bibliometric analysis.

Furthermore, it is of paramount importance to recognize that the effectiveness of a search formula hinges on its comprehensiveness because an overly simplistic approach may unintentionally omit pertinent publications. In the study by Zhou *et al*[1], the authors' reliance on the phrases "IBD" OR "ulcerative colitis" OR "Crohn's disease" is insufficient for capturing the entirety of relevant research. Additionally, in Zhou *et al*'s study, the publication period spanned from 2001 to 2021[1]; however, the exact commencement and conclusion dates were not provided. Moreover, there is some inconsistency in the description of the screening timeframe, with the Methods section indicating 2001-2021 while the AB and results sections mention 2002-2022. Hence, further clarification regarding the precise timeframe for the inclusion of literature is warranted.

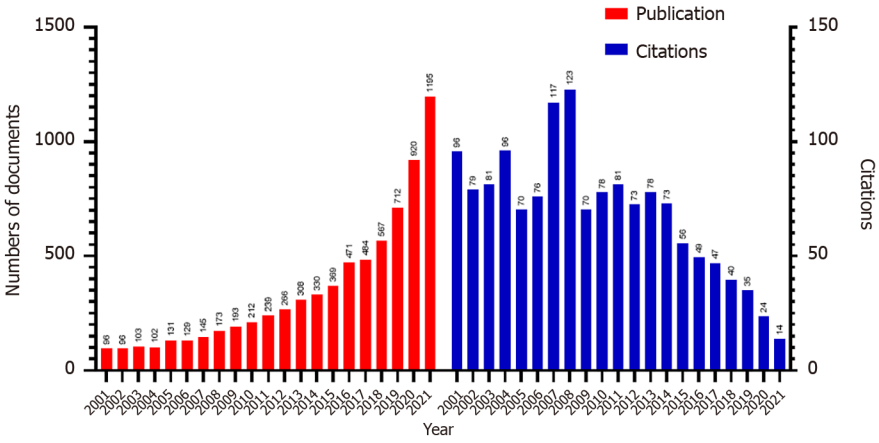
As part of our contribution to refining the retrieval strategy, we posit that it is prudent to incorporate synonymous terms and nomenclature associated with "IBD". In our opinion, the authors should additionally incorporate the subsequent terms into the search equation: "Intestinal inflammation" and "gut inflammation." Similarly, for "intestinal barrier," the search formula included "intestinal barrier", "gut barrier", "mucosal barrier," "intestinal permeability", "epithelial barrier", "intestinal tight junction", "intestinal epithelium", and "intestinal integrity". Furthermore, numerous terms exhibit both plural and singular variations. The authors could consider employing various wildcards, such as "*", which signifies that it can replace any number of characters. For instance, "IBD*" would return the terms "IBD" and "IBDs". The flowchart of the literature screening is indicated in Figure 1. Our suggested retrieval formula is summarized in Supplementary material.

The refined search query, incorporating a more extensive array of relevant terminologies, undeniably facilitated a more exhaustive exploration of the pertinent literature. Our search, conducted from January 1, 2001 to December 31, 2021, retrieved a total of 9831 records. After the careful exclusion of literature of diverse types and non-English studies, we ultimately preserved 7344 articles for our analysis. The annual publication trends are elucidated in Figure 2 and the corresponding author's countries and country scientific production are manifested in Figure 3.



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Figure 1 Flowchart of literature screening.

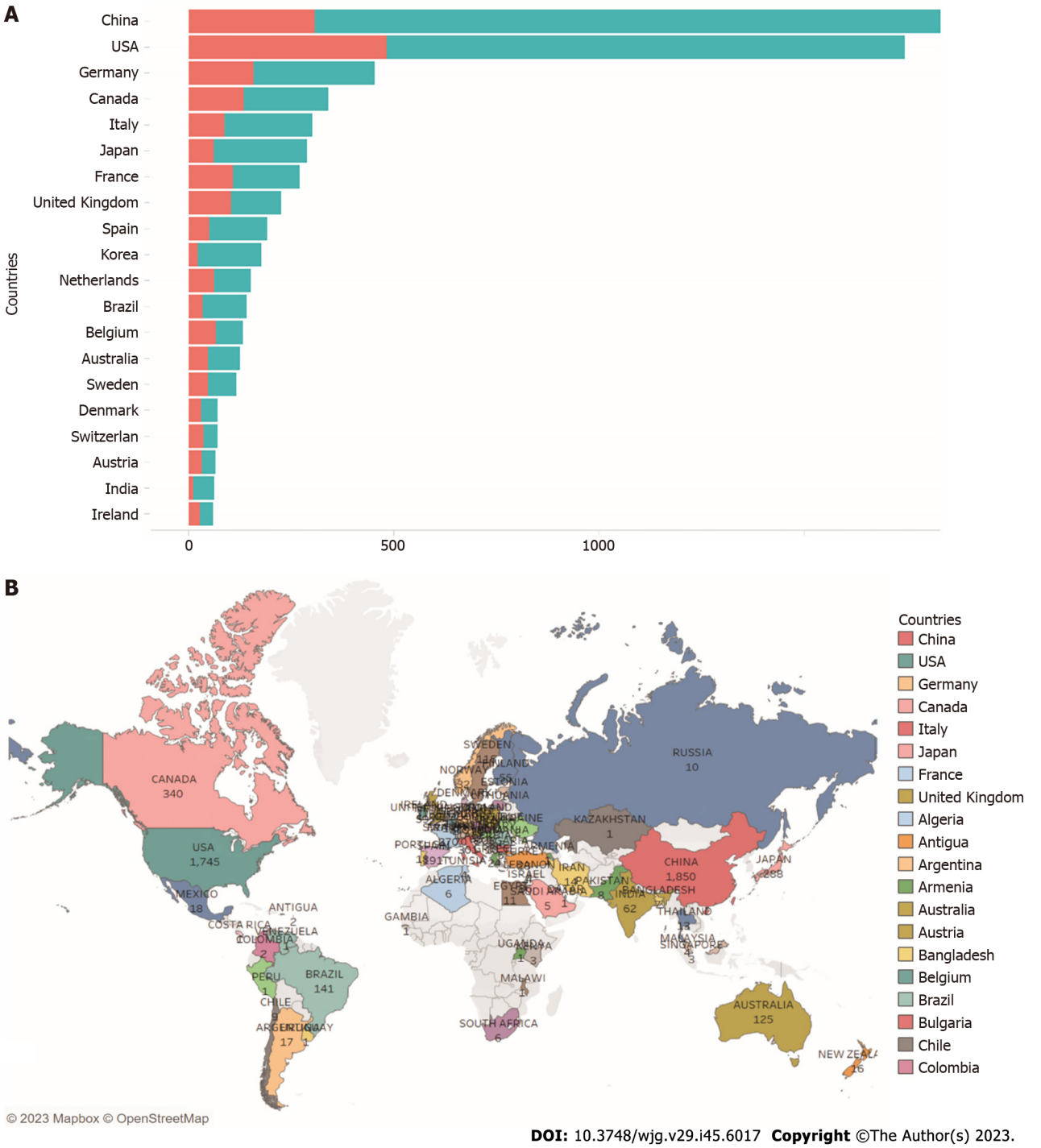


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Figure 2 Annual publication trends and country publications.

In contrast to the findings presented by Zhou *et al*[1], our study, which used a more refined retrieval scope focusing on "TI/AK/AB," identified a larger pool of related studies on the intestinal barrier in IBD (7344 *vs* 4482). It is noteworthy that such a substantial change in the number of publications can exert a significant impact on various quantitative data points, including those related to the most prolific countries (as depicted in Figure 3), institutions, and authors. Therefore, to mitigate potential bias, it is imperative to carefully formulate an appropriate retrieval formula when conducting bibliometric analyses. From our perspective, seeking expert consultation to tailor search keywords to the specific domain of study is an essential and prudent step.

It is crucial to underscore that the significant fluctuations in publication numbers can wield a profound influence on various quantitative aspects, including publication counts, citations, the most prolific countries/regions, institutions, authors, cited authors, journals, cited academic journals, keyword co-occurrence, clusters, and bursts. This underscores the critical importance of meticulously devising an appropriate retrieval formula, which serves as the bedrock for unbiased bibliometric analysis. In our considered view, soliciting expert consultation on search keywords tailored to the



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Figure 3 Corresponding author's countries and country scientific production. A: Corresponding author's countries; B: Country scientific production. The data were analyzed using R package "bibliometrix" by R software (version: 4.3.0).

specific domain of study is an indispensable step in this process.

In conclusion, while we extend our commendations to Zhou *et al*[1] for their meticulous work, we firmly believe that our proposal complements the precision and accuracy of the data analysis of trends in research on "intestinal barrier in IBD" during the last two decades.

FOOTNOTES

Co-corresponding authors: Yan-Dong Miao and Fang Zhang.

Author contributions: Miao YD contributed to conceptualization, manuscript writing, review, and editing, funding acquisition, and project administration; Zhang F contributed to conceptualization, manuscript writing, review, and editing, and supervision; Luan WY wrote the original draft; Yang Z, Chen XD, and Zhang TT performed data analysis and prepared the figures. Miao YD and Zhang F were

designated as co-corresponding authors. Miao YD was responsible for the formulation or evolution of overarching research goals and aims, specifically critical review, commentary, or revision in both pre- or post-publication stages, management and coordination for the research activity planning and execution, and acquisition of the financial support for the project leading to this publication, while Zhang F was responsible for reviewing and editing the draft, oversight, and leadership for the research activity planning and execution, including mentorship external to the core team. This designation reflects their equal contributions and shared responsibility in overseeing the project and correspondence related to this research. All authors approved the final manuscript.

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EDITORIAL

Emerging space for non-polyethene-glycol bowel preparations in inflammatory bowel disease-related colonoscopy: Veering toward better adherence and palatability

Raffaele Pellegrino, Antonietta Gerarda Gravina

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Abstract

Patients with inflammatory bowel diseases (IBDs) require repeated endoscopic evaluations over time by colonoscopy to weigh disease activity but also for different and additional indications (*e.g.*, evaluation of postoperative recurrence, colorectal cancer surveillance). Colonoscopy, however, requires adequate bowel preparation to be of quality. The latter is achieved as long as the patient takes a certain amount of product to have a number of bowel movements suitable to clean the colon and allow optimal visualization of the mucosa during endoscopy. However, significant guidelines recommend preparations for patients with IBD not excelling in palatability. This recommendation originates from the fact that most of the studies conducted on bowel preparations in patients with IBD have been done with isosmolar preparations based on polyethylene glycol (PEG), for which, therefore, more safety data exist. As a result, the low-volume non-PEG preparations (*e.g.*, magnesium citrate plus picosulphate, oral sulphate solutions) have been set aside for the whole range of warnings to be heeded because of their hyperosmolarity. New studies, however, are emerging, leaning in overall for a paradigm shift in this matter. Indeed, such non-PEG preparations seem to show a particularly encouraging and engaging safety profile when considering their broad potential for tolerability and patient preference. Indeed, such evidence is insufficient to indicate such preparations in all patients with IBD but may pave the way for those with remission or well-controlled disease. This article summarizes the central studies conducted in IBD settings using non-PEG preparations by discussing their results.

Key Words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Bowel preparation; Colonoscopy; Polyethylene glycol; Low-volume

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Core Tip: Preparations based on polyethylene glycol (PEG) are most recommended for patients with inflammatory bowel disease (IBD) undergoing colonoscopy. However, these solutions are not always palatable because they often require the intake of large volumes of solution, making it difficult for the patient to complete the entire preparation. This leads to a reduction in the quality of the endoscopic examination. Low-volume non-PEG-based, although excluded in the major guidelines for patients with IBD, are emerging as potentially safe in this setting, especially in remission or mild disease conditions.

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INTRODUCTION

Quality colonoscopy in the inflammatory bowel disease (IBD) patient has adequate bowel preparation as an essential prerequisite. Colonoscopy is, in fact, an imperative examination of IBD and encompasses a long list of indications, among which initial diagnosis, patient follow-up to weigh response to therapy, and colorectal cancer surveillance are recognized [1-4]. Bowel preparation requires the patient to take an oral formulation of varying constitution and volume to obtain a functional number of bowel movements to determine the cleanliness of rectal, colonic and ileal segments that can be explored by colonoscopy[5]. However, new retrograde modalities are emerging (*i.e.* colonic lavage)[6]. The pharmacodynamics of bowel preparations require patient compliance so that the patient ingests the entire formulation and follows any complementary indications (*i.e.* a low-fiber diet).

As a result, it tends to follow that patients' preferred preparations are those with lower volume, good taste (palatable), suitable dose dilution over time (*e.g.*, split dose), and good safety profile such that they do not experience symptoms while taking the product[7].

However, the widely recommended preparations for patients with IBD are low (< 3 L) or high volume (at least 3 L) polyethylene glycol (PEG)-based preparations[5]. This choice, while not axiomatically pursuing the goal of palatability, is necessary because most studies in IBD are available for this type of preparation and because safety studies have shown a significantly better safety profile for PEG solutions than for non-PEG-based solutions (*e.g.*, magnesium citrate plus picosulphate, oral sulfate solutions)[8].

This paradigm, which has been going on for several years, has the potential to change the view of the new studies that are increasingly emerging on non-PEG-based and low-volume solutions.

WHAT PROFILE DO NON-PEG SOLUTIONS HAVE IN IBD?

Non-PEG preparations ordinarily have osmotic power, which partly affects their safety profile compared to isosmolar PEG solutions, especially for electrolyte disturbances[5,9] and mucosal damage[5]. PEG preparations are based on the principle that they, being nonabsorbable and isosmolar fluids, result in minimal electrolyte and fluid absorption or secretion by the gastrointestinal wall. Therefore, to achieve a purgative effect, they must generally be used in large volumes[10]. The opposite principle applies to non-PEG preparations in that their cleansing effect is based precisely on osmotic potency rather than on accumulating nonabsorbable fluids[10]. Despite this, in a general sense, low-volume non-PEG preparations have several potential advantages for the patient employing them for colonoscopy.

First and foremost, these preparations do not have the disadvantage of requiring large volumes of preparation associated with lower adherence and patient preference or gastrointestinal overload symptoms (*e.g.*, nausea, vomiting, abdominal pain) that often prevent completion of the preparation[11]. Additionally, PEG solutions are often poorly palatable due to their salty taste[12]. The more excellent tolerability of non-PEG low-volume solutions compared with PEG-based preparations has also been demonstrated in a large meta-analysis of more than 20 randomized placebo-controlled trials in which, in terms of efficacy, these were also found to be non-inferior to PEG solutions[13].

However, several studies are already available for non-PEG low-volume preparations in IBD (Table 1). These studies have produced compelling results in both clinical trial settings and real-world ones on such bowel preparation efficacy/effectiveness rates and safety.

For example, the prospective CLEAN study in 2019 provided some results about sodium, magnesium and potassium sulphate (trisulphate solutions), sodium phosphate and sodium picosulphate preparations in 119 IBD patients[14]. In all the above regimens, patients had followed a split regimen and a low-fiber diet before colonoscopy in more than half of the cases. In only 18 cases, however, the indication was a flare-up of IBD, and, as a result, most of the patients had a Mayo endoscopic subscore[15] of less than 2 (*i.e.* endoscopically inactive IBD). Picosulphate preparation showed, in this study, a higher mean Boston bowel preparation scale[16] than 2 or 4 L PEG solutions (*i.e.* increased cleaning capacity). The safety profile, within the limit of low numbers, was acceptable in comparison with PEG preparations. Regarding tolerability, however, sodium picosulfate showed substantially more excellent palatability as well as less nausea, vomiting, and bloating.

Table 1 Summary of studies that examined non-polyethylene glycol-based bowel preparations in patients with inflammatory bowel diseases

Ref.	Bowel preparation	IBD sample size	IBD type, *n*	Sample age	IBD duration	Disease activity trend	Split regimen as Y/N or %	Clear diet as Y/N or %
Briot *et al* [14], 2019	MPS, NaP, PicoS	12 (MPS), 26 (NaP), 80 (PicoS)	CD: 8 (MPS), 14 (NaP), 57 (PicoS); UC: 4 (MPS), 11 (NaP), 21 (PicoS); Undetermined IBD: 1 (NaP), 2 (PicoS)	Median: 42.9 (MPS), 41 (NaP), 40.8 (PicoS)	13.9 yr (MPS), 12.9 yr (NaP), 10.6 yr (PicoS)	Remission, mild	58.3% (MPS), 84.6% (NaP), 55.6% (PicoS)	100% (MPS), 100% (NaP), 95.1% (PicoS)
Mohsen *et al*[17], 2021	PicoS + magnesium citrate + PEG	61	N/A	Mean: 39.7	N/A	Unknown	Y	Y
Rueda García *et al* [18], 2023	PicoS	31	CD: 18, UC: 12: Undetermined IBD: 1	Mean: 51.7	N/A	Mild	Y	Y
Kim *et al* [19], 2022	OST	55	CD: 18, UC: 37	Mean: 44.4	93.2 mo	Remission, mild	Y	Y
Lee *et al* [20], 2023	OSS	92	UC: 92	Mean: 47.9	7 yr	Remission, mild	Y	Y

CD: Crohn's disease; IBD: Inflammatory bowel disease; MPS: Sodium, magnesium, and potassium sulphates; N: No; N/A: Not applicable; NaP: Sodium phosphate; PEG: Polyethylene glycol; PicoS: Sodium picosulphate; OSS: Oral sulphate solution; OST: Oral sulphate tablet; UC: Ulcerative colitis; Y: Yes.

Mohsen *et al*[17] also later weighed the efficacy and safety of an osmotically active solution of sodium picosulphate and magnesium citrate (with PEG added) in 56 patients with IBD. These determined less abdominal pain than the PEG-ascorbate comparison, albeit with a greater serum increase in magnesium and no significant differences in efficacy and safety between the groups. This study, however, did not operate a particular stratification by colonoscopy indication type and IBD activity. Another trial (*i.e.* EII-PREP) showed promising results regarding sodium picosulphate but, unfortunately, in only 31 IBD patients[18].

In a subsequent trial targeting patients with inactive IBD, Kim *et al*[19] reported a lower bubble score rate in the novel oral sulphate table preparations and higher palatability (including a willingness to reuse the preparation for subsequent colonoscopy) than the PEG-ascorbate comparison. The problem of bubbles is another technical aspect to consider when discussing mucosal visibility, so much so that the use of simethicone for this purpose is bleached out by European guidelines and also found beneficial for IBD[5,8].

The safety and efficacy profile was comparable. Lee *et al*[20] also reported a similar efficacy and safety profile (even considering changes in serum electrolytes) between novel oral sulphate solution and PEG-ascorbate in 92 patients with inactive ulcerative colitis.

WHAT PROBLEMS PLAGUE NON-PEG PREPARATIONS, AND WHAT IS THE MISSING PIECE OF THE PUZZLE TO ALLOW THEM FULL USE IN IBD?

Studies evaluating non-PEG preparations, specifically in the population with IBD *vs* the counterpart PEG preparations, have shown some homogeneity in reporting good palatability, tolerability, and safety results.

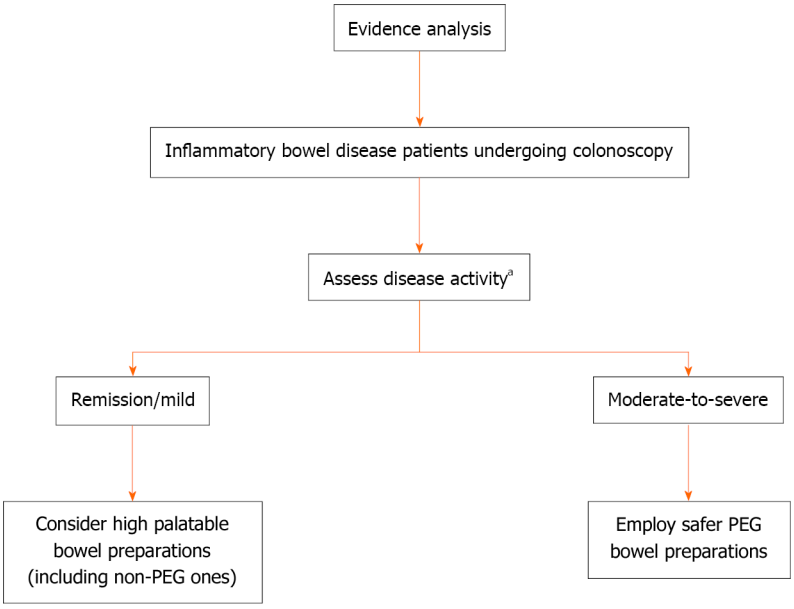
However, there are some drawbacks to consider. Such studies are generally conducted in low sample size settings. This phenomenon poses the problem of requiring more and more evidence from studies with larger samples and, therefore, greater statistical power and generalizability. The absence of recommendations for non-PEG preparations by major guidelines also limits the use of these preparations in clinical practice and thus counteracts the conduct of real-world studies weighing the real-life effectiveness of these preparations by adding new data[5].

In addition, in the available studies, the different disease activity IBD subgroups are not well represented, sometimes unreported and not always weighted by scores widely recognized as usable in IBD (*e.g.*, partial and total Mayo scores, Crohn's disease index of severity, Harvey-Bradshaw index[15]).

Moreover, most available studies enrolled patients with mild or no endoscopic activity. This severely limits interpretation in cases of moderate and moderate-severe endoscopic activity. Indeed, the problem does not arise for severe acute activity since, in those cases, at most, rectoscopy is enough to perform endoscopic evaluation and exclude over-infections (*e.g.*, cytomegalovirus).

One of the reasons for European guidelines to deny the indication of non-PEG solutions in IBD is that they have been described to be associated with a risk of mucosal damage about ten times higher than PEG solutions[5].

However, this recommendation, although seemingly supportable, encapsulates in the definition of IBD the broad spectrum of different degrees of endoscopic activity. Therefore, a conditional stratification of recommendations that



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Figure 1 Stratification hypothesis on the indication of non-polyethylene glycol-based bowel preparations in patients with inflammatory bowel diseases. [a]Disease activity should be determined by instruments explicitly designed for inflammatory bowel diseases (*e.g.*, partial Mayo score, Crohn's disease index of severity, Harvey-Bradshaw index). PEG: Polyethylene glycol.

considers a balance of the efficacy/safety profile for individual indications for colonoscopy should probably be advocated. In other words, one cannot treat an indication for assessment of disease activity (perhaps in a patient with moderate-severe clinical and biochemical activity) and an oncologic surveillance colonoscopy performed in a long-term remission setting in the same way. In the former case, safety weighs more than efficacy since the primary purpose of the endoscopist is to consider disease activity to select treatment, and the mucosal status is already objectively compromised variably. In the second case, where the disease is already well controlled, and the patient has a colon much closer to that of the healthy population, it is undoubtedly efficacy a parameter to observe (especially in patients without safety concerns) as a colonoscopy with a lower quality of bowel preparation may significantly reduce the adenoma detection rate[21]. This evaluation becomes even more relevant compared to high-volume centers performing high-quality chromoendoscopy for surveillance colonoscopy in IBD patients.

A large proportion of patients with IBD receive the diagnosis at a young age and are largely destined for repeated endoscopic evaluations over time. The most recent treat-to-target strategies[22] place endoscopic outcomes among the pillars for eventual IBD treatment discontinuation or switch. For such patients, therefore, under conditions where there are no particular safety red flags, palatable bowel preparations should be reserved, and research should move in this direction. The current evidence, even better if evaluated meta-analytically, could potentially argue for a modification of the recommendations in future guideline updates, probably by including those with IBD in stable remission, or at most in already known mild endoscopic activity, in the pool of patients who can take advantage of low-volume non-PEG preparations. These evaluations are tentative and need to be weighed by *ad hoc* meta-analyses that consider the efficacy-safety ratio of non-PEG low-volume solutions against the current standard of care to assess whether the formulation of recommendations (even with low evidence level) is possible.

CONCLUSION

New evidence has emerged regarding using non-PEG-based bowel preparations for colonoscopy in patients with IBD. Although limited by initial data, these preparations appear to possess sustainable and potentially safe use in patients with IBD in remission or with mild activity with no particular safety concerns of adverse events or post-colonoscopy flare-ups. However, net of this, major guidelines, including the European ones, indicate and recommend only PEG solutions for patients with IBD, encompassing all patients with IBD in this indication without making stratifications based on disease activity. Future guidelines for bowel preparation should weigh whether the currently available studies allow such stratification (Figure 1) by opening the possibility for patients with well-controlled IBD to the use of more palatable preparations for better patient compliance, greater validity of colonoscopy with adequate bowel preparation, and ultimately for greater patient comfort.

FOOTNOTES

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REVIEW

Frailty in end-stage liver disease: Understanding pathophysiology, tools for assessment, and strategies for management

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Abstract

Frailty and sarcopenia are frequently observed in patients with end-stage liver disease. Frailty is a complex condition that arises from deteriorations across various physiological systems, including the musculoskeletal, cardiovascular, and immune systems, resulting in a reduced ability of the body to withstand stressors. This condition is associated with declined resilience and increased vulnerability to negative outcomes, including disability, hospitalization, and mortality. In cirrhotic patients, frailty is influenced by multiple factors, such as hyperammonemia, hormonal imbalance, malnutrition, ascites, hepatic encephalopathy, and alcohol intake. Assessing frailty is crucial in predicting morbidity and mortality in cirrhotic patients. It can aid in making critical decisions regarding patients' eligibility for critical care and transplantation. This, in turn, can guide the development of an individualized treatment plan for each patient with cirrhosis, with a focus on prioritizing exercise, proper nutrition, and appropriate treatment of hepatic complications as the primary lines of treatment. In this review, we aim to explore the topic of frailty in liver diseases, with a particular emphasis on pathophysiology, clinical assessment, and discuss strategies for preventing frailty through effective treatment of hepatic complications. Furthermore, we explore novel assessment and management strategies that have emerged in recent years, including the use of wearable technology and telemedicine.

Key Words: End-stage liver disease; Frailty; Liver cirrhosis; Malnutrition; Sarcopenia

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Core Tip: Frailty is a common condition in patients with cirrhosis, and it is associated with increased morbidity and mortality. This review provides a comprehensive overview of the etiology, pathophysiology, assessment, and management of frailty in cirrhosis. It places particular emphasis on the management of frailty during complications, while also delving into the future of managing this condition.

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INTRODUCTION

Frailty is a complex and ever-evolving syndrome that arises from a combination of deteriorating events across various physiological systems. This results in a reduction in the body's physical ability to withstand stressors, leading to decreased resilience and increased susceptibility to adverse outcomes such as disability, hospitalization, and mortality[1-3]. Half of the patients diagnosed with cirrhosis of any cause exhibit frailty[4]. This condition has been shown to have a significant impact on the health outcomes of individuals with liver diseases, leading to higher rates of morbidity and mortality[5]. Despite its importance, there is still a lack of knowledge in the field of frailty in liver cirrhosis and an absence of universal assessment and treatment protocols, especially in the presence of decompensation with ascites and encephalopathy. This review offers an analysis of the pathophysiological mechanisms that give rise to the clinical manifestations of frailty, various assessment tools employed to evaluate frailty in patients with cirrhosis, and highlights the management options available for frail cirrhotic patients including future therapeutic options for this condition.

DEFINITION OF FRAILTY

Although frailty, sarcopenia, malnutrition, and cachexia are distinct terms in literature, they are deeply interconnected [6]. All four terminologies can be used to express patients with muscle loss and measures targeting management of any of these conditions can improve the others[7]. Here is a breakdown of each terminology (Table 1).

PATHOPHYSIOLOGY OF FRAILTY IN END-STAGE LIVER DISEASE

The precise reason for frailty in liver disease is not known, but it is thought to result from the failure of several organ systems, including the neuromuscular, endocrine, immune, and skeletal muscle systems[17] (Figure 1). In this section, we outline a brief overview of different factors that have been cited in literature as potential causes of frailty in cirrhosis[17,18].

Hyperammonemia

Hyperammonemia is a major factor that induces sarcopenia and frailty in patients with liver cirrhosis[19-21]. There are multiple mechanisms through which hyperammonemia can contribute to frailty in individuals with cirrhosis. These mechanisms include upregulation of myostatin expression inhibiting skeletal muscle mass, mitochondrial dysfunction, heightened production of reactive oxygen species that impede protein synthesis, and enhanced proteolysis[22,23].

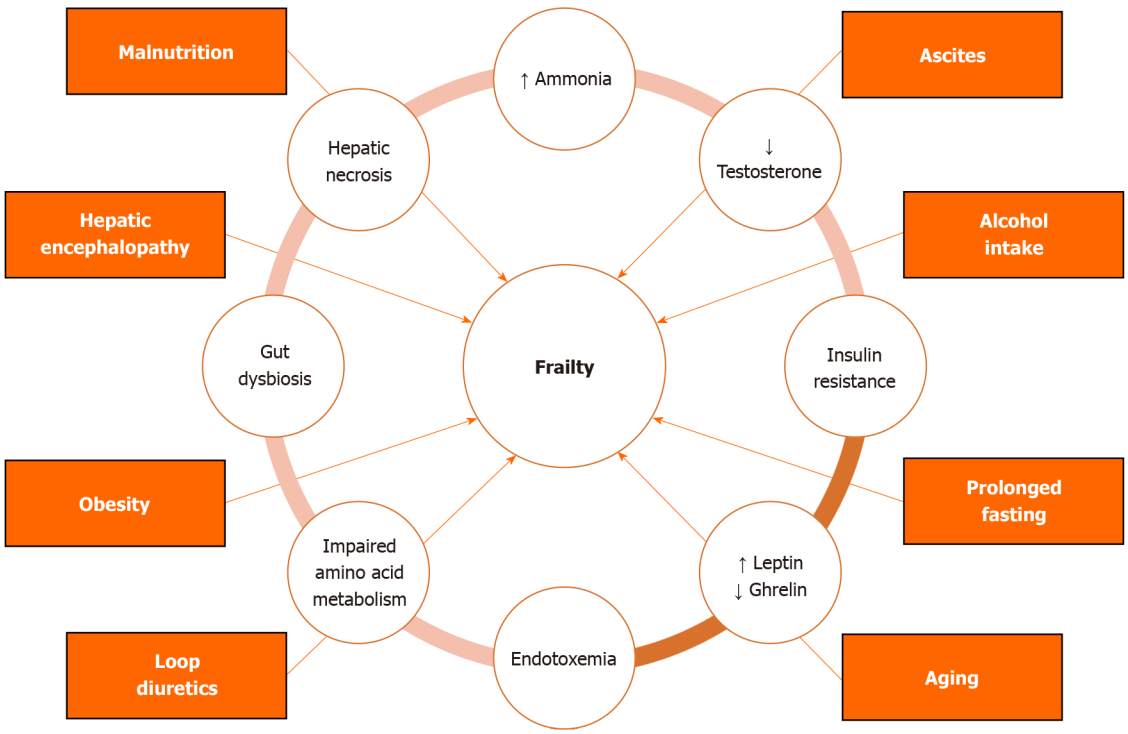
Hormonal disturbance

Testosterone plays a crucial role in activating the Akt/mTOR pathway, which is responsible for the development and maintenance of muscle tissue[24]. Individuals with cirrhosis often experience low levels of circulating testosterone. This can be attributed to the detrimental effects of cirrhosis on the hypothalamic-pituitary-gonadal axis[25]. Additionally, an increased activity of the hepatic aromatase enzyme converts testosterone into estrogen, further contributing to the decline in testosterone levels and subsequent loss of muscle mass[26]. Moreover, research has shown that androgens suppress the expression of myostatin, a key inhibitor of muscle mass[27,28]. This explains the elevated levels of myostatin in cirrhosis patients which contributes to their muscle mass loss[29].

Insulin resistance is a common occurrence in individuals with cirrhosis, as supported by the literature[30]. Insulin resistance can lead to muscle loss through several mechanisms. Insulin has anabolic effects on muscle tissue. But, when there is insulin resistance, it reduces its ability to stimulate protein synthesis, resulting in muscle mass loss[31]. Insulin resistance can also impair mitochondrial function, leading to decreased energy availability and further promoting muscle loss[32]. Finally, individuals with insulin resistance may be less physically active, which can cause muscle atrophy and a decline in muscle function[33]. All these factors contribute to the development of muscle mass loss and frailty in patients with cirrhosis.

Table 1 Definition of frailty, different types of sarcopenias, malnourishment, and cachexia

	Definition
Frailty	A condition where patients undergo a reduction in their physical abilities and become more vulnerable to health-related challenges, leading to negative health consequences. It is a multifaceted concept that involves different aspects such as physical, psychological, social, and environmental factors[8]
Malnourishment	An imbalance in the consumption of nutrients, whether it be a deficiency or an excess, can have detrimental effects on the body's tissues and overall physical form[9]
Cachexia	A metabolic syndrome that is complex and linked to an underlying illness. It is distinguished by the reduction of muscle mass, with or without a decrease in fat mass[10]
Sarcopenia	A debilitating syndrome that is marked by a gradual and widespread decline in both skeletal muscle mass and strength[11]
Dynapenia	The pre-sarcopenia stage, in which only muscle strength is reduced[12]
Primary sarcopenia	The loss of anatomical skeletal muscle mass in the aging population[13]
Secondary sarcopenia	The loss of skeletal muscle mass in various chronic diseases[14]
Compound sarcopenia	The combination of primary (*i.e.*, age-related) and secondary (*i.e.*, disease-related) sarcopenia. It occurs in older patients with chronic diseases[15]
Sarcopenic obesity	A state of decreased muscle mass in the setting of increased fat mass. The muscle wasting can be obscured by increased muscle mass, making specialized testing and management necessary[16]



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Figure 1 Pathophysiology of frailty in advanced liver disease.

Leptin and ghrelin are two hormones that play a crucial role in regulating energy balance, appetite, and body weight [34]. Leptin, which is produced mainly by adipose tissue, functions by suppressing hunger and promoting satiety[35]. In individuals with cirrhosis, there is typically an elevation in serum leptin levels, which can contribute to frailty by decreasing appetite, altering energy balance, and causing muscle wasting[36]. Conversely, ghrelin, which stimulates hunger, gastric motility, and gastric acid secretion, is found to be reduced in liver cirrhosis compared to healthy individuals[37,38]. These changes can affect satiety, digestive functions, and muscle mass, which predispose individuals with cirrhosis to frailty.

Proinflammatory mediators

Liver diseases trigger proinflammatory mediators release due to hepatic necrosis and endotoxemia. In advanced liver disease, the death of liver cells triggers the recruitment of immune cells such as macrophages and neutrophils[39]. This

leads to an increase in the production of substances promoting inflammation, such as cytokines, resulting in inflammation both locally and systemically[40]. Additionally, patients with cirrhosis have elevated levels of proinflammatory mediators due to endotoxemia[41]. This occurs when bacterial endotoxins circulate in the bloodstream due to impaired gut barrier function[42], portosystemic shunts[43], compromised Kupffer cell function[44], and altered gut microbiota [45]. The presence of endotoxemia triggers the release of tumor necrosis factor-alpha, interleukin-6, and reactive oxygen species which promote systemic inflammation, cause oxidative stress, and impair mitochondrial function[43,46]. The presence of these proinflammatory mediators can give rise to various frailty components, including muscle wasting, loss of appetite, fatigue, energy deficiency, and impaired immune function[18].

Gut microbiota dysbiosis

The human gut is a complex ecosystem that harbors a diverse array of microbial species, which engage in multifaceted interactions with our body[47]. One of the key benefits provided by the gut microbiota is their remarkable ability to ferment indigestible substances, such as dietary fibers, yielding short-chain fatty acids (SCFAs) like acetate, propionate, and butyrate. Moreover, the gut microbiota plays a pivotal role in generating a wide range of metabolites, including secondary bile acids and indols[48]. These metabolites, along with bacterial components, can circulate throughout the human body, exerting their influence on organs beyond the gut itself[49]. In the context of cirrhosis, there is a notable prevalence of gut dysbiosis, which involves an imbalance in the gut microbiota. This imbalance is marked by a decrease in the diversity of bacterial species and an overall reduction in the number of bacteria present in the gut[50,51].

In cirrhotic patients, dysbiosis plays a significant role in frailty development through various mechanisms[52-55]. Firstly, the presence of a high proportion of harmful bacteria in the gut can lead to systemic inflammation as bacterial lipopolysaccharides translocate through the compromised gut barriers[56,57]. This inflammation can cause muscle wasting and energy deficiency, further contributing to frailty. Secondly, individuals with cirrhosis often have a lower abundance of beneficial gut bacteria, which reduces their capacity to contribute to metabolism. This reduction leads to a decrease in the production of SCFAs, which are not only essential as an energy source but also serve as metabolic mediators in skeletal muscles[58-60]. The decrease in SCFAs may contribute to the observed skeletal muscle loss in cirrhotic patients and further exacerbate the development of frailty[61]. Lastly, small intestinal bacterial overgrowth (SIBO) is common in cirrhosis, and the increased population of gut microbes can lead to heightened competition with the human body for nutrients[62]. This competition can worsen the loss of muscle mass and frailty, as evidenced by the link between SIBO and malnutrition in cirrhosis[63].

Impaired amino acids metabolism

Amino acid metabolism is a crucial process that plays a vital role in maintaining energy balance, promoting muscle growth, and regulating critical processes such as inflammation and insulin sensitivity[64,65]. When this process is disrupted, it can lead to the development of frailty and degenerative diseases[66]. Individuals with liver cirrhosis experience imbalances in body metabolism, resulting in higher levels of fatty acid oxidation and gluconeogenesis. This leads to an imbalance in amino acid metabolism, causing changes in amino acid levels in the blood[67,68]. As a consequence, there are low levels of branched-chain amino acids (BCAAs) in the blood, which are essential for muscle tissue. BCAAs are crucial for protein synthesis in skeletal muscles and serve as a preferred source of energy. Therefore, low levels of BCAAs in the blood can lead to muscle degradation and impaired muscle strength[69]. Moreover, patients with end-stage liver disease (ESLD) have impaired ammonia detoxification in the liver, leading to high levels of ammonia in the blood. To compensate for this, the body breaks down BCAAs stored in skeletal muscles to help eliminate ammonia [70]. This constant consumption of BCAAs further contributes to low levels of BCAAs in the blood, leading to sarcopenia and frailty. Fortunately, supplementing with BCAAs has shown significant benefits for individuals with liver cirrhosis and frailty. In a recent 16-wk randomized controlled trial, BCAA supplementation was found to improve the liver frailty index (LFI), serum albumin levels, and quality of life in frail compensated cirrhotic patients[69]. This finding is consistent with previous studies that have used BCAAs as a therapy for sarcopenia in liver cirrhosis[71,72].

PREDISPOSING FACTORS FOR FRAILTY IN CIRRHOSIS

Malnutrition

Cirrhosis often leads to malnutrition, which is associated with negative consequences[73]. Malnutrition in cirrhosis has various contributing factors, including inadequate oral intake, early satiety caused by ascites, imbalance in ghrelin and leptin levels, maldigestion, malabsorption, gut microbial dysbiosis, and encephalopathy[73,74]. Frailty is strongly associated with malnutrition. Research has shown that malnourished patients have a 3.381 times higher risk of developing frailty compared to well-nourished patients[75].

Ascites

In cirrhotic patients, ascites can cause several symptoms such as loss of appetite, difficulty moving, reduced stomach capacity, and poor digestion[76] (Table 2). These symptoms can ultimately result in malnutrition and frailty. However, therapeutic paracentesis has been shown to effectively counteract these effects and provide several benefits for cirrhotic patients, including improving appetite, decreasing feelings of fullness, enhancing caloric intake, and improving exercise tolerance[77]. Large-volume paracentesis can increase fasting gastric volumes, which leads to better tolerance of nutrient ingestion and improved caloric intake[78]. This could potentially be explained by the procedure providing more space for

Table 2 Pathophysiology, effects, and management recommendations for frailty predisposing factors in cirrhosis

Predisposing factor	Pathophysiology	Morbidity and mortality	Recommendations
Ascites	Loss of appetite; Difficult ambulation; Reduced stomach capacity; Poor digestion	Odds of frailty were higher in ascitic than non-ascitic patients [adjusted odd ratio 1.56, 95% confidence interval (CI): 1.15-2.14][129]. Ascitic patients identified as frail had a 29% waitlist mortality rate, higher than the 17% rate for non-frail patients[129]	Large volume paracentesis with iv albumin; Salt intake not < 5 g NaCl/d to preserve food palatability
Hepatic encephalopathy (HE)	Decreased voluntary oral intake; Decreased capacity for ambulance and exercise	Odds of frailty were higher in HE than in non-HE patients (odd ratio 2.45, 95%CI: 1.80-3.33)[129]. Waitlist mortality was higher for HE patients identified as frail (30%) than non-frail (20%)[129]	Enteral nutrition with precautions to avoid aspiration and hyperglycemia; Parenteral nutrition if indicated; Avoid unnecessary protein restriction
Alcohol intake	Decreased oral intake; Gastrointestinal upset; Vitamin and mineral deficiency; Increased resting energy expenditure; Alcohol direct toxic muscular and neurologic effects	Frail alcoholic liver disease patients had a significantly higher risk of death or liver transplantation compared to non-frail patients ($P < 0.001$)[130]	Alcohol abstinence; Healthy diet with approximately 30 kcal/kg to 40 kcal/kg per day; Small and frequent meals; Enteral feeding in severe disease
Sarcopenic obesity	Challenging to diagnose; Physical disability due to decreased muscle size and high muscle fat	MASLD cirrhotic patients have an increased risk of worsening frailty over time and higher waitlist mortality than non-MASLD patients[131]	Structured exercise program to help preserve muscle mass; If caloric restriction is necessary, maintain adequate protein intake (1.2-1.5 g/kg/d)
Prolonged fasting	Accelerated catabolic state with Increased muscle breakdown		Limit fasting period to a maximum of 12 h; Daily calorie intake should be divided into 4-6 meals; Late evening snacks
Loop diuretics	May worsen muscle mass loss	Loop diuretics inversely correlated with skeletal muscle mass in cirrhotic patients ($P < 0.0001$) and high doses were independently associated with mortality [126]	Regular frailty assessments are recommended for patients who have been on prolonged courses of loop diuretics, particularly when the dosage exceeds 20 mg/d; Spironolactone may be a preferable option for long-term use due to its promising efficacy in treating sarcopenia
Aging	Combined muscle loss due to aging and hepatic illness (compound sarcopenia)	Elderly sarcopenic patients with cirrhosis have longer hospital stays, higher hospitalization costs, and increased risk of in-hospital mortality[15]	Frequent frailty assessment and management in elderly patients with cirrhosis

MASLD: Metabolic dysfunction-associated steatotic liver disease.

the stomach to relax within the peritoneal cavity with albumin infusion helping relieve gastric wall edema[78].

Salt restriction is currently a crucial component of ascites treatment at all stages, as it helps prevent sodium overload and promotes the mobilization of fluid retention[79,80]. Previous studies have indicated that a tight restriction of sodium intake (< 5 g salt daily) can result in a reduced need for diuretics, faster resolution of ascites, and shorter hospital stays [81-83]. However, strict sodium restriction may not always be the optimal approach for managing ascites in cirrhotic patients. A study by Gauthier *et al*[81] provides an example of this. The researchers conducted a trial on patients who were receiving diuretic treatment. They compared the resolution of ascites in a group with strict salt restriction (only 1230 mg salt daily) to a group with no sodium restriction[81]. Surprisingly, despite the rapid resolution of ascites in the restricted salt group at the beginning of the study, there was no significant difference between the two groups after 90 d [81].

While strict sodium restriction has demonstrated short-term benefits, it may increase the risk of sarcopenia and frailty in cirrhotic patients due to the unpleasant taste of food and the additional effort required for food acquisition and preparation, ultimately resulting in a loss of appetite[81,84-86]. This can lead to malnutrition and an increased risk of developing ascites. Two randomized controlled trials have provided strong evidence that adhering to a strict low-salt diet increases the risk of malnutrition and can worsen ascites in cirrhotic patients[87,88]. In a study conducted by Gu *et al*[87], the disappearance of ascites in cirrhotic patients was compared between a group with restricted salt intake and a group with unrestricted salt intake. The results demonstrated that cirrhotic patients in the unrestricted salt group (8.8 g NaCl/d) consumed more calories than those in the restricted salt group (4.2 g NaCl/d), leading to higher albumin levels with better nutritional status and rapidly resolving ascites[87]. These findings suggest that strict salt restriction may not always be the optimal approach for managing ascites in cirrhotic patients. Instead, individualized dietary recommendations are necessary to ensure adequate patient nutrition and prevent complications[84]. For this reason, leading hepatology scientific societies recommend a daily salt intake of no less than 5 g NaCl/d (equivalent to 2 g Na/d) for cirrhotic patients with ascites[79,89-92]. To meet the recommended salt intake, it is highly recommended to avoid consuming canned foods or pre-packaged meals. Instead, prioritize the consumption of fresh, home-cooked dishes that are rich in fruits, vegetables, and dairy products[93]. Furthermore, to enhance the flavor of meals it is advised to substitute salt with a

variety of herbs and spices[84].

Hepatic encephalopathy

Patients with hepatic encephalopathy are at a high risk of malnutrition and frailty due to a decrease in voluntary oral intake[94]. This can result in longer hospital stays, worsening of their condition, and increased susceptibility to infections [95]. When dealing with such cases, enteral nutrition is advised, but it is important to take precautions to avoid the risk of aspiration and hyperglycemia[7]. Parenteral nutrition, on the other hand, should only be used for patients who are unable to tolerate enteral feeding or cannot meet their caloric and nutrient needs orally[7].

It is important to note that protein intake should not be restricted in patients with encephalopathy[7]. While there used to be a debate about whether protein supplementation could worsen hepatic encephalopathy, recent research has shown that protein does not cause encephalopathy and may even improve cognitive function in the long term[96,97]. On the contrary, restricting protein intake could exacerbate frailty[7]. Therefore, to avoid the risk of worsening frailty, it is generally recommended that patients consume protein from a diverse range of sources, including vegetables and dairy products[98]. The American Association for the Study of Liver Diseases (AASLD) suggests that adults with cirrhosis should consume 1.2-1.5 g of protein per kilogram of ideal body weight daily[7,97]. However, for critically ill cirrhotic patients, the recommended protein intake is slightly higher, ranging from 1.2-2.0 g/kg of ideal body weight daily[7]. For children with cirrhosis, a protein intake of up to 4 g/kg of body weight per day is both safe and successful in improving anthropometric measurements without the development of hepatic encephalopathy[99].

Alcohol intake

Alcohol consumption has a significant and complex impact on nutritional status, affecting both nutrient intake and absorption. Initially, alcohol itself contributes to approximately 50% of daily caloric intake, leading to a decreased intake of other essential nutrients[100]. Moreover, alcohol-induced anorexia, nausea, vomiting, and gastritis exacerbate the situation by further reducing oral intake of nutrients. Alcohol also interferes directly with nutrient absorption by damaging the mucosal lining of the stomach and small intestine[101]. This damage impairs the absorption of important nutrients such as protein, vitamins A, B1, B12, folic acid, and zinc, leading to deficiencies of these micronutrients and vitamins in individuals with alcohol-associated liver disease (ALD)[98,100]. Furthermore, chronic alcohol consumption has been found to increase resting energy expenditure, which over time can lead to significant muscle wasting and frailty [102]. These factors cause malnutrition and frailty in alcohol-related cirrhosis, which has a detrimental impact on survival rates and quality of life, with an increased risk of various complications. These include variceal bleeding, development of ascites and hepatic encephalopathy, susceptibility to infections, longer hospital stays, and occurrence of hepatorenal syndrome[103].

Given the multifaceted impact of alcohol on liver diseases and nutritional status, lifelong abstinence is recommended [104]. Additionally, it is crucial to address nutritional deficiencies and promote a healthy diet in individuals with ALD [105]. This can help to mitigate the negative effects of alcohol on nutrient intake and absorption, as well as prevent muscle wasting and frailty. To prevent these issues, current guidelines recommend approximately 30 kcal/kg to 40 kcal/kg per day for these patients, with an emphasis on small, frequent meals as some patients may not be able to tolerate large meals three times a day[106]. In severe cases of ALD, supplementation in the form of enteral nutrition should be considered to ensure adequate nutrition and prevent complications associated with malnutrition[107].

Metabolic dysfunction-associated steatotic liver disease and sarcopenic obesity

Sarcopenic obesity is a condition where there is a simultaneous loss of skeletal muscle and gain of adipose tissue, which is a common finding in patients with cirrhosis[108]. This condition is characterized by a decrease in muscle size and an increase in muscular fat, known as myosteatosis. It can be challenging to diagnose, particularly in patients with metabolic dysfunction-associated steatotic liver disease (MASLD), as the presence of morbid obesity can obscure the signs of sarcopenia, making it difficult to detect[109]. Sarcopenic obesity has detrimental effects on the morbidity and mortality of patients with liver cirrhosis[108]. It poses a greater risk of physical impairment and disability than either sarcopenia or myosteatosis alone, and it is considered a negative prognostic marker for the progression of liver cirrhosis and outcomes of liver transplantation[110]. Additionally, both sarcopenia and myosteatosis are associated with a higher risk of long-term mortality in cirrhosis[108].To manage sarcopenia and MASLD, increasing physical activity and following a healthy diet can be helpful[111].

Currently, there are no specific exercise recommendations available for cirrhotic patients with sarcopenic obesity[112]. However, several studies have indicated that exercise can have positive effects on these patients[113-115]. These studies have shown that exercise can lead to a reduction in body weight and fat mass, as well as an improvement in skeletal muscle mass and physical capacity in patients with cirrhosis. These positive outcomes are particularly advantageous for patients with sarcopenic obesity. In a randomized controlled trial conducted by Román *et al*[114], functional capacity, body composition, and the risk of falls were measured in patients with cirrhosis before and after a moderate exercise program. The results of the study demonstrated that the exercise group experienced significant improvements in functional capacity, increased muscle mass, decreased body fat, and a reduced risk of falls, while no changes were observed in the control group[114]. Studies have also shown that elderly men who engage in moderate-to-vigorous exercise for at least thirty minutes a day have a lower risk of developing sarcopenic obesity[116]. Therefore, it can be concluded that a moderate exercise program can be beneficial for patients with cirrhosis, especially those with sarcopenic obesity. Additionally, while cirrhotic patients with sarcopenic obesity have increased body mass index (BMI) and body fat, it is important to be cautious when recommending weight loss to affected patients, in order not to exacerbate frailty [16]. If caloric restriction is deemed necessary, it is imperative to closely monitor the body composition, muscle strength,

and physical activity levels of these individuals using appropriate assessment tools[117]. Moreover, it is recommended to implement a comprehensive approach that includes ensuring adequate protein intake (1.2-1.5 g/kg/d) and applying a structured exercise program to help preserve muscle mass and promote overall health[7].

Prolonged fasting

Individuals with cirrhosis experience a more rapid breakdown of their body tissues due to starvation than healthy individuals[118]. After an overnight fast, the type of energy sources utilized by cirrhotic patients is comparable to that of healthy individuals who have been fasting for 2-3 d[119]. This implies that cirrhotic patients who undergo medical procedures, such as gastrointestinal endoscopy, with prolonged fasting are at risk of developing a severe catabolic state, which can lead to the breakdown of their body tissues[118]. Therefore, it is recommended that patients with liver cirrhosis limit their fasting period to a maximum of 12 h[120,121]. It is also advised that their daily calorie intake should be divided into 4-6 meals, which can include snacks at night[122,123]. This dietary approach can help maintain stable blood sugar levels and prevent the breakdown of body tissues due to prolonged fasting[118].

Diuretics

The evidence supporting the notion that loop diuretics may worsen sarcopenia and frailty is mounting. For instance, a study discovered that bumetanide and furosemide administration had an adverse effect on myogenic differentiation and exercise-induced muscle hypertrophy[124]. Additionally, loop diuretics have been associated with a decrease in thigh and arm circumference among heart failure patients, regardless of the severity of their disease[125]. Furthermore, a retrospective study involving 266 cirrhotic patients found that high doses of loop diuretics were associated with rapid muscle mass loss and poor survival rates, independent of liver disease severity[126]. This study found that therapeutic dosages of loop diuretics were inversely correlated with skeletal muscle mass in cirrhotic patients, as demonstrated by both simple ($r = -0.27$, $P < 0.0001$) and multiple regression analyses ($t = -3.07$, $P = 0.002$)[126]. Patients receiving more than 20 mg of loop diuretics per day had lower overall survival rates compared to those receiving < 20 mg (median, 66 mo *vs* 97 mo; $P = 0.002$), and higher doses of loop diuretics were independently associated with mortality among cirrhotic patients [hazard ratio, 1.86; 95% confidence interval (CI): 1.03-3.24; $P = 0.039$][126]. Compared to loop diuretics, spironolactone has been suggested to have potential benefits in preventing muscle mass loss, enhancing muscle blood flow, and boosting contractile power[127]. Given these findings, future research needs to investigate the impact of different types of diuretics on muscle health, particularly in patients who are on long-term or high-dose loop diuretic therapy, with regular assessments of frailty.

Aging and compound sarcopenia

The loss of muscle mass due to aging, known as primary sarcopenia, and the loss of muscle mass due to chronic illness, known as secondary sarcopenia, combine to form a health condition called compound sarcopenia[15]. This condition can have a significant impact on the clinical outcomes of older adults with chronic diseases[128]. In patients with hospitalized cirrhotic patients, compound sarcopenia has been associated with increased length and cost of hospital stay with detrimental effects on patient survival[15]. As a result, it is crucial to regularly assess and provide aggressive treatment for elderly patients with sarcopenia.

INFLUENCE OF FRAILTY ON CIRRHOSIS PATIENTS

Depression and reduced quality of life

Frailty is a condition that is associated with reduced cognitive abilities, increased risk of falls, and lower quality of life [132-134]. Depression is a common occurrence in patients with ESLD and is closely linked to frailty, rather than the severity of liver disease[135]. A prospective cohort study was conducted on 542 patients with ESLD who were referred for liver transplantation to investigate the relationship between frailty, depression, and the severity of liver disease[135]. The study found a significant association between frailty and depression, with an odds ratio of 2.78, $P < 0.001$. However, no significant association was found between the Model for End-Stage Liver Disease (MELD) score and depression[135]. This highlights the importance of addressing frailty as a potential risk factor for depression in patients with ESLD.

In another study, the relationship between frailty and disability was examined in cirrhotic individuals receiving outpatient care. Disability was evaluated through the measurement of individuals' capacity to carry out essential activities of daily living (ADLs), such as feeding and bathing, as well as more complex tasks known as instrumental ADLs (IADLs), which encompass activities like shopping and managing finances. The study found a strong link between frailty and disability in patients with cirrhosis[136]. The LFI was used to measure frailty, and each point increase in the LFI was associated with a higher likelihood of experiencing difficulty with ADLs and IADLs[136]. The odds of experiencing current difficulty with at least one ADL and IADL were 3.3-fold and 4.6-fold higher, respectively, for each point increase in the LFI[136]. In participants who initially did not have any baseline disability, the study revealed that for every point increase in the LFI, the odds of having trouble with at least one ADL and IADL after 6 mo were 2.6 times and 1.7 times higher, respectively[136]. These findings suggest that even a slight increase in frailty can significantly impact the ability to perform essential tasks and responsibilities, thereby affecting both patients and their caregivers' quality of life.

Increased risk of cirrhosis complications and hospitalization

Frailty is a distinctive risk factor that is independently associated with a range of cirrhosis-related complications, such as

ascites, encephalopathy, hepatorenal syndrome, and sepsis, which often necessitate hospitalization[129,137,138]. Moreover, frailty has been linked to an increased risk of acquiring nosocomial infections[139]. Patients with significant frailty may require prolonged ICU and hospital stays, and they are more prone to respiratory complications and sepsis [140]. In a prospective study involving 373 pre-transplant patients, researchers found that gait speed, a measure of frailty, played a significant and influential role in the risk of hospitalization for various complications related to cirrhosis[138]. The study found that for every 0.1 m/s decrease in gait speed, there was a 22% increase in the number of hospital days ($P < 0.001$), indicating a robust correlation between frailty and the need for hospital care[138].

Increased risk of non-home discharge

Frailty has been identified as a significant risk factor for non-home discharge among hospitalized patients, which can lead to increased healthcare and financial burden[139]. The results of a prospective study conducted on 211 cirrhotic patients from three Liver transplantation centers revealed that frailty was strongly associated with discharge to physical rehabilitation, a skilled nursing facility, or hospice, rather than being discharged to the patient's home[139]. The study's odds ratio indicated that for every one-point increase in the LFI, the likelihood of non-home discharge increased by 1.81 times, (95%CI: 1.14-2.86)[139].

Increased risk of mortality

Frailty is an established factor that significantly increases the likelihood of severe complications and mortality among liver cirrhotic patients, both before and after liver transplantation[141]. In a comprehensive study conducted across nine transplant centers in the United States, researchers assessed frailty in pretransplant patients on the waitlist and found a significant correlation between the presence of frailty, as measured by the LFI, and an increased risk of mortality. Specifically, patients with frailty had an adjusted risk of death that was nearly twice as high as those without frailty (sub-hazard ratio 1.82, 95%CI: 1.31-2.52)[129]. Furthermore, a systematic review examining the impact of frailty on post-transplant mortality revealed that frailty had a negative effect on post-transplant outcomes. The review suggested that severe frailty was associated with a two-fold reduction in early survival and a 50% reduction in late survival[140].

ASSESSMENT OF FRAILTY IN LIVER DISEASES

The early and prompt assessment of frailty in cirrhotic patients is crucial for healthcare providers to enhance comprehensive care for ESLD patients[142]. Frailty has been observed to be a valuable predictor of outcomes both before and following therapeutic interventions. For instance, frailty has been associated with post-transjugular intrahepatic portosystemic shunt (TIPS) and post-liver transplant morbidity and mortality[143,144]. Additionally, early identification of frailty is also crucial as it is possible to reverse frailty to some extent, and identifying it early on enables healthcare providers to intervene more effectively to enhance the health outcomes of patients with frailty[145]. Therefore, all patients with this ailment need to undergo a frailty assessment to aid in making critical decisions regarding their life and death, including determining their eligibility for critical care and transplantation and prioritizing prehabilitation services such as nutrition, physiotherapy, and psychotherapy[146].

Frailty reassessment is also crucial to monitor the response to treatment of cirrhotic patients who have been diagnosed with frailty. Patients with well-compensated liver cirrhosis should be reassessed at least once a year, while those with decompensated cirrhosis or those receiving active management for these conditions should be reassessed more frequently, every 8 wk to 12 wk[7]. This will help healthcare providers to identify any changes in the patient's condition and adjust their treatment plan accordingly, which can improve the patient's quality of life and overall health outcomes [7].

Assessing frailty in cirrhotic patients requires the use of various assessment tools, each with its own methodology, time requirements, and limitations[146]. It is important to note that clinical interpretation of these tools can also vary among cirrhotic patients. Therefore, healthcare providers should choose the most appropriate assessment tool for each patient and interpret the results accurately. In the following paragraphs, we will summarize the three most used assessment tools for evaluating frailty in cirrhotic patients, including their strengths and limitations.

Fried frailty index

The fried frailty index (FFI) is a commonly used assessment tool for frailty that encompasses both subjective and objective components. It states self-reported fatigue, weight loss, and limited physical activity, along with objective measurements of walking speed and grip strength[8]. It is a quick assessment that can be completed in less than 10 min. The use of FFI has been linked to predicting morbidity and mortality in patients with liver cirrhosis. Higher FFI scores have been associated with elevated MELD scores, reduced albumin levels, ascites, and hepatic encephalopathy[147]. Moreover, the degree of physical weakness observed in individuals on the liver transplant waiting list, as determined by the FFI, is a noteworthy indicator of total hospitalized days per year, regardless of the severity of their liver disease[148]. Additionally, a one-unit increase in FFI leads to a 50% increase in mortality rates among those on the waiting list[149]. Despite these benefits, the accuracy of the FFI may be questionable when assessing frailty in decompensated cirrhosis. In a cohort of 685 pre-transplant patients, the FFI was found to have no association with survival among hepatic encephalopathy patients[147]. This could be attributed to the challenges that HE patients face in reporting subjective FFI components and the suboptimal performance of objective components such as grip strength and walking speed[147].

Clinical frailty scale

The clinical frailty scale (CFS) is a 1-min consistent method utilized to evaluate frailty in patients[150]. It is a comprehensive subjective clinical assessment of frailty that is user-friendly and has demonstrated its ability to anticipate mortality or the requirement for institutionalized care[151]. The scale rates individuals' level of comorbidity, function, and dependence on others for daily activities, ranging from 1 (very fit) to 9 (terminally ill)[151]. Frail patients (CFS > 4) were associated with increased rates of unplanned hospitalization or death among outpatient cirrhotic patients[150]. Additionally, CFS has linked frailty to acute kidney injury and hepatorenal syndrome in patients with hospitalized liver cirrhotic patients[137]. However, the CFS provides only a brief overview of frailty and is not detailed enough to track changes in frailty resulting from therapeutic interventions[146].

LFI

The LFI is a valuable tool for assessing frailty in hepatic patients, as it combines three performance-based evaluations: grip strength of the hand, the duration taken to perform five chair stands, and the duration of maintaining three different balance positions[152]. This quick test (3-5 min) is specifically designed to assess frailty in hepatic patients, making it a reliable and efficient tool for healthcare professionals[152]. A higher LFI score indicates a greater degree of frailty, and an LFI cut-off of > 4.62 has been identified as the most effective in distinguishing between cirrhotic patients who are at high risk for rehospitalization within 30 d and those who are not[153]. This makes the LFI a crucial tool for identifying patients who require additional care and support to prevent rehospitalization. Moreover, healthcare professionals can use the LFI to assess a patient's frailty before transplantation, identifying those who are at high risk for waitlist mortality and prolonged hospital stay after transplantation[154]. Incorporating LFI into the subjective clinical evaluation has been demonstrated to correctly reclassify the survival status of 34% of waitlist patients[154].

In addition to assessing frailty in hospitalized patients, the LFI is also employed to diagnose frailty in out-patient individuals with cirrhosis, making it a versatile tool that can be used in a variety of healthcare settings[155]. Compared to the Karnofsky Performance Status scale, which only measures one aspect of frailty, the LFI is a more comprehensive and accurate tool for assessing the risk of mortality as it captures multiple components of frailty[156].

MANAGEMENT OF FRAILTY IN LIVER DISEASES

In the management of frailty in liver cirrhosis, a continuous process is involved, which includes multiple interventions and regular reassessment of the patient to monitor their response and guide the next steps. In this section, we will provide a summary of the interventions for treating frailty in cirrhosis, as depicted in Figure 2.

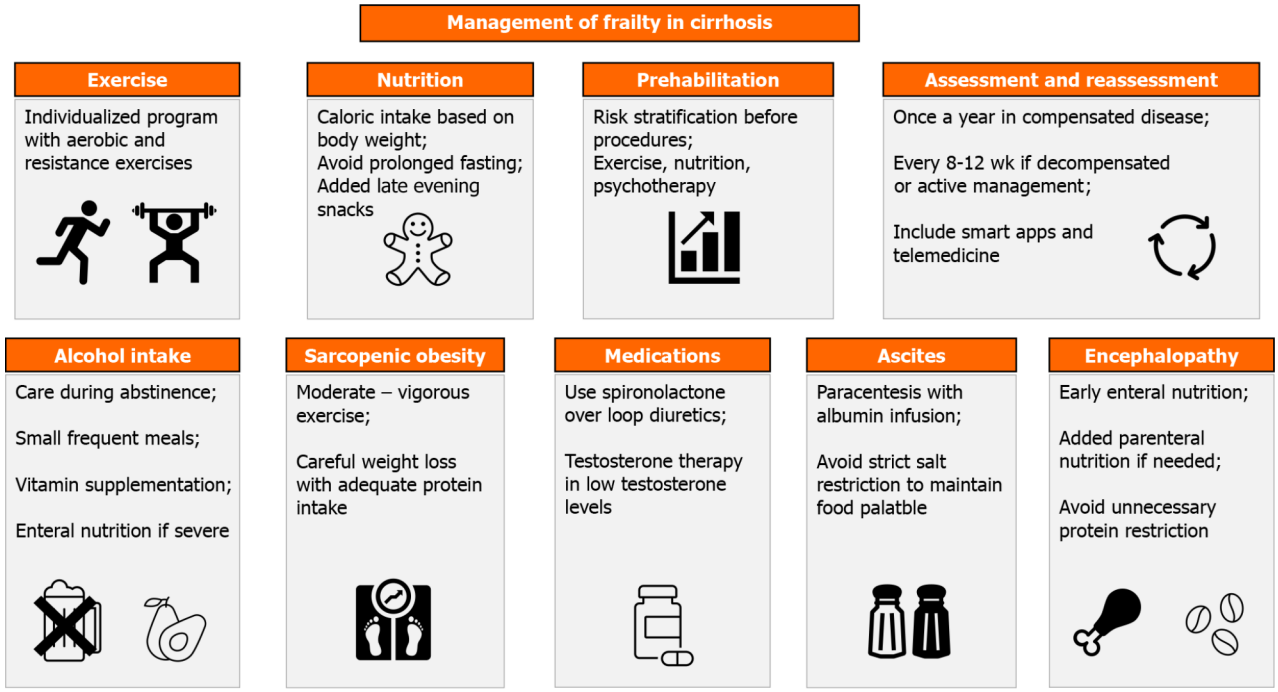
Exercise

Recent studies have shown that a sedentary lifestyle is linked to lower survival rates among decompensated cirrhotic patients. In fact, low moderate-vigorous activity has been associated with increased mortality among liver transplant wait-listed patients[157]. Conversely, exercise interventions have the potential to safely improve exercise capacity, peak oxygen consumption, muscle mass and function, and quality of life, while decreasing the hepatic venous pressure gradient in individuals with cirrhosis[158,159]. This can even reverse the process of frailty in cirrhotic patients.

A study was conducted to evaluate the feasibility and benefits of a 6-wk exercise program for cirrhotic patients who were waiting for a liver transplant[160]. The program involved supervised exercise on a stationary bike three times a week. The results of the study showed that the exercise program was both feasible and beneficial for these patients[160]. Specifically, 56% of the patients (9 out of 16) were able to complete the full 6-wk program, and the exercise group showed a significant improvement in oxygen consumption compared to the control group[160].

Although exercise has potential benefits for cirrhotic patients, incorporating it into their routine can be challenging due to the risk of complications. This is especially true for decompensated patients, for whom there have been few studies on the safety and effects of exercise[161]. Cirrhotic patients, especially those who are decompensated, face numerous challenges in performing exercises, such as muscle wasting, fatigue, fluid retention, risks of falls, bleeding tendency, and portal hypertension[162,163]. To fully benefit from exercise, it is crucial to customize the exercise program to the individual patient's needs and risk of complications[7]. For instance, patients experiencing muscle mass loss may benefit from incorporating resistance training into their program, while those with a history of falls should exercise with caution when engaging in aerobic activities[7]. Moreover, while regular exercise has been found to decrease chronic portal hypertension, it is important to note that in acute situations, exercise can lead to an increase in portal pressure. Therefore, it is crucial to apply appropriate primary or secondary prophylaxis for variceal rupture to mitigate any potential risks [162].

To enhance the physical frailty and quality of life of both compensated and decompensated cirrhotic patients, it is generally recommended to incorporate a combination of aerobic and resistance exercises into their routine for at least 12 wk[161]. However, to prevent possible complications and maximize the benefits of exercise, an individualized exercise and nutrition plan should be created based on the patient's degree of frailty. This approach involves categorizing patients into one of three groups: absent/mild, moderate, or severe frailty, and creating an individualized plan for each patient accordingly[164]. For patients with severe frailty, an inpatient rehabilitation program is recommended as they are likely to derive the greatest benefit from it[164]. This approach ensures close monitoring of the patient's progress and helps in avoiding complications. Patients with moderate frailty are advised to participate in home-based exercises, with a focus on enhancing ADLs[164]. For patients with mild or no frailty, it is recommended that they engage in moderate-intensity exercise for a minimum of 150 min/wk[164]. These individuals should gradually work on improving their physical



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Figure 2 Different strategies for treating frailty in cirrhosis.

capacity and strength. Regular reassessment of all patients is necessary to modify their exercise programs based on their current state[164]. By following these guidelines, cirrhotic patients can safely and effectively improve their physical health and quality of life.

Nutrition

Studies have shown that interventions aimed at improving nutrition, such as providing nutritional education, protein-energy supplementation, and oral nutritional support, can effectively enhance frailty[165]. The Practice Guidance by the AASLD recommends developing a personalized nutrition plan for all individuals with cirrhosis, considering their current nutritional status[7]. To tailor the nutritional plan for cirrhotic patients, AASLD suggests considering their weight and BMI. For individuals with a BMI between 30-40 kg/m^2, the recommended energy intake is 25-35 kcal/kg/d, while for those with a BMI ≥ 40 kg/m^2, the recommended energy intake is 20-25 kcal/kg/d[7]. Moreover, AASLD recommends a safe protein intake of 1.2-1.5 g/kg/d for adults with cirrhosis, which should come from a diverse range of sources[7]. A recent randomized controlled trial showed that intensive nutrition therapy administered at home for six months can enhance frailty and sarcopenia in patients with decompensated cirrhosis[166]. The study found that the intervention group showed a greater improvement in the LFI compared to the control group (0.8 *vs* 0.4; $P < 0.001$)[166]. AASLD highlights the importance of identifying and overcoming obstacles to proper nutrition for patients with cirrhosis who are frail[7]. This may involve loosening restrictions on salt intake in cases of diet unpalatability, encouragement of enteral feeding in encephalopathy, prevention of prolonged fasting hours, and providing late evening snacks[7].

Prehabilitation

Prehabilitation, also known as preoperative habilitation, involves a range of interventions that are implemented before a medical procedure or treatment to mitigate or prevent any adverse effects that may arise[167]. In the context of liver transplantation, prehabilitation aims to identify high-risk patients as early as possible and enhance their physical capacity before surgery[168]. This is achieved through various interventions, including exercise, nutrition, and psychological stress management[169]. According to a recent systematic review of eight studies, prehabilitation could potentially boost the aerobic capacity of patients who are awaiting orthotopic liver transplantation and is deemed a safe and feasible approach [170]. The review also revealed notable improvements in several metrics, such as VO2 peak, 6-min walking distance, hand grip strength, LFI, and quality of life[170]. Despite the evidence supporting the role of prehabilitation before liver transplantation, there is limited research on the benefits of prehabilitation for frail cirrhotic patients after liver transplantation. While a single study has demonstrated that an exercise training program can lead to post-transplant short-term benefits, such as reduced 90-d readmission rates and shorter hospital stays[171], further research is needed to confirm the potential long-term benefits of prehabilitation after liver transplantation.

Medications

Although pharmacological interventions are not commonly used in frailty treatment, there is evidence to suggest that testosterone supplementation may be a safe and effective option for enhancing muscle mass and strength in males with cirrhosis and low testosterone levels. A 54-wk randomized controlled trial conducted by Sinclair *et al*[172] concluded that

testosterone supplementation can safely enhance muscle mass and strength in this population. However, there is limited information regarding the effect of testosterone therapy in treating frailty after liver transplantation. A single retrospective study suggested that short testosterone therapy may be useful in treating frailty after liver transplantation [173]. In this study, administering a single dose of testosterone replacement therapy along with regular exercise has been associated with patient and graft survival rates of 93.8% and 87.5% at one and five years, respectively[173]. Large randomized controlled trials are necessary to validate the safety and potential benefits of administering testosterone as a treatment for frailty following liver transplantation.

FUTURE APPROACHES IN FRAILTY MANAGEMENT

Emerging information technologies for frailty assessment

Frailty assessment tools are now available online, making it easier to assess frailty in the geriatric population. These web-based tools are user-friendly and can be accessed through internet-connected devices[174]. In addition, smartphone applications have shown great potential in evaluating and quantifying physical activity and patient mobility, which are crucial indicators of frailty. For instance, a recent app was used to prehabilitate liver transplant candidates, and it was found that the app's training level matched that of a physical therapist in 89% of cases[175]. The app also motivated patients through videos and gamification features, leading to a 35% increase in physical activity performance among participants[175]. However, these tools require further advancements to fully comprehend the current patient frailty status, especially in cirrhotic patients who require special clinical evaluation. Validation studies are also needed to ensure that these smart tools can be integrated into clinical decision-making for patients.

A novel tool called the tele-liver frailty index (TeLefI) has been proposed for frailty assessment and follow-up in cirrhotic patients[176]. It utilizes telemedicine to virtually measure frailty in liver transplant candidates with cirrhosis. Wang *et al*[176] introduced this tool by comparing frailty assessment using in-person LFI and then by TelefI assessment tool[176]. The telemedicine-based TeLefI tool has been statistically validated in predicting LFI > 4.4, indicating that it can effectively identify patients who require more frequent follow-up or in-person assessment[176]. This tool has the potential to revolutionize frailty assessment and follow-up in cirrhotic patients, especially those who live in remote or underserved areas. This has the potential to not only decrease healthcare expenses but also enhance patient outcomes. While this tool has shown promising results, more research is necessary to fully understand the benefits and limitations of this novel approach to frailty assessment.

Potential therapeutic targets

Targeting pathways that contribute to the progression of frailty shows promise as a potential treatment approach[17]. In fact, there are several medications that could potentially be used to treat frailty in cirrhosis. Here are some examples (Table 3).

Metformin: In general, the research on the effectiveness of metformin in treating frailty is inconclusive. Although some studies indicate that metformin may not be useful in decreasing the occurrence of frailty[177,178], other studies suggest that it could be advantageous in managing age-related illnesses and ailments including frailty[179-181]. The reason for this protective effect may be attributed to the reduction of insulin resistance, which is involved in the pathophysiology of frailty[182]. Additionally, the correlation discovered between metformin usage and lower levels of proinflammatory cytokines, regardless of blood glucose levels, may also contribute to the mechanism[183]. However, further investigation is necessary to gain a complete understanding of metformin's potential in treating frailty in cirrhosis.

Rifaximin: Rifaximin is an antibiotic that boasts a highly favorable safety profile. Its primary function is to specifically target and eradicate harmful bacteria in the intestinal tract[184]. Due to its low absorption rate, rifaximin is considered an excellent choice for patients who require a safe and effective treatment option[185]. One of the key benefits of rifaximin is its ability to modify the composition of the gut microbiota, resulting in a notable decrease in harmful bacterial taxa. This, in turn, helps to prevent hyperammonemia, bacterial endotoxemia, and translocation, which have been identified as contributing factors to muscle loss in cirrhosis patients[62]. In a recent uncontrolled study, the long-term use of rifaximin in cirrhosis patients had a positive impact on their nutritional status[186]. Furthermore, the study demonstrated that rifaximin enabled these patients to maintain a consistent amount of muscle mass[186]. These findings shed light on the potential of rifaximin as an intervention for addressing malnutrition and potentially mitigating muscle loss in individuals with cirrhosis. However, further research is needed, specifically randomized controlled trials, to establish the efficacy of rifaximin in treating frailty in liver cirrhosis. Additionally, probiotics and fecal microbial transplantation are being explored as potential therapies for frailty induced by gut dysbiosis in liver cirrhosis[62]. However, their effectiveness in human subjects is still under investigation, and there is a lack of comprehensive studies in this area.

Myostatin antagonists: Myostatin is a type of signaling molecule that falls under the transforming growth factor-β superfamily[187]. It has a crucial function in skeletal muscle metabolism, and it works as an inhibitor of muscle mass, leading to a decrease in muscle size[188]. Higher levels of myostatin are linked to poorer survival rates in cirrhosis patients, and increased serum myostatin levels are associated with decreased muscle mass[29]. Studies have demonstrated that blocking myostatin can result in muscle hypertrophy and the reversal of muscle atrophy in both young and old mice[189-191], making it a potential solution to prevent muscle wasting in liver cirrhosis patients[192]. There exist various therapeutic interventions that can counteract the impact of myostatin, such as monoclonal antibodies, myostatin propeptide, and follistatin[190,193-196]. Additional studies are needed to ascertain the efficiency and safety of

Table 3 Comparison of different frailty suggested medications in cirrhotic patients

Suggested medication	Target of action	Side effects	Dose used in clinical trials	Clinical trial results
Metformin	Insulin resistance; Proinflammatory cytokines	Gastrointestinal upset; Non responders[209]	-	-
Rifaximin	Gut dysbiosis	-	1200 mg daily for 24 wk	No significant changes in skeletal muscle index[186]
Myostatin antagonists	Hyperammonemia; Muscle mass inhibition	Spontaneous bone fractures[210]; Vascular (nasal and gum bleeding, telangiectasia)[211]	-	-
L-Carnitine	Hyperammonemia; Proinflammatory cytokines (antioxidant)	Gastrointestinal upset	(1000 mg/d) + exercise for 6 mo [201]	No significant changes in muscle mass, leg, and handgrip strength[201]
L-ornithine L-aspartate	Hyperammonemia	Gastrointestinal upset	6 g three times daily for 2 wk [207]	No significant increase in prealbumin level after use[207]
Testosterone therapy	Testosterone deficiency	Cardiovascular diseases; Prostate cancer; Erythrocytosis[212]	Intramuscular injection of testosterone undecanoate 1000 mg at 0, 6, 18, 30, 42 wk[172]	The intervention group had increased muscle and bone mass with lower fat mass[172]

myostatin antagonists in this specific context.

Carnitine: L-carnitine, an endogenous compound with antioxidant properties, has been shown to promote muscle growth by increasing muscle blood flow[197]. Carnitine also has an essential role in fatty acid metabolism, and its deficiency leads to increased hepatic steatosis, hyperammonemia, cardiac and skeletal muscle disease[198]. Despite its potential benefits, there is limited information available on the effectiveness of L-carnitine in treating frail cirrhotic patients. Two retrospective studies have suggested that taking L-carnitine supplements may help prevent the loss of skeletal muscle mass in individuals with liver cirrhosis[199,200]. On the contrary, a clinical trial involving the administration of a daily dose of 1000 mg of L-carnitine for over six months to liver cirrhosis patients did not result in significant changes in muscle mass, handgrip, and leg strength[201]. Further research is necessary to evaluate the potential benefits of L-carnitine in the treatment of frailty in cirrhosis.

L-ornithine L-aspartate: L-ornithine L-aspartate (LOLA) has been proven to be an effective treatment for hepatic encephalopathy as it helps to reduce ammonia levels[202]. It achieves this by stimulating the production of urea in the liver and promoting muscle glutamine synthesis[203,204]. Administering LOLA to mice with non-alcoholic steatohepatitis has been shown to enhance muscle protein function[205]. By decreasing ammonia levels and improving muscle function in mice, LOLA has been suggested as a potential treatment for sarcopenia and frailty in individuals with cirrhosis[206]. However, a randomized controlled trial administering LOLA of 6 g three times daily for 2 wk to a group of 17 liver cirrhotic patients did not produce a significant increase in prealbumin levels[207]. Therefore, future research is necessary to validate the beneficial role of LOLA in treating frailty in patients with decompensated cirrhosis.

Hormonal therapy: Sinclair *et al*[172] conducted a randomized controlled trial that lasted for 12 mo, which showed notable enhancements in muscle mass, bone mineral mass, and a decrease in total fat mass among cirrhotic patients who received testosterone therapy. Nevertheless, further research is required to ascertain the safety and effectiveness of this therapy, particularly due to potential adverse effects such as an increased risk of hepatocellular carcinoma, cardiovascular diseases, and elevated hematocrit levels[208]. Additionally, the efficacy and safety of testosterone therapy after liver transplantation require further investigation.

TIPS and frailty

There is an ongoing debate surrounding the use of TIPS as a potential therapy for sarcopenia and frailty in liver cirrhosis patients. While several studies have shown promising results in terms of improving muscle mass and nutritional status, as well as reducing the risk of hepatic encephalopathy and mortality in sarcopenic patients[213-215], other studies have raised concerns about the use of TIPS as a therapy for sarcopenia and frailty, particularly in severe cases. These studies have demonstrated that these conditions are associated with a higher risk of post-TIPS complications, such as non-home discharge, prolonged hospital stay, hepatic encephalopathy, and even mortality[143,216,217].

Moreover, while all studies conducted have focused on sarcopenic assessment after TIPS, only one novel study has included the effect of TIPS on frailty parameters[218]. In this study, 12 cirrhosis patients showed improvement in skeletal muscle mass six months after TIPS compared to their baseline measurements before the procedure[218]. However, there was no improvement in LFI, handgrip strength, or physical performance measurements[218]. This study adds to the concerns about using TIPS as a potential therapy for cirrhotic frail patients. To address these concerns and obtain more conclusive evidence, we strongly recommend that future studies on this topic prioritize larger sample sizes and control groups.

CONCLUSION

Frailty is a complex medical condition that arises from multiple predisposing factors in patients with cirrhosis. It has a significant impact on the morbidity and mortality of cirrhotic patients and can even predict the outcomes of therapeutic interventions. Therefore, it is crucial to properly assess, reassess, and intervene in cirrhotic patients to prevent, treat, or even reverse this hazardous process. Looking ahead, future research should explore the possibility of utilizing telemedicine and smart apps for frailty assessment to make clinical decisions for patient treatment and follow-up. This could potentially improve patient outcomes and reduce healthcare costs. Furthermore, future research must investigate the safety and long-term potential benefits of therapeutic strategies, including TIPS, prehabilitation, and medications for managing frailty in cirrhotic patients, especially after liver transplantation.

FOOTNOTES

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MINIREVIEWS

Surgical complications after pancreatic transplantation: A computed tomography imaging pictorial review

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Abstract

Pancreatic transplantation is considered by the American Diabetes Association and the European Association for the Study of Diabetes an acceptable surgical procedure in patients with type 1 diabetes also undergoing kidney transplantation in pre-final or end-stage renal disease if no contraindications are present. Pancreatic transplantation, however, is a complex surgical procedure and may lead to a range of postoperative complications that can significantly impact graft function and patient outcomes. Postoperative computed tomography (CT) is often adopted to evaluate perfusion of the transplanted pancreas, identify complications and as a guide for interventional radiology procedures. CT assessment after pancreatic transplantation should start with the evaluation of the arterial Y-graft, the venous anastomosis and the duodenojejunostomy. With regard to complications, CT allows for the identification of vascular complications, such as thrombosis or stenosis of blood vessels supplying the graft, the detection of pancreatic fluid collections, including pseudocysts, abscesses, or leaks, the assessment of bowel complications (anastomotic leaks, ileus or obstruction), and the identi-

fication of bleeding. The aim of this pictorial review is to illustrate CT findings of surgical-related complications after pancreatic transplantation. The knowledge of surgical techniques is of key importance to understand postoperative anatomic changes and imaging evaluation. Therefore, we first provide a short summary of the main techniques of pancreatic transplantation. Then, we provide a practical imaging approach to pancreatic transplantation and its complications providing tips and tricks for the prompt imaging diagnosis on CT.

Key Words: Diabetes mellitus; Type 1; Pancreas transplantation; Complications; Computed tomography; Diagnostic imaging

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Core Tip: Pancreatic transplantation is a complex surgical procedure and, similarly to any major surgical intervention, may lead to a range of postoperative complications that can significantly impact graft function and patient outcomes. Computed tomography offers non-invasive and accurate visualization of the transplanted pancreas and surrounding structures, providing detailed anatomical information and aiding in the detection of complications.

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INTRODUCTION

Pancreatic transplantation is considered by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) an acceptable surgical procedure in patients with type 1 diabetes (T1D) also undergoing kidney transplantation [*i.e.* simultaneous pancreas and kidney (SPK) transplant] in pre-final or end-stage renal disease if no contraindications are present[1]. Pancreatic transplantation has been considered for years the only treatment for T1D that consistently establishes an insulin-independent, normoglycemic state, with 5-year graft survival of 83%, 55% and 70%, in case of SPK, pancreatic transplantation alone (PTA) or pancreas after a kidney transplantation (PAK), respectively [2-4]. The main indication of PTA is presence of T1D and normal or near-normal renal function in patients who suffer from hypoglycemia unawareness, which results in impaired quality of life or with difficulty adhering to the requirement of insulin injection[5]. In few selected cases, transplantation of pancreatic islet – a less invasive procedure consisting in the transplantation of islets of Langerhans in the recipient hepatic portal system – is indicated as an alternative to pancreatic transplantation by ADA and EASD[1]. However, intrahepatic islet transplantation for T1D is limited because of the need of multiple infusions and poor islet viability after transplantation, and more recently intracutaneous transplantation of islets has also been investigated[6]. In a 20-year span from 2000 to 2020, only about 4365 islet allotransplants have been performed according to a recent worldwide survey[7], while the number of pancreatic transplantations in the 10-year span from 2010 to 2019 is of about 23000 procedures[8].

Pancreatic transplantation is a complex surgical procedure and, similarly to any major surgical intervention, may lead to a range of postoperative complications that can significantly impact graft function and patient outcomes[9,10]. The occurrence of postoperative complications may vary depending on factors such as the type of transplantation (SPK, PAK or PTA) and the specific patient population. Cross-sectional imaging – *i.e.*, Doppler ultrasound (US), contrast-enhanced US (CEUS) and computed tomography (CT) – plays a pivotal role in the evaluation of the transplanted pancreas. US is the preferred initial imaging modality for evaluating the transplanted pancreas due to its safe, non-invasive, radiation-free, simple, quick, and repeatable approach. However, US is often affected by factors such as intestinal gas interference and operator proficiency, and partial thrombosis may be easily missed[11]. CT offers non-invasive and accurate visualization of the transplanted pancreas and surrounding structures, providing detailed anatomical information and aiding in the detection of complications. Indeed, CT allows for the identification of vascular complications, such as thrombosis or stenosis of blood vessels supplying the graft as well as bleeding or pseudoaneurysms, the detection of pancreatic fluid collections, including pseudocysts, abscesses, or leaks, the assessment of bowel complications (anastomotic leaks, ileus or obstruction), while it has a limited role in the evaluation of graft rejection[12,13]. Magnetic resonance imaging (MRI) is rarely uncommonly used for examination the assessment of pancreatic graft-related complications, and it is preferred particularly in patients with declining renal function. The main adoption of CT compared to MRI is based on different reasons, including wider availability of CT compared to MRI particularly in the emergency and urgent settings, the lower acquisition time of CT exams compared to MRI which is important in imaging acutely ill and intensively monitored patients, but also to the lower spatial and temporal resolution, creating difficulties in the evaluation of the enteric anastomosis and vascular complications. Contrast medium administration in CT should not be a contraindication in kidney transplant recipients. Fananapazir *et al*[14] demonstrated that the incidence of acute kidney injury in patients with transplanted kidney submitted to CT scans with low-osmolality iodine-based contrast material was 7% when considering the threshold of ≥ 0.3 mg/dL for the increase in serum creatinine levels. The incidence of contrast induced nephropathy after contrast-enhanced CT was similar (6.1%) in a study by Cheungpasitporn *et al*[15]. However, in a study involving

about 6175 patients, McDonald *et al*[16] demonstrated lack of significant difference in the onset if contrast induced nephropathy between patients with a solitary kidney, including kidney transplant recipients (4.1%), and those with bilateral kidneys (4.2%). The assessment of serum creatinine for calculating the estimated glomerular filtration rate is recommended before contrast medium administration within 7 d before CT in patients with an acute disease, an acute deterioration of a chronic disease or in hospitalized patients, and preventive hydration protocols need to be considered in at-risk patients as indicated by guidelines[17-19].

This pictorial review is aimed at illustrating CT findings of surgical-related complications after pancreatic transplantation. A comprehensive knowledge of surgical techniques allows understanding the postoperative anatomic changes that are identified on CT images. Therefore, this review will first a short summary of the main techniques of pancreatic transplantation and, then, will discuss imaging tips and tricks for the prompt diagnosis of complications after pancreatic transplantation on CT.

SURGICAL TECHNIQUES

After organ procurement from a deceased donor, a meticulous graft back-table surgery is necessary before pancreatic transplantation. Main steps are: Splenectomy, removal of the excess fat surrounding the pancreas and ligation of small vessels and lymphatics along the inferior margin of the pancreatic tail, coursing anteriorly around the surface of the neck and head of the pancreas to the proximal duodenal staple line. A suture of the vessels at the root of the mesentery and inferior mesenteric vein is performed followed by an oversewing of the mesenteric staple line. A vascular preparation is necessary using a Y conduit of donor iliac artery which is anastomosed to the superior mesenteric and splenic arteries of the graft (Figures 1 and 2). Assessment of blood supply to the entire pancreas graft exclusively *via* cross-circulation between splenic artery and superior mesenteric artery is mandatory in order to guarantee blood supply to all the pancreas graft. By flushing the superior mesenteric artery and not looking for back-flushing through the gastroduodenal artery is possible to recognize the need for vascular reconstruction of the gastroduodenal artery to guarantee blood supply to head of the pancreas graft and duodenal segment, and possibly reduce the incidence of duodenal complications [20]. A duodenal shortening with stapler and oversewing of the staple line is then completed. At this stage, the graft is ready for implantation.

Through a midline incision, the pancreas allograft is usually placed intraperitoneally, on the right side with the head in a cranial position, and receives arterial inflow from the iliac artery (Figure 1). Venous anastomosis can be performed through systemic vein technique with the graft portal vein (PV) anastomosed to the recipient inferior vena cava or iliac vein, or through the PV technique with the graft PV anastomosed to the recipient inferior vena cava or iliac vein. In the PV technique, the graft PV is connected to the recipient superior mesenteric vein. Systemic drainage theoretically may lead to hyperinsulinemia[21], while portal drainage could allow a more physiological "first pass" effect through the liver since insulin is immediately extracted by the liver. However, the arterial anastomosis to the iliac artery tends to be more difficult using the PV technique and it requires a very long Y graft. Moreover, obesity, thickened mesentery, or an inadequate caliber of the superior mesenteric vein can make the portal drainage even harder[22]. Long-term studies comparing the two techniques have not demonstrated clear metabolic advantages with portal drainage and the use of the PV has remained marginal over the years[23]. For the exocrine pancreas drainage, anastomosis between the donor duodenum and recipient small bowel loop (*i.e.*, jejunal or an ileal loop) is performed side-to-side with a circular stapler. After firing the stapler (trans-oral anvil delivery system EEA), the end of the donor duodenum is closed using a linear stapler. Alternatively, a bladder diversion can be performed[24]. Bladder drainage can be particularly advantageous in case of PTA, for the assessment of the concentration of urinary amylase as a marker of rejection. Disadvantages of this technique include both urologic complications such as hematuria (16%), leaks (14%), reflux pancreatitis (11%), recurrent urinary infections (10%), urethritis (3%), urethral stricture/disruption (3%), and metabolic complications due to the urinary loss of the bicarbonate-rich pancreatic juice, including hyperchloremic metabolic acidosis and dehydration[23, 25]. When these complications became intractable, conversion from bladder to enteric drainage is often necessary[26]. Enteric drainage is currently the predominant technique in pancreatic transplantation[23].

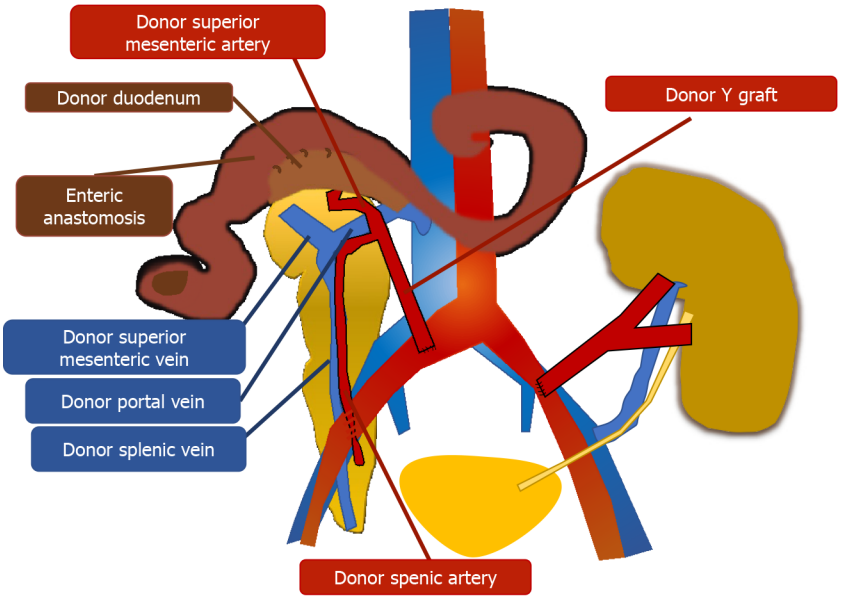
Usually, recipients start receiving immunosuppression during surgery, consisting in antibody induction and a triple drug immunosuppressive therapy is started immediately postoperatively. Most of the Centers utilize a regimen consisting of tacrolimus, mycophenolate mofetil and steroid.

Heparin prophylaxis is recommended with intravenous heparin administered as a single dose during surgery after pancreas revascularization and it is continued after surgery with low molecular weight heparin or continuous infusion, at the discretion of the Transplant Centre.

Some days after transplantation, a CT scan is advisable to early recognize vascular thrombosis or any signs of vascular alterations, not yet clinically relevant.

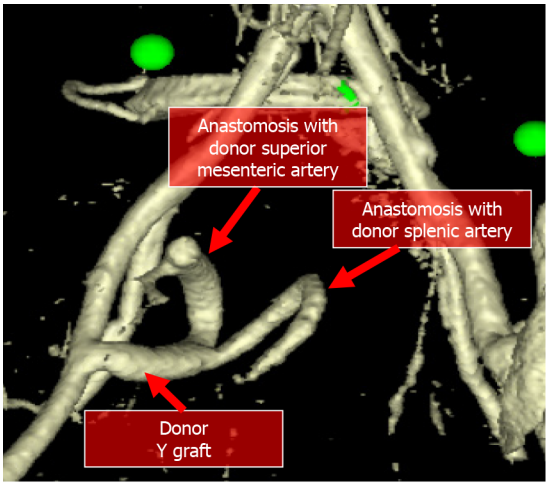
COMPLICATIONS AFTER PANCREATIC TRANSPLANTATION

Postoperative surgical complications of pancreatic transplantation are still common despite many improvements in surgical techniques, and may be distinguished based on time of onset[27-28]. Postoperative monitoring of the pancreatic graft by CT is requested in about 89% of cases according to a recent series of 230 pancreatic transplantations and is helpful for optimizing patient management[29].



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Figure 1 Schematic imaging representation of simultaneous pancreas and kidney transplantation.



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Figure 2 Volume rendering reformat of computed tomography images in the arterial phase demonstrates the Y conduit of donor iliac artery to the superior mesenteric and splenic arteries of the graft.

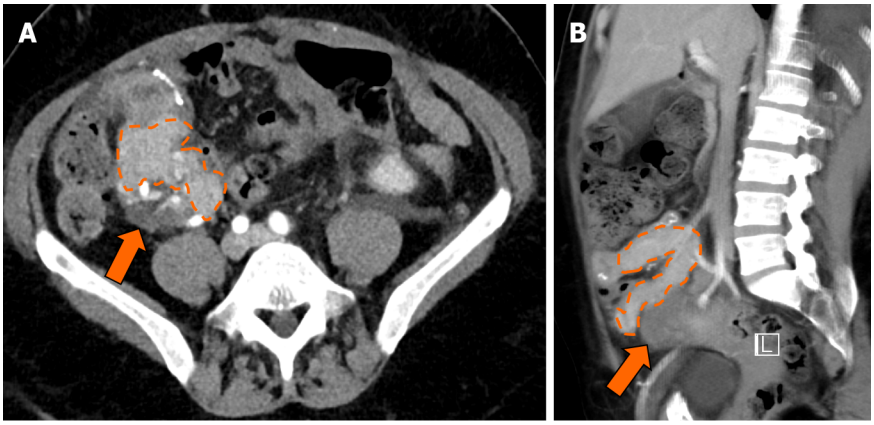
The main reasons for requesting imaging are related to the indication per protocol even without acute clinical indication, sudden progressive hyperglycemia, persistent or abdominal tenderness[29]. However, the adoption of CT needs to be patient-tailored particularly in the context of SPK, due to the potentially nephrotoxic effect of contrast agents which is reported to be as low as 5.6% in kidney transplant recipients with the use of hypo-osmolar contrast agent[30].

Initial complications of pancreatic transplantations are largely related to technical factors, including vascular thrombosis, bleeding, enteric anastomotic leak or graft pancreatitis, and urologic complications[8]. Late complications include pseudocyst formation, post-transplant lymphoproliferative disease, pseudoaneurysms, artero-venous fistulas and rejection (Figure 3).

Graft thrombosis is the most common complication after transplant and may lead to graft failure in about 3.7%, 4.1% and 5.9% of SPK, PAK and PTA, respectively[8]. Graft loss due to infection, pancreatitis, bleeding, leaks, and other reasons occur in up to 0.5%, 0.5%, 0.5%, 0.4%, and 0.6%, respectively[8].

Graft thrombosis

Graft thrombosis occurs in 7%-34% of patients after pancreas transplant, with high body mass index being a risk factor, may affect arteries or veins and can be partial or complete[10,12,29,31]. Partial graft thrombosis occurs in about 25% of cases after pancreatic transplantation, is subclinical in the majority of cases, and may resolve spontaneously with medical therapy[29]. Venous thrombosis is far more common than arterial thrombosis, but arterial thrombosis is the most



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Figure 3 A 51-year-old woman, one month after simultaneous kidney-pancreatic transplantation is admitted to the emergency department for abdominal pain; final diagnosis at biopsy of the pancreatic graft was rejection. A: Contrast enhanced computed tomography in the axial plane shows a minimally inhomogeneous pancreatic graft (dotted orange line) with peripancreatic fluid (arrows) posteriorly to the graft; B: Sagittal plane of the same contrast enhanced computes tomography.

dangerous leading to rapid graft loss[29,32]. Acute rejection and CT finding of pancreatitis are risk factors for graft thrombosis[12].

Contrast-enhanced CT with angiographic phase and venous phase demonstrates thrombosis as a filling defect in a vessel during the vascular phase, and allows to clearly indicate the extension of the filling defect. Arterial thrombosis (Figure 4) may lead to graft dysfunction, pancreatitis, leakage of pancreatic enzymes, sepsis, necrosis and even emphysematous transformation of the graft if it is left untreated. Pancreatic graft venous thrombi (Figure 5) can remain relatively localized possibly maintaining normal graft function, or may propagate into the PV, iliac vein, or vena cava (in case of systemic venous drainage) or superior mesenteric veins (in case of portal venous drainage). In case of vascular thrombosis, a decreased enhancement of the transplanted pancreas usually occurs dure to the organ ischemia, and the main differential diagnosis between arterial and venous thrombosis is made by direct visualization of the thrombus in the vessel.

Percutaneous thrombectomy, followed by anticoagulation, is considered a therapeutical option to remove the thrombus, with low complication rate[33].

Graft thrombosis may be graded on contrast-enhanced CT based on the system proposed by Hakeem *et al*[12] into:

Grade 0 – Lack of thrombosis.

Grade 1 – Peripheral thrombosis: Thrombus is located at the transected margin of the superior mesenteric vein/splenic vein or superior mesenteric artery and it is present only in a single branch.

Grade 2 – Intermediate nonocclusive thrombosis.

Venous: Thrombus extends into parenchymal vessels/main trunk of the superior mesenteric vein or splenic vein to the superior mesenteric vein/splenic vein confluence but not into the PV.

Arterial: Thrombus extends into the main trunk of the superior mesenteric artery/splenic artery to the "Y" graft but not into the Y graft.

Grade 3 – Central occlusive thrombosis.

Interestingly, despite the diagnosis of vascular thrombosis seems quite straightforward Hakeem *et al*[12] showed some discrepancies among radiologists in the detection with 28 new thrombosis identified on retrospective imaging review, with the grade 1 thrombosis being underestimated at initial diagnosis by the reporting radiologists.

Pancreatitis

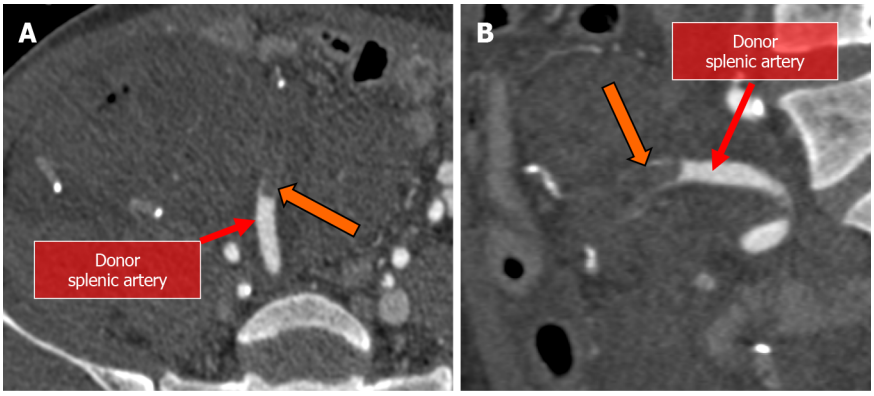
Graft pancreatitis is amongst the most frequent complications following pancreatic transplantation, the third most common cause for re-operation following bleeding and pancreas graft thrombosis, and a common histologic feature identified in up to 61% of rejected allografts[34,35]. Graft pancreatitis is distinguished in early if it occurs within 3 mo and later after 3 mo, while physiological acute graft pancreatitis occurs within the first 72 h after reperfusion of the transplanted organ secondarily to an acute inflammatory response related to ischemic reperfusion injury[36].

Common findings in CT include focal or diffuse enlargement of the pancreatic parenchyma, with edematous changes (usually is noted a decreasement in HU value), indistinct pancreatic margins due to inflammation, and surrounding fat stranding (Figure 6). CT imaging allows to differentiate between edematous and necrotizing pancreatitis, to assess the presence of collections and to evaluate long-term evolutions including pseudocyst and walled-off necrosis[37].

Notably, graft pancreatitis and vascular thrombosis may occur simultaneously; indeed, pancreatitis can predispose to vascular thrombosis, and vascular thrombosis can also lead to graft inflammation, thus leading to some overlapping CT imaging features as shown on Figure 7.

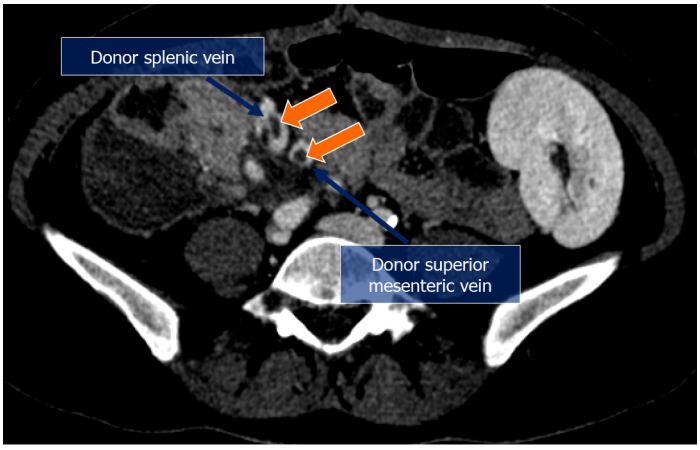
Peripancreatic collection and Infection

Infection may occur in the form of peripancreatic fluid collections (Figure 6), pseudocysts (Figure 8), leakage at the level



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Figure 4 A 46-year-old woman, few days after surgery had low prothrombin time; final diagnosis was graft arterial thrombosis. A: Contrast enhanced computed tomography in the angiographic phase demonstrated arterial thrombosis of the donor splenic artery. In this patient, the transplanted pancreas was explanted thereafter; B: Coronal plane of the same contrast enhanced computed tomography in the angiographic phase.



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Figure 5 A 35-year-old woman is admitted to the emergency department for abdominal pain; final diagnosis was venous thrombosis. Contrast enhanced computed tomography in the venous phase demonstrates venous thrombi (orange arrows) in the donor splenic vein and in the donor superior mesenteric vein.

of the enteric anastomosis (Figure 9), or infection of the abdominal wall surgical wound[21,38].

Fluid collections are the most common abnormality after pancreatic transplantation, may occur early or late after transplant, are clinically significant in about 16% of patients, and may lead to superinfection[38,39]. Intraabdominal collections also include seroma, hematoma, lymphocele, urinoma, or pseudocyst[11,38].

CT demonstrates an abnormal fluid collection of low attenuation with surrounding rim-enhancement and possible intralesional gas[38]. Soft tissue edema and fat stranding in the adjacent tissues may also be present. Percutaneous drainage is safe and effective for management of peripancreatic fluid collections after pancreas transplant[38].

Enteric or Pancreatic Leaks

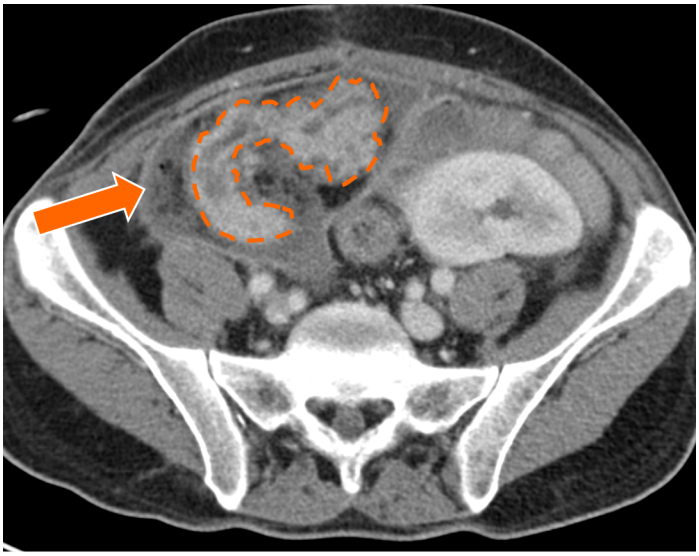
Duodenal leaks represent about 2.5%-2.9% of complications after pancreatic transplantation[40,41] and represent the cause of re-laparotomy in about 2.1% of cases[34]. Enteric leaks after pancreatic transplantation are usually characterized by extravasation of pancreatic juice from the duodenojejunostomy site leading to focal peritonitis and collections (Figure 9), and eventually abscess formation[26]. Duodenal leaks usually occur early in the postoperative course, but may also be seen late post-transplant and increase 6-mo graft loss risk with a hazard ratio of 13.9[40-42]. Pancreatic duct fistula after focal pancreatitis or ischemia leading to duct disruption, may also result in the development of a peripancreatic collection[42].

Bleeding

In the early postoperative period, the occurrence of bleeding is the most common cause of reoperation[29].

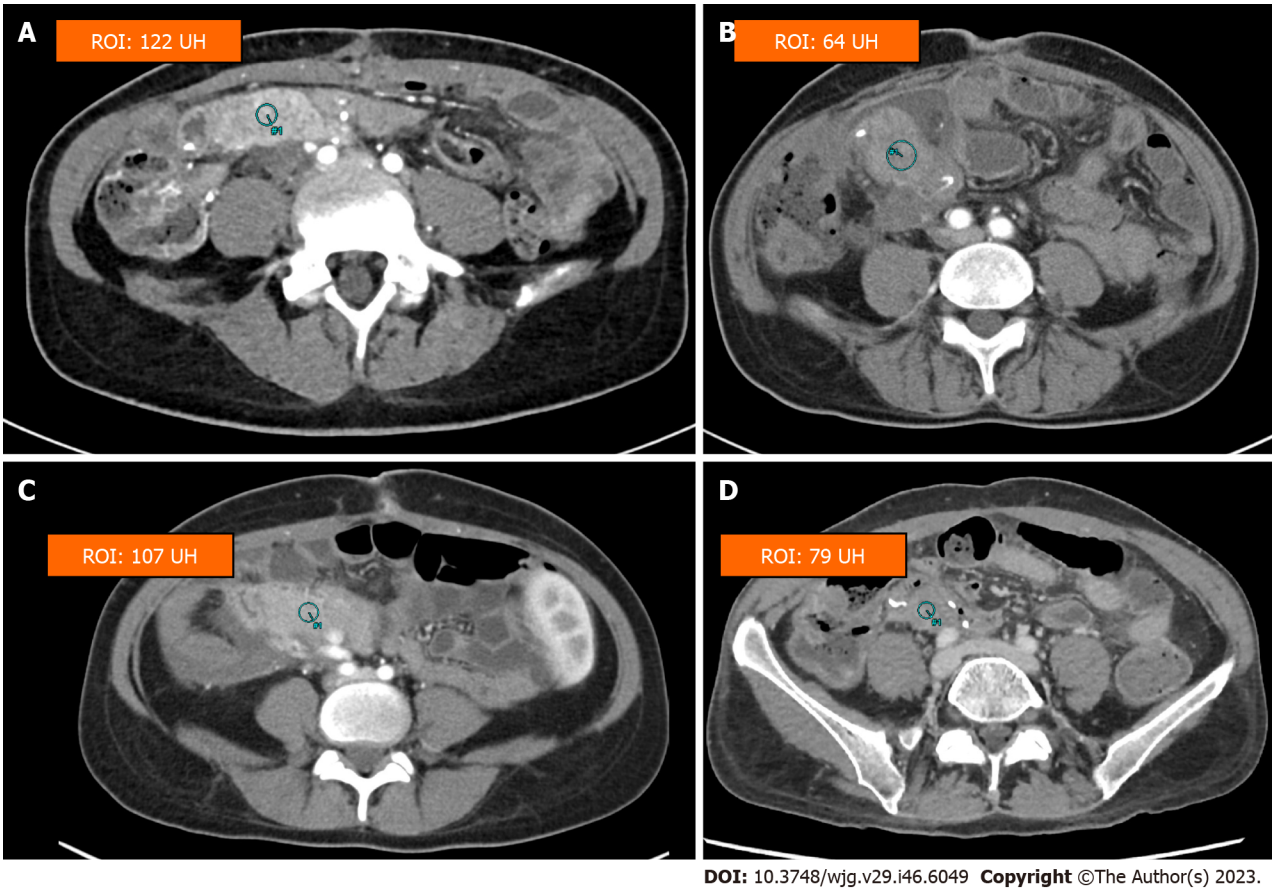
The source of bleeding in the early post-operative period may be:

Intra-abdominal: It is usually related to damage of the peripancreatic vessels or vascular anastomosis, enhanced by the antithrombotic prophylaxis or antithrombotic therapy in patients with vascular thrombosis.



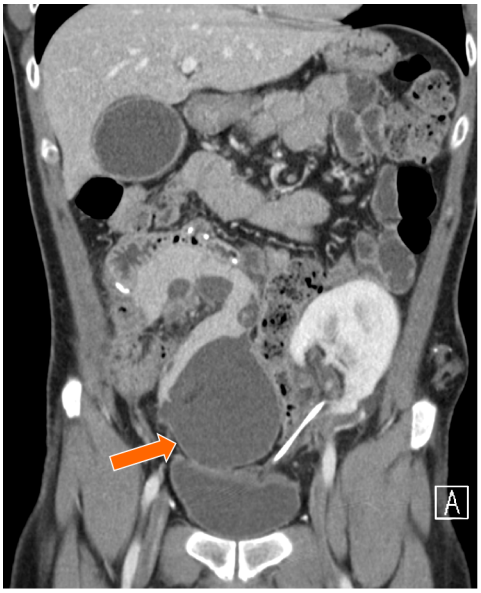
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Figure 6 A 35-year-old man is admitted to the emergency department presenting hyperpyrexia and abdominal tenderness; final diagnosis was edematous pancreatitis with acute peripancreatic fluid collection. Contrast enhanced computed tomography in the axial plane shows inhomogeneous transplanted pancreas (dotted orange lines) with slightly enlarged Wirsung duct and acute peripancreatic fluid collection (arrow) with thick wall and fat stranding surrounding the transplanted pancreas. A diagnosis of edematous pancreatitis was made.



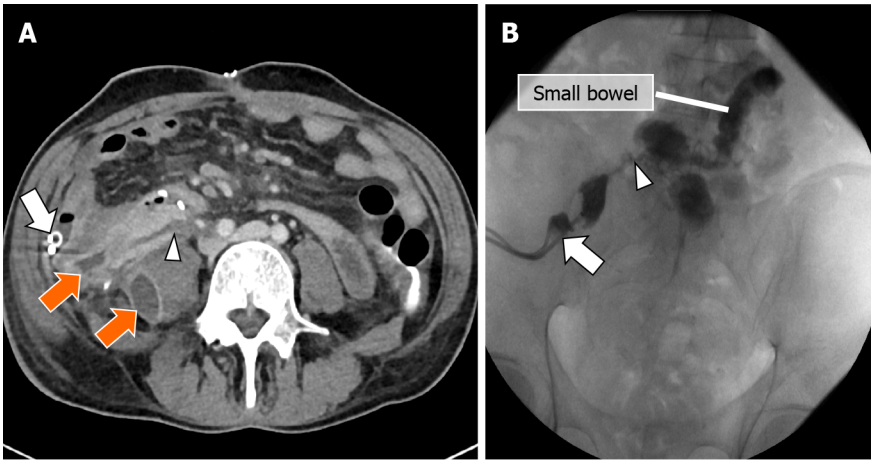
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Figure 7 Contrast enhanced computed tomography in four different patients after pancreatic transplantation. A region of interest is drawn in the pancreas in the four patients. A: Normal pancreatic parenchyma; B: Inflammatory pancreatitis; C: Decreased parenchymal enhancement from venous thrombosis; D: Decreased parenchymal enhancement from arterial thrombosis.



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Figure 8 A 35-year-old man after four weeks from edematous pancreatitis. Contrast enhanced computed tomography in the coronal plane shows an encapsulated fluid collection (arrow) of homogeneously low attenuation surrounded by a well-defined enhancing wall consistent with pancreatic pseudocyst.



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Figure 9 A 43-year-old man admitted to the emergency department for hyperpyrexia and abdominal tenderness; final diagnosis was enteric leakage. A: Contrast enhanced CT demonstrated the presence of an enteric leakage as a fistulous tract (arrowhead) from the small bowel at the level of the duodenojejunostomy, which resulted into fluid collections (orange arrows). Abdominal drainage (white arrow) was inserted and the analysis of the fluid was consistent with the diagnosis of enteric leakage; B: Fluoroscopic image demonstrate the presence of an enteric leakage as a fistulous tract (arrowhead) from the small bowel at the level of the duodenojejunostomy. Abdominal drained is also evident (white arrow).

Digestive: It comes from the digestive anastomosis or the staple line of the duodenal ends. Gastrointestinal bleeding after pancreas transplant usually occur at the duodenojejunostomy due to reduced blood flow to the graft or due to ulcers at the anastomosis. Digestive bleeding may resolve after conservative measures such as correction of coagulation abnormalities, heparin withdrawal and transfusion; surgical revision may be indicated if the bleeding does not resolve.

Precontrast CT may indicate the presence of an acute hematoma as a hyperattenuating (usually above 60 UH) collection on preconstrast CT in case of intrabdominal bleeding or hyperattenuating content in the GI lumen in case of gastrointestinal bleeding. In case of active bleeding, contrast-enhanced CT acquired in the arterial and venous phases demonstrates pooling/extravasation of the contrast agent within the hematoma or within the bowel lumen and may allow to identify the culprit vessel in case of intrabdominal hemorrhage.

Bleeding due to other arterial complications (*e.g.* arterio-venous fistula, pseudoaneurysm, arterioenteric fistula, arterio-ourinary fistula) have been reported but are infrequent, with occurrence of pseudoaneurysm being more common as a late complication[32,43]. Management can be endovascular or surgical and should be individualized[43].

Pseudoaneurysm may develop particularly at the level of anastomoses as a consequence of chemical damage due to leak of pancreatic enzymes[26]. On CT imaging, pseudoaneurysm appears as a saccular enhancing outpouching from the injured artery, with enhancement similar to other arteries, but does not increase in size on delayed phases and follows the



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Figure 10 A 42-year-old woman admitted in the hospital for elevation of pancreatic amylase 6 mo after transplant; final diagnosis was venous stenosis. Contrast enhanced computed tomography in the sagittal plane with Maximum Intensity Projection reconstruction demonstrates venous stenosis (arrowhead) at the anastomotic site. The pancreatic graft is enlarged with inhomogeneous enhancement which likely reflects graft dysfunction.

blood pool on all phases. Arterio-venous fistula is a very rare complication of pancreatic transplantation[32,43] which may result from the postoperative or post-biopsy laceration of both arterial and venous walls and may potentially lead to major bleeding or graft loss if untreated[43-45]. On CT, arterio-venous fistula will be demonstrated on arterial phase as enlarged Y-graft arteries with an early opacification of the donor draining vein.

Graft vascular stenosis

Vascular stenosis is relatively uncommon with an incidence of about 2.5% and more commonly occur at the anastomotic level[40]. Early detection of vascular stenosis is critical to avoid complications, including thrombosis, ischemia, and graft dysfunction[46]. The multiplanar reconstruction of the vascular tree provides a reliable method of visualizing the entire vascular anastomoses, detecting a focal decrease in caliber of the vessel (Figure 10).

CONCLUSION

In conclusion, pancreatic transplantation is a complex surgery with high morbidity often related to postoperative complications, which may occur during hospitalization, early in the first three months or in the late period. Cross-sectional imaging with CT plays a critical initial role in the diagnosis and management of postoperative complications in many transplanted patients who present to the emergency department for suspected transplant dysfunction. Therefore, radiologists should be aware of surgical techniques and normal imaging appearance of pancreatic transplantation and should be well-trained to recognize identify acute findings and provide key imaging information to optimize patient management.

FOOTNOTES

Author contributions: D'Alessandro C, made the literature search, wrote the first draft of the manuscript, and prepared most of the figures; Todisco M conceptualized the manuscript, helped with figures preparation and provided inputs; Di Bella C and Furian L wrote the first draft of the manuscript for the surgical paragraph, provided inputs and revised the draft of the manuscript; Crimì F provided inputs and revised the draft of the manuscript; Quaia E conceptualized the manuscript, provided inputs, and revised the draft of the manuscript; Vernuccio F conceptualized the manuscript, helped with the literature search, wrote the outline of the manuscript, prepared some of the figures and extensively revised the draft of the manuscript.

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ORIGINAL ARTICLE

Basic Study

Exosome-mediated transfer of circRNA563 promoting hepatocellular carcinoma by targeting the microRNA148a-3p/metal-regulatory transcription factor-1 pathway

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Abstract

BACKGROUND

Mesenchymal stem cells (MSCs) exert anti-oncogenic effects *via* exosomes containing non-coding RNA (ncRNA), which play important roles in tumor biology. Our preliminary study identified the interaction of the ncRNA hsa_circ_0000563 (circ563) and the circ563-associated miR-148a-3p in exosomes, as miR-148a-3p and its target metal-regulatory transcription factor-1 (MTF-1) are implicated in hepatocellular carcinoma (HCC) progression.

AIM

To identify the clinical significance, functional implications, and mechanisms of circ563 in HCC.

METHODS

The expression levels of miR-148a-3p and MTF-1 in exosomes derived from MSC and HCC cells were compared, and their effects on HCC cells were assessed. Using a dual-luciferase reporter assay, miR-148a-3p was identified as an associated microRNA of circ563, whose role in HCC regulation was assessed *in vitro* and *in vivo*.

RESULTS

The silencing of circ563 blocked the HCC cell proliferation and invasion and induced apoptosis. Co-culturing of HCC cells with MSC-derived exosomes following circ563 overexpression promoted cell proliferation and metastasis and elicited changes in miR-148a-3p and MTF-1 expression. The tumor-promoting effects of circ563 were partially suppressed by miR-148a-3p overexpression or MTF-1 depletion. Xenograft experiments performed in nude mice confirmed that circ563-enriched exosomes facilitated tumor growth by upregulating the ex-

pression of MTF-1. In HCC tissues, circ563 expression was negatively correlated with miR-148a-3p expression but positively correlated with MTF-1 levels.

CONCLUSION

MSCs may exhibit anti-HCC activity through the exosomal circ563/miR-148a-3p/MTF-1 pathway, while exosomes can transmit circ563 to promote oncogenic behavior by competitively binding to miR-148a-3p to activate MTF-1.

Key Words: Exosome; Cell communication; Noncoding RNA; Metal-regulatory transcription factor-1; Mesenchymal stem cells

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Core Tip: We identified the functional implications and mechanisms of exosomal hsa_circ_0000563 (circ563) in hepatocellular carcinoma (HCC). Mesenchymal stem cells suppressed HCC proliferation and invasion *via* their exosomes. The circ563 interacts with miR-148a-3p, a molecule involved in HCC progression, and both are identified at different levels in exosomes. The tumor-promoting effects of circ563 were partially suppressed by miR-148a-3p overexpression or depletion of metal-regulatory transcription factor-1 (MTF-1). Xenograft experiments performed in nude mice confirmed that circ563-enriched exosomes facilitated tumor growth *via* MTF-1 upregulation. Our findings provide new insights into circ563/miR-148a-3p/MTF-1 signaling as a potential therapeutic target for HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a fatal disease and the most common form of primary liver cancer. Although novel chemotherapeutic agents and treatment modalities have been clinically implemented, the survival rate of patients with HCC has not yet improved[1]. Therefore, a deeper understanding of the mechanisms facilitating HCC progression is paramount for the development of more effective therapeutic strategies.

The tumor microenvironment (TME) has been an important aspect of tumor research. Recent studies indicate that mesenchymal stem cells (MSCs) are essential stromal cells in the TME and exert their effects *via* exosomes, as demonstrated by multiple studies[2,3]. Furthermore, the efficacy of therapeutic MSC transplantation has been reported in both experimental and clinical research[4-6]. As a result, MSC-derived exosomes have attracted significant attention due to their implications in intercellular communication.

Exosomes are small extracellular vesicles approximately 30-150 nm in diameter that are formed by cells through a series of regulatory processes. These vesicles are enriched in bioactive molecules like non-coding RNA (ncRNA) with crucial biological functions, whose delivery to recipient cells is involved in various physiological and pathological processes, including carcinogenesis and metastasis[7,8]. Circular RNA (circRNA) is a new class of endogenous ncRNA, which has selectively conserved microRNA (miRNA) target sites and exerts regulatory effects in cancer biology by regulating miRNA gene expression[9,10]. Such interactions between circRNA and miRNA are known as the competitive endogenous RNA theory[11]. miRNAs represent the ncRNA of 20-25 nucleotides, which mediate post-transcriptional gene silencing by binding to the 3'-untranslated regions of target mRNAs. Exosomal circRNA and miRNA are involved in tumor initiation and progression[7,8,12,13]; however, their expression profiles, functions, and dysregulation in HCC require further clarification.

This study found that MSCs suppressed HCC proliferation and invasion *via* their exosomes. The ncRNA circ563 interacts with miR-148a-3p, and both are identified at different levels in exosomes. Metal-regulatory transcription factor-1 (MTF-1) as the direct target of miR-148a-3p is implicated in HCC progression. Therefore, further studies should be performed to determine the possible role and significance of circ563/miR-148a-3p/MTF-1 axis in the development of HCC.

MATERIALS AND METHODS

Cell lines and culture

The human HCC cell lines Hep3B (RRID: CVCL_0326, iCell Bioscience, Shanghai, China) and SNU387 (RRID: CVCL_0250, Procell, Wuhan, China), MSC (Procell, Wuhan, China), and the human embryonic kidney cell line HEK 293T (RRID: CVCL_0045, Cell Research, Shanghai, China) were cultured according to the manufacturer's instructions. The cell

lines used in this study were authenticated, and no cellular cross-contamination or mycoplasma infection was detected.

Isolation and characterization of exosomes

The exosomes were isolated as previously described[14], and their morphology was observed under a transmission electron microscope. The size distribution of exosomes was analyzed using the NanoSight LM10 system (NanoSight, Amesbury, United Kingdom) equipped with fast video capture and particle-tracking software.

Exosome internalization assay

MSCs were modified to express fused CD63-eGFP *via* lentiviral transduction following the manufacturer's instructions. The HCC cells were seeded on glass coverslips coated with poly-L-lysine (Sigma, Shanghai, China). MSC-derived exosomes were co-cultured with HCC cells for 24 h at 37 °C before washing and fixation at room temperature. The uptake of labeled exosomes by recipient cells was observed under a fluorescence microscope (Leica TCS SP8 X, Leica, Shanghai, China).

Western blotting

Exosomes were harvested for protein extraction. Equal amounts of protein were subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis and then electroblotted onto a polyvinylidene difluoride membrane (Merck, Nantong, Jiangsu, China) followed by western immunoblotting. Membranes were incubated with primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (ZSGB-BIO, Beijing, China). The bands were visualized using a Tanon 5200 Chemiluminescent imaging system (Molecular Devices, Beijing, China) and analyzed using ImageJ software (version 1.52, National Institutes of Health, Bethesda, United States). The following primary antibodies were used: Anti-TSG101, anti-CD9, and anti-CD81 (Abcam, Shanghai, China).

Real-time polymerase chain reaction

Total RNA was extracted using the Ultrapure RNA Kit (CoWin Biotech, Beijing, China) according to the manufacturer's protocol. Equal amounts of the samples were amplified, and real-time polymerase chain reaction (RT-PCR) was performed as previously described[14]. The following primer pairs were used: MiR-148a-3p, forward: 5′-CTCAGTGCAC-TACAGAACTTTGT-3′, reverse: 5′-ATCCAGTGCAGGGTCCGAGG-3′; MTF-1, forward: 5′-GAAAAGCCATTTCG-GTGCGA-3′, reverse: 5′-CAGCCATTACTGGGGCAGAA-3′ (DingGuo Biotechnology, Beijing, China); hsa_-circRNA_0000562 (circ562), forward: 5′-TTTAGTACACAGCATGGCTCA-3′, reverse: 5′-ATTAGCACCCAT-TTTCTTCAGT-3′; hsa_circRNA_0000563 (circ563), forward: 5′-TTGGAATTTAACATGCTTGC-3′, reverse: 5′-GCATTAGCACCCATTTTCTT-3′; hsa_circRNA_00001110 (circ1110), forward: 5′-TCACTTGGGTTCATTCTATTCA-3′, reverse: 5′-GCTTGTCTTTTCTTCTGTTTCC-3′; U6, forward: 5′-GCTCGCTTCGGCAGCACA-3′, reverse: 5′-GAACGCTTCACGAATTTGCGTG-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward: 5′-AGAAGGCT-GGGGCTCATTTG-3′, reverse: 5′-AGGGGCCATCCACAGTCTTC-3′; and actin, forward: 5′-CCCTATAAAAC-CCAGCGGCG-3′, reverse: 5′-TCGTCGCCCACATAGGAATC-3′ (Tsingke Biotechnology, Qingdao, China). Data were analyzed using the comparative CT method, and the $2^{-\Delta\Delta Ct}$ method showed the difference between treatment and control conditions.

Transfection assay

Overexpression plasmids of miR-148a-3p, knockdown plasmids of MTF-1, and control empty vectors were obtained from GenePharma (Shanghai, China). Small interfering RNA targeting circ562 (sense: 5′-GGCUCAGCAAAAUG-GAUGAAAdTdT-3′; antisense: 5′-UUUCAUCCAUUUUGCUGAGCCdTdT-3′), circ563 (sense: 5′-GCUU-GCACAAGAAAUGGAUGAdTdT-3′; antisense: 5′-UCAUCCAUUUCUUGUGCAAGCdTdT-3′), and circ1110 (sense: 5′-GGUUCAUUCUAUUCAGUGACUdTdT-3′; antisense: 5′-AGUCACUGAAUAGAAUGAACCdTdT-3′) were synthesized by Hippobio (Zhejiang, China). The cells with stable overexpression of miR-148a-3p (5′-UCAG-UGCACUACAGAA-CUUUGU-3′) or circ563 or with stable knockdown of MTF-1 (5′-GCGGTACCAATGTACCTTTGA-3′) were transfected with lentiviral vectors for *in vitro* and *in vivo* studies. The cells in 6-well plates were transfected using Lipofectamine 2000 (Invitrogen, Shanghai, China), according to the manufacturer's instructions.

Cell viability assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded into 96-well plates (approximately 1500 cells/well) before treatment. At the end of each treatment, the MTT reagent was added to the medium. The cells were incubated for an additional 4 h, and the absorbance of the samples was measured at 490 nm using a plate reader (Thermo Fisher Scientific, MA, United States). Experiments were performed in triplicate, and data were expressed as the mean optical density ± SD.

Colony formation assay

The cells (approximately 1000 cells/well) were seeded into six-well plates with different treatments, either simultaneously or subsequently. After 14 d, the cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet, and the individual clones (> 50 cells/clone) were counted. Each treatment was performed in triplicate. Data were expressed as the mean ± SD.

Transwell assays

Transwell assays were performed to assess cell invasion and migration abilities in 24-well plates with BD chambers (8-mm pores; BD Biosciences, Shanghai, China). Approximately 1 × 10^5 cells/well were seeded into the upper chambers and cultured in a serum-free medium. The medium containing 30% serum was placed in the lower chambers. After migration through the Transwell membrane, the cells were fixed with 4% paraformaldehyde and stained with crystal violet (Solarbio, Beijing, China). Compared with the invasion assay, the Transwell chambers for the migration assay were not coated with Matrigel. All Transwell treatments were conducted in triplicate.

Flow cytometry

Following different treatments, cell apoptosis was detected using the phycoerythrin (PE) Annexin V Apoptosis Detection Kit I (BD Pharmingen, China) according to the manufacturer's instructions. The cells were harvested after trypsin digestion, centrifuged, and washed with annexin V binding buffer three times. The cells were then stained with PE annexin V and 7-amino-actinomycin D at room temperature for 15 min and analyzed using flow cytometry (BD FACSAria II, Shanghai, China), and the results were analyzed using FlowJo software (version 10; Ashland, OR, United States).

Luciferase reporter assay

A wild-type (wt) or mutant circ563 fragment was constructed and cloned into plasmids containing luciferase. The recombinant circ563-wt/mt plasmids were co-transfected into HEK 293T cells with either a negative control or a miR-148a-3p mimic using Lipofectamine 2000 (Invitrogen Inc., CA, United States). After 48 h, the luciferase activity was measured using the Dual-Luciferase Reporter 1000 Assay System (Promega, Madison, WI, United States). The firefly luciferase enzyme activity was normalized to the Renilla luciferase activity, and the ratio of firefly to Renilla luciferase activity was evaluated. The experiments were independently performed in triplicate.

Patients and specimens

Human HCC tissues and paired tumor-adjacent noncancerous liver tissues were obtained from 14 patients with HCC who underwent tumor resection at the Shandong Provincial Hospital, China. None of the patients had undergone radio- or chemotherapy before surgery. Informed consent was obtained from each participant and approved by the Institutional Review Board. The study was approved by the Institutional Medical Ethics Committee of Shandong Provincial Hospital. Patients aged ≥ 18 years and diagnosed with HCC without any local or systemic treatment were included in the study. By contrast, patients with other types of cancers, liver failure, and taking medications that affect liver function were excluded.

HCC tumor-xenograft mouse model

The animal study was approved by the Animal Ethics Committee of Shandong Provincial Hospital. The animal protocol was designed to minimize pain or discomfort during the experiments. The animals were acclimatized to laboratory conditions (23 °C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for 2 wk before experimentation. Five-week-old BALB/c nude mice (Provincial Laboratory Animal Center, Jinan, China) were used for this study. Following different treatments, the Hep3B cells were prepared at 3 × 10^6 cells/100 μL. Initially, the HCC-bearing nude mice were prepared to develop subcutaneous tumors. After four weeks, circ563-enriched exosomes (approximately 10^6) were injected intratumorally into the tumor mass twice a week for two weeks. The tumor size was measured weekly, and the tumor volume was calculated using the following formula: Volume (mm^3) = tumor length × $width^2$/2. After six weeks, all mice were sacrificed to compare the tumor volumes and weights. The tumors were fixed in 10% formalin and embedded in paraffin for subsequent immunohistochemical examination.

Immunohistochemistry and scoring

The sections were stained with an anti-MTF-1 sary antibody (Zhongshan Biotechnology Co., Beijing, China). The slides were imaged and analyzed using Aipathwell software (Servicebio, Wuhan, China). The staining intensity was characterized as not present (0), weak but detectable above control (1), moderate (2), and very strong (3). The H-scores were calculated as follows: H-score = ∑(pi × i) = (percentage of weak intensity cells × 1) + (percentage of moderate intensity cells × 2) + (percentage of strong intensity cells × 3). Data were presented as the mean ± SD.

Statistical analysis

Statistical analyses were performed using SPSS 21.0 software. Data were presented in bar plots as the mean ± SD of at least three independent experiments. A P value of < 0.05 (two-tailed) was considered significant. Student's t-test was performed to determine whether the two groups were significantly different. Pearson's correlation analysis was used to assess the correlations between circ563, miR-148a-3p, and MTF-1.

RESULTS

MSCs and MSC-derived exosomes exhibiting tumor-suppressive effects on HCC with corresponding changes in miR-148a-3p and MTF-1 levels

HCC cell proliferation was inhibited by co-culturing with MSCs, which was consistent with the reduced cell viability, migration, and invasion observed under these conditions (Figures 1A-G and Supplementary Figures 1A-G). However, as shown in Figure 1H and Supplementary Figure 1H, apoptosis was induced in HCC cells. To further verify the regulatory role of MSC-derived exosomes in HCC, the exosomes were isolated and characterized by specific marker expression (Figure 1I) and particle size analysis (Figure 1J). In addition, we observed and verified the uptake of labeled exosomes in HCC cells (Figure 1K and Supplementary Figure 1I). MSC-derived exosomes also exerted substantial anti-oncogenic effects in HCC cells by blocking cell proliferation, migration, and invasion while inducing apoptosis (Figures 1L-S and Supplementary Figures 1J-Q). Therefore, MSCs inhibit HCC proliferation and metastasis *via* their exosomes.

A previous study from our laboratory demonstrated that exosomal miR-148a-3p functions as a tumor suppressor in HCC progression, with MTF-1 being its direct target[14]. In the present study, MSC-derived exosomes displayed increased miR-148a-3p and decreased MTF-1 expression levels compared with HCC-derived exosomes (Figure 1T and Supplementary Figure 1R). Co-culturing HCC cells with either MSCs or MSC-derived exosomes led to the corresponding changes in miR-148a-3p and MTF-1 expression levels (Figure 1U and Supplementary Figure 1S). This finding indicates that exosomes contain bioactive materials involved in the regulation of miR-148a-3p and MTF-1 expression in HCC cells.

Characterization of circ563 and its expression in clinical HCC

A search of the CircInteractome databases (https://circinteractome.nia.nih.gov/) yielded three potential circRNAs [circ563, hsa_circRNA_0000562 (circ562), and hsa_circRNA_00001110 (circ1110)] that are most likely to bind to miR-148a-3p based on potential complementary sequences. Further analysis was performed using dual-luciferase reporter assays and RT-PCR. The RT-PCR analyses showed that circRNA was positively correlated with MTF-1 but negatively correlated with miR-148a-3p (Figure 2A). The overexpression of miR-148a-3p considerably inhibited the luciferase activity of circ563-wt. However, the luciferase activity of circ563-mutant-type (mt) cells transfected with miR-148a-3p mimics remained unaffected, thus indicating that miR-148a-3p is a target miRNA of circ563 (Figure 2B). Subsequently, knockdown experiments were performed to investigate whether circ563 affects the biological processes of HCC cells. si-circ563 was transfected into Hep3B and SNU387 cells, both strongly expressing circ563, and the knockdown efficiency was determined by RT-PCR (Figure 2C and Supplementary Figure 2A). Exosomal circ563 (exo-circ563) expression levels were also compared with those of MSCs (Figure 2D and Supplementary Figure 2B). Silencing of circ563 expression blocked the proliferation and invasion of HCC cells and increased their apoptosis rate (Figures 2E-I and Supplementary Figures 2C-G).

Next, circ563 expression was analyzed in human HCC tissues, which showed higher circ563 levels with downregulated miR-148a-3p and upregulated MTF-1 expression levels compared with tumor-adjacent tissues (Figures 3A-C). Correlation analyses further revealed the relationships between circ563, miR-148a-3p, and MTF-1 levels in HCC (Figures 3D-F). These findings suggest that circ563 may be involved in potentiating HCC cell growth and metastasis, considering the observed correlation with miR-148a-3p and MTF-1.

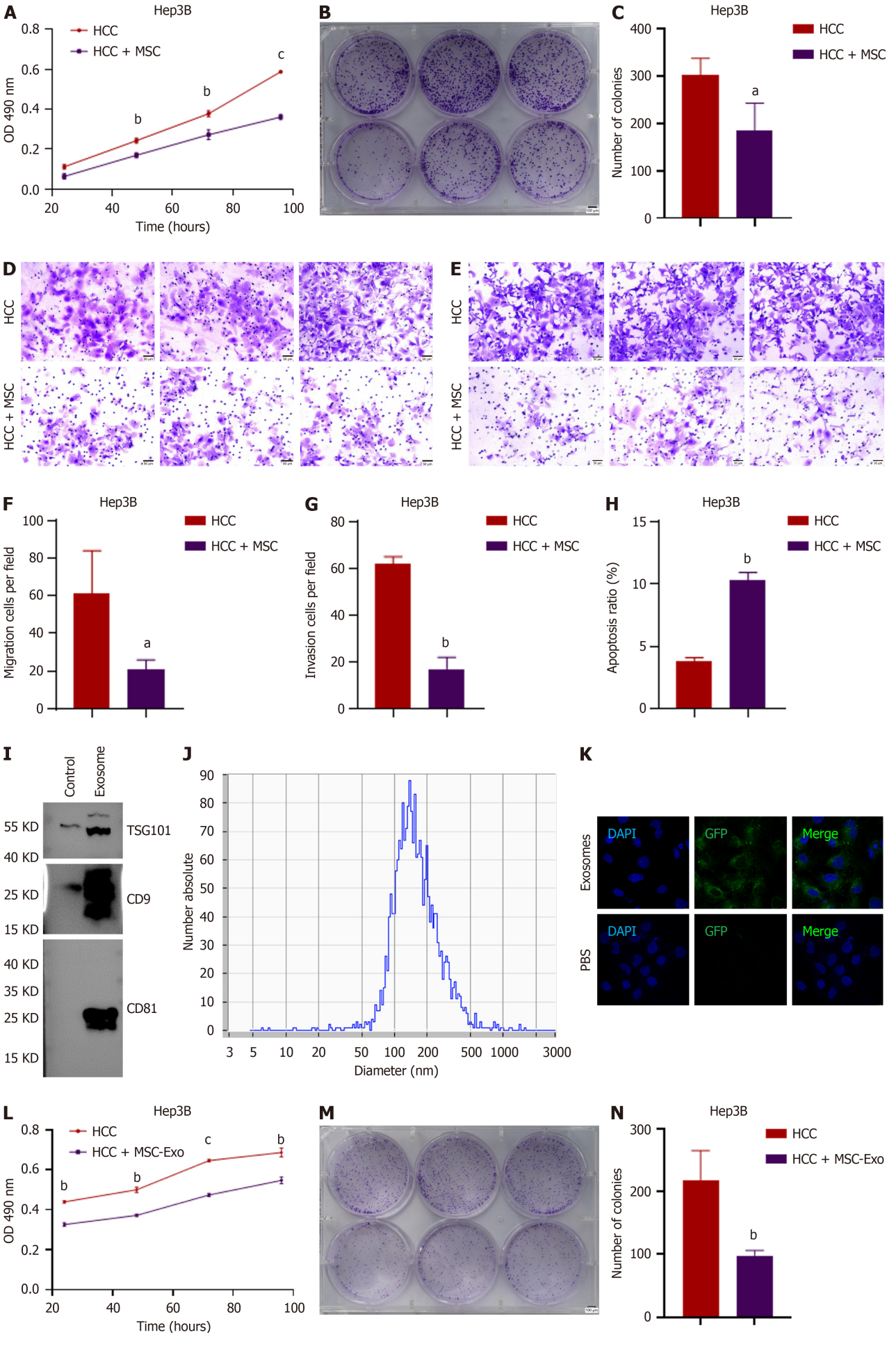
Exo-circ563 potentiating HCC progression by sequestering miR-148a-3p that regulates MTF-1

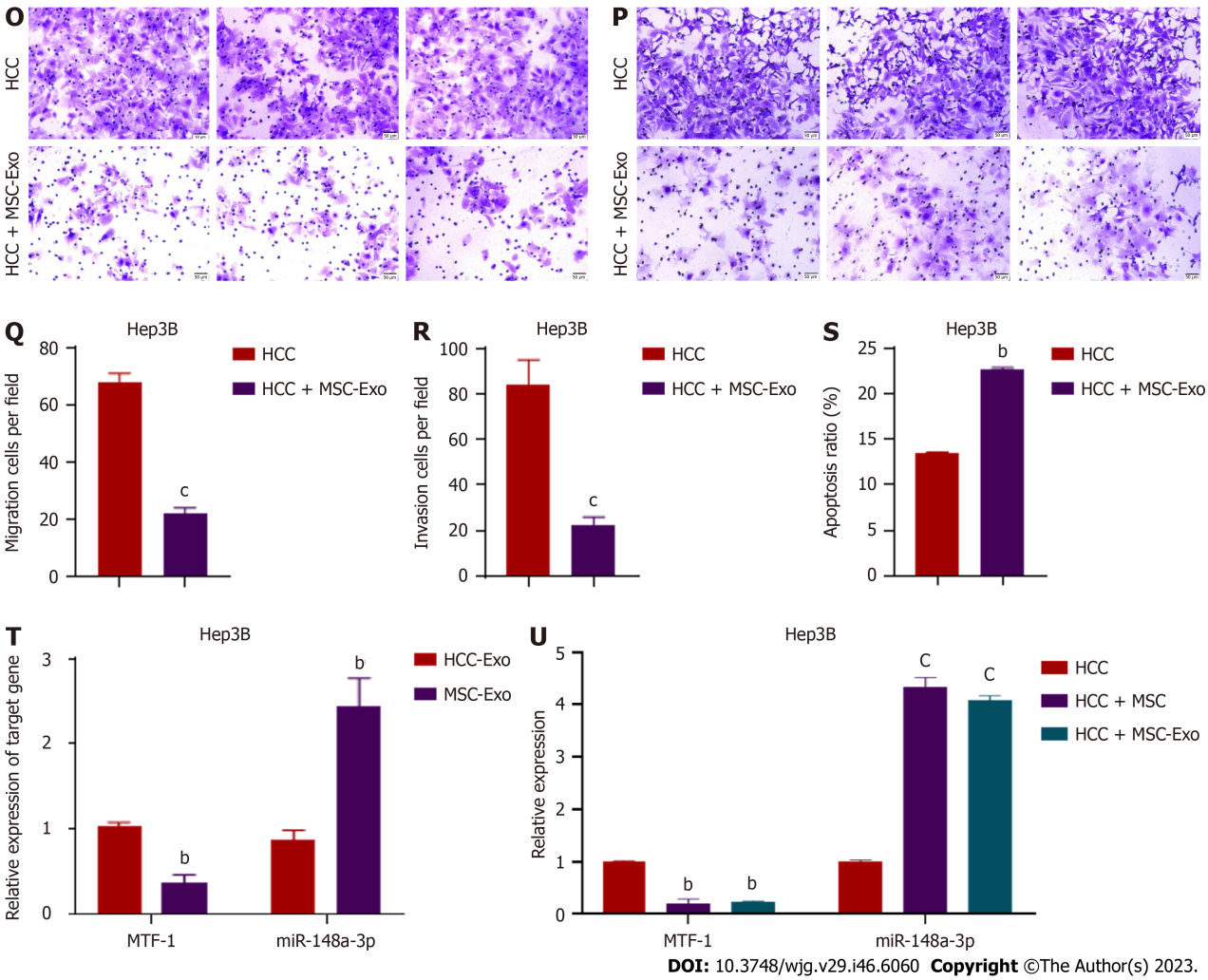
To further explore whether exo-circ563 exerts an oncogenic role, we suppressed the expression of circ563 in HCC cells and detected a reduction in exo-circ563 levels using RT-PCR (Figure 4A). The HCC cells co-treated with exosomes containing downregulated circ563 levels led to a marked reduction in cell proliferation and invasion and enhanced apoptosis (Figures 4B-I and Supplementary Figures 3A-H).

Consistent with previous results, exo-circ563 overexpression facilitated HCC cell progression. The exo-circ563 overexpression efficiency and upregulation were ascertained through RT-PCR (Figures 4J and K). We co-cultured HCC cells with MSC-derived exosomes following the circ563 overexpression and observed an increase in HCC cell proliferation and metastasis and a suppression of apoptosis (Figures 4L-S and Supplementary Figures 3I-P). These effects were partially prevented by the overexpression of miR-148a-3p or depletion of MTF-1 (Figure 5 and Supplementary Figure 4). These data indicate the involvement of a circ563-dependent mechanism in regulating miR-148a-3p expression as described by the competitive endogenous RNA theory.

circ563 accelerating HCC tumorigenesis in vivo

To further confirm the significance of exo-circ563 in HCC tumorigenesis and progression, *in vivo* experiments were performed. The upregulation of circ563 dramatically accelerated tumor growth in nude mice as evidenced by the detection of larger tumors. This effect could be reversed by miR-148a-3p overexpression or MTF-1 depletion (Figures 6A-C). Therefore, the effects of circ563, by facilitating HCC progression *via* miR-148a-3p and MTF-1 regulation, were also present *in vivo*. Correspondingly, the expression of MTF-1 in murine tumor tissues had changed similarly to tumor growth (Figures 6D and E). All these results show that circ563 accelerates HCC progression by sponging miR-148a-3p to elicit MTF-1-dependent oncogenic effects *via* the circ563/miR-148a-3p/MTF-1 axis (Figure 7).


A
Hep3B
HCC
HCC + MSC
OD 490 nm
Time (hours)
B
C
Hep3B
HCC
HCC + MSC
Number of colonies
D
HCC
HCC + MSC
E
HCC
HCC + MSC
F
Hep3B
HCC
HCC + MSC
Migration cells per field
G
Hep3B
HCC
HCC + MSC
Invasion cells per field
H
Hep3B
HCC
HCC + MSC
Apoptosis ratio (%)
I
Control
Exosome
55 KD
40 KD
TSG101
25 KD
15 KD
CD9
40 KD
35 KD
25 KD
15 KD
CD81
J
Number absolute
Diameter (nm)
K
Exosomes
PBS
DAPI
GFP
Merge
L
Hep3B
HCC
HCC + MSC-Exo
OD 490 nm
Time (hours)
M
N
Hep3B
HCC
HCC + MSC-Exo
Number of colonies



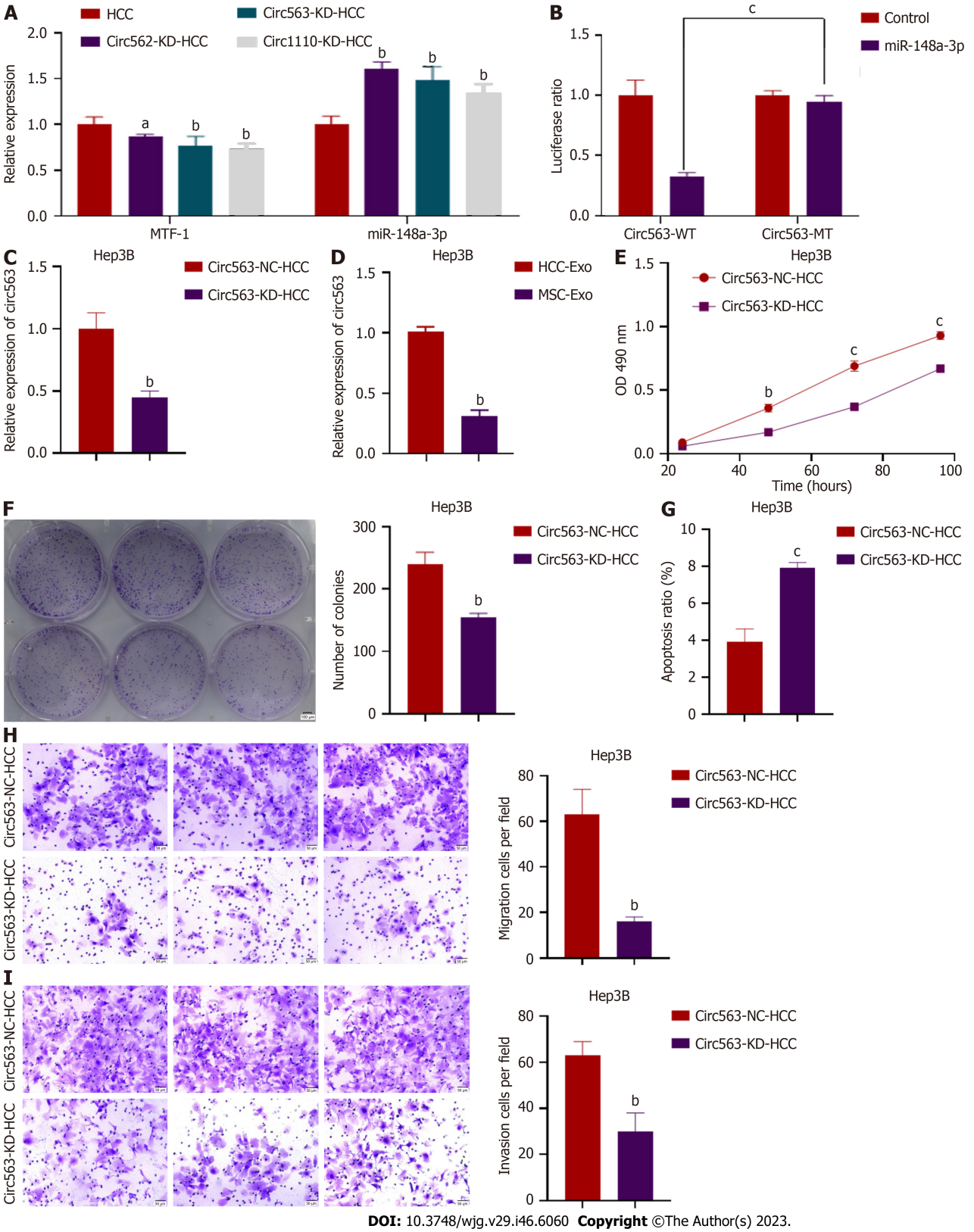
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Figure 1 Effects of mesenchymal stem cells or mesenchymal stem cell-derived exosome co-incubation on Hep3B cells. A-C: The proliferation of Hep3B cells was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide and colony formation assays; D-G: Invasion and metastasis were determined using the Transwell assay. Scale bar = 500 μm; H: Cell apoptotic rates were detected after co-incubation using flow cytometry; I: Exosomes were isolated, and specific exosome markers were identified using western blotting; J: The particle size analyzer showed that the average diameter of the vesicles was within the diameter range of exosomes; K: The uptake of labeled exosomes was observed and verified; L-S: Hep3B cell viability and metastasis were both suppressed after exposure to mesenchymal stem cell (MSC)-derived exosomes, and apoptosis was enhanced; T: Comparison of miR-148a-3p and metal-regulatory transcription factor-1 (MTF-1) expression levels in MSC- and hepatocellular carcinoma (HCC)-derived exosomes; U: Assessment of miR-148a-3p and MTF-1 expression by real-time polymerase chain reaction in HCC cells co-cultured with MSC and MSC-derived exosomes. Quantitative data from three independent experiments are shown as the mean ± SD (error bars). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. HCC: Hepatocellular carcinoma; miRNA: MicroRNA; MSC: Mesenchymal stem cell; MTF-1: Metal-regulatory transcription factor-1; PBS: Phosphate buffered saline.

DISCUSSION

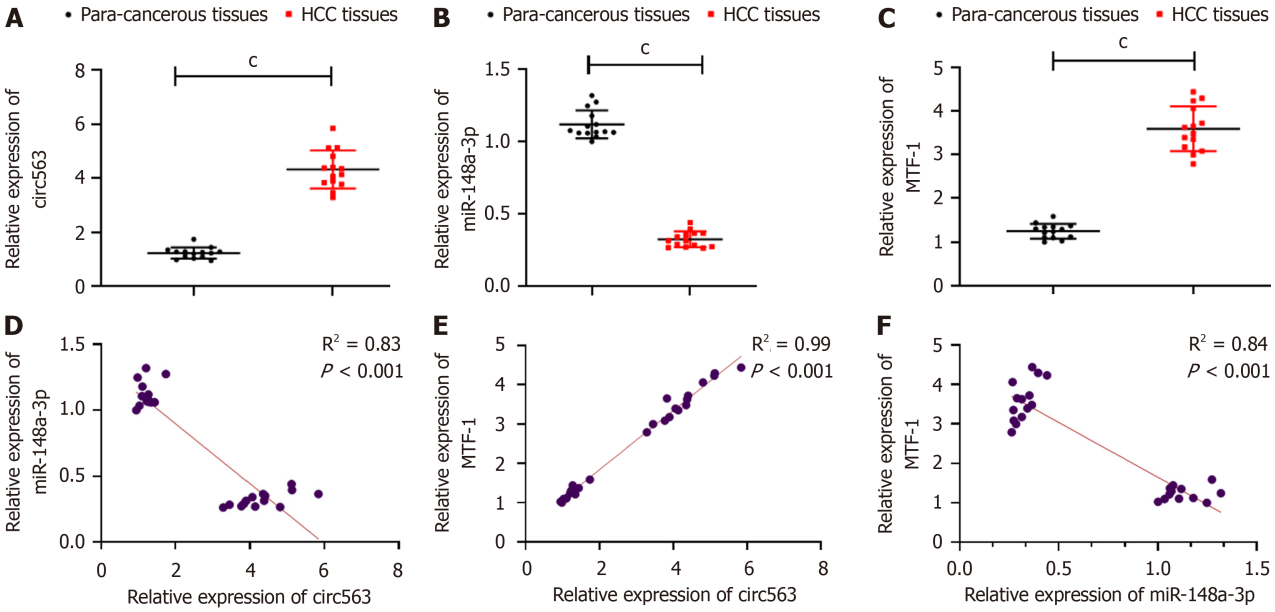
Liver cancer remains a global health challenge, and its incidence is expected to reach over 1 million cases by 2025[15]. HCC is the most common primary liver cancer. It is characterized by aggressive progression, broad metastasis, frequent recurrence, and high mortality. Therefore, a better understanding of the processes involved in HCC tumorigenesis and metastasis is urgently needed to discover new treatment strategies and improve patient prognosis.

Recent studies have established a comprehensive role of TME in disease development, making it one of the most promising areas of oncological research[16,17]. Moreover, HCC cells can alter their surrounding microenvironment to promote their growth and metastasis[18,19]. The crosstalk between tumor cells and their microenvironment is important for MSC recruitment to HCC tissues, which has also been observed in HCC animal models[19,20]. Although MSCs were initially considered as oncological delivery vehicles due to their migratory and homing capacity toward tumors[21], increasing evidence suggests that they can modulate tumorigenesis *via* the exosomes[2,3]. In the present study, co-incubation with MSCs inhibited the proliferation and invasion of HCC cells but enhanced their apoptotic capabilities. Further investigation on the mediating role of MSC-derived exosomes in the regulation of HCC cells also led to the discovery of their anti-oncogenic potential by blocking the development of HCC cells. Based on our findings, an exosome-based adjuvant intervention could offer a novel therapeutic approach to HCC by delivering the protected "cargo" directly to the tumor site.



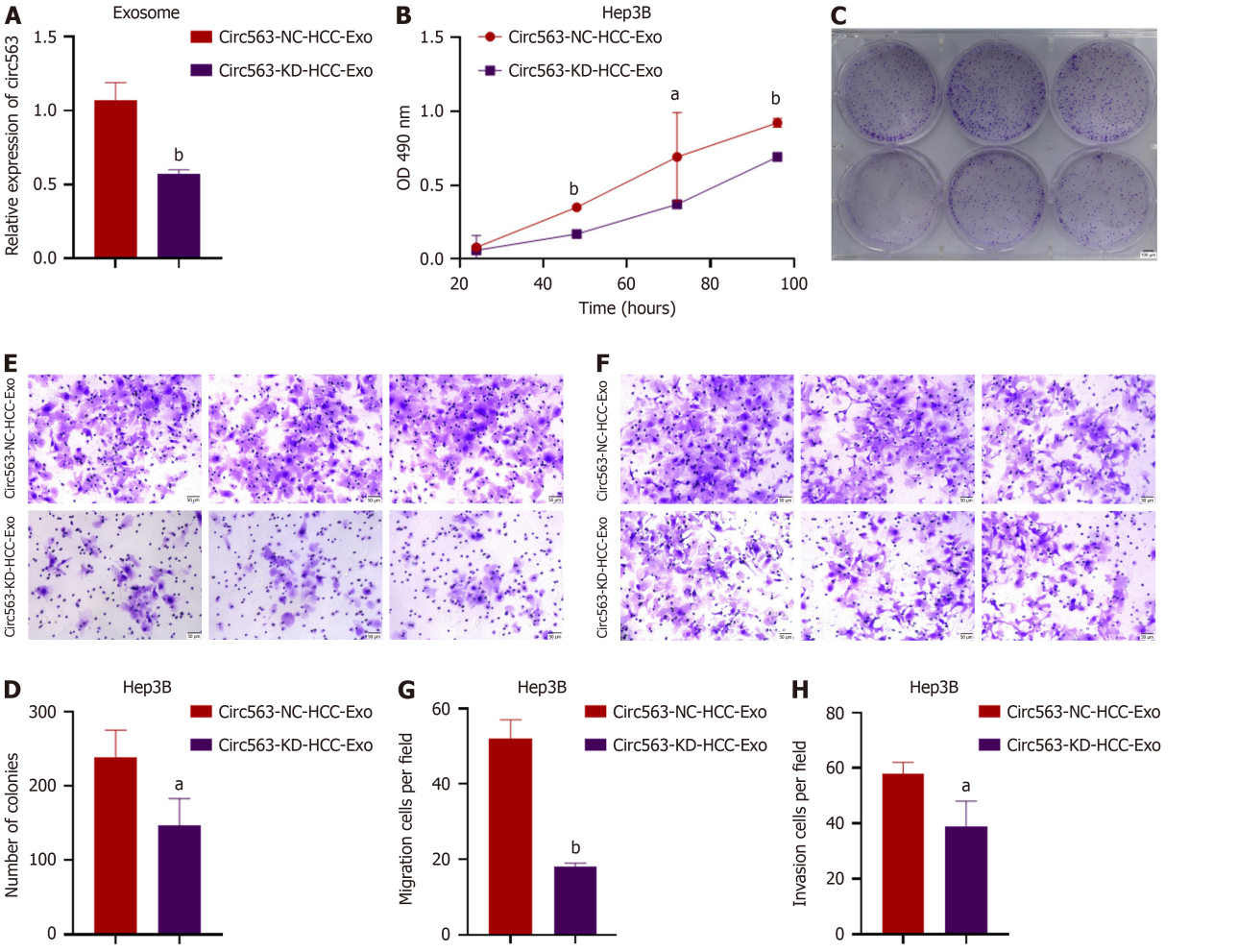
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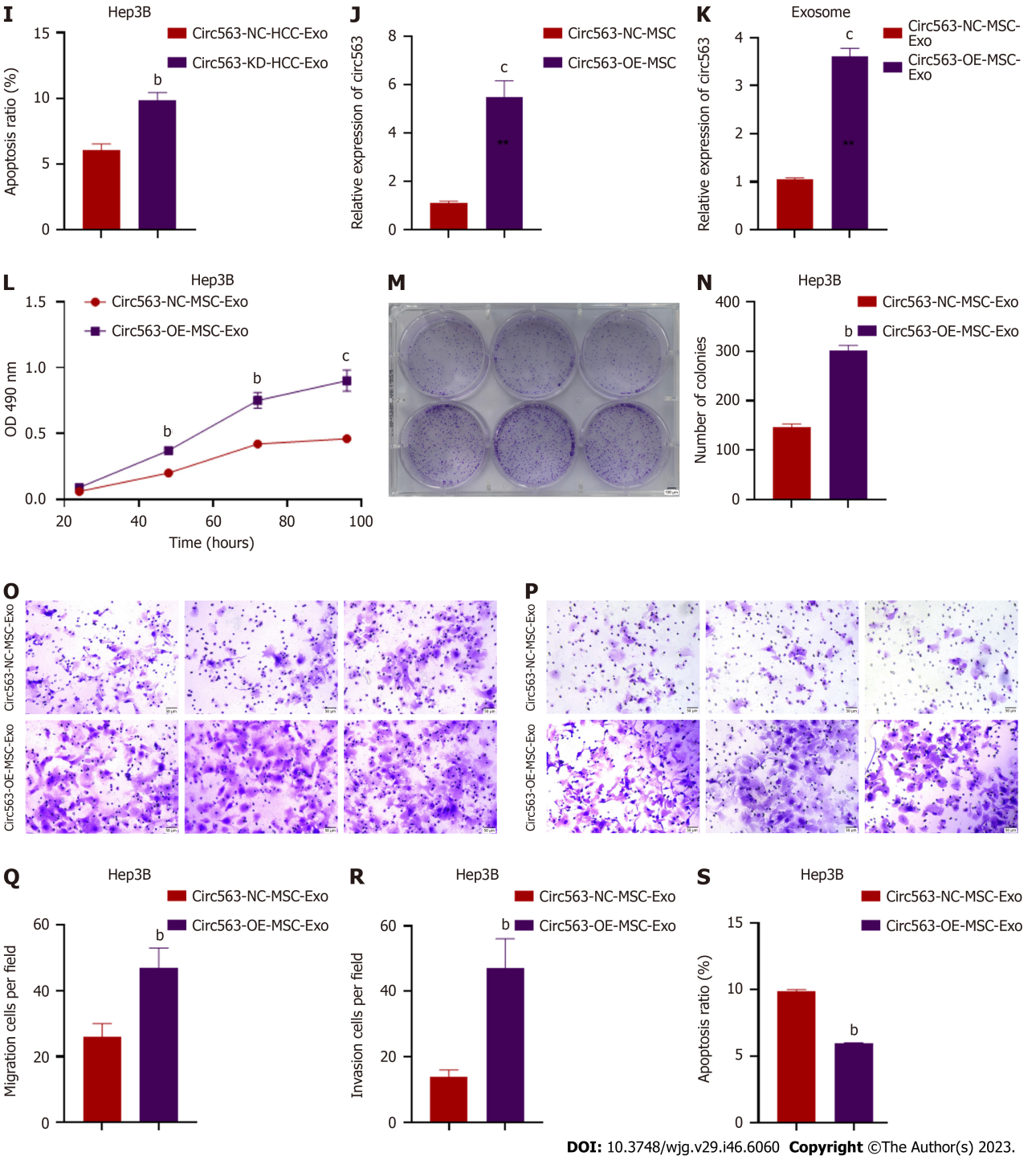
Figure 2 Identification of hsa_circRNA_0000563. A: Three circular RNAs (circRNAs) (circ562, circ563, and circ1110) were predicted to most likely bind to miR-148a-3p, and the miR-148a-3p and metal-regulatory transcription factor-1 (MTF-1) levels in hepatocellular carcinoma (HCC) cells transfected with the predicted circRNA were detected by quantitative real-time polymerase chain reaction (RT-PCR); B: A dual-luciferase reporter assay was performed to confirm the direct binding between circ563 and miR-148a-3p based on their complementary sequences; C: The knockdown efficiency was verified by RT-PCR; D: The expression levels of circ563 in exosomes were assessed by RT-PCR; E and F: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and colony-forming assay results showed that circ563 silencing suppressed cell proliferation and reduced the number of colonies; G: Knockdown of circ563 enhanced Hep3B cell apoptosis as determined by flow cytometry; H and I: Transwell assays revealed that reducing the levels of circ563 impaired the migratory potential and invasiveness of HCC cells. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. circRNA: Circular RNA; HCC: Hepatocellular carcinoma; Exo: Exosome.



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Figure 3 Clinical relevance of circ563 in hepatocellular carcinoma. To further delineate the role of circ563, hepatocellular carcinoma and paired tumor-adjacent tissues were subjected to real-time polymerase chain reaction. A-C: Enhanced circ563 expression in hepatocellular carcinoma (HCC) tissue was detected compared with that in tumor-adjacent tissues, in line with miR-148a-3p downregulation and metal-regulatory transcription factor-1 (MTF-1) upregulation; D-F: Correlation analyses indicated the relationships between circ563, miR-148a-3p, and MTF-1 expression in HCC. °$P < 0.001$. circRNA: Circular RNA; HCC: Hepatocellular carcinoma; MTF-1: Metal-regulatory transcription factor-1.

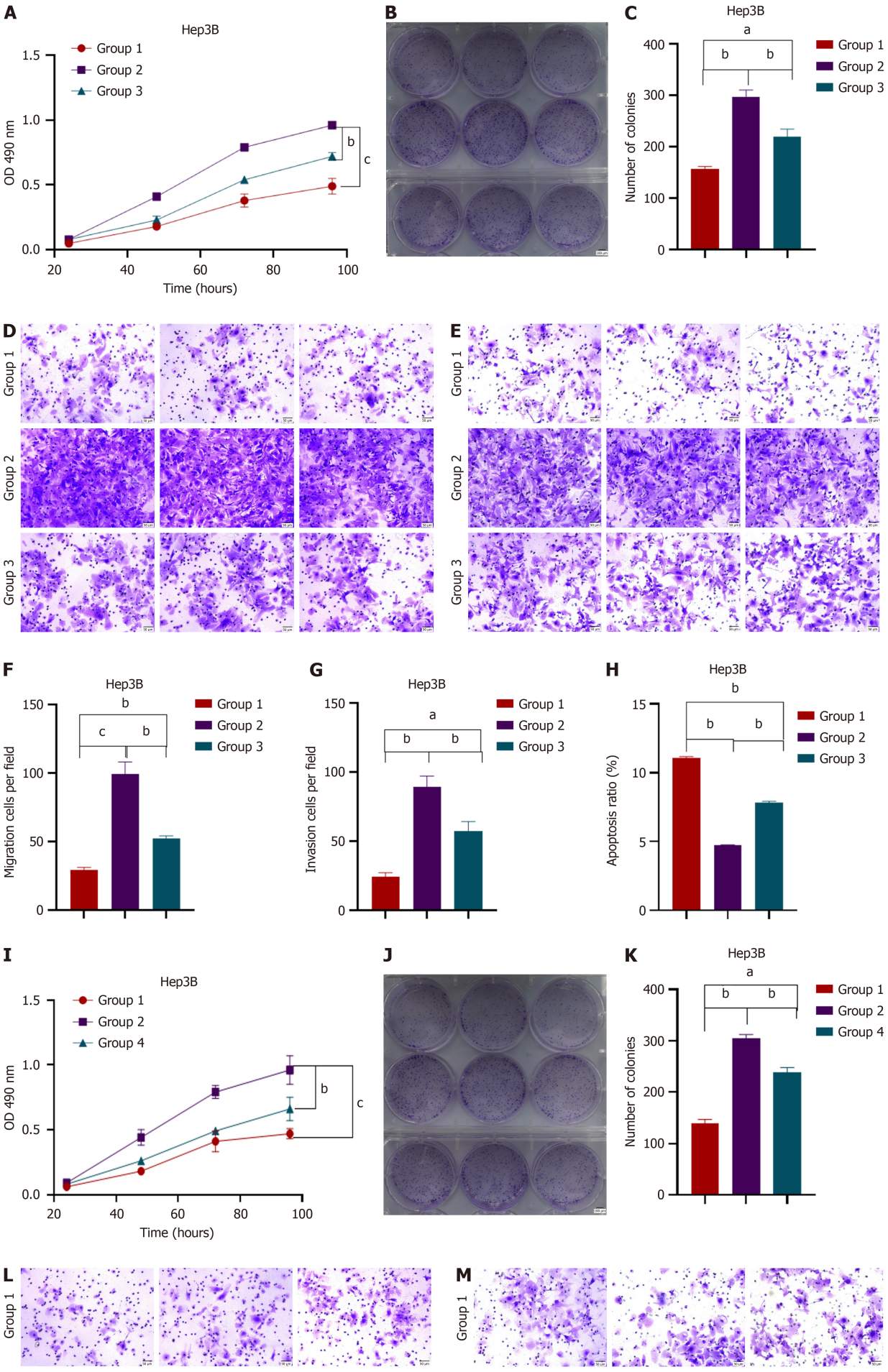


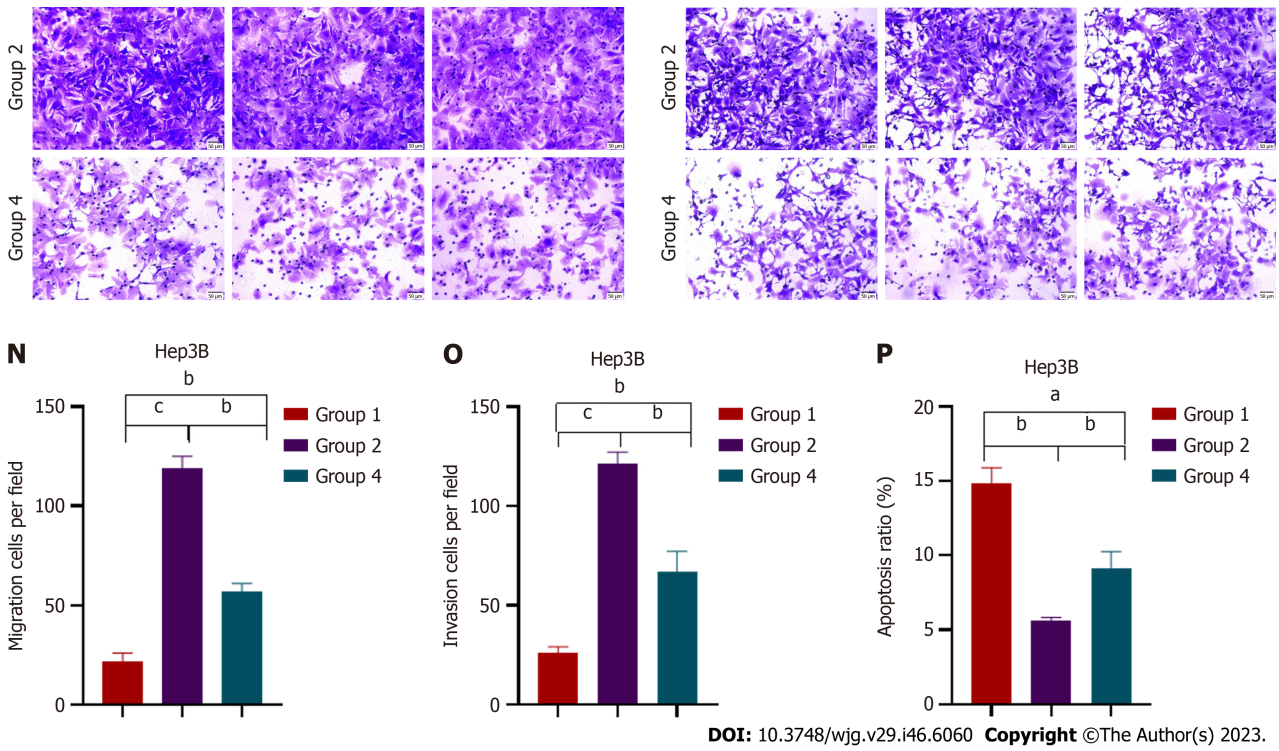


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Figure 4 Exosomal circ563 facilitating hepatocellular carcinoma progression. A: First, a knockdown experiment was conducted in Hep3B cells, and the circ563 levels in Hep3B-derived exosomes were quantified by polymerase chain reaction. Hep3B cell function was assessed after co-treatment with exosomes isolated from the culture medium of circ563-knockdown Hep3B cells; B-D: Downregulation of exosomal circ563 (exo-circ563) reduced Hep3B cell proliferation and the number of colonies formed; E-H: Decreased exo-circ563 levels suppressed the migratory activity and invasiveness of the Hep3B cells; I: The downregulation of exo-circ563 was correlated with apoptosis induction; J: The efficiency of circ563 overexpression in the mesenchymal stem cells (MSCs) was determined by quantitative real-time polymerase chain reaction; K: The upregulation of circ563 in MSC-derived exosomes was confirmed; L-R: The hepatocellular carcinoma (HCC) cell proliferation, metastasis, and apoptosis rates were assessed following exosome treatment. Exo-circ563 significantly induced HCC cell proliferation, migration, and invasion; S: Flow cytometry analysis showed that the percentage of apoptotic cells was significantly decreased. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. exo-circ563: Exosomal circ563; HCC: Hepatocellular carcinoma; MSC: Mesenchymal stem cell; Exo: Exosome.

In addition, the exosomes from MSCs have shown tumor-homing capabilities in xenograft mouse models[22]. The uptake of labeled exosomes from MSCs into HCC cells was also reported in the present study. Interestingly, the exosome function most likely depends on the biological materials they encapsulate[23,24]. Mounting evidence suggests that exosomal ncRNAs (miRNA, lncRNA, and circRNA) are actively involved in the initiation and progression of various diseases by regulating cellular proliferation, differentiation, angiogenesis, and immune response[25-27].


A
Hep3B
Group 1
Group 2
Group 3
OD 490 nm
Time (hours)
B
C
Hep3B
Number of colonies
D
Group 1
Group 2
Group 3
E
F
Hep3B
Migration cells per field
G
Hep3B
Invasion cells per field
H
Hep3B
Apoptosis ratio (%)
I
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Hep3B
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Figure 5 Oncogenic effect of exosomal circ563 prevented by either miR-148a-3p upregulation or metal-regulatory transcription factor-1 knockdown. A-P: Exosomal circ563 promoted Hep3B cell proliferation and invasion, which were reversed by miR-148a-3p overexpression or metal-regulatory transcription factor-1 depletion. Group 1: Co-culturing Hep3B cells with mesenchymal stem cell (MSC)-derived exosomes; Group 2: Co-culturing Hep3B cells with exosomes derived from circ563-overexpressing MSCs; Group 3: Co-culturing Hep3B cells after miR-148a-3p upregulation with exosomes derived from circ563-overexpressing MSCs; and Group 4: Co-culturing Hep3B cells after metal-regulatory transcription factor-1 knockdown with exosomes derived from circ563-overexpressing MSCs. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

circRNAs, a subset of ncRNA that is enriched and more stable in exosomes[28], were differentially expressed in tumor tissues than in normal tissues, suggesting their involvement in tumorigenesis and disease development[29]. *In vitro* and *in vivo* experiments revealed that circRNA commonly sponges miRNA, a major class of small ncRNA closely associated with HCC[30,31], and mediates post-transcriptional gene silencing by binding to the 3′-untranslated region or open reading frames of target mRNA. Previously, our data revealed that exosomal miR-148a-3p functions as a tumor suppressor in HCC with MTF-1 as its direct target. MTF-1 maintains metal homeostasis to protect cells against injury by excess metals and exerts oncogenic potential by manipulating metal or redox homeostasis, enhancing angiogenesis, and inducing tumor development in HCC. The current findings confirmed an increase in miR-148a-3p and a decrease in MTF-1 expression levels in MSC-derived exosomes compared with HCC-derived exosomes. Following HCC cell co-culture with MSC or MSC-derived exosomes, consistent changes were observed in the miR-148a-3p and MTF-1 expression levels. This finding indicates that exosomes may contain specific bioactive materials involved in regulating the expression of miR-148a-3p and MTF-1.

Considering this, we searched the CircInteractome databases and identified hsa_circ_0000563 (circ563) as the specific target that competitively binds to or sponges miR-148a-3p. Circ563 has been implicated in the changes in the pathogenesis of atherosclerosis with significantly lower expression in patients with coronary artery disease than in those assigned as controls. However, the significance and mechanistic function of circ563 in carcinoma, especially in HCC, are yet to be studied[32]. In the current study, the loss-of-function of circ563 in HCC alters cell proliferation, invasion, and apoptosis, as well as miR-148a-3p and MTF-1 expression. Elevated levels of circ563 were correlated with more aggressive tumor behavior, highlighting its oncogenic role in HCC, leading to the conclusion that circ563 activation might be a global event during hepatocarcinogenesis. Intracellularly, circ563 competitively binds to miR-148a-3p, resulting in the activation of MTF-1 in a pattern opposite to that of miR-148a-3p alone. In addition, circ563 enrichment was identified in HCC-derived exosomes with the corresponding changes in the levels of miR-148a-3p and MTF-1. This observation suggests that: (1) Circ563 originating from cells can be delivered by exosomes into the peripheral circulation and recipient cells; and (2) Circ563 affects cell function and accelerates HCC development, given its possible correlation with miR-148a-3p and MTF-1 expression.

Furthermore, rescue experiments demonstrated that the potentiating effects of exo-circ563 could be partially blocked by miR-148a-3p upregulation or MTF-1 knockdown. The oncogenic role of circ563 was verified *in vivo*, as exosomes enriched with circ563 facilitated HCC cell growth in nude mice. Additionally, we assessed the expression patterns of circ563, miR-148a-3p, and MTF-1 in HCC tissues and compared them with those in tumor-adjacent tissues; the trends were consistent with those in HCC cells and exosomes. Collectively, the relative stability, high abundance, and conserved nature of exosomal circRNA across species make them promising candidates as oncogenic biomarkers; a better

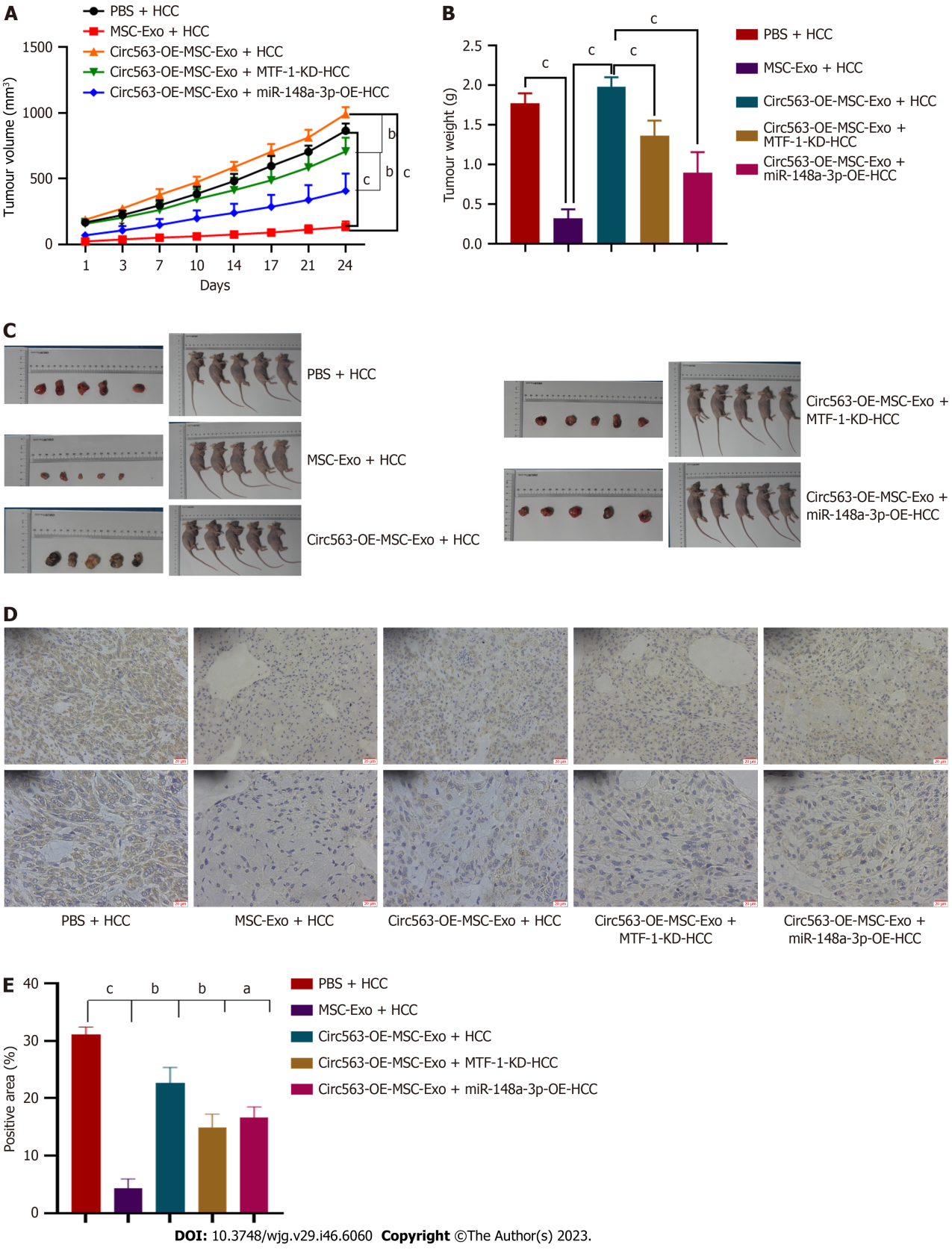
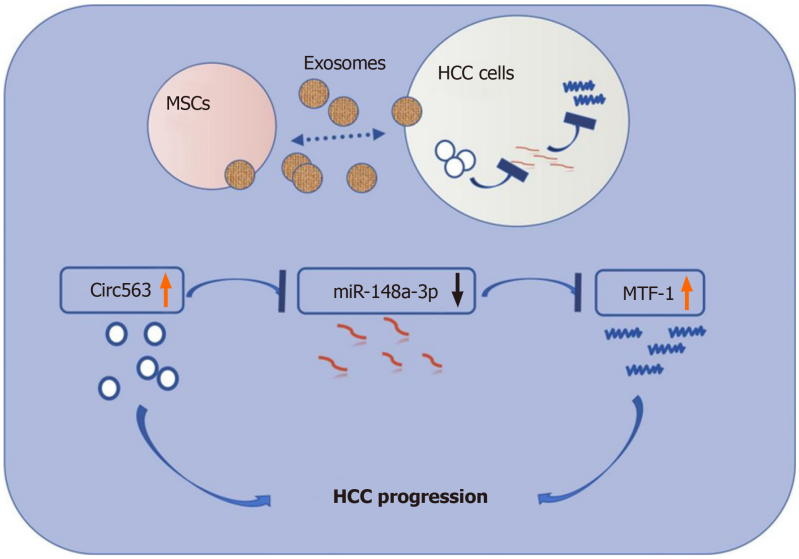


Figure 6 Circ563 promoting hepatocellular carcinoma tumor growth *in vivo*. Mouse xenografts were generated to verify the role of circ563 *in vivo*. Hepatocellular carcinoma cells stably transfected with miR-148a-3p overexpression, metal-regulatory transcription factor-1 knockdown, or control vectors were implanted into the subcutaneous tumors of mice. The xenografts were treated with exosomes after four weeks. A-C: Accelerated growth of circ563-overexpressing Hep3B-derived xenografts is shown with increased tumor volume and weight compared with the control group. Both miR-148a-3p overexpression and metal-regulatory transcription factor-1 (MTF-1) depletion partially reversed the increase in tumor volume and weight; D and E: MTF-1 staining reveals a similar trend with cell growth. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. exo-circ563: Exosomal circ563; HCC: Hepatocellular carcinoma; MSC: Mesenchymal stem cell; Exo: Exosome; MTF-1: Metal-regulatory transcription factor-1.



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Figure 7 The schematic model of circ 563/miR-148a-3p/metal-regulatory transcription factor-1 in hepatocellular carcinoma. MTF-1: Metal-regulatory transcription factor-1; HCC: Hepatocellular carcinoma; MSCs: Mesenchymal stem cells.

understanding of the exo-circ563-mediated intercellular network could facilitate the development of therapeutic strategies for patients with HCC.

CONCLUSION

We clarified the regulatory mechanisms of circ563, which can sponge miR-148a-3p to elicit MTF-1-dependent oncogenic effects, eventually acting as a tumor-potentiating factor in HCC. Thus, the ncRNA circ563 could be a promising therapeutic target, and liquid biopsy of serum exosomes targeting circ563 can help diagnose HCC and predict the prognosis of affected patients.

ARTICLE HIGHLIGHTS

Research background

Mesenchymal stem cells (MSCs) exert anti-oncogenic effects *via* exosomes containing non-coding RNA (ncRNA), and the efficacy of MSC-derived exosome therapies has been demonstrated. Our preliminary study identified the interaction of the ncRNA hsa_circ_0000563 (circ563) and the circ563-associated miR-148a-3p which are both enclosed in exosomes, as miR-148a-3p and its target metal-regulatory transcription factor-1 (MTF-1) are implicated in hepatocellular carcinoma (HCC) progression.

Research motivation

This study is to identify the clinical significance, functional implications, and mechanisms of circ563 in HCC.

Research objectives

This study aims to investigate the role of circ563 in modulating HCC functions.

Research methods

The expression levels of miR-148a-3p and MTF-1 in exosomes derived from MSC and HCC cells were compared, and their effects on HCC cells were assessed. Using a dual-luciferase reporter assay, miR-148a-3p was identified as an associated miRNA of circ563, whose role in HCC regulation was assessed *in vitro* and *in vivo*.

Research results

Circ563 silencing blocked the HCC cell proliferation and invasion and induced apoptosis. Co-culturing of HCC cells with MSC-derived exosomes following circ563 overexpression contributed to cell proliferation and metastasis and elicited changes in miR-148a-3p and MTF-1 expression. The tumor-promoting effects of circ563 were partially suppressed by miR-148a-3p overexpression or MTF-1 depletion. Xenograft experiments confirmed that circ563-enriched exosomes facilitated tumor growth by upregulating the expression of MTF-1. In HCC tissues, circ563 expression was negatively

correlated with miR-148a-3p expression but positively correlated with MTF-1 levels.

Research conclusions

Our findings suggested MSCs may exhibit anti-HCC activity through the exosomal circ563/miR-148a-3p/MTF-1 pathway.

Research perspectives

The study presents a new dataset related to HCC. We clarified the regulatory mechanisms of circ563, which can sponge miR-148a-3p to elicit MTF-1-dependent oncogenic effects in HCC. Thus, the ncRNA circ563 could be a potential therapeutic target, and liquid biopsy of serum exosomes targeting circ563 may help predict the prognosis of patients with HCC.

FOOTNOTES

Author contributions: Yang Z designed and coordinated the study, and wrote the manuscript; Lyu ZZ, Li M, Yang MY, and Han MH performed the experiments; Lyu ZZ, Li M, Han MH, and Yang Z acquired and analyzed the data; Lyu ZZ, Han MH, and Yang Z interpreted the data; and all authors approved the final version of the article.

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Institutional animal care and use committee statement: The animal study was approved by the Animal Ethics Committee of Shandong Provincial Hospital (protocol code NSFC: NO.2019-021; approval date: 02/19/2019).

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

Data sharing statement: The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

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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SCIENTOMETRICS

Hotspots and frontiers of the relationship between gastric cancer and depression: A bibliometric study

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Abstract

BACKGROUND

A significant relationship between gastric cancer (GC) and depression has been found in the last 20 years. However, there is no comprehensive information that helps researchers find popular and potential research directions on GC and depression.

AIM

To determine the research status and hotspots by bibliometric analysis of relevant publications on the relationship between GC and depression.

METHODS

We used the Web of Science Core Collection to search and collate the literature on GC and depression from 2000 to 2022 on 31 May, 2023. Then, visualization analysis was performed using VOSviewer software (version 1.6.19) and the Bibliometrix package in R software.

RESULTS

We retrieved 153 pertinent publications from 2000 to 2022. The annual publication count showed an overall upward trend. China had the most prominent publications and significant contributions to this field (n = 64, 41.83%). Before 2020, most studies focused on "the effect of GC on the development and progression of

depression in patients." The latest research trends indicate that "the effect of depression on the occurrence and development of GC and its mechanism" will receive more attention in the future.

CONCLUSION

The study of "the effect of depression on the occurrence and development of GC and its mechanism" has emerged as a novel research theme over the past two years, which may become a research hotspot in this field. This study provides new insights into the hotpots and frontiers of the relationship between GC and depression, potentially guiding researchers toward hot research topics in the future.

Key Words: Gastric cancer; Depression; Bibliometric analysis; Visualization; Web of Science

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Core Tip: Gastric cancer (GC), the most common malignant tumour in the digestive system, has the third-highest mortality rate and the fifth-highest morbidity rate among all cancers. In recent years, some researchers have paid attention to the impact of depression on the occurrence and development of GC and tried to explore the interaction mechanism, which has become an emerging research trend in GC and depression. Bibliometric analysis is a popular and rigorous method for quantitative analysis of large volumes of scientific literature data. It is necessary to investigate the relationship between GC and depression. However, as far as we know, there is no bibliometric study on GC and depression. This study shows the hotspots and frontiers of GC and depression on a global level.

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INTRODUCTION

Gastric cancer (GC), the most common malignant tumour in the digestive system, has the third-highest mortality rate and the fifth-highest morbidity rate among all cancers[1]. As the most common mental disorder, depression is particularly prevalent in cancer patients in recent studies[2-4]. At present, the discussion on the pathogenesis of depression is quite rich, mainly including miRNAs that disorderly expressed[5], receptors and their gene abnormality[6], cerebral structural and functional changes[7]. On the one hand, cancer patients often experience painful emotional reactions, some of which can manifest as depression. On the other hand, depression affects the attitude of cancer patients toward cancer and adherence to drug treatment and the endocrine and immune function, which in turn affects cancer progression[8]. There is increasing evidence that mental disorders such as depression are associated with the incidence and progression of cancer[8-11].

The current research status on the relationship between cancer and depression is still unknown. Studies have focused on research topics such as breast cancer and depression[11,12], lung cancer and depression[13], and colorectal cancer and depression[14]. In recent years, some researchers have paid attention to the impact of depression on the occurrence and development of GC and tried to explore the interaction mechanism, which has become an emerging research trend in GC and depression.

Bibliometric analysis is a popular and rigorous method for quantitatively analyzing large volumes of scientific literature data. Based on the databases of academic publications, such as Pubmed, Web of Science, and Scoups, can reveal the status, hotspots, and emerging trends in a particular research field[15]. It can provide comprehensive information that helps researchers find popular and potential research directions in specific disciplines.

It is necessary to investigate the relationship between GC and depression. However, as far as we know, there is no bibliometric study on GC and depression. Therefore, this study aims to provide an overview of the research status on the relationship between GC and depression by bibliometric analysis. Furthermore, we tried to propose the hotspots, evolution trends, and future research advancement patterns in this field. In addition, future research patterns are forecast based on evaluating bibliometric results in this field.

MATERIALS AND METHODS

Data sources

We selected the Web of Science Core Collection (WOSCC) for the literature collection. WOSCC is the world's leading citation database. It contains records of articles from the highest-impact journals worldwide, including open-access journals, conference proceedings, and books. Notably, the coverage of specific titles extends back to the year 1900[16,17].

This comprehensive and extensive database provides a robust foundation for our bibliometric analysis.

Search strategies

We focused on the WOSCC, collecting the literature on "GC and depression". Then, we searched and exported the relevant articles to the WOSCC on 31 May, 2023. Our search strategy was as follows: Topic = (stomach neoplasm OR stomach cancer OR stomach tumour stomach carcinoma OR gastric carcinoma OR gastric carcinoma or gastric cancer OR gastric neoplasm OR gastric tumour AND TS = depression OR depressive disorder OR depressive symptom. The retrieval time range was from 1 January, 2000 to 31 December, 2022. We included only articles, reviews, and systematic reviews to facilitate further literature content analysis, excluding irrelevant publications.

Bibliometric analysis

We used an Excel spreadsheet to collect bibliometric indicators: The total number of publications, the year of publication, the top ten countries, the Journal Citation Reports (JCR) Quartile rankings of the source journals, the top 5 citations, and the research types.

In addition, we utilized the Bibliometrix package of R-studio software to analyze the included literature data and Biblioshiny for data visualization. In this study, we examined the top ten countries contributing to the field of GC and depression and the annual publication trend for each country.

Visualized analysis

We used VOSviewer software (Version 1.6.19) for keyword co-occurrence analysis to visualize networks of keywords. The network focuses on understanding a particular field's knowledge composition and structure by studying the links between keywords in the article. By drawing the keywords co-occurrence visualization maps, we can identify the hotspots and frontiers in "GC and depression".

Classification of publications

For the classification of publications, we divided the research on the correlation and interaction mechanism between GC and depression into two groups: "GC to depression" and "depression to GC." Based on the above grouping, we used the 2022 JCR Quartile rankings to classify the publications within the two groups. The journal sources not existing in the JCR Quartile rankings were excluded. Additionally, we classified the two research groups according to the classification standard of medical studies[18], which is based on research methodologies.

RESULTS

Analysis of annual publications

In the WOSCC, we retrieved 153 publications on "GC and depression". The number of publications on this topic has generally increased from 2000 to 2022, indicating a growing interest in this field among researchers. A more rapid growth in the number of publications was observed from 2019 to 2021, with 2021 recording the highest annual number of articles (n = 29) (Figure 1).

Top active countries

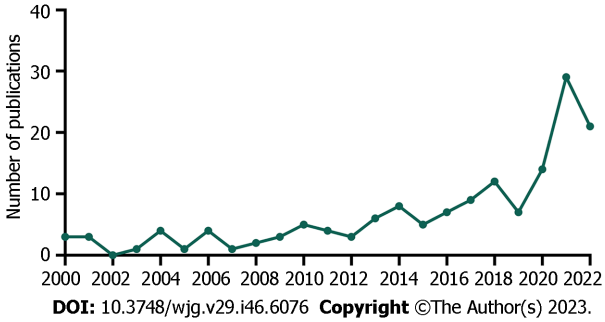
Over the past 22 years, at least 21 countries have published relevant papers on "GC and depression". The top ten countries issued 140 articles, accounting for 90.32% of the total publications in this field (Figure 2A). The most significant number of publications was from China (n = 64), the total number of publications is 7287986, followed by South Korea (n = 28), the total number of publications is 1368778, and the third was the United States (n = 16), the total number of publications is 210673. From the trend of annual publications of the top ten countries, China has the most significant increase and the fastest growth rate, followed by South Korea and the United States (Figure 2B).

Hotspots and frontiers

We imported the publication related to "GC and depression" in the WOSCC into VOSviewer (version 1.6.19) software, selected all keywords (including author keywords and keywords plus) that occurred more than five times, and divided these keywords into five clusters with a different color (Figure 3). The five cluster are "effect of treatments on GC patients with mental disorders" (cluster 1, red), "epidemiological researches on GC and depression" (cluster 2, green) "diagnosis of GC causing mental disorders and the interaction mechanism" (cluster 3, blue) "outcomes of GC patients" (cluster 4, yellow) and "social support of mental disorders in GC patients" (cluster 5, purple).

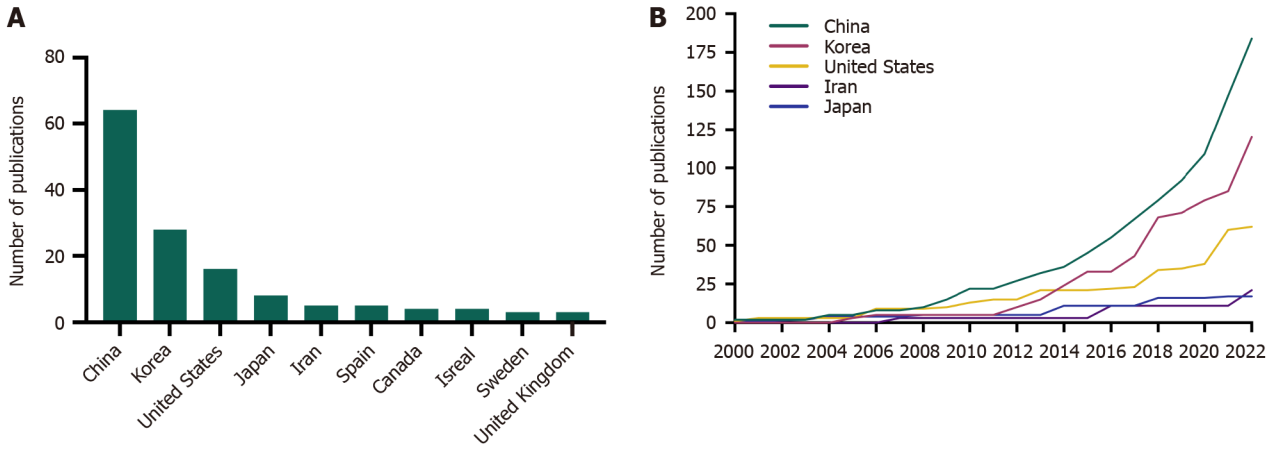
In cluster 1, the high-frequency keywords are quantity of life, depression scale, gastrectomy, and chemotherapy. In cluster 2, the high-frequency keywords are risk, prevalence, epidemiology, and mortality. In cluster 3, the high-frequency keywords are diagnosis, anxiety, expression, and apoptosis. In cluster 4, the high-frequency keywords are survivors, symptoms, association, and outcomes. In cluster 5, the high-frequency keywords are adjustment and social support. These results indicate that "GC and depression" included five research directions from 2000 to 2022.

Overlay visualization is shown in Figure 4. The keywords colored purple mean the average year of the appearance of the keyword is earlier than other colors. Keywords colored yellow means the average year of the appearance of the keyword is later. Before 2012, the hospital anxiety and depression scale of patients with GC was the most concerned topic (purple). Then, researchers gradually turned to the psychological adjustment, quality of life, and diagnosis of patients



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Figure 1 The number of publications from the Web of Science containing gastric cancer and depression per year from 2000 to 2022.



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Figure 2 Top active countries with the most publications on gastric cancer and depression. A: The top 10 most published countries; B: Annual publication trends for the top 5 most published countries.

with GC (green). In recent years, researchers have paid more and more attention to the association between GC and depression, the mechanism of interaction between the two, and the risk factors (yellow) (Figure 4).

Baseline characteristics of publications on the relationship between GC and depression

From 2000 to 2022, the number of publications on "GC to depression" and "depression to GC" showed an increased trend. Compared with the first research on "depression to GC" published in 2004, the research on "GC to depression" published earlier, was published in 2001. In addition, the number of publications of "GC to depression" was higher than "depression to GC" before 2020 and was lower after 2020 (Figure 5).

From 2000 to 2022, the number of publications on "GC to depression" is 48, and "depression to GC" is 27. China is the most active country in both groups, followed by South Korea. Furthermore, China has a higher proportion of publications in "depression to GC" than other countries (n = 17, 63%) (Figure 6).

JCR Quartile rankings and top-cited publications on the relationship between GC and depression

We analyzed the JCR Quartile rankings results for the two groups. The JCR Quartile rankings of publications on "GC to depression" were distributed as follows: Q1 (26%), Q2 (43%), Q3 (17%), and Q4 (14%). However, the JCR Quartile rankings of publications on "depression to GC" were Q1 (43%), Q2 (28%), Q3 (24%), and Q4 (5%). Compared with "GC to depression", there were more publications on "depression to GC" in Q1. Furthermore, publications on "depression to GC" in Q4 only accounted for 5%, which was less than "GC to depression" (Figure 7).

We summarized the top five most frequently cited publications in "GC to depression" and "depression to GC", respectively. Brintzenhofe-Szoc *et al*[19], published in *Psychosomatics* in 2009, had the most cited frequency in "depression to GC" (frequency of cited = 226). Lee *et al*[20], published in the *Journal of Neurogastroenterology and Motility* in 2015, had the most cited frequency in "depression to GC" (frequency of cited = 84) (Tables 1 and 2).

Study types of publications on the relationship between GC and depression analysis

Our analysis revealed that the most prevalent research method in "GC to depression" was the epidemiological research method, among which cross-sectional studies accounted for 40%, followed by cohort studies. In "depression to GC", the most used is the basic research method, including cell, gene, animal, and biochemical research methods (Figure 8).

Table 1 Top cited list of the top 5 highly cited papers related to "gastric cancer to depression" from 2001 to 2020

Ranking	Authors	Title	Journal	Time cited
1	Brintzenhofe-Szoc *et al* [19]	Mixed Anxiety/Depression Symptoms in a Large Cancer Cohort: Prevalence by Cancer Type	*Psychosomatics*	226
2	Nordin *et al*[36]	Predicting anxiety and depression among cancer patients: a clinical model	*European Journal of Cancer*	121
3	Hong and Tian[37]	Prevalence of anxiety and depression and their risk factors in Chinese cancer patients	*Supportive Care in Cancer*	118
4	Tavoli *et al*[54]	Anxiety and depression in patients with gastrointestinal cancer: does knowledge of cancer diagnosis matter?	*BMC Gastroenterology*	98
5	Kim *et al*[40]	Prevalence and prognostic implications of psychological distress in patients with gastric cancer	*BMC Cancer*	68

Table 2 Top cited list of the top 5 highly cited papers related to "depression to gastric cancer" from 2001 to 2020

Ranking	Authors	Title	Journal	Time cited
1	Lee *et al*[20]	The Effect of Emotional Stress and Depression on the Prevalence of Digestive Diseases	*Journal of Neurogastroenterology and Motility*	84
2	Shi *et al*[38]	Catecholamine up-regulates MMP-7 expression by activating AP-1 and STAT3 in gastric cancer	*Molecular Cancer*	72
3	Bica *et al*[39]	Depression as a Risk Factor of Organic Diseases: An International Integrative Review	*Journal of Nursing Scholarship*	38
4	Nan *et al*[55]	Effects of depression on parameters of cell-mediated immunity in patients with digestive tract cancers	*World Journal of Gastroenterology*	23
5	Huang *et al* [44]	Depression accelerates the development of gastric cancer through reactive oxygen species-activated ABL1 (Review)	*Oncology Reports*	21

DISCUSSION

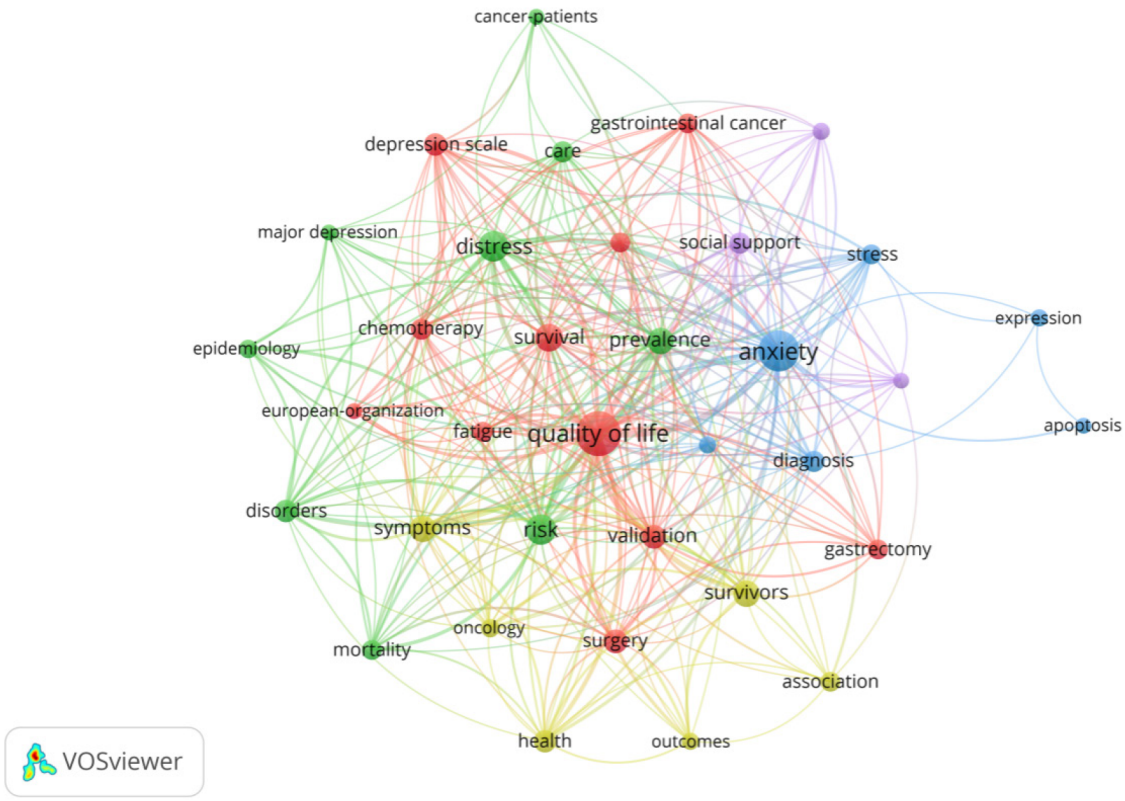
General status, hotspots, and frontiers in the relationship between GC and depression

This study is the first bibliometric analysis of the relationship between GC and depression, providing a potential opportunity to explore the interaction mechanism further. This study analyzed the global publications on GC and depression from 2000 to 2022. The results showed an increasing trend in the annual number of publications in this field. In addition, we could conclude that China and South Korea are the most productive countries in GC and depression. According to the global statistics of GC in 2020, the incidence of GC in China and South Korea is among the top five, and the disease burden is heavy[21]. Therefore, there is more research on GC in China and South Korea than in other countries, and the increase in annual publication volume is relatively significant.

The keyword co-occurrence analysis of the research on GC and depression revealed the distribution of research topics in this field from 2000 to 2022. Combining the overlay visualization, we could present the research hotspots and frontiers in the field. Most researchers focus on which cancer patients are often accompanied by depression and the incidence of depression in GC patients. Kouhestani *et al*[22] estimated the prevalence of depression in GC patients globally based on WHO region classification. They found that 37% of GC patients were accompanied by depression. Regionally, the Eastern Mediterranean region has the highest prevalence of depression in GC patients among all WHO regions. The results suggested that depression is high among GC patients.

Depression could affect the overall health of GC patients. GC patients with depression might significantly impact their quality of life, prognosis, and survival rate[23]. Therefore, researchers have also shown significant interest in the quality of life of GC patients with depression and its improvement. The diagnosis and treatment of GC might impact the psychological condition of patients[24]. Some interventions, such as proper social support[25] and multidisciplinary cooperative continuous nursing[26], could relieve the depression of GC patients.

The research on the interaction mechanism between GC and depression from the perspective of epidemiological[27,28], cellular[29,30], and genetic[30,31]levels became an emerging research topic. This trend might be due to the many kinds of research on hot topics published during this period, revealing innovative explanations emerging from new research fields. For example, the gut-brain axis theory established bidirectional interactions among the brain, the gut, and the gut microbiome[32,33]. Furthermore, many prospective epidemiological studies have shown that depression is a risk factor for cancer[12,34]. In addition, some studies have confirmed that depression is a risk factor for digestive carcinoma and proposed a mechanism for it[35]. These innovative explanations provided new ideas for exploring the interaction mechanism of GC and depression.



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Figure 3 The network visualization map of keywords co-occurrence in gastric cancer and depression during 2000-2022 based on VOSviewer.

The difference in publications between the two groups

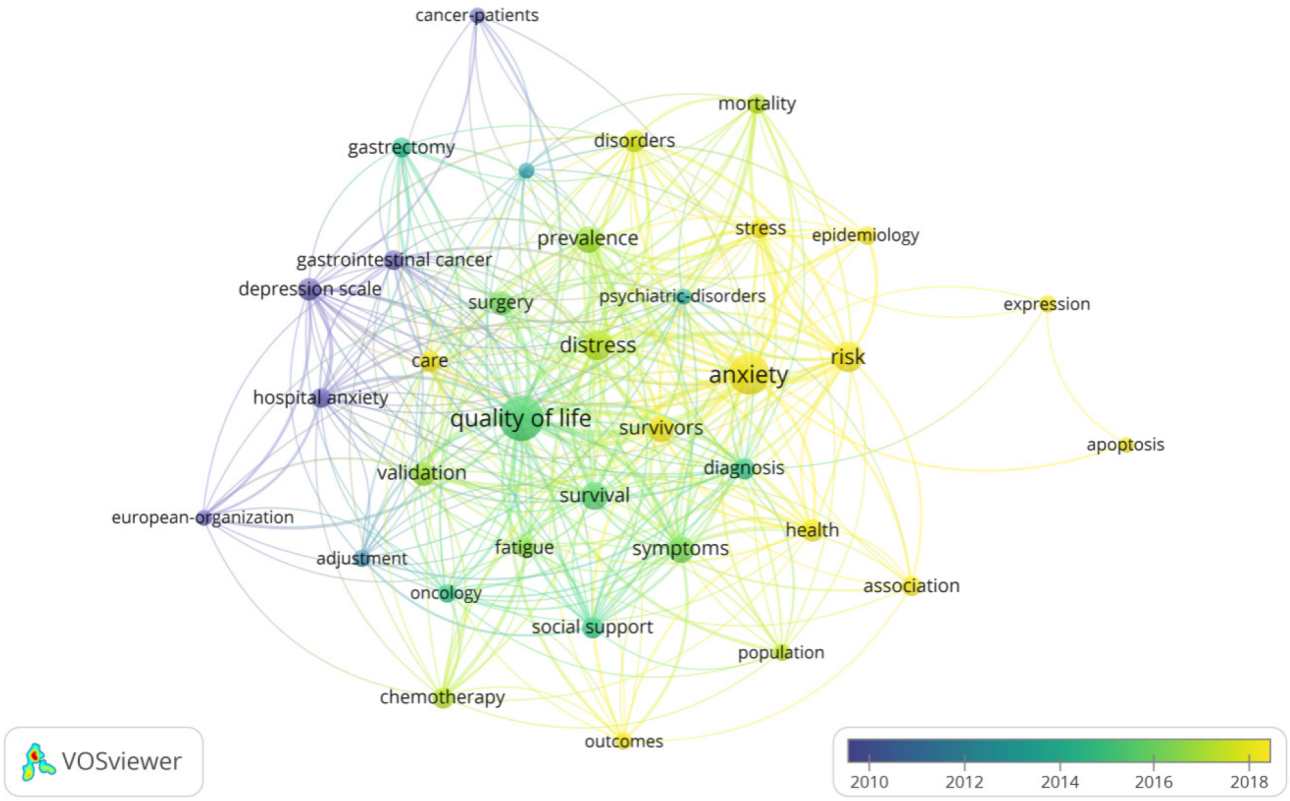
This study indicated that in "GC to depression", the top five most highly cited publications are epidemiological studies, such as the prevalence, mortality, and risk factors of GC patients with depression. This result was also consistent with cluster 2 in the keyword co-occurrence analysis (Figure 3, green). The publication most frequently cited was Brintzenhofe-Szoc *et al*[19], entitled "Mixed Anxiety/Depression Symptoms in a Large Cancer Cohort: Prevalence by Cancer Type", published in *Psychosomatics* in 2009. This study found that mixed anxiety/depression has a high incidence of gastric, pancreatic, head and neck, and lung cancer. The second most cited publication was by Nordin *et al*[36] from the *European Journal of Cancer*. This study indicated that levels of anxiety and depression at diagnosis predict a similar status 6 mo later, and the hospital anxiety and depression scale, combined with a single question about social support, maybe a suitable screening tool for clinical use. The third most cited publication was by Hong and Tian[37] from *Supportive Care in Cancer*. This study showed that depression level was high among Chinese cancer patients. Patients with lung, esophagus, cervix, liver, and stomach cancers were the high-risk groups for depression.

In "depression to GC", researchers mainly focus on the mechanism. The most highly cited was by Lee *et al*[20], entitled "The Effect of Emotional Stress and Depression on the Prevalence of Digestive Diseases", published in the *Journal of Neurogastroenterology and Motility* in 2015. This study showed depression was associated with peptic ulcer disease, adenoma/colon cancer, and GC. Depression was an independent risk factor for gastric adenoma/GC and might be a cause of GC. The second cited publication was by Shi *et al*[38] from *Molecular Cancer*. This study found that up-regulation of matrix metalloproteinase-7 expression through beta 2-adrenergic receptors (AR)-mediated signaling pathway is involved in the invasion and metastasis of GC. The third cited publication was by Bica *et al*[39] from the *Journal of Nursing Scholarship*. This study indicated that mechanisms connecting depression to physical illness appear to involve alterations in the hypothalamic-pituitary axis, unhealthy lifestyle, chronic or acute stressors including posttraumatic stress, an increase in C-reactive protein in men, taking antidepressant medication, and social and emotional loneliness.

We further analyzed the publications on "GC to depression" and "depression to GC". We found that the number of publications in China ranked first in both groups, especially in "depression to GC". Compared with other countries, China focused on the impact of depression on GC patients and the mechanism of it earlier. In addition, we found that research on "depression to GC" was mainly published in Q1 journals, indicating that this research topic might become an exciting research direction in the future.

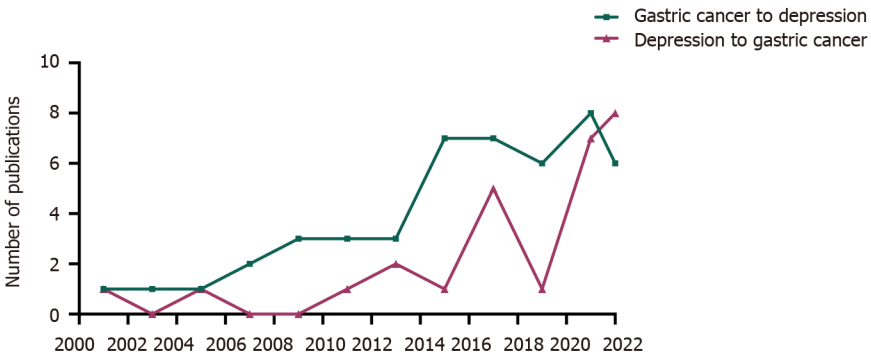
The difference in research types between the two groups

In "GC to depression", the most research type was epidemiological study, among which 40% were cross-sectional studies. For example, Kim *et al*[40] designed a cross-sectional study and found that depression is common in patients with all stages of GC and is associated with worse outcomes. Kwon *et al*[27] designed a case-control study and identified a



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Figure 4 The overlay visualization map of keywords co-occurrence in gastric cancer and depression during 2000-2022 based on VOSviewer.



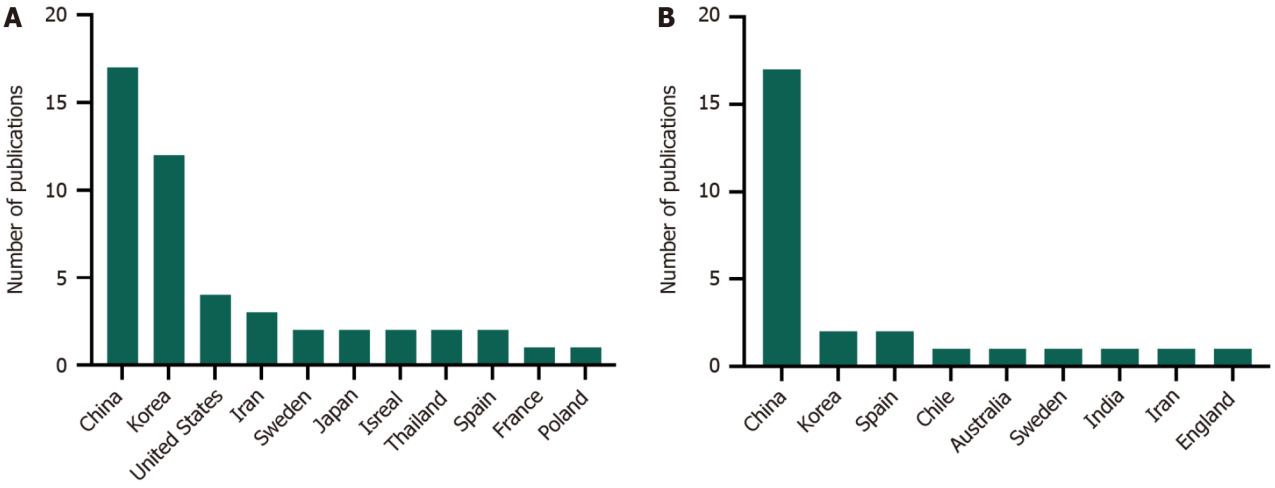
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Figure 5 Comparison of the annually published papers on "gastric cancer to depression" and "depression to gastric cancer".

significant relationship between GC and depression among South Korean adults, especially among female patients between 60 and 69 years old of high income and living in metropolitan regions. In addition, Liu and Wang *et al*[41] conducted a prospective cohort study and found that postoperative depression gradually worsened, relating to poor prognosis, and the degree of malignancy in GC patients is positively correlated with the severity of depression.

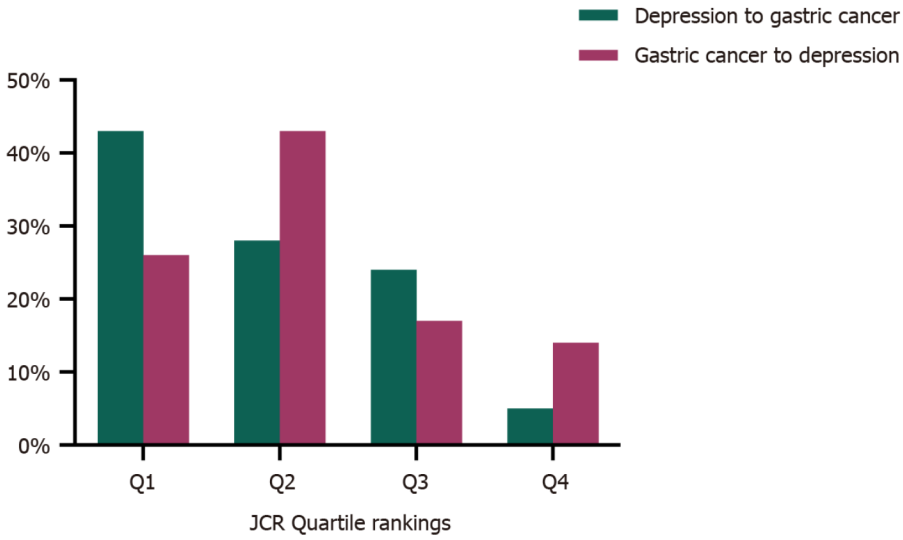
In "depression to GC" was mainly basic studies. In recent years, there has been a significantly increased number of publications on the mechanism of depression to GC. This trend might be due to recent epidemiological evidence indicating that mental disorder is a risk factor for GC, but the mechanism is still unclear[20,42,43]. There are four basic study types in "depression to GC": cell study, genetic study, biochemistry, and animal study. This result showed that researchers mainly focused on how depression affects the occurrence and development of GC and conducted research based on animal, cell, gene, and biochemistry levels.

As mentioned above, Shi *et al*[38] confirmed the effect of depression on GC at the genetic level in 2010. Other researchers have found that oxidative stress (OS) is related to GC and depression. Huang *et al*[44] found that high levels of reactive oxygen species (ROS) can activate ABL1 in response to OS. That triggered ABL1 subsequently contributed to the development of GC *via* interactions with the downstream targets and corresponding signaling pathways. Based on this study, they further explored the mechanism and found that ROS-activated ABL1 mediates inflammation by



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Figure 6 Comparison of the number of publications on "gastric cancer to depression" and "depression to gastric cancer" in different countries. A: "Gastric cancer (GC) to depression"; B: "Depression to GC".



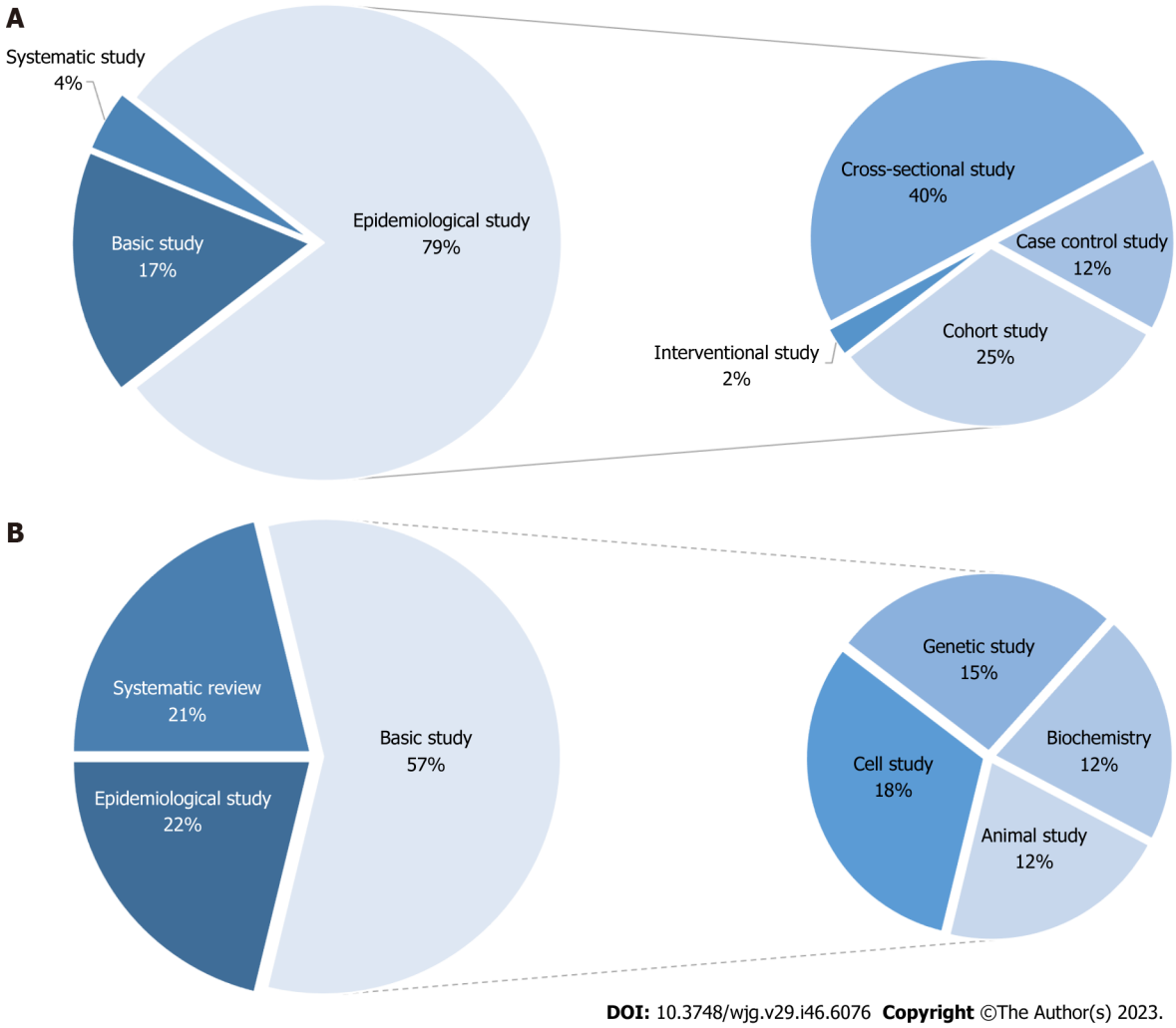
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Figure 7 Published journal sources for "gastric cancer to depression" and "depression to gastric cancer" studies Journal Citation Report divisional statistics. JCR: Journal Citation Report.

regulating NF-kappa B1 and STAT3, which subsequently leads to the development of GC and GC-related depression[45]. In addition, Pan *et al*[46], based on the study by Shi *et al*[38], found that catecholamine-induced neuroendocrine phenotypes of GC cells led to depression-accelerated GC invasion and metastasis *via* the beta(2)-AR/metastasis-associated with colon cancer 1 (MACC1) axis, while beta(2)-AR antagonist or MACC1 silencing could reverse it, showing promising potential therapeutic strategies for improving the outcome of GC patients with comorbid depression. These studies built a foundation for future research on the mechanism of "depression to GC".

Research prospection

The causal relationship between depression and GC merits deeper investigation, which could yield valuable insights into the mechanisms at play. As mentioned above, there have already been population, individual, cellular, molecular, and genetic studies, and some researchers have established animal models. However, there is still a lack of interaction mechanism research on GC and depression at a systematic level. We noticed that depression belongs to the abnormalities of the central nervous system, and GC belongs to the diseases of the digestive system. However, there is still a lack of research on the mechanism of "depression to GC" at the systemic level. As mentioned above, researchers have focused on the association between the central nervous system and other system diseases. For example, Guida *et al*[31] found that changes in gut bacterial composition might cause altered responses in affective behaviors *via* several concurring cellular and molecular mechanisms. Their findings offered the first step towards new insights into microbiota's obscure role in central nervous system functioning. Extricating these pathways may lead to new therapeutic approaches in pathologies



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Figure 8 Classification of "gastric cancer to depression" and "depression to gastric cancer" study types. A: "Gastric cancer (GC) to depression"; B: "Depression to GC".

showing comorbidity between gastrointestinal disorders and psychiatric illness. Van Kessel *et al*[32] found that an abundance of bacterial tyrosine decarboxylase in the proximal small intestine could explain the increased dosage regimen of levodopa treatment in Parkinson's patients. This study suggested that there was an association between the central nervous system and the digestive system. In addition, Mediavilla[47] also found that the importance of gut-brain communication in health and disease, especially the orexin/hypocretin system, bidirectional crosstalk, plays a significant role in various gastrointestinal disorders.

In addition, the nervous system is essential in regulating immune responses to various diseases[48]. Reiche *et al*[49] proposed that stress and depression impair the immune response and might promote the initiation and progression of some types of cancer. Therefore, studying the bidirectional communication between the neuroendocrine and immune systems could contribute to new clinical strategies. Kuol *et al*[50] presented three approaches to the neuro-immune interaction in cancer progression: lymphoid organs innervation, neurotransmitters, and immune cells in cancer, tumor-associated immune cells, and the nervous system. Cortese *et al*[51] indicated that the interaction between nerves and immune cells is critical to cancer growth. There is an interaction between cancer progression and the nervous system[52]. Martyn *et al*[53] showed that Schwann cells, which are the most prevalent neuroglia within the peripheral nervous system, could attract different subsets of immune regulators and augment their ability to suppress the effector T cells and up-regulate invasiveness of tumor cells. These studies provided a new perspective for further exploration[54,55].

Based on this, we speculate that the researcher can also explore the mechanism of depression affecting GC from the perspective of the association between the central nervous and other systems in the future. Furthermore, providing nuanced insights into the severity of depression could enrich the study's findings. Given that depression spans a spectrum ranging from mild to severe, it would be advantageous to elucidate the varying degrees of depression and their potential correlation with the incidence of GC.

CONCLUSION

This study shows the hotspots and frontiers of GC and depression on a global level. According to the increased trend of publications, the number of publications in the future might continue to increase. Between 2000 and 2022, China and South Korea contributed the most to the relationship between GC and depression. The "effect of treatments on GC patients with mental disorders" and "epidemiological research on GC and depression" have always been the research hotspots in the field. The "interaction mechanism between GC and depression" has emerged as a research frontier in recent years, which can be divided into two groups: "GC to depression" and "depression to GC".

The primary research type of "GC to depression" was epidemiological study represented by cross-sectional studies. In contrast, the primary research type of "depression to GC" was basic study, which focused on the mechanism, and it has shown a significantly increased trend in the past two years. "The mechanism of depression effect on the occurrence and development of GC" will be a frontier in the research field of GC and depression in the future. This study is also the starting point for further discussion. At the same time, since its mechanism was still unclear, it shows the necessity of further analysis.

ARTICLE HIGHLIGHTS

Research background

Depression is particularly prevalent in cancer patients in recent studies, and gastric cancer (GC) is the most common malignant tumour in the digestive system. There is increasing evidence that mental disorders such as depression are associated with the incidence and progression of cancer. Some researchers have paid attention to the impact of depression on the occurrence and development of GC, which has become an emerging research trend in GC and depression.

Research motivation

Present the research status and explore the hotspots for frontier studies using bibliometric analysis of relevant publications on the relationship between GC and depression.

Research objectives

We focused on the Web of Science Core Collection, collecting 153 pieces of literature on "GC and depression". The retrieval time range was from 1 January, 2000 to 31 December, 2022. We included only articles, reviews, and systematic reviews.

Research methods

We used an Excel spreadsheet to collect bibliometric indicators. In addition, we utilized the Bibliometrix package of R-studio software to analyze the included literature data and Biblioshiny for data visualization.

Research results

The annual publication count showed an overall upward trend. China had the most prominent publications and significant contributions to this field. The effect of depression on the occurrence and development of GC and its mechanism will receive more attention in the future.

Research conclusions

The effect of depression on the occurrence and development of GC and its mechanism may become a research hotspot. This study provides new insights into the hotpots and frontiers of the relationship between GC and depression.

Research perspectives

This study shows the number of publications on GC and depression in the future might continue to increase. This study is the starting point for further research on the mechanism of depression's effect on the occurrence and development of GC. Since the mechanism was still unclear, it shows the necessity of further analysis.

FOOTNOTES

Co-first authors: Jia-Yu Liu and Ji-Qi Zheng.

Co-corresponding authors: Jian-Ning Zhang and Wen-Pei Tang.

Author contributions: Liu JY and Zheng JQ were responsible for literature search, study design, data collection, data interpretation, and writing; Zhang JN, Tang WP and Yin CL were responsible for study design and provided feedback on all manuscript texts. Liu JY and Zheng JQ contributed equally to this work as co-first authors; Zhang JN and Tang WP contributed equally to this work as co-corresponding authors. The reasons for designating Liu JY and Zheng JQ as co-first authors, and Zhang JN and Tang WP as co-corresponding authors are threefold. First, the research was performed as a collaborative effort, and the designation of co-corresponding authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete

the study and the resultant paper. This also ensures effective communication and management of post-submission matters, ultimately enhancing the paper's quality and reliability. Second, the overall research team encompassed authors with a variety of expertise and skills from different fields, and the designation of co-corresponding authors best reflects this diversity. This also promotes the most comprehensive and in-depth examination of the research topic, ultimately enriching readers' understanding by offering various expert perspectives. Third, Liu JY and Zheng JQ contributed efforts of equal substance throughout the research process. The choice of these researchers as co-corresponding authors acknowledges and respects this equal contribution, while recognizing the spirit of teamwork and collaboration of this study. In summary, we believe that designating Zhang JN and Tang WP as co-corresponding authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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LETTER TO THE EDITOR

Albumin-bilirubin score in non-malignant liver diseases should be properly validated

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Abstract

The albumin-bilirubin (ALBI) score to assess the risk of decompensation in patients with initially compensated cirrhosis may improve their prognostic evaluation. This letter critically evaluates the research, which utilizes the ALBI score to forecast decompensation in cirrhosis patients over a three-year period. This score was initially developed to assess liver function in hepatocellular carcinoma, its prognostic utility for non-malignant liver diseases has now been explored, recognizing decompensation as a pivotal event that significantly affects patient's survival. Some concerns regarding the methodology of this research may be raised, particularly the exclusive use of radiological diagnosis, potentially including patients without definite cirrhosis and thus skewing the decompensation risk assessment. The reported predominance of variceal bleeding as a decompensating event conflicts with established literature, that often reports ascites as the initial decompensation manifestation. The letter highlights the absence of details on esophageal varices and their management, which could introduce bias in evaluating the ALBI score's predictive power. Furthermore, the letter points out the small sample size of patients with high-risk ALBI grades, potentially compromising the score's validity in this context. We suggest prospective future research to investigate the dynamic changes in the ALBI score over time to reinforce the validity of the ALBI score as a predictor of decompensation in non-malignant liver disease.

Key Words: Albumin-bilirubin score; Decompensated cirrhosis; Liver disease; Non-

malignant liver disease; Portal hypertension

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Core Tip: The albumin-bilirubin (ALBI) score was initially proposed to evaluate liver function in patients with hepatocellular carcinoma. It proposed to validate the ALBI score to assess the risk of decompensation in patients with compensated cirrhosis. We provide a comment to highlight the preliminary nature of the evidence reported by the authors. Further studies are needed to validate the ALBI score to predict decompensation in patients with cirrhosis.

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TO THE EDITOR

We read with great interest the study by Navadurong *et al*[1], who identified the albumin-bilirubin (ALBI) score for predicting decompensation in patients with initially compensated cirrhosis in a 3-year period.

The ALBI grade was initially proposed by Johnson *et al*[2] to assess liver function in patients with hepatocellular carcinoma; subsequently, it has been proposed as a prognostic tool in patients with non-malignant liver diseases. Here, the occurrence of decompensation in patients with cirrhosis is important in the prognostic assessment because after the first episode of decompensation, the patients' survival significantly declines compared to patients with compensated cirrhosis, with a median survival of 19 and 107 mo in patients with decompensated and compensated diseases, respectively[3].

We would like to commend the authors for the effort. However, we believe that some methodological issues may have limited the strength of the study's conclusion. First, relying solely on radiological tools to diagnose cirrhosis overlooks the comprehensive assessment of this complex condition, which should include clinical, laboratory, and histological data for a more accurate diagnosis and treatment plan. Hence, patients without definite cirrhosis, in whom the decompensation risk is inconsistent, may have been included.

Second, the study reports variceal bleeding as the main cause of decompensation. This result is somehow conflicting with the literature, as ascites is most frequently reported as the first decompensating event[4,5]. We observed that the inclusion criteria did not consider the presence and characteristics of esophageal varices, such as their size, presence of red marks, and prophylactic measures for first bleeding (beta-blockers, elastic band ligation). Hence, it is impossible to ascertain whether the association of ALBI grade with decompensation risk remains independent from these potential biases. As some studies previously suggested that the ALBI grade is correlated with hepatic venous pressure gradient, we believe that future studies aimed at assessing more on this correlation and clinical outcomes could be an area of interest [6].

Lastly, as reported by the authors, the number of patients with high-risk ALBI grade and occurrence rate of decompensating events were few. This limitation could have decreased the validity of the score in this setting. Therefore, it might have been of interest to assess whether longitudinal modifications of the ALBI score, as previously reported for other well-established prognostic indexes, could have gauged its prognostic relevance[7].

We believe that using the ALBI grade as proposed by the authors is fascinating; however, the study conclusions may be regarded as preliminary, and we concur with the authors' suggestion that the role of the ALBI grade in non-malignant liver disease as a predictor of decompensation should be confirmed in prospective, larger studies before being considered a validated tool.

FOOTNOTES

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LETTER TO THE EDITOR

Paying attention to the value of thrombelastography and the impact of postreperfusion syndrome on outcomes of liver transplantation

Yu-Li Wu, Lu Che, Yi-Qi Weng

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Abstract

Only limited information is available about the connection between massive blood transfusion and postoperative survival rates in pediatric liver transplantation. The aim of Gordon's study was to examine the potential impact of perioperative transfusion on postoperative complications and death in young children receiving pediatric living-donor liver transplantation (PLDLT). The authors concluded that transfusion of a red blood cell volume higher than 27.5 mL/kg during the perioperative period is associated with a significant increase in short- and long-term postoperative morbidity and mortality after PLDLT. However, viscoelastic coagulation monitoring was not utilized in the study; instead, only conventional coagulation monitoring was conducted. Overall, the choice of blood coagulation monitoring method during blood transfusion can have a significant impact on patient prognosis. Several studies have shown that the viscoelastic coagulation testing such as thrombelastography (TEG) is highly sensitive and accurate for diagnosing coagulation dysfunction. Indeed, a TEG-guided blood transfusion strategy can improve prognosis. Moreover, postreperfusion syndrome is one of the most common complications of liver transplantation and an important factor affecting the prognosis of patients and should also be included in regression analysis.

Key Words: Liver transplantation; Child; Blood transfusion; Thrombelastography; Reperfusion Injury; Prognosis

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Core Tip: The influence of blood transfusion strategies based on different coagulation testing methods on the outcomes of pediatric liver transplantation cannot be ignored. Additionally, postreperfusion syndrome during liver transplantation can have an important impact on the prognosis of pediatric patients and should be accounted for when studying risk factors for postoperative mortality.

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TO THE EDITOR

We read with interest the recent article by Gordon *et al*[1] titled "Perioperative blood transfusion decreases long-term survival in pediatric living donor liver transplantation (LDLT)". The original study sought to ascertain whether blood transfusions are related to early and late postoperative complications and mortality in children undergoing LDLT. The authors concluded that in pediatric LDLT, perioperative red blood cell transfusions exceeding 27.5 mL/kg are a direct cause of reduced patient and graft survival as well as an independent risk factor for death. We are grateful for the contribution of the authors, who performed a long-term postoperative follow-up and collected a large amount of data, providing important information that avoiding or reducing blood transfusions improves postoperative survival in children with liver transplantation, which can help to inform clinical decision-making. However, we believe that the following aspects need to be discussed.

In the study, viscoelastic coagulation monitoring was not used, and only conventional coagulation monitoring was carried out, and we think that this may have a certain impact on the results. The conventional coagulation test (CCT) includes the international normalized ratio (INR), prothrombin time, activated partial prothrombin time, thrombin time, and platelet count, among others. The viscoelastic coagulation test is used to measure and analyze the viscoelastic properties of blood clot formation. It involves measuring and converting the viscoelasticity produced by the interaction between fibrin strands and platelets during the blood coagulation process into digital data, which are then graphically plotted for analysis. Currently, there are two types of equipment available for conducting viscoelastic coagulation tests: classical thrombelastography (TEG) and rotational thromboelastometry. The coagulation system is responsible for maintaining the balance between clot formation and dissolution in the blood, and monitoring the coagulation process during a blood transfusion is crucial to ensure proper blood clotting and minimize the risk of complications such as bleeding or clotting disorders. Studies have shown that TEG during liver transplantation can effectively monitor the hypercoagulable state of patients and the subsequent risk of embolism; in contrast, the ability of the INR to monitor the hypercoagulable state and predict the risk of embolism is poor[2]. A randomized controlled trial showed that in patients with liver cirrhosis and severe coagulation dysfunction before invasive surgery, the TEG-guided transfusion strategy significantly reduced the use of blood products and did not increase bleeding complications compared with the standard strategy (blood transfusion guided by the INR and platelet count)[3]. In another randomized clinical trial, Bonnet *et al*[4] found that a transfusion algorithm based on thromboelastometry coagulation assessment reduced the total number of blood product units transfused during liver transplantation, especially the amount of fresh frozen plasma transfused. Therefore, the value of the CCT in liver transplantation is questionable; it is time-consuming and cannot fully reflect the complex changes in coagulation in patients with liver disease over time. Viscoelastic coagulation tests can provide comprehensive information from coagulation initiation to fibrinolysis, clot strength, and stability; they are more sensitive and accurate than the CCT in the diagnosis of coagulation disorders and can help to prevent complications and improve patient outcomes.

Like any other test, TEG has certain limitations. It measures blood coagulation outside the body, rather than the coagulation of blood while it is flowing within the vasculature; therefore, TEG does not reflect the function of the endothelium in coagulation[5]. In addition, the TEG testing equipment is costly and requires more professional training for operators to use it effectively. The factors mentioned above may limit the prevalence of TEG usage. However, the viscoelastic coagulation assay was recommended in the recent clinical guidelines by the European Society of Anesthesiology to reduce the rate of blood product transfusion during liver transplantation[6]. This guideline pointed out that the preoperative viscoelastic coagulation assay might help to predict blood loss and blood transfusion during liver transplantation[6].

In addition, the Gordon *et al*[1] study did not include all events that had an impact on prognosis in regression analysis, such as postreperfusion syndrome (PRS). PRS is defined as a significant decrease of over 30% in the mean arterial pressure compared with that at the end of the anhepatic phase, and this decrease has to last at least 1 minute and occur in the first 5 min after liver graft reperfusion[7]. Decreased body temperature in children before reperfusion and prolonged graft cold ischemia time are independent risk factors for PRS in pediatric liver transplantation[7]. Metabolic acidosis, hyperkalemia, hypocalcemia, and the release of many proinflammatory cytokines into the systemic circulation by the transplanted liver releasing after reperfusion are possible mechanisms for PRS[8]. PRS is one of the most common complications during liver transplantation and can lead to delayed recovery of graft function, prolonged hospitalization, and increased mortality and seriously affect quality of life in the postoperative period[7,9]. Therefore, we believe that PRS

may have an important impact on the prognosis of children and should be included in regression analysis.

In summary, considering the advantages of viscoelastic coagulation monitoring, we should pay attention to the value of using TEG in liver transplantation. Additionally, PRS can have an important impact on the prognosis of pediatric patients who undergo liver transplantation and should be considered when exploring risk factors for postoperative mortality.

FOOTNOTES

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REVIEW

Age-specific causes of upper gastrointestinal bleeding in children

Marija Kocic, Petar Rasic, Vuk Marusic, Dragan Prokic, Djordje Savic, Maja Milickovic, Ivana Kitic, Tanja Mijovic, Adrijan Sarajlija

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Abstract

The etiology of upper gastrointestinal bleeding (UGIB) varies by age, from newborns to adolescents, with some of the causes overlapping between age groups. While particular causes such as vitamin K deficiency and cow's milk protein allergy are limited to specific age groups, occurring only in neonates and infants, others such as erosive esophagitis and gastritis may be identified at all ages. Furthermore, the incidence of UGIB is variable throughout the world and in different hospital settings. In North America and Europe, most UGIBs are non-variceal, associated with erosive esophagitis, gastritis, and gastric and duodenal ulcers. In recent years, the most common causes in some Middle Eastern and Far Eastern countries are becoming similar to those in Western countries. However, variceal bleeding still predominates in certain parts of the world, especially in South Asia. The most severe hemorrhage arises from variceal bleeding, peptic ulceration, and disseminated intravascular coagulation. Hematemesis is a credible indicator of a UGI source of bleeding in the majority of patients. Being familiar with the most likely UGIB causes in specific ages and geographic areas is especially important for adequate orientation in clinical settings, the use of proper

diagnostic tests, and rapid initiation of the therapy. The fundamental approach to the management of UGIB includes an immediate assessment of severity, detecting possible causes, and providing hemodynamic stability, followed by early endoscopy. Unusual UGIB causes must always be considered when establishing a diagnosis in the pediatric population because some of them are unique to children. Endoscopic techniques are of significant diagnostic value, and combined with medicaments, may be used for the management of acute bleeding. Finally, surgical treatment is reserved for the most severe bleeding.

Key Words: Upper gastrointestinal bleeding; Age-specific; Epidemiology; Pediatric; Unusual cause

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Core Tip: This review provides general and comprehensive epidemiological data, overviewing the most common causes of upper gastrointestinal bleeding (UGIB) in children, in different age groups. The relevant literature in English on pediatric UGIB was searched until 2022, with special reference to age-related causes, unusual and rare causes, and risk factors. The literature search was performed using Medline *via* PubMed database (www.pubmed.gov), Google Scholar (www.scholar.google.com) and Cochrane Database, using the following terms: "neonates and infants" and "upper gastrointestinal bleeding"; "children" and "upper gastrointestinal bleeding"; "children" and "upper gastrointestinal bleeding" and "unusual causes"; "infants and children" and "upper gastrointestinal hemorrhage" and "unusual causes".

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INTRODUCTION

Upper gastrointestinal bleeding (UGIB) represents one of the most urgent conditions in the pediatric population. The etiologies of UGIB vary by age, from newborns to adolescents, with substantial overlap between age groups[1]. The origin of UGIB in children can be traced to any location from the esophagus to the ligament of Treitz, which represents the point of transition between the foregut and midgut[1,2] (Figure 1). Approximately 20% of all GI bleeding in children arises at those sites[3]. A significant share of patients with UGIB has a benign clinical course since approximately 80% present with self-limited bleeding[2]. About three-fourths (73%) of the patients present with hematemesis, followed by melena (21% of patients) and coffee-ground emesis (6% of patients)[1,4]. Nevertheless, some patients may also experience abdominal pain, nausea, dyspepsia, or vertigo[4,5]. Furthermore, hematochezia might be a rare sign of UGIB, except in neonates and infants who have rapid passage through the GI tract[3].

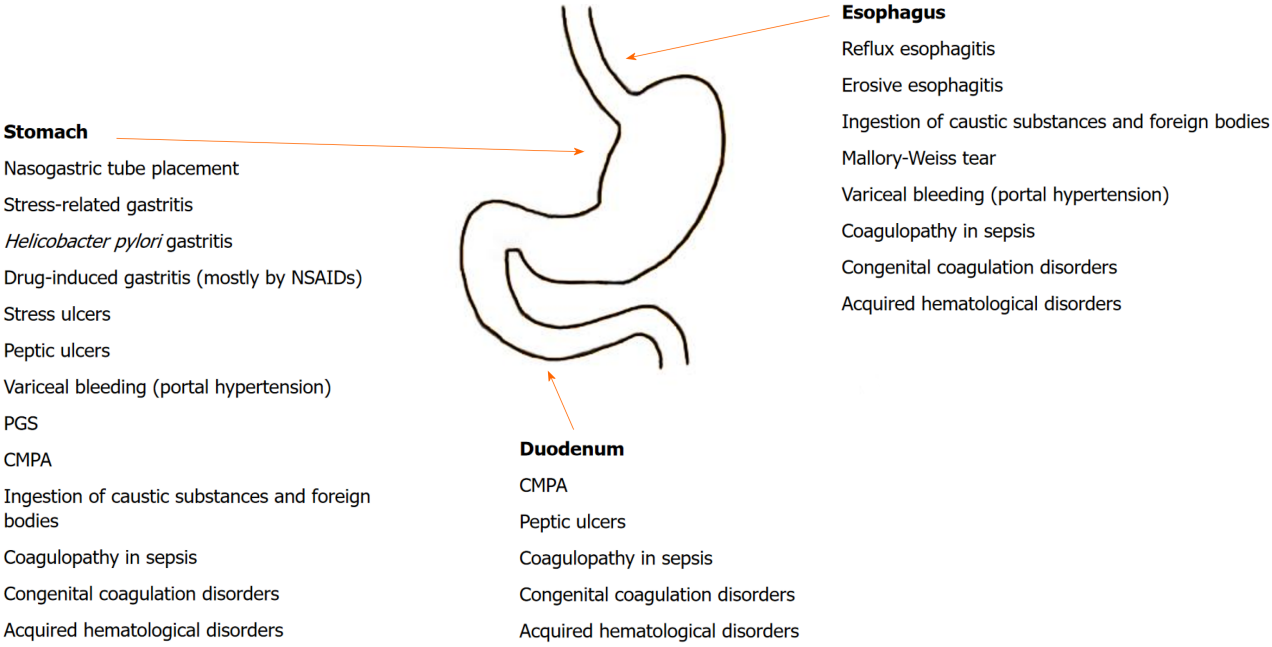
Differential diagnosis of UGIB is based on age, clinical presentation, and the amount of bleeding[6]. It must also include non-GI sources such as ingested maternal blood, epistaxis, and hemoptysis as well as food-mimicking hematemesis, coffee ground emesis, or melena[1,5]. Certain types of food may confuse children and parents due to a very similar appearance to blood in vomitus (*e.g.*, red food coloring, red candies, fruit-flavored drinks, fruit juices, and beets). Similarly, the melena-like appearance of the stool may be caused by some drugs like bismuth subsalicylate or iron supplements and food such as grape juice, spinach, beets, or blueberries[6].

Therefore, the anamnesis obtained from parents and the affected patient, if possible, must include information about associated signs and symptoms, dietary habits, and medicament use, and the attempt to quantify the bleeding. Large-volume UGIB might be encountered in non-steroidal anti-inflammatory drug (NSAID)-induced gastritis, but more frequently in variceal bleeding, both in young children and adolescents[6,7].

The initial approach in the treatment of patients with significant GI bleeding involves the establishment of adequate oxygen support, placement of central or peripheral intravenous catheters, hemodynamic stabilization, transfusion of blood and blood products, as well as correction of any underlying coagulopathies[5]. Endoscopy *per se*, is both a diagnostic and therapeutic procedure that is especially important in an emergency setting[8,9]. The frequency of endoscopic therapeutic interventions is especially high in patients with esophageal varices or bulbar peptic ulcer hemorrhage[8].

EPIDEMIOLOGICAL CHARACTERISTICS OF UGIB IN CHILDREN

There is a relative paucity of data regarding the exact incidence of UGIB in children. Based on the study conducted in France in 2010, the incidence of UGIB in the pediatric population is 1-2 per 10000 children[10]. The risk of UGIB is especially high in some subpopulations such as critical care patients. Critical illness alone may be a risk factor for stress-related GI ulceration and bleeding related to systemic hypoxemia, low gastric pH, impaired splanchnic perfusion, and dysregulated mucosal cytoprotection[11]. Besides, the use of medications such as NSAIDs, aspirin, corticosteroids, and



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Figure 1 Anatomic locations of upper gastrointestinal bleeding in children, with corresponding common causes. CMPA: Cow's milk protein allergy; NSAIDs: Non-steroidal anti-inflammatory drugs; PGS: Prolapse gastropathy syndrome.

selective serotonin reuptake inhibitors may be additional risk factors for severe UGIB[1,5]. Moreover, other comorbidities such as cerebral palsy increase the risk of erosive esophagitis primarily due to a higher prevalence of gastroesophageal reflux[4]. Few studies have provided critical care statistics related to UGIB in children[11-15]. The cumulative incidence of UGIB in the pediatric critical care population of Canada was shown to be 10.2%[12]. Moreover, a prospective cohort study from Thailand, which included 110 pediatric intensive care unit (PICU) patients, who required mechanical ventilation longer than 48 h, revealed that the incidence of UGIB was 51.8%, among which 3.6% of the cases presented with clinically significant bleeding[13]. According to the Belgian literature review published in 2011, the incidence of significant UGIB in children admitted to the PICU was 0.4%-1.6%[14]. However, an earlier prospective comparative study from 1992 conducted in a PICU of a tertiary care university-based facility in the United States found that the prevalence of UGIB was as high as 25%[15].

UGIB may be a life-threatening condition in cases of large blood volume loss, and worldwide mortality rates in children reach up to 15%[1,4]. A study from the United States showed that the overall mortality of PICU patients was 4.8%, with significantly higher mortality observed in patients with UGIB than in those without (16% *vs* 1.3%; $P < 0.001$) [15]. To reduce the risk of GI bleeding in children hospitalized in the PICU for critical asthma, stress ulcer prophylaxis (SUP) is commonly prescribed. A retrospective, multicenter cohort study conducted in the United States from 2010 to 2019 included children 3 years to 17 years of age admitted to the PICU for critical asthma. Of the 30177 children, 10387 (34.4%) received SUP throughout the 10-year period. Gastritis was noted in 32 (0.1%) subjects, and rates did not differ for patients who had and had not received SUP (0.11% *vs* 0.1% respectively; $P = 0.706$). Moreover, no episodes of major GI bleeding events were documented for the entire study sample regardless of SUP exposure[16]. These data raise a question of a need for the routine use of SUP in this population and advocate the consideration of a more individualized approach to the prevention of GI bleeding.

MOST COMMON CAUSES OF UGIB IN THE PEDIATRIC POPULATION

Various factors influence the occurrence of UGIB in different regions and countries around the world[1,2,10,17]. According to the literature, the origin of the UGIB in the pediatric population differs in developing and developed countries[1-3,8,18-22]. Based on causes, the UGIB may be classified into two major groups: Variceal and non-variceal[5]. Previously reported data from a study conducted in the Middle East in 2012, suggested that the most common cause of UGIB is esophageal varices (in 39% of patients with UGIB)[23]. Similarly, data from India in 1996 also indicated variceal bleeding as the most common UGIB, affecting 95% of patients. The majority (92%) of these cases occurred due to extrahepatic portal vein obstruction (EHPVO)[22]. However, the data obtained from an Iranian study conducted 2012-2014 indicated that the most common etiologies of UGIB among all patients admitted for GI bleeding were prolapse gastropathy syndrome (PGS) and esophagitis, with rates of 18.6% and 15.9%, respectively, followed by esophageal varices, gastritis, and coagulopathy, with frequency of 7.1% for each[18]. Moreover, a large cross-sectional study from Turkey from 2020 showed that the most common causes of UGIB were esophagitis (47%), peptic ulcer (18.1%), and esophageal varices (11.1%)[8]. In China, a 10-year retrospective multicenter cohort study conducted 2003-2012 reported

Table 1 Common causes of upper gastrointestinal bleeding in neonates[1,3,5,24]

Cause	Bleeding source location
Swallowed blood	Non-GI bleeding
Nasogastric tube placement	Stomach
Stress-related gastritis	Stomach
CMPA	Stomach, duodenum
NEC	Lower GI tract
Vitamin K deficiency	Distinct locations
DIC	Distinct locations
Congenital coagulation disorders[1]	Distinct locations

[1]Hemophilia, Von Willebrand disease, *etc.*

CMPA: Cow's milk protein allergy; DIC: Disseminated intravascular coagulation; GI: Gastrointestinal; NEC: Necrotizing enterocolitis.

erosive gastritis as the most frequent endoscopic finding in children with UGIB (33.5%), followed by duodenal ulcer (23.2%). The same study showed that the prevalence of erosive gastritis decreased with children's age (correlation coefficient = -0.787), while duodenal ulcer showed an increasing trend regarding age (correlation coefficient = 0.958)[21].

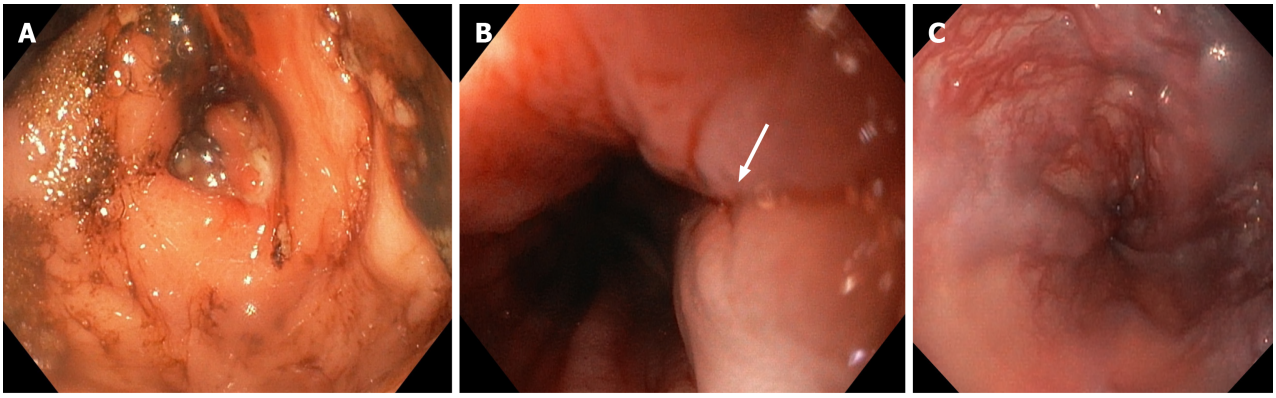
According to the literature, the most common causes of UGIB in North America and Europe are PGS (12.7%), gastric erosions and ulcers (10.8%), erosive esophagitis (9.5%), and duodenal erosions/ulcers (8.2%), whereas less common are esophageal varices (6.3%) and Mallory-Weiss tear (3.8%)[3,4]. A retrospective cohort, single-institution study conducted in the United Kingdom 2009-2014 by Nasher *et al*[20] included 32 patients with UGIB, with an average age at presentation of 5.9 ± 5.5 years in males and 8.2 ± 6.0 years in females. Over one-half (59.3%) of patients underwent an upper GI endoscopy procedure, which identified esophageal varices and esophagitis (occurring in 26% of patients each) as the most common findings, followed by gastritis/duodenitis (identified in 15.8% of patients). Duodenal ulcer was found in 2 patients who had evidence of *Helicobacter pylori* (*H. pylori*) on biopsy and in 1 with a previous history of NSAID intake. Furthermore, 1 patient had a rare cause of UGIB, a gastric vascular malformation[20]. Moreover, a 7-year retrospective cohort study conducted from 2007 to 2013 in the northeast of Romania which included 103 patients with UGIB showed that erosive gastritis was the most common cause of UGIB (occurring in 33.9% of included patients). The other causes comprised esophagitis (14.6%), duodenitis (11.6%), duodenal ulcer (10.7%), gastric ulcer (5.8%), esophageal varices (4.8%) and Mallory-Weiss syndrome (1.9%), whereas 16.5% of patients had multicausal UGIB. Furthermore, the particular bleeding source was determined in 34.9% of patients, a possible one in 39.8%, while in 25.2% the source has not been ascertained. Finally, in the same study, NSAID consumption and *H. pylori* infection were documented in 17.5% and 36.9% of patients, respectively[19].

CAUSES OF UGIB IN NEONATES

The most common causes of UGIB in newborns include coagulation disorders such as vitamin K deficiency, cow's milk protein allergy (CMPA), stress-related gastritis, sepsis, and trauma related to nasogastric tube placement[1,3,5,6,24] (Table 1). CMPA is a common food allergy both in neonates and infants, with an incidence estimated as 1.8%-7.5% in the 1st year of life[25]. Since there is no specific diagnostic test, the diagnosis of CMPA is primarily established by clinical evaluation and the elimination diet is the preferable treatment option[26].

UGIB in neonates in the neonatal ICU (NICU) setting is not uncommon. A retrospective-prospective cohort study conducted in Finland in 2000 which included 189 newborns treated in the NICU found that approximately 20% of patients had signs of GI bleeding. Mechanical ventilation was determined to be the crucial risk factor [odds ratio (OR) = 4.06; 95% confidence interval: 1.21-12.3], with 53% of mechanically ventilated patients having gastric mucosal lesions[27].

Furthermore, vitamin K deficiency must always be considered in neonates having UGIB. Vitamin K deficiency bleeding is usually categorized into three major groups: Early-onset (1st 24 h of life), classical (2nd to 7th d), and late-onset (2nd to 12th wk). Special attention should be given to the late-onset form, which exerts a greater burden in low- and middle-income countries where the median incidence is 80/100000, compared with high-income countries where the median incidence is significantly lower, 8.8/100000[28]. Besides, all neonates with hematemesis should also be screened for other bleeding disorders such as maternal thrombocytopenic purpura, hemophilia, and Von Willebrand disease, which are well-known risk factors for possible GI bleeding. Furthermore, the large amount of UGIB in newborns may also be caused by gastric erosion due to the placement of a nasogastric tube, sepsis, and/or disseminated intravascular coagulation[5]. When major UGIB occurs in neonates, parenteral vitamin K and proton pump inhibitors administration are recommended empirically[29]. Moreover, it is very important to initially exclude ingestion of blood of maternal origin that may be swallowed during delivery or from cracked nipples, which can be perceived as UGIB. Therefore, alkali denaturation testing (known as the Apt test) should be performed whenever available to distinguish maternal from fetal blood[30]. If maternal blood ingestion is excluded, another source of UGIB should be sought in this age group.



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Figure 2 The most common causes of upper gastrointestinal bleeding in children from 1-year-old to 3-years-old. A: Gastric ulcer in a 3-year-old boy who presented with hematemesis; B: Mallory-Weiss tear in a 3-year-old boy; C: Esophageal varices grade II in a 2-year-old boy with extrahepatic portal vein obstruction after cardiac surgery.

Finally, preterm infants represent a delicate pediatric population, in whom bright red blood in nasogastric aspirate may be a presenting sign of necrotizing enterocolitis (NEC)[3], although the source of hemorrhage caused by NEC is mostly located in the lower GI tract[31,32]. NEC usually develops in preterm infants with usual onset between 2 wk and 3 wk of life. On the other hand, full-term infants comprise about 10% of all babies with NEC, and in this subgroup NEC may appear within the 1st wk of life[33]. Because of insufficient reliable data due to inconsistencies in diagnosis and/or data collection of different studies, the precise incidence of NEC is undetermined[32]. When diagnosed, NEC must be treated rapidly with nasogastric decompression, proper antimicrobial therapy, and other supportive measures. If there is a suspicion of advanced NEC, surgical treatment is necessary[3].

CAUSES OF UGIB IN INFANCY

In younger infants, during the early months of life, the causes of UGIB are related to reflux and erosive esophagitis, PGS, CMPA, and stress-related gastritis[1,3,17,18,25] (Table 2). Gastritis with subsequent mucosal bleeding and stress ulcers are especially common causes of UGIB in infants hospitalized in PICU[3,27]. Less frequent causes include caustic and foreign body ingestion, coagulation disorders, medication-induced bleeding (mostly caused by NSAIDs), and rarely peptic ulcers and esophageal varices[5,17,34]. The presence of melena, particularly in the youngest children, points to significant GI hemorrhage[10,20]. The source of bleeding in these patients usually can be identified by flexible endoscopy, though drug-induced ulceration should be suspected from detailed medical history data and usually does not require endoscopic confirmation. An age-related retrospective study, which was conducted 2000-2010 in Iran and included children and adolescents aged 0-18 years, showed that erosive esophagitis was the most common cause of GI bleeding in all patients. This was also the most common finding in patients less than 1-year-old, with a prevalence of 37%, followed by gastritis (25.9%)[17]. On the other hand, the same study identified peptic ulcer disease (PUD) as a cause of UGIB in only 7.4% of infants, which significantly differed from older children. In concordance with these findings are the results from a national case-crossover study, conducted in France over a 2-year period, where a similar prevalence of the most common UGIB causes in this age group was reported[10].

CAUSES OF UGIB IN TODDLERS

The most common causes of UGIB in children from 1-year-old to 3-years-old include reflux and erosive esophagitis, gastritis, caustic ingestions, peptic ulcer (Figure 2A), esophageal varices, vomiting-induced bleeding similar to ones from Mallory-Weiss tear (Figure 2B), and PGS[1,3,4,18] (Table 3). PGS represents a retrograde prolapse of a part of gastric mucosa from the proximal stomach into the distal esophagus[35]. A study by Cleveland *et al*[4] found the erosion caused by prolapse of a portion of the gastric cardia along the lesser curvature found in PGS represents a more common source of bleeding than PUD or nonspecific gastritis in children.

It is well known that NSAIDs (including aspirin) and corticosteroids can increase the risk of UGIB, causing impairment of the gastric mucosal barrier by reduction and alteration of mucous secretion and promoting tissue fragility[5,36]. A group of French researchers performed a national case-crossover study with a total of 177 children with UGIB involved. The study reported that UGIB in one-third of the cases occurred due to exposure to NSAIDs at doses used for analgesic or antipyretic purposes. According to the age groups, the observed risk was more than four times higher in children up to 7-years-old (OR = 14.1) than in patients 8-years-old to 16-years-old (OR = 3.4)[10]. Although gastric ulcers have been associated with the chronic use of NSAIDs, reports from a single center in the United States over a 1-year period described UGIB in patients aged 16 mo to 36 mo (median age of 23.5 ± 9.0 mo) who developed hematemesis within 24 h

Table 2 Common causes of upper gastrointestinal bleeding in infants[1,3,5,17,26]

Cause	Bleeding source location
Erosive esophagitis	Esophagus
Reflux esophagitis	Esophagus
Ingestion of caustic substances and foreign bodies	Esophagus, stomach
Erosive gastritis	Stomach
Stress ulcers	Stomach
Drug-induced gastritis (most commonly by NSAIDs)	Stomach
PGS	Stomach
CMPA	Stomach, duodenum

CMPA: Cow's milk protein allergy; NSAIDs: Non-steroidal anti-inflammatory drugs; PGS: Prolapse gastropathy syndrome.

Table 3 Common causes of upper gastrointestinal bleeding in toddlers[1,4,18]

Cause	Bleeding source location
Erosive esophagitis	Esophagus
Mallory-Weiss tear	Esophagus
Ingestion of caustic substances and foreign bodies	Esophagus, stomach
Variceal bleeding associated with portal hypertension	Esophagus, stomach
Erosive gastritis	Stomach
Peptic ulcers	Stomach
PGS	Stomach
Drug-induced gastritis (most commonly by NSAIDs)	Stomach

NSAIDs: Non-steroidal anti-inflammatory drugs; PGS: Prolapse gastropathy syndrome.

after receiving just one or two age- and weight-appropriate doses of ibuprofen. In each of those patients, esophagogastroduodenoscopy demonstrated an antral gastric ulcer[36].

Furthermore, it is reported that the consumption of NSAIDs combined with infection with *H. pylori* is associated with a higher risk of gastroduodenal ulceration and bleeding[19]. A study from Tunis showed that the severity of gut mucosal damage in a population with a high prevalence of *H. pylori* infection is correlated with NSAID level intake, especially in children younger than 24 mo. However, the presence of *H. pylori* infection did not worsen gut mucosal injury severity in patients receiving NSAIDs[37].

Foreign body ingestion represents a significant risk factor for major complications, including UGIB. The occurrence of UGIB in these patients was shown to be time-related regarding the diagnosis establishment. Ingestion of a button battery is especially hazardous because it often results in secondary complications such as mucosal erosions, bleeding, gastric outlet obstruction, mediastinitis, or abscess formation[34,38]. Even in the case of a button battery lodging in the esophagus only transiently, severe erosion and ongoing necrosis of the esophagus and surrounding tissues may occur after the removal. Therefore, batteries that are in the esophagus must be removed within 2 h to avoid potentially fatal complications such as perforation and, the most severe one, aorto-esophageal fistula formation and subsequent bleeding [34]. Exsanguination from aorto-esophageal fistula has been reported in infants and toddlers[39].

Severe UGIB bleeding may be caused by esophageal varices rupture, which develops as a result of portal hypertension [40,41]. The most frequent causes of portal hypertension and its complications in children are EHPVO (Figure 2C) and liver cirrhosis. Liver cirrhosis in infancy is most commonly caused by biliary atresia (BA) and metabolic disorders, whereas in older children, the most common causes are alpha-1-antitrypsin deficiency, autoimmune hepatitis, primary sclerosing cholangitis, and Wilson's disease[42]. In patients with BA, esophageal varices may develop earlier compared to other causes of cirrhosis in children, with the median age of the first bleeding at 17 mo[43]. The management of variceal bleeding comprises both prophylaxis and acute emergency treatment and includes the use of drugs (*e.g.*, propranolol, vasopressin, and somatostatin), endoscopic procedures (*e.g.*, periodic surveillance endoscopies, endoscopic sclerotherapy, and band ligation), interventional radiology (*e.g.*, transjugular intrahepatic portosystemic shunt), and surgery (*e.g.*, portosystemic shunting)[44].

CAUSES OF UGIB IN CHILDREN OLDER THAN 3-YEARS-OLD

In children older than 3-years-old, bleeding most commonly arises from Mallory-Weiss tear, reflux and erosive esophagitis, erosive gastritis (Figure 3A), chronic PUD, caustic ingestions, esophageal varices, gastric varices (Figure 3B), and foreign body ingestion[1,4,5,24]. Besides, younger children and adolescents may experience bleeding from severe coagulopathy due to hematological disorders such as leukemia and idiopathic thrombocytopenic purpura[3] (Table 4).

PUD in children is similar to that in adults; however, there are some differences, especially in the prevalence of etiologies, clinical presentation, and complications. PUD is usually categorized as primary when associated with *H. pylori* and hypersecretory conditions including Zollinger-Ellison syndrome (ZES), short-bowel syndrome, cystic fibrosis, hyperparathyroidism, *etc*. Primary PUD is more often found in the duodenum, whereas secondary ulcers are more frequently localized in the stomach. On the other hand, secondary PUD is usually associated with systemic disease and drug ingestion and comprises stress ulcers, drug-induced ulcers, ulcers associated with infections other than *H. pylori*, Crohn's disease (CD) ulcers, ulcers associated with foreign body ingestion, *etc*[45]. A retrospective analysis performed in a single Endoscopy Center in China in 2021 included 173 children who were found to have upper GI ulcer. Primary ulcer was found in 148 (85.6%) patients and secondary in 25 (14.4%) patients. Of those with secondary ulcers, foreign body in the digestive tract was the most common cause, and was observed in 17 children (68%), followed by Henoch-Schönlein purpura (HSP) in 5 children (20.0%) and CD in 3 children (12.0%)[46].

H. pylori infection has been found in 50% of the world's population (approximately in 70% of the population in developing countries and 30%-40% in developed countries)[47] (Figure 3C). In children, the majority of *H. pylori* infections are asymptomatic, regardless of being associated with microscopic gastric inflammation[48]. Spontaneous eradication of the infection is noted mainly in infants and young children, and it decreases with age[49]. Besides, due to a rising resistance of *H. pylori* to antimicrobials and lack of symptom improvement in the absence of PUD, testing for *H. pylori* presence and application of eradication therapy are recommended only for the subset of patients with a high suspicion of PUD[47]. Therapy should be based on antibiotic resistance profiles and tailored accordingly using sufficiently high doses and treatment durations of 10-14 d, to achieve an initial eradication success rate of ≥ 90%[48]. In case of unknown susceptibility, high-dose triple therapy with proton pump inhibitors, amoxicillin, and metronidazole for 14 d is recommended as first-line therapy[48]. In a retrospective cohort study from the Middle East (conducted 1993-2002), which included 521 patients younger than 18-years-old who presented with UGIB, 24 (5%) children were diagnosed by endoscopy with PUD. The average age was 15 (range: 5-18 years). Primary PUD was found in 79% of the children, and according to the available histopathological data, the main finding was antral gastritis highly associated with *H. pylori*[50]. Moreover, a retrospective cohort study conducted in Japan 1995-2001, which included 283 patients aged 9 mo to 16 years, showed that the prevalence of *H. pylori* was highest in children with nodular (antral) gastritis (98.5%) and duodenal PUD (83%). *H. pylori* infection strongly correlated with gastric and duodenal ulcers in the age group of 10-years-old to 16-years-old compared to children 9-years-old or younger, in whom this correlation was not identified (OR = 12.1 *vs* 4.1 for duodenal ulcer, and OR = 8.2 *vs* 0.7 for gastric ulcer). Besides, researchers identified a low frequency of hematemesis or tarry stools (occurring in 9% of all patients), suggesting that there was no association with significant acute GI blood loss in the majority of patients[51]. Moreover, it has been reported that several hematological diseases may be associated with *H. pylori* infection, such as iron deficiency anemia, immune thrombocytopenia, and vitamin B12 deficiency, which can lead to additional GI disorders and subsequent UGIB[52].

EHPVO represents an important cause of portal hypertension in developing countries, and some data suggest that it is responsible for the occurrence of up to 80% of portal hypertension in children[53]. It commonly presents as incidental splenomegaly on physical examination (in 43.3% of cases) and in 40% as UGIB manifested as hematemesis and/or melena due to gastroesophageal varices[41]. Gastroesophageal varices caused by portal hypertension as a consequence of EHPVO were shown to be the most common source of significant UGIB in children above 3-years-old in South Asian countries[22,54]. A retrospective cohort study from India, conducted from 2002 to 2012, found 30 patients with EHPVO (5-years-old to 14-years-old). The peculiarity of these results was that delivery at home, which was one of the risk factors, was reported in all 30 cases, while the other risk factors, umbilical sepsis and a history of cow dung application over the umbilical cord were identified in 27% and 6.7% of patients, respectively. Almost 75% of all patients in that study had UGIB. Endoscopy findings revealed esophageal varices in all enrolled patients, gastric varices in 38.5%, and portal hypertensive gastropathy in 20%[55].

BA is a common cause of portal hypertension in older children as well as in children 3-years-old or younger. A cross-sectional multicentric study conducted between May 2006 and December 2009, as a part of the Childhood Liver Disease Research and Education Network, enrolled a total of 163 patients with BA (aged 1-25 years). The 43/163 (26.3%) patients with portal hypertension had complications, and 19.6% manifested with esophageal varices bleeding. Among the 32 patients with esophageal varices bleeding, most (34.4%) had their first bleeding episode between 6-years-old and 10-years-old, followed by equal portions (21.8% each) between birth and 2-years-old, between 2-years-old and 5-years-old, and ≥ 10-years-old[40].

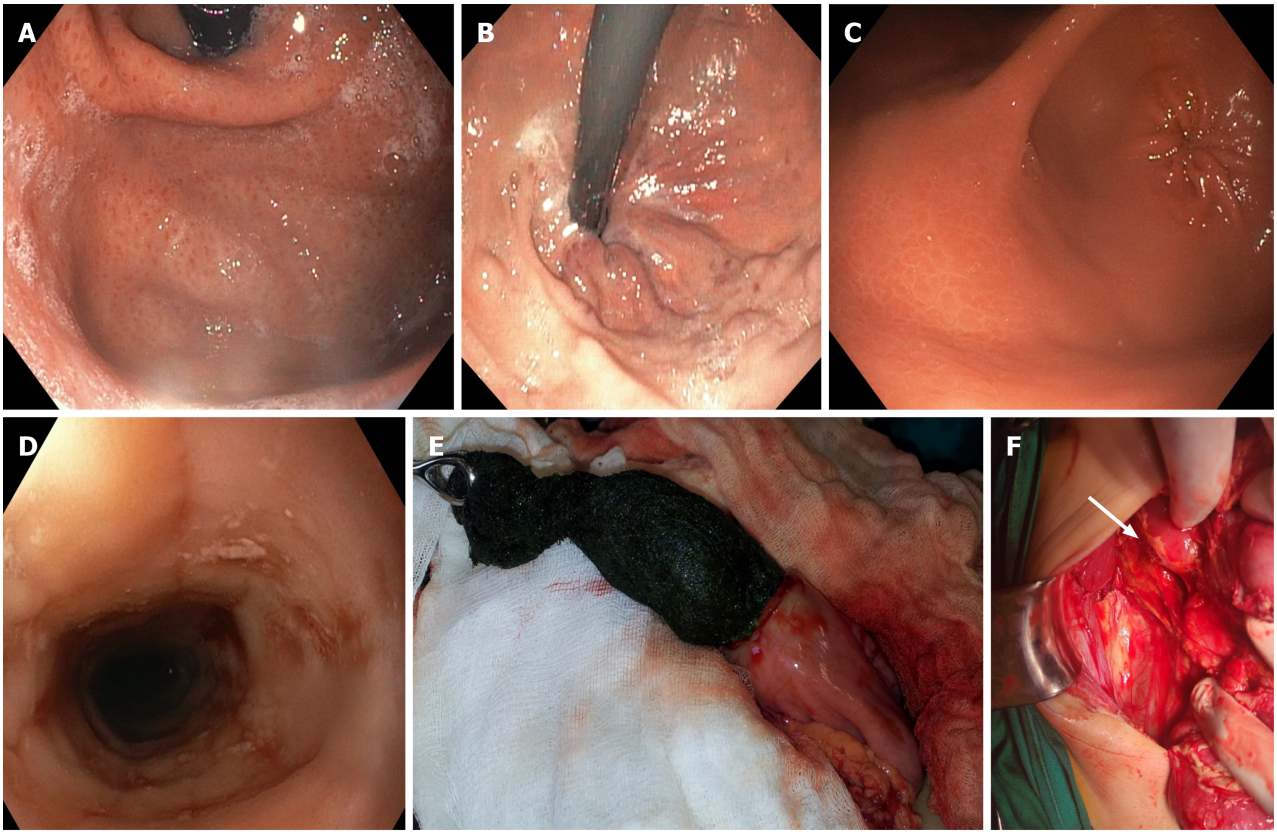
UNUSUAL CAUSES OF UGIB

Unusual causes of UGIB (Table 5) are as clinically challenging in pediatric patients as in their adult counterparts. The incidence of unusual etiologies could be higher in pediatric practice since most of the rare diseases initially present during childhood. Unusual causes comprise various congenital anomalies, acquired diseases, and unusual foreign body ingestion.

Table 4 Common causes of upper gastrointestinal bleeding in children ages > 3 yr[1,4,5,24,50]

Cause	Bleeding source location
Erosive esophagitis	Esophagus
Reflux esophagitis	Esophagus
Mallory-Weiss tear	Esophagus
Variceal bleeding associated with portal hypertension	Esophagus, stomach
Erosive gastritis	Stomach
Stress ulcers	Stomach
Drug-induced gastritis (most commonly by NSAIDs)	Stomach
Helicobacter pylori gastritis	Stomach
Peptic ulcers	Stomach, duodenum
Acquired hematological disorders (ITP, leukemia, *etc*.)	Distinct locations

ITP: Idiopathic thrombocytopenic purpura; NSAIDs: Non-steroidal anti-inflammatory drugs.



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Figure 3 Causes of upper gastrointestinal bleeding in children older than 3-years-old. A: Erosive gastritis in a 14-year-old girl with cerebral palsy; B: Gastroesophageal varices type 2 in a 6-year-old girl with chronic liver disease due to extrahepatic portal vein obstruction; C: Macronodular gastritis in a 16-year-old girl due to *Helicobacter pylori* infection; D: Eosinophilic esophagitis in a 16-year-old boy; E: Gastric trichobezoar in a 9-year-old boy; F: Traumatic perforation of the duodenum as a consequence of a handlebar injury in a 6-year-old boy. The patient was referred to the hospital two days after injury and the surgery was performed on the day of admission.

Unusual UGIB causes affecting the esophagus

UGIB is reported as a very rare clinical presentation in patients with alimentary tract duplications, including in newborns, infants, and older children[56-58]. The esophagus is, after the ileum, the second most common spot of occurrence of these congenital anomalies[56,59]. Alimentary tract duplications are usually diagnosed in infancy and childhood, with 60% of patients presenting at ages younger than 2 years[60]. The majority of esophageal duplications are related to the right side of the esophagus and do not communicate with its lumen[61]. However, they may sometimes contain gastric epithelium

which can provoke peptic ulceration, intra-cystic hemorrhage, and fistulation into the esophagus, presenting with hematemesis or melena[62].

Furthermore, it is not uncommon for eosinophilic gastrointestinal diseases (EGIDs) to initially present with GI bleeding [63]. Eosinophilic esophagitis (EoE) (Figure 3D), with an estimated prevalence of 10 to 57 per 100000, is the most common type of EGIDs, a group of chronic GI disorders, characterized by GI symptoms and pathological eosinophilic infiltration of various parts of the GI tract, without secondary causes of tissue eosinophilia[64]. Ozdogan *et al*[63] reported on 3 patients aged 8, 10, and 12 years who had hematemesis as the initial sign of EoE.

Unusual UGIB causes affecting the stomach

Gastric duplications (GDs) comprise only 7% of all GI duplications[59]. These congenital anomalies are commonly located in the region of the greater curvature. The cystic type comprises around 80% of GDs and they do not communicate with the gastric lumen, unlike the remaining 20% representing tubular GDs which are contiguous with the stomach and usually communicate with the gastric lumen. According to the study by Li *et al*[65] which included 319 patients with GD, vomiting and abdominal pain were the most common manifestations of these anomalies. GI hemorrhage was shown to be the third most common symptom, occurring in 16.3% of children.

Eosinophilic gastritis is even rarer than EoE, with a prevalence estimated at 6.6 per 100000[66]. Ozdogan *et al*[63] reported on 2 patients aged 7 and 14 years with eosinophilic gastritis who initially presented with hematemesis.

Some gastric tumors may also lead to GI bleeding. Gastrointestinal stromal tumors (GISTs) are an extremely rare cause of UGIB, described in several case series and reports[67]. They are most commonly located in the stomach and the median age at diagnosis is reported to be between 60 years and 69 years[68]. Patients 16-years-old or younger represent only 1.4%, and those less than 21-years-old represent 2.5% of all gastric GIST patients[67,69]. Authors from the United States have reported on a case of an 11-year-old girl who presented with anemia and guaiac-positive stool 1 mo after tonsillectomy. Because her anemia was thought to be secondary to blood loss from her tonsillectomy, iron therapy was started. Since 2 wk thereafter her hemoglobin decreased again, esophagogastroduodenoscopy was performed and demonstrated 7-8 large submucosal masses in the antrum and distal body of the stomach, with deep ulceration in one of the masses. Endoscopic ultrasound-guided fine needle aspiration confirmed the diagnosis of epithelioid GIST[69].

Furthermore, there are scarcely reported cases of primary gastric lymphomas in children. Authors from Korea reported on a case of a 10-year-old patient with a family history of gastric adenocarcinoma, who presented with recurrent hematemesis and melena. Esophagogastroduodenoscopy revealed diffuse antral ulcers, while a tissue biopsy showed a diffuse large B cell non-Hodgkin lymphoma[70]. Other rare gastric tumors such as Schwannoma causing GI bleeding have been reported anecdotally in toddlers and adolescents[71,72]. Moreover, Corasaniti *et al*[73] reported on a case of a young infant who presented with bilious vomiting, melena, and anemia due to a giant gastric polyp, histologically proven to be focal foveolar hyperplasia.

Bezoars (including trichobezoars, phytobezoars, pharmacobezoars, lactobezoars, *etc*.) are indigestible conglomerations trapped in the GI tract[74]. They are most commonly located in the stomach, although they may be observed in the esophagus, duodenum, and other segments of the bowel[75]. Trichobezoars (Figure 3E) are associated with trichotillomania and trichophagia in psychiatric patients. Bhatia *et al*[76] reported on 24 cases of trichotillomania attending the psychiatry outpatient department and found that the majority of cases (54.2%) belonged to the age group of 6-years-old to 10-years-old. In 2021, LaGrandeur *et al*[77] reported on a case of a seemingly healthy school-aged girl who presented with recidivant hematemesis, in whom a gastric trichobezoar was revealed during endoscopy.

Unusual UGIB causes affecting the duodenum

Duplications of duodenum contain gastric mucosa in up to 20% of cases. This could lead to intracystic hemorrhage or perforation of the cyst with subsequent GI bleeding and peritonitis[56]. Diagnostics of the alimentary tract duplication rely on ultrasonography, GI contrast study, computed tomography, and magnetic resonance imaging. Additional testing including scintigraphy may be useful in detecting ectopic gastric mucosa[61].

Varices in the duodenum are rarely present as a cause of UGIB, in comparison to varices located in the esophagus and stomach which are well-known complications of portal hypertension. Duodenal varices are mostly reported in adult patients. Hiçsönmez *et al*[78] reported on a curious case of a 12-year-old girl with severe bleeding from duodenal varices secondary to EHPVO and subsequent cavernous transformation of the portal vein.

Duodenal trauma (Figure 3F) in the pediatric population is infrequent, comprising 2%-10% of blunt abdominal trauma cases and only 0.14%-0.16% of all injured children per year[79,80]. Bicycle accidents are the most frequent cause of blunt trauma, representing between 5% and 14% of total closed abdominal injuries. A case study from Spain in 2019 described an adolescent boy with duodenal perforation after being injured by a bicycle handlebar, presenting with hematemesis, abdominal pain, and swelling and tenderness in the upper right abdominal quadrant and epigastrium. An exploratory laparotomy revealed perforation in the posterior wall of the third part of the duodenum and the patient was treated with the resection of the third portion of the duodenum and Roux-en-Y duodenojejunostomy[81].

Some unexpected, potentially dangerous foreign bodies used in everyday life may lead to severe GI damage. Nguyen *et al*[82] reported on a case of a 6-year-old girl who presented with abdominal pain, coffee-ground emesis, and melena due to uncooked pasta lodged in the duodenum and creating an erosion.

Hemobilia is a rare cause of UGIB both in adults and children. The bleeding from and into the biliary tract usually follows trauma to the hepatobiliary-pancreatic system[83]. Some of the rare causes of hemobilia reported in the pediatric population are parasitic infestation with Ascaris lumbricoides, liver abscess (occurring predominately in African and Asian regions), GD, pancreatitis, choledochal cyst, gall bladder polyps, and Von Willebrand disease[83,84].

Unusual UGIB causes affecting distinct locations

Dieulafoy's lesions are vascular malformations comprising abnormally enlarged arteries with the potential for massive GI bleeding. These anomalies, which are thought to be congenital, account for 1%-5.8% of all cases of acute UGIB and are most commonly located at the lesser curvature of the stomach[85-87]. However, they were shown to be extragastric in 33% of cases, affecting the duodenum, colon, esophagus, rectum, or jejuno-ileum[86]. Dieulafoy's lesions are more common in males than females (2:1 ratio), and the mean age at presentation is within the 5th decade of life (range: 50-70 years)[88]. This disorder may be underdiagnosed in any age group, and there are only several sporadic cases and case series available in the literature, comprising children of ages from preterm neonates to 18-years-old[89-92]. The signs and symptoms are related to blood loss, either due to intermittent or massive acute GI hemorrhage. Melena is the most common form of presentation, followed by hematemesis, hemoptysis, hematochezia, and iron deficiency anemia. In most severe cases, bleeding from Dieulafoy's lesions may lead to hemodynamic instability[93]. Since these lesions have a major potential for life-threatening hemorrhage, they should be included in the differential diagnosis of massive UGIB in all age groups.

ZES is an extremely rare condition caused by gastrin-producing tumors (gastrinomas), with an overall incidence of 0.1-3 per 1000000. Only 2% of gastrinoma cases occur in the pediatric population[94]. These tumors are usually located in the duodenal wall or in the pancreas. Hypergastrinemia causes non-healing or recurrent ulcers in 85% of cases localized in the duodenum. However, these ulcers may also appear in the stomach, jejunum, or multiple locations[95]. Authors from India reported on a case of a 12-year-old boy with a refractory peptic ulcer caused by gastrinoma in the pancreatic head. Upper GI endoscopy displayed multiple linear ulcers both in the mid and lower parts of the esophagus involving the gastroesophageal junction, as well as multiple superficial ulcers in the antrum and three parts of the duodenum[94]. Also, Zaatar *et al*[96] reported on a 7-year-old boy who presented with mild to moderate iron deficiency anemia, with fasting hypergastrinemia (serum gastrin of 200-500 pg/mL). Investigation revealed a positive stool guaiac test and elevated gastric acid secretion, while endoscopy showed multiple small gastric fundal ulcerations and severe gastritis. However, the investigation towards ZES produced negative findings.

Peutz-Jeghers syndrome is an autosomal dominant inherited disorder, occurring with an incidence of 1 to 50000-200000, characterized by GI hamartomas and mucocutaneous pigmentations due to melanin deposition[97,98]. This rare disorder usually presents with painless rectal bleeding. Nevertheless, authors from Indonesia reported in 2022 on the case of a 5-year-old patient who presented with abdominal pain, dark-red bloody stools, and anemia due to bleeding from scar ulcers in the duodenal region and erosive inflammation of the upper and lower GI tract along with multiple polyps. The patient had multiple black spots on the lips and mucous membranes (conjunctiva and buccal mucosa) and a family history of similar symptoms, which led to the diagnosis of Peutz-Jeghers syndrome[98].

The inflammation in CD may affect any part of the GI tract. Whilst in lower GI it commonly manifests as hematochezia, CD is rarely associated with UGIB. Though it has been rarely reported, when found on endoscopy, the source of UGIB bleeding in patients with CD is commonly a secondary deep ulcer or multiple ulcers[46,99].

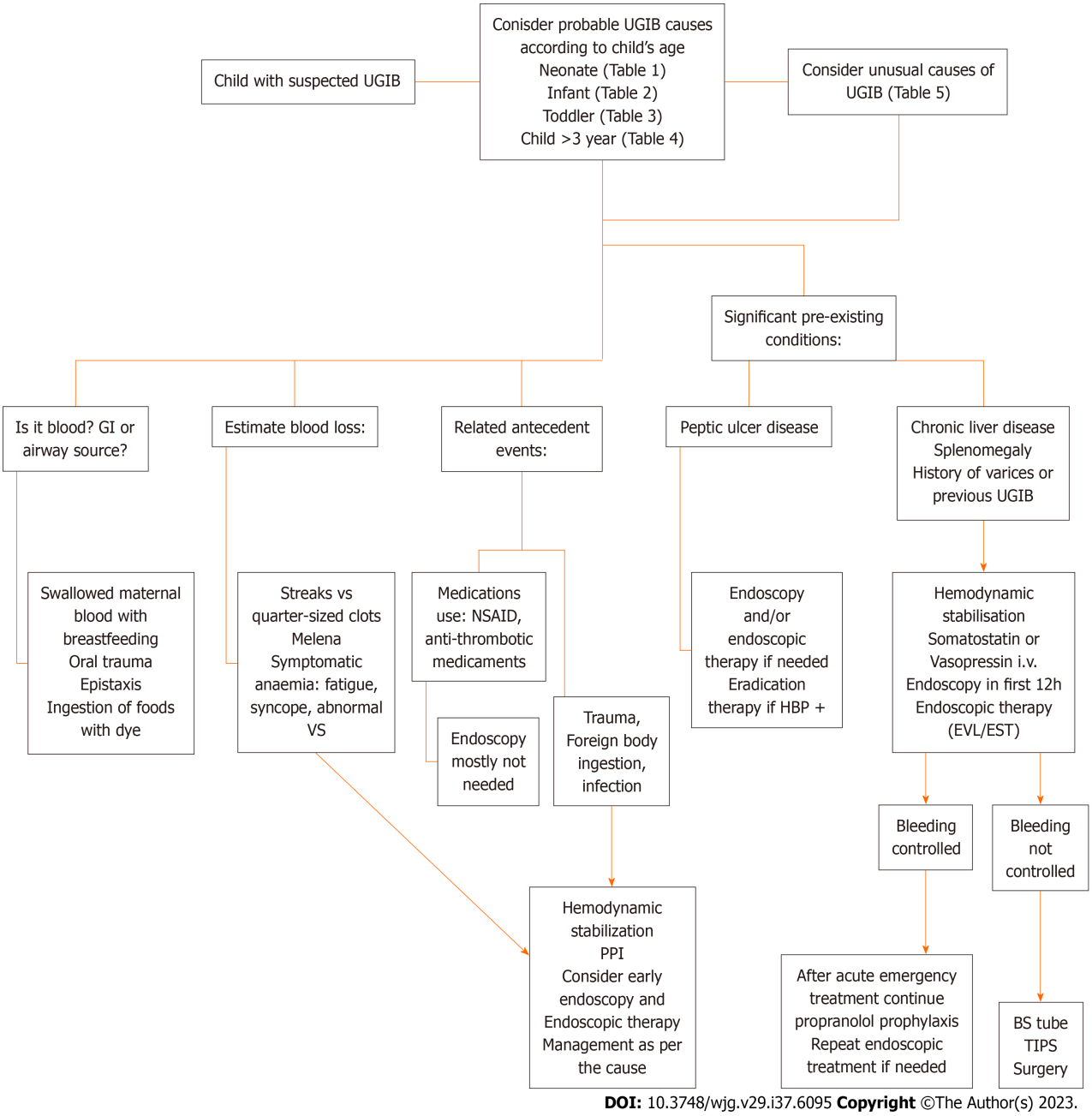
HSP is considered the most common type of vasculitis in children, affecting 9-22 children per 100000 annually[100]. Although over two-thirds of patients with HSP have GI symptoms during acute illness, these symptoms are usually transient. Most patients (90%) with HSP are children younger than 10 years, with a peak incidence in the 6th year of life [101]. However, it can be also diagnosed in infants, adolescents, and adults[100,102]. HSP has been shown to present with a milder course in infants and children younger than 2 years[102,103]. Authors from Taiwan retrospectively analyzed 158 children with HSP from 1987 to 1998. A total of 104 boys and 54 girls (male:female = 1.9:1; ranging in age from 2-13 years) were enrolled in that study. The main GI manifestations included abdominal pain (88%), GI bleeding (75%), and vomiting (25%). Hematemesis, leukocytosis (> 20000/cm^3), high C-reactive protein (> 50 mg/L), and hemorrhagic erosive duodenitis were found to correlate with prolonged hospitalization[104]. A retrospective study conducted in China from 2017 to 2019, analyzed 99 children who had GI bleeding as a complication of HSP. The age at onset ranged from 2-16 years, and the majority (72%) of patients were males. Among all patients with HSP and GI bleeding, 37 had hematemesis, 71 had hematochezia, and 9 had both symptoms. No significant difference was found in sex and age distributions between those who had mild and those who had severe bleeding (P > 0.05)[105]. Furthermore, authors from Italy conducted a retrospective study 1998-2002, which included 95 boys and 55 girls diagnosed with HSP, and concluded that males were affected more often than females (male:female = 1.8:1)[101]. The age and sex distributions showed that most patients (91%) were less than 10-years-old, which is in concordance with previous reports. Out of 77 (51%) children who had GI involvement, GI bleeding was present in 27 children (18%). Among these patients, GI bleeding was revealed by fecal occult blood test and/or melena in 23 (15%), while 3 (2%) had melena alone and 1 had hematemesis.

Obscure GI bleeding

Obscure GI bleeding is a type of bleeding that persists or recurs without an obvious etiology despite upper endoscopy and colonoscopy evaluation[24]. Since it is usually caused by small bowel lesions, it does not represent UGIB. Small bowel capsule endoscopy has a high diagnostic yield and safety in the investigation of obscure GI bleeding and it has been strongly recommended by the European Society of Gastrointestinal Endoscopy as the first-line investigation[106]. Nevertheless, some authors suggest that it may be appropriate to consider an endoscopic second look before performing a capsule endoscopy[107].

CONCLUSION

In the pediatric population, the etiology of UGIB is diverse and mostly age-related. Since there is a significant etiologic



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Figure 4 Clinical approach to children with upper gastrointestinal bleeding. BS: Blakemore-Sengstaken; EST: Endoscopic sclerotherapy; EVL: Esophageal varices ligation; GI: Gastrointestinal; PPI: Proton pump inhibitor; TIPS: Transjugular intrahepatic portosystemic shunt; UGIB: Upper gastrointestinal bleeding; VS: Vital signs; NSAIDs: Non-steroidal anti-inflammatory drugs.

overlap between the age groups, the differential diagnosis must take into account a wide variety of conditions, including rare causes. Even if the hemorrhage is non-life threatening, UGIB is distressing for children and their families. Therefore, all cases of GI bleeding require prompt and thorough investigation based on the use of existing diagnostic algorithms with the utmost goal being discernment of the origin of hemorrhage. Clinically significant UGIB is commonly caused by variceal bleeding in low- and middle-income countries, whereas non-variceal etiology is more common in high-income countries.

We emphasize that a properly taken anamnesis is very important for differentiating the true cause of bleeding from conditions mimicking hematemesis and melena. The information about dietary habits, medicaments use, other underlying diseases, and the possibility of foreign body ingestion are crucial leads to the right diagnosis. The identification of unusual causes of UGIB prevents delay in the diagnosis of rare and potentially treatable conditions. Finally, exact and timely recognition of the cause of UGIB in the pediatric population may significantly preclude morbidity and mortality. Therefore, we have created a comprehensive diagnostic-therapeutic flow-chart that, combined with the information provided in the Tables of this article, may help clinicians in the initial management of the child with UGIB (Figure 4).

Table 5 Unusual causes of upper gastrointestinal bleeding in children, based on age

Cause	Age of onset	Bleeding source location
Esophageal duplications[60,62]	All ages	Esophagus
Eosinophilic esophagitis[63]	Children ≥ 3 yr	Esophagus
Gastric duplications[65]	All ages	Stomach
Eosinophilic gastritis	Children ≥ 3 yr	Stomach
Gastric GIST[69]	Children ≥ 3 yr	Stomach
Lymphoma[70]	Children ≥ 3 yr	Stomach
Schwannomas[71,72]	Toddlers, children ≥ 3 yr	Stomach
Focal foveolar hyperplasia[73]	Infants	Stomach
Bezoar[77]	Children ≥ 3 yr	Stomach
Duplications of duodenum[56,60]	All ages	Duodenum
Duodenal varices[78]	Children ≥ 3 yr	Duodenum
Blunt duodenal trauma[81]	Children ≥ 3 yr	Duodenum
Food as a foreign body	Children ≥ 3 yr	Duodenum
Hemobilia[83,84]	All ages	Biliary tract
Dieulafoy's lesions[89-92]	All ages	Distinct locations
Peptic ulcers in ZES[94]	Children ≥ 3 yr	Distinct locations
Peutz-Jeghers syndrome[98]	Children ≥ 3 yr	Distinct locations
Crohn's disease[99]	Children ≥ 3 yr	Distinct locations
HSP[101,104,105]	Toddlers, children ≥ 3 yr	Distinct locations

GIST: Gastrointestinal stromal tumor; HSP: Henoch-Schönlein purpura; ZES: Zollinger-Ellison syndrome.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Cohort Study

Comparison of fecal calprotectin levels and endoscopic scores for predicting relapse in patients with ulcerative colitis in remission

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Abstract

BACKGROUND

Although the usefulness of endoscopic scores, such as the Mayo Endoscopic Subscore (MES), Ulcerative Colitis Endoscopic Index of Severity (UCEIS), and Ulcerative Colitis Colonoscopic Index of Severity (UCCIS), and biomarkers such as fecal calprotectin (FC) for predicting relapse in ulcerative colitis (UC) has been reported, few studies have included endoscopic scores for evaluating the entire colon.

AIM

To compare the usefulness of FC value and MES, UCEIS, and UCCIS for predicting relapse in patients with UC in clinical remission.

METHODS

In total, 75 patients with UC in clinical and endoscopic remission who visited our institution between February 2019 and March 2022 were enrolled. The diagnosis of UC was confirmed based on the clinical presentation, endoscopic findings, and histology, according to the current established criteria for UC. Fecal samples were collected the day before or after the colonoscopy for measurement of FC. Endoscopic evaluations were performed using MES, UCEIS, and UCCIS. The primary outcome measure of this study was the assessment of the association between relapse within 12 mo and MES, UCEIS, UCCIS, and FC. The secondary outcome was the comparison between endoscopic scores and biomarkers in en-rolled

patients with UC with mucosal healing.

RESULTS

FC and UCCIS showed a significant correlation with UCEIS (r = 0.537, P < 0.001 and r = 0.957, P < 0.001, respectively). Receiver-operating characteristic analysis for predicting MES 0 showed that the area under the curve of UCCIS was significantly higher than that of FC (P < 0.01). During the 1-year observation period, 18 (24%) patients experienced a relapse, and both the FC and UCCIS of the relapse group were significantly higher than that of the remission group. The cut-off values for predicting relapse were set at FC = 323 mg/kg and UCCIS = 10.2. The area under the curve of the receiver-operating characteristic analysis for predicting relapse did not show a significant difference between FC and UCCIS. The accuracy of the endoscopic scores and biomarkers in predicting relapse was 86.7% for UCCIS, 85.3% for UCEIS, 76.0% for FC, and 73.3% for MES.

CONCLUSION

The three endoscopic scores and FC may predict UC relapse during clinical remission. Among these scores, UCEIS may be the most useful in terms of ease of evaluation and accuracy.

Key Words: Ulcerative colitis; Mayo Endoscopic Subscore; Ulcerative Colitis Endoscopic Index of Severity; Ulcerative Colitis Colonoscopic Index of Severity; Fecal calprotectin; Relapse

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Core Tip: We evaluated the usefulness of fecal calprotectin and endoscopic scores, including the Mayo Endoscopic Subscore, Ulcerative Colitis Endoscopic Index of Severity (UCEIS), and Ulcerative Colitis Colonoscopic Index of Severity, in patients with ulcerative colitis (UC) in remission. All three endoscopic scores and fecal calprotectin are useful for predicting relapse in UC. The UCEIS is easy to evaluate and appears to be highly accurate in predicting relapse.

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INTRODUCTION

With the advances in treatment options for ulcerative colitis (UC), achieving mucosal healing has become a key therapeutic goal[1]. Mucosal healing is evaluated using endoscopic scores, such as the Mayo Endoscopic Subscore (MES) and Ulcerative Colitis Endoscopic Index of Severity (UCEIS)[2,3]. Although endoscopic examination is the most direct method for the evaluation of mucosal healing, frequent endoscopic examinations are not recommended owing to the associated costs and potential risks, along with the physical burden and psychological stress on the patient. Consequently, biomarkers are used as a method of evaluating mucosal status as an alternative to endoscopic examination[4]. Biomarkers such as fecal calprotectin (FC), immunological fecal occult blood test, and leucine-rich alpha-2 glycoprotein have been reported to be useful in UC[5-12]. Particularly, FC has shown a significant correlation with endoscopic scores and reflects mucosal activity in UC[5,6]. In addition, FC is widely used in clinical practice and often employed as a marker in large-scale clinical trials of new therapeutic agents to determine their therapeutic efficacy[13-15].

The endoscopic score can predict the prognosis of UC, with higher scores indicating higher rates of subsequent hospitalizations and surgeries[16-18]. A previous report on patients with UC with mucosal healing showing an MES of 1 or less showed that the subsequent relapse rate was significantly higher in the MES 1 group than in the MES 0 group[19]. Thus, while the endoscopic score has been shown to contribute to the prediction of subsequent relapse, biomarkers have also been identified as effective predictors[20-25]. Particularly, there are many reports on the prediction of relapse in UC using FC[20-24].

As previously mentioned, biomarkers reflect the endoscopic scores and contribute to the subsequent prediction of prognosis. In this study, we analyzed the relative efficacy of endoscopic scores against that of biomarkers in predicting relapse. Considering the possibility that this analysis may require a more detailed endoscopic score than just MES and UCEIS, we also incorporated the Ulcerative Colitis Colonoscopic Index of Severity (UCCIS), which provides a comprehensive assessment of the overall colorectal score[26,27].

MATERIALS AND METHODS

Patients and disease assessments

In total, 75 patients with UC in clinical remission who visited the Hamamatsu University School of Medicine between February 2019 and March 2022 were enrolled. These patients were diagnosed with UC based on their clinical presentation, endoscopic findings, and histology according to the current established criteria for UC[28]. Patients diagnosed with enteritis, including Crohn's disease and inflammatory bowel disease unclassified, were excluded.

In this study, the clinical activity of UC was evaluated using the clinical activity index (CAI) according to Rachmilewitz [29]. Endoscopic scores for UC were assessed using MES, UCEIS, and UCCIS[2,3,26]. MES was evaluated according to the following criteria: 0, normal or inactive disease; 1, mild disease with erythema, decreased vascular pattern, and mild friability; 2, moderate disease with marked erythema, absence of vascular patterns, friability, and erosions; and 3, severe disease with spontaneous bleeding and ulceration[2]. The UCEIS score was evaluated by calculating the sum of three descriptors: vascular pattern (score 0-2), erosions and ulcers (score 0-3), and bleeding (score 0-3)[3]. The UCCIS score was assessed using the following descriptors in the five segments of the ascending colon, transverse colon, descending colon, sigmoid colon, and rectum: vascular pattern (score 0-2), granularity (score 0-2), erosions and ulcers (score 0-4), and bleeding/friability (score 0-2). These descriptor scores were then applied to the following formula: UCCIS = 3.1 × sum (vascular pattern across five segments) + 3.6 × sum (granularity across five segments) + 3.5 × sum (ulceration across five segments) + 2.5 × sum (bleeding/friability across five segments)[26]. Clinical remission was defined as CAI 4 or less, and mucosal healing was defined as MES 0 or MES 1. Patients who met these criteria were included in this study.

Biomarker measurement

Fecal samples were collected in plastic tubes for FC measurement and stored at -20 ℃ until shipment to the laboratory (SRL Inc., Tokyo, Japan). The measurements were performed using a Phadia 250 Immunoassay Analyzer (HITACHI Ltd., Tokyo, Japan) and Elia A Calprotectin 2 reagent (Phadia GmbH, Freiburg, Germany) using fluorescence enzyme immunoassay principles. As colonoscopic preparation could influence the results of FC, fecal samples were collected the day before or after the colonoscopy.

Study design

This retrospective, single-center observational study aimed to evaluate whether MES, UCEIS, UCCIS, and FC serve as predictors of clinical relapse. The primary outcome measure was the assessment of the association between relapse within 12 mo and MES, UCEIS, UCCIS, and FC. The secondary outcome was the comparison between endoscopic scores and biomarkers in the enrolled patients with UC with mucosal healing.

Patients enrolled in this study made outpatient visits at intervals of 3 or more months. These patients were outpatients for more than 12 mo or until relapse. Clinical relapse was defined as an increase in CAI above baseline due to the worsening of diarrhea and abdominal pain or frequent or bloody stools requiring modification or addition of treatment. Changes in treatment were made at the discretion of each attending physician.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, N.Y., United States) and EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan)[30]. Differences were assessed using the Mann-Whitney U test or Student's *t*-test. Correlations were analyzed using Spearman's correlation coefficient. Receiver-operating characteristic (ROC) analysis was performed for endoscopy score and relapse prediction. The cumulative non-failure rate was evaluated using Kaplan-Meier analysis with the log-rank test. $P < 0.05$ was considered statistically significant.

Ethical statement

The study protocol was reviewed and approved by the ethics committee of Hamamatsu University School of Medicine (No. 20-322). This study was conducted in accordance with the Good Clinical Practice principles in adherence to the Declaration of Helsinki.

RESULTS

Patient characteristics

In total, 75 patients with UC were enrolled in this study. The baseline patient characteristics are shown in Table 1. The median patient age and disease duration were 49 years and 8 years, respectively. A total of 43 patients had an MES of 0, and 32 had an MES of 1. UCEIS scores ranged from 0 to 3, and the median UCCIS and FC values were 0 and 174 mg/kg, respectively.

Association between FC and endoscopic score

First, the association between endoscopic score and FC was assessed in enrolled patients with UC with MES 0 and 1. FC and UCCIS were significantly higher in the MES 1 group than in the MES 0 group ($P < 0.001$ and $P < 0.001$, respectively; Figure 1A and B). Both FC and UCCIS showed a significant correlation with UCEIS ($r = 0.537$, $P < 0.001$ and $r = 0.957$, $P < 0.001$, respectively; Figure 1C and D). A significant correlation was also observed between FC and UCCIS ($r = 0.506$, $P <$

Table 1 Baseline patient characteristics

Characteristic	All, *n* = 75
Age in yr, median [IQR]	49 [36, 62]
Male/Female, *n* (%)	45 (60.0)/30 (40.0)
Disease duration in yr, median [IQR]	8 [5, 13]
Disease extent, *n* (%)	
Extensive colitis	45 (60.0)
Left-sided colitis	24 (32.0)
Proctitis	6 (8.0)
CAI by the Rachmilewitz index, median [IQR]	0 [0, 1]
MES, *n* (%)	
MES 0	43 (57.3)
MES 1	32 (42.7)
UCEIS, *n* (%)	
UCEIS 0	39 (52.0)
UCEIS 1	17 (22.7)
UCEIS 2	13 (17.3)
UCEIS 3	6 (8.0)
UCCIS, median [IQR]	0 [0, 6.7]
FC in mg/kg, median [IQR]	174 [43, 810]
Medication used during the study, *n* (%)	
Oral 5-ASA	48 (64.0)
Suppository steroids	2 (2.7)
Systemic steroids	9 (12.0)
Immunomodulators	23 (30.7)
Biologics	30 (40.0)

5-ASA: 5-aminosalicylic acid; CAI: Clinical activity index; FC: Fecal calprotectin; IQR: Interquartile range; MES: Mayo Endoscopic Subscore; UCCIS: Ulcerative Colitis Colonoscopic Index of Severity; UCEIS: Ulcerative Colitis Endoscopic Index of Severity.

0.001; Figure 1E). ROC analysis to predict MES 0 showed cut-off values of FC 385 mg/kg and UCCIS 6.6, with an area under the curve (AUC) of 0.858 [95% confidence interval (CI): 0.770-0.946] and 0.987 (95%CI: 0.969-1.000; Table 2). The AUC of UCCIS was significantly higher than that of FC ($P < 0.001$; Figure 2).

Association between FC and endoscopic scores, and clinical relapse

In total, 18 (24.0%) patients experienced clinical relapse during the 1-year follow-up period. The baseline FC and UCCIS values were significantly higher in the relapse group than in the remission group ($P < 0.001$ and $P < 0.001$, respectively; Figure 3A and B). In the ROC analysis for predicting clinical relapse, the cut-off value for FC was 323 mg/kg, and the AUC was 0.813 (95%CI: 0.698-0.927; Figure 3C). The cut-off value for UCCIS was 10.2, and the AUC was 0.823 (95%CI: 0.697-0.949), with no significant difference (Figure 3C).

Kaplan-Meier analysis of remission rate grouped by cut-off value

Kaplan-Meier analysis was used to assess the remission maintenance rate by grouping by each endoscopic score and cut-off value. When the endoscopic score was grouped by MES 0 and 1 and UCEIS ≤ 1 and ≥ 2, a significant difference was observed in the log-rank test ($P < 0.001$ and $P < 0.001$, respectively; Figure 4A and B). The analysis also revealed significant differences between the FC < 323 and FC ≥ 323 groups and UCCIS < 10.2 and UCCIS ≥ 10.2 groups using the log-rank test ($P < 0.001$ and $P < 0.001$, respectively; Figure 4C and D). Regarding the accuracy of relapse prediction, UCCIS had the highest accuracy at 86.7%, followed by UCEIS at 85.3% (Table 3). The accuracies of FC and MES were 76.0% and 73.3%, respectively.

Table 2 Receiver-operating characteristic analysis of fecal calprotectin and Ulcerative Colitis Colonic Index of Severity for predicting Mayo Endoscopic Subscore 0

Factor	FC	UCCIS
Cut-off value	385	6.6
AUC (95%CI)	0.858 (0.770-0.946)	0.987 (0.969-1.000)
PPV	0.793	0.992
NPV	0.804	0.994
Sensitivity	0.719	0.992
Specificity	0.804	0.985
Accuracy	0.800	0.947

AUC: Area under the curve; CI: Confidence interval; FC: Fecal calprotectin; NPV: Negative predictive value; PPV: Positive predictive value; UCCIS: Ulcerative Colitis Colonoscopic Index of Severity.

Table 3 Comparison of accuracy of relapse prediction between fecal calprotectin levels, Ulcerative Colitis Colonoscopic Index of Severity, Mayo Endoscopic Subscore, and Ulcerative Colitis Endoscopic Index of Severity

Factor	Sensitivity	Specificity	PPV	NPV	Accuracy
FC ≥ 323	0.500	0.933	0.833	0.737	0.760
UCCIS ≥ 10.2	0.750	0.898	0.667	0.930	0.867
MES 1	0.833	0.702	0.469	0.930	0.733
UCEIS ≥ 2	0.722	0.895	0.684	0.911	0.853

FC: Fecal calprotectin; MES: Mayo Endoscopic Subscore; NPV: Negative predictive value; PPV: Positive predictive value; UCCIS: Ulcerative Colitis Colonoscopic Index of Severity; UCEIS: Ulcerative Colitis Endoscopic Index of Severity.

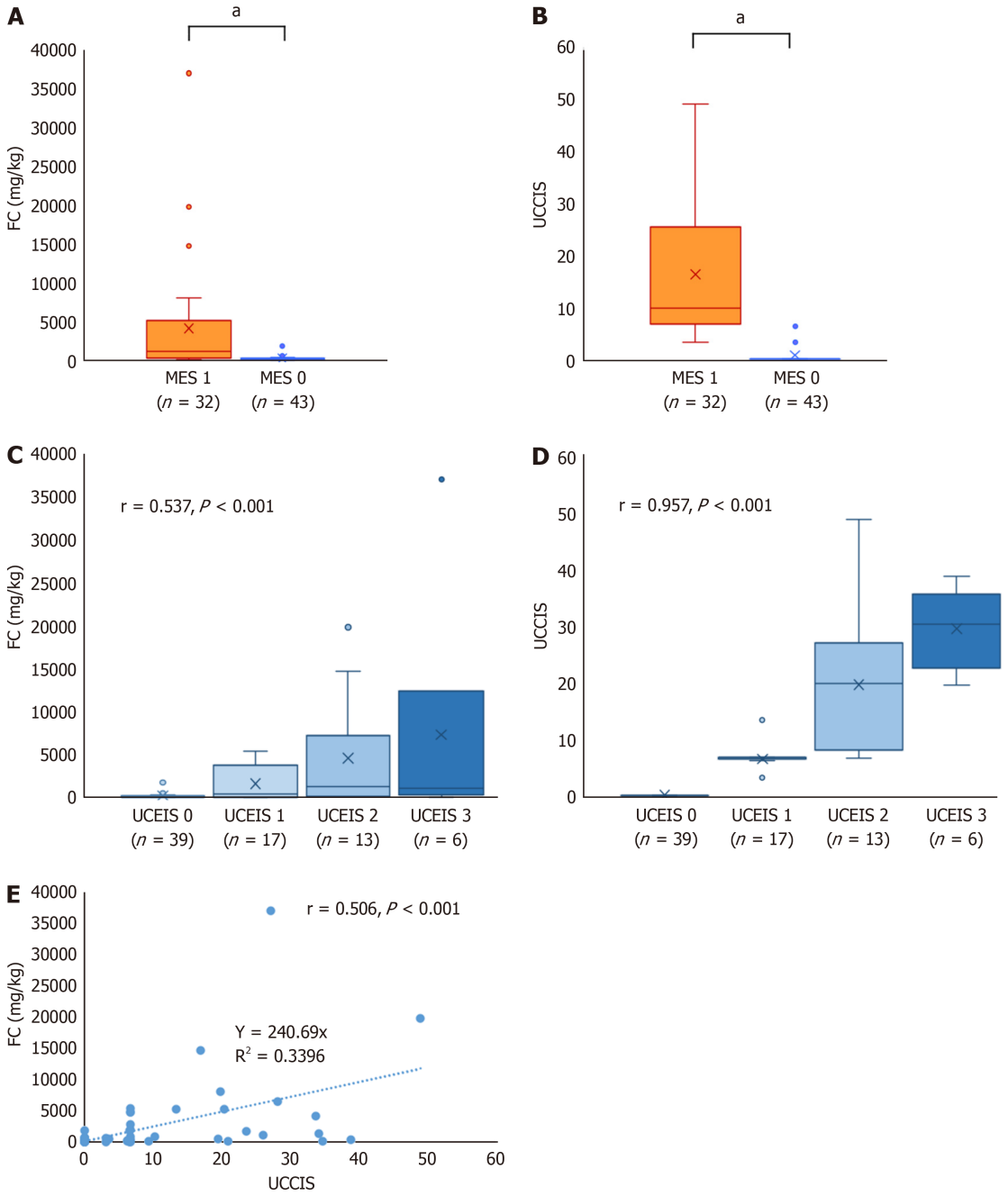
DISCUSSION

This study showed that FC, MES, UCEIS, and UCCIS are useful for predicting relapse in patients with UC in clinical remission. Endoscopic and biomarker assessment must be used in current clinical practice for UC, in which achievement of mucosal healing is the goal of treatment because endoscopic scores and biomarkers have been reported to contribute to subsequent prognosis in patients with UC[16-25]. MES, a simple endoscopic score, is often used in large-scale clinical trials and real-world clinical practice. Although the simplicity of MES makes it easy to use, it cannot be used for detailed scoring[2]. On the other hand, UCEIS, which evaluates vascular, bleeding, and erosion/ulcer patterns, is capable of providing a more detailed evaluation compared to MES[3]. However, the assessment of MES and UCEIS is performed on the most active lesions, located in the sigmoid colon or rectum, thus only assessing localized areas. There are several reports on endoscopic scores that evaluate the activity of the entire colon. UCCIS, like UCEIS, is calculated by scoring each item and substituting those scores into the formula[26]. Although UCCIS evaluates the entire colon, its complexity of scoring poses considerable challenges.

Biomarkers quantify activity and enable detailed evaluation of inflammation[21]. In Japan, endoscopic examination and biomarker measurements cannot be performed in the same month. As previously mentioned, each endoscopic score and biomarker has its own advantages and disadvantages. To the best of our knowledge, no studies have yet compared the abilities of MES, UCEIS, UCCIS, and FC, a representative biomarker, to predict relapse.

In this study, we investigated the prediction of relapse and evaluated the relationship between FC, UCEIS, and UCCIS in patients with a mucosal healing score of MES 1 or less. A few reports on biomarkers have evaluated the association between biomarkers and endoscopic scores in the entire severity range of MES, from 0 to 3. Guardiola *et al*[31] reported that FC is useful for evaluating UC activity, including histological evaluation, in patients with UC who are in clinical and endoscopic remission. Previously, we reported a significant correlation between FC and UCCIS in UC with an MES ≤ 1 (r = 0.653, P < 0.001)[32]. In that study, FC showed a significant correlation with UCEIS and UCCIS, indicating that FC is a sensitive biomarker that reflects endoscopic activity even among patients who have achieved mucosal healing.

Regarding the prediction of relapse, which is the main purpose of this study, it was found that FC, MES, UCEIS, and UCCIS are all useful for predicting relapse within 1 year. Several reports on the prediction of recurrence using endoscopic scores have shown that MES 1 was associated with a significantly higher risk of relapse compared to MES 0 and MES 1 [19]. We have also previously shown the usefulness of MES for relapse prediction in the analysis that examined the relapse prediction ability of fecal occult blood test[33]. Conversely, Yamamoto *et al*[34] reported that a similar analysis did not show a significant difference in predicting 1-year relapse, suggesting that the relapse prediction ability of MES is

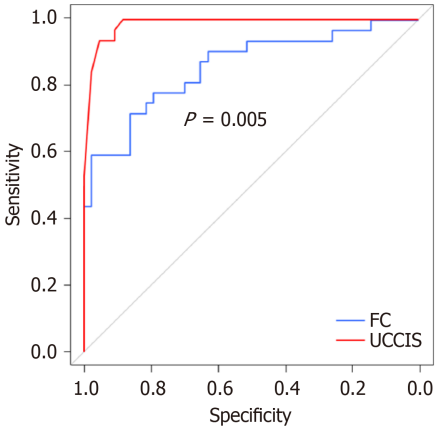


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Figure 1 Association between fecal calprotectin and endoscopic scores. A: Differences in fecal calprotectin (FC) levels between Mayo Endoscopic Subscore (MES) groups; B: Differences in the Ulcerative Colitis Colonic Index of Severity (UCCIS); C: Correlation with the Ulcerative Colitis Endoscopic Index of Severity; D: Relationship between FC and UCCIS; E: Correlation between FC and UCCIS. [a]*P* < 0.001.

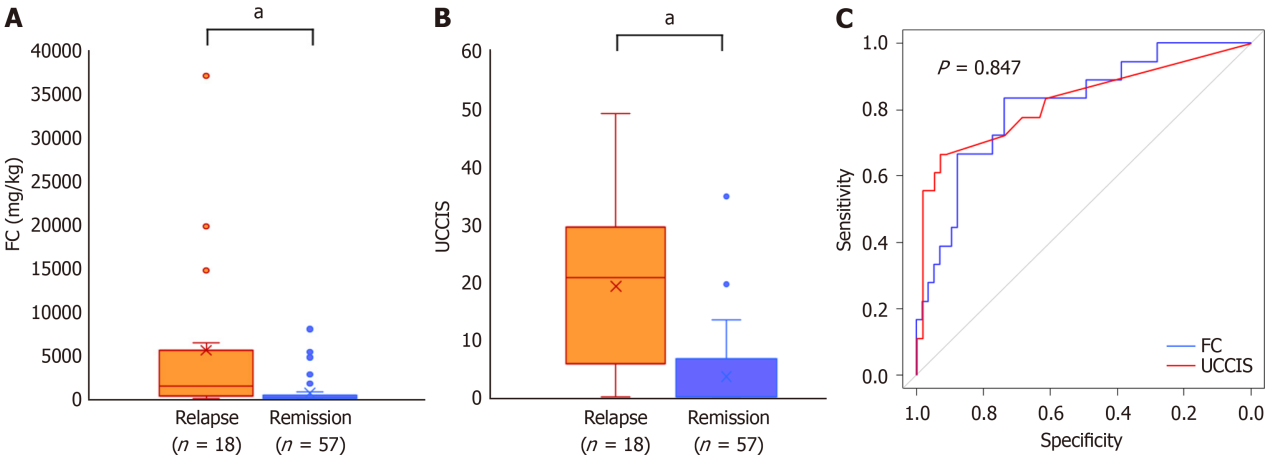
controversial. Arai *et al*[35] examined relapse prediction using UCEIS and reported that UCEIS is useful in mid- to long-term relapse prediction. We previously reported recurrence prediction using UCCIS, and the analysis was performed over a long-term observation period of 2 years and 5 years[36].

The cut-off of UCEIS in this study was set at 2, and the analysis was performed accordingly. This was because other UCEIS scores were also grouped and analyzed; however, the analysis grouped by scores of 2 or more and 1 or less showed the most accurate results. Arai *et al*[35] also reported that grouping based on a UCEIS cut-off of 2 or higher and 1 or lower was useful, and the cut-off value of UCEIS 2 was considered to be valid. Moreover, we did not perform multivariate analysis because UCEIS and UCCIS have a strong correlation close to 1, and including both these variables would have rendered the statistical analysis inconsequential. Instead, we examined the sensitivity, specificity, positive predictive values, negative predictive values, and accuracy. Regarding accuracy, both UCEIS and UCCIS exhibited an accuracy of 80% or more and were considered to be useful scores for predicting relapse. However, the UCCIS is an extremely complicated scoring system in which four items are evaluated across five colonic segments, and the scores are substituted into a formula. Therefore, it is not realistic to use this score in clinical practice. Hence, the UCEIS emerges as a preferable endoscopic scoring system in predicting relapse, owing to its accuracy and ease of use in clinical practice.



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Figure 2 Receiver-operating characteristic analysis of fecal calprotectin and Ulcerative Colitis Colonic Index of Severity for predicting Mayo Endoscopic Subscore 0. FC: Fecal calprotectin; UCCIS: Ulcerative Colitis Colonic Index of Severity.



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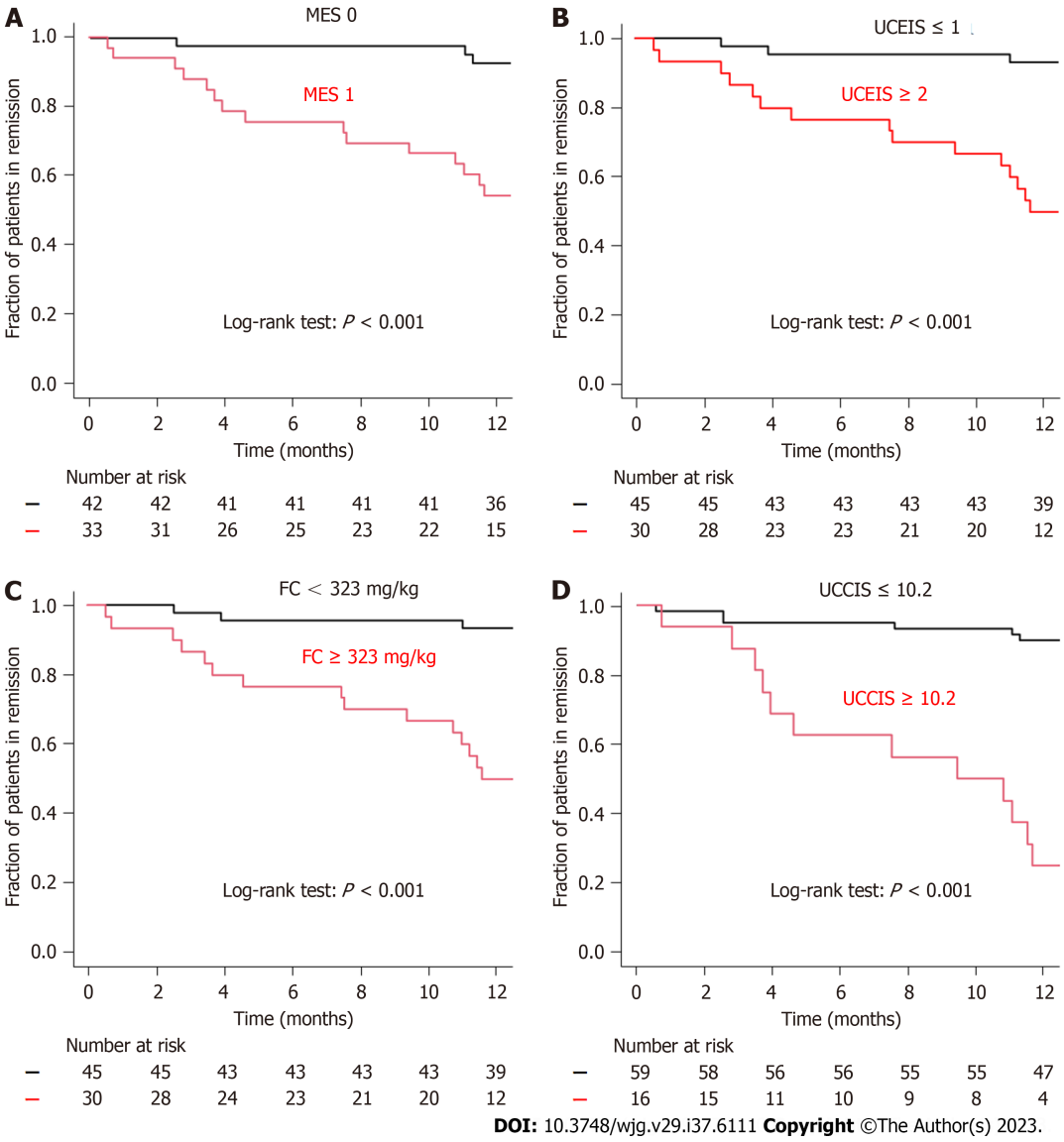
Figure 3 Differences between fecal calprotectin and Ulcerative Colitis Colonic Index of Severity in terms of predicting relapse and the receiver-operating characteristic analysis for predicting relapse. A: Difference in fecal calprotectin (FC) levels between the relapse and remission groups; B: Difference in Ulcerative Colitis Colonic Index of Severity (UCCIS) between the relapse and remission groups; C: Receiver-operating characteristic analysis of FC and UCCIS for predicting relapse within 1 year. [a]$P < 0.001$. FC: Fecal calprotectin; UCCIS: Ulcerative colitis colonic index of severity.

Intensifying treatment based on the UCEIS score in real-world clinical practice could help prevent relapse; hence, further prospective studies in this regard are desired.

The strength of this study is that endoscopic examination and biomarker measurements were performed simultaneously. However, currently, biomarkers and endoscopic measurements cannot be performed together in clinical practice. Nevertheless, several limitations to this study must be acknowledged. First, it was a single-center retrospective analysis conducted in a small number of patients. Second, our results were not compared with other biomarkers, such as leucine-rich alpha-2 glycoprotein; histological findings were also not considered. Third, biomarker and endoscopic evaluations were not performed at the time of relapse.

CONCLUSION

In conclusion, MES, UCEIS, UCCIS, and FC were useful for predicting relapse in patients with UC in clinical remission. Among the three endoscopic scores evaluated, UCEIS may be the most useful in terms of ease of evaluation and predictive accuracy.



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Figure 4 Kaplan-Meier analysis of relapse-free rates. A: Mayo Endoscopic Subscore; B: Ulcerative Colitis Endoscopic Index of Severity; C: Fecal calprotectin levels; D: Ulcerative Colitis Colonic Index of Severity. FC: Fecal calprotectin; MES: Mayo endoscopic subscore; UCCIS: Ulcerative colitis colonic index of severity; UCEIS: Ulcerative colitis endoscopic index of severity.

ARTICLE HIGHLIGHTS

Research background

The goal of ulcerative colitis (UC) treatment is to achieve mucosal healing, for which endoscopic evaluation is recommended. To avoid endoscopy, fecal calprotectin (FC), which may be an alternative biomarker for UC, was reported to be useful in evaluating patients. Although endoscopic scores and FC, in addition to traditional biomarkers and the Mayo Endoscopic Subscore (MES), are useful for predicting relapse in patients with UC in remission, no studies have compared the predictive abilities of the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) and the Ulcerative Colitis Colonoscopic Index of Severity (UCCIS), which assesses the entire colon.

Research motivation

To evaluate whether FC and MES, UCEIS, and UCCIS are useful for predicting relapse in patients with UC in clinical remission.

Research objectives

Overall, 75 patients with UC in clinical remission, with a clinical activity index (CAI) according to Rachmilewitz score was ≤ 4, underwent colonoscopic examination and FC measurements.

Research methods

We assessed whether the enrolled patients experienced UC relapse within 12 mo after endoscopic examination and FC

measurement. Clinical relapse was defined as an increase in CAI above baseline due to worsening of diarrhea and abdominal pain or frequent or bloody stools, requiring modification or addition of treatment. We also evaluated the association between endoscopic scores and FC.

Research results

Cut-off values and areas under the curve (AUC) for FC and UCCIS in the receiver-operating characteristic analysis to predict clinical relapse were 323 mg/kg, 0.813 [95% confidence interval (CI)]: 0.698-0.927], and 10.2, for FC, AUC, and UCCIS, respectively.

The AUC was 0.823 (95%CI: 0.697-0.949). Univariate analysis was performed using these cut-off values (FC < 323 mg/kg *vs* ≥ 323 mg/kg; UCCIS < 10.2 *vs* ≥ 10.2; MES 0 *vs* 1; and UCEIS ≤ 1 *vs* ≥ 2). The accuracy of relapse prediction was the highest with UCCIS, followed by UCIES, FC, and MES.

Research conclusions

MES, UCEIS, UCCIS, and FC were useful for predicting relapse in patients with UC in clinical remission.

Research perspectives

UCCIS comprehensively evaluates the endoscopic activity of UC, helping to predict its relapse. However, its complexity poses a challenge. Among the three endoscopic scores, UCEIS may be the most useful in terms of ease of evaluation and accuracy.

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FOOTNOTES

Author contributions: Ishida N made the concept of this study; Ishida N and Sugimoto K designed the study; Ishida N, Ito T, Takahashi K, Asai Y, Miyazu T, Higuchi T, Tamura S and Tani S collected the data; Yamade M, Iwaizumi M, and Hamaya Y analyzed the data; Ishida N and Sugimoto K wrote the article; and Hamaya Y and Osawa S provided critical insights regarding article preparation.

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Informed consent statement: Informed consent from patients was obtained in the form of an opt-out form on the hospital website.

Conflict-of-interest statement: The authors have no conflicts of interest related to the manuscript.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Impact of guideline adherence on the prognosis of Barcelona clinic liver cancer stage B hepatocellular carcinoma

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Abstract

BACKGROUND

Patients with Barcelona clinic liver cancer (BCLC) stage B hepatocellular carcinoma (HCC) are considerably heterogeneous in terms of tumor burden, liver function, and performance status. To improve the poor survival outcomes of these patients, treatment approaches other than transarterial chemoembolization (TACE), which is recommended by HCC guidelines, have been adopted in real-world clinical practice. We hypothesize that this non-adherence to treatment guidelines, particularly with respect to the use of liver resection, improves survival in patients with stage B HCC.

AIM

To assess guideline adherence in South Korean patients with stage B HCC and study its impact on survival.

METHODS

A retrospective analysis was conducted using data from 2008 to 2016 obtained from the Korea Central Cancer Registry. Patients with stage B HCC were categorized into three treatment groups, guideline-adherent, upward, and downward, based on HCC guidelines recommended by the Asian Pacific Association for the Study of the Liver (APASL), the European Association for the Study of the Liver (EASL), and the American Association for the Study of Liver Diseases (AASLD). The primary outcome was HCC-related deaths; tumor recurrence served as the secondary outcome. Survival among the groups was compared using the Kaplan-Meier method and the log-rank test. Predictors of survival outcomes were identified using multivariable Cox regression analysis.

RESULTS

In South Korea, over the study period from 2008 to 2016, a notable trend was observed in adherence to HCC guidelines. Adherence to the EASL guidelines started relatively high, ranging from 77% to 80% between 2008 and 2012, but it gradually declined to 58.8% to 71.6% from 2013 to 2016. Adherence to the AASLD guidelines began at 71.7% to 75.9% from 2008 to 2010, and then it fluctuated between 49.2% and 73.8% from 2011 to 2016. In contrast, adherence to the APASL guidelines remained consistently high, staying within the range of 90.14% to 94.5% throughout the entire study period. Upward treatment, for example with liver resection, liver transplantation, or radiofrequency ablation, significantly improved the survival of patients with BCLC stage B HCC compared to that of patients treated in adherence to the guidelines (for patients analyzed according to the 2000 EASL guidelines, the 5-year survival rates were 63.4% *vs* 27.2%, $P < 0.001$), although results varied depending on the guidelines. Progression-free survival rates were also significantly improved upon the use of upward treatments in certain groups. Patients receiving upward treatments were typically < 70 years old, had platelet counts > 10^5/μL, and serum albumin levels ≥ 3.5 g/dL.

CONCLUSION

Adherence to guidelines significantly influences survival in South Korean stage B HCC patients. Curative treatments outperform TACE, but liver resection should be selected with caution due to disease heterogeneity.

Key Words: Hepatocellular carcinoma; Barcelona clinic liver cancer stage B; Guideline adherence; Liver neoplasms; Transarterial chemoembolization; Liver resection

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Core Tip: The current hepatocellular carcinoma (HCC) guidelines do not recommend curative treatments, except liver transplantation, for patients with Barcelona clinic liver cancer stage B HCC. Our study suggests survival benefits for selected patients aged < 70 years, with platelet counts > 10^5/μL and albumin levels ≥ 3.5 g/dL, even if the liver function corresponds to Child-Pugh score B7, beyond the Milan criteria and outside the up-to-7 criteria. As for the B2 group of the Kinki criteria, which presents a highly diverse population of patients with stage B HCC, curative strategies should be considered with caution through a multidisciplinary approach.

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INTRODUCTION

Hepatocellular carcinoma (HCC), the most common type of primary liver cancer and the second leading cause of cancer mortality, is a significant worldwide public health issue. In 2020, liver cancer was the second most common cause of premature death from cancer among persons aged 30 to 69 years, even in high-income countries[1]. In South Korea, HCC has the second highest mortality rate across all age groups and places a heavy burden on the working-age population, with considerable economic consequences[2].

To ensure effective management and treatment of HCC, various international guidelines have been drawn up, including those from the Asian Pacific Association for the Study of the Liver (APASL), the European Association for the Study of the Liver (EASL), and the American Association for the Study of Liver Diseases (AASLD)[3-7]. The AASLD and EASL guidelines are based on the Barcelona Clinic Liver Cancer (BCLC) staging system, which considers factors such as tumor characteristics (number, size, vascular invasion, and extrahepatic localization), liver function [Child-Pugh score (CPS)], and performance status (PS) as defined by the Eastern Cooperative Oncology Group scale to determine appropriate treatment options and predict patient prognosis. Patients with stage B, which typically includes those with multinodular tumors, a CPS of A or B, a PS of 0, and no vascular invasion or extrahepatic spread exhibits extreme heterogeneity with tumor size and number, liver function and PS. It encompasses patients with single tumors larger than 5 cm and those with multiple tumors, leading to differences in tumor burden. Varying degree of impairment in liver function and PS, and preference introduce additional diversity in treatment approaches.

The BCLC staging system strongly recommends transarterial chemoembolization (TACE) for patients with stage B HCC. However, in East-Asia there is a notable deviation from this recommendation, with liver resection being considered a viable treatment option for patients with stage B HCC. Nonrandomized controlled trials performed in East Asian populations have revealed that around half of patients with stage B HCC undergo TACE, while an equal proportion receive liver resection. After sensitivity analysis, liver resection demonstrated superior survival outcomes to TACE for patients with stage B HCC[8]. This reveals the potential benefits of adopting non-adherent treatment modalities to improve the prognosis of patients with stage B HCC. Consequently, HCC guidelines have continuously evolved in

response to global clinical evidences[9-13].

The Korea Central Cancer Registry (KCCR), established in 1980 by the Ministry of Health and Welfare, is a hospital-based nationwide cancer registry. Its primary goal is to accurately record cancer incidence in South Korea, facilitating cancer research and treatment planning through the development of a comprehensive cancer database. Each year, all newly diagnosed cancer patients are registered within this system[14].

This study aims to evaluate the adherence to each set of HCC guidelines (EASL, AASLD, and APASL) in South Korea between 2008 and 2016, using data from the KCCR. Additionally, we aim to assess the impact of non-adherence to guidelines on the survival outcomes of patients with stage B HCC. By identifying specific patient subgroups that benefit from treatment that deviates from the guidelines, this study could significantly contribute to the refinement of guidelines to allow improved real-world management of patients with stage B HCC.

MATERIALS AND METHODS

Study population and study outcomes

This was a retrospective multicenter cohort study that included 13838 treatment-naïve patients with HCC registered in the KCCR from 2008 to 2016 and followed up until December 2019. The diagnosis of HCC was made based on pathological findings of surgical specimens or liver biopsies, or radiologic findings of liver dynamic computed tomography or magnetic resonance imaging. Stage B HCC was defined as multinodular tumors with a CPS of A or B, PS of 0, and no vascular invasion or cancer-related symptoms, in accordance with the BCLC staging system. A total of 650 patients with BCLC stage B HCC were selected and divided into three groups based on compliance with the EASL (2000, 2012), AASLD (2005, 2010), and APASL (2010) guidelines[3-7], guideline-adherent, upward, and downward treatment, excluding 1298 patients with critical missing value (Figure 1).

The primary endpoint was HCC-related death, and the secondary endpoint was tumor recurrence after the initial HCC treatment. HCC-related survival was measured from the date of the first treatment until HCC-related death or the last follow-up. Progression-free survival (PFS) was measured from the date of the first treatment to the date of the second treatment. Tumor recurrence was determined when the period between consecutive treatments was longer than one month.

Definition of guideline adherence

Guideline adherence was defined differently for each guideline based on the grades of evidence and recommendations (Supplementary Table 1)[3-7,15]. Among non-adherent treatments, upward treatment referred to more aggressive or curative treatments than those recommended in the BCLC staging system or updated treatments with proven efficacy. Downward treatment referred to moving from left to right in the BCLC staging system or treatments under clinical trials with no proven efficacy. All guidelines recommended TACE as standard therapy for unresectable, large, or multifocal stage B HCC. The APASL guidelines state that liver resection can be considered if HCC is confined to the liver, anatomically resectable, and satisfactory liver function reserve is present.

Anthropometric and laboratory evaluation

Data on the anthropometric parameters age, sex, body mass index (BMI, kg/m^2), etiology (hepatitis B or C, alcohol consumption), presence of diabetes mellitus and hypertension, ascites, CPS, and Mayo End-Stage Liver Disease (MELD) score were collected. Levels of serum creatinine, sodium, and alanine aminotransferase (ALT), platelet counts, serum albumin levels, total bilirubin levels, and international normalized ratio (INR) were recorded as laboratory parameters. Data on tumor number, maximum tumor diameter, and alpha-fetoprotein (AFP) levels were collected as tumor factors. All laboratory parameters were measured using a conventional automated analyzer.

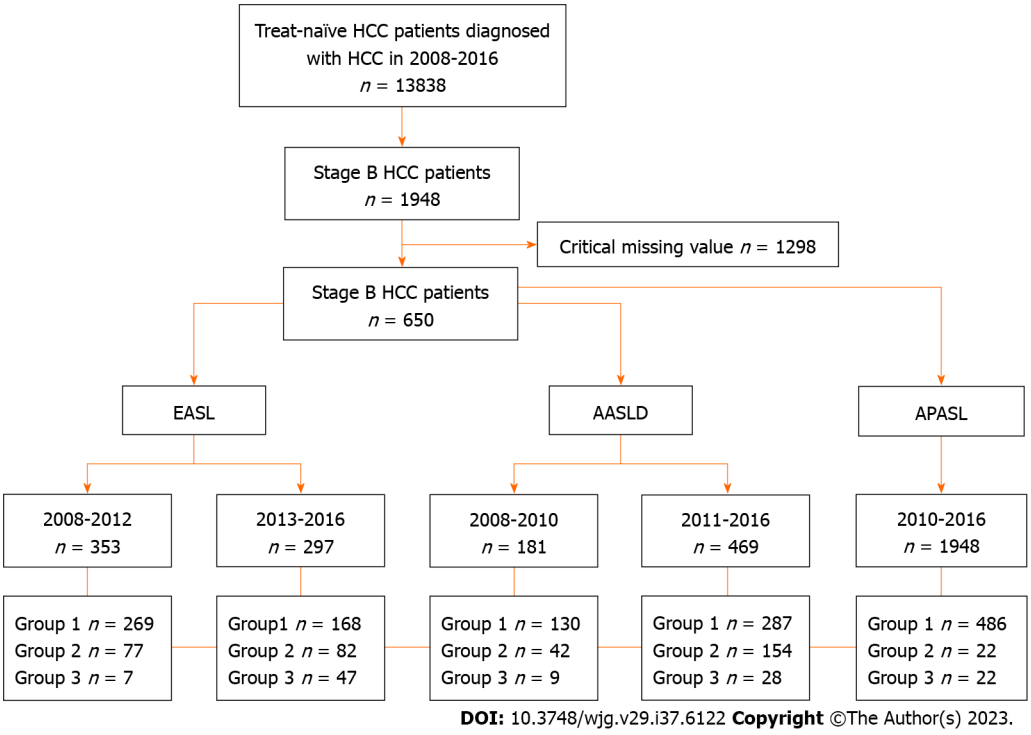
Statistical analysis

All statistical analyses were performed using SAS software version 9.4 (SAS Institute Inc., Cary, NC, United States). Continuous variables with normal distribution (age, BMI, CPS, MELD score, serum creatinine, sodium, ALT, platelets, serum albumin, total bilirubin, INR, tumor number, maximum tumor diameter, and AFP) are expressed as mean ± SD. The χ^2-test with Fisher's exact test was used to compare categorical variables (sex, etiology, and ascites). HCC-related survival and PFS was compared using the Kaplan-Meier method with the log-rank test. Univariate Cox regression analysis was performed and multivariate Cox regression analysis was conducted using selected variables sorted through stepwise selection to identify reliable predictors of survival in patients with stage B HCC. The modified Bolondi or Kinki subclassification system was used to categorize patients based on liver function and tumor status as follows: B1 (CPS of 5-7 and within up-to-7), B2 (CPS of 5-7 and beyond up-to-7), and B3 (CPS of 8, 9, and any tumor status) (Table 1)[16,17]. Propensity score matching (PSM) analysis was performed for variables, such as age, etiology, platelet count, serum albumin level, tumor burden, and MELD score to balance differences of baseline characteristics between patients who underwent liver resection and TACE during the subgroup analysis based on Kinki criteria. The results are presented as hazard ratios (HRs) with 95% confidence intervals (95%CIs). Statistical significance was set at $P < 0.05$.

Table 1 Modified Bolondi or Kinki subclassification system

Subclassification	B1	B2	B3	
Child-Pugh score	5-7	5-7	8, 9	
'Beyond Milan' and within 'up to 7 criteria'	In	Out	Any	
			In	Out
Concept of treatment strategy	Curative	Non-curative	Curative intent if within up-to-7 criteria	Palliative, no treatment
Treatment option	Resection	TACE with DC beads	Transplantation	HAIC
	RFA	HAIC	RFA	Superselective TACE with DC beads
	Superselective cTACE	Sorafenib	Superselective cTACE	

RFA: Radiofrequency ablation; cTACE: Conventional transarterial chemoembolization; TACE: Transarterial chemoembolization; DC: Drug-eluting; HAIC: Hepatic arterial infusion chemotherapy.



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Figure 1 Flow chart of classification according to hepatocellular carcinoma guidelines. Group 1: Guideline-adherent group; Group 2: Upward treatment group; Group 3: Downward treatment group. AASLD: American Association for the Study of Liver Diseases; APASL: Asian Pacific Association for the Study of the Liver; EASL: European Association for the Study of the Liver; HCC: Hepatocellular carcinoma.

RESULTS

Baseline characteristics and distribution of treatment strategies according to each HCC guideline

The baseline characteristics of the patients treated in accordance with the EASL, AASLD, and APASL guidelines, are detailed in Tables 2 and 3, and Supplementary Table 2. Of the 353 patients analyzed according to the 2000 EASL guidelines, 76.2% received guideline-compliant treatment, and 21.8% received upward treatment. Of the patients analyzed according to the 2012 EASL guidelines, 27.6% received upward treatment; the seven patients who received downward treatment were excluded from the analysis due to low sample size. Patients in the upward treatment group, compared to guideline-adherent patients, had a younger average age (57.5 *vs* 60.7 years) and lower rates of diabetes (13.0% *vs* 29.4%). They also had lower ALT levels, CPS, MELD scores, and tumor numbers, as well as higher sodium levels, platelet counts, and serum albumin levels. Of the patients assessed according to the 2005 AASLD guidelines, nine downward treated patients were excluded due to the low sample size, and the 26.1% of patients who received upward treatment had fewer tumors compared with guideline-adherent patients. Of the patients analyzed according to the 2010

Table 2 Baseline characteristics of patients with hepatocellular carcinoma between 2008 and 2016 according to the 2000 and 2012 European Association for the Study of the Liver guidelines

Variables	2000 EASL guidelines (HCC patients, 2008-2012)			2012 EASL guidelines (HCC patients, 2013-2016)			
	Guideline-adherent	Upward treatment	*P* value	Guideline-adherent	Upward treatment	Downward treatment	*P* value
No. of patients	269.0	77.0		168.0	82.0	47.0	
Age (yr)	60.7 ± 10.4	57.5 ± 10.2	0.017	62.6 ± 10.5	60.9 ± 9.7	64.8 ± 9.0	0.100
Male sex (*n*, %)	226 (84.0)	68 (88.3)	0.469	146 (86.9)	70 (85.4)	40 (85.1)	0.921
BMI (kg/m^2)	24.5 ± 4.3	24.6 ± 3.1	0.919	23.8 ± 3.3	23.6 ± 4.2	23.2 ± 3.4	0.626
DM (*n*, %)	79 (29.4)	10 (13.0)	0.004	51 (30.4)	24 (29.3)	14 (29.8)	0.984
Hypertension (*n*, %)	98 (36.4)	28 (38.4)	0.991	58 (34.5)	40 (48.8)	19 (40.4)	0.095
Etiology							
Hepatitis B (*n*, %)	166 (61.7)	51 (66.2)	0.469	99 (58.9)	51 (62.2)	27 (57.4)	0.729
Hepatitis C (*n*, %)	36 (13.4)	8 (10.4)	0.487	26 (15.5)	5 (6.1)	4 (8.5)	0.014
Alcohol (*n*, %)	98 (36.4)	30 (39.0)	0.685	72 (42.9)	41 (50.0)	21 (44.7)	0.566
Ascites (*n*, %)	23 (8.6)	1 (1.3)	0.085	19 (11.3)	9 (11.0)	7 (14.9)	0.522
Creatinine (mg/dL)	1.0 ± 0.6	0.9 ± 0.2	0.160	0.9 ± 0.5	1.0 ± 0.6	0.9 ± 0.3	0.624
Sodium (mmol/L)	139.4 ± 3.2	140.3 ± 2.9	0.021	138.7 ± 2.9	139.1 ± 3.8	137.8 ± 2.7	0.074
Alanine aminotransferase (IU/L)	49.2 ± 35.8	43.5 ± 32.8	0.206	50.7 ± 44.0	43.7 ± 26.3	43.9 ± 30.9	0.297
Platelet count (10^9/L)	143.8 ± 71.5	172.8 ± 68.1	0.002	146.4 ± 69.6	175.8 ± 74.4	173.6 ± 91.6	0.005
Serum albumin (g/dL)	3.8 ± 0.5	4.1 ± 0.6	< 0.001	3.8 ± 0.5	4.1 ± 0.5	3.6 ± 0.6	< 0.001
Total bilirubin (mg/dL)	1.1 ± 1.2	1.0 ± 0.6	0.475	1.0 ± 0.9	0.9 ± 0.8	1.1 ± 0.7	0.173
INR	1.1 ± 0.1	1.1 ± 0.2	0.017	1.1 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	0.064
Child-Pugh score	5.5 ± 0.7	5.2 ± 0.5	0.025	5.5 ± 0.9	5.3 ± 0.7	5.8 ± 1.0	0.021
MELD score	8.8 ± 2.5	7.8 ± 2.2	0.002	8.7 ± 2.7	8.0 ± 2.5	8.8 ± 2.4	0.164
Alpha-fetoprotein (ng/mL)	1772.6 ± 7218.6	1826.7 ± 5996.8	0.952	3757.3 ± 19911.7	3186.6 ± 20583.6	2553.0 ± 9928.3	0.918
Numbers of tumor	3.7 ± 1.3	2.8 ± 1.2	< 0.001	3.7 ± 1.3	3.0 ± 1.3	4.0 ± 1.4	< 0.001
Maximum tumor diameter (cm)	4.8 ± 3.0	4.8 ± 2.2	0.989	5.1 ± 3.1	5.9 ± 3.4	6.0 ± 3.7	0.070

Data are presented as mean ± SD or number (%). BMI: body mass index; DM: Diabetes mellitus; EASL: European Association for the Study of the Liver; HCC: Hepatocellular carcinoma; INR: International normalized ratio; MELD: Mayo End-Stage Liver Disease.

AASLD guidelines, 32.8% received upward treatment; these patients were younger (average age: 59.6 *vs* 62.6 years) and had lower rates of diabetes (21.4% *vs* 30.3%) and fewer tumors than guideline-adherent patients. In contrast, of the patients assessed according to the 2010 APASL guidelines, only 4.2% received upward treatment, with the vast majority (91.7%) treated in line with the guidelines. The upward treatment group had higher BMI and serum sodium levels than the treatment-adherent group (Supplementary Table 2).

With respect to treatment strategies, among the 155 patients who received treatment upward of the EASL guidelines, 72.9% underwent liver resection, 9.7% received a liver transplant, and 8.4% had radiofrequency ablation (RFA). According to the AASLD guidelines, 200 patients received upward treatment, with 56.5% of these patients undergoing liver resection, 7.5% receiving a liver transplant, and 7% undergoing RFA. Additionally, 58 patients were classified as undergoing upward treatment due to CPS B liver function while receiving transcatheter chemotherapy (TACE, drug-eluting bead TACE, transarterial radioembolization). Under APASL guidelines, most patients with stage B HCC (486 of 530) adhered to guidelines, with 94 of the guideline-adherent patients undergoing liver resections. Among the 22 patients receiving upward treatment, 50% received a liver transplant and 50% underwent RFA (Table 4). These findings underscore the diverse treatment approaches available for stage B HCC and the need for personalized management strategies.

Table 3 Baseline characteristics of patients with hepatocellular carcinoma between 2008 and 2016 according to the 2005 and 2010 American Association for the Study of Liver Diseases guidelines

Variables	2005 AASLD guidelines (HCC patients, 2008-2010)			2010 AASLD guidelines (HCC patients, 2011-2016)		
	Guideline-adherent	Upward treatment	*P* value	Guideline-adherent	Upward treatment	*P* value
No. of patients	130	46		287	154	
Age (yr)	60.8 ± 8.9	58.3 ± 10.2	0.286	62.6 ± 10.8	59.6 ± 10.0	0.005
Male sex (*n*, %)	106 (81.5)	40 (87)	0.498	252 (87.8)	130 (84.4)	0.319
BMI (kg/m^2)	24.6 ± 4.5	24.2 ± 2.6	0.533	24.1 ± 3.4	24.0 ± 3.9	0.811
DM (*n*, %)	32.3	19.6	0.131	87 (30.3)	33 (21.4)	0.046
Hypertension (*n*, %)	40.8	30.4	0.289	107 (37.3)	63 (40.9)	0.456
Etiology						
Hepatitis B (*n*, %)	86 (66.2)	30 (34.8)	0.908	164 (57.1)	93 (60.4)	0.656
Hepatitis C (*n*, %)	14 (10.8)	5 (10.9)	0.985	43 (15.0)	16 (10.4)	0.087
Alcohol (*n*, %)	47 (36.2)	19 (41.3)	0.596	117 (40.8)	68 (44.2)	0.492
Ascites (*n*, %)	4 (3.1)	4 (9.5)	0.156	14 (4.9)	36 (23.4)	< 0.001
Creatinine (mg/dL)	1.0 ± 0.7	0.9 ± 0.2	0.345	0.9 ± 0.4	0.9 ± 0.7	0.786
Sodium (mmol/L)	140.0 ± 3.0	139.8 ± 4.0	0.736	139.1 ± 2.8	138.6 ± 3.6	0.092
Alanine aminotransferase (IU/L)	51.3 ± 36.6	42.6 ± 25.3	0.138	48.5 ± 40.7	42.7 ± 27.0	0.112
Platelet count (10^9/L)	146.1 ± 70.3	151.3 ± 74.1	0.669	151.2 ± 67.8	158.3 ± 79.1	0.326
Serum albumin (g/dL)	3.9 ± 0.4	3.8 ± 0.7	0.063	3.9 ± 0.5	3.8 ± 0.7	0.146
Total bilirubin (mg/dL)	0.9 ± 0.4	1.6 ± 2.5	0.005	0.9 ± 0.4	1.2 ± 1.2	< 0.001
INR	1.1 ± 0.1	1.2 ± 0.2	0.074	1.1 ± 0.1	1.1 ± 0.2	< 0.001
Child-Pugh score	5.3 ± 0.5	5.8 ± 1.2	< 0.001	5.3 ± 0.5	5.8 ± 1.1	< 0.001
MELD score	8.6 ± 2.0	9.2 ± 3.5	0.186	8.2 ± 2.1	9.1 ± 3.3	< 0.001
Alpha-fetoprotein (ng/mL)	1516.5 ± 5731.8	440.0 ± 1635.9	0.212	2442.3 ± 10522.8	3862.8 ± 23036.1	0.379
Numbers of tumor	3.7 ± 1.3	3.0 ± 1.3	0.001	3.7 ± 1.3	3.1 ± 1.3	< 0.001
Maximum tumor diameter (cm)	4.3 ± 1.9	4.1 ± 1.9	0.579	5.1 ± 3.0	5.5 ± 3.5	0.169

Data are presented as mean ± SD or number (%). AASLD: American Association for the Study of Liver Diseases; BMI: Body mass index; DM: Diabetes mellitus; HCC: Hepatocellular carcinoma; INR: International normalized ratio; MELD: Mayo End-Stage Liver Disease.

Changes in guideline adherence over time

Over the study period (2008-2016), there was a discernible trend in adherence rates to HCC guidelines. Adherence to the EASL guidelines initially ranged from 77% to 80% (2008-2012) but showed a downward tendency to 58.8% to 71.6% (2013-2016). Similarly, adherence to the AASLD guidelines started at 71.7% to 75.9% (2008-2010) and subsequently varied between 49.2% and 73.8% (2011-2016). In contrast, adherence to the APASL guidelines was consistently high, at 90.1% to 94.5% throughout the study period (Figure 2).

Factors affecting HCC-related mortality according to guideline adherence

According to the 2000 EASL guidelines, Patients who underwent upward treatment had significantly better 5-year survival rates than those who received guideline-adherent treatment (63.4% *vs* 27.2%, log-rank $P < 0.001$, Figure 3A). Risk factors for HCC-related deaths included > 4 tumors and a maximum tumor diameter > 10 cm. Upward treatment (HR 0.448, 95%CI: 0.310-0.647, $P < 0.001$) and a higher platelet count (> 10^5/μL; HR 0.672, 95%CI: 0.507-0.890, $P = 0.006$) were associated with significantly improved HCC-related survival (Table 5). According to 2012 EASL Guidelines, upward treatment demonstrated the best survival outcome of all the treatment groups (5-year survival rates: 57.3% *vs* 35.2%, log-rank $P < 0.001$, Figure 3B). Risk factors for HCC-related deaths included > 70 years of age, male sex, total bilirubin level > 1.2 mg/dL, AFP > 200 ng/mL, > 4 tumors, maximum tumor diameter > 5 cm, and downward treatment. However, upward treatment (HR 0.720, 95%CI: 0.478-1.086, $P = 0.117$) did not significantly improve HCC-related survival (Table 5). With respect to the 2005 AASLD Guidelines, patients who underwent upward treatment had significantly better 5-year

Table 4 Distribution of treatment strategies according to each hepatocellular carcinoma guideline

Treatment strategy	2000 EASL		2012 EASL			2005 AASLD		2010 AASLD		2010 APASL		
	Adherence	Upward	Adherence	Upward	Downward	Adherence	Upward	Adherence	Upward	Adherence	Upward	Downward
Total, *n* (%)	269 (76.2)	77 (21.8)	168 (56.6)	82 (27.6)	47 (15.8)	130 (71.8)	46 (15.5)	287 (61.2)	154 (32.8)	486 (91.7)	22 (4.7)	22 (4.7)
Liver resection	0	56 (72.7)	0	57 (63.4)	0	0	26 (56.5)	0	87 (56.5)	94 (19.3)	0	0
Liver transplantation	0	11 (14.3)	0	4 (4.9)	0	0	6 (13.0)	0	9 (5.8)	0	11 (50)	0
Radiofrequency ablation	0	6 (7.8)	0	7 (8.5)	0	0	4 (8.7)	0	10 (6.5)	0	11 (50)	0
TACE	261 (97)	0	168 (100)	0	0	130 (100)	10 (21.7)	269 (93.7)	45 (28.2)	6	0	0
TACE with drug-eluting beads	0	4 (5.2)	0	10 (12.2)	0	0	0	12 (4.2)	2 (1.3)	14 (2.9)	0	0
Radioembolization (Yttrium-90)	0	0	0	4 (4.9)	0	0	0	0	1 (0.6)	4 (0.8)	0	0
Chemotherapy	8 (1.9)	0	0	0	13 (27.7)	0	0	3 (0.9)	0	0	0	20 (90.7)
Radiation therapy	0	0	0	0	2 (4.3)	0	0	0	0	0	0	0
No treatment	0	0	0	0	0	0	0	0	0	0	0	2 (9.1)

Data are presented as number (%). AASLD: American Association for the Study of Liver Diseases; APASL: Asian Pacific Association for the Study of the Liver; EASL: European Association for the Study of the Liver; HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization.

survival rates than those who received guideline-adherent treatment (63% *vs* 30%, log-rank $P < 0.001$, Figure 4A). Risk factors for HCC-related death included > 4 tumors and a maximum tumor diameter > 5 cm. Upward treatment (HR 0.465, 95%CI: 0.322-0.670, $P < 0.001$) and a platelet count > 10^5/μL (HR 0.684, 95%CI: 0.518-0.904, $P = 0.008$) significantly improved HCC-related survival outcomes in patients with HCC between 2008 and 2010. For patients assessed under the 2010 AASLD guidelines, patients who underwent upward treatment demonstrated better 5-year survival rates than those who received guideline-adherent treatment (50% *vs* 29.3%, log-rank $P < 0.001$, Figure 4B). Factors associated with HCC-related deaths included > 70 years of age, CPS > 7, > 4 tumors, and a maximum tumor diameter > 5 cm. Upward treatment (HR 0.478, 95%CI: 0.333-0.685, $P < 0.001$) and serum albumin levels > 3.5 g/dL (HR 0.596, 95%CI: 0.416-0.855, $P = 0.005$) were associated with improved HCC-related survival (Table 6). With respect to the 2010 APASL guidelines, patients who received guideline-adherent treatment showed the highest survival rates among all the groups (1-year survival rates: 84.1%, 77.3%, and 36.4%, in the guideline-adherent, upward, and downward treatment groups, respectively, log-rank $P < 0.001$, Supplementary Figure 1). Risk factors for HCC-related death included > 70 years of age, INR > 1.2, total bilirubin level > 1.2 mg/dL, > 4 tumors, a maximum tumor diameter > 5 cm, and downward treatment. Upward treatment (HR 0.704, 95%CI: 0.372-1.333, $P = 0.281$) was not associated with better survival outcomes (Supplementary Table 3), which may be attributed to the relatively limited number of patients in the upward treatment group compared with the guide-adherent group.

Table 5 Univariate and multivariate Cox regression analysis for hepatocellular carcinoma -related death according to 2000 and 2012 European Association for the Study of the Liver guidelines

Variables	2000 EASL guidelines (HCC patients, 2008-2012)				2012 EASL guidelines (HCC patients, 2013-2016)			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age (≥ 70 yr)	1.339 (1.004-1.783)	0.046	1.300 (0.961-1.758)	0.089	1.838 (1.336-2.529)	< 0.001	1.765 (1.269-2.687)	0.001
Male sex	1.239 (0.875-1.754)	0.227			0.592 (0.401-0.874)	0.008	0.610 (0.409-0.910)	0.153
BMI (≥ 25kg/m^2)	0.890 (0.695-1.140)	0.356			0.829 (0.606-1.135)	0.242		
DM	1.153 (0.880-1.510)	0.301			1.027 (0.740-1.424)	0.875		
Hypertension	1.072 (0.836-1.375)	0.583			1.026 (0.757-1.391)	0.867		
Etiology								
Hepatitis B	0.902 (0.704-1.156)	0.415			0.852 (0.624-1.165)	0.316		
Hepatitis C	1.056 (0.743-1.501)	0.763			1.270 (0.822-1.962)	0.282		
Alcohol	1.109 (0.866-1.420)	0.393			0.960 (0.711-1.295)	0.787		
Ascites								
Mild	2.499 (1.543-4.048)	< 0.001			1.552 (0.962-2.503)	0.072		
Moderate to severe	1.302 (0.486-3.509)	0.597			1.736 (0.767-3.929)	0.186		
Creatinine (> 1 mg/dL)	1.283 (0.975-1.689)	0.076			1.123 (0.786-1.605)	0.524		
Sodium (> 135 mmol/L)	0.620 (0.411-0.937)	0.024			0.615 (0.408-0.929)	0.021		
Alanine aminotransferase (> 80 IU/L)	1.166 (0.813-1.672)	0.403			0.886 (0.556-1.412)	0.610		
Platelet count (> 10^5/μL)	0.685 (0.529-0.886)	0.004	0.672 (0.507-0.890)	0.006	0.735 (0.526-1.025)	0.07		
Serum albumin (≥ 3.5 g/dL)	0.694 (0.523-0.921)	0.011			0.605 (0.428-0.855)	0.004		
Total bilirubin (> 1.2 mg/dL)	1.529 (1.153-2.027)	0.003			1.510 (1.096-2.081)	0.012	1.391 (0.998-1.938)	0.051
INR (> 1.2)	1.303 (0.982-1.729)	0.067			1.333 (0.904-1.967)	0.147		
Child-Pugh score (≥ 7)	1.808 (1.187-2.754)	0.006			1.586 (0.994-2.530)	0.053		
MELD score (> 9)	1.463 (1.112-1.926)	0.007			1.438 (1.035-1.998)	0.030		
Alpha-fetoprotein (≥ 200 ng/mL)	1.287 (0.993-1.668)	0.056			1.626 (1.196-2.211)	0.002	1.392 (1.001-1.936)	0.049
Numbers of tumor (> 3)	1.810 (1.419-2.309)	< 0.001	1.685 (1.293-2.196)	< 0.001	1.654 (1.219-2.244)	0.001	1.673 (1.178-2.275)	0.003
Maximum tumor diameter (cm)								
< 2	Ref				Ref			
2-5	0.642 (0.409-1.008)	0.054	0.969 (0.608-1.545)	0.894	1.489 (0.807-2.750)	0.203	1.725 (0.908-3.276)	0.096

5-10	1.061 (0.666-1.689)	0.803	1.862 (1.136-3.055)	0.0138	1.694 (0.912-3.148)	0.095	2.378 (1.228-4.603)	0.010
> 10	2.511 (1.328-4.748)	0.005	4.377 (2.268-8.448)	< 0.001	4.023 (2.051-7.892)	< 0.001	4.358 (2.120-8.956)	< 0.001
Treatment								
Guideline adherence	Ref				Ref			
Upward	0.372 (0.263-0.525)	< 0.001	0.448 (0.310-0.647)	< 0.001	0.631 (0.426-0.935)	0.022	0.720 (0.478-1.086)	0.117
Downward					1.974 (1.362-2.859)	< 0.001	1.838 (1.257-2.687)	0.002

95%CI: 95%confidence interval; BMI: Body mass index; DM: Diabetes mellitus; EASL: European Association for the Study of the Liver; HCC: Hepatocellular carcinoma; HR: Hazard ratio; INR: International normalized ratio; MELD: Mayo End-Stage Liver Disease.

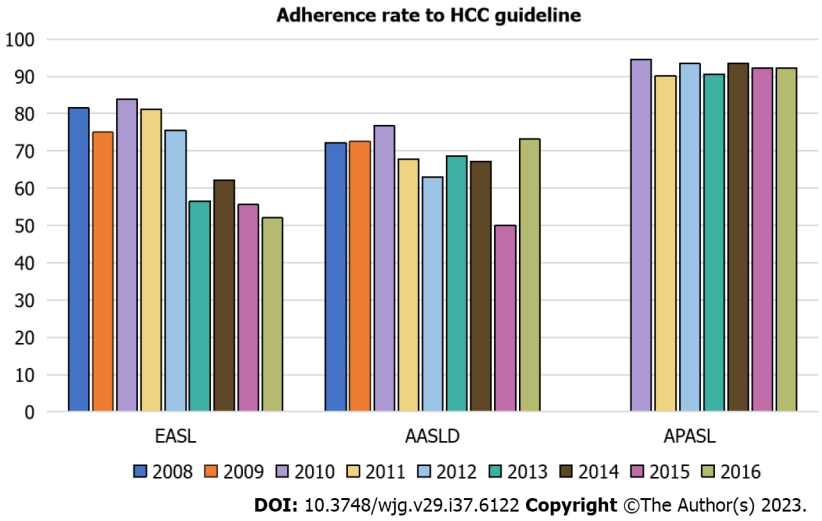


Figure 2 Changes in the rate of adherence to hepatocellular carcinoma guidelines over time. AASLD: American Association for the Study of Liver Diseases; APASL: Asian Pacific Association for the Study of the Liver; EASL: European Association for the Study of the Liver; HCC: Hepatocellular carcinoma.

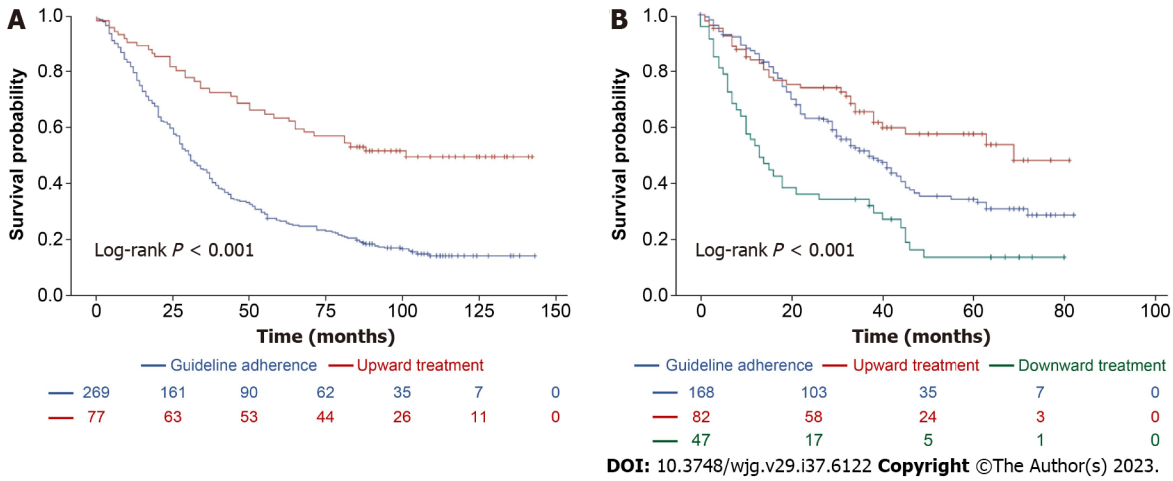


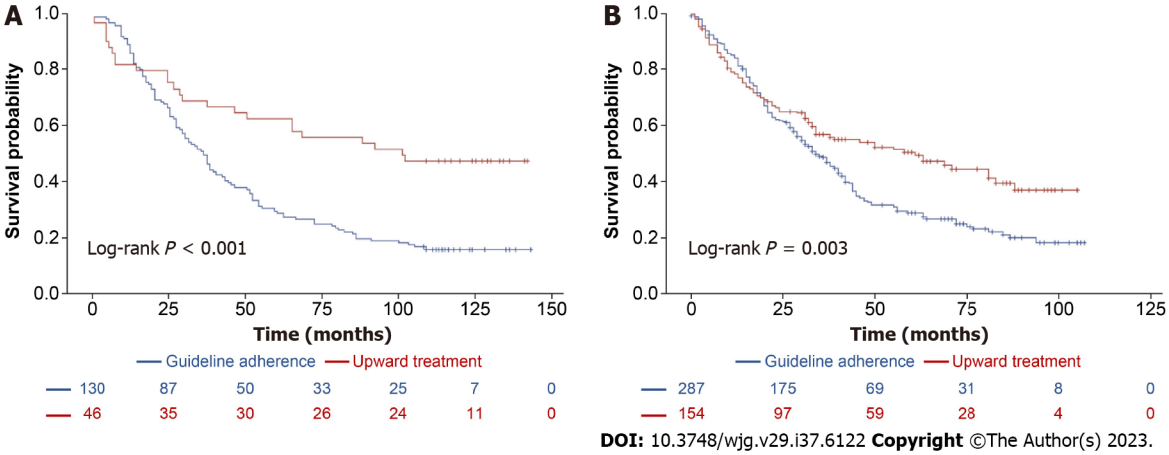
Figure 3 Kaplan-Meier survival curves of hepatocellular carcinoma-related deaths for hepatocellular carcinoma patients according to European Association for the Study of the Liver guideline. A: Kaplan-Meier curve of hepatocellular carcinoma (HCC)-related deaths between 2008 and 2012 according to the 2000 European Association for the Study of the Liver (EASL) guidelines; B: Kaplan-Meier curve of HCC-related deaths between 2013 and 2016 according to the 2012 EASL guidelines.

Table 6 Univariate and multivariate Cox regression analysis for hepatocellular carcinoma-related death according to 2005 and 2010 American Association for the Study of Liver Diseases guidelines

Variables	2005 AASLD guidelines (HCC patients, 2008-2010)				2010 AASLD guidelines (HCC patients, 2011-2016)			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age (≥ 70 yr)	1.229 (0.809-1.865)	0.334	1.210 (0.896-1.635)	0.214	1.775 (1.376-2.289)	< 0.001	1.587 (1.206-2.089)	0.001
Male sex	1.237 (0.776-1.972)	0.371			0.832 (0.602-1.152)	0.268		
BMI (≥ 25 kg/m^2)	1.164 (0.819-1.654)	0.397			0.827 (0.650-1.053)	0.123		
DM	0.990 (0.681-1.438)	0.958			1.236 (0.954-1.600)	0.109		
Hypertension	1.129 (0.798-1.596)	0.493			1.101 (0.868-1.398)	0.428		
Etiology								
Hepatitis B	0.867 (0.608-1.235)	0.429			0.828 (0.652-1.051)	0.121		
Hepatitis C	1.068 (0.633-1.801)	0.807			1.225 (0.884-1.698)	0.223		
Alcohol	1.155 (0.815-1.637)	0.417			0.994 (0.785-1.259)	0.961		
Ascites								
Mild	1.984 (0.872-4.516)	0.102			1.907 (1.303-2.791)	0.001		
Creatinine (> 1 mg/dL)	1.322 (0.901-1.940)	0.153			1.206 (0.919-1.582)	0.176		
Sodium (≥ 135 mmol/L)	0.876 (0.445-1.724)	0.702			0.573 (0.407-0.807)	0.002		
Alanine aminotransferase (> 80 IU/L)	1.021 (0.614-1.699)	0.937			0.968 (0.670-1.400)	0.865		
Platelet count (> 10^5/μL)	0.590 (0.414-0.839)	0.003	0.684 (0.518-0.904)	0.008	0.736 (0.568-0.952)	0.020		
Serum albumin (≥ 3.5 g/dL)	0.865 (0.573-1.306)	0.491			0.533 (0.408-0.696)	< 0.001	0.596 (0.416-0.855)	0.005
Total bilirubin (> 1.2 mg/dL)	1.619 (1.102-2.378)	0.014			1.509 (1.162-1.959)	0.002		
INR (> 1.2)	1.229 (0.838-1.802)	0.291			1.421 (1.054-1.915)	0.021		
Child-Pugh score (≥ 7)	1.430 (0.667-3.065)	0.358			1.822 (1.288-2.577)	0.001	2.429 (1.434-4.114)	0.001
MELD score (> 9)	1.465 (1.018-2.108)	0.040			1.521 (1.163-1.988)	0.002		
Alpha-fetoprotein (≥ 200 ng/mL)	1.436 (1.004-2.055)	0.048			1.319 (1.029-1.692)	0.029		
Numbers of tumor (> 3)	1.458 (1.036-2.051)	0.030	1.570 (1.208-2.040)	0.001	1.830 (1.443-2.320)	< 0.001	1.870 (1.426-2.452)	< 0.001
Maximum tumor diameter								
< 2	Ref				Ref			
2-5	0.767 (0.408-1.442)	0.411	0.967 (0.606-1.543)	0.889	0.974 (0.623-1.522)	0.908	1.294 (0.794-2.107)	0.310
5-10	0.925 (0.475-1.800)	0.818	1.792 (1.093-2.939)	0.021	1.459 (0.929-2.291)	0.101	2.210 (1.338-3.650)	0.002

> 10	4.267 (0.918-19.839)	0.064	3.437 (1.784-6.623)	< 0.001	2.692 (1.578-4.594)	< 0.001	3.261 (1.834-5.797)	< 0.001
Treatment								
Guideline adherence	Ref				Ref			
Upward	0.442 (0.283-0.691)	< 0.001	0.465 (0.322-0.670)	< 0.001	0.678 (0.524-0.879)	0.003	0.478 (0.333-0.685)	< 0.001

95%CI: 95% confidence interval; AASLD: American Association for the Study of Liver Diseases; BMI: body mass index; DM: diabetes mellitus; HCC: Hepatocellular carcinoma; HR: Hazard ratio; INR: International normalized ratio; MELD: Mayo End-Stage Liver Disease.



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Figure 4 Kaplan-Meier survival curves of hepatocellular carcinoma-related deaths for hepatocellular carcinoma patients according to American Association for the Study of Liver Diseases guideline. A: Kaplan-Meier curve of hepatocellular carcinoma (HCC)-related deaths between 2008 and 2010 according to the 2000 American Association for the Study of Liver Diseases (AASLD) guidelines; B Kaplan-Meier curve of HCC-related deaths between 2011 and 2016 according to the 2010 AASLD guidelines.

These findings suggest that adherence to different guidelines and specific treatment choices played a crucial role in the prognosis of patients with HCC, with common risk factors, including tumor characteristics, patient age, and liver function influencing survival outcomes.

Comparison of PFS according to guideline adherence

For patients analyzed according to the 2000 EASL guidelines, there was no significant difference in PFS between the guideline-adherent and upward treatment groups (Supplementary Figure 2A). However, with respect to the 2012 EASL guidelines, the guideline-adherent group had markedly improved 1-year PFS compared with the upward treatment group (60.5% *vs* 39.8%, log-rank $P < 0.001$, Supplementary Figure 2B). Between 2013 and 2016, upward treatment (HR 0.648, 95%CI: 0.461-0.909, $P = 0.012$) and serum albumin levels ≥ 3.5 g/dL (HR 0.74, 95%CI: 0.568-0.964, $P = 0.026$) were associated with improved PFS (Supplementary Table 5).

For patients assessed under the 2005 AASLD guidelines, no significant difference in PFS was observed between guideline-adherent and upward treatment groups (Supplementary Figure 3A). However, with respect to the 2010 AASLD guidelines, upward treatment was associated with superior 1-year PFS than guideline adherence (58.6% *vs* 38.9%, log-rank $P < 0.001$, Supplementary Figure 3B). Between 2011 and 2016, upward treatment (HR 0.556, 95%CI: 0.426-0.726, $P < 0.001$), and serum albumin levels ≥ 3.5 g/dL (HR 0.689, 95%CI: 0.511-0.928, $P = 0.014$) were associated with improved PFS (Supplementary Table 5).

With respect to the 2010 APASL guidelines, the upward treatment group exhibited the highest 1-year PFS rate (75%, 44.8%, and 31.3% in upward, guideline-adherent and downward treatment groups, respectively, log-rank $P = 0.028$, Supplementary Figure 4). Risk factors for tumor progression included > 70 years of age, > 4 tumors, a maximum tumor diameter > 5 cm, and downward treatment. Compared to guideline adherence, between 2010 and 2016, upward treatment (HR 0.561, 95%CI: 0.313-1.004, $P = 0.052$) and a platelet count > 10^5/μL (HR 0.740, 95%CI: 0.587-0.932, $P = 0.011$) were associated with a significant improvement in PFS (Supplementary Table 6).

In summary, regardless of the specific guidelines followed, factors such as adherence to guidelines, treatment choice (especially upward treatment), serum albumin levels, and platelet count consistently played pivotal roles in determining the prognosis of patients with HCC, particularly in terms of PFS.

Subgroup analysis of the impact of guideline adherence on overall survival according to BCLC subclassification

Participants were categorized into BCLC stage B1 (40.6%, *n* = 263), B2 (55.1%, *n* = 357), and B3 (4.3%, *n* = 28). Among B1 and B2 patients (96.7% of the total), a significant portion received upward treatment (66.7% and 70%, respectively, Supplementary Tables 7 and 10).

In the B1 group, patients who received upward treatment had a significantly higher 5-year survival rate compared with those whose treatment adhered to guidelines (71.1% *vs* 41.4%, log-rank $P < 0.001$, Figure 5A). Upward treatment was associated with a significant improvement in survival outcomes (HR 0.470, 95%CI: 0.288-0.766, $P = 0.002$), even after PSM at a 1:1 ratio for variables, such as platelet count, serum albumin level, MELD score, number of tumors, and maximum tumor diameter (Supplementary Tables 8 and 9, Supplementary Figure 5). In the B2 group, a similar trend was observed, with a higher 5-year survival rate in patients receiving upward treatment compared with those whose treatment adhered to guidelines (51.2% *vs* 21.6%, log-rank $P < 0.001$, Figure 5B). Upward treatment remained a robust indicator of improved survival (HR 0.553, 95%CI: 0.317-0.965, $P = 0.037$, Supplementary Tables 11 and 12, Supplementary Figure 6) after 1:1 PSM for variables, such as age, etiology, sodium level, platelet count, serum albumin level, MELD score, number of tumors, and maximum tumor diameter.

Interestingly, despite the Kinki criteria recommending TACE, hepatic arterial infusion chemotherapy, and systemic chemotherapy as treatment options for patients with B2 HCC, liver resection, liver transplantation, or RFA resulted in superior outcomes for over 70% of patients with B2 HCC compared with following the guidelines. These findings highlight the potential benefits of individualized treatment approaches beyond guideline recommendations for certain BCLC subgroups.

DISCUSSION

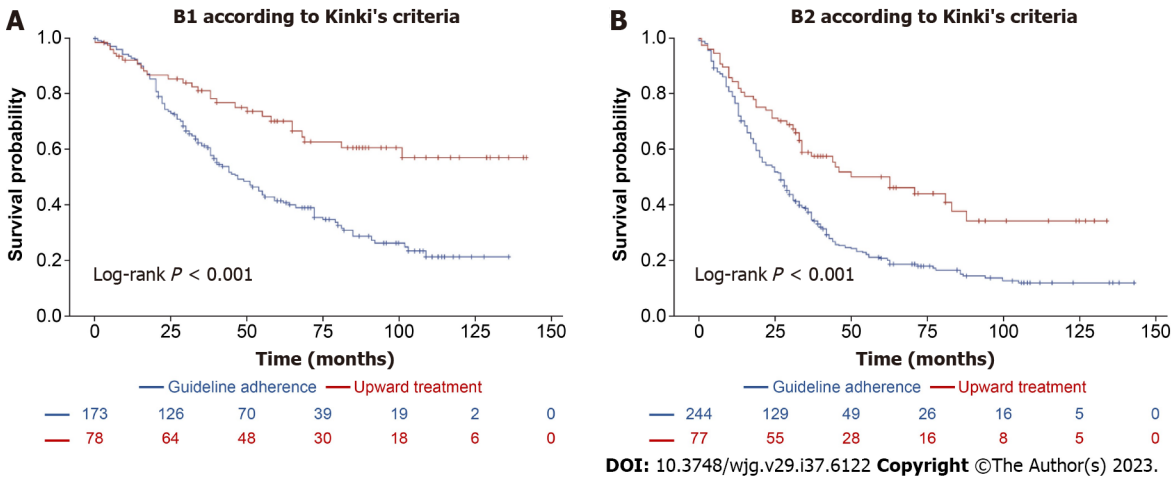
This large-scale, longitudinal study examined real-world data from patients with stage B HCC in South Korea over an 8-year period. As this was a nationwide multicenter study using data from the KCCR, random and representative selection of patients with HCC was performed. The adherence rate to guidelines for the treatment of stage B HCC has not increased over time, highlighting a gap between official recommendations and clinical practice. This study examines the implications of treatment decisions for patients with stage B HCC.

Notably, the present study revealed that liver resection is a common treatment option for stage B HCC in South Korea, deviating from EASL and AASLD guidelines. This reflects the tendency of Asian countries to adopt more aggressive HCC treatment strategies than Western countries[18-20]. Furthermore, curative treatments, including liver resection, yield better survival outcomes than TACE in certain patients. Prognostic factors for patients with stage B HCC after curative treatment included age, tumor number, maximum tumor diameter, and underlying liver function, aligning with prior large-scale studies[22-24]. Overall, these findings suggest that curative treatments may significantly improve the prognosis of patients with stage B HCC, even after accounting for potential selection bias.

Achieving significant increases in EASL and AASLD guideline adherence rates over time remains elusive in East Asian countries. One plausible explanation for this lies in the complex and multifaceted nature of HCC, which often requires tailored treatment strategies that may not always align with the standard guidelines. Moreover, the preference for curative or aggressive treatments for stage B HCC in East Asian countries may be attributed to the higher incidence of HCC in these countries compared with that in Western countries, largely due to a higher prevalence of chronic hepatitis B. This has necessitated the development of specialized treatment approaches. The establishment of specialized liver centers and multidisciplinary teams has resulted in the cultivation of expertise in various treatment modalities. Over time, the tradition of aggressive HCC treatment, including liver resection and transplantation, has become ingrained based on continuous research and clinical trials, leading to innovative strategies. Moreover, variations in healthcare infrastructure, clinical practice, demographics and differences in treatment preferences could all fundamentally make the differences for guideline non-adherence across regions in different countries.

In 2022, the BCLC group updated their recommendations for HCC treatment, sub-classifying stage B HCC patients into three groups based on tumor characteristics and potential treatment responses; those eligible for extended liver transplantation criteria despite multiple HCCs, those suitable for TACE due to well-defined HCC nodules and preserved portal flow, and those with diffuse, infiltrative, and extensive HCC that may benefit from systemic therapy[25]. However, the updated BCLC staging system still does not recommend liver resection as a feasible therapy for stage B HCC due to the lack of prospective studies. Notably, a Chinese randomized controlled trial and a South Korean retrospective cohort study have demonstrated potential survival benefits of liver resection over TACE in selected patients with multiple HCCs [26]. In a South Korean retrospective cohort study, two periods (2003-2005 and 2008-2010) were compared to assess changing treatment trends. The results indicated that patients with stage 0-C HCC who underwent curative treatments in the later cohort achieved superior 5-year survival outcomes to those who received non-curative therapy[27]. The potential survival benefits of liver resection over TACE in selected patients with stage B HCC have been verified through systematic reviews and meta-analyses[28,30-33]. Considering real-world scenarios [21-24,29] that demonstrate superior outcomes with liver resection can provide robust evidence for the adoption of curative treatments in patients with more advanced HCC. However, careful patient selection is required, considering individual patient characteristics and institutional expertise, to maximize the survival benefit.

Patients with chronic liver disease are at an increased risk of post-hepatectomy liver failure; however, advances in preoperative assessments such as portal hypertension evaluation, future liver remnant volume or function prediction, portal vein embolization, surgical techniques, and postoperative management have expanded the possibilities of liver resection even in more advanced stages of HCC. As a result, portal hypertension, multifocal HCCs, and portal vein



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Figure 5 Kaplan-Meier survival curves of hepatocellular carcinoma-related deaths for hepatocellular carcinoma patients according to Barcelona clinic liver cancer subclassification. A: Kaplan-Meier curve of hepatocellular carcinoma (HCC)-related deaths between 2008 and 2016 in substage B1 HCC patients according to the European Association for the Study of the Liver (EASL) guidelines; B: Kaplan-Meier curve of HCC-related deaths between 2008 and 2016 in substage B2 HCC patients according to the EASL guidelines.

thrombosis are now recognized as manageable challenges in HCC treatment. Overall, the importance of multidisciplinary evaluation and meticulous planning in the selection of treatment strategies for stage B HCC cannot be overstated; where technically feasible, surgical resection remains a vital option.

Our study has several limitations that warrant consideration when interpreting the results. First, given its retrospective nature, there is a possibility that treatment strategies were influenced by physician or patient preferences, introducing inherent bias. To establish the safety and effectiveness of curative treatments for stage B HCC, well-designed prospective studies are essential. Second, our study excluded certain patients with stage B HCC who may benefit from alternative treatments or systemic therapy according to the 2022 BCLC staging system, due to the limited number of participants. This exclusion could impact the generalizability of our findings. Third, we were unable to account for potential confounding factors such as tumor location, pathology, degree of differentiation, and imaging characteristics, as these data were not available in the KCCR. These factors can influence treatment choices and prognosis, potentially affecting our results. Lastly, due to the small sample size, we did not conduct a survival analysis comparing the B3 group with the B1 and B2 groups. While our study offers valuable insights into stage B HCC treatment and prognosis, well-designed prospective studies that overcome these limitations are necessary for a more comprehensive understanding of HCC and its management.

We propose that the eligibility criteria for liver resection be expanded to patients with stage B HCC in selected patients aged < 70 years, with platelet counts > 10^5/μL, and serum albumin levels ≥ 3.5 g/dL, even in cases where the liver function corresponds to CPS B7 or the HCC status is beyond the Milan criteria and outside the up-to-7 criteria. However, careful patient selection by considering liver function, tumor location, and tumor burden is crucial.

CONCLUSION

The present study verified the discrepancy between guideline recommendations and real-world clinical practice in the treatment of stage B HCC, with liver resection often chosen against guideline recommendations, resulting in improved survival for selected patients. Multidisciplinary evaluation is crucial for the selection of appropriate curative treatments in patients with stage B HCC, considering patient characteristics and institutional expertise. Prospective studies are required to further assess the clinical implications of curative treatments in stage B HCC.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is a major global health concern, and the second leading cause of cancer mortality worldwide. Treatment guidelines are based on the Barcelona Clinic Liver Cancer staging system, but in East Asian countries, liver resection is often preferred to transarterial chemoembolization for stage B HCC due to better survival outcomes.

Research motivation

The need for regional adaptations in HCC treatment guidelines to improve the prognosis of patients with stage B HCC.

Research objectives

This study aims to evaluate adherence to international HCC guidelines in South Korea using data from 2008-2016, investigate the treatment strategies for stage B HCC, analyze the impact of guideline non-adherence on survival, and identify patient subgroups who may benefit from guideline deviation to improve real-world management.

Research methods

In this retrospective analysis, data from the Korea Central Cancer Registry from 2008 to 2016 were utilized. Patients with stage B HCC were categorized into groups based on treatment adherence to HCC guidelines from Asian Pacific, European, and American associations for the study of liver diseases. The primary outcome was HCC-related deaths, with tumor recurrence as a secondary outcome; statistical analysis was performed using Kaplan-Meier curves with log-rank tests and multivariable Cox regression analysis to analyze survival outcomes and predictors.

Research results

The adherence to European Association for the Study of the Liver and American Association for the Study of Liver Diseases HCC treatment guidelines exhibit a declining trend over time in South Korea. Curative treatments, which were a deviation from guideline recommendations, led to significantly improved survival rates. Patients receiving upward treatments were < 70 years of age, and had platelet counts > 10^5/μL and serum albumin levels ≥ 3.5 g/dL.

Research conclusions

This study, based on real-world data in South Korea, revealed a persistent gap between treatment guideline recommendations and real clinical practice for patients with stage B HCC; liver resection, which was often chosen against guideline recommendations, resulted in improved survival for selected patients.

Research perspectives

These findings suggest that expanding the eligibility criteria for liver resection in specific patient groups may be beneficial. The study also highlights the need for careful patient selection through a multidisciplinary approach when considering curative treatments for stage B HCC. However, prospective studies are needed to further evaluate the clinical implications of curative treatments in stage B HCC.

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FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study

Risk factors and a predictive nomogram for lymph node metastasis in superficial esophageal squamous cell carcinoma

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Abstract

BACKGROUND

Superficial esophageal squamous cell carcinoma (ESCC) is defined as cancer infiltrating the mucosa and submucosa, regardless of regional lymph node metastasis (LNM). Endoscopic resection of superficial ESCC is suitable for lesions that have no or low risk of LNM. Patients with a high risk of LNM always need further treatment after endoscopic resection. Therefore, accurately assessing the risk of LNM is critical for additional treatment options.

AIM

To analyze risk factors for LNM and develop a nomogram to predict LNM risk in superficial ESCC patients.

METHODS

Clinical and pathological data of superficial ESCC patients undergoing esophagectomy from January 1, 2009 to January 31, 2016 were collected. Logistic regression analysis was used to predict LNM risk factors, and a nomogram was developed based on risk factors derived from multivariate logistic regression analysis. The receiver operating characteristic (ROC) curve was used to obtain the accuracy of the nomogram model.

RESULTS

A total of 4660 patients with esophageal cancer underwent esophagectomy. Of these, 474 superficial ESCC patients were enrolled in the final analysis, with 322 patients in the training set and 142 patients in the validation set. The prevalence of LNM was 3.29% (5/152) for intramucosal cancer and increased to 26.40% (85/322) for submucosal cancer. Multivariate logistic analysis showed that tumor size, invasive depth, tumor differentiation, infiltrative growth pattern, tumor budding, and lymphovascular invasion were significantly correlated with LNM. A nomogram using these six variables showed good discrimination with an area under the ROC curve of 0.789 (95%CI: 0.737-0.841) in the training set and 0.827 (95%CI: 0.755-0.899) in the validation set.

CONCLUSION

We developed a useful nomogram model to predict LNM risk for superficial ESCC patients which will facilitate additional decision-making in treating patients who undergo endoscopic resection.

Key Words: Superficial esophageal squamous cell carcinoma; Lymph node metastasis; Risk factors; Nomogram; Predictive model

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Core Tip: This is a retrospective study to identify risk factors for lymph node metastasis (LNM) in superficial esophageal squamous cell carcinoma (ESCC) and to develop a nomogram model for predicting LNM. A total of 474 superficial ESCC patients who underwent esophagectomy were enrolled. Multivariate logistic analysis showed that tumor size, invasive depth, tumor differentiation, infiltrative growth pattern, tumor budding, and lymphovascular invasion were significantly correlated with LNM. A predictive nomogram using these six variables showed good performance and will facilitate the treatment choice for superficial ESCC patients.

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INTRODUCTION

Superficial esophageal squamous cell carcinoma (ESCC) is defined as esophageal cancerous lesions infiltrating the mucosa and submucosa, regardless of lymph node metastasis (LNM)[1]. Endoscopic resection has been the first treatment choice for superficial ESCC patients with no or low risk of LNM and has an *en* bloc rate of more than 90%[2,3]. However, additional treatment is recommended for patients with high risk of LNM, especially for patients with positive lymphovascular invasion (LVI) and positive vertical margins[4]. Assessing pathological characteristics after endoscopic resection and predicting the risk of LNM is critical for additional treatment strategies.

Recent studies have established several predictive models to identify LNM risk for superficial ESCC patients[5-8]. However, some limitations existed in these models. For example, some studies did not incorporate critical factors, such as tumor budding and tumor infiltrative growth (INF) pattern, into LNM risk prediction. In some studies, the submucosa was considered as an entire layer[5,6] and the depth was not classified as submucosa 1 (SM1), SM2 or more[7,8]. Therefore, we aimed to establish a nomogram predictive model based on comprehensive pathological features obtained from esophagectomy to improve the predictive performance of such models.

MATERIALS AND METHODS

Patients

Patients who underwent esophagectomy at West China Hospital of Sichuan University from January 1, 2009, to January 31, 2016, were enrolled. The inclusion criteria were as follows: (1) Histopathological diagnosis of esophageal cancer; and (2) patients who received esophagectomy. The exclusion criteria were as follows: (1) Not pT1 stage tumor; (2) the histologic type was not squamous cell carcinoma; (3) number of dissected lymph nodes < 12; (4) history of previous malignancies; (5) incomplete clinical data; and (6) lesions with low-grade intraepithelial neoplasia or high-grade intraepithelial neoplasia. This retrospective study was approved by the Institutional Review Board of West China Hospital of Sichuan University (No. 2015-159). Informed consent was signed before the surgery.

Data collection

General clinical and endoscopic features, such as age, sex, tumor location, and endoscopic type, were retrospectively collected. Tumor location was defined as: (1) Upper: 15 to 24 cm from the incisors; (2) middle: 24 to 32 cm from the incisors; and (3) lower: 32 cm from the incisors to the cardia. Endoscopic types were identified according to the Paris classification criteria for superficial tumors[9]. The pathologic diagnosis was independently confirmed by two experienced pathologists. If the diagnosis is inconsistent, a third expert pathologist will re-examine the specimen, and the final diagnosis will be made when two or more pathologists agree on the diagnosis.

Data regarding pathological characteristics of specimens, such as tumor size, invasion depth, differentiation grade, INF pattern, tumor budding, LVI, and number of dissected lymph nodes, were collected. The vertical invasion depth of submucosal invasion was measured from the muscularis mucosae according to the Japanese guidelines[10]. The invasion depth was classified into four layers: Muscularis mucosae (MM), upper third of submucosa (SM1), middle third of submucosa (SM2) and lower third of submucosa (SM3) according to the Japanese Classification of Esophageal Cancer [10]. The differentiation grade was grouped as well differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3)[11]. The tumor INF pattern was carefully observed and classified into three groups according to previous reports[10,12]: INF-a (expansive type, expansive growth of tumor nests downward continuously from the epithelium as a whole), INF-b (intermediate type between INF-a and INF-c), and INF-c (infiltrative type, tumor infiltrates with a way of single cell or small tumor nests, or trabecular arrangement of tumor cells on the leading edge of the tumor). Tumor budding is defined as a single tumor cell or a small tumor nest consisting of up to 4 cells at the front of the tumor invasion [13]. In this study, tumor budding was assessed on hematoxylin and eosin-stained slides at the front of tumor invasion. Tumor budding was categorized into three types: No budding, low-grade tumor budding (1 to 4 budding foci at a 20 × objective lens) and high-grade tumor budding (≥ 5 budding foci at a 20 × objective lens) according to a previous study [14]. For LVI, two experienced pathologists observed the same specimen to improve diagnostic accuracy.

Statistical analysis

Continuous variables are presented as mean ± SD and are compared with a *t* test in the case of a normal distribution. Categorical data are presented as percentages and are compared with the chi-square test or Fisher's exact test. All variables associated with LNM at a significant level were enrolled in the stepwise multivariate logistic analysis. All data were statistically analyzed by SPSS 22.0 software (IBM SPSS, Chicago, IL, United States). *P* values < 0.05 were considered statistically significant.

R software (version 4.1.3) with the rms package was used to formulate a predictive nomogram using variables derived from multivariate logistic analysis. The pROC package was used to formulate the receiver operating characteristic (ROC) curve, and the area under the curve (AUC) was used to evaluate the predictive performance of this nomogram as previously reported[15]. The nomogram can convert each regression variable to a scale of 0-100 points based on the regression coefficient. Finally, the predicted probabilities were derived from the total points obtained from each independent variable.

RESULTS

Clinicopathologic characteristics

In total, 4660 esophageal cancer patients underwent esophagectomy from January 1, 2009 to January 31, 2016 of which, 474 superficial ESCC patients were enrolled in the final analysis (Figure 1). The clinicopathologic characteristics of enrolled patients are presented in Table 1. Most of the superficial ESCC patients were male (77.4%), the average age was 60 (range, 38-84) years, and the average tumor size was 23 (range, 3-73) mm. Most tumors (63.9%) were located in the middle third of the esophagus. According to the endoscopic appearance, 309 (65.2%) tumors presented as flat lesions. Regarding invasion depth, 152 (32.1%) patients had intramucosal cancer, 80 (16.9%) patients had SM1 cancer, and 242 (51.0%) patients had tumors deeper than SM1. Regarding tumor differentiation, 103 (21.7%) patients, 279 (58.9%) patients, and 92 (19.4%) patients had well differentiated, moderately differentiated, and poorly differentiated tumors, respectively. INF-b was the most common INF pattern, with 232 (48.9%) cases reported as such. The total tumor budding rate was 13.5% (64/474), and the LVI rate was 5.7% (27/474). Overall, 90 of the 474 (16.48%) patients had lymph node metastasis (LNM), and the LNM rate was 3.29% (5/152) in T1a tumors and 26.40% (85/322) in T1b tumors. The average number of dissected lymph nodes was 18.0 (range, 12-53) (Table 1).

Risk factors for LNM

The 474 enrolled patients were randomly grouped into a training set and a validation set at a ratio of 7:3. Comparisons of clinicopathological characteristics between the LNM+ and LNM- groups are presented in Table 2. Variables such as tumor size, invasion depth, tumor differentiation, INF pattern, tumor budding and LVI were significantly associated with LNM in both the training set and validation set according to the univariate analysis (Table 2). Furthermore, multivariate logistic regression analysis also showed that tumor size, invasion depth, tumor differentiation, INF pattern, tumor budding, and LVI were independent risk factors for LNM (Table 3).

Development and validation of the nomogram

Subsequently, a nomogram was developed based on six independent risk factors derived from the multivariate analysis (Figure 2). The point of each factor was proportional to its own β-coefficient resulted from logistic regression. Finally, the

Table 1 Patient clinicopathologic characteristics

Characteristics	No. of patients, *n* (%)
Gender	
Male	367 (77.4)
Female	107 (22.6)
Age (yr), median (range)	60 (38-84)
Tumor size (mm), median (range)	23 (3-73)
Tumor location	
Upper third	28 (5.9)
Middle third	303 (63.9)
Lower third	143 (30.2)
Paris classification	
0-I	95 (20.0)
0-II	309 (65.2)
0-III	70 (14.8)
Depth of invasion	
MM	152 (32.1)
SM1	80 (16.9)
SM2	106 (22.3)
SM3	136 (28.7)
Differentiation	
Well	103 (21.7)
Moderate	279 (58.9)
Poor	92 (19.4)
INF pattern	
INF-a	196 (41.4)
INF-b	232 (48.9)
INF-c	46 (9.7)
Tumor budding	64 (13.5)
LVI	27 (5.7)
LNM	
Yes	90 (19.0)
No	384 (81.0)
Dissected LN, median (range)	18.0 (12-53)

MM: Muscularis mucosae; SM1: Upper third of submucosa; SM2: Middle third of submucosa; SM3: Lower third of submucosa; INF pattern: Infiltrative growth pattern; LVI: Lymphovascular invasion; LNM: Lymph node metastasis; LN: Lymph node.

total points of each factor were added and visually corresponded to a predictive value for LNM. The ROC curve showed that this nomogram had good predictive performance both in the training set and in the validation set, with AUCs of 0.789 (95%CI: 0.737-0.841) and 0.827 (95%CI: 0.755-0.899), respectively (Figure 3).

DISCUSSION

Endoscopic resection has become one of the preferred treatment methods for superficial ESCC. Compared to surgery, it has fewer complications and a shorter recovery time[16]. Guidelines and studies have indicated that endoscopic

Table 2 Comparisons of clinicopathological characteristics between lymph node metastasis positive and lymph node metastasis negative group

Variable	Training set (*n* = 332)		*P* value	Validation set (*n* = 142)		*P* value
	LNM(-)	LNM(+)		LNM(-)	LNM(+)	
Gender			0.298			0.913
Male	201	51		84	20	
Female	68	12		31	7	
Age (yr), median (range)	60 (42-80)	60 (45-84)	0.204	60 (38-78)	58 (47-76)	0.522
Tumor size, (cm), mean ± SD	2.23 ± 1.21	2.65 ± 0.99	0.008	2.07 ± 0.97	2.61 ± 0.86	0.008
Tumor location, *n* (%)			0.095			0.702
Upper third	14	6		7	1	
Middle third	181	47		62	13	
Lower third	74	10		46	13	
Paris classification, *n* (%)			0.282			0.158
0-I	46	14		26	9	
0-II	186	37		74	12	
0-III	37	12		15	6	
Depth of invasion, *n* (%)			< 0.001			< 0.001
MM	100	3		47	2	
SM1	53	6		19	2	
> SM1	116	54		49	23	
Differentiation, *n* (%)			0.015			0.029
Well	77	8		15	3	
Moderate	148	38		80	13	
Poor	44	17		20	11	
INF pattern, *n* (%)			0.001			0.014
INF-a	125	13		53	5	
INF-b	123	41		52	16	
INF-c	21	9		10	6	
Tumor budding, *n* (%)			0.009			0.009
No	241	48		103	18	
Low	19	8		8	5	
High	9	7		4	4	
LVI, *n* (%)			0.005			0.004
Yes	10	8		4	5	
No	259	55		111	22	

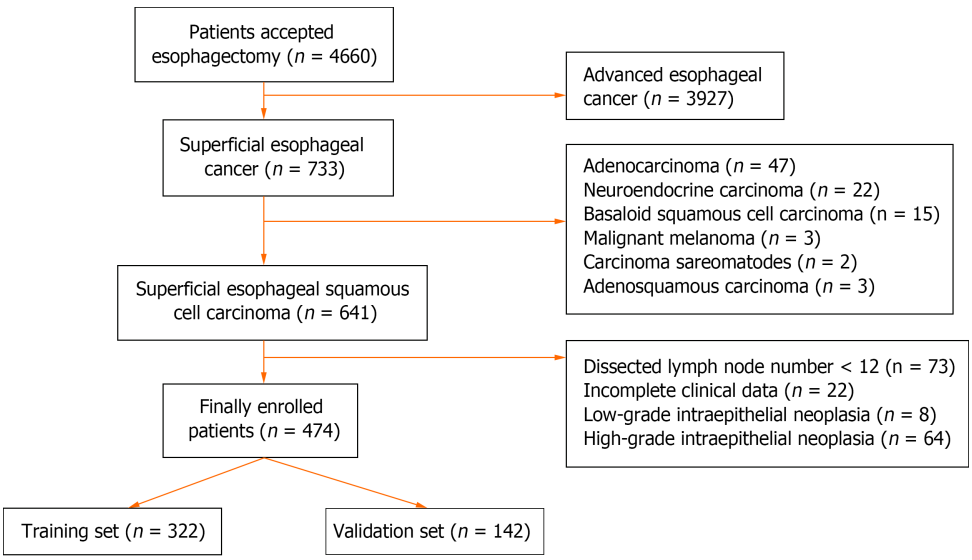
LNM: Lymph node metastasis; MM: Muscularis mucosae; SM1: Upper third of submucosa; INF pattern: Infiltrative growth pattern; LVI: Lymphovascular invasion.

submucosal dissection (ESD) and endoscopic submucosal tunnel dissection (ESTD) can be used to treat lesions limited to the muscularis mucosa and submucosal lesions with invasion depths ≤ 200 μm, which have no or an extremely low risk of lymph node metastasis[2,17-19]. The *en bloc* resection rate of ESD for superficial ESCC is 98.2%-100%, and the curative resection rate is 78.2%-96.1%[17-19]. However, patients with a high risk of LNM or noncurative endoscopic resection always need further treatment[4]. Therefore, summarizing post-ESD pathological characteristics and identifying risk factors for predicting LNM are critical to guide post-ESD treatment. In this study, we enrolled superficial ESCC patients who underwent esophagectomy and lymph node dissection and collected detailed pathological information, such as

Table 3 Multivariate logistic analysis of risk factors for lymph node metastasis in superficial esophageal squamous cell carcinoma

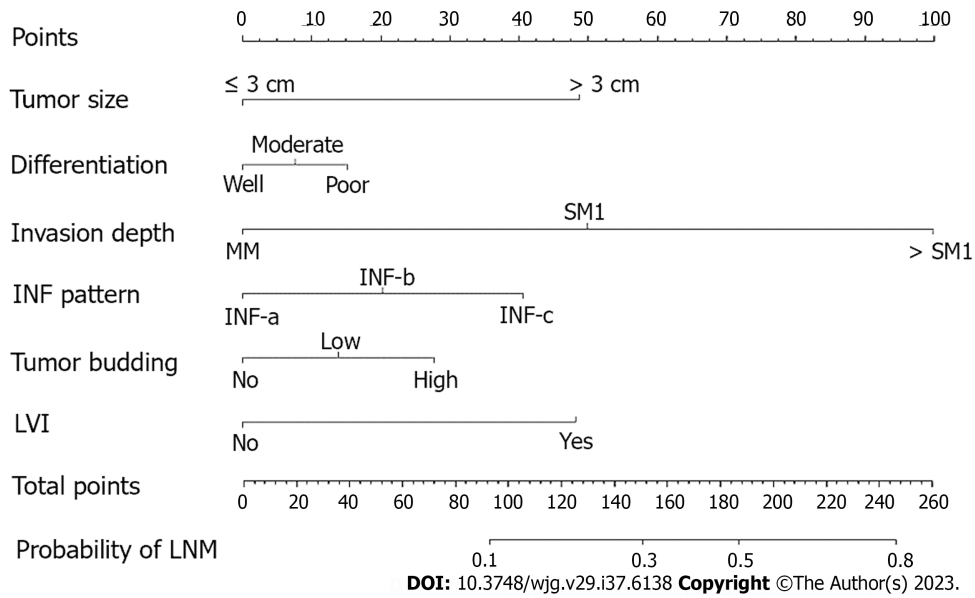
Factors	Training set			Validation set		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Tumor size (cm)	1.365	1.081-1.723	0.009	1.750	1.139-2.688	0.011
Invasive depth						
MM	Reference			Reference		
SM1	3.774	0.907-15.696	0.068	2.474	0.325-18.856	0.382
> SM1	15.517	4.707-51.158	0.001	11.031	2.463-49.401	0.002
Tumor differentiation						
Well or cis	Reference			Reference		
Moderate	2.423	0.807-9.451	0.072	0.715	0.179-2.856	0.635
Poor	3.670	1.465-9.198	0.006	4.078	0.977-17.026	0.054
INF pattern						
INF-a	Reference			Reference		
INF-b	3.205	1.637-6.274	0.001	3.262	1.114-9.552	0.031
INF-c	4.121	1.566-10.843	0.004	6.360	1.623-24.922	0.008
Tumor budding						
Low	2.114	0.875-5.108	0.096	3.815	0.978-14.813	0.054
High	3.905	1.387-10.995	0.010	4.769	1.315-17.290	0.017
No	Reference			Reference		
LVI						
No	Reference			Reference		
Yes	3.767	1.422-9.979	0.008	4.408	1.346-14.438	0.014

MM: Muscularis mucosae; SM1: Upper third of submucosa; INF pattern: Infiltrative growth pattern; LVI: Lymphovascular invasion.



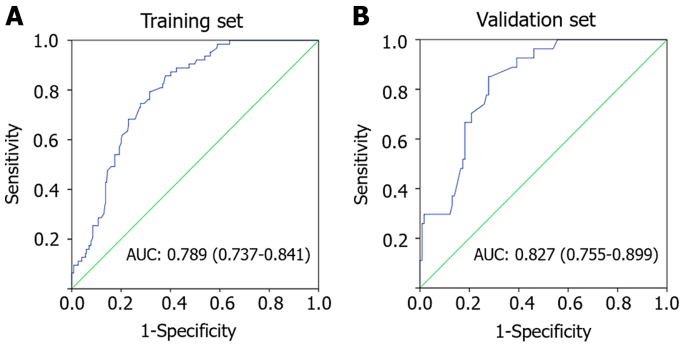
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Figure 1 Flowchart of enrollment process.



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Figure 2 Nomogram for predicting the probability of lymph node metastasis in superficial esophageal squamous cell carcinoma patients. Calculate the total points of different characteristics, and drop a vertical line from the total points row to obtain the probability of lymph node metastasis. MM: Muscularis mucosae; SM1: Upper third of submucosa; SM2: Middle third of submucosa; SM3: Lower third of submucosa; INF pattern: Infiltrative growth pattern; LVI: Lymphovascular invasion; LNM: Lymph node metastasis.



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Figure 3 Receiver operating characteristics curve of the nomogram for predicting lymph node metastasis. A: Receiver operating characteristics (ROC) curve in the training set; B: ROC curve in the validation set. AUC: Area under the curve.

tumor budding and tumor infiltrative growth pattern, to comprehensively analyze and identify the risk factors for LNM, providing favorable evidence for post-ESD treatment decisions.

Our findings in this study indicated that superficial ESCC patients with positive LNM were more likely to have larger tumors, deeper invasion, poorer differentiation, more INF-c infiltrative patterns, more high-grade tumor budding, and more positive LVI both in both the training and validation sets. Some previous studies have likewise indicated that tumor size is positively correlated with LNM risk[5-8]. Ruan *et al*[8] found that tumor size > 2 cm was an independent risk factor for LNM in superficial ESCC. Our results showed that patients with tumor size > 3 cm had a higher risk of LNM (Figure 2). In another study, it was reported that a 1-cm increase in esophageal tumor length increased the LNM risk by 3.55 times[20]. Therefore, it is necessary to measure the area of cancer cells after the ESD procedure. If possible, pathological recovery should be performed to determine tumor size after the ESD procedure since it is crucial for predicting LNM risk.

In addition, we found that tumor invasion depth is associated with LNM risk. It was reported that the LNM rates of T1a and T1b superficial ESCC were 6.2%-8.0% and 20.0%-29.3%, respectively[21-23]. Similarly, in our study, the LNM rate in T1a tumor was 3.29% (5/152) and increased to 26.40% (85/322) in T1b tumor. Further multivariate analysis showed that an invasion depth deeper than SM1 (OR: 15.517, 95%CI: 4.707-51.158) is an independent risk factor for LNM, which has been confirmed by other studies[5,6]. The esophageal submucosa is an area rich in lymphovascular network, and once intruded into the submucosa, tumor cells are more likely to infiltrate vasculature[24,25]. In our study, LVI positivity (OR: 3.767, 95%CI: 1.422-9.979) was also an independent risk factor for LNM. Therefore, additional treatment, such as surgery or chemoradiotherapy, should be recommended for post-ESD patients with submucosal invasion deeper than 200 μm and positive LVI.

It has been well established that the grade of tumor budding is positively correlated with the rate of LNM in solid cancers, including gastrointestinal cancers[26-28]. We found that high-grade tumor budding (OR: 3.905, 95%CI: 1.387-10.995) was positively correlated with LNM risk in superficial ESCC. Similarly, Li *et al*[29] checked tumor budding in pT1b ESCC by using Pan-CK immunohistochemical (IHC) staining and found that the tumor budding level is an excellent predictor of LNM and patient survival time. However, there is no gold standard for differentiating the threshold value of tumor budding in superficial ESCC specimens, and a clear and standardized method for distinguishing and reporting tumor budding in superficial ESCC is urgently needed. In addition, tumor budding status in post-ESD specimens should be carefully assessed and reported.

The Japanese guidelines recommend that tumor infiltrative growth patterns should be reported in esophageal cancer [10]. Some studies have identified infiltrative type c (INF-c) as associated with deep tumor invasion, poor tumor differentiation, and a high risk of LNM[12,30,31]. In our study, multivariate logistic regression identified INF-b (OR: 3.205; 95%CI: 1.637-6.274), INF-c (OR: 4.121, 95%CI: 1.566-10.843) and poor differentiation (OR: 3.670, 95%CI: 1.465-9.198) as independent risk factors for LNM. Invasion of surrounding tissue by cancer cells is a key step in tumor progression and metastasis[32]. As the tumor grows, the morphology and behaviour of cancer cells at the tumor front undergo epithelial mesenchymal transition, detaching from the tumor body and infiltrating deep into the submucosa or even deeper[30]. Poorly differentiated tumors are more likely to have LVI and LNM in superficial ESCC, as previously reported[6,8]. Therefore, a detailed assessment of tumor differentiation, INF pattern, tumor budding, and LVI is critical for predicting LNM risk in post-ESD patients.

Overall, we analyzed the risk factors for LNM in superficial ESCC patients by evaluating detailed pathological characteristics and developed a nomogram by incorporating six variables, including tumor size, invasion depth, tumor differentiation, tumor budding, tumor infiltrative growth pattern and LVI. This nomogram showed good predictive performance, with an AUC of 0.789 (95%CI: 0.737-0.841) in the training set and 0.827 (95%CI: 0.755-0.899) in the validation set. Although the data for this nomogram come from surgical specimens, we believe it is also applicable to post-ESD patients, as all six enrolled predictors can be easily obtained from post-ESD specimens. The use of this predictive nomogram will facilitate the assessment of LNM, thus providing references for guiding post-ESD treatment. However, this is a single-center retrospective study. More multicenter studies are needed to further confirm the reliability of the nomogram.

In addition, this study has some limitations. First, this is a retrospective study, and bias in case selection cannot be avoided. Second, differences in surgical procedures and chronological differences in pathological diagnostic criteria may affect the consistency of the results. D2-40 and CD34 IHC staining were not used in LVI diagnoses in this study, which would lead to underestimating the positivity of LVI. Finally, the LNM rate of superficial ESCC in this study cannot accurately represent the overall LNM rate because patients who underwent ESD were excluded because their postoperative LNM rate could not be calculated.

CONCLUSION

In conclusion, we identified the risk factors for LNM in superficial ESCC patients and developed a useful nomogram for predicting LNM risk by integrating all significant risk factors. This nomogram model will facilitate decision-making regarding additional treatment options in post-ESD patients.

ARTICLE HIGHLIGHTS

Research background

Endoscopic resection of superficial esophageal squamous cell carcinoma (ESCC) is limited to lesions that have no or low risk of lymph node metastasis (LNM). Patients with a high risk of LNM always need further treatment after endoscopic resection.

Research motivation

Accurately assessing the LNM risk is critical for additional treatment choices for superficial ESCC patients who underwent endoscopic resection.

Research objectives

This study aimed to analyze the risk factors for LNM and develop a LNM predictive nomogram for superficial ESCC patients.

Research methods

Clinical and pathological data from superficial ESCC patients underwent esopha-gectomy from January 1, 2009, to January 31, 2016, were collected and analyzed. A nomogram was performed using R software (version 4.1.3) based on six risk factors of LNM.

Research results

A total of 474 superficial ESCC patients were enrolled. The prevalence of LNM was 3.29% for intramucosal cancer and

increased to 26.40% for submucosal cancer. A nomogram incorporating six variables, including tumor size, invasion depth, tumor differentiation, tumor budding, tumor infiltrative growth pattern, and lymphovascular invasion, was successfully developed.

Research conclusions

We developed a useful nomogram model to predict LNM risk for superficial ESCC patients, which will facilitate additional treatment decisions for patients who underwent endoscopic resection.

Research perspectives

The nomogram model is a simple and useful tool to facilitate the prediction of LNM risk for superficial ESCC patients.

FOOTNOTES

Author contributions: Wang J and Deng K designed and performed the research; Wang J, Zhang X and Gan T collected and analyzed the data; Wang J and Zhang X wrote the manuscript; Deng K, Rao NN and Yang JL supervised the report and revised the manuscript.

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of West China Hospital of Sichuan University, approval No. 2015(159).

Informed consent statement: All study participants who underwent esophagectomy were provided informed written consent prior to surgery.

Conflict-of-interest statement: The authors declare no conflicts of interest for this article.

Data sharing statement: No additional data are available.

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ORIGINAL ARTICLE

Basic Study

5-methoxytryptophan induced apoptosis and PI3K/Akt/FoxO3a phosphorylation in colorectal cancer

Tian-Lei Zhao, Yue Qi, Yi-Fan Wang, Yi Wang, Hui Liang, Ya-Bin Pu

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Abstract

BACKGROUND

Colorectal cancer (CRC) is a highly prevalent malignancy worldwide, and new therapeutic targets urgently need to be found to prolong patient survival. 5-methoxytryptophan (5-MTP) is a tryptophan metabolite found in animals and humans. However, the effects of 5-MTP on proliferation and apoptosis of CRC cells are currently unknown.

AIM

To investigate the effects of 5-MTP on the proliferation, migration, invasion, and apoptosis abilities of CRC cells. Additionally, we seek to explore whether 5-MTP has the potential to be utilized as a drug for the treatment of CRC.

METHODS

In order to evaluate the effect of 5-MTP on CRC cells, a series of experiments were conducted for evaluation. Colony formation assay and Cell Counting Kit 8 assays were used to investigate the impact of 5-MTP on the proliferation of CRC cell lines. Cell cycle assays were employed to examine the effect of 5-MTP on cellular growth. In addition, we investigated the effects of 5-MTP on apoptosis and reactive oxygen species in HCT-116 cells. To obtain a deeper understanding of how 5-MTP affects CRC, we conducted a study to examine its influence on the PI3K/Akt signaling pathway in CRC cells.

RESULTS

This article showed that 5-MTP promoted apoptosis and cell cycle arrest and inhibited cell proliferation in CRC cells. In many articles, it has been reported that PI3K/Akt/FoxO3a signaling pathway is one of the most important signaling pathways involved in internal regulating cell proliferation and differentiation.

Nevertheless, 5-MTP combined with PI3K/Akt/FoxO3a signaling pathway inhibitors significantly promoted apoptosis and cell cycle arrest and inhibited cell proliferation in CRC cells compared with 5-MTP alone in our study.

CONCLUSION
Therefore, there is strong evidence that 5-MTP can be used as an effective medicine for CRC treatment.

Key Words: Colorectal cancer; 5-methoxytryptophan; Apoptosis; Cell cycle arrest; PI3K/Akt signaling pathway

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Core Tip: Colorectal cancer (CRC) is insensitive to radiotherapy and has poor therapeutic efficacy, and there is an urgent need to find new therapeutic targets to prolong patient survival. 5-methoxytryptophan (5-MTP) is a tryptophan metabolite present in both animals and humans. 5-MTP has a wide range of physiological functions such as stabilizing endothelial function, anti-inflammation, and antioxidant to prevent cellular damage. Our study found that 5-MTP combined with an inhibitor of the PI3K/Akt/FoxO3a signaling pathway significantly promoted apoptosis and cell cycle arrest and inhibited cell proliferation in CRC cells compared with 5-MTP alone.

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INTRODUCTION

The incidence and mortality of colorectal cancer (CRC) have declined over the past 30 years[1]. However, CRC morbidity and mortality are rising among young adults[2,3]. Patients with advanced CRC have a poor prognosis. Pathologic classification is used to assess prognosis and inform the treatment of CRC[4]. Great efforts have been made to develop noninvasive biomarkers to detect early cancers and/or reflect individual cancer risk, which is critical for reducing CRC mortality[5,6]. However, little progress has been made in improving disease-free survival in CRC patients. Because the pathomechanism of CRC progression is not fully understood, more studies are needed to discover and develop effective medicines for CRC treatment.

Traditional Chinese medicine (TCM) has a long history of treating malignant tumors and is of great significance in reducing the recurrence and metastasis rate, reducing adverse reactions to chemotherapy, prolonging survival, and improving quality of life[7,8]. Also, some previous study demonstrate the significant change of tryptophan after TCM treatment in cancer patients[9,10]. However, the potential anti-cancer role of tryptophan-related metabolites is still yet to be elucidated. 5-methoxytryptophan (5-MTP) is a tryptophan metabolite found in animals and humans[11]. Tryptophan is first hydroxylated by tryptophan type 1 or type 2 hydroxylases (TPH-1, TPH-2) to generate 5-hydroxytryptophan, then methylase hydroxyindole-O-methyltransferase further methylates 5-hydroxytryptophan to 5-MTP[12]. 5-MTP has anti-inflammatory and anti-fibrotic effects and has become an indispensable therapeutic factor in diseases such as myocardial infarction and renal fibrosis[11,12]. However, there have not been any reports of CRC, so more specific roles need to be further teased out.

In addition, 5-MTP was demonstrated for the first time to inhibit proliferation, invasion, and migration at the CRC cell level; promote apoptosis, reactive oxygen species (ROS) levels, and cell cycle arrest; and combined with PI3K/Akt/p-FoxO3a signaling pathway to play a stronger therapeutic role. Therefore, 5-MTP may be used as an adjuvant new strategy for treating CRC.

MATERIALS AND METHODS

Cell culture

The three major kinds of human colon cancer cell lines, including HCT-116, HCT15, and SW480, were purchased from American Type Culture Collection (https://www.atcc.org/) and cultured in McCoy's 5A (Gibco, United States), RPMI-1640 (Gibco, United States) and DMEM medium (Gibco, United States), respectively, supplemented with 10% fetal bovine serum (Gibco, United States) and 1% penicillin/streptomycin (Sangon Biotech, China). The colon cancer cell lines were incubated in incubator (Thermo Scientific, United States) with 5% CO_2 at 37 °C. We added 5-MTP (MedChemExpress, United States) in different concentrations during cell culture.

Cell cycle assays

Cells were digested to make single-cell suspensions at logarithmic phase and uniformly seeded in 12-well plates at 1 × 10^5 cells per well. After 48 h, cells were collected and washed with 1 mL of precooled phosphate buffered saline (PBS). The cell pellet was resuspended with 1 mL of precooled 70% ethanol and fixed overnight at 4 °C. On the second day, 70% ethanol was discarded by centrifugation at 1000 × *g* for 5 min at 4 °C, washed with 1 mL of precooled PBS. Each sample was added with 500 μL propyl iodide (PI) staining solution, gently mixed, incubated at 37 °C in the dark for 30 min, and the cells were filtered with a 400-mesh cell strainer to detect the cell cycle of each group by flow cytometry.

Apoptosis assays

Colon cancer cells in the logarithmic growth phase were seeded in 6-well plates, and 5-MTP-treated cells were added after the cells attached. Cells in each group were digested with ethylene diamine tetraacetic acid-free trypsin, washed with PBS, and collected at 1000 × *g* for 5 min. Binding buffer 100 μL was used to resuspend cells, and fluorescein isothiocyanate staining solution 5 μL and PI staining solution 10 μL were added. They were blown and mixed well by the pipette. The cells were allowed to stand at room temperature for 15 min. Before loading the machine, 400 μL of binding buffer solution was added to each tube and mixed well so that the final system was 500 μL. Apoptosis of cells in each group was detected by flow cytometry.

ROS assays

To test the ROS level in each group of cells, the colon cancer cells were inoculated into 6-well plates at logarithmic growth phase. The 2′,7′-dichlorofluorescin diacetate (Sangon Biotech, China) was added, incubated in a cell culture incubator at 37 °C for 20 minutes, and observed under a confocal microscope. Similarly, Hoechst 33342 Viable Cell Staining Solution (Sangon Biotech, China) was added and incubated in a 37 °C cell culture incubator for 10 min and observed under a confocal microscope.

JC-10 assays

Cells were treated with 5-MTP at various concentrations for 48 h at 12-well plate. Before incubation, 25 μL of 200X JC-10 concentrate (Sangon Biotech, China) was added to a 5 mL assay buffer to dilute JC-10. 500 μL of JC-1 solution was added to each well and incubated at 37 °C for 20 min. After incubated, washed twice with staining buffer, added 500 μL cell culture medium, and observed under an inverted fluorescence microscope.

Western blot assays

CRC cell lines were treated with 5-MTP for 48 h at logarithmic growth phase before protein extraction. Then, cells were washed with precooled PBS, 500 μL of RIPA buffer (Beyotime, China) was added to each dish, scraped by cell scraper, and transferred to a new centrifuge tube, lysed on ice for 30 min and centrifuged (12000 × *g*, 15 min, centrifugation radius 30 cm) to collect the supernatant. After adjusting the protein concentration, 4 × loading buffer was added and placed in a metal bath for heating denaturation (95 °C, 10 min). Sodium-dodecyl sulfate gel electrophoresis electrophoresis was performed to separate proteins (80 V), transferred by wet membranes (300 mA, 90 min), and blocked with 5% skimmed milk overnight (4 °C). Primary antibodies were used to incubate overnight (4 °C), including anti-AKT (1:1000, Cell Signaling Technology, #4685), anti-p-AKT (1:1000, Cell Signaling Technology, #13038), anti-FoxO3a (1:1000, Cell Signaling Technology, #12829), anti-p-FoxO3a (1:1000, Cell Signaling Technology, #9466), anti-β-actin (1:1000, Cell Signaling Technology, #4970), anti-Bax (1:1000, Cell Signaling Technology, #41162), anti-Bcl2 (1:1000, Cell Signaling Technology, #15071), anti-PARP (1:1000, Cell Signaling Technology, #9532), anti-Caspase3 (1:1000, Cell Signaling Technology, #9662), anti-Bim (1:1000, Cell Signaling Technology, #2933), anti-P27 (1:1000, Cell Signaling Technology, #3688) and anti-Cyclin D1 (1:1000, Cell Signaling Technology, #55506). Membranes were washed, incubated with secondary antibodies (1:10000) for 1 h at room temperature, and finally developed with ECL (Jiapeng Biotech, China), cassette luminescence, and band data analysis was performed by ImageJ software.

Colony formation assay

Cells were seeded in six-well plates at 800 cells/well in cell suspension, and 5-MTP was added when the cells were completely attached and cultured in an incubator for about 10 d, and > 50 cells/colony were considered as one clone, stained with crystal violet, photographed and counted.

Cell Counting Kit 8 assays

After adjusting the cell density of each group to 1 × 10^4 cells/mL, 200 μL of cell suspension was added to a 96-well plate and cultured at 37 °C for 0, 24, 48, and 72 h. Following this, 10 μL of Cell Counting Kit 8 (CCK-8) solution was added to each well. After incubation at 37 °C for 2 h, absorbance values were measured using a microplate reader (Flash Biotech, China) at 450 nm.

Migration and invasion assays

Invasion assays were performed at 37 °C using transwell chambers coated with matrigel. The cell density was adjusted to 4 × 10^5 cells/mL, 100 μL was added to the upper transwell chamber, and then 600 μL of medium containing 10% foetal bovine serum was added to the lower chamber. After incubation at 37 °C for 24 h, the cells on the lower surface of the membrane were fixed with 4% paraformaldehyde (Sangon Biotech, China) for 20 min and stained with 500 μL, 1% crystal violet (Sangon Biotech, China) for 30 min. Five fields were randomly selected to observe the number of cells in invasion

using an inverted light microscope and photographed.

Statistical analysis

GraphPad Prism (v9.0.0) software was used for data processing. Measurement data were expressed as mean ± SD. An independent sample *t*-test was performed between two groups. A one-way analysis of variance was used to compare the means between multiple groups. $P < 0.05$ was considered with statistically significant.

RESULTS

5-MTP inhibited human CRC cell proliferation

ATL was selected for further analysis, and its structure is shown in Figure 1A. To validate the role of 5-MTP in CRC, we selected HCT116, HCT15, and SW480 for subsequent experiments. In HCT116, HCT15, and SW480 cells, the results of CCK8 and colony formation assay showed that 5-MTP inhibited CRC cell proliferation gradually with increasing drug concentration (Figures 1B-J). These data suggested that 5-MTP inhibited human CRC cell proliferation.

5-MTP induced apoptosis and ROS in HCT-116 cells

Next, we examined the effects of 5-MTP on CRC cell apoptosis by measuring levels of JC-1 and ROS, among others. In HCT-116 cells, Hoechst staining showed that 5-MTP inhibited CRC cell activity progressively (Figures 2A and B). Flow cytometry results showed that 5-MTP significantly inhibited JC-1 levels and promoted the apoptosis rate and ROS levels (Figures 2C-H).

5-MTP induced cell cycle arrest in HCT-116 cells

Flow cytometry results showed that the peak value became higher in the G2/M phase, that is, 5-MTP-induced HCT116 cell cycle arrest (Figures 3A and B). These results demonstrated that 5-MTP induced cell cycle arrest in HCT-116 cells.

5-MTP inhibited migration and invasion in HCT-116 cells

To further investigate the effect of 5-MTP on CRC cells, invasion and migration assays showed that 5-MTP inhibited CRC cell invasion and migration (Figures 4A-C). Ly294002 (PI3K) inhibitors have been shown to play a role in various tumors, including proliferation, metastasis, and apoptosis[13-15]. However, the effects of 5-MTP combined with ly294002 on invasion and migration in CRC cells are further enhanced is unknown. It was found that 5-MTP combined with ly294002 resulted in significantly less cell invasion and migration than 5-MTP (Figures 4D and F).

5-MTP induced dose-response and time course inhibition of PI3K/Akt/FoxO3a signaling pathway in HCT-116 cells

The PI3K/Akt/FoxO3a signaling pathwa plays an important role in various pathological processes in various tumors, including proliferation, metastasis, and apoptosis[16-18]. However, Figure 4 confirmed that 5-MTP combined with ly294002 inhibited CRC cell invasion and migration. The next experimental results showed that 5-MTP inhibited the relative protein levels of p-Akt/t-AKT and p-FoxO3a/t-FoxO3a with increasing drug concentration in HCT116 cells (Figures 5A-D). It was further found that the relative protein levels of p-Akt/t-AKT and p-FoxO3a/t-FoxO3a were also inhibited over time when the 5-MTP concentration was fixed, confirming the previous conclusions (Figures 5E-H). Meanwhile, western blot results showed that Caspase3, PARP, and BAX protein levels were increased, while Bcl2 protein levels were significantly decreased (Figures 6A-D). These results are the same as those in the Figure 1. Overall, our data indicated that 5-MTP induced dose-response and time course inhibition of PI3K/Akt/FoxO3a signaling pathway in HCT-116 cells.

Apparent correlations between 5-MTP-induced cell cycle and apoptosis-associated proteins or inhibition of PI3K/Akt/FoxO3a signaling pathway in HCT-116 cells

To further verify that 5-MTP combined with PI3K/Akt/p-FoxO3a signaling pathway inhibitors played a better therapeutic effect, ly294002 (PI3K) inhibitor and AKT small interfering RNA (siRNA) were transfected into HCT116 cells. The results showed that the relative protein levels of p-Akt/AKT and p-FoxO3a/FoxO3a were significantly decreased in the 5-MTP combined with the ly294002 group compared with the 5-MTP group. At the same time, we also found that Cyclin D1 and P27 protein levels were significantly decreased while Bim was significantly increased in the 5-MTP combined ly294002 group compared with the 5-MTP group, suggesting that 5-MTP-induced cell cycle-related proteins are associated with the PI3K/Akt/p-FoxO3a signaling pathway (Figures 7A-D). It was further found that the 5-MTP combined with the AKT siRNA group came to the same conclusion as the 5-MTP group (Figures 7E-H).

DISCUSSION

CRC is a highly prevalent disease in countries around the world, and the incidence increases with age, accounting for nearly one-third of patients over 75 years of age[19,20]. The number of patients with early-onset CRC no more than 50 years of age also cannot be ignored[3,21]. The main treatments for CRC are surgery, radiotherapy, and chemotherapy [22]. However, 35% of patients are found to be in the advanced stage and lose the chance of radical surgery[23]. With the

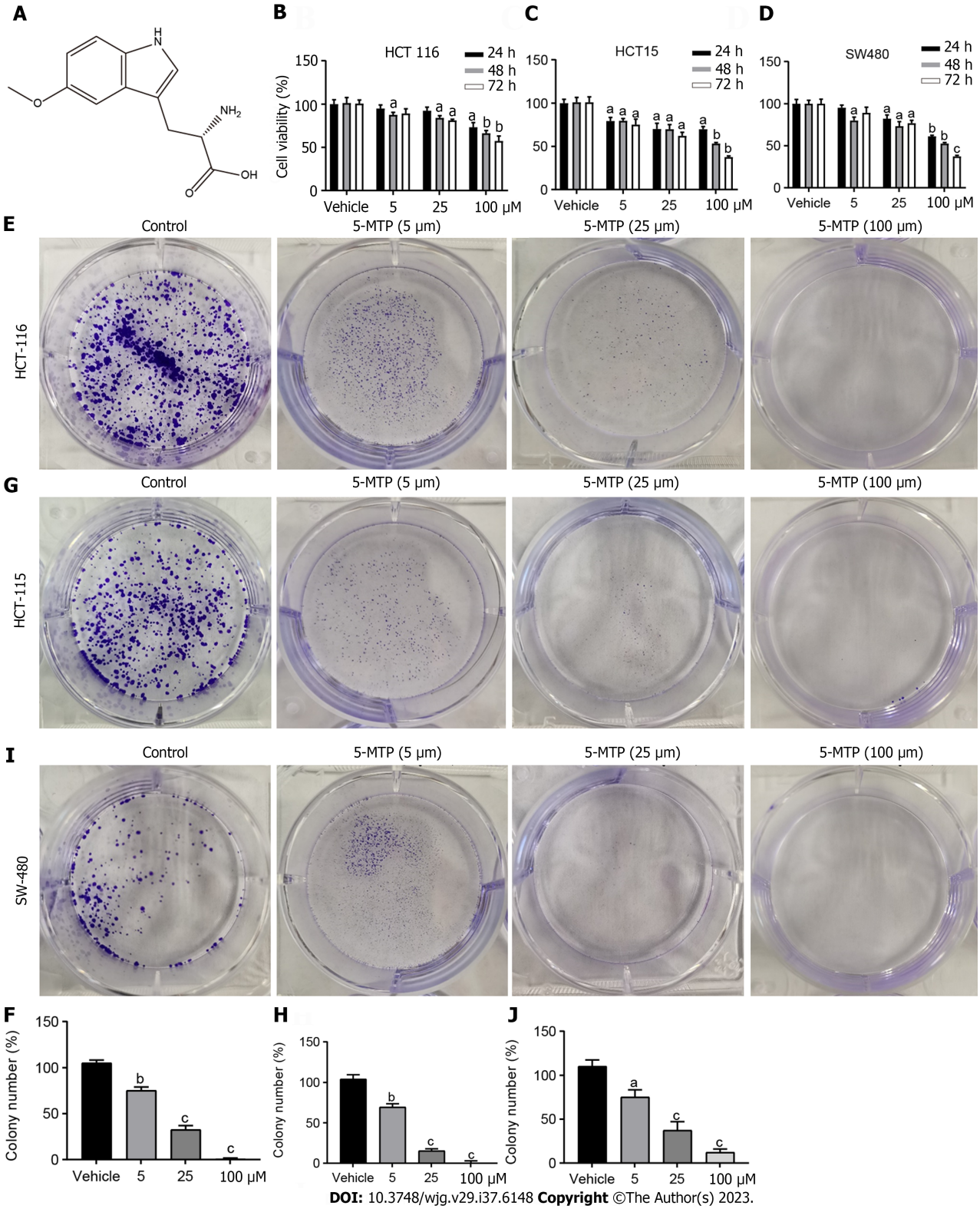
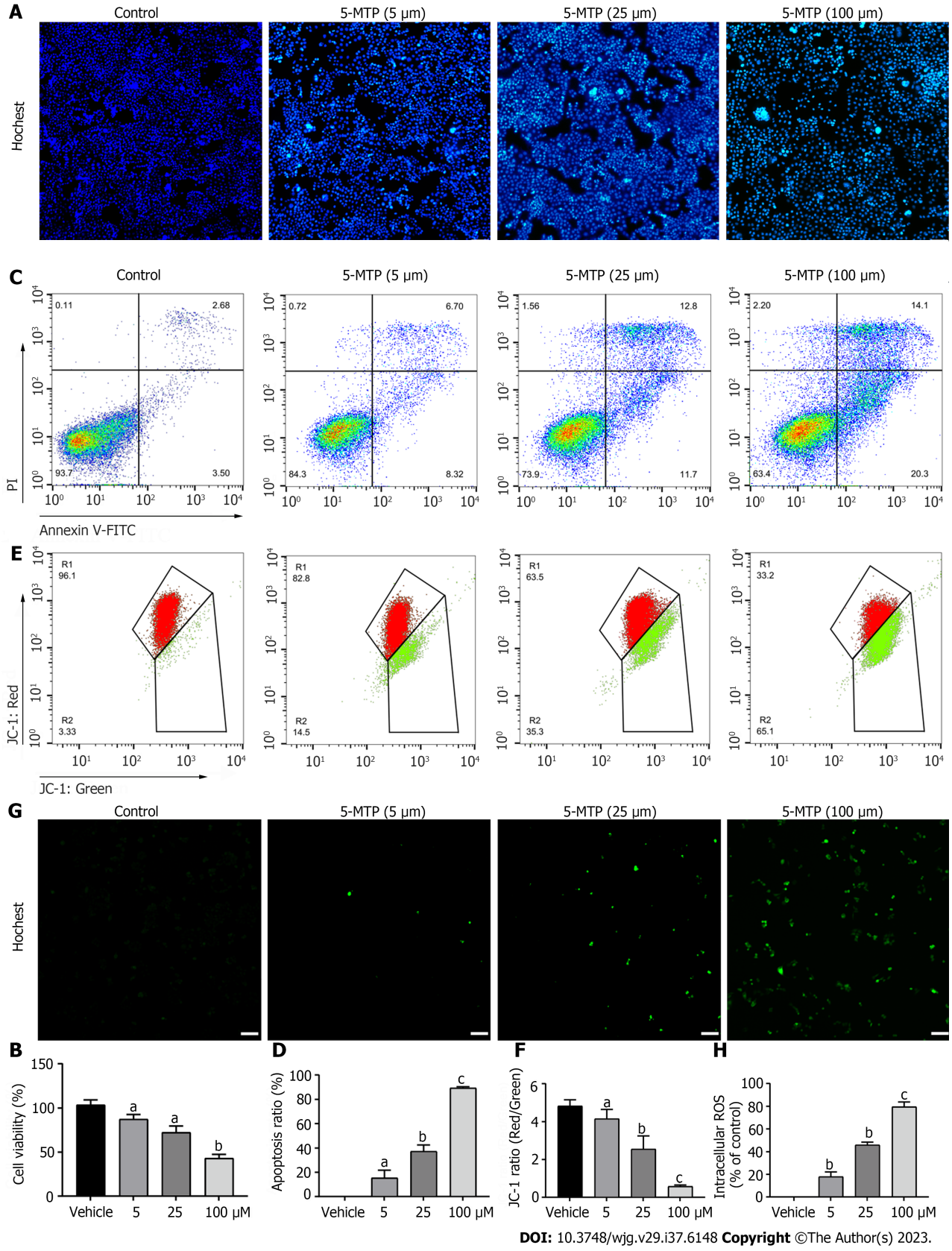


Figure 1 5-methoxytryptophan inhibited human colorectal cancer cell proliferation. A: The structure of 5-methoxytryptophan (5-MTP) is shown; B-D: The Cell Counting Kit 8 assays were used to measure the viability of the colorectal cancer cells treated with 5-MTP at the dose of 5, 25 and 100 μM; E-J: Colony formation assay were used to measure the viability of the HCT-116 (E and F), HCT-15 (G and H), SW480 (I and J) cells treated with 5-MTP at the dose of 5, 25 and 100 μM. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. 5-MTP: 5-Methoxytryptophan.

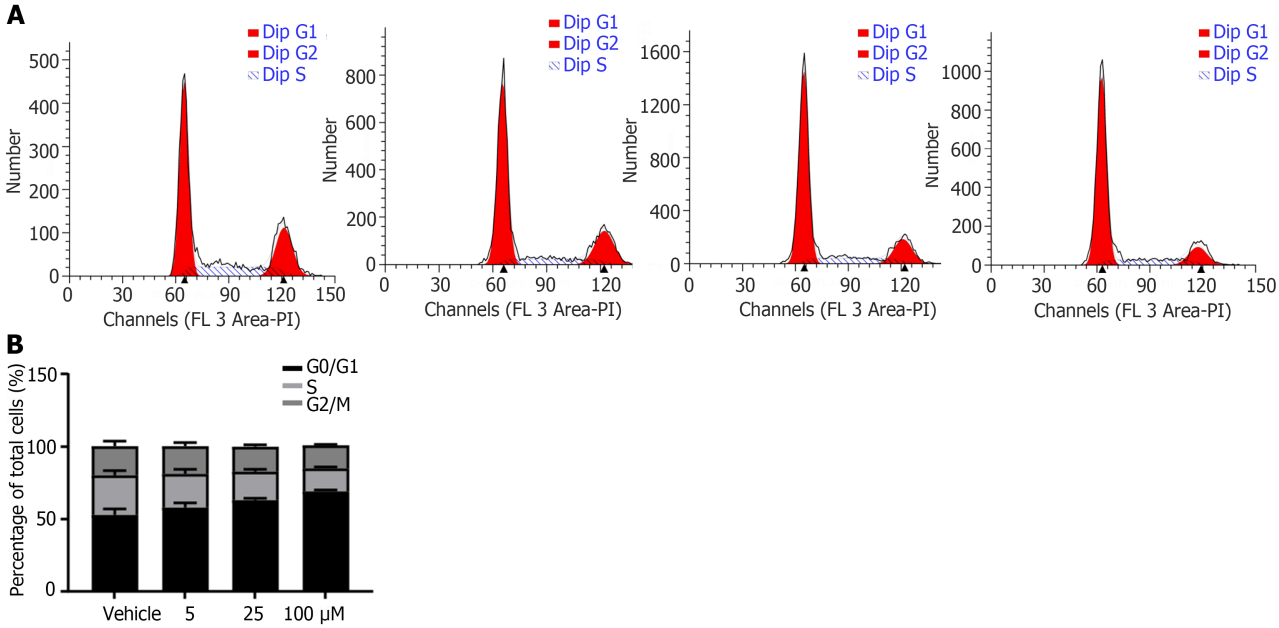
in-depth study of the formation, development, and treatment of CRC from the molecular and genetic levels, adjuvant therapy with TCM monomers optimizes the balance between tumor cell killing and non-targeted effects with the advantage of specific selection combined with oncogenic sites[24,25].

5-MTP, with a molecular formula of $C_{12}H_{14}N_2O_3$ and a molecular weight of 234.251, is a newly identified tryptophan metabolite produced by cells, such as fibroblasts, renal epithelial, smooth muscle and vascular endothelial cells[11,26,27]. Current studies have shown that 5-MTP has various physiological functions, such as stabilizing endothelial function, anti-



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Figure 2 5-methoxytryptophan induced apoptosis and reactive oxygen species in HCT-116 cells. A and B: The Hoechst staining was used to measure the viability of HCT-116 cells treated with 5-methoxytryptophan (5-MTP) at the dose of 5, 25 and 100 μM; C and D: Apoptosis was detected by flow cytometry by double staining with PI and Annexin V of HCT-116 cells treated with 5-MTP at the dose of 5, 25 and 100 μM; E and F: Flow cytometry were used to analyses the JC-1 level of HCT-116 cells treated with 5-MTP at the dose of 5, 25 and 100 μM; G and H: 2',7'-dichlorofluorescin diacetate assays were used to test the reactive oxygen species production of HCT-116 cells treated with 5-MTP at the dose of 5, 25 and 100 μM. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. 5-MTP: 5-Methoxytryptophan; ROS: Reactive oxygen species; PI: Propyl iodide.



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Figure 3 5-methoxytryptophan induced cell cycle arrest in HCT-116 cells. A and B: The HCT-116 cells were treated with 5-methoxytryptophan at the dose of 5, 25, and 100 μM. The cycle arrest was detected by flow cytometry.

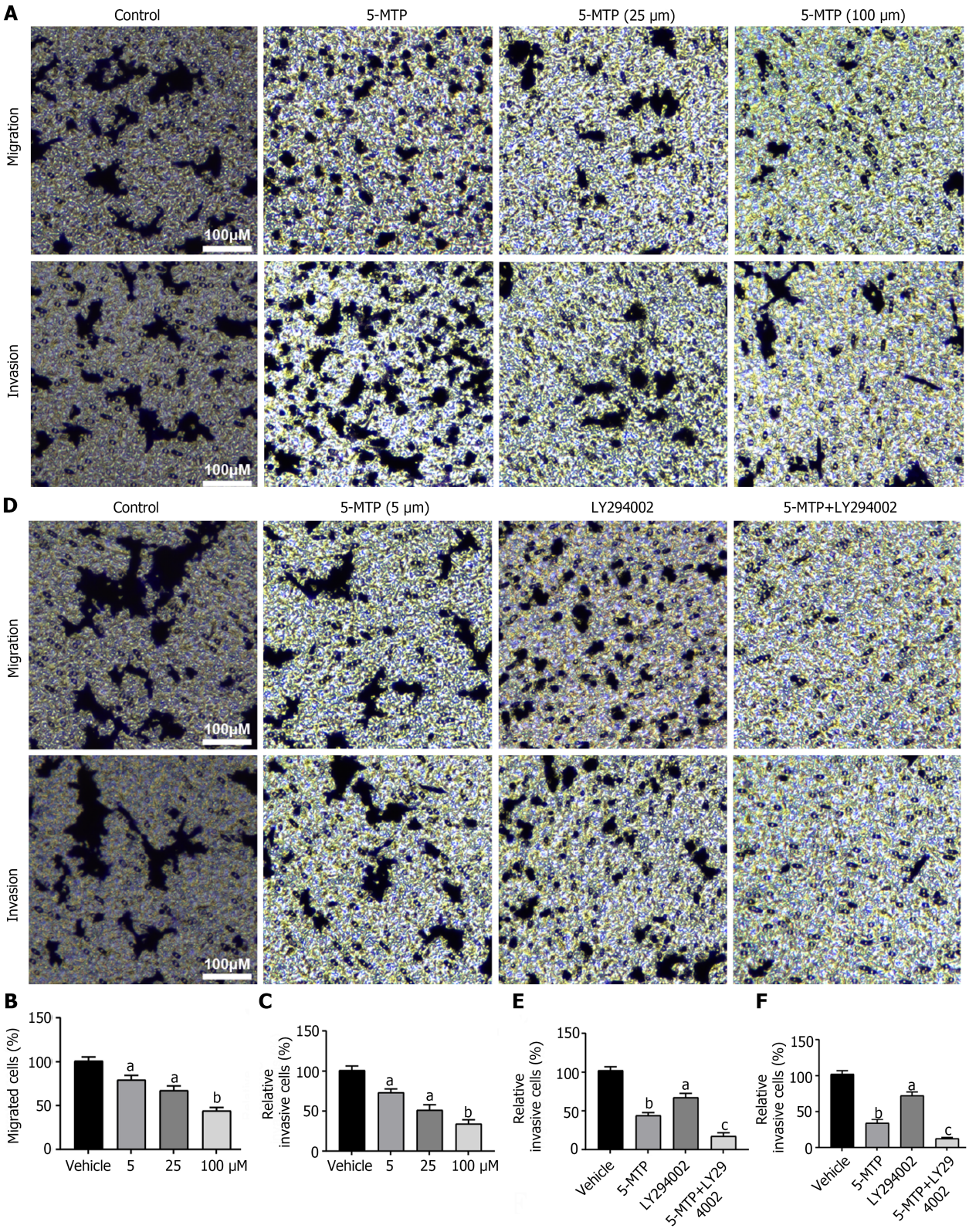
inflammation, and anti-oxidation[11,28]. 5-MTP has been shown to be involved in regulating inflammatory responses and can maintain endothelial cell tight junctions to some extent[29]. Proinflammatory factors inhibit the expression of TPH-1, a key enzyme in 5-MTP synthesis in endothelial cells, reducing 5-MTP production, which leads to endothelial barrier disruption[30]. In addition, 5-MTP plays an important vaso-protective function by regulating vascular permeability, controlling systemic inflammation, and defending against systemic inflammation and multiple organ failure[31]. It has been found that 5-MTP may regulate cardiomyocyte growth-associated proteins, cytoskeleton, redox, and protein folding, thereby promoting wound healing, and preventing damaged cell death by maintaining redox balance and reducing endoplasmic reticulum stress[32].

PI3K/Akt signaling pathway is one of the most important signaling pathways involved in internal regulating cell proliferation and differentiation[33]. Akt is essential for the regulation of cell migration and growth[34]. P-Akt is the active form of Akt and is closely associated with CRC[35]. P-Akt plays an important role in CRC progression[36]. FOXO family members are major effector proteins of the PI3K/Akt signaling pathway[37]. The FOXO family is a subclass of the FOX family that contains four isoforms (FOXO1, FOXO3, FOXO4, and FOXO6), all of which structurally contain a Forkhead DNA-binding domain that binds to the same DAF-16-binding element binding site within the target gene promoter through a DNA domain, and thus regulates target gene expression[38]. FOXO3a has been found to be an important transcription factor for genes involved in cell cycle progression, apoptosis, metabolism, differentiation, and autophagy[39]. PI3K/Akt regulates the expression of a series of genes related to cell proliferation, apoptosis, and cycle arrest downstream of FOXO by working on phosphorylation sites on FOXO protein[40]. FOXO3A is involved in the biological effects of CRC, and this process may be related to cell proliferation and apoptosis[18]. For example, many FOXO factors are phosphorylated by Akt in the presence of growth factors, resulting in their translocation from the nucleus to the cytoplasm; PI3K/AKT pathway mediates hyperglycemia-induced apoptosis in ventricular myocytes of neonatal rats through translocation of FOXO3a to the nucleus[17,41].

In this study, the efficacy of 5-MTP in the treatment of CRC by *in vitro* cell experiments was investigated, and the effects of 5-MTP on CRC cells under different conditions were examined using CCK-8, colony formation, apoptosis rate detection, JC-1 level, ROS level and cell cycle detection. The results showed that 5-MTP promoted apoptosis and cell cycle arrest and inhibited cell proliferation in CRC cells. In subsequent experiments, the effect of 5-MTP combined with PI3K/Akt/p-FoxO3a signaling pathway inhibitors in the treatment of CRC was focused, confirming that combined PI3K/Akt/p-FoxO3a signaling pathway inhibitors played a more effective role in the treatment of CRC. There was a correlation between 5-MTP-induced cyclin-related proteins and PI3K/Akt/p-FoxO3a signaling pathway.

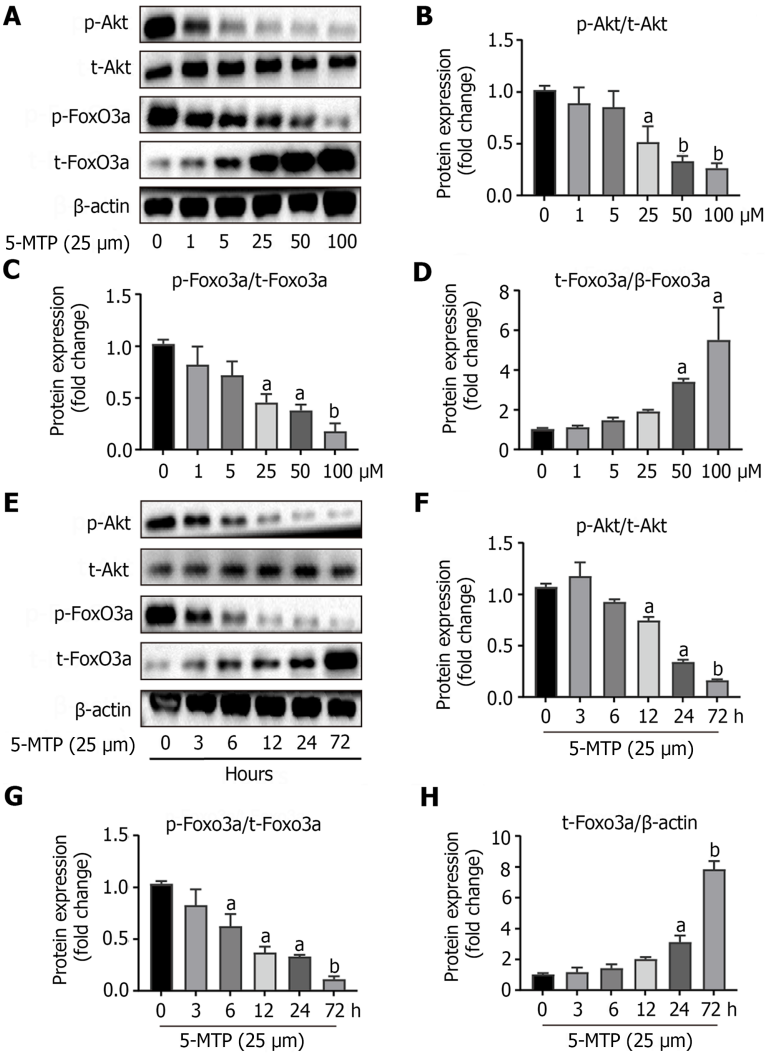
CONCLUSION

In summary, 5-MTP could inhibit proliferation and promote apoptosis of CRC cells, and combined ly294002 or AKT inhibitors played a more effective role in treating CRC. This study provided theoretical guidance for the clinical treatment of CRC with 5-MTP. However, there were some limitations, and *in vivo* experiments such as mice are still needed for further validation in the future.



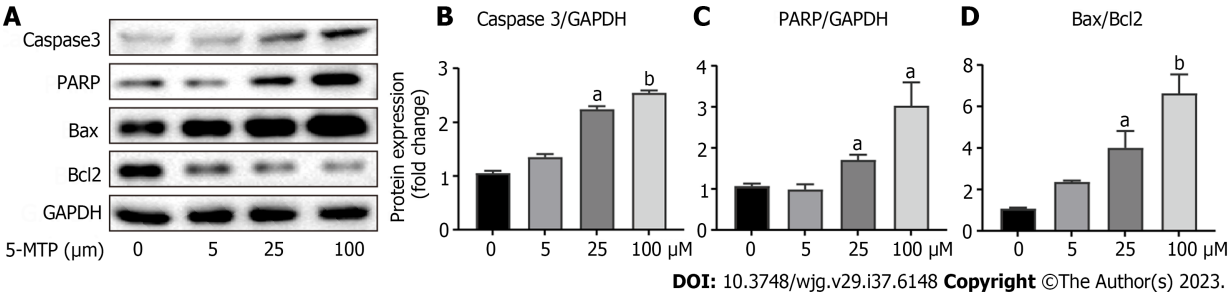
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Figure 4 5-methoxytryptophan inhibited migration and invasion in HCT-116 cells. A-C: Detection of migration and invasion in HCT-116 cells were treated with 5-methoxytryptophan (5-MTP) at the dose of 5, 25 and 100 μM by transwell test; D-F: The HCT-116 cells were treated with 5-MTP and/or LY294002. Detection of migration and invasion in HCT-116 cells by transwell test. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. 5-MTP: 5-Methoxytryptophan.



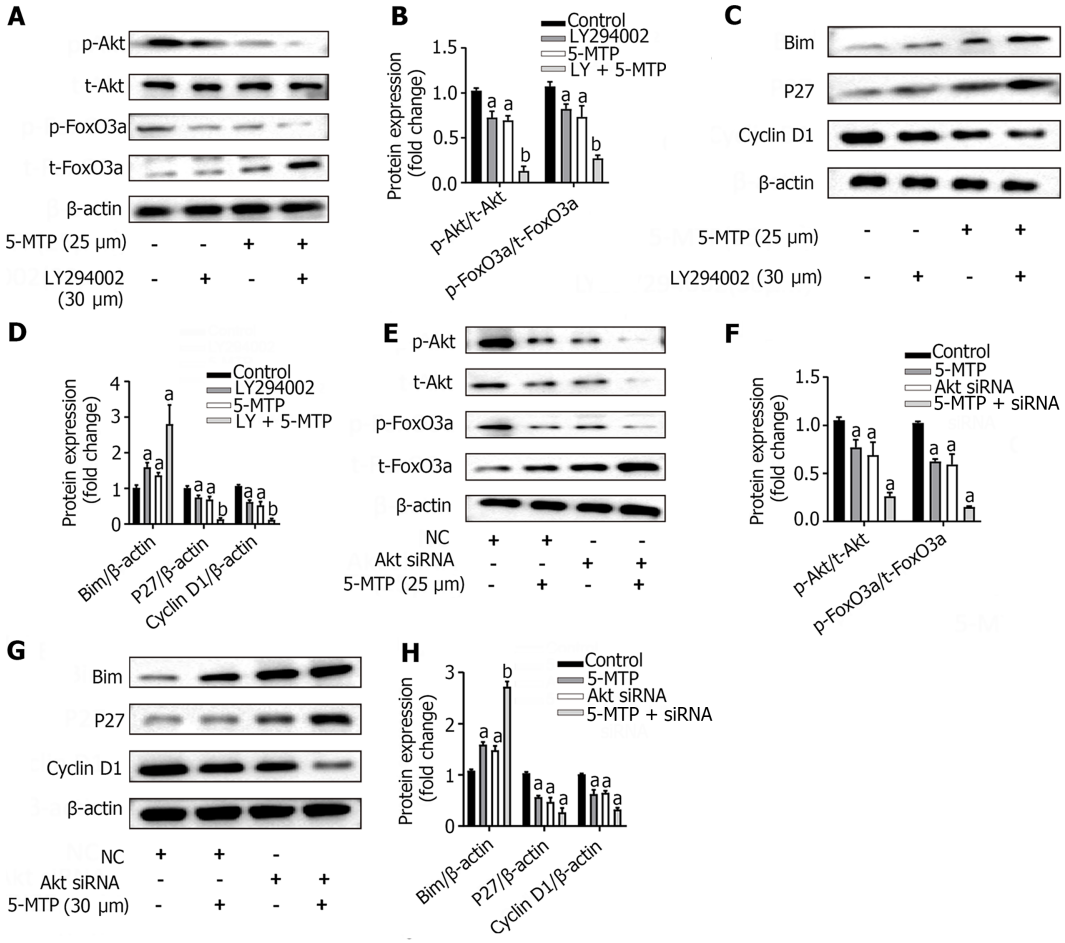
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Figure 5 5-methoxytryptophan induced dose-response and time course inhibition of PI3K/Akt/FoxO3a signaling pathway in HCT-116 cells. A-D: The HCT-116 cells were treated with 5-methoxytryptophan (5-MTP) at the dose of 0, 1, 5, 25, 50 and 100 μM. The expression of PI3K/Akt/FoxO3a signaling pathway protein was detected by western blotting; E-H: The HCT-116 cells were treated with the dose of 25 μM of 5-MTP at the time of 0, 3, 6, 12, 24 and 72 h. The expression of p-Akt, t-AKT, p-FoxO3a and t-FoxO3a was detected by western blotting. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. 5-MTP: 5-Methoxytryptophan.



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Figure 6 5-methoxytryptophan induced apoptosis in HCT-116 cells. A-D: The expression of apoptosis-associated protein was detected by western blotting. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. 5-MTP: 5-Methoxytryptophan; Bcl2: B-cell lymphoma-2.



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Figure 7 Apparent correlations between 5-methoxytryptophan-induced cell cycle and apoptosis-associated proteins or inhibition of PI3K/Akt/FoxO3a signaling pathway in HCT-116 cells. A-D: The HCT-116 cells were treated with 5-methoxytryptophan (5-MTP) and/or LY294002. The expression of PI3K/Akt/FoxO3a signaling pathway and cycle arrest protein was detected by Western blotting; E-H: The HCT-116 cells were treated with the dose of 25 μM of 5-MTP and/or AKT siRNA. The expression of p-Akt, t-AKT, p-FoxO3a, t-FoxO3a, Bim, Cyclin D1 and P27 was detected by western blotting. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. 5-MTP: 5-Methoxytryptophan; NC: Normal control; siRNA: Small interfering RNA.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is a highly prevalent malignant tumor. Research is needed to find and develop effective drugs for the treatment of CRC. 5-methoxytryptophan (5-MTP) is a tryptophan metabolite found in animals and humans. The effect of 5-MTP on the proliferation and apoptosis of CRC cells is still unclear.

Research motivation

Our work explored the effects of 5-MTP on the proliferation, migration, invasion and apoptosis of CRC cells. We tried to explore the potential of 5-MTP as a drug for the treatment of CRC.

Research objectives

Here, we studied that 5-MTP combined with PI3K/Akt/FOXO3a signaling pathway inhibitor can significantly promote apoptosis and cell cycle arrest of CRC cells, and inhibit cell proliferation. 5-MTP can be used as an effective drug in the treatment of CRC.

Research methods

In this study, a series of experiments were carried out. Colony forming assay and Cell Counting Kit were used to detect the effect of 5-MTP on the proliferation of CRC cell lines. The effect of 5-MTP on cell growth was detected by cell cycle analysis. In addition, the effects of 5-MTP on apoptosis and reactive oxygen species of HCT-116 cells were studied. In order to further explore the effect of 5-MTP on CRC, we studied the effect of 5-MTP on PI3K/Akt signaling pathway in CRC cells.

Research results

5-MTP can promote apoptosis and cell cycle arrest of CRC cells, and inhibit cell proliferation. However, compared with 5-MTP alone, 5-MTP combined with PI3K/Akt/FOXO3a signaling pathway inhibitors significantly promoted apoptosis and cell cycle arrest of CRC cells, and inhibited cell proliferation. It provides new insights into the mechanism of drug action.

Research conclusions

5-MTP combined with PI3K/Akt/FOXO3a signaling pathway inhibitor significantly promoted apoptosis and cell cycle arrest of CRC cells, and inhibited cell proliferation.

Research perspectives

This study confirmed that 5-MTP combined with PI3K/Akt/FOXO3a signaling pathway inhibitor could significantly promote the apoptosis and cell cycle arrest of CRC cells, and inhibit cell proliferation. These results provide a new direction for the drug treatment of CRC. However, the study of mechanism in this study is relatively limited. Therefore, further analysis, nude mouse experiments and more cell experiments are needed to explore its mechanism.

FOOTNOTES

Co-first authors: Tian-Lei Zhao and Yue Qi.

Co-corresponding authors: Hui Liang and Ya-Bin Pu.

Author contributions: Pu YB and Liang H conceived and designed the study; Zhao TL and Qi Y performed the experiments and prepared the manuscript, they made the same contribution to this work and should share the first authorship; Wang YF and Wang Y analyzed the data; Pu YB and Liang H conceived, designed this study, and revised the manuscript; Pu YB and Liang H have the same contribution to this work, they should be listed as co-corresponding authors; and all authors have read and approved the final version of the manuscript.

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

Data sharing statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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LETTER TO THE EDITOR

Clinical characteristics and outcomes of autoimmune pancreatitis based on serum immunoglobulin G4 levels: A single-center, retrospective cohort study

Fouad Jaber, Khaled Elfert, Saqr Alsakarneh, Azizullah Beran, Mohammed Jaber, Manesh Kumar Gangwani, Yazan Abboud

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Abstract

Autoimmune pancreatitis (AIP) is a complex, poorly understood disease gaining increasing attention. "Clinical Characteristics and Outcome of AIP Based on Serum IgG4 levels," investigated AIP with a focus on serum immunoglobulin (Ig) G4 levels. The 213 patients with AIP were classified according to serum IgG4 levels: Abnormal (elevated) and normal. Patients with higher IgG4 levels exhibited a more active immune system and increased relapse rates. Beyond IgG4, the IgA levels and age independently contributed to relapse risk, guiding risk assessment and tailored treatments for better outcomes. However, limitations persist, such as no IgA correlation with IgG4 levels, absent data on autoantibody-positive AIP cases critical for Asian diagnostic criteria, and unexplored relapse rates in high serum IgG AIP by subtype. Genetic factors and family histories were not addressed. As the understanding and referral of seronegative AIPs increase, there's a growing need for commercially available, highly sensitive, and specific

autoantibodies to aid in diagnosing individuals with low or absent serum IgG4 levels.

Key Words: Autoimmune pancreatitis; Relapse; Immunoglobulin G; Immune System, Immunoglobulin A; Outcomes

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Core Tip: The study on autoimmune pancreatitis (AIP) based on serum immunoglobulin (Ig) G4 levels offers valuable insights into this complex condition. Elevated IgG4 and IgA levels in patients with AIP were associated with more active immune system and higher relapse rates, highlighting the potential of IgG4 as a biomarker. However, limitations include the lack of analysis on IgA levels in relation to IgG4 levels, the absence of data on autoantibodies, and the lack of reporting on family history and genetic factors. As awareness of AIP grows, there is a need for highly sensitive and specific autoantibodies to aid in diagnosis, especially for IgG4-negative AIP patients.

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TO THE EDITOR

We read with great interest a recent article published in your esteemed journal, titled "Clinical Characteristics and Outcome of Autoimmune Pancreatitis Based on Serum IgG4 levels" by Zhou *et al*[1]. Autoimmune pancreatitis (AIP) is a complex and poorly understood condition that has garnered considerable attention in recent years. This study by Zhou *et al* offers valuable insights into the characteristics and outcomes of AIP, focusing on the role of serum immunoglobulin (Ig) G4 levels[1]. We believe that the findings presented in this research hold significant clinical implications and merit further discussion and dissemination.

The authors meticulously investigated a cohort of 213 patients with AIP, and their decision to categorize them into two groups based on serum IgG4 levels, the abnormal group with high IgG4 levels and the normal group, is particularly noteworthy[1]. By comparing these groups, the study reveals several compelling findings that deserve attention from the medical community.

Firstly, in line with other studies[2-4], this study highlights that patients with AIP and elevated IgG4 levels have distinct clinical features, such as a higher relapse rate[1]. This observation contributes to our understanding of the heterogeneity within the population of patients with AIP and highlights the potential importance of serum IgG4 levels as a biomarker of disease activity.

Furthermore, identifying factors associated with AIP relapse is of utmost importance for clinical management. The multivariate analyses performed in this study suggest that not only serum IgG4 levels but also IgA levels and patient age play independent roles in predicting relapse[1]. This information could help physicians stratify risks and adjust treatment strategies for patients, ultimately improving their long-term outcomes.

However, a few limitations are worth mentioning. While the study found an association between IgA levels and higher relapse rates[1], no further analysis of IgA levels relative to serum IgG4 levels was performed. One study mentioned that serum IgA and IgM levels were lower in patients with high-level serum IgG4 AIP than in patients with normal serum level IgG4 AIP[5], while another study reported an inverse correlation between serum IgG4 and IgM or IgA in 20 cases of AIP[6]. Further stratification based on IgA levels could expand our knowledge of the association between IgG4 and IgA in AIP.

Furthermore, the proportion of patients with AIP with positive autoantibodies was not discussed in this study[1]. While serum IgG levels and anti-nuclear antibody positivity were previously part of the classical criteria for AIP[7], neither the current international consensus diagnostic criteria for AIP[8] nor the Japanese revised clinical diagnostic criteria for AIP[9] included these two elements. Nonetheless, some studies have reported lower IgG4 levels in patients with positive serum autoantibodies compared to patients without autoantibodies. This finding may contribute to demonstrating the presence of AIP with an association of autoantibodies alone in a subset of patients. Furthermore, in one study, higher serum IgM and IgA levels were observed in serum autoantibody-positive (+) patients with AIP compared to serum autoantibody-negative (-) patients with AIP, suggesting that examining the properties of high serum IgG4 AIP and serum autoantibodies could provide valuable insights[5]. With increasing understanding and prevalence of seronegative AIP among general clinicians, there is a growing demand for commercially available autoantibodies with superior sensitivity and specificity to aid in the identification and diagnosis of AIP in individuals with low or absent serum IgG4 levels.

Another limitation to consider is that the study did not examine relapse rates in patients with high serum IgG levels based on the type of AIP[1]. Previous research has suggested different relapse rates, with type 1 AIP in patients with high serum IgG4 having higher rates (20%-40%) compared to type 2 AIP[5,10,11]. The lack of this information limits our

understanding of how serum IgG levels may impact relapse risk in different AIP subtypes.

Finally, Zhou *et al*[1] reported neither family history nor genetic factors. It is important to note that HLA-DRB1 haplotypes are associated with AIP susceptibility[12] as well as other diseases, such as rheumatoid arthritis[13]. This genetic aspect requires further study to better understand the complex interplay between genetics and AIP.

In conclusion, the research conducted by Zhou *et al*[1] sheds light on the clinical aspects of AIP and highlights the importance of serum IgG4 levels as a prognostic indicator. It also provides valuable insights into risk factors for relapse, which can serve as a basis for more targeted therapeutic interventions. As AIP continues to be a challenge for physicians worldwide, studies such as these contribute significantly to our knowledge and have the potential to improve patient care.

FOOTNOTES

Author contributions: Jaber F conceived the research; Jaber F, Alsakarneh S, Elfert K designed the research workflow; Jaber F, Alsakarneh S, Abboud Y, and Jaber M wrote the final manuscript; Beran A and Gangwani MK supervised the project; all authors have read and agreed to the final version of the manuscript.

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LETTER TO THE EDITOR

Liver decompensation after rapid weight loss from semaglutide in a patient with non-alcoholic steatohepatitis -associated cirrhosis

Matthew Peverelle, Jonathan Ng, James Peverelle, Ryan D. Hirsch, Adam Testro

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Abstract

There is rapidly increasing uptake of GLP-1 (glucagon-like peptide-1) agonists such as semaglutide worldwide for weight loss and management of non-alcoholic steatohepatitis (NASH). remains a paucity of safety data in the vulnerable NASH cirrhotic population. We report herein the first documented case of liver decompensation and need for liver transplant waitlisting in a patient with NASH-cirrhosis treated with semaglutide. Rapid weight loss led to the development of ascites and hepatic encephalopathy and an increase in the patients Model for Endstage Liver Disease-Na (MELD-Na) score from 11 to 22. Aggressive nutritional supplementation was commenced and the semaglutide was stopped. Over the following months she regained her weight and her liver recompensated and her MELD-Na decreased to 13, allowing her to be delisted from the transplant waitlist. This case serves as a cautionary tale to clinicians using semaglutide in the cirrhotic population and highlights the need for more safety data in this patient group.

Key Words: Semaglutide; Non-alcoholic steatohepatitis; Cirrhosis; Non-alcoholic steatohepatitis cirrhosis; Glucagon-like peptide 1 agonists; Weight loss

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Core Tip: Patients with NASH cirrhosis who lose weight rapidly with semaglutide are at risk of liver decompensation. This complication requires the immediate cessation of semaglutide and aggressive nutritional rehabilitation with supplemental protein feeds and micronutrients. Restoration of lost weight can lead to liver recompensation; however, consideration of liver transplantation should be given to patients who fail to respond to treatment.

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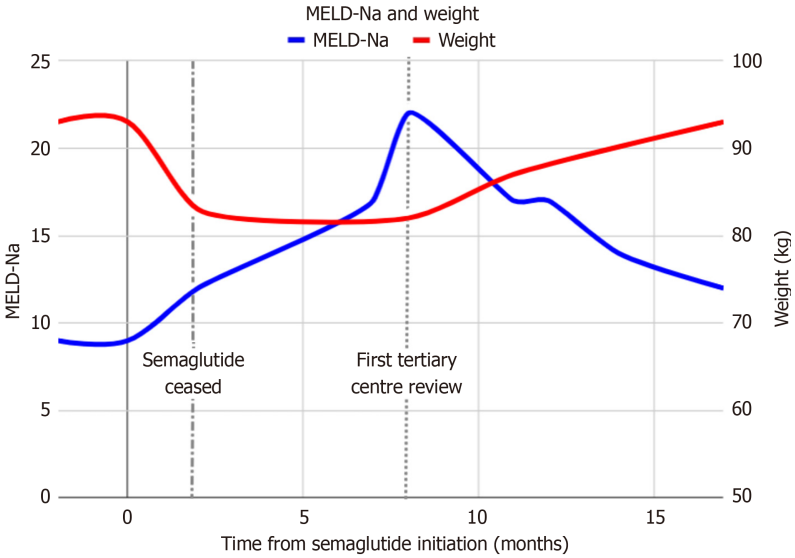
TO THE EDITOR

We read with interest the systematic review and meta-analysis by Zhu *et al*[1] reporting on the efficacy and safety of semaglutide in patients with non-alcoholic fatty liver disease (NAFLD). Analyzing three randomized control trials involving 458 patients, they found that semaglutide was effective at improving histologic and radiologic markers of non-alcoholic steatohepatitis (NASH) activity but not histologic fibrosis. The risk of serious adverse events was similar compared to placebo, and importantly, no cases of hepatic decompensation occurred.

We herein present a case of liver decompensation in a patient with NASH cirrhosis after the use of semaglutide. A 68-year-old female with compensated NASH cirrhosis [Model for Endstage Liver Disease-Na (MELD-Na) score 11] was prescribed semaglutide 2.4 mg once weekly to manage her diabetes and obesity. Her other medications at the time included salbutamol for asthma. The semaglutide led to 10 kg weight loss (11% body weight) within 8 wk of treatment before it was stopped. After approximately 8% body weight loss she developed new onset hepatic encephalopathy (HE) requiring the use of lactulose and rifaximin. She also developed ascites requiring diuretic therapy (spironolactone 100 mg and frusemide 40 mg; further increases limited by postural hypotension) and two large-volume paracenteses. Patient adherence to prescribed medications was confirmed by her family during this time. Due to her rapid weight loss, her semaglutide was stopped. Despite stabilization of her weight, she continued to decompensate and her MELD-Na continued to rise (Figure 1). On referral to our service her MELD-Na was 22 (bilirubin 40 μmol/L, creatinine 44, international normalized ratio 1.6, Na 128). Investigations for alternate causes of decompensation including infection, alcohol consumption, hepatocellular carcinoma and portal vein thrombosis were negative. We concluded her liver decompensation was most likely secondary to semaglutide-induced rapid weight loss and malnutrition. She was commenced on high energy and high protein supplementation consisting of 60 g Sustagen twice-daily and micronutrient replacement with thiamine. She also underwent assessment for liver transplantation. Over the following 3 mo, she was reviewed each month by a transplant hepatologist and dietitian to assess her clinical progress, nutritional intake and adherence to treatment. Over this period, she managed to regain 5 kg (6%) of her ideal body weight, and this was associated with an improvement in her ascites and HE and a reduction in MELD-Na to 19 (Figure 1). By 6 mo her weight had returned to baseline, she no longer required abdominal paracentesis and her MELD-Na was 13. She was de-listed from the transplant waitlist and remains compensated at last follow-up.

Our case highlights the potential risk of rapid weight loss with semaglutide in the vulnerable NASH cirrhosis population. In our case, the rapidity of weight loss was significantly greater compared to the studies included in Zhu *et al*'s[1] meta-analysis (10 kg after 8 wk *vs* 6.5 kg after 48-72 wk). Loomba *et al*'s[2] study, included within the meta-analysis, involved patients with NASH cirrhosis and did not report any cases of hepatic decompensation. Of note, our patients pre-semaglutide MELD-Na score was higher (11 for our patient *vs*. 7.6 in the study group), potentially conferring a greater predisposition to decompensation. It is interesting to note that despite stabilization of her weight after stopping se-maglutide, our patient continued to decompensate until she was reviewed at our tertiary centre, as illustrated in Figure 1. Rapid weight loss is an established precipitant of hepatic decompensation in the post bariatric surgery population[3], with pathophysiological mechanisms thought to involve endogenous free-fatty acid oxidative damage, mitochondrial dysfunction and gut dysbiosis leading to hepatic inflammation and fibrosis[4-6]. However, it should be noted that decompensation has generally occurred later (up to 5 years post-surgery) and degree of excess weight loss (*i.e.* the amount of weight above the ideal body weight) was up to 110%[7]. Furthermore, the use of glucagon-like peptide 1 agonists such a semaglutide causes delayed gastric emptying, which may impact the absorption of concomitantly administered oral medications and therefore their efficacy. This may have contributed to our patients' diuretic-refractory ascites. In patients with liver cirrhosis, it is therefore important to consider inadequate absorption of medical therapies as a contributor to failure to respond to standard treatment.

Clinicians should consider the use of semaglutide cautiously in patients with underlying NASH cirrhosis and should strictly adhere to the prescribing information and dose escalation protocols as recommended and consider using a lower dose of the drug. Failure to follow strict dose escalation protocol may lead to significant gastrointestinal side effects including nausea and vomiting, which may precipitate weight loss and decompensation. Furthermore, clinicians must exercise a low threshold for cessation should weight loss occur rapidly (≥ 1% of body weight/week) or signs of liver decompensation develop.



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Figure 1 Patient's weight change with semaglutide use and MELD-Na score over time.

FOOTNOTES

Author contributions: Peverelle M, Ng J and Peverelle J designed research and collected data; Hirsch RD analyzed data and designed figures; Testro A analyzed and interpreted data; All authors contributed to the drafting of the manuscript and all authors have given final approval for its submission.

Conflict-of-interest statement: Dr. Peverelle has nothing to disclose.

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OPINION REVIEW

Challenges involved in the application of artificial intelligence in gastroenterology: The race is on!

Chrysanthos D Christou, Georgios Tsoulfas

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Abstract

Gastroenterology is a particularly data-rich field, generating vast repositories of data that are a fruitful ground for artificial intelligence (AI) and machine learning (ML) applications. In this opinion review, we initially elaborate on the current status of the application of AI/ML-based software in gastroenterology. Currently, AI/ML-based models have been developed in the following applications: Models integrated into the clinical setting following real-time patient data flagging patients at high risk for developing a gastrointestinal disease, models employing non-invasive parameters that provide accurate diagnoses aiming to either replace, minimize, or refine the indications of endoscopy, models utilizing genomic data to diagnose various gastrointestinal diseases, computer-aided diagnosis systems facilitating the interpretation of endoscopy images, models to facilitate treatment allocation and predict the response to treatment, and finally, models in prognosis predicting complications, recurrence following treatment, and overall survival. Then, we elaborate on several challenges and how they may negatively impact the widespread application of AI in healthcare and gastroenterology. Specifically, we elaborate on concerns regarding accuracy, cost-effectiveness, cybersecurity, interpretability, oversight, and liability. While AI is unlikely to replace physicians, it will transform the skillset demanded by future physicians to practice. Thus, physicians are expected to engage with AI to avoid becoming obsolete.

Key Words: Artificial intelligence; Machine learning; Gastroenterology; Cost-effectiveness; Interpretability; Accuracy

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Core Tip: Currently, artificial intelligence (AI) and machine learning (ML) have several applications in the prevention, diagnosis, treatment, and prognosis of various gastrointestinal diseases, including gastroesophageal reflux disease, esophageal cancer, gastric cancer, gastrointestinal bleeding, inflammatory bowel diseases, polyps, colorectal cancer, and others. Despite their promising results, AI/ML applications in gastroenterology are hindered by several challenges, including accuracy, cost-effectiveness, cybersecurity, interpretability, oversight, and liability concerns. In this opinion review, we elaborate on these challenges and present different ways to overcome them.

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INTRODUCTION

Gastroenterology is a particularly data-rich field, with data being omnipresent. Several gastroenterology norms, including patient electronic records, imaging, endoscopy images and videos, capsule endoscopy videos, genomic analyses, microbiome data, and histopathology images, generate vast data repositories[1-3]. Conforming with big data features, these repositories provide a fruitful ground for artificial intelligence (AI) applications and, more specifically, AI's subfield, machine learning (ML)[4]. By learning from these data, ML algorithms are able to predict future data points [5]. AI/ML applications have been described in many studies as the potential solution to long-standing healthcare challenges, including the need for cost reduction, enhancing diagnostic accuracy, facilitating individualized and evidence-based care, and more importantly, significantly ameliorating the morbidity and mortality associated with various diseases[6-8].

In this opinion review, we first present the current status of the application of AI in the field of gastroenterology. Then, we pose a series of challenges that these applications face and explore ways to overcome them. Specifically, we elaborate on the following challenges: Accuracy concerns, cost-effectiveness, cybersecurity, interpretability, oversight, and liability.

CURRENT STATUS

Physicians use experience, intuition, and quantifiable and non-quantifiable variables for decision-making. ML algorithms, on the other hand, perform a series of precise calculations of all the quantifiable variables to perform a certain task. Computer systems, in general, and AI/ML tools, particularly, surpass, by far, physicians in their ability to quantify multiple correlations, even in fields where the physicians hold in-depth expertise. Thus, the application of ML tools is particularly useful for performing tasks requiring extensive analytical skills such as unraveling correlations in datasets, following laboratory results trends for long periods of time, and recognizing patterns in various imaging modalities. AI classifiers commonly employed in ML tools in gastroenterology are support vector machines (SVMs), artificial neural networks (ANNs), and convolutional neural networks (CNNs). SVMs are used in supervised learning. Data points of the instances are mapped in a high-dimensional space, where the hyperplane that separates the instances based on their assigned label, retaining the highest performance, is selected[9]. SVMs can be used for linear and non-linear problems using kernel functions[10]. ANNs are inspired by the human brain and consist of an input and output layer with at least one hidden layer in between[11]. As the architectures of ANNs became more sophisticated with the addition of multiple hidden layers and even more layer connections, the concept of deep neural networks (DNNs) emerged[12,13]. A particular type of DNN, the CNN, has found profound application in gastroenterology since the demonstrated high performance in image interpretation. CNNs are based on convolution, where the image is processed using a series of filters or kernels to detect patterns within the image, such as edges and textures[14].

PREVENTION

Table 1 presents a series of studies where AI/ML models have been applied in the field of gastroenterology. These models could be integrated into the clinical setting and follow real-time patient data trends. Then, these models could flag patients at high risk of developing a plethora of gastrointestinal diseases. A notable example is ColonFlag, an ML algorithm used to stratify the risk of developing colorectal cancer. Impressively, the tool has been shown to identify patients with colorectal cancer who would otherwise have avoided screening and to identify patients at the early stages of the disease 18 to 24 mo prior to the usual diagnosis time[15,16]. Other recent studies focus on the stratification of gastric cancer development by employing electronic health care records[17,18]. These models demonstrate how AI/ML models

Table 1 Studies employing artificial intelligence/machine learning tools in the field of gastroenterology

Ref.	Parameters employed/study design	AI classifier	Sample size, control group, validation	Outcomes	Performance[1]
Prevention					
Goshen *et al*[15]	EHR data/prospective validation	LR, DT, GB	688 flagged patients, NA, NA	High risk of CRC development	19 (7.5%) CRCs were found in 254 colonoscopies
Holt *et al*[16]	EHR data/case-control study	LR, DT, GB	1893641 patients, 15 controls matched to 1 case, NA	Early detection of CRC	0.536-0.624$^{c\text{-index}}$
Huang *et al*[17]	EHR data/case-control study	LR, LASSO, SVM, KNN, RF	1035 patients, 3 controls matched to 1 NCGC case, 10-fold CV	Early detection of NCGC	LR: 72.4^{acc}, 79.3^{sen}, 70.4^{spe}. LASSO: 75.1^{acc}, 80^{sen}, 73.6^{spe}. SVM: 75.1^{acc}, 76.3^{sen}, 74.7^{spe}. KNN: 78.1^{acc}, 68.9^{sen}, 80.8^{spe}. RF: 72.2^{acc}, 77.8^{sen}, 70.6^{spe}
Briggs *et al*[18]	EHR data/case-control study	RF, SVM, LR, NB, EGBDT	40348, 7471 cases/32877 controls), hold-out validation (75:25), 5-fold CV	Early detection of oesophago-gastric cancer	87-89^{acc}, 0.81-0.87$^{c\text{-index}}$
Diagnosis					
Manandhar *et al*[20]	Gut microbiome data (fecal 16S metagenomic data)/case-control study	RF, SVM, DT, ANN	1429 patients, 729 IBD and 700 non-IBD patients, hold-out validation	Diagnosis of IBD	77-84^{acc}, 0.41-0.82^{sen}, 77-84^{sen}, 46-64^{spe}
Papa *et al*[21]	Gut microbiome data/case-control study	SVM, RF	105 children and young adults, 91 with IBD and 24 controls, 10-fold CV	Diagnosis of IBD in child population	0.83-0.91$^{c\text{-index}}$
Pace *et al*[22]	Laboratory results, clinicopathological parameters/retrospective cohort study	ANN	159 patients, 103 with gastroesophageal reflux and 56 controls, 20-fold CV	Diagnosis of gastroesophageal reflux disease	78-100^{acc}
Yepes *et al*[25]	Expression levels of 1046/315 microRNAs in gastric samples/retrospective cohort study	SVM, RF	648 gastric samples, 479 cancer and 169 controls, leave-one-out CV	Classification of non-tumor mucosa *vs* tumor sample	SVM: 94^{acc}, RF: 89.3^{acc}
Huang *et al*[26]	Serum expression levels of miR-21-5p, miR-22-3p, and miR-29c-3p/retrospective cohort study	Several	192, 122 cancer samples and 70 controls, leave-one-out CV	Identification of patients with gastric cancer	56-93^{acc}
Alizadeh *et al*[27]	Serum expression levels of miR-663a, miR-1469, miR-92a-2-5p, miR-125b-3p, and miR-532-5p/retrospective cohort study	ANN	671, NR, 5-fold CV	Identification of patients with pancreatic ductal adenocarcinoma	93^{acc}, 93^{sen}, 92^{spe}
Afshar *et al*[28]	Serum expression levels of miR-6726-5p, miR-7111-5p, miR-1247-3p, and miR-614/retrospective cohort study	ANN	200, 50 with colorectal cancer and 150 healthy controls, hold-out validation (70:15:15)	Identification of patients with colorectal cancer	100^{acc}, 1$^{c\text{-index}}$, 100^{sen,spe}
Duttagupta *et al*[29]	Expression levels of 847 microRNAs in the peripheral blood/case-control study	SVM	40, 20 patients with ulcerative colitis and 20 controls, 10-fold CV	Identification of patients with ulcerative colitis	92.3-92.8^{acc}, 87.8-89.5^{sen}, 96.2-96.8^{spe}
Treatment					
Morilla *et al*[30]	Colonic microRNA profiles (9 microRNAs) and five clinical factors/retrospective cohort study	DNN	76 patients, 22 responders and 54 non-responders, hold-out validation (47:29)	Prediction of response to treatment of patients with active severe ulcerative colitis	80-93[1], 0.80-0.91$^{c\text{-index}}$
Takiyama *et al*[31]	Esophago-gastro-duodenoscopy imaging/retrospective cohort study	CNN	17080 images, 363 larynx/2142 esophagus/13048 stomach/1528 duodenum, hold-out validation	Anatomical classification among the larynx, esophagus, stomach, and duodenum	0.99-1.00$^{c\text{-index}}$
Rogers *et al*[35]	Data from baseline impedance, nocturnal baseline impedance, and acid exposure time/retrospective cohort study	DT	335 patients, 210 with gastroesophageal reflux and 115 controls, NR	Prediction of response to treatment with proton pump inhibitors for patients with gastroeso-	0.31-0.938$^{c\text{-index}}$

				phageal reflux disease	
Takayama *et al* [36]	Clinicopathological parameters, treatment data/retrospective cohort study	ANN	90 patients, 32 non-responders and 58 remission-effect, hold-out validation (54:36)	Prediction of the need for operation for UC patients treated with cytoapheresis	96^{sen}, 97^{spe}
Das *et al*[37]	Laboratory results, clinicopathological parameters/retrospective cohort study	ANN	587 patients, 246 patients with major stigmata-emergent endoscopy to 162 patients, hold-out validation (194:193:200)	Prediction of major stigmata of recent hemorrhage	89^{acc}, 89-96^{sen}, 63-89^{spe}
				Prediction of the need for emergent endoscopy	61-81^{acc}, 61-94^{sen}, 48-82^{spe}
Chu *et al*[38]	Laboratory results, clinicopathological parameters/retrospective cohort study	Several	189 patients, NR, hold-out validation (122:67)	Prediction of the source of GIB	69.7-94.3^{acc}, 0.658-0.999$^{c\text{-}index}$, 90.1-98.0^{sen}, 89-100^{spe}
				Prediction of the need for blood resuscitation	64.7-94.1^{acc}, 0.381-0.993$^{c\text{-}index}$, 90.3-93.9^{sen}, 18.4-95.5^{spe}
				Prediction of the need for emergent endoscopy	62.7-83.3^{acc}, 0.404-0.913$^{c\text{-}index}$, 80.1-89.1^{sen}, 13.8-85.7^{spe}
				Prediction of disposition	58.4-89.7^{acc}, 0.324-0.972$^{c\text{-}index}$, 81.9-92.9^{sen}, 18.4-90.9^{spe}
Loftus *et al*[39]	Laboratory results, clinicopathological parameters/retrospective cohort study	ANN	147 patients, 41% of patients with severe lower GIB and 13 patients needed surgical intervention, hold-out validation (103:44)	Prediction of severe lower GIB	0.979$^{c\text{-}index}$
				Prediction of the need for surgical intervention	0.954$^{c\text{-}index}$
Ayaru *et al*[40]	Laboratory results, clinicopathological parameters/retrospective cohort study	GB	300 patients, 88 with severe bleeding, 53 with recurrent bleeding, and 35 requiring intervention, hold-out validation (170:130)	Prediction of severe lower GIB	78-83^{acc}
				Prediction of recurrent bleeding	88-88^{acc}
				Prediction of the need for intervention	88-91^{acc}
Prognosis					
Sato *et al*[41]	Laboratory results, clinicopathological parameters, tumor characteristics/retrospective cohort study	ANN	395 patients, 281 alive at 1-year-89 alive at 5 years, hold-out validation (53:27:20)	1-yr and 5-yr survival of patients with esophageal cancer following surgery	0.883-0.884$^{c\text{-}index}$, 78.1-80.7^{sen}, 84.7-86.5^{spe}
Shung *et al*[42]	Laboratory results, clinicopathological parameters/retrospective cohort study	GB	2357 patients, 1109 requiring either transfusion or hemostatic intervention, hold-out validation (1958:399)	Stratification of risk of patients with gastrointestinal bleeding	0.91$^{c\text{-}index}$
Zhou *et al*[43]	Laboratory results, clinicopathological parameters, tumor characteristics/retrospective cohort study	Several	2012 patients, 405 patients with recurrence, hold-out validation (80:20)	Recurrence of gastric cancer following surgery	0.77-0.80$^{c\text{-}index}$

[1]Test set results are reported.

Acc: Accuracy (%); c-index: under the receiver operating curve or C-index; Sen: Sensitivity (%); Spe: Specificity (%); ANN: Artificial neural network; CD: Chron's disease; CNN: Convolutional neural network; CRC: Colorectal cancer; CV: Cross-validation; DNN: Deep neural network; DT: Decision tree; EGBDT: Extreme gradient boosting decision tree; EHR: Electronic healthcare record; GB: Gradient boosting; IBD: Inflammatory bowel disease; KNN: K-nearest neighbor; LASSO: Least absolute shrinkage and selection operator; LR: Logistic regression; NA: Not applicable; NB: Naïve Bayes; NBI: Narrow-band imaging; NCGC: Noncardia gastric cancer; NPV: Negative predictive value; NR: Not recorded; RF: Random forest; SVM: Support vector machine; UC: Ulcerative colitis.

can be used at the primary care level to lessen the burden of a plethora of gastrointestinal diseases significantly.

DIAGNOSIS

Currently, endoscopy constitutes the gold standard in the diagnosis of various gastrointestinal diseases. Nevertheless, endoscopy is an unpleasant intervention for the patient, has a significant cost, and is related to a series of potential complications[19]. AI/ML models utilizing non-invasive parameters that provide comparably accurate diagnoses could

either replace, minimize, or refine the indications of endoscopy. Two studies have used data from the microbiota and microbiome to identify patients with inflammatory bowel disease (IBD)[20,21]. Although these models could not replace endoscopy in the diagnosis of IBD, they can be used to identify patients requiring endoscopic evaluation. However, in the diagnosis of gastroesophageal reflux disease, the employment of ML tools could substitute the use of endoscopy for patients at low risk of developing cancer[22]. These efforts demonstrate how ML tools could be used to significantly reduce the cost and complications related to endoscopy, avoiding, at the same time, an unpleasant experience for the patient.

The introduction of next-generation sequencing has widened our capabilities regarding detecting host genetic factors related to certain diseases and allowed us to unveil the complex hereditary background of several diseases by clarifying the cascade of gene interactions and the impact of the environment[23,24]. This progress allowed for the emergence of novel biomarkers, including microRNAs. In gastroenterology, various studies have employed microRNA profiling data to develop tools able to diagnose various gastrointestinal diseases. Specifically, tools have been developed that classify non-tumor from tumor mucosa from gastric samples[25]. In addition, by employing microRNA profiling, data from serum models have been developed that identify patients with gastric cancer[26], pancreatic ductal adenocarcinoma[27], colorectal cancer[28], and ulcerative colitis[29]. Finally, a model has been developed using microRNA profiling from colonic samples to predict the response to treatment of patients with active severe ulcerative colitis[30]. These models again demonstrate how AI/ML tools can provide a non-invasive endoscopy alternative.

Except for replacing endoscopy, AI/ML tools could significantly enhance the endoscopy's clinical outcomes. Such models could be used for developing Computer Aided Detection or Diagnosis systems (CAD systems) to facilitate the interpretation of endoscopy images. First, these CAD systems could aid the endoscopist to navigate in the gastrointestinal tract. Existing models have demonstrated outstanding performance[31]. These CAD systems could be used as a "third eye" for the endoscopist, an "eye" that does not get tired, is not susceptible to any distractions, and can identify lesions missed by the endoscopist. Following the identification of lesions, these models could be used to classify the lesions, for example, as benign or malignant. In capsule endoscopy, CAD systems could automatically identify and then classify lesions from capsule endoscopy videos and then provide the images to the gastroenterologist for review. Such models could significantly decrease the cost associated with the labor hours required to review the capsule endoscopy videos. Several models have been developed for a plethora of gastrointestinal diseases, including IBD, gastric cancer, small bowel lesions (ulcerative/hemorrhagic), colon cancer, and others[6,32]. Another application of CAD systems is proposing an ideal site for biopsy when needed. CAD systems also find a particular application in colonoscopy in classifying benign from malignant polyps[33]. By avoiding the removal of benign polyps and correctly removing all malignant polyps with the assistance of CAD systems, the cost-efficiency of coloscopy significantly increases. Notably, regarding polyp classification, the reported negative predictive value in the literature of CAD systems is above the 90% recommended by the American Society of Gastrointestinal Endoscopy for adenoma detection[34].

TREATMENT

In addition to diagnosis, AI/ML-based tools can be employed to facilitate treatment allocation for various gastrointestinal diseases. Data regarding impedance and acid exposure time were employed in a study to develop an ML tool predicting treatment response[35]. Other studies focusing on IBD have developed tools that predict treatment response to specific drugs or the need for operation[35,36]. A different group of studies, which focused on gastrointestinal bleeding management, achieved the development of models identifying patients in need of a transfusion, emergent endoscopy, emergent surgery, and intensive care unit triage[37-40]. These studies demonstrate how AI/ML tools could be used to create frameworks for individualized, evidence-based treatment allocations.

PROGNOSIS

AI/ML algorithms have also been employed in the development of predictive tools able to predict complications, recurrence following treatment, and overall survival. Examples of such studies include a study developing an ML tool to predict overall survival in patients with esophageal cancer[41], a model predicting in-hospital death for patients with gastrointestinal bleeding[42], and an ML model predicting the chance of recurrence in operated gastric cancer patients [43]. Such models could be used for family and patient counseling.

ACCURACY CONCERNS

The accuracy of a developed AI/ML tool largely depends on the quality of the training data. The notion "garbage in, garbage out" is particularly true in ML models. Flaws and weaknesses of the datasets, such as wrongly labeled instances, low image quality, small dataset size and variability of instances, and discrepancies in the data collection processes, will be inadvertently built into the end model. For example, capsule endoscopy has significantly lower image resolution compared with other types of endoscopy images, and thus, a model trained from capsule endoscopy imaging is expected to have lower classification accuracy. Standardization of data collection methods is essential to acquire datasets of clean, high-quality data that are representative of a diverse patient population. A main challenge encountered in ML

development is class imbalance. Most of the time, normal findings represent the majority class, while patients with a disease are usually the minority class. This problem is amplified in multi-class situations where accurate classification of rare classes is particularly challenging. A series of techniques to undersample the majority class and oversample the minority class in medical datasets have been proposed that, to some extent, resolve this issue[44]. In addition, in a study, researchers developed a model able to add polyps in the endoscopic images, which can then be used for training ML models[45]. Such methodologies of creating synthetic instances could again resolve the imbalance challenge. A different challenge in ML model development is underfitting, which can be defined as the incapability of the model to capture the variability of the data[46]. On the other hand, overfitting can be described as the inability of the model to retain its performance in datasets other than the training dataset[47]. In other words, the model's performance is significantly superior in the training set and drops in new datasets. CNNs, which are widely used in gastroenterology, are vulnerable to overfitting[14]. There is a series of proposed methodologies to resolve underfitting and overfitting challenges, including early stopping/dropout, penalty method/regularization, adjusting the learning rate and the optimizer, weight matrix initialization, data augmentation, and batch normalization[46,48]. Another intriguing point is that ML models are trained from datasets not practically available in the clinical setting. The different training variables are derived at various time points during the treatment of the patient and are not readily available at a given time. These missing parameters could become a shortcoming anchoring the use of ML in the clinical setting. All the above challenges have a common "true" solution. Physicians should be included in the development of ML models from the early stages of the procedure. Effective communication among physicians, the developing team, and the investors could address and resolve any potential shortcomings of these models. The key is to involve physicians in all the stages of the process. Physicians should be involved in problem identification to ensure that the developed tool addresses actual needs and in data collection and annotation to ensure that the data are labelled correctly, which is crucial in supervised learning. Additionally, we believe that the following steps should be followed during model development to avoid potential drawbacks: Physicians should provide a detailed description of the problem at hand to the developing team, explain what is requested by the model (the endpoint), describe the features thoroughly, cooperate to identify and engineer features of high predictive value, discuss the sample size needed based on the nature of the task (binary classification, multi-class classification, regression), ensure that the patients included in the developing procedure are a representative sample of the targeted group, conduct a rigorous clinical validation before the clinical application of the model, provide input on how the tool can be seamlessly be integrated into the existing clinical workflows without causing disruption, discuss the prospects of real-time learning, where the model continues to learn and improves following clinical application, and provide insights into ethical considerations including ensuring patient privacy and avoiding potential biases. Even, following the model's application in the clinical setting is essential to establish a framework of continuous monitoring and feedback from healthcare professionals to address model shortcomings and improve the model's efficiency. Finally, when applying AI/ML-based tools in the clinical setting is crucial to collect data on the impact of the use of such tools on the clinical outcome, the well-being of the patient, and the patient-physician relation. Failing to cooperate efficiently and to communicate what exactly is expected from the model could result in an end product that does not meet the expectations of the physicians and jeopardizes the safety of the patients.

COST-EFFECTIVENESS

Unfortunately, resources are not infinite. Especially in an era of technological advancement, with other cutting-edge technologies, such as three-dimensional printing and bioprinting, virtual and augmented reality, regenerative medicine, and robotics that also promise to facilitate patient care, AI must prove that it is worth investing in. In reality, AI, which has been at the forefront for many decades, has not yet been able to provide the expected results in healthcare. This is evident during AI winters, where investors demonstrate their frustration by cutting funding[4]. The expectations are often placed extremely high, and from their perspective, AI overpromises and constantly underdelivers. As mentioned above, effective communication among the physicians, the developing team, and the investors is crucial in agreeing on the expectations and avoiding disappointment in the end product. Nevertheless, even if physicians, developers, and investors are satisfied, the true success of the model should be assessed in association with the clinical outcome. Thus, conducting a series of cost-effective analyses is crucial to determine whether AI applications' benefits in healthcare are worth the associated cost. The costing of new technologies is generally challenging due to the lack of pre-existing data, and it involves bottom-up costing approaches, which typically require more resources and are time-consuming[49]. The cost associated with new technologies tends to drop over time; thus, cost estimation studies will need to be updated throughout the lifecycle of the AI application. Despite its importance, the topic of the cost-effectiveness of the application of AI in medicine is poorly investigated, mainly due to the lack of real AI applications in the clinical setting. There are several ways to improve the cost-effectiveness of implementing AI/ML-based technologies in healthcare. First, a series of models has been developed and patented with a high performance in performing several tasks, such as CAD systems that classify benign from malignant polyps. Thus, employing existing or pre-trained models rather than developing new tools from scratch could significantly decrease the cost of adopting these technologies in clinical practice. When, however, healthcare institutions decide to invest in developing AI/ML-based tools from scratch, a plethora of approaches could be adopted to improve the cost-effectiveness, including the utilization of collaborative platforms and open-source tools, running models on existing hardware when possible to reduce the need of specialized hardware, use of existing datasets when available to avoid data acquisition costs, and finally focus on developing tools that allow for the automatization of routine, time-consuming tasks while prioritizing high-incidence conditions with the potential for significant cost savings. One of the few studies addressing this issue is a recent study in the field of gastroenterology, where researchers

performed a Markov model microsimulation of AI application in screening colonoscopy and concluded that at the United States population level, AI application could prevent more than seven thousand colorectal cancer cases and more than two thousand related deaths, and provide a yearly saving of 290 million dollars[50]. These results are very promising, and as soon as AI models are widely applied in the clinical setting, studies proving their cost-effectiveness must be conducted.

CYBERSECURITY

Cybersecurity is a challenge not exclusive to AI but to the tendency of digitalization in general. Nevertheless, AI introduces further concerns since it requires a wholly electronic tracking of patient records. This raises concerns that a huge amount of sensitive data is vulnerable to massive disclosures[51]. For example, a transfer of 1.6 million patient records to Google DeepMind was ruled illegal in the United Kingdom[52]. Additionally, due to the sensitive nature of data (including financial data), healthcare facilities are essentially attractive targets for cyberattacks. The introduction of AI in healthcare allows for the emergence of new vulnerabilities. Imagine an intrusion into an ML, which is now manipulated into making wrong decisions or even introducing malevolent data. For example, in a study, the researchers demonstrated how a DNN was used to add or remove lung tumors from computed tomography scans at a level of accuracy that remained undetected by specialized radiologists[53]. Imagine a similar intrusion that will introduce colorectal cancer images during real-time colonoscopy. Such incidents could severely undermine any trust in AI in healthcare and have severe consequences on patient outcomes. How could patients and physicians be confident about any AI model suggestions, knowing that they can be manipulated? Evidently, the information technology infrastructure will need to be expanded in order to accommodate AI applications and shield these models from potential threats.

INTERPRETABILITY

Interpretability in ML refers to the degree of explainability and transparency of the model[54]. In other words, whether the logic behind the model's decisions is understandable or not. Imagine interpretability as a spectrum, where at the "white" end, we find "white-box" models, where their inner workings and decision-making processes are fully explainable and interpretable. At the other end of the spectrum, we find "black-box" models, which are characterized by a highly complex architecture to the extent that we are unable to comprehend their inner mechanisms and decision-making processes. In simple tasks, for example, when the features and target variable have a linear relation, white box models demonstrate a sufficiently high performance, rendering the use of black box models redundant. However, in complex tasks with high-dimensional data, black boxes exhibit superior performance to white boxes due to their ability to capture intricate patterns and relationships within the data. However, the application of black box models in healthcare introduces a series of challenges, including undermining the trust towards AI, inability to oversight any biases or prejudiced decisions towards minority groups, failure to justify our AI-assisted decision to patients, and violation of fundamental ethical tenets that physicians should hold a basic comprehension of the devices that they use. Avoiding using "black-box" models due to the above challenges could significantly handicap the potential of AI applications in healthcare. Thus, we should aim for methodologies that allow for a level of reasoning of "black-box" model predictions. Introducing a level of interpretability in black boxes applied in healthcare is crucial to enhance patient and physician trust in these tools and facilitate clinical decision-making. Interpretability can be divided into global and local. Global interpretability offers a level of explainability of the model as a whole[55]. Ways to improve global interpretability include feature importance analysis, surrogate models, and interactive visualization tools developed to allow users to explore how the various features influence the model's predictions[55-58]. For example, a model stratifying the risk of colorectal cancer development could provide its prediction along with a notification that the prediction is based mainly on the patient's sex, age, and blood count. On the other hand, local interpretability provides the reasoning behind individual predictions[55]. Methodologies to enhance local interpretability include surrogate models, Shapley values, saliency regions, and visualization techniques[55-58]. Shapley values could be used to demonstrate how each value of each feature contributes to the model's prediction, while saliency maps provide a *post-hoc* visualization to comprehend which parts of the input were used by the model to reach its decision and are particularly helpful for CNNs to visualize which parts of the image were used in the model's interpretation. For example, a saliency map would highlight exactly which parts of a biopsy that the model focused on to reach its diagnosis. Providing a level of explainability for "black-box" models is key to gaining the trust of both physicians and patients and will play a pivotal role in determining which models will dominate the industry.

OVERSIGHT AND LIABILITY CONCERNS

As acknowledged by the Food and Drug Administration (FDA), applications regarding AI/ML-based software marketing in healthcare have increased exponentially[59,60]. In early 2021, the FDA published the Artificial Intelligence/Machine Learning (AI/ML)-Based Software as a Medical Device (SaMD) Action Plan, while in 2023, it published the Marketing Submission Recommendations for a Predetermined Change Control Plan for Artificial Intelligence/Machine Learning (AI/ML)-Enabled Device Software Functions[59,61]. Similarly, the European Union has announced its first regulation on

AI applications, the EU AI Act[62]. Evidently, governments feel the need for AI oversight and regulation. Regarding law and regulation, the true challenge for the future is to find the golden mean between too little oversight, which may have devastating and severe consequences for the well-being of our patients, and too strict regulation, which will handicap innovation. AI/ML-based tools are unique in the way that they constantly evolve following the initial audit and marketing. Thus, as the model encompasses more data and is retrained, it diverges from the original product to the extent that it can be considered an entirely different product at different time points. Who ensures the safety and credibility of the product at different time points? Evidently, a lifecycle regulatory framework is needed that provides post-marketing oversight, or alternative time-limited authorizations should be granted with periodic audits when several modifications have been made to the initial product[63].

Liability is the main physician-related concern that could undermine the widespread application of AI in healthcare. Even if the research succeeds in providing highly accurate, reliable AI models, for AI to impact the clinical outcome, physicians will need to accept and adopt these models in their everyday lives. Liability could be the main concern that discourages physicians from doing so. To evade malpractice liability, a physician is expected to provide care at the same level as a competent, same-specialty physician, considering the available resources[64]. However, the introduction of AI makes liability a far more complicated matter. An intriguing concept to consider is the potential legal binding of AI's decisions in the future. As AI/ML-based software continues to advance and grow, it is reasonable to assume that they may eventually outperform physicians, at least in certain tasks. In such a scenario, how can physicians justify ignoring the decisions presented by AI, especially when AI's decisions are solely data-driven and lack any subjective bias?

Furthermore, who should be held liable if following the AI model's decision leads to patient harm? Currently, no legal precedent clarifies liability in cases where a patient suffers an injury due to an inaccurate output generated by AI/ML-based software[65]. In a published legal analysis, the authors present a series of scenarios based on the various combinations of whether the physicians follow the AI recommendation, whether the AI recommendation is correct, and whether a patient injury occurs. The authors' conclusion highlights that since existing laws protect physicians from liability when adhering to standard care, it incentivizes them to downplay AI's actual usefulness, potentially turning AI into a mere confirmatory tool instead of a tool to enhance the quality of care[65]. Until a comprehensive legal framework regarding liability is established, healthcare facilities have valid concerns and may hesitate to adopt AI technologies due to fears and uncertainties about potential liabilities that they may expose the facility and its staff to.

CONCLUSION

In this opinion review, we initially elaborate on the current status of the application of AI/ML-based software in gastroenterology. Currently, AI/ML-based models have been developed in the following applications: Models integrated into the clinical setting following real-time patient data flagging patients at high risk for developing a gastrointestinal disease, models employing non-invasive parameters that provide accurate diagnoses aiming to either replace, minimize, or refine the indications of endoscopy, models utilizing genomic data to diagnose various gastrointestinal diseases, CAD systems facilitating the interpretation of endoscopy images, models to facilitate treatment allocation and predict the response to treatment, and finally, models in prognosis predicting complications, recurrence following treatment, and overall survival that can be used for family and patient counseling. Then, we elaborate on several challenges and how they may negatively impact the widespread application of AI in healthcare and gastroenterology, offering possible ways to overcome them. Specifically, we have elaborated on concerns regarding accuracy, cost-effectiveness, cybersecurity, interpretability, oversight, and liability.

It is worth noting that the vast majority of AI research originates from high-income countries[66]. Despite being described as the solution that could narrow the gap in quality of care between high and low-income countries, AI may become one more brick in the wall of inequality. To avoid that, future AI initiatives should include middle and low-income countries. In any healthcare system where AI/ML-based software will be integrated, it is essential to simultaneously integrate equally sophisticated oversight mechanisms to ensure the safety of patients; otherwise, physicians may encounter unintended ramifications.

As mentioned before, AI/ML models' decision-making is based on quantifiable parameters. However, clinicians rely on non-quantifiable parameters for decision-making. It is crucial to state that overreliance on AI could impair our critical thinking and reasoning. Thus, the ultimate goal is not AI-driven but AI-assisted clinical practice. AI could be the means to augment our intelligence by embracing the inherent complexity of medicine. Envision the hospital of the future where hospitals are fully robotic, AI and robots conduct the majority of procedures, 3D printers are used to print almost everything, from medical equipment to artificial parts, telemedicine allows patients to stay at home, virtual and augmented reality will assist physicians at all procedures, and biosensors are placed at birth diagnosing all diseases at an early stage and alerting physicians[67-69]. It is unlikely that AI will replace physicians. Nevertheless, it is also unlikely that the skillset demanded by future physicians will be in any way similar to the present. In this upcoming transformation of healthcare with the integration of cutting-edge technologies, AI is expected to be at the center. Thus, physicians are expected to engage with AI to avoid becoming obsolete.

FOOTNOTES

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REVIEW

Gene targeted and immune therapies for nodal and gastrointestinal follicular lymphomas

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Abstract

Follicular lymphoma (FL) is the most common indolent B-cell lymphoma (BCL) globally. Recently, its incidence has increased in Europe, the United States, and Asia, with the number of gastrointestinal FL cases expected to increase. Genetic abnormalities related to t(14;18) translocation, BCL2 overexpression, NF-κB pathway-related factors, histone acetylases, and histone methyltransferases cause FL and enhance its proliferation. Meanwhile, microRNAs are commonly used in diagnosing FL and predicting patient prognosis. Many clinical trials on novel therapeutics targeting these genetic abnormalities and immunomodulatory mechanisms have been conducted, resulting in a marked improvement in therapeutic outcomes for FL. Although developing these innovative therapeutic agents targeting specific genetic mutations and immune pathways has provided hope for curative options, FL treatment has become more complex, requiring combinatorial therapeutic regimens. However, optimal treatment combinations have not yet been achieved, highlighting the importance of a complete understanding regarding the pathogenesis of gastrointestinal FL. Accordingly, this article reviews key research on the molecular pathogenesis of nodal FL and novel therapies targeting the causative genetic mutations. Moreover, the results of clinical trials are summarized, with a particular focus on treating nodal and gastrointestinal FLs.

Key Words: Gastrointestinal follicular lymphoma; Genetic mutation analysis using next-generation sequencing; MicroRNA; Gene targeted therapy; Immune therapy

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Core Tip: Recently, the incidence of follicular lymphoma (FL) has increased in Asia, Europe, and the United States, with the number of gastrointestinal FL cases expected to increase. This article reviews the recent literature on the molecular origins of, innovative treatments for, and clinical trial findings on FL, focusing on gastrointestinal FL. Genetic factors [*e.g.*, t(14;18) translocation and B-cell lymphoma 2 overexpression] drive FL growth, while microRNAs aid in its diagnosis and prognosis. Although recent trials have reported enhanced treatment outcomes, curative therapies remain elusive, and combinatorial regimens have not been optimized. Hence, gastroenterologists require a more comprehensive understanding of gastrointestinal FL pathogenesis to facilitate improved therapeutic outcomes.

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INTRODUCTION

Follicular lymphoma (FL) is the most common form of indolent B-cell lymphoma (BCL)[1]. In recent years, FL incidence has rapidly increased in Europe, the United States, and Asia[2], and the number of advanced-stage gastrointestinal FL cases is expected to increase in the near future. Although it responds well to treatment, it is prone to relapse and is refractory; hence, FL is regarded as an incurable disease with a poor prognosis. The NF-κB pathway is an intracellular signaling pathway activated by cell surface receptor stimuli in B-cell receptor (BCR)- and Toll-like receptor (TLR)-expressing cells. Indeed, the constant activation of this pathway contributes to lymphoma development. Histone methylation and acetylation of gene promoters regulate gene expression; abnormalities in gene expression can influence lymphoma development and proliferation. In addition, the t(14;18) translocation, found in most FLs, causes the overexpression of BCL2, eliciting an anti-apoptotic effect that mediates cell immortalization. Recently, genome-wide association studies (GWAS) have identified several loci involved in FL development.

Approximately 150 microRNAs (miRNAs) participate in the development of FL *via* enhanced cell proliferation. These include the miR-17-92 cluster, the importance of which has been evaluated in FL diagnosis and prognosis.

Rituximab, a monoclonal antibody against the CD20 surface antigen expressed in more than 90% of BCL-expressing cells, was developed approximately 20 years ago, and its combination with cyclophosphamide, doxorubicin, vincristine, and prednisolone (CHOP) represents a standard chemotherapy that continues to be the mainstay of treatment. Nevertheless, the past decade has witnessed considerable advancements in developing new FL therapeutic agents targeting various oncogenes.

This article reviews the latest findings in nodal FL, particularly related to molecular genetic analysis and diagnosis while summarizing the progress and future perspectives of novel FL therapeutics targeting various genetic abnormalities. The article concludes with a personal view of gastroenterologists' role in treating advanced-stage FL of gastrointestinal origin.

INCIDENCE AND EPIDEMIOLOGY

FL is the leading indolent (low-grade) BCL, accounting for 10%-20% of all non-Hodgkin lymphomas[1]. Its incidence is increasing rapidly in Western and Asian countries[2]. In particular, the incidence of FL in Japan is increasing[3]. Gastrointestinal FL (GI-FL) occurs in the lymphoid tissue of the gastrointestinal tract and is derived from the FL of B-cell lymphocytes and primarily arises from the lymph follicles (lymphoid apparatus) in the submucosa of the gastrointestinal tract. Histologically, GI-FL contains a folliculocentric lymphoma cell population; FL is categorized histopathologically as grade 1, 2, 3a, or 3b, with grade 3b usually identified and treated as an aggressive medium- or high-grade lymphoma. Most patients with grade 3b FL present with enlarged lymph nodes, and approximately 70%-85% are diagnosed with an advanced-stage III/IV disease, often associated with significant bone marrow involvement. Although the incidence of GI-FL is low and relatively rare compared with that of other lymphomas, it is more common in middle-aged and older adults, with no apparent difference in incidence between men and women; however, the peak incidence in certain age groups is not reported.

SYMPTOMS

The symptoms of gastrointestinal FL vary among individuals, ranging from asymptomatic with very mild symptoms (most common) to well-defined symptoms, depending on the stage of the disease, degree of disease progression, and tumor location. The symptoms of GI-FL include: Discomfort or pain in the upper abdomen, often worsening after meals or at night; gastrointestinal symptoms: Nausea, vomiting, loss of appetite, bloating, upset stomach, diarrhea, and constipation; weight loss due to anorexia and impaired nutrient absorption; anemia due to intestinal bleeding and tumor

growth (anemia-induced fatigue and shortness of breath may occur); and edema due to the compression on lymphatic vessels and blood vessels, particularly in the abdomen and lower limbs.

Among the 249 GI-FL cases reported in a previous study, 150 with clinical symptoms were asymptomatic at onset (43.4%), 9.3% presented with vague gastrointestinal symptoms, including abdominal discomfort or heartburn, 28.7% had abdominal pain, 8.0% had symptoms of bowel obstruction, such as nausea and vomiting, and 6.0% had melena, such as tar-like black stools, a symptom of upper gastrointestinal bleeding[4].

DIAGNOSIS OF GI-FL

GI-FLs are diagnosed using a combination of methods. The following section details those most commonly used.

Endoscopy

Endoscopy represents the most important diagnostic test for GI-FL as it is useful for gross diagnosis and facilitates the extraction of biopsies for pathological examination and histological analysis. Gastric malignant lymphomas are divided into several gross types[5]. Colorectal malignant lymphomas often present with characteristic polyp-like endoscopic findings, termed multiple lymphomatous polyposis (MLP)[6], which are useful for diagnosis. The frequency of GI-FL detection has increased rapidly in Japan owing to the widespread and generalized use of small bowel endoscopy, advances in endoscopic equipment, improved endoscopist techniques, and increased opportunities for health screening [7].

Histopathological examination

Tissue samples obtained from biopsies are assessed pathologically using a microscope. GI-FL is diagnosed based on the presence of folliculocentric lymphoma cell populations and other histological features characteristic of FLs. Moreover, testing for specific proteins through immunostaining is performed. The following is a list of parameters used in the pathological diagnosis of FL.

Follicular-like structures: FLs are characterized by the formation of follicular-like structures within the tumor that are B-cell aggregates forming follicles within tumors. Additional abnormal follicular structures can be present in FL.

Grade: FLs are classified into three grades (1-3) by assessing the degree of formation of follicular-like structures within the tumor and the atypia (morphological abnormalities) of lymphoma cells.

Immunohistopathological examinations: Immunostaining findings showing CD20 and CD10 positivity and BCL2 overexpression are characteristic of FL and essential for the histological diagnosis of FL.

In FLs, lymphoma cells express the CD20 surface protein, a specific B-cell marker and an important indicator for diagnosing FL. CD10 is also expressed by FL cells. BCL2 is a protein rarely expressed in normal lymphoid tissues but is overexpressed in FLs. It is involved in the regulation of apoptosis (cell death) and is a characteristic immunohistochemical marker in FLs.

Chromosomal abnormalities: The t(14;18) translocation is frequently observed in FLs, causing the fusion of *BCL2* with the immunoglobulin H chain (*IGH*) gene, leading to overexpression of BCL2. Pathologists confirm IGH reconstruction as a basis for a definitive FL diagnosis.

Molecular genetic analyses

Molecular genetic analyses provide important information regarding the pathogenesis and prognosis of FL. This section details the molecular genetic analyses of FLs.

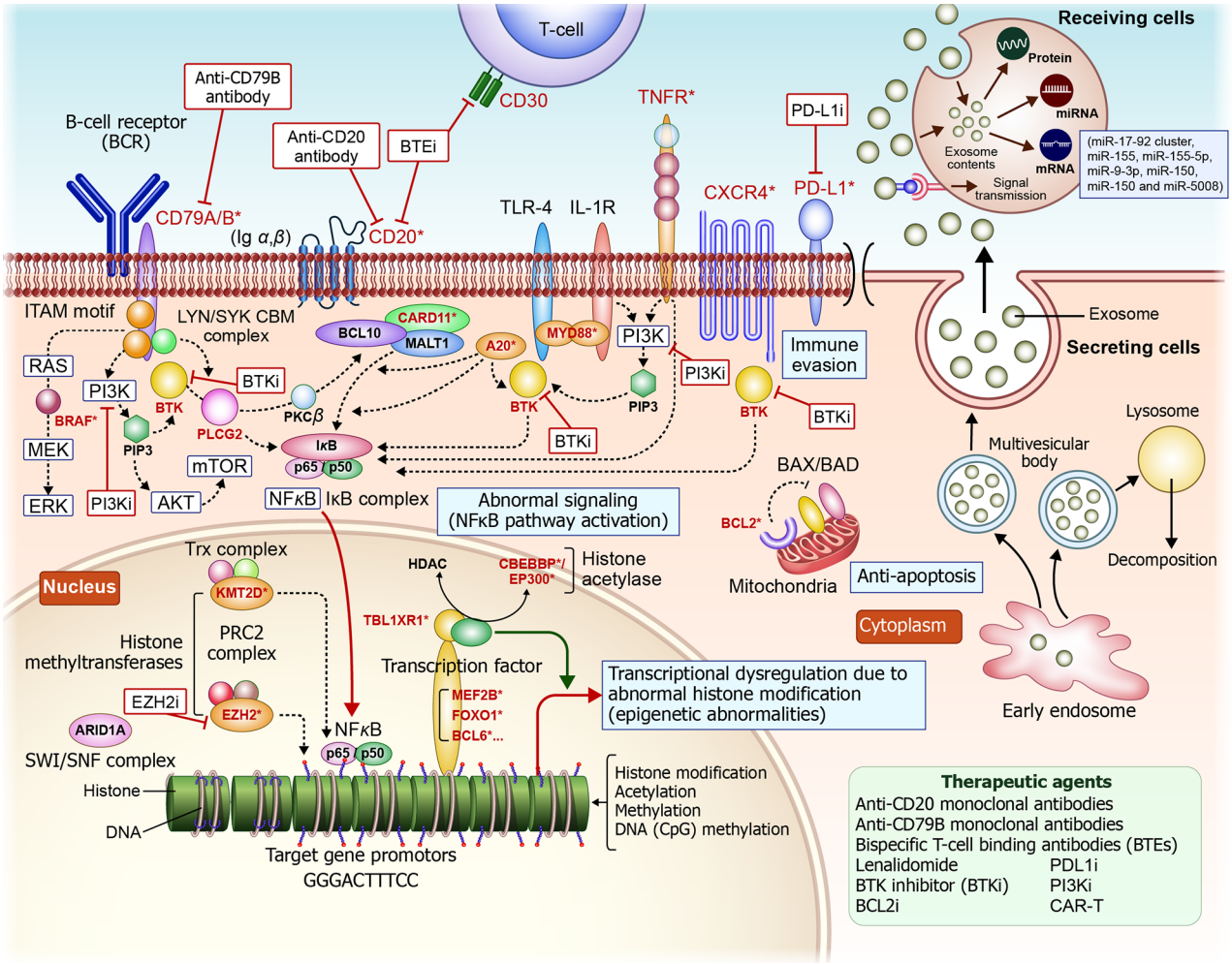
Detection of t(14;18) translocation: As mentioned in the section on pathological diagnosis above (section 1.3), t(14;18) translocation is a key component of FL diagnosis.

Detection of gene rearrangements using polymerase chain reaction: In FLs, there are rearrangements of *IGH* and other genes. These can be detected *via* polymerase chain reaction, allowing for the evaluation of the presence and frequency of specific gene rearrangements.

Genetic mutation analysis using next-generation sequencing: In FL, specific genetic mutations are associated with disease progression and prognosis. Next-generation sequencing (NGS) enables rapid and large-scale DNA sequencing to identify such mutations and assess their significance in FL. In particular, numerous genetic abnormalities have been characterized for BCL using NGS. These abnormalities in BCL impact proteins involved in tumorigenesis, resulting in: (1) Constant activation of the NF-κB signaling pathway; (2) Abnormal functioning of epigenome-related enzymes; (3) Abnormal expression of apoptosis-related factors; and (4) Immune system evasion (Figure 1)[8].

Constant activation of the NF-κB signaling pathway: This activation is involved in lymphoma development. Mutations in NF-κB pathway-related factors such as CD79B, MYD88, A20/tumor necrosis factor alpha-induced protein 3 (TNFAIP3), and CARD11 occur in active B-cell (ABC)-type diffuse large BCL (DLBCL).

Functional abnormalities in epigenome-related enzymes: Genetic abnormalities related to histone methyltransferases (such as KMT2D and EZH2) and histone acetylases (such as EP300 and CREBBP) occur in FL- and germinal center B-cell (GCB)-type DLBCL.



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Figure 1 Follicular lymphoma-related genes involved in follicular lymphoma pathogenesis and proliferation, and the action of novel therapeutic agents targeting them. MicroRNAs are secreted from the follicular lymphoma cytoplasm as exosomes, taken up by recipient cells, and regulate gene expression (right side of the figure).

Abnormalities in apoptosis-related factors: Chromosome t(14;18) reciprocal translocations, found in most FLs and 20%-30% of DLBCLs, promote the expression of BCL with anti-apoptotic properties and are involved in cell immortalization.

Immune system evasion: Abnormalities in genes related to immune evasion, such as PDL1/L2, are detected in DCLBL-like forms, Hodgkin lymphomas, and adult T-cell lymphomas. These abnormalities possibly aid lymphoma cells in evading immune attacks. The following findings have recently been reported for each NGS-discovered BCL-related gene involved in FL development and proliferation:

Polatuzumab vedotin is a first-in-class anti-CD79B antibody-drug conjugate (ADC) that combines an anti-CD79B monoclonal antibody with a tubulin polymerization inhibitor. CD79B is specifically expressed in B-cells. Polatuzumab vedotin binds to CD79B to deliver the chemotherapeutic agents and destroy B-cells. Three major clinical trials have investigated the efficacy and safety of polatuzumab vedotin. The G029376 trial demonstrated its efficacy in relapsed/refractory (r/r) FL and DLBCL[9]. The GO29365 trial tested the efficacy of polatuzumab vedotin + bendamustine and rituximab (BR) or bendamustine and obinutuzumab therapy in patients with r/r FL or DLBCL[10]. The JO40762 (P-DRIVE) study evaluated the complete response rate in patients with r/r DLBCL treated with polatuzumab vedotin + BR therapy[11]. Comprehensive genetic analyses classified DLBCL into ABC-type DLBCL and GCB-type DLBCL. *MYD88* and *CD79B* mutations are highly prevalent in ABC-type DLBCL and contribute to the constant activation of the NF-κB pathway, resulting in lymphoma development. In addition, ABC types with mutations in *MYD88* and *CD79B* in BCLs have a poorer prognosis[12]. These classifications are important for predicting prognosis and treatment response.

A20/TNFAIP3 is a cytoplasmic zinc finger protein that inhibits NF-κB activity mediated by tumor necrosis factor (TNF). AIP3 deficiency is observed in ABC-type BCL, MALT lymphoma, and Hodgkin lymphoma. A20, also known as TNFAIP3, is involved in the negative regulation of the NF-κB pathway. A20 induces a suppressive inflammatory response and exhibits anti-apoptotic effects while affecting immune homeostasis. Mutations and deletions in *A20* are frequent in non-GCB-typey DLBCL and DLBCL. *A20* gene abnormalities are also reported in certain DLBCLs[13]. Deletions, inactivating mutations, and methylation of the *A20* promoter at 6q23 inactivate A20 in various lymphomas. A20 inactivation is a key feature of lymphomas, and loss of A20 expression occurs in FLs[14]. Meanwhile, BCL10 is an essential regulator of NF-κB activation and is implicated in B-cell proliferation[15].

KMT2D is a histone lysine methyltransferase important in suppressing BCLs. Deletion of KMT2D increases the number of germinal center B-cells and promotes B-cell proliferation in mice. The overexpression of BCL2 combined with KMT2D deletion increases the incidence of lymphomas derived from germinal center B-cells. Therefore, KMT2D can function as a tumor suppressor and may be involved in reconstructing epigenetics that promote lymphoma development. Accordingly, targeting KMT2D is an effective approach for alleviating the early stages of tumorigenesis[16]. Tazemetostat is an orally administered EZH2 inhibitor that selectively inhibits a specific epigenetic-related protein, EZH2, to regulate the expression of cancer-related genes and inhibit cancer cell growth[17]. Tazemetostat was approved in Japan based on the results of clinical trials in patients with r/r B-cell non-Hodgkin lymphoma with EZH2 mutations[18,19]. Mutations in EZH2 occur in 7%-27% of FLs[20,21]; these patients may be eligible for treatment with tazemetostat. However, considering that FL is a refractory disease with frequent relapses, new treatment strategies are required. CREBBP, EZH2, MEF2B, and EP300 mutations occur in 33%, 27%, 15%, and 9% of FLs, respectively. Genome-wide analysis *via* NGS has shown that *EZH2, CREBBP*, and *MLL2*—histone-modifying genes—are frequently mutated in FL with essential roles in lymphomagenesis. *IGH-BCL2* translocations and CREBBP mutations are early events, whereas MLL2 and TNFSFR14 mutations are late events during disease evolution. In the 2008 WHO classification, three new variants were included: (1) Pediatric FL; (2) Primary intestinal FL; and (3) *In situ* FL[22].

MiRNAs

MiRNAs are non-coding RNAs involved in regulating gene expression. The aberrant expression of specific miRNAs is reported in FL. Hence, analyses of miRNAs provide essential information for predicting disease mechanisms and prognoses.

Relationship between exosomes and miRNAs: Exosomes are small vesicles released by cells containing various biomolecules (proteins, ribonucleic acids, and lipids) with myriad biological roles, including transmitting information between cells and expulsing unwanted intracellular components. MiRNAs are produced intracellularly and regulate gene expression by binding to mRNAs and regulating their stability and translation efficiency. The miRNAs produced inside cells can be wrapped in exosomes and released to the outside of the cell, facilitating the transport of miRNAs to other cells in remote areas, where they participate in information transfer and cell–cell interactions. This interaction is an important mechanism of information transfer and disease progression. The transport of miRNAs by exosomes is a form of extracellular signal transduction. When a sender cell receives a specific signal or is subjected to pathological conditions, the miRNA profile of the released exosomes changes, influencing the effect on recipient cells. Therefore, miRNA transduction by exosomes is important in regulating disease progression and cell–cell interactions (Figure 1).

MiRNAs associated with FL: Among the miRNAs related to FL are miR-17, miR-18a, miR19-a, miR-20a, miR-19b-1, and miR92a of the miR-17-92 cluster, and others are miR-155, miR-21, miR-155-5p, miR-150, miR-9-3p, miR-29 family, miR-21, miR-5008, miR-7e-5p, miR-451, miR-338-5p, miR-142, miR-376 cluster, and approximately 150 others (Table 1).

The miR-17-92 cluster is overexpressed in FLs and contains several miRNAs, including miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a. These are involved in cancer cell proliferation, cell cycle regulation, and apoptosis inhibition. The miRNA-17-92 cluster was first identified in malignant BCLs and has important roles in the immune system, heart and lung development, and oncogenic events[23]. Overexpression of the miR-17-92 cluster in MCL negatively regulates the expression of various BCR suppressor molecules, resulting in the overactivation of BCR signaling [24]. In a genetic analysis of 33 cases of small intestinal DLBCL and gastrointestinal MALT lymphomas, miRNA levels were reduced by more than two-thirds compared with those in normal tissues (13 species in the decreased group) and increased by more than 1.5-fold (15 species in the increased group), indicating the existence of miRNAs that were upregulated and downregulated[25], in association with FL development and tumor tissue growth. In patients with B-cell non-Hodgkin lymphoma (B-NHL), members of the miR-17-92 cluster are differentially expressed among the B-NHL subtypes, with the most prominent differences between FL and germinal center B-cell-like (GBC) subtypes[26]. MiR-18, miR-19a, and miR-92a were overexpressed in patients with B-NHL with better overall survival (OS), and high levels of miR-19a and miR-92a significantly reduced event-free survival (EFS). Meanwhile, miR-17-92 overexpression reduces the incubation period required for xenograft tumor visualization, and its knockout reduces tumor formation. MiR-17-92 expression in FL differs significantly from that in GBC, and miR-19a could have marked effects on OS and EFS in patients with B-NHL[26].

MiR-155 is a miRNA encoded by the *MIR155* host gene or *MIR155HG* in humans. MiR-155 is involved in various physiological and pathological processes[27,28]. *In vivo* regulation of miR-155 expression from external molecular sources might suppress malignant tumor growth[29] and viral infections[30] while encouraging the advancement of cardiovascular diseases[31]. *MIR155HG* was initially identified as a gene that is transcriptionally activated *via* promoter insertion into a retroviral integration site commonly found in BCLs, earlier known as the B-cell integration cluster. RNA polymerase II carries out the transcription of *MIR155HG*. The resultant RNA is approximately 1500 nucleotides long and is capped and polyadenylated; single-stranded miR-155—a 23-nucleotide segment—is retained in exon 3 and undergoes processing from the parent RNA molecule[32]. Sequence analysis of small RNA clone libraries compared with those from other organ systems shows that miR-155-5p is one of five miRNAs (miR-142, miR-144, miR-150, miR-155, and miR-223) specific to hematopoietic cells, including B-cells, T-cells, monocytes, and granulocytes[33].

MiR-155 is transferred from leukemic cells to healthy B-cells *via* gap junctions and promotes their transformation into tumor-like cells. In addition, immature B-cells deficient in miR-155 evade apoptosis due to elevated BCL2 protein levels. This protein is involved in B-cell malignancy and is regulated by miR-155. Lawrie *et al*[34] reported that miR-155, miR-210, miR-106A, miR-149, and miR-139 are differentially regulated in non-neoplastic lymph nodes and DLBCL/FL. Meanwhile, Roehle *et al*[35] reported that miR-155, miR-21, and miR-221 are more highly expressed in ABC- than GCB-

Table 1 MicroRNAs involved in follicular lymphoma development and proliferation

MicroRNA(s)	Role in FL development	Sequence (5' to 3')	Ref.
miR-17	Involved in cell proliferation and apoptosis	UAAAGUGCUUACAGUGCAGGUAG	[23-26]
miR-18a	Role in angiogenesis and tumorigenesis	UAAGGUGCAUCUAGUGCAGAUAG	[26]
miR-19a	Promotes cell survival	UGUGCAAAUCUAUGCAAAACUGA	[26]
miR-20a	Involved in cell cycle regulation	UAAAGUGCUCAUAGUGCAGGUAG	
miR-19b-1	Promotes cell survival	UGUGCAAAUCCAUGCAAAUCUGA	
miR-92a	Role in angiogenesis	UAUUGCACUUGUCCCGGCCUGU	[26]
miR-155	B-cell transformation in follicular lymphoma	UUAAUGCUAAUUGUGAUAGGGGU	[27-35]
miR-21	Overexpressed and promotes tumor progression	UAGCUUAUCAGACUGAUGUUGA	[35]
miR-155-5p	Role for diagnosis of FL	UUAAUGCUAAUCGUGAUAGGGGU	[33,36]
miR-150	Influences B-cell differentiation	UCUCACAUUGGUCUACAAUCU	[33,36,41]
miR-9-3p	Role for diagnosis of FL and in B-cell malignancies	UCUUUGGUUAUCUAGCUGUAUGA	[36-40]
miR-29 family	Epigenetic regulation and tumorigenesis	Varies by family member	[42]
miR-5008	Regulation of endogenous mRNA levels	Sequence is not available	[43]
miR-7e-5p	Downregulation by c-MYC over-expression arise poor prognosis of FL	UGAGGUAGGAGGUUGUAUAGUU	[44]
miR-451	Involved in cell cycle progression	AAACCGUUACCAUUACUGAGUU	[45]
miR-338-5p	Possible role in cellular proliferation	UAAUGACUGCACUGACCUUUGA	[45]
miR-142	Role in hematopoiesis and B-cell function	UGUAGUGUUUCCUACUUUAUGGA	[46]
miR-376 cluster	Involvement in cellular differentiation	Varies by cluster member	[42]

FL: Follicular lymphoma.

type cell lines. Notably, the expression levels of miR-155 and miR-21 were higher in non-malignant ABC cells than in GCB cells. Moreover, Arzuaga-Mendez *et al*[36] reported that miR-155 expression is a valuable biomarker for the diagnosis and differentiation of lymphoma subtypes, specifically miR-155-5p and miR-9-3p for the diagnosis of FL, and miR-150 and miR-17-92 cluster for the differential diagnosis of FL and DLBCL. Additionally, miR-9-3p is associated with tumorigenesis, functioning as an oncogene in papillary thyroid cancer[37,38] and a tumor suppressor in gastric cancer[38,39]. It also modulates breast cancer by influencing growth and drug resistance[38,40]. Lower levels of miR-150 and higher levels of its target, FOXP1, are associated with reduced OS, indicating that miR-150 might be an effective biomarker that can be quantified in formalin-fixed paraffin-embedded tissue. In addition, the MYC/miR-150/FOXP1 axis in malignant B-cells helps determine the aggressiveness and high-grade transformation of FL[41].

An array-based assay including 76 non-Hodgkin B-cell mixed lymphomas was performed to investigate the etiology of different BCLs using miRNAs. The miR-29 family and miR-17-92 cluster are regulated by *MYC* genes associated with MYC proteins in BCLs. A network analysis was used to demonstrate that a small set of differentially expressed miRNAs associated with MYC can either upregulate or downregulate a select group of hub proteins, including BCL2, CDK6, MYB, ZEB1, CTNNB1, BAX, and XBP1 in BCLs[42]. MiR-21 is associated with cancer progression and metastasis; it is also highly expressed in FLs and has been implicated in the regulation of cell proliferation and apoptosis.

Larrea *et al*[43] identified 21 genes with altered miRNA-binding sites in transformed FL (tFL), that is, FL that is transformed to have more aggressive tumor cells. More than 40% of these mutations are present only in patients with tFL. Mutations in BCL2 and EZH2 reduce the binding efficiency of miR-5008 and miR-5008, regulating endogenous mRNA levels[43].

Additionally, the downregulation of miR-7e-5p by c-MYC overexpression is associated with a poor prognosis in patients with FL[44]. Furthermore, a study reported several abnormally altered miRNAs in FL, specifically, 39 significantly downregulated and 27 significantly upregulated miRNAs. MiR-451 was the most downregulated (345-fold decrease), while miR-338-5p was the most upregulated (172-fold increase) compared with those in healthy donors[45].

Recurrent mutations in hsa-miR-142 and editing of the hsa-miR-376 cluster were observed in diffuse large B-cell and FLs. A direct physical interaction between miRNA and mRNA was observed using argonaute-2 photoactivated ribonucleoside-enhanced cross-linking and immunoprecipitation experiments. This integrated analysis revealed several regulatory pathways associated with lymphoma pathogenesis, including the Ras, PI3K-Akt, and MAPK signaling pathways affected by lymphoma mutations. The regulation of mRNAs *via* miRNAs is highly relevant to lymphoma pathogenesis[46]. The onset of malignant tumors, such as malignant lymphomas and other cancers, as well as the proliferation of tumor cells, is regulated quantitatively by miRNAs, not qualitatively. The intricate mechanisms regulating expression are balanced through elaborate and judicious positive and negative regulation.

MiRNAs exhibit duplicity among subgroups of malignant lymphomas. Many miRNAs regulating tumor cell growth overlap between DLBCL and FL. Aberrantly expressed miRNAs have been identified in patients with hematological malignancies, suggesting that they may be novel clinical diagnostic and prognostic biomarkers. Hershkovitz-Rokah *et al* [47] experimentally validated miRNA expression signatures in DLBCL and FL. The authors identified unique miRNA expression patterns for each disease, with some overlap and abnormal expression of miRNAs between them. Their analysis also led to the discovery of several relevant miRNA–mRNA pairs in each disease. Furthermore, potential regulatory pathways were highlighted *via* gene ontology analysis. Specific miRNAs (miR-15a, miR-16, miR-17, miR-106, miR-21, miR-155, and miR-34a-5p) were aberrantly expressed in DLBCL tumor tissues; their specific expression patterns proved useful for DLBCL diagnosis. The overexpression of these miRNAs influences FL pathogenesis. However, given the highly complex functions and interactions of miRNAs, various aspects remain to be elucidated. Understanding how these specific miRNAs are involved in the development and progression of FL is essential. In particular, genes and signaling pathways targeted by these miRNAs should be identified, as their regulation can alter cancer cell behavior[47].

MiRNAs are promising biomarkers for FL diagnosis and prognosis and are relatively stable in the blood and tissues. Hence, they can be measured using relatively simple experimental techniques. This could have potential applications for non-invasive diagnosis and prognosis of FL. Notably, the functions of individual miRNAs are complex and can each affect numerous genes and signaling pathways, requiring comprehensive and cumulative interpretation of research results to enable the development of effective treatments and new biomarkers.

Indeed, miRNAs represent potential new treatment targets for FL as they can be easily produced with simple 20-30 nucleotide sequences. Hence, if they can be administered at high concentrations to tumors *via* intravascular infusion, transarterial injection, or direct injection, miRNAs can elicit therapeutic effects by upregulating or downregulating relevant oncogenes.

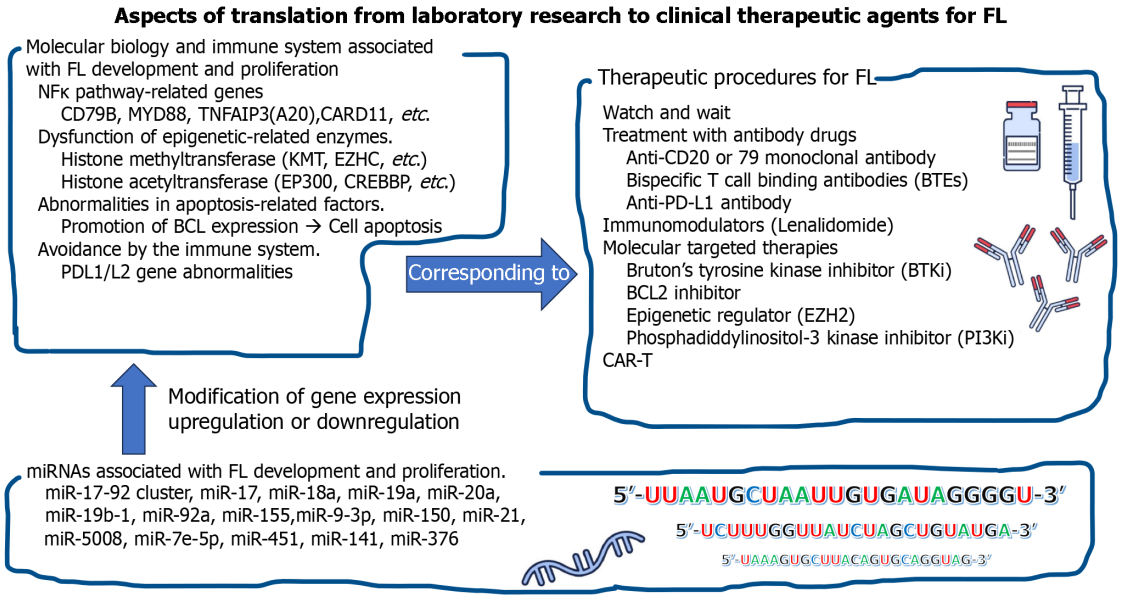
GWAS

GWAS examine the association between genetic polymorphisms (SNPs) and specific diseases. In FL, GWAS have identified genetic variants associated with the risk of disease development. For example, Skibola *et al*[48] conducted a large GWAS comprising 4523 cases and 13344 controls of European descent and identified five non-human leukocyte antigen (HLA) FL susceptibility loci and mutations in the *HLA* gene associated with FL risk: 11q23.3 near CXCR5, 11q24.3 near ETS1, 3q28 at LPP, 18q21.33 near BCL2, and 8q24.21 near PVT1. In addition, four HLA-DRβ1 multichain amino acids and independent signals of HLA class II rs17203612 and HLA class I rs3130437 were associated with FL risk. Therefore, mutations at several common loci outside the HLA region significantly contribute to FL risk[48]. Indeed, mutations at specific gene loci other than HLA have been identified for each of the five subtypes of non-Hodgkin lymphoma, namely, DLBCL, FL, chronic lymphocytic leukemia (CLL), MZL, and primary central nervous system lymphoma (PCNSL)[45]. The WEE1 Locus is a "potential therapeutic marker"[49]. Moore *et al*[50] estimated the odds ratios (ORs) and 95% confidence intervals (CIs) using logistic regression for the subjects' height and the risk of developing the four non-Hodgkin lymphoma subtypes. A slight significant difference was observed in an increased risk of developing CLL (OR = 1.08, 95%CI: 1.00-1.17, $P = 0.049$), whereas no significant association was observed with DLBCL, FL, or MZL[50].

Epidemiological studies have shown that the risk of non-Hodgkin lymphoma, including FL, is higher in patients with autoimmune disease. This association has been analyzed in a GWAS[51,52]. No association was reported between B-cell-mediated autoimmune diseases and the risk of FL or MZL[51]. Moreover, there was no association between autoimmune diseases and the risk of developing non-Hodgkin lymphoma; however, non-Hodgkin lymphoma shared more genetic etiologies with autoimmune diseases ($P = 0.0041$) than solid tumors[52]. In particular, genes involved in apoptosis and telomere length are associated with autoimmune diseases and non-Hodgkin lymphoma[52]. Additionally, Choi *et al*[53] constructed a site-specific polygenic risk score (PRS) using GWAS and identified risk variants for nine tumor types, including FL. They also estimated the hazard ratio for each tumor type in relation to the PRS in 400807 people of European descent from the United Kingdom Biobank; significant capacity reactivity was observed, with a two-fold higher risk in FL[53].

An association between other tumor types and blood lipid levels was reported by Kleinstern *et al*[54]. The authors analyzed the association between each non-Hodgkin lymphoma subtype and lipid traits using GWAS. High-density lipoprotein (HDL) cholesterol positively correlated with FL, DLBCL, and MZL, whereas triglyceride (TG) negatively correlated with MZL. There was no strong correlation between the OR and *P* value. Meanwhile, Wang *et al*[55] analyzed 2686 cases of FL through GWAS using SNP2HLA; the risk of developing FL increased with an increasing number of homozygous HLA class II loci. This supports the role of HLA zygosity in non-Hodgkin lymphoma cases and indicates that different immune pathways could underlie the etiology of non-Hodgkin lymphoma[55].

The association between prognostic predictors of FL and various loci has also been evaluated using a GWAS[56]. In a multicenter meta-analysis of 586 patients diagnosed with FL in Denmark, Sweden, and the United States, loci strongly associated with lymphoma-specific mortality were observed at SNPs on 17q24 (rs10491178)[56]. In addition, two high-binding SNPs in interleukin-8 (rs4073) were associated with OS[56]. Conde *et al*[57] showed that multiple independent loci on the X chromosome could be involved in the etiology of FL. The Xq21.1 signal was also observed in DLBCL, indicating the sharing of a susceptibility locus with FL[57]. Multiple loci involved in the development of B-cell non-Hodgkin lymphoma, including FL, have been identified. For example, several loci, beyond the five loci reported by Waller *et al*[58], are associated with developing non-Hodgkin lymphoma; a GWAS identified approximately 150 Loci associated with non-Hodgkin lymphoma development[58]. However, these loci have been identified in fragments; therefore, several other important loci could remain unknown. Further GWAS studies and advances in analysis methods will lead to the discovery of more new loci associated with the development of FL, elucidation of the mechanisms underlying the interactions that lead to lymphoma development, development of new therapeutic agents targeting these loci, and the era of FL cure (Figure 2).



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Figure 2 Genetic mutations that cause follicular lymphoma development and microRNAs that regulate onset and tumor cell growth. Novel therapeutic agents have been developed to target FL-associated gene mutations involved in follicular lymphoma (FL) pathogenesis and proliferation. MicroRNAs are secreted from the FL cytoplasm as exosomes and internalized into recipient cells where they are transduced and regulate gene expression *via* upregulation or downregulation. FL: Follicular lymphoma; BCR: B-cell receptor; BTE(i): Bispecific T-cell binding antibodies (inhibitor); PD-L1(i): Programmed death ligand 1 antibody (inhibitor); miRNA: MicroRNA; mRNA: Messenger RNA; PI3K(i): Phosphatidylinositol-3 kinase (inhibitor); BTK(i): Bruton's tyrosine kinase (inhibitor); AKT: Protein kinase B; mTOR: Mechanistic target of rapamycin; EZH2(i): Enhancer of zeste homolog 2 (inhibitor).

Imaging studies

Imaging studies are used to assess the disease stage. Common imaging modalities include chest radiography, ultrasonography, computed tomography, and magnetic resonance imaging. These assess the lymph node and visceral enlargement, presence of metastases, and location and size of the lesion.

Additional tests

Additional tests could be required in advanced stages or under certain circumstances. Bone marrow biopsies and cerebrospinal fluid tests help confirm the extent of the disease and the sites involved. Several diagnostic specialists (pathologists, hematologists, and radiologists) are involved in the diagnosis process to increase accuracy. Once the diagnosis is confirmed, an appropriate treatment strategy (chemotherapy, radiotherapy, immunotherapy, or surgery) is selected based on the disease stage and general health of the patient.

CHOICE OF TREATMENTS

The following factors influence treatment selection for GI-FL[59,60]: (1) Lesion characteristics and progression: The lesions' location, size, and spread are assessed to determine the characteristics and progression of the disease. This provides a basis for determining disease risk and prognosis; (2) Establishing a pathological diagnosis: The pathological diagnosis of FL should be based on appropriate laboratory and histological evaluations. A pathological diagnosis forms the basis for choosing the most appropriate treatment[61]; and (3) Accurate staging assessment: Staging of the disease is based on accurate diagnosis and evaluation. The stage is important for assessing disease progression and developing a proper treatment plan[59]. Treatment decisions are determined by histological subtype and the extent of the disease[62], that is, stage (Lugano classification) (Table 2)[60]. Although histological grade is a good predictor of prognosis in nodal FL, this alone cannot determine the treatment. Stage, which indicates the extent and spread of the disease, determines the treatment; both stage and histological grade can be used concurrently. In primary GI-FL, personalized treatment choices are determined based on the individual circumstances of each case and the changes over time, in addition to the Lugano classification and histological grade[7].

For advanced stages (III and IV), irradiation is used if the disease is confined to a small number of areas that can be irradiated; this is augmented with chemotherapy with or without immunotherapy depending on the extent of organ involvement, spread, number of sites, and presence of distant metastases. In stages III and IV, if there are one, two, or three fewer lesions and irradiation is feasible in the area, radiation plus the extent of organ-specific invasion, spread, number of sites, and presence of distant metastases may be considered. Additionally, chemotherapy, chemotherapy plus immunotherapy (or in some cases a triple therapy), and, if appropriate, for example, if the patient has symptoms of obstruction of the digestive tract, where resection would improve symptoms and patient quality of life, surgical resection

Table 2 Lugano staging of gastrointestinal tract lymphoma[60]

Stage	
I	Tumor confined to GI tract
	Primary site or multiple, non-contiguous lesions
II	Tumor extends into the abdomen from primary GI site
	Nodal involvement
II_1	Local: Paragastric in cases of gastric lymphoma and para-intestinal for intestinal lymphoma
II_2	Distant: Mesenteric in case of an intestinal primary lymphoma; otherwise paraaortic, paracaval, pelvic, or inguinal
IIE	Penetration of serosa involving adjacent organs or tissues: Enumerate sites of involvement, *e.g.*, IIE (pancreas), IIE (large intestine), IIE (post-intestinal wall)
	Where there is nodal involvement and penetration involving adjacent organs, the stage is denoted using a subscript (1 or 2) and E, *e.g.*, II_1E (pancreas)
IV	Disseminated extra-nodal involvement or a GI tract lesion with supradiaphragmatic nodal involvement

GI: Gastrointestinal.

can be considered. Treatment should be customized to individual cases considering the specific requirements and constraints. In GI-FL, the characteristics of the gastrointestinal tract should be considered, and treatment measures are tailored to the disease status of each patient[7]. GI-FL treatment should be personalized based on the disease stage and patient's general health status. If necessary, the treatment plan should be reviewed regularly and modified based on disease progression, treatment efficacy, and patient response.

TREATMENT

"Watch-and-wait" approach

A watch-and-wait approach may be adopted in some patients to treat GI-FL. In this approach, patients diagnosed in the early stages of the disease are not immediately administered treatment but are regularly observed and monitored. When GI-FL is detected early, patients have a good prognosis. In these cases, a good 10-year prognosis is possible with surgical resection alone or with R-CHOP treatment (a combination of chemotherapy and targeted therapy). However, "watch-and-wait" is often the mainstay of treatment owing to the side effects and reduced patient quality of life. Schmatz *et al*[63] compared 63 patients with stage I GI-FL in treatment and "watch-and-wait" groups; there was no difference in progression-free survival (PFS) or OS. In another study, patients with GI-FL and low tumor volume were divided into a "watch-and-wait" group (15 patients) and a rituximab combined with chemotherapy group (14 patients); no difference in prognosis was observed[64]. Given the broad distribution of GI-FL at the time of diagnosis, localized treatment options are often deemed inappropriate. However, certain pathological features, including lower tumor extension and invasiveness than those in nodal FL, suggest that “watch-and-wait” is a viable option. This approach is promising in some subtypes of GI-FL with a favorable prognosis. Iwamuro *et al*[65] recently reported a long-term retrospective study for localized (stage I or II) GI-FL patients managed with a watch-and-wait strategy, revealing 5-year and 10-year event-free survival rates of 91.1% and 86.9%, respectively. Hence, this strategy is reasonable for GI-FL patients in early disease stages. However, the individual patient’s condition prior to treatment, the overall effect of possible treatments, and their side effects should be considered. The “watch-and-wait” approach should be explained to the patient as an option before choosing the treatment.

Radiotherapy

Radiotherapy can be used to treat localized lesions, particularly when the lesions are localized or when used in combination with other treatment modalities. Radiotherapy uses high-energy radiation to destroy tumor cells. However, several factors have led to radiotherapy not being effectively adopted for treating nodal or gastrointestinal FL[66,67].

Wound avoidance: Radiotherapy can affect the surrounding tissues, particularly in the gastrointestinal tract, an area prone to wounding; therefore, other treatment options could be chosen to avoid wounding. Wound avoidance can reduce treatment-related complications and improve quality of life.

Dose limitation: The gastrointestinal tract is an area where radiation doses must be limited as it is sensitive to radiation damage, which could be associated with an increased risk of complications and side effects. Therefore, elective treatments may be preferred.

Advances in chemotherapy: Following the introduction of drug combinations and target-directed therapies, chemotherapy has proven more effective and selective than radiotherapy.

Risk of side effects and complications: Radiotherapy risks severe side effects and complications in some patients. Particularly in treating the gastrointestinal tract, radiotherapy can significantly impact daily activities, such as food intake and bowel movements. To minimize these risks, other treatment options are preferred.

These factors tend to reduce the use of radiotherapy in favor of more elective treatments and a multifaceted approach centered on chemotherapy. However, the indications of radiotherapy and the balance of benefits and risks must be assessed for each case. Radiotherapy could be beneficial for certain conditions to prevent FL progression.

Treatment agents

Several novel FL agents are used primarily for treating nodal FL. This is the mainstay of treatment for advanced primary GI-FL, which is expected to show an increased incidence in the near future.

Antibodies: Monoclonal antibodies: Immunotherapy, particularly with rituximab and other anti-CD antibodies, is exceedingly effective in managing FL and constitutes a vital and superior component of modern treatment regimens. The success rate of rituximab monotherapy is 67% in untreated FL and 46% in patients with relapsed FL[68]. A German low-grade lymphoma study showed that rituximab plus CHOP was superior to CHOP alone in patients with advanced-stage FL and that OS was significantly improved[69]. In addition to rituximab, several other antibody-based agents have been developed, including tafatasitamab[70], polatuzumab vedotin[9], loncastiximab tecilin[71], maglorimab[72], and obinutumab[73]. Further, the combination of tafacitumab and lenalidomide[70] and polatuzumab vedotin[9] is effective. These therapies are promising for treating FL and other lymphomas; hence, clinical trials are ongoing. Additionally, biosimilars of rituximab have been approved, including CT-P10, which has shown efficacy and safety comparable to those of rituximab[74]. These developments are important for the treatment of FL and could contribute to reducing healthcare costs.

Bispecific T-cell-binding antibodies: Bispecific T-cell-binding antibodies (BTEs) are potent therapeutics that target specific tissues and cells by binding multiple antigens. They are commonly used to treat FL, with CD3 and CD20 being the primary targets. Mosnetuzumab and glofitumab are notable BTEs. Mosnetuzumab, a CD20 × CD3 bispecific antibody, demonstrated a high overall response rate (ORR) of 66% and a complete response rate (CRR) of 49% in a phase I study on r/r FL[75]. When combined with lenalidomide, an ORR of 92% and a CRR of 77% were achieved. Glofitamab, another BTE, showed positive results in a phase I study on r/r B-cell non-Hodgkin lymphoma, including painless lymphoma, with an ORR of 65.7% and CRR of 57.1%. Higher response rates were observed when the drug was combined with obinutuzumab[76]. The anti-CD3 and anti-CD20 BTE, epcoritamab, showed promising efficacy in phase I/II trials of r/r non-Hodgkin lymphoma and FL with a high ORR and CRR[77,78]. Similarly, odronestamab, a CD20 × CD3 bsAb, showed impressive results in phase I trials of r/r FL[79,80]. Overall, BTE is an effective immunotherapeutic strategy for FL. Further progress is expected from ongoing clinical trials.

Anti-PD-L1 antibodies: Anti-PD-L1 antibodies such as atezolizumab and pembrolizumab have shown promising efficacy in FL by enhancing T-cell function and antibody-dependent cellular cytotoxicity (ADCC) in NK cells. In a phase I study that combined atezolizumab and obinutumab, the ORR was found to be 54% (CRR: 23%) in patients with r/r FL and DLBCL, while an ORR of 17% (CRR: 4%) was observed for DLBCL alone. The PFS reached 9 mo for FL and 3 mo for DLBCL[81]. Another trial using pembrolizumab in combination with rituximab exhibited an ORR of 67% and a CRR of 50% in patients with r/r FL. The median PFS and 3-year OS rates were 12.6 mo and 97%, respectively. During the median follow-up duration of 35 mo, 23% of the patients maintained remission[82]. By enhancing T-cell function and NK cell ADCC, PD-1 blockade represents a fundamental mechanism of action that could further improve the therapeutic efficacy of anti-PD-1 antibody therapy in patients with FL.

Immunomodulatory drugs: Lenalidomide is an oral immunomodulatory drug used to treat FL and exhibits tumor-killing and immunomodulatory properties[83]. Clinical trials have shown that combining lenalidomide and rituximab (R2) has a higher ORR and a longer time to progression than lenalidomide alone[84]. The phase III AUGMENT trial showed a significant improvement in PFS with R2 compared with that with rituximab alone[85]. The phase IIIb MAGNIFY trial investigated the extension of R2 treatment and confirmed an ORR of 69% and a PFS of 40 mo[86]. The phase II GALEN trial tested a single arm of lenalidomide and obinutuzumab and reported an ORR of 95%, 2-year PFS of 65%, and OS of 87%[87]. Meanwhile, the phase III RELEVANCE trial showed no superiority of R2 over chemoimmunotherapy for frontline FL[88]. The phase II E2408 trial assessed the efficacy of three different approaches: Bendamustine/rituximab with bortezomib (BR) induction with R2 maintenance, BR induction with rituximab maintenance, and BR induction with maintenance using the proteasome inhibitor bortezomib and rituximab. The trial found that all three groups demonstrated similar and high CRRs[89]. Separately, a clinical trial utilizing lenalidomide and obinutuzumab, as the frontline therapy for patients with advanced untreated FL, reported encouraging results, with an ORR of 98%, CRR of 92%, and 2-year PFS of 96%[90]. Network meta-analyses of randomized controlled trials comparing treatment efficacy[91] were conducted to determine the optimal treatment regimens, sequences, and combination therapies. The evolution and diversification of therapeutics for FL have expanded the combinations of drugs with different mechanisms of action and treatment sequences; however, further clinical trials are needed to determine the most effective treatment regimen and sequence.

Molecular targeted therapies (small-molecule compounds): Bruton's tyrosine kinase inhibitors: Bruton's tyrosine kinase (BTK) plays a crucial role in regulating B-cell differentiation and activation in immune cells. For blood cancers like B-cell non-Hodgkin lymphoma and CLL, including FL, BTK inhibitors (BTKis) are currently under examination for their potential therapeutic benefits. First-generation ibrutinib, second-generation acalabrutinib and zanubrutinib, and third-generation piltobrutinib have demonstrated high effectiveness against B-cell non-Hodgkin lymphoma and CLL[92-96].

However, zanubrutinib monotherapy has limited efficacy in r/r FL[92]. Combination therapy with ibrutinib and rituximab resulted in better response rates in patients with r/r FL[95]. Acarabrutinib showed good efficacy and was well-tolerated by untreated and r/r FL patients[97]. In patients with r/r FL, the combination of zanubrutinib and obinutuzumab showed better PFS than obinutuzumab alone[98]. BTKis exhibited superior activity when used in combination therapies; hence, future studies should focus on evaluating various combinations of novel BTKis and other agents in patients with FL.

Proapoptotic pathway inhibitors (BCL2 inhibitors): Venetoclax, a potent BCL2 inhibitor, exhibits strong binding to BCL2, an anti-apoptotic protein often found in high levels in various blood cancers. By releasing apoptosis-promoting proteins, venetoclax triggers rapid and irreversible apoptosis in blood cancer cells. In a phase I trial of patients with FL, venetoclax monotherapy demonstrated an ORR of 38% and median PFS of 11 mo[99]. The phase II CONTRALTO trial compared different treatment groups, with the combination of venetoclax and rituximab presenting a CRR of 17%, venetoclax plus BR showing a CRR of 75%, and BR combined with venetoclax having a CRR of 69%[100]. Moreover, a phase I/II study reported promising results with the combination therapy of venetoclax and ibrutinib, showing an ORR of 69% and CRR of 25%[101]. BCL2 inhibitors have great potential for improving outcomes in patients with FL caused by BCL2 overexpression.

Epigenetic regulators: Drugs targeting the epigenetic regulation of gene expression, such as DNA methylation, are promising in treating blood cancers. Tazemetostat, an inhibitor of epigenetic regulator (EZH2) and histone methyltransferase, has shown positive outcomes. In a phase II study, tazemetostat treatment resulted in a higher ORR in patients with EZH2 mutations than in wild-type patients[19]. Patients with high-risk FL exhibited particularly favorable responses. The combination therapy of tazemetostat with rituximab and R2 is currently under investigation. Treatment with vorinostat, a histone deacetylase inhibitor, resulted in an ORR of 49% and median PFS of 20 mo in a phase II study of patients with r/r FL[102]. In a phase II study, vorinostat, in combination with rituximab, achieved an ORR of 50% and CRR of 41%[103]. Mocetinostat, another histone deacetylase inhibitor, showed limited efficacy, with an ORR of 12% in a phase II trial in patients with r/r FL[104].

EZH2 and histone deacetylase inhibitors hold promise as epigenetic regulators for the treatment of FL and are expected to yield improved therapeutic outcomes, particularly for tFL.

Phosphatidylinositol-3 kinase inhibitors: The BCR signaling pathway is homeostatically activated in B-cell tumors, leading to the development of inhibitors targeting this pathway. Phosphatidylinositol-3 kinase (PI3K) is a lipid kinase that mediates the phosphorylation of inositol ring 3 of inositol phospholipids—a membrane component[105]. Class I PI3Ks play an important role in signaling and are subdivided into four isoforms, α, β, ϒ, and δ[106]. Idelalisib, which selectively inhibits the δ isoform, is used in the treatment of r/r FL; it has shown the highest efficacy among PI3K inhibitors[107]. Nevertheless, most patients experience adverse events, and life-threatening adverse events limit the use of these agents[108,109]. Duvelisib, a dual inhibitor of PI3K-δ and PI3K-γ, has demonstrated impressive efficacy (70%); however, its use is also associated with a relatively high occurrence of adverse events (grade 3 or higher)[110,111]. It is the only PI3K inhibitor approved for treating r/r FL. Umbralisib is a selective PI3K-δ and casein kinase-1-ε (CK1ε) inhibitor and a fourth-generation PI3Ki with a potential therapeutic role in FL[112,113]. Parsaclisib is a potent PI3K isoform inhibitor that effectively treats r/r FL; however, it is also associated with frequent adverse events[114,115]. Zandelisib, a novel PI3-Kδ inhibitor, has shown good efficacy in the treatment of r/r indolent non-Hodgkin lymphoma with minimal adverse events[116]. However, clinical trials of these inhibitors are ongoing and require careful patient selection and implementation owing to the high frequency of adverse events.

PI3K/Akt/mTOR signaling pathway inhibitors: The PI3K/Akt/mTOR signaling pathway promotes abnormal regulation of tumor cell growth, metabolism, and survival[117]. Dual inhibitors aimed at this pathway have been developed and exhibit encouraging therapeutic effects, including high efficacy at minimal doses and low drug resistance [118]. Temsirolimus, a TOR inhibitor, when used with lenalidomide, showed synergistic effects in untreated advanced lymphomas, particularly in r/r classical Hodgkin lymphoma. However, hematological adverse events were frequent, with three grade 5 adverse events reported[119]. Further development of this novel PI3K/Akt/mTOR dual inhibitor and additional clinical trial data will enhance therapeutic outcomes and promote its use in FL treatment.

Cell-based therapies: CAR-T cell therapy is an innovative treatment for r/r hematological malignancies involving genetically engineered autologous T cells with chimeric antigen receptors (CARs) to target cancer cells. To date, CAR-T cell therapy has shown high efficacy; however, it is limited by hematological toxicity, including post-treatment cytoreduction[120,121]. In addition, several other challenges exist in the development of CAR-T therapies, including complex logistics, manufacturing constraints, toxicity concerns, and economic burdens[122]. Clinical trials have contrasted the effectiveness and safety of three cellular therapies: Autologous, allogeneic, and CAR-T cell therapies[123]. Recent clinical trials have evaluated autologous anti-CD19 CAR-T agents, such as axicabtagene ciloleucel (axi-cell), tisagenlecleucel (tisa-cell), and lisocabtagene maraleucel (liso-cell), all of which have shown remarkable efficacy against r/r DLBCL and FL. Various clinical trials have suggested that axi-cells can achieve an ORR of 94%, CRR of 79%, and sustained remission rate of 40%[124,125]. Tisa-cells have also shown substantial efficacy, achieving an ORR of 86% and CRR of 69% after a median follow-up of 17 mo[126-128]. Liso-cells, which are autologous anti-CD19 CAR-T cells, displayed high efficacy in r/r DLBCL where hematopoietic stem cell transplantation is not planned[129] and may be utilized in r/r FL in the future. The TRANSFORM and PILOT studies have also highlighted the potent efficacy of liso-cells in second-line treatment of r/r large BCL, leading to their approval as a third-line treatment option for aggressive BCL[130]. Despite these challenges, CAR-T-cell therapy could be developed as a fundamental treatment modality for FL.

Response-adapted post-induction strategy: The FOLL12 study compared standard rituximab maintenance therapy with experimental post-induction therapy for patients with FL. Experimental therapy ranged from observation of patients with

the complete metabolic response (CMR) and minimal residual disease (MRD) negativity to administering four doses of rituximab to patients with CMR and MRD positivity until MRD became negative, to one dose of ibritumomab tiuxetan for non-CMR patients, followed by three doses of the standard treatment with rituximab maintenance. With a median follow-up of 53 mo, patients in the standard treatment group had significantly better PFS than those in the experimental group (3-year PFS, 86% *vs* 72%; $P < 0.001$). However, for patients with FL who responded positively to induction therapy, the standard 2-year rituximab maintenance therapy extended PFS following the remission induction[131].

SUMMARY OF GI-FL TREATMENTS

Similar to that for nodal FL, the treatment strategy for GL-FL should be carefully considered based on Lugano stage and the histological grade. Furthermore, personalized treatment choices should be made based on the circumstances of individual patients and their changes over time[7]. The possibility of gastrointestinal perforation due to tumor reduction should always be considered, as this could be a lethal side effect. Moreover, the watch-and-wait strategy has been the mainstay option for GI-FL treatment. However, with the current advances in novel therapeutic agents, many advanced cases are increasingly being treated aggressively with chemotherapy, immunotherapy, or a combination of the two. Surgical resection and postoperative chemotherapy, immunotherapy, or a combination of these, could be an option in some instances, such as gastrointestinal obstruction[7]. Therefore, the therapeutic approach should be more individualized in GI-FL. Depending on the site and stage of the lesion and the patient's general health, a combination of surgery, chemotherapy, radiotherapy, and immunotherapy may be chosen. Conservative surgical or endoscopic treatment of the gastrointestinal tract may be an option depending on the tumor's specific anatomy and histological characteristics.

CONCLUSION

Molecular genetic analysis of FL has revealed overexpression of the *BCL2* gene, rearrangement of the *IGH* gene, and genetic mutations in NF-κB pathway-related factors. To this end, genetic abnormalities related to histone methyltransferases and histone acetylases have been characterized. Approximately 150 miRNAs involved in the development and proliferation of FL, including the miR-17-92 cluster, miR-155, miR-155-5p, miR-9-3p, miR-150, miR-155, and miR-5008, have been identified. Their role as prognostic predictors has been investigated. Recently, GWAS have identified several loci involved in FL development.

There has been significant progress in the development of novel FL therapeutics targeting the genes responsible for FL pathogenesis and tumor growth, including anti-CD20, CD79 monoclonal antibodies, BTEs, anti-PD-L1 antibodies, lenalidomide, and immunomodulators such as lenalidomide, BTKis, BCL2 inhibitors, EZH2 inhibitors, PI3K inhibitors, dual inhibitors of the PI3K/Akt/mTOR signaling pathway, and CAR-T cell therapy. The efficacy of these novel agents is demonstrated in numerous clinical trials, not only as single agents but also in combination. Recent research focuses on identifying the best combination and the order of treatments in multi-drug therapy. The number of advanced cases of primary GI-FL and nodal cases is expected to increase. Gastroenterologists must be trained and offered sufficient practice in treating these patients, especially those in advanced stages.

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Artificial intelligence system for the detection of Barrett's esophagus

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Abstract

BACKGROUND

Barrett's esophagus (BE), which has increased in prevalence worldwide, is a precursor for esophageal adenocarcinoma. Although there is a gap in the

detection rates between endoscopic BE and histological BE in current research, we trained our artificial intelligence (AI) system with images of endoscopic BE and tested the system with images of histological BE.

AIM
To assess whether an AI system can aid in the detection of BE in our setting.

METHODS
Endoscopic narrow-band imaging (NBI) was collected from Chung Shan Medical University Hospital and Changhua Christian Hospital, resulting in 724 cases, with 86 patients having pathological results. Three senior endoscopists, who were instructing physicians of the Digestive Endoscopy Society of Taiwan, independently annotated the images in the development set to determine whether each image was classified as an endoscopic BE. The test set consisted of 160 endoscopic images of 86 cases with histological results.

RESULTS
Six pre-trained models were compared, and EfficientNetV2B2 (accuracy [ACC]: 0.8) was selected as the backbone architecture for further evaluation due to better ACC results. In the final test, the AI system correctly identified 66 of 70 cases of BE and 85 of 90 cases without BE, resulting in an ACC of 94.37%.

CONCLUSION
Our AI system, which was trained by NBI of endoscopic BE, can adequately predict endoscopic images of histological BE. The ACC, sensitivity, and specificity are 94.37%, 94.29%, and 94.44%, respectively.

Key Words: Barrett's esophagus; Artificial intelligence system; Endoscopy; Narrow-band imaging; Gastroesophageal reflux disease

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Core Tip: The prevalence of Barrett's esophagus (BE) diagnosed by endoscopy significantly differs from BE diagnosed by histology (7.8% *vs* 1.3%). Current research showed that image-enhanced endoscopy can only increase the detection ability for dysplasia lesions in BE. Our artificial intelligence prediction system, which was trained by endoscopic BE images with the Olympus narrow-band imaging system, still provided good prediction results for images of histological BE. The accuracy, sensitivity, and specificity are 94.37%, 94.29%, and 94.44%, respectively, in the final test, which indicates that endoscopic BE images have characteristics similar to images of histological BE.

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INTRODUCTION

Barrett's esophagus (BE), which is characterized by a columnar lined esophagus with documented intestinal metaplasia[1,2], is a precursor for esophageal adenocarcinoma (EAC)[3,4]. The prevalence of BE has been increasing in western countries[5,6] for decades, and this trend has also recently been observed in Asian countries[7]. The annual incidence of EAC is approximately 0.3% in non-dysplasia BE[8], while the annual incidence of EAC increases to 0.76% in low-grade dysplasia BE[9] and to 6% in high-grade dysplasia BE[10]. Although the importance of the detection of BE in patients is recognized by endoscopists worldwide, the prevalence of BE diagnosed by endoscopy significantly differs from BE diagnosed by histology[7] (7.8% *vs* 1.3%).

The methods of biopsy in Seattle protocol[11] have been under debate, as random four-quadrant biopsies at 2-cm intervals are time-consuming and intolerable under some situations. A recent study showed that targeted biopsy using image-enhanced endoscopy and molecular biomarkers can increase the detection ability for dysplasia lesions in BE[12]. The above issues indicate that there is a large degree of interobserver disagreement in the recognition and biopsy methods of BE. One previous study showed that the prevalence of histological BE is 2.6% in a health examination center data[13] but that the utilization of biopsy during endoscopy is far less in daily practice in Taiwan. We propose that an artificial intelligence (AI) system should be helpful for promoting awareness of BE in daily endoscopic practice.

AI systems are already applied in many fields of modern medicine, which started with diabetic retina observation[14,15], diabetes mellitus care[16], and the detection and classification of Alzheimer's disease[17] and later extended to the fields of gastroenterology and endoscopy. AI systems can help detect and differentiate the classification of colon polyps [18,19], gastric cancer[20], neoplastic and non-neoplastic small bowel lesions[21], pancreatic lesions[22,23], BE[24-26], and EAC[24,26,27]. Despite recent improvements in the AI prediction of BE and EAC, the accuracy (ACC) of such models has

Table 1 Baseline characteristics of development and test sets

NBI image number	Training set, *n* = 771		Label balance, *n* = 1187		Valid set, *n* = 193		Test set, *n* = 160	
	n	%	*n*	%	*n*	%	*n*	%
No Barrett's esophagus	563	73.02	563	47.43	141	73.06	90	56.25
Barrett's esophagus	208	26.98	624	52.57	52	26.94	70	43.75

NBI: Narrow-band imaging.

remained approximately 90%; one of the insurmountable problems is that there are insufficient images of histologically proven BE to train AI models. Our healthcare system faced the same dilemma, and we tried to conquer this problem by establishing a relationship between images of endoscopic BE and images of histological BE.

We trained our AI model with images of endoscopic BE and endoscopic images from patients without BE and then tested our AI prediction system using cases both with and without histologically proven BE. We also compared the outcomes of our prediction system with data from similar recent studies.

MATERIALS AND METHODS

Data acquisition and preparation

The endoscopic images of patients with typical gastroesophageal reflux symptoms were collected from Chung Shan Medical University Hospital (Taichung City, Taiwan) and Changhua Christian Hospital (Changhua County, Taiwan) retrospectively from January 1, 2020 to June 30, 2020, resulting in 724 cases, with 86 patients having complete histological results. The collection of clinical data was de-identification and sent for further image annotation. This study reviewed and approved by the institutional review board (IRB) on May 28, 2020 with IRB number CS1–20075 (the application of artificial intelligence in advanced endocopy) and conducted under IRB regulations to ensure the rights and welfare of the participants.

The images utilized in this study were obtained from the hospitals' endoscopy systems, which were operated by professional physicians. The narrow-band imaging (NBI) mode in the Olympus endoscopy system was selected for capturing images. We collected both histological BE images and other image data from patients with typical symptoms of gastroesophageal reflux disease (GERD) to establish a study dataset.

Data annotation

The development set of this study consisted of 964 images. Three senior endoscopists who were instructing physicians of the Digestive Endoscopy Society of Taiwan independently annotated the images in the development set to determine whether each image was classified as endoscopic BE. After a majority vote among the three senior endoscopists, the final classification was determined. The test set consisted of 160 images of 86 cases, and the categorization was based on the histological results.

Dataset

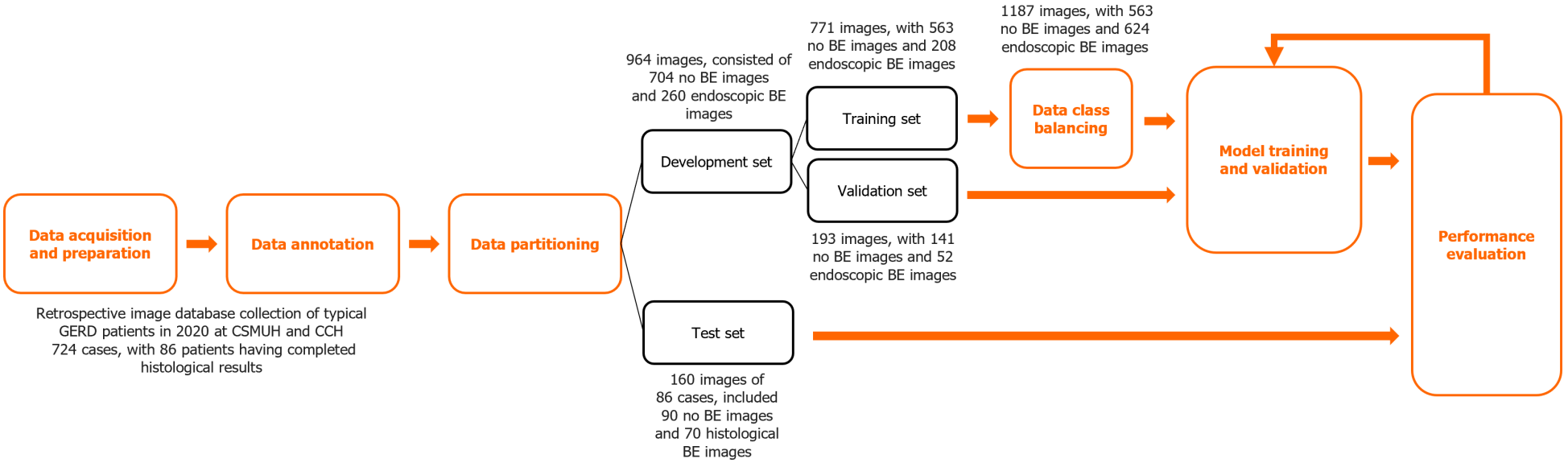
The development set consisted of 704 images without BE and 260 endoscopic BE images. To avoid overfitting, the image dataset was divided using the holdout method, with 80% of the data employed as the training set and 20% of the data employed as the validation set for model training and development. The test set, which consists of complete histological result images, was used to evaluate the predictive performance of the model. The test set included 90 images without BE and 70 histological BE images. These details are shown in Table 1.

Model design

This section describes the deep learning model architecture utilized in the study to accomplish the binary classification task for BE. First, the image size was resized to 224 pixel × 224 pixel for inputting into the model. To address the issue of class imbalance in the dataset, this study employed a resampling technique to augment the data from the minority class. Data class balancing was performed only on the training set. The original training dataset consisted of 771 images, of which 563 images did not have BE, and 208 images showed endoscopic BE. After data augmentation, the training dataset expanded to 1,187 images, of which 563 did not show BE and 624 images showed endoscopic BE. The effects of data augmentation are listed in the Supplementary Table 1.

In this study, a transfer learning technique was applied for model training. Six pretrained models were compared, including EfficientNetV2B1, EfficientNetV2B2, EfficientNetV2B3, DenseNet201, ResNet50, and VGG16. Ultimately, EfficientNetV2B2 was selected as the backbone architecture for further evaluation due to its better prediction ACC.

The deep neural network in this study employed the sigmoid function as the activation function in the output layer. An early stopping technique was utilized to monitor the maximization of validation ACC, which allowed the model to stop training at a suitable convergence point. After completion of the training, the best model weights were saved. To further enhance the model's generalization ability, the development set was repeatedly sampled to create multiple



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Figure 1 Flow chart of study design and data acquisition. BE: Barrett's esophagus; GERD: Gastroesophageal reflux disease; CSMUH: Chung Shan Medical University Hospital; CCH: Changhua Christian Hospital.

training sets for model training and validation, with the aim of developing an optimal AI model. A flow chart of the study design and data acquisition is shown in Figure 1.

Classification performance evaluation

The evaluation metrics applied in this study include ACC, sensitivity (SEN), and specificity (SPE), which determine the classification performance of the model. ACC indicates the classification ACC of the model, SEN reveals the proportion of actual positive samples correctly identified as positive, and SPE indicates the proportion of actual negative samples correctly identified as negative.

RESULTS

We collected endoscopic images of the gastroesophageal junction in a total of 724 cases, with 86 patients having complete histological results. There were 771 original images in the training set, including 563 images that did not show BE and 208 images that showed endoscopic BE (26.98% of the total number of images). A total of 193 original images were included in the validation set, including 141 images without BE and 52 images with endoscopic BE (26.94% of the validation images). Due to the imbalanced distribution in these two categories and the limited number of endoscopic BE images, we augmented the BE images in the training set to obtain a total of 624 images of endoscopic BE, which provided 52.57% of

Table 2 Comparison of performance using different pretrained models

Pre-trained model	Training			Validation			Test		
	ACC	SEN	SPE	ACC	SEN	SPE	ACC	SEN	SPE
EfficientNetV2B1	0.9196	0.8702	0.9378	0.7876	0.6346	0.8440	0.7625	0.6857	0.8222
EfficientNetV2B2	0.9702	0.9663	0.9716	0.8497	0.7500	0.8865	0.8500	0.8286	0.8667
EfficientNetV2B3	0.9170	0.8798	0.9307	0.7824	0.6154	0.8440	0.8125	0.7429	0.8667
ResNet50	0.8962	0.7885	0.9361	0.8290	0.5577	0.9291	0.7063	0.5429	0.8333
DenseNet201	0.8755	0.8173	0.8970	0.7772	0.5577	0.8582	0.7312	0.6143	0.8222
VGG16	0.2698	1.0000	0.0000	0.2694	1.0000	0.0000	0.4375	1.0000	0.0000

ACC: Accuracy; SEN: Sensitivity; SPE: Specificity.

Table 3 Confusion matrices

Confusion matrices		Predicted class					
		Training, *n* = 771		Validation, *n* = 193		Test, *n* = 160	
		BE	No BE	BE	No BE	BE	No BE
Actual class	BE	206	2	50	2	66	4
	No BE	2	561	3	138	5	85

BE: Barrett's esophagus.

the images in the training set. These baseline characteristics are shown in Table 1.

Comparisons of different pre-trained models

We compared the prediction outcomes in different pre-trained models, which included EfficientNetV2B1, EfficientNetV2B2, EfficientNetV2B3, ResNet50, DenseNet201, and VGG16. ACC was comparable in the training process of most of the pre-trained models, with the exception of VGG16, which had an ACC of only 26.98%. The same trends were observed in the validation set, with an ACC of 78.76%, 84.97%, 78.24%, 82.90%, 77.72%, and 26.94% for EfficientNetV2B1, EfficientNetV2B2, EfficientNetV2B3, ResNet50, DenseNet201, and VGG16, respectively. For final testing, EfficientNetV2B2 achieved the best ACC of 85%, which was superior to the other pre-trained models; hence, we chose EfficientNetV2B2 as the training model for our BE prediction system. Detailed data on SEN, SPE, and ACC for the pre-trained model comparisons are shown in Table 2.

Results of AI image-based model performance

For the training set, two images were incorrectly classified as having no BE among 208 images of endoscopic BE. For images that showed no BE, two images were incorrectly classified as having BE, while the other 561 images were correctly classified as having BE. Overall, ACC, SEN, and SPE were 99.48%, 99.04%, and 99.64%, respectively.

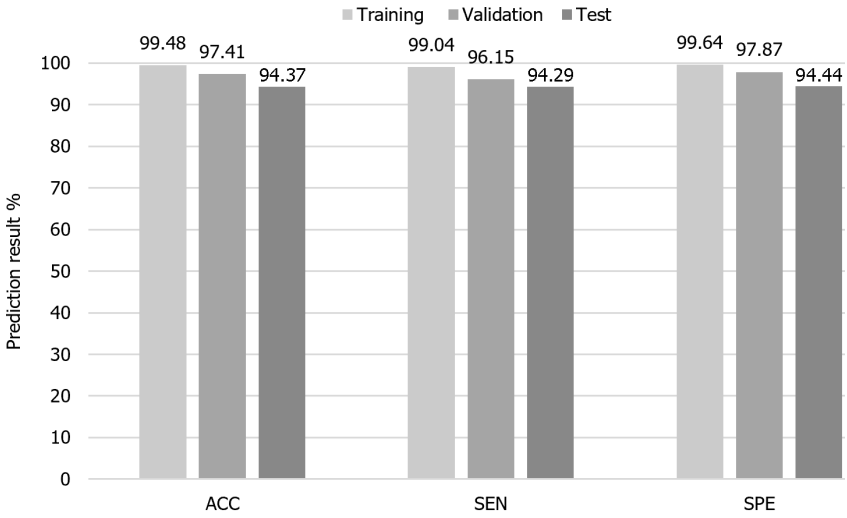
For the validation set, two images were incorrectly classified as having no BE among 52 images of endoscopic BE, while three images were incorrectly classified as having BE among 141 non-BE images. Overall, ACC, SEN, and SPE were 97.41%, 96.15%, and 97.87%, respectively.

For the test set, four images were incorrectly classified as having no BE among 70 images of histological BE, and five images were incorrectly classified as having BE among 90 non-BE images (as proven by histology) in the final test. The AI BE prediction system had an ACC of 94.37%, a SEN of 94.29%, and a SPE of 94.44%. Detailed information is provided in Table 3 and Figure 2.

Representative images of BE with successful detection (panel A), no BE with successful detection (panel B), BE with false detection (panel C), and no BE with false detection (panel D) are demonstrated in Figure 3.

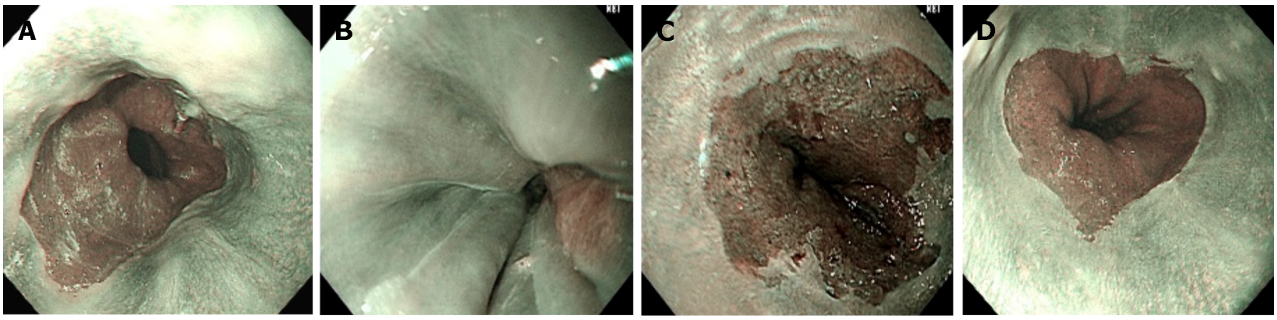
DISCUSSION

We used only NBI images from the Olympus endoscopy system in our study instead of white light endoscopy images because our previous data showed the superiority of NBI images over white light endoscopy images for the differentiation of GERD classification[28]. However, some studies have shown that NBI images do not display differences in detecting BE esophagus compared to white light endoscopy images[29,30], while another recent convincing study



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Figure 2 Artificial intelligence image-based Barrett's esophagus prediction model performance. ACC: Accuracy; SEN: Sensitivity; SPE: Specificity.



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Figure 3 Narrow-band images of the artificial intelligence prediction system. A: Barrett's esophagus with successful detection; B: Normal esophagus with successful detection; C: Barrett's esophagus with false detection; D: Normal esophagus with false detection.

showed benefits in neoplasia detection with an NBI system[12]. The concept that targeted biopsies using NBI enable the detection of more dysplastic areas and thus reduce the number of biopsies required is widely accepted by the Japan Esophageal Society[12,31]. As a result, we exclusively utilized NBI still images in our AI prediction system training.

Our study is unique in that training of the pretrained model and selected AI prediction model was performed with images of endoscopic BE rather than images of histological BE. The AI prediction system was still able to maintain a high ACC in the final prediction test that used images of histological BE. This finding indicates that the images of endoscopic BE have characteristics similar to those of histological BE in our study. The final prediction results using the EfficientNetV2B2 training model showed that SEN, SPE, and ACC were 94.29%, 94.44%, and 94.37%, respectively. Previous data have highlighted that ACC is currently unsatisfied in AI systems of BE endoscopic interpretation[32], but the AI prediction of early cancer or dysplasia were impressive. Our database employed larger real-case numbers and more original images than previous studies and showed improved SEN, SPE, and ACC. We believe that the data augmentation technique limited the ACC in the final test results due to the large number of training images with similar detailed characteristics.

The most similar recent studies focused on BE neoplasia detection. Ebigbo *et al*[33] demonstrated an ACC of 89.9% using only 14 test patients in 2020, while Hussein *et al*[26] improved the ACC to 93% with 118 patients in 2022. Another study by Abdelrahim *et al*[25] in 2023, which utilized real-time video for the identification of BE dysplasia, showed a similar ACC of 92% with 75 cases.

Our study selected 771 original images from 579 patients as the training set, 193 original images from 145 patients as the validation set, and 160 images from 86 patients as the test set; the final ACC was 94.37%. Detailed information on these results is provided in Table 4. We focused on building an AI reminder system that helps endoscopists decide whether to perform a biopsy when BE is suspected instead of a system that reminds endoscopists which BE lesions may already have dysplastic changes.

Table 4 A comparative summary of the state-of-the-art approaches for the binary classification of Barrett's esophagus

Ref.	Validation (train:valid:test)	Valid	Test
Ebigbo *et al*[33], 2020	Holdout; Image (129:N/A:62); Test patients:14	ACC: N/A	ACC: 89.9%
		AUC: N/A	AUC: N/A
		SEN: N/A	SEN: 83.7%
		SPE: N/A	SPE: 100.0%
		PPV: N/A	PPV: N/A
		NPV: N/A	NPV: N/A
Hussein *et al*[26], 2022	Holdout; Video (64:11:44); Patients: 118	ACC: N/A	ACC: N/A
		AUC: N/A	AUC: 93%
		SEN: N/A	SEN: 91%
		SPE: N/A	SPE: 79%
		PPV: N/A	PPV: N/A
		NPV: N/A	NPV: N/A
Abdelrahim *et al*[25], 2023	Holdout; Image (816:471:N/A); Video (161:N/A:75); Case (161:34:75)	ACC: 94.7%	ACC: 92.0%
		AUC: 0.898	AUC: 0.964
		SEN: 95.3%	SEN: 93.8%
		SPE: 94.5%	SPE: 90.7%
		PPV: 83.6%	PPV: 88.2%
		NPV: 98.6%	NPV: 95.1%
Our approach, 2023	Holdout image; Image (771:193:160); Case (579:145:86)	ACC: 97.41%	ACC: 94.37%
		AUC: 97.01%	AUC: 94.37%
		SEN: 96.15%	SEN: 94.29%
		SPE: 97.87%	SPE: 94.44%
		PPV: 94.00%	PPV: 92.96%
		NPV: 98.57%	NPV: 95.51%

ACC: Accuracy; AUC: Area under the curve; N/A: Not applicable; NPV: Negative predictive value; PPV: Positive predictive value; SEN: Sensitivity; SPE: Specificity.

Limitations

Due to the lack of awareness regarding the diagnosis of BE, cases of histological BE are relatively rare in central Taiwan. First, we initiated this study and collected endoscopic images of the lower esophagus and esophagogastric junction in two medical centers in central Taiwan. The endoscopic BE dataset was developed using the Delphi method, which involved blind voting on endoscopic images by three endoscopists, instead of histological BE images, which can lead to selection bias due to the previously known difference in prevalence between endoscopic BE and histologic BE[7] (7.8% *vs.* 1.3%). Second, the method of biopsy was not recorded in all cases, which meant that some of the cases involved random biopsies, while other cases were NBI-targeted biopsies. Third, the images in our database were obtained from an Olympus endoscopic system with lower esophageal still NBI pictures. It is not known whether our system can adequately perform for white light or other endoscopic systems. The main limitations were attributed to the limited cases and limited original images in our study and the limited number of cases of histological BE. However, our AI prediction system, which was trained by endoscopic BE images, still provided good prediction results for images of histological BE.

CONCLUSION

Our AI prediction system can provide good prediction results for images of histological BE obtained with Olympus NBI after training with images of endoscopic BE.

ARTICLE HIGHLIGHTS

Research background

The prevalence of endoscopic Barrett's esophagus (BE) differs significantly from histological BE. We believe the endoscopic characteristics are similar with endoscopic BE and histological BE.

Research motivation

We want to train an artificial intelligence (AI) system to identified images of BE under endoscopic environments.

Research objectives

To construct an AI system for the detection of endoscopic images of histological BE.

Research methods

Endoscopic narrow-band images of 724 cases, were collected from two medical centers at central Taiwan, with 86 patients having pathological results. Images of endoscopic BE was classified using independent annotation by three senior endoscopists, who were instructing physicians of the Digestive Endoscopy Society of Taiwan. The test set consisted of 160 endoscopic images in 86 histological BE cases.

Research results

EfficientNetV2B2 [accuracy (ACC): 0.85] was selected as the backbone architecture from six training model due to better ACC result. In the final test, the AI system obtained 94.37%, 94.29%, and 94.44%, in ACC, sensitivity, and specificity respectively.

Research conclusions

Our AI prediction system can provide good prediction results after training with images of endoscopic BE.

Research perspectives

Our result implies that images of endoscopic BE share similar characteristics with images of histological BE even in the perspectives of AI system. The gap from endoscopic BE to histological BE maybe comes from biopsy or sampling bias. This opinion needs further prospective studies to confirm. Meanwhile, a better AI prediction system for endoscopic video BE detection is an ongoing task in the near future.

FOOTNOTES

Co-corresponding authors: Chi-Chih Wang and Ming-Hseng Tseng.

Author contributions: Tsai MC and Wang CC were responsible for the conception and design of the study; Yen HH, Tsai HY, Huang YK, Luo YS, Sung WW, and Tseng MH were responsible for the acquisition, analysis, or interpretation of data; Tsai MC and Wang CC were responsible for drafting the manuscript; Edy Kornelius and Lin CC were responsible for critically revising the manuscript for important intellectual content; Tsai HY and Tseng MH were responsible for the statistical analyses; Tseng MH and Sung WW were responsible for obtaining the funding; Tseng MH and Wang CC were responsible for supervising the study; Wang CC and Tseng MH contributed equally to this work as co-corresponding authors. The reasons for designating Wang CC and Tseng MH as co-corresponding authors are as follows. The research was performed as a collaborative effort, and the designation of co-corresponding authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper. This also ensures effective communication and management of post-submission matters, ultimately enhancing the paper's quality and reliability. The overall research team encompassed authors with a variety of expertise and this also promotes the most comprehensive and in-depth examination of the research topic, ultimately enriching readers' understanding by offering various expert perspectives. Wang CC contributed to the study design, endoscopic image collection, and endoscopic image interpretation while Tseng MH constructed the AI model. The choice of these researchers as co-corresponding authors acknowledges and respects this equal contribution, while recognizing the spirit of teamwork and collaboration of this study. In summary, we believe that designating Wang CC and Tseng MH as co-corresponding authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

Institutional review board statement: The collection of clinical data was reviewed and approved by the institutional review board (IRB) with IRB number CS1–20075 and conducted under IRB regulations to ensure the rights and welfare of the participants.

Informed consent statement: The institutional review board (IRB) has agreed to waive informed consent.

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

Data sharing statement: Dataset available from the corresponding author.

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ORIGINAL ARTICLE

Observational Study

Clinical value of the Toronto inflammatory bowel disease global endoscopic reporting score in ulcerative colitis

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Abstract

BACKGROUND

Endoscopic evaluation in diagnosing and managing ulcerative colitis (UC) is becoming increasingly important. Several endoscopic scoring systems have been established, including the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) score and Mayo Endoscopic Subscore (MES). Furthermore, the Toronto Inflammatory Bowel Disease Global Endoscopic Reporting (TIGER) score for UC has recently been proposed; however, its clinical value remains unclear.

AIM

To investigate the clinical value of the TIGER score in UC by comparing it with the UCEIS score and MES.

METHODS

This retrospective study included 166 patients with UC who underwent total colonoscopy between January 2017 and March 2023 at the Affiliated Hospital of Qingdao University (Qingdao, China). We retrospectively analysed endoscopic scores, laboratory and clinical data, treatment, and readmissions within 1 year. Spearman's rank correlation coefficient, receiver operating characteristic curve, and univariate and multivariable logistic regression analyses were performed using IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, NY, United States) and GraphPad Prism version 9.0.0 for Windows (GraphPad Software, Boston, Massachusetts, United States).

RESULTS

The TIGER score significantly correlated with the UCEIS score and MES (r = 0.721, 0.626, both P < 0.001), showed good differentiating values for clinical severity among mild, moderate, and severe UC [8 (4–112.75) *vs* 210 (109–219) *vs* 328 (219–426), all P < 0.001], and exhibited predictive value in diagnosing patients with severe UC [area under the curve (AUC) = 0.897, P < 0.001]. Additionally, the TIGER (r = 0.639, 0,551, 0.488, 0.376, all P < 0.001) and UCEIS scores (r = 0.622, 0,540, 0.494, and 0.375, all P < 0.001) showed stronger correlations with laboratory and clinical parameters, including C-reactive protein, erythrocyte sedimentation rate, length of hospitalisation, and hospitalisation costs, than MES (r = 0.509, 0,351, 0.339, and 0.270, all P < 0.001). The TIGER score showed the best predictability for patients' recent advanced treatment, including systemic corticosteroids, biologics, or immunomodulators (AUC = 0.848, P < 0.001) and 1-year readmission (AUC = 0.700, P < 0.001) compared with the UCEIS score (AUC = 0.762, P < 0.001; 0.627, P < 0.05) and MES (AUC = 0.684, P < 0.001; 0.578, P = 0.132). Furthermore, a TIGER score of ≥ 317 was identified as an independent risk factor for advanced UC treatment (P = 0.011).

CONCLUSION

The TIGER score may be superior to the UCIES score and MES in improving the accuracy of clinical disease severity assessment, guiding therapeutic decision-making, and predicting short-term prognosis.

Key Words: Ulcerative colitis; Toronto Inflammatory Bowel Disease Global Endoscopic Reporting score; Ulcerative Colitis Endoscopic Index of Severity; Mayo Endoscopic Subscore; Endoscopy; Severity

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Core Tip: The manuscript introduces the clinical value of the Toronto Inflammatory Bowel Disease Global Endoscopic Reporting (TIGER) score for ulcerative colitis (UC). Our study, for the first time, validated that the TIGER score accurately reflects disease activity and is significantly correlated with laboratory parameters in patients with UC. We also defined TIGER score thresholds for upgraded treatment and 1-year readmission, providing treatment strategies and personalised disease management for patients with UC.

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INTRODUCTION

Ulcerative colitis (UC) is a multifactorial disease that is characterised by continuous mucosal inflammation of the colon and rectum with an increasing incidence, resulting in a high-cost burden worldwide[1,2]. Endoscopy is the principal technique for visualising lesions in the intestinal mucosa and is regarded as the gold standard for evaluating mucosal inflammation[3]. It can reflect endoscopic disease activity and plays a vital role in assessing therapeutic effects, colorectal cancer surveillance, and UC management[4-6]. These data indicate that the endoscopic score, as a concretisation of endoscopic evaluation, may be a prognostic indicator in UC[7,8]. Several endoscopic scoring systems have been developed, including the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) score and Mayo Endoscopic Subscore (MES), which are commonly used in clinics and trials[9,10]. The UCEIS score, proposed by Travis *et al*[9] in 2012, ranges between 0 and 8, with higher scores indicating increased endoscopic severity, whereas the MES, created by Schroeder *et al* [10] in 1987, categorises severity into a 4-point scoring grade where patients with normal or inactive, mild, moderate, or severe disease are given scores of 0, 1, 2, or 3, respectively. Recently, the extent of mucosal inflammation has been emphasised, and the controversy on the accuracy of the UCEIS score and MES has arisen since both tools provide final scores aimed only at the most severely inflamed segment without highlighting the number of segments exhibiting moderate-to-severe inflammation[11,12]. Endoscopic scores focusing only on the severity during the medical treatment may be flawed because of the presence of segmental remission[13,14]. Therefore, attempts have been made in the past 10 years to assess disease extent and score the entire colonic mucosa[15,16]. In 2022, Zittan *et al*[17] proposed a reliable and useful endoscopic score, the Toronto Inflammatory Bowel Disease Global Endoscopic Reporting (TIGER) score. The TIGER score, constructed for both UC and Crohn's disease, can accurately and comprehensively evaluate the disease severity in all colonic segments and optimise treatment strategies in patients with UC[17].

However, whether the TIGER score has better clinical value than the UCEIS score and MES remains unclear. Therefore, this study aimed to analyse the clinical value of the TIGER score in UC by comparing its relationship with disease severity, predictive potential of treatment options, and prognosis with those of the UCEIS score and MES.

MATERIALS AND METHODS

Study design and patients

This retrospective study included 166 patients aged 18–75 years with a confirmed diagnosis of UC who were initially admitted to the Affiliated Hospital of Qingdao University (Qingdao, China) between January 2017 and March 2023. The following were the exclusion criteria: (1) Patients who did not undergo a colonoscopy, those with inadequate bowel preparation, or those with difficulty in undergoing a full colonoscopy, including the terminal ileum; and (2) presence of comorbidities, such as gastrointestinal neoplasia, infectious bowel disease, previous colorectal surgery, severe cardiac or pulmonary disease, and haemopathy. Figure 1 presents the selection process of the patients.

This study was conducted in accordance with the principle of the Declaration of Helsinki (World Medical Association, 2013) and was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (Approval number: QYFY WZLL 28085). Additionally, the Ethics Committee of the Affiliated Hospital of Qingdao University waived the requirement for written informed consent due to this study's retrospective design.

Clinical and laboratory parameters

Demographic and clinical data, including age, sex, body mass index (BMI), symptoms (abdominal pain, diarrhoea, bloody stool, and fever), disease duration, medical history, assessment of disease severity, treatment programs, length of hospitalisation, hospitalisation costs, and baseline endoscopy results, were collected. Laboratory data, including C-reactive protein (CRP) level, erythrocyte sedimentation rate (ESR), white blood cell (WBC) count, neutrophil (NE) count, lymphocyte (LYM) count, platelet (PLT) count, haemoglobin (Hb) level, albumin (Alb) level, urea nitrogen (BUN) level, uric acid level, and creatinine (Cr) level, were also collected. Based on the Montreal classification system, the extent of UC was classified into three types as follows: Proctitis, left-sided colitis, and extensive colitis defined as E1, E2, and E3, respectively[18]. Furthermore, disease severity in patients with UC was assessed using the Truelove and Witts Severity Index[19].

Endoscopic scores

Colonoscopy images were read independently by two experienced gastroenterologists (Ding XL and Liu AL) who were blinded to the clinical information of the patients, and the TIGER score, UCEIS score, and MES were calculated. When the results were inconsistent, a third senior physician (Tian ZB) confirmed the diagnosis and made the final decision. The TIGER score of each segment was evaluated by summing the following five items: General mucosal appearance, ulcer/erosion size, percentage of ulcer/erosion surface area, percentage of affected surface area per segment, and degree of narrowing[17]. Segments with a TIGER score of ≥ 5, which represent moderate-to-severe endoscopic disease activity, received an additional 100 points[17,20]. The total TIGER score was the sum of the five segmental scores (terminal ileum, ascending, transverse, descending colon, and rectum), and the first digit implied the number of moderate-to-severe endoscopic active segments[17]. The UCEIS score was determined using the following three descriptors with a total score ranging from 0 to 8: Erosion and ulcers, bleeding, and vascular pattern[9]. Furthermore, the MES was scored between 0 and 3 according to the following parameters: Erythema, vascular pattern, bleeding, friability, and ulcerations[10].

Statistical analysis

Statistical analyses and the construction of charts were performed using IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, NY, United States) and GraphPad Prism version 9.0.0 for Windows (GraphPad Software, Boston, Massachusetts, United States), respectively. Quantitative variables with a normal distribution were presented as mean (± SD), while those with a non-normal distribution were presented as median [interquartile range (IQR)]. Qualitative variables were presented as numbers (percentages). The correlations between the TIGER score and the UCEIS score, MES, and laboratory and clinical parameters were tested using Spearman's rank correlation coefficient (r). For continuous variables, the two-sample t-tests or Mann–Whitney U test was performed to compare two groups, and the ANOVA or Kruskal–Wallis H test was used to compare multiple groups, as appropriate. For categorical variables, the chi-square test was used for the univariate analysis. Multivariable logistic regression analysis was used to identify independent risk factors. Furthermore, we used the area under the curve (AUC) of the receiver operating characteristic (ROC) curve to compare the discrimination abilities across different scoring systems. Two-sided hypothesis tests were considered, and statistical significance was set at $P < 0.05$.

RESULTS

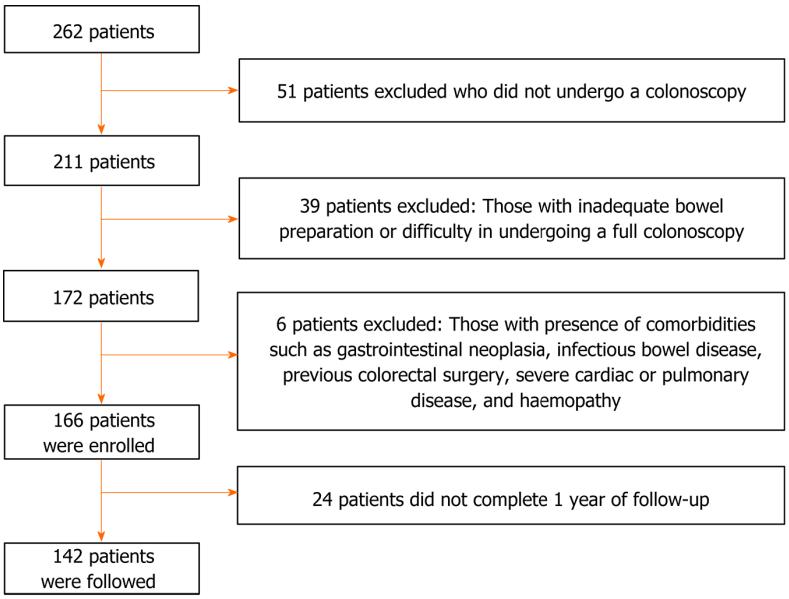
Baseline characteristics of patients with UC

Overall, 166 patients were selected in this study. Notably, 98 (59%) males and 68 (41%) females were included, with a mean age of 42.29 ± 12.05 years. The patients with UC were categorised into mildly, moderately, and severely active groups comprising 44 (26.5%), 51 (30.7%), and 71 (42.8%) patients, respectively, according to Truelove and Witts Severity Index[19]. The median TIGER score, UCEIS score, and MES were 214, 5, and 3, respectively. Furthermore, 161 (97.0%), 40 (24.1%),18 (10.8%), 3 (1.8%), 2 (1.2%), 2 (1.2%), and 1 (0.6%) patients received 5-aminosalicylates (5-ASAs) orally or rectally, used systemic corticosteroids, were treated with biologics (including infliximab or vedolizumab), used immunomodulators, received tofacitinib, used thalidomide, and underwent colorectal surgery, respectively (Table 1).

Table 1 Patient demographic and clinical characteristics

Characteristics	Values (*n* = 166)
Gender, *n* (%)	
Male	98 (59.0)
Female	68 (41.0)
Age (yr)	42.29 ± 12.05
BMI (kg/m^2)	22.49 ± 3.47
Duration (mo)	36.0 (12.0, 96.0)
Montreal classification, *n* (%)	
E1 = proctitis	21 (12.7)
E2 = left-sided colitis	47 (28.3)
E3 = extensive colitis	98 (59.0)
Truelove and Witts Severity Index, *n* (%)	
Mild	44 (26.5)
Moderate	51 (30.7)
Severe	71 (42.8)
Total TIGER score	214 (109, 324)
UCEIS score	5 (3, 6)
MES	3 (2, 3)
Treatment, *n* (%)	
5-ASAs	161 (97.0)
Systemic corticosteroids	40 (24.1)
IFX	8 (4.8)
VDZ	10 (6)
Immunomodulators	3 (1.8)
Tofacitinib	2 (1.2)
Thalidomide	2 (1.2)
Colectomy	1 (0.6)
Length of hospitalization (d)	7.00 (4.75-10.00)
Hospitalisation costs (CNY)	9088.11 (6788.40-12500.72)
Readmission within 1 yr, *n* (%)	48 (33.8)
CRP (mg/L)	3.58 (1.44-9.96)
ESR (mm/60 min)	10 (6-18)
WBC (× 10^9/L)	6.95 (5.53-8.62)
NE (× 10^9/L)	4.29 (3.03-5.59)
LYM (× 10^9/L)	1.81 (1.45-2.27)
PLT (× 10^9/L)	273.50 (216.75-365.50)
Hb (g/L)	127.00 (104.75-143.00)
Alb (g/L)	42.65 (37.50-54.10)
BUN (mmol/L)	3.96 (3.01-5.02)
UA (μmol/L)	296.39 ± 103.49
Cr (μmol/L)	57.50 (49.00-69.00)

BMI: Body mass index; TIGER: Toronto Inflammatory Bowel Disease Global Endoscopic Reporting; UCEIS: Ulcerative Colitis Endoscopic Index of Severity; MES: Mayo Endoscopic Subscore; 5-ASAs: 5-Aminosalicylates; IFX: Infliximab; VDZ: Vedolizumab; CRP: C-reactive protein; CNY: Chinese Yuan; ESR: Erythrocyte sedimentation rate; WBC: White blood cell; NE: Neutrophil count; LYM: Lymphocyte count; PLT: Platelet count; Hb: Haemoglobin; Alb: Albumin; BUN: Urea nitrogen; UA: Uric acid; Cr: Creatinine.



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Figure 1 Selection process of the patients.

Correlations among the TIGER score, UCEIS score, and MES

The TIGER score was strongly correlated with the UCEIS score (r = 0.721, P < 0.001) and moderately correlated with MES (r = 0.626, P < 0.001). Additionally, a moderate correlation was observed between the UCEIS score and MES (r = 0.681, P < 0.001).

Comparison of the endoscopic scores in different clinical severities

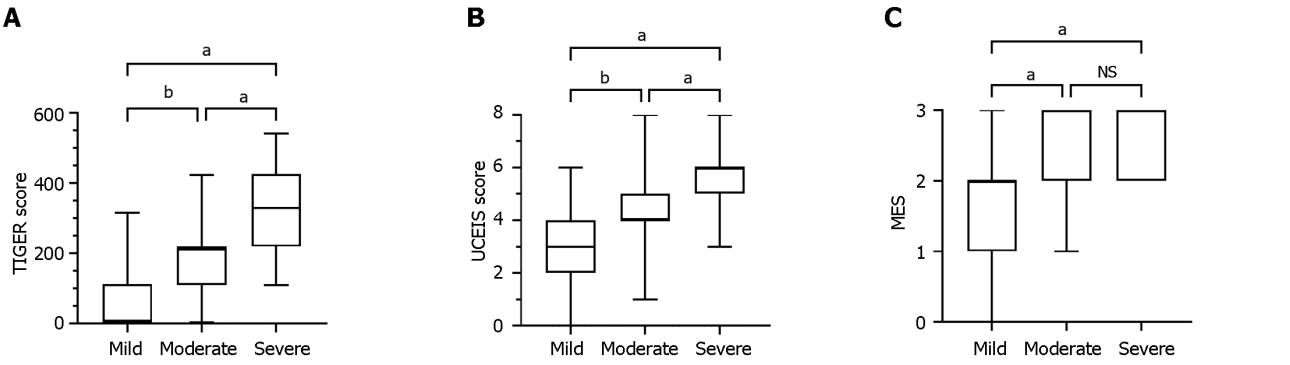
Using the Truelove and Witts Severity Index[19], the performance of the three endoscopic scoring systems in patients with different clinical severities was evaluated. Significant differences were observed in the three endoscopic scoring systems among patients with different disease severities (all P < 0.001). Comparison within each group revealed that both the TIGER and UCEIS scores showed significant distinctions from each other [median (IQR): 8 (4–112.75) *vs* 210 (109–219) *vs* 328 (219–426), all P < 0.001; 3 (2–4) *vs* 4 (4–5) *vs* 6 (5–6), all P < 0.001], although no significant difference was found in the MES between moderate and severe disease severities (P > 0.05) (Figure 2).

Comparison of the diagnostic value of the endoscopic scores for patients with severe UC

To evaluate the diagnostic performance of the three endoscopic scoring systems for severe UC, assessed using the Truelove and Witts Severity Index[19], the patients were further categorised into mild-to-moderate (n = 95) and severe (n = 71) groups, and the diagnostic value was analysed using the ROC curve. The results revealed that the TIGER score exhibited superior diagnostic performance, with an AUC, sensitivity, specificity, and cut-off value of 0.897, 80.3%, 83.2%, and 217.5, respectively (P < 0.001), demonstrating a good diagnostic capability for severe UC. The UCEIS score ranked second, with an AUC, sensitivity, specificity, and cut-off value of 0.839, 81.7%, 72.6%, and 4.5, respectively (P < 0.001), whereas the MES exhibited relatively poor accuracy for diagnosing severe patients with UC, showing an AUC, sensitivity, specificity, and cut-off value of 0.727, 74.6%, 67.4%, and 2.5, respectively (P < 0.001) (Figure 3).

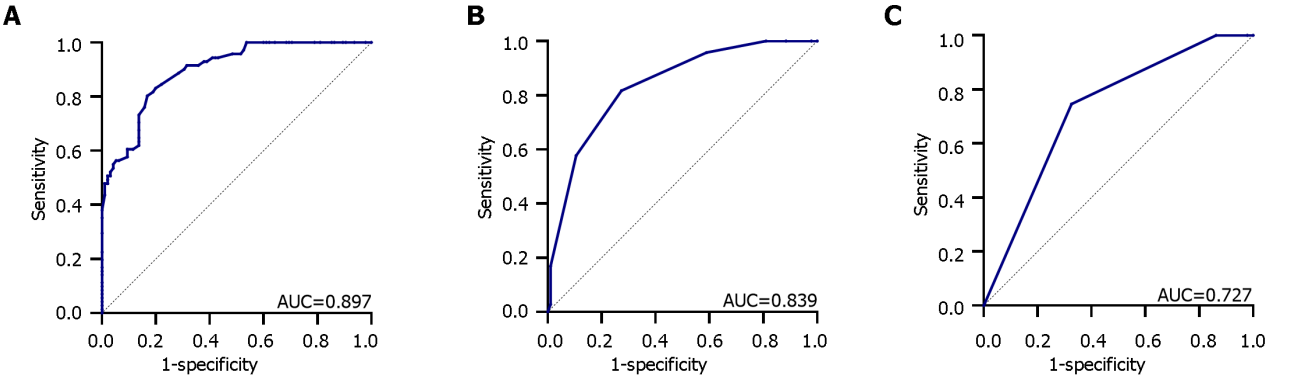
Correlations between the three endoscopic scores and laboratory/clinical parameters

The three scoring systems were positively correlated with CRP level, ESR, WBC count, NE count, PLT count, length of hospitalisation, and hospitalisation costs (all P < 0.001) but were negatively correlated with Hb, Alb, and BUN levels (all P ≤ 0.001). Furthermore, the correlations between the TIGER score and CRP level, ESR, WBC count, NE count, PLT count, Alb level, and BUN level (r = 0.639, 0.551, 0.387, 0.458, 0.429, -0.422, and -0.320, all P < 0.001) were higher than those between MES and the respective parameters (r = 0.509, 0.351, 0.268, 0.310, 0.248, -0.278, and -0.251, all P ≤ 0.001). However, the correlation between the TIGER score and Hb level was lower than that of the UCEIS score but higher than the MES (all P ≤ 0.001). The correlations between the TIGER score and CRP level, ESR, length of hospitalisation, and hospitalisation costs were similar to those of the UCEIS score (all P < 0.001) and higher than those of the MES (all P <



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Figure 2 Comparisons of endoscopic scores among the mild (*n* = 44), moderate (*n* = 51), and severe (*n* = 71) patients. A: Comparison of Toronto Inflammatory Bowel Disease Global Endoscopic Reporting score among different severities of ulcerative colitis (UC); B: Comparison of Ulcerative Colitis Endoscopic Index of Severity score among different severities of UC; C: Comparison of Mayo Endoscopic Subscore among different severities of UC. $^{a}P < 0.0001$; $^{b}P < 0.001$; NS: Not significance; TIGER: Toronto Inflammatory Bowel Disease Global Endoscopic Reporting; UCEIS: Ulcerative Colitis Endoscopic Index of Severity; MES: Mayo Endoscopic Subscore.



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Figure 3 Comparison of the diagnostic value of endoscopic scores for patients with severe ulcerative colitis. A: The diagnostic value of Toronto Inflammatory Bowel Disease Global Endoscopic Reporting score for severe patients; B: The diagnostic value of Ulcerative Colitis Endoscopic Index of Severity score for severe patients; C: The diagnostic value of Mayo Endoscopic Subscore for severe patients. AUC: Area under the curve.

0.001). No correlations were observed among BMI, disease duration, LYM count, or Cr level in any of the three scoring systems (all $P > 0.05$) (Table 2).

The relationship between the endoscopic scores and advanced treatment

Overall, 113 (68.1%) and 53 (31.9%) patients received 5-ASAs alone and advanced treatment during this admission or within 1 month of discharge, respectively, classifying them into the 5-ASAs-alone and advanced treatment groups, respectively. Advanced treatments included systemic corticosteroids, biologics, immunomodulators, thalidomide, and surgery. Notably, patients in the advanced treatment group demonstrated significantly higher TIGER score [median (IQR): 328 (220.5–426) *vs* 116 (8.5–218); $Z = -7.210$, $P < 0.0001$], UCEIS score [median (IQR): 6 (4–6) *vs* 4 (3–5); $Z = -5.543$, $P < 0.0001$], and MES [median (IQR): 3 (2–3) *vs* 2 (2–3); $Z = -4.272$, $P < 0.0001$], as shown in Figure 4A-C.

ROC analysis was performed in patients with UC to compare the predictive capability for advanced treatment. The TIGER score had an AUC of 0.848 with a sensitivity, specificity, and cut-off value of 69.8%, 86.7%, and 317, respectively ($P < 0.001$), showing the best predictive potential for treatment escalation. The UCEIS score had an AUC of 0.762, with a sensitivity, specificity, and cut-off value of 60.4%, 83.2%, and 5.5, respectively ($P < 0.001$), indicating a moderate predictive capability. Furthermore, the AUC of the MES was 0.684 with a sensitivity, specificity, and cut-off value of 73.6%, 60.2%, and 2.5, respectively ($P < 0.001$), indicating lower predictive potential than the TIGER and UCEIS scores for advanced therapies (Figure 4D-F).

Based on the ROC curves (Figure 4D-F), we selected the TIGER score, UCEIS score, and MES of 317, 5.5, and 2.5, respectively, as the cut-off values and patients were classified into the low- and high-score groups. The TIGER score, UCEIS score, MES, extent of UC, CRP level, Hb level, and Truelove and Witts Index exhibited significant differences between the 5-ASAs-alone and advanced treatment groups (all $P < 0.001$; Table 3). Furthermore, the TIGER score of ≥ 317 [odds ratio (OR): 3.891; 95% confidence interval (95%CI): 1.360–11.136; $P = 0.011$) and extent of E3 (OR: 6.488; 95%CI: 1.617–26.027; $P = 0.008$) were the significant risk factors for treatment escalation (Table 4).

Table 2 Correlations between three endoscopic scores and laboratory/clinical parameters

Parameters	TIGER score		UCEIS score		MES	
	Spearman coefficient (*r*)	*P* value	Spearman coefficient (*r*)	*P* value	Spearman coefficient (*r*)	*P* value
BMI	-	0.146	-	0.035	-	0.191
Duration	-	0.660	-	0.604	-	0.814
CRP	0.639	< 0.001	0.622	< 0.001	0.509	< 0.001
ESR	0.551	< 0.001	0.540	< 0.001	0.351	< 0.001
WBC	0.387	< 0.001	0.319	< 0.001	0.268	< 0.001
NE	0.458	< 0.001	0.369	< 0.001	0.310	< 0.001
LYM	-	0.349	-	0.823	-	0.790
PLT	0.429	< 0.001	0.395	< 0.001	0.248	< 0.001
Hb	-0.353	< 0.001	-0.388	< 0.001	-0.245	0.001
Alb	-0.422	< 0.001	-0.306	< 0.001	-0.278	< 0.001
BUN	-0.320	< 0.001	-0.295	< 0.001	-0.251	0.001
UA	-	0.233	-0.164	0.035	-	0.510
Cr	-	0.305	-	0.399	-	0.144
Stay	0.488	< 0.001	0.494	< 0.001	0.339	< 0.001
Costs	0.376	< 0.001	0.375	< 0.001	0.270	< 0.001

BMI: Body mass index; TIGER: Toronto Inflammatory Bowel Disease Global Endoscopic Reporting; UCEIS: Ulcerative Colitis Endoscopic Index of Severity; MES: Mayo Endoscopic Subscore; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; WBC: White blood cell; NE: Neutrophil count; LYM: Lymphocyte count; PLT: Platelet count; Hb: Haemoglobin; Alb: Albumin; BUN: Urea nitrogen; UA: Uric acid; Cr: Creatinine.

Relationship between the endoscopic scores and 1-year readmission

A 1-year follow-up was conducted on 142 patients to investigate the relationship between the endoscopic scores and 1-year readmission (Figure 1). Notably, 142 patients were classified into the non-readmission (n = 94) and readmission (n = 48) groups to compare the predictive value of the assessed endoscopic scoring systems for readmission within 1 year. Mann–Whitney test results revealed that the readmission group had a higher TIGER [median (IQR): 319.5 (210–425.75) *vs* 210 (106–222.5); Z = -3.889, P < 0.001] and UCEIS [median (IQR): 5 (4–6) *vs* 4 (3–6); Z = -2.529, P = 0.011] scores than the non-readmission group, whereas no significant difference was observed in the MES [median (IQR): 3 (2–3) *vs* 2(2–3); Z = -1.701, P = 0.089] between the two groups (Figure 5A-C).

ROC curves were drawn to compare the predictive value of the scoring systems for readmission within 1 year (Figure 5D-F). The AUC of the TIGER score was 0.700, indicating a sensitivity, specificity, and cut-off value of 60.4%, 73.4%, and 220.5, respectively (P < 0.001), demonstrating a predictive capability for readmission. The AUC of the UCEIS score was 0.627, with a sensitivity, specificity, and cut-off value of 43.8%, 74.5%, and 5.5, respectively (P < 0.05), exhibiting inferior predictive ability for readmission. Conversely, MES showed no significant predictive value compared with the two endoscopic scores mentioned above, with an AUC of 0.578 (P = 0.132).

DISCUSSION

In this study, we compared the clinical utility of the TIGER score, UCEIS score, and MES and identified their roles in predicting disease burden and short-term clinical outcomes.

The three indices consistently reflected endoscopic findings, and the TIGER score strongly and moderately correlated with the UCEIS score and MES, respectively. Xu *et al*[20] indicated that the TIGER score correlated with the UCEIS score and MES, with correlation coefficients of 0.6193 and 0.4527, respectively, similar to our results. These findings demonstrate a better correlation between the TIGER and UCEIS scores, which we attribute to the better definition and grading of the descriptors in the two scoring systems, such as more detailed scoring criteria for erosions and ulcers[21].

Furthermore, using the Truelove and Witts Severity Index[19], we discovered that the TIGER and UCEIS scores could distinguish between the different UC severities. Notably, the TIGER score demonstrated optimal diagnostic performance for severe UC. This superior performance may be because the TIGER score assesses total bowel segments and considers both inflammation and the extent of UC, whereas the UCEIS score and MES exclude the extent of UC. Interestingly, the

Table 3 Univariable analysis of risk factors for the advanced treatment, *n* (%)

Variables	5-ASAs-alone group (*n* = 113)	Advanced treatment group (*n* = 53)	*P* value
Age			0.373
< 40 yr	45 (39.8)	25 (47.2)	
≥ 40 yr	68 (60.2)	28 (52.8)	
Sex			0.054
Male	61 (54.0)	37 (69.8)	
Female	52 (46.0)	16 (30.2)	
Extent			< 0.001
E1 or E2	65 (57.5)	3 (5.7)	
E3	48 (42.5)	50 (94.3)	
CRP			< 0.001
< 5 mg/L	85 (75.2)	14 (26.4)	
≥ 5 mg/L	28 (24.8)	39 (73.6)	
Hb			< 0.001
≥ 110 g/L	94 (83.2)	26 (49.1)	
< 110 g/L	19 (16.8)	27 (50.9)	
Truelove and Witts index			< 0.001
Mild or moderate	84 (74.3)	11 (20.8)	
Severe	29 (25.7)	42 (79.2)	
TIGER score			< 0.001
< 317	98 (86.7)	16 (30.2)	
≥ 317	15 (13.3)	37 (69.8)	
UCEIS score			< 0.001
< 6	94 (83.2)	21 (39.6)	
≥ 6	19 (16.8)	32 (60.4)	
MES			< 0.001
≤ 2	68 (60.2)	14 (26.4)	
> 2	45 (39.8)	39 (73.6)	

TIGER: Toronto Inflammatory Bowel Disease Global Endoscopic Reporting; UCEIS: Ulcerative Colitis Endoscopic Index of Severity; MES: Mayo Endoscopic Subscore; 5-ASAs: 5-Aminosalicylates; CRP: C-reactive protein; Hb: Haemoglobin.

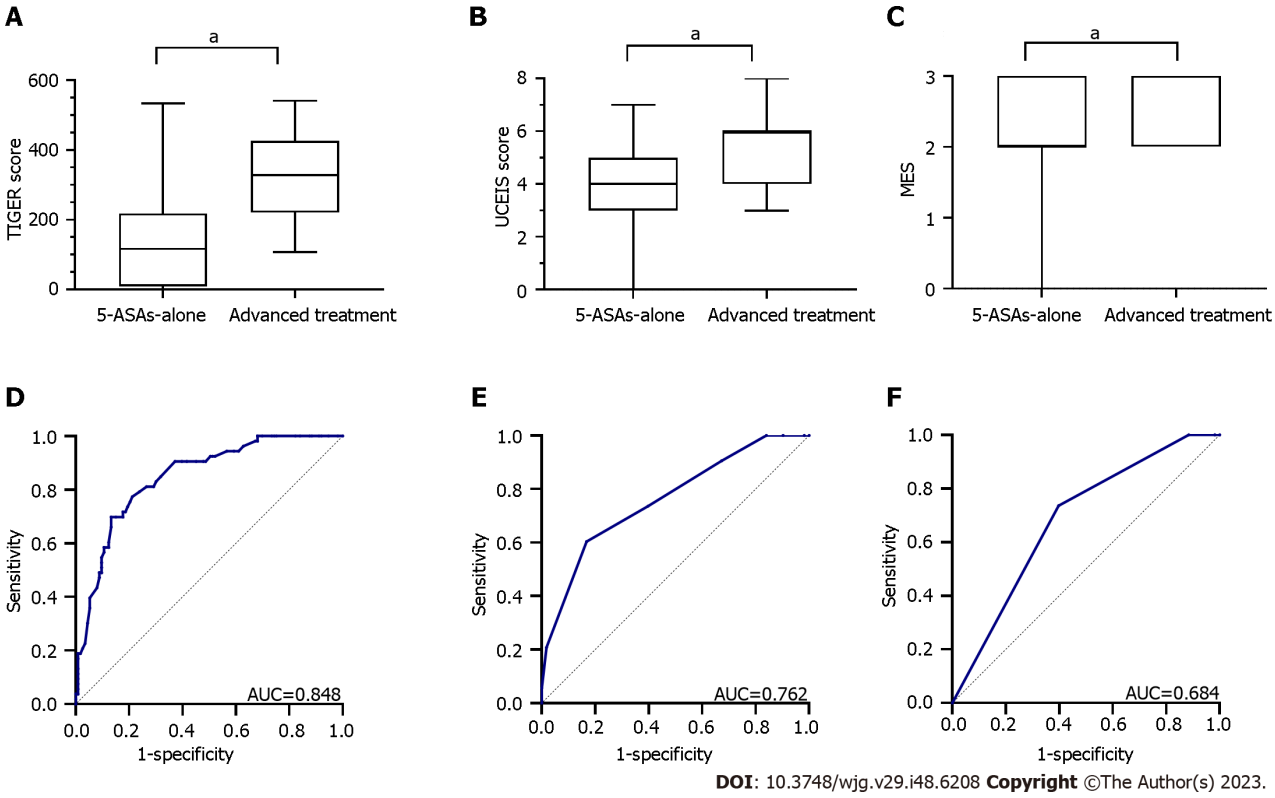
extent is one of the dimensions used to evaluate endoscopic severity and can influence the overall severity of UC[22]. Osada *et al*[23] demonstrated that total colonoscopy could provide complete information on patients with UC and improve the accuracy of clinical disease assessment, which is consistent with our conclusion. Nevertheless, because of factors including discomfort and complications, total colonoscopic studies included fewer acute severe cases, which might have resulted in different results. Gomes *et al*[24] revealed a poor correlation between total colonoscopic findings and clinical manifestations. Moreover, a finer categorization and larger scale of the scoring system may be more advantageous and accurate in reflecting inflammatory burden and treatment response[22,25]. The UCEIS score, ranging from 0 to 8, provides a larger scale and finer gradings of ulcers and bleeding than the MES. Song *et al*[26] also demonstrated that the UCEIS score was superior to MES in diagnosing UC severity. Therefore, we infer that the TIGER score can provide a detailed description of the ulcers (size and percentage of surface) and localised inflammation in relation to the bowel segment and a wide range of scores between 0 and 560, resulting in optimal performance when reflecting the overall severity[17].

In this study, we observed that the TIGER score was significantly correlated with the clinical parameters of active inflammation, particularly CRP levels, and the burden parameters including length of stay and cost, whereas the MES exhibited disadvantages in evaluating the clinical activity of UC compared to the TIGER and UCEIS scores. Previous studies have demonstrated that objective blood markers, including CRP, ESR, WBC, PLT, Alb, and Hb, are relevant to UC endoscopic severity and disease activity[27,28]. Additionally, the association between endoscopic scores and inflam-

Table 4 Multi-variable logistic regression analysis for the risk factors of the advanced treatment

Variables	Odds ratio	95%CI	*P* value
Extent, E3	6.488	1.617-26.027	0.008
CRP ≥ 5 mg/L	2.554	0.956-6.819	0.061
Hb < 110 g/L	1.736	0.615-4.899	0.297
Truelove and Witts Index, Severe	1.390	0.452-4.278	0.566
TIGER score ≥ 317	3.891	1.360-11.136	0.011
UCEIS score ≥ 6	2.171	0.736-6.403	0.160
MES > 2	0.945	0.335-2.667	0.914

95%CI: 95% confidence interval; CRP: C-reactive protein; Hb: Haemoglobin; TIGER: Toronto Inflammatory Bowel Disease Global Endoscopic Reporting; UCEIS: Ulcerative Colitis Endoscopic Index of Severity; MES: Mayo Endoscopic Subscore.

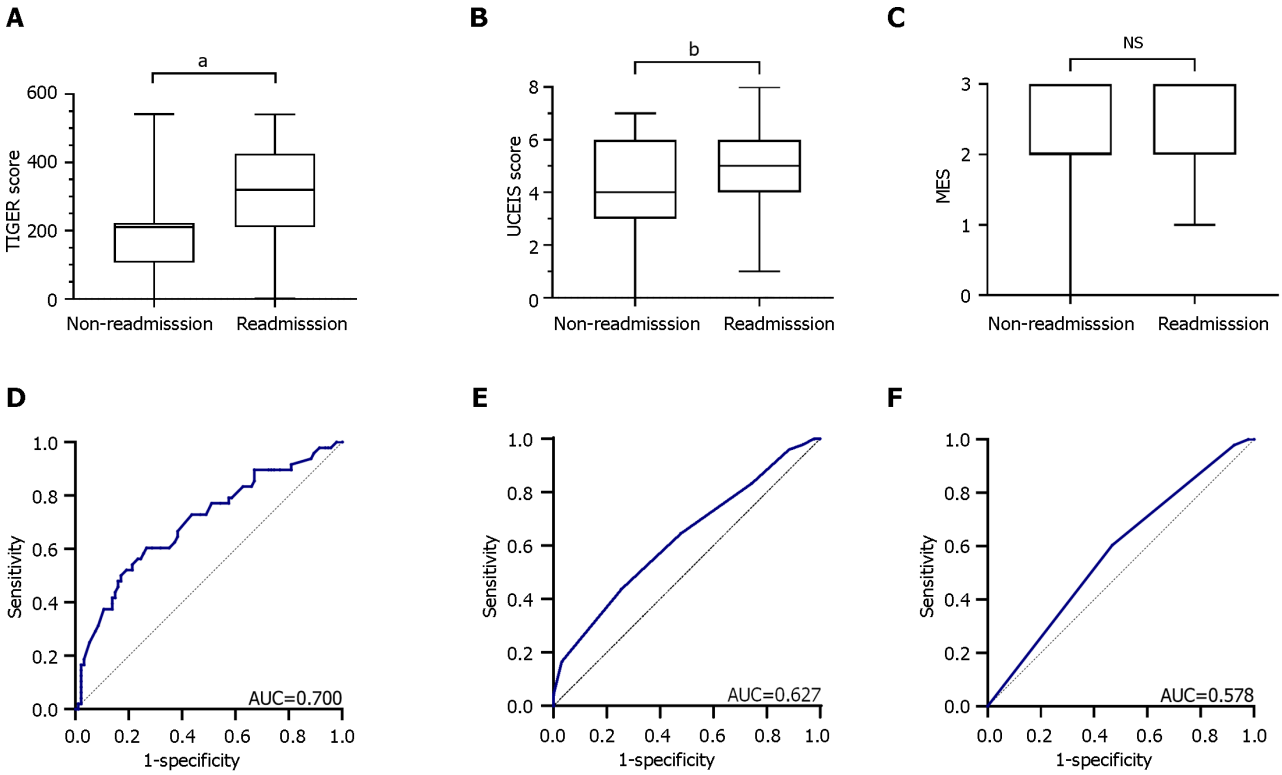


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Figure 4 Comparisons of endoscopic scores between the 5-aminosalicylates-alone group (*n* = 113) and the advanced treatment group (*n* = 53). A: Comparisons of Toronto Inflammatory Bowel Disease Global Endoscopic Reporting (TIGER) score between 2 groups; B: Comparisons of Ulcerative Colitis Endoscopic Index of Severity (UCEIS) score between 2 groups; C: Comparisons of Mayo Endoscopic Subscore (MES) between 2 groups; D: The predictive value of TIGER score for advanced treatment recently; E: The predictive value of UCEIS score for advanced treatment recently; F: The predictive value of MES for advanced treatment recently. [a]*P* < 0.0001. AUC: Area under the curve.

matory burden has been confirmed in other studies[29,30]. Recent studies have shown that CRP, a typical acute-phase protein, reflects the inflammatory state of the entire colon, which aligns with our findings that demonstrate the existence of a correlation between the TIGER score and CRP levels[31]. Additionally, Zittan *et al*[17] reported that the TIGER score was positively correlated with faecal calprotectin levels and the inflammatory bowel disease (IBD) Disk score, indicating that the disease condition and burden of UC may be observed using the TIGER score. Inflammatory biomarkers can exacerbate damage to the epithelial barrier and imbalance of the intestinal mucosal immune system and influence the synthesis of related protein synthesis in UC[32,33], which may be manifested in endoscopic mucosal inflammation and reflected by the TIGER score since it contains a clear description of mucosal appearance and ulcer conditions and could precisely describe and assess the entire intestine.

Five-ASAs are still recommended as the standard treatment and maintenance strategies for patients with mild-to-moderate UC, whereas systemic corticosteroids, thiopurines, biologics, immunomodulators, and tofacitinib are



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Figure 5 Comparisons of the endoscopic scores between the non-readmission group (*n* = 94) and the readmission group (*n* = 48). A: Comparisons of Toronto Inflammatory Bowel Disease Global Endoscopic Reporting (TIGER) score between 2 groups; B: Comparisons of Ulcerative Colitis Endoscopic Index of Severity (UCEIS) score between 2 groups; C: Comparisons of Mayo Endoscopic Subscore (MES) between 2 groups; D: The predictive value of TIGER score for 1-year readmission; E: The predictive value of UCEIS score for 1-year readmission; F: The predictive value of MES for 1-year readmission. [a]*P* < 0.001; [b]*P* < 0.05; ns: No significance; AUC: Area under the curve.

considered upgraded treatment options for those with moderate-to-severe disease activity or those with 5-ASAs failure or intolerance[34,35]. In this study, we observed that the TIGER score had a superior predictive potential for advanced treatment in patients with UC and demonstrated that a TIGER score of ≥ 317 was an independent risk factor for indicating that patients with three or more segments involved in moderate-to-severe endoscopic activity were more likely to upgrade their treatment programs. This could be a useful indication of escalating treatment. Severe endoscopic activity and extensive colitis may represent a severe degree of disease, leading to therapy escalation and poor prognosis[36-38], which could explain why patients with higher TIGER scores were at a higher risk of advanced treatment in this study. Bálint *et al*[39] suggested that a scoring system should provide additional information on the localization and extent of the disease and argued that this could guide treatment choices, which is consistent with the above mentioned results.

Meanwhile, a high readmission rate indicates increased disease severity and poor prognosis in UC[40]. Therefore, the prediction of readmissions could help clinicians develop healthcare plans and manage patients. We observed that the TIGER score in the readmission group was higher than that in the non-readmission group and it displayed the best predictive capability for readmission within 1 year. These results indicate that the TIGER score could help predict short-term prognosis in patients with UC. Higher TIGER scores indicate more severe UC, which may also be associated with a higher readmission rate[40]. Notably, the UCEIS score was observed to be an independent risk factor for the 1-year readmission, demonstrating that endoscopic scores might be associated with early readmission[41].

The TIGER score requires further validation in clinical practice before its broad adoption. Five descriptors in each of the five segments were evaluated during the endoscopic examination to assess the mucosal inflammation of the entire intestine, resulting in 25 items that required scoring. For segments with a TIGER score of ≥ 5, 100 bonus points were counted, and the segmental scores were summed to determine the final total TIGER score. With the recent development of high-performance computers, advanced optical technologies, molecular imaging, and artificial intelligence algorithms, a computer-aided diagnostic system for patients with IBD to improve endoscopic assessment has become possible[42-45]. Although the TIGER score requires calculation and total colonoscopy, it can be intelligently calculated currently and may be automatically scored in the future by analysing the entire colonic mucosa.

Furthermore, we validated, for the first time, that the TIGER score could accurately reflect disease activity and significantly correlate with laboratory parameters in patients with UC. Moreover, we also defined TIGER score thresholds for upgraded treatment and 1-year readmission, providing treatment strategies and personalised disease management for patients with UC. Additionally, this study included a larger cohort of patients with UC than a previous study[17].

However, this study had some limitations. First, this was a single-centre retrospective study. Second, some patients with acute severe UC could not undergo total colonoscopy and were excluded from the study. Therefore, multicenter prospective studies are warranted.

CONCLUSION

The TIGER score is a useful scoring method that provides an overall intestinal evaluation of endoscopic activity and demonstrates a significant correlation with the UCEIS score, MES, and laboratory indices, particularly CRP levels. Furthermore, the TIGER score may be superior to the UCEIS and MES scoring systems in improving the accuracy of clinical disease severity assessment, guiding therapeutic decision-making to some extent, and predicting short-term clinical outcomes.

ARTICLE HIGHLIGHTS

Research background

Endoscopy is crucial in the diagnosis, assessment, and management of ulcerative colitis (UC). Several endoscopic scoring systems have been established to make endoscopic evaluation quantified and objective, including the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) score and Mayo Endoscopic Subscore (MES). The Toronto Inflammatory Bowel Disease Global Endoscopic Reporting (TIGER) score for UC, which considers the extent of UC involvement and reflects the number of segments with moderate-to-severe inflammation, was proposed in 2022.

Research motivation

Although the TIGER score is a novel and reliable tool for reflecting complete endoscopic inflammation, its clinical value remains unclear.

Research objectives

To assess the clinical value of the TIGER score by comparing it with the UCEIS score and MES.

Research methods

We performed a retrospective study that included 166 patients with UC who underwent total colonoscopy. Spearman's rank correlation coefficient was used to estimate the linear associations of three scores and laboratory/clinical parameters. The receiver-operating characteristic curve was performed to compare the predictive potentials of the three scores for predicting severe UC, patients' recent advanced treatment, and 1-year readmission. Univariate and multivariable logistic regression analyses were performed to investigate the independent risk factors for treatment escalation.

Research results

The TIGER score showed a significant correlation with the UCEIS score, MES, and laboratory indices, particularly C-reactive protein levels. Additionally, the TIGER score exhibited the best predictive capability for diagnosing patients with severe UC, upgrading treatment options, and 1-year readmission and a TIGER score of ≥ 317 was found to be an independent risk factor for treatment escalation in UC.

Research conclusions

The TIGER score exhibits an advantage in assessing the disease severity of UC, guiding treatment decisions, and predicting short-term prognosis compared to the UCEIS score and MES.

Research perspectives

The TIGER score may have significant clinical utility in evaluating, treating, and managing patients with UC, although multicenter prospective studies are required to promote its use.

FOOTNOTES

Author contributions: Ding XL, Liu XY, and Tian ZB designed this study; Zhang LJ evaluated and reviewed the statistical methods; Ding XL, Liu XY, Liu AL, Zhang XF, and Wu J collected data; Liu XY analysed the data; Liu XY edited the manuscript; Ding XL, Liu AL, Tian ZB revised the manuscript; and all authors have read and approved the final manuscript.

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (Approval No. QYFY WZLL 28085).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: All authors declare no potential conflicts of interest related to this study.

Data sharing statement: The data used in this study can be obtained from the corresponding author upon reasonable request.

STROBE statement: The authors have read the STROBE statement, and the manuscript was revised in accordance with the STROBE Statement—checklist of items.

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ORIGINAL ARTICLE

Basic Study

Single-cell analysis identifies phospholysine phosphohistidine inorganic pyrophosphate phosphatase as a target in ulcerative colitis

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Abstract

BACKGROUND

Ulcerative colitis (UC) is a chronic gastrointestinal disorder characterized by inflammation and ulceration, representing a significant predisposition to colorectal cancer. Recent advances in single-cell RNA sequencing (scRNA-seq) technology offer a promising avenue for dissecting the complex cellular interactions and molecular signatures driving UC pathology.

AIM

To utilize scRNA-seq technology to dissect the complex cellular interactions and molecular signatures that underlie UC pathology.

METHODS

In this research, we integrated and analyzed the scRNA-seq data from UC patients. Moreover, we conducted mRNA and protein level assays as well as pathology-related staining tests on clinical patient samples.

RESULTS

In this study, we identified the sustained upregulation of inflammatory response pathways during UC progression, characterized the features of damaged endo-

thelial cells in colitis. Furthermore, we uncovered the downregulation of phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP) has a negative correlation with signal transducer and activator of transcription 3. Significant downregulation of LHPP in UC patient tissues and plasma suggests that LHPP may serve as a potential therapeutic target for UC. This paper highlights the importance of LHPP as a potential key target in UC and unveils its potential role in inflammation regulation.

CONCLUSION

The findings suggest that LHPP may serve as a potential therapeutic target for UC, emphasizing its importance as a potential key target in UC and unveiling its role in inflammation regulation.

Key Words: Ulcerative colitis; Single-cell RNA sequencing; Phospholysine phosphohistidine inorganic pyrophosphate phosphatase

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Core Tip: Ulcerative colitis (UC), a chronic inflammatory bowel disease linked to colorectal cancer, was investigated using single-cell RNA sequencing technology. The study unveiled sustained upregulation of inflammatory response pathways and characterized damaged endothelial cells during UC progression. Notably, the downregulation of phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP) exhibited a negative correlation with signal transducer and activator of transcription 3. LHPP's significant downregulation in UC patient tissues and plasma suggests its potential as a therapeutic target. The findings highlight LHPP as a key target in UC and emphasize its role in inflammation regulation, offering insights for potential therapeutic interventions.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) characterized by mucosal inflammation and ulceration primarily affecting the colon and rectum[1]. It is a multifactorial[2,3]. Individuals with UC often experience debilitating symptoms such as abdominal pain, bloody diarrhea, diarrhea, rectal bleeding, and an increased risk of colorectal cancer (CRC), severely impacting their quality of life. Extensive research over the years has yielded valuable insights into the pathogenesis of UC, highlighting the importance of aberrant immune responses and the gut microbiome in disease development and progression[4-6]. However, a comprehensive understanding of the cellular and molecular mechanisms underlying UC development and progression remains elusive, and there is a growing need for more precise and targeted therapeutic interventions[7].

Recent advances in single-cell RNA sequencing (scRNA-seq) technology have revolutionized our ability to dissect the heterogeneity of cell populations within complex tissues[8-10]. This revolutionary technique empowers researchers to discern distinct cell subsets, unveil novel cellular pathways, and delve into the dynamics of immune responses with unparalleled resolution[11,12]. In the context of UC, scRNA-seq offers a unique opportunity to decipher the intricate cellular interactions and molecular signatures driving disease pathology[13-17].

Phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP), a histidine phosphatase protein, has been implicated in diverse biological processes, including tumor suppression in hepatocellular carcinoma[18], increasing the expression of cleaved-poly (ADP-ribose) polymerase and cleaved-Casp3 protein to promote apoptosis[19], and inflammation regulation and immune response modulation. Recent studies have suggested its potential correlation with survival of CRC patients[20]. Another study reports that the loss of LHPP in intestinal epithelial cells correlate with colitis in mice, suggests the involvement of LHPP in IBD[21,22]. However, the precise involvement and impact of LHPP in the context of UC remain an unexplored territory, offering a promising avenue for further investigation.

In this study, we integrated the current single-cell sequencing data of UC[15,16], shedding light on the specific cell types, transcriptional profiles, and immune signaling pathways that play pivotal roles in UC pathogenesis. By interrogating the single-cell landscape of UC-affected colonic tissues, we identified sustained upregulation of inflammatory response pathways during the progression of UC and characterized the features of damaged endothelial cells (EC) in colitis. Through integrated analysis of UC disease databases utilizing single-cell data, we uncovered that LHPP exhibits sustained downregulation in UC, displaying a negative correlation with signal transducer and activator of transcription 3 (*STAT3*). Notably, our experimental results in UC patients corroborated that when *STAT3* expression is upregulated, LHPP expression is downregulated, suggesting a potential role for *STAT3* in transcriptionally inhibiting LHPP expression. Furthermore, LHPP not only exhibited decreased expression in the intestinal tissue of UC patients but also displayed reduced expression in their plasma samples. This observation suggests that LHPP may serve as a critical

factor in the pathogenesis of UC.

MATERIALS AND METHODS

Construction of cell atlas of healthy and UC human clonal tissues

The raw data of healthy and UC human clonal tissues were from GSE125527 in GEO database. The R package Seurat (version 4.0.2) was used for construction of cell atlas of human clonal tissues. In brief, the function "CreateSeuratObject" was used to load gene expression matrix of each sample. The function 'SCTransfrom' was used for finding high variable genes, normalization and scaling of the gene expression matrix for each sample, the 'PrepSCTIntegration' and 'FindIntegrationAnchors' functions were used for selecting the anchors for integration all samples. The function "IntegrateData" was used for the following integration. After integration, the function "ScaleData" was used to scale the integrated expression matrix, then the principle component analysis and Dimensionality reduction of dataset were performed by the functions "RunPCA" and "RunUMAP", the functions "FindNeighbors" and "FindClusters" were used to cell clustering and identification. The function 'FindAllMarkers' (|avg_log2FC| ≥ 0.5 and p_val_adj ≤ 0.05) was used to calculate marker genes for each cell type.

Identification of differential expression genes (DEGs) between healthy and UC group across tissues and cell compartments

The 'FindMarkers' function in Seurat was used to identify differentially expressed genes in diseased *vs* healthy group (diseased/healthy) of each cell type, which were based on normalized data and the Wilcoxon test. The screening criteria for significantly differentially expressed genes were selected by BH-adjusted *P* value < 0.05 and $|\log_2 FC| > 0.25$.

Pseudo-time trajectory inference

To characterize the mobilization and of EC during UC, the R package Monocle2 was used to perform pseudotime trajectory inference for EC of healthy and UC tissues. The top 3000 high variable genes were used to calculate the pseudotime. The functions "plot_pseudotime_heatmap" and "plot_genes_in_pseudotime" were used to perform time-related gene analysis.

Gene set score analysis

The gene sets were downloaded from MSigDB (https://www.gsea-msigdb.org/gsea) and the 'AddModuleScore' function of Seurat was used to calculate gene set scores.

Patients and tissues

The biopsy samples were collected from 12 heathy control subjects and 15 UC patients from the Department of Gastroenterology, The Third Affiliated Hospital of Zhejiang Chinese Medical University (Hangzhou, China, ZSLL-KY-2023-031-01). The protocol was approved by the Institutional Ethics Committee of The Third Affiliated Hospital of Zhejiang Chinese Medical University and was in concordance with the Helsinki Declaration. Biopsy specimens were collected and washed twice with phosphate-buffered saline, then frozen by liquid nitrogen.

Quantitative real-time polymerase chain reaction (PCR)

Colonic samples were subjected to physical homogenization and total RNA extracted by TRIzol Reagent (Invitrogen). Then cDNA was synthesized using the high-capacity reverse transcription kit (Thermo Fisher) following the manufacturer's instructions. The qPCR reactions were performed using TaqMan gene expression and assays on ABI QuantStudio 5 (Applied Biosystems, Thermo-Fisher).

Western blotting

Tissues were lysed using SDS lysis buffer (containing 100 mmol/L Tris-HCl (pH = 7.0), 1% SDS and 2% 2-mercaptoethanol, supplemented with 1 x protease inhibitors from Roche) and incubated at 105 ℃ for 10 min. Total protein was extracted and quantified using the BCA Kit (Abcam). Equal amounts proteins were subjected to SDS-PAGE electrophoresis and subsequently transferred onto PVDF membranes (Millipore). After blocking with 5% skim milk, the membranes were treated overnight at 4 ℃ with the following antibodies: Mouse monoclonal antibody against GAPDH (1: 2000 dilution; 0411; sc47724); Rabbit monoclonal antibody against *STAT3* (1: 1000 dilution; abcam; ab68153). Rabbit monoclonal antibody against *STAT3* (1: 500 dilution; abcam; ab254788). Following incubation with HRP-conjugated secondary antibodies, the PVDF membrane was visualized using an enhanced chemiluminescence (ECL) kit (Thermo).

Enzyme-linked immunosorbent assay (ELISA)

The protein levels of IL6 (Biolegend) and LHPP (Abbexa) in the blood were determined using ELISA according to the manufacturer's instructions. Following incubation with the detection kit, the plate was analyzed at 450 nm using a chemiluminescence immunoassay system (Dxl800Access, Beckman, United States).

Statistical analysis

All data were statistical analyzed using GraphPad Prism software version 8.0. The data were presented as the mean ±

SEM. Comparisons were conducted using the two-tailed student's *t* test or one-way ANOVA. *P* values < 0.05 were considered statistically significant.

RESULTS

Single-cell transcriptomics identified major cell types in human colon microenvironment

To systematically elucidate the microenvironmental changes occurring during the development of UC, we conducted an analysis utilizing publicly available single-cell datasets from healthy individuals and UC patients. Following quality control (Supplementary Figures 1 and 2), a total of 64643 high quality cells were filtered from healthy (n = 8) and UC (n = 8) individuals, we identified eight major cell types, which were $CD4^+$ T cells (37.44%), $CD8^+$ T cells (4.70%), natural killer T cells (NKT) (15.00%), natural killer cells (7.98%), B cells (13.81%), myeloid immune cells (8.80%), and EC (2.45%) (Figure 1A, Supplementary Figure 1). The accuracy of cell identification was confirmed by the expression of canonical marker genes and top marker gene annotation of each cell type (Figure 1B-D). The distribution between healthy and inflamed tissues indicated that there is no specific cell types generated during the inflammatory process (Figure 1E, Supplementary Figure 2).

Regarding alterations in cellular composition, we observed that during UC development, the proportions of $CD8^+$ T cells and NKT cells were increased (Figure 1F and G). This suggests a pronounced activation response by the host immune system in the face of inflammation, mobilizing these immune cell populations to target and eliminate diseased cells. The heightened presence of $CD8^+$ T cells, renowned for their cytotoxic capabilities, signifies a concerted effort by the immune system to target and eliminate inflamed UC tissues. NKT cells, with their unique properties bridging the innate and adaptive immune responses, may also play a crucial role in anti-inflammatory. This bolstering of cytotoxic immune cells within the colon microenvironment is an encouraging sign, suggesting that the immune system is actively engaged in combating the UC. However, the observed decline in the proportions of $CD4^+$ T cells and B cells is a matter of concern (Figure 1F and G). $CD4^+$ T cells play a vital role in mediating diverse immune responses, including antigen presentation to cytotoxic CD8+ T cells and regulation of immune tolerance. A reduction in their presence could suggest a potential weakening of the immune system's ability to recognize inflammatory cells. Similarly, B cells, which are instrumental in antibody production and antigen presentation, also exhibited diminishing proportions. This decline might impede the cytotoxic ability against UC of $CD8^+$ T and NKT cells. The reduction in $CD4^+$ T cells and B cells might be indicative of an evolving immune evasion strategy employed by UC cells, where the colon microenvironment becomes less conducive to an effective immune response and gains a tendency of the chronic, long-term inflammation.

Concurrently, we observed a progressive increase in the proportion of ECs within the colon microenvironment (Figure 1F and G). This phenomenon underscored the significant role of angiogenesis in the UC's progression. The increasing angiogenesis has been proved a strong link with inflammation in various inflammatory diseases. Angiogenesis and inflammation cooperative with each other, and hypoxia acts as a common stimulus for both[23]. Furthermore, a robust vascular network can facilitate the intravasation and extravasation of inflammation factors, enabling metastasis to distant sites within the body.

These findings offer valuable insights into the alterations taking place within the microenvironment during the progression of UC. The body responds to inflammation through a series of immune cell activities. The augmented angiogenesis underscores the significance of vascularization in the inflammation microenvironment.

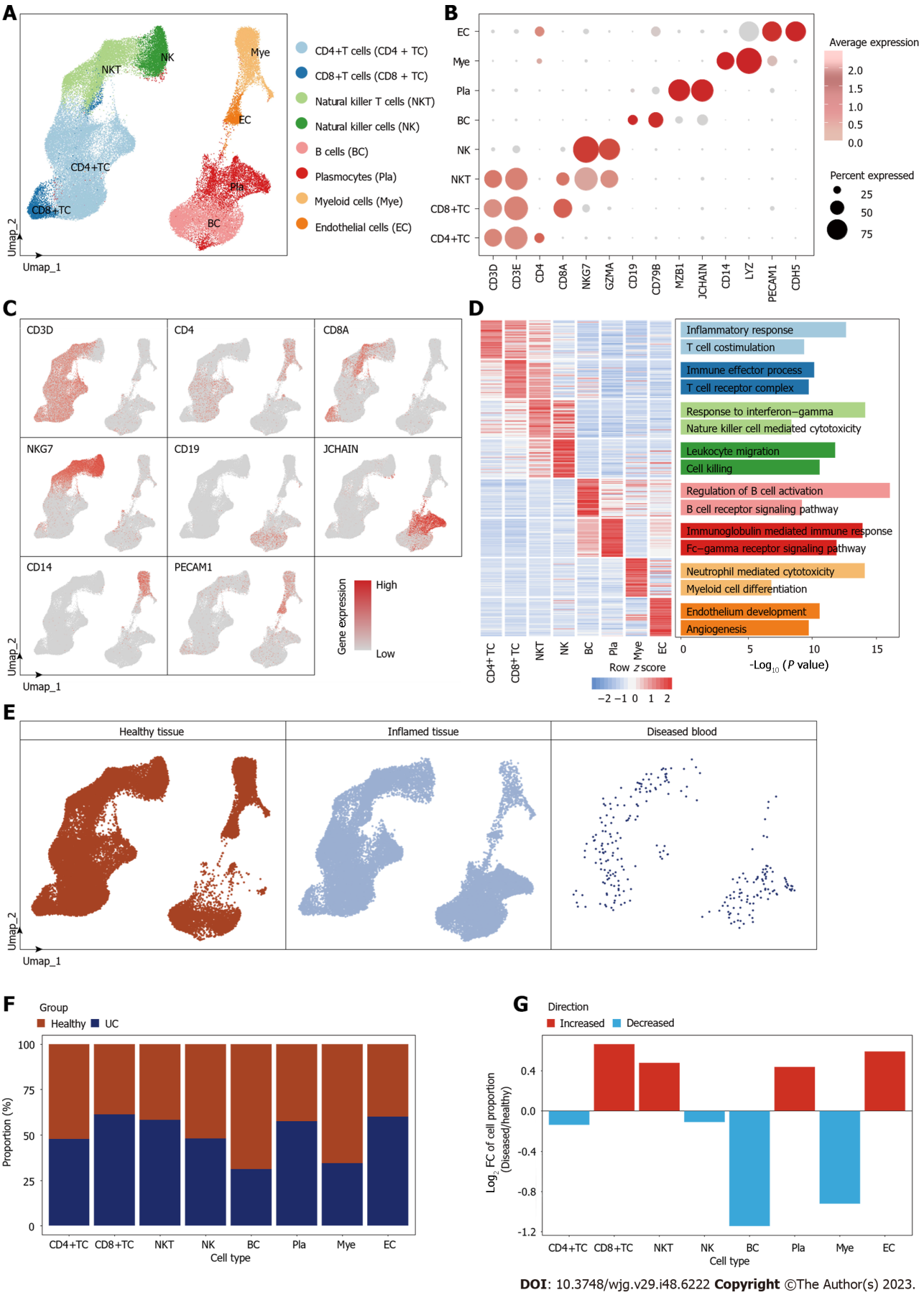
Characterization of cell-type-specific transcriptomic changes in UC progression

We systematically investigated the differential gene expression profiles across all cell types during the process of UC development. Overall, we identified 706 upregulated and 1471 downregulated differential genes. Notably, ECs exhibited the highest number of both upregulated and downregulated DEGs. These trends in differential gene expression to some extent reflect the inflammatory response of these cells during the progression of UC (Figure 2A-D).

Upon examining the upregulated differential genes within each cell type, we identified a substantial association with the regulation of the innate immune response, such as "regulation of innate immune response", "cytokine Signaling in the Immune system", and "neutrophil degranulation". These findings suggested an elevated immune response and immune cell infiltration within the inflamed tissue. During the UC, the body recruited immune cells to the inflamed tissue for the purpose of clearing infections, necrotic cells, or other pathological cells. Notably, the upregulation of the "epithelial to mesenchymal transition" pathway was also observed, hinting at the possibility that sustained inflammation may induce tissue fibrosis. Additionally, the upregulation of the "Angiogenesis" pathway signifies the presence of neovascularization within the inflamed tissue, which aligns with our previous observation of an increased proportion of ECs. These findings underscored the dynamic response of the body to inflammation, involving the recruitment of immune cells to the site of inflammation, concurrent local tissue fibrosis, and angiogenesis.

In the realm of downregulated genes, a notable decrease in the expression of genes associated with various metabolic processes, including "xenobiotic metabolic process", "oxidative phosphorylation", and "fatty acid metabolic process". These findings indicated that inflammation may lead to a functional decline in affected tissues. Moreover, the downregulation of genes related to "chromatin organization", "cell activation" and "intracellular protein transmembrane transport" indicated alterations in cellular states within the inflamed tissue. These observations collectively imply that inflammation induced substantial changes in cellular function and identity.

These findings of differential gene expression profiles across diverse cell types during UC process has unveiled intriguing insights into the dynamic molecular changes occurring within the inflammation microenvironment, corresponding with the high frequency DEGs across all cell types (Figure 2E and F). The activation of inflammation-related



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Figure 1 Cell type identification and cell proportion diversity during ulcerative colitis. A: Umap plot showing different cell types distribution in human clonal tissues; B: Dot plot showing the gene expression levels of the classic marker genes of each cell type; C: Umap plots showing the expression profiles of

indicated cell-type-specific marker genes. The color key from gray to red indicates low to high gene expression levels; D: Heatmap showing the gene expression levels of the top 50 cell-type-specific marker genes for each cell type, with corresponding functional annotations on the right. The color key from blue to red represents low to high gene expression levels; E: Umap plots showing distribution of each group of human clonal tissues; F: Bar plot showing the cell proportion distribution of each cell type in healthy and ulcerative colitis tissues; G: Bar plot showing the fold change level of cell proportion during lesion. CD4+ TC: $CD4^+$ T cells; CD8+ TC: $CD8^+$ T cells; NKT: Natural killer T cells; NK: Natural killer cells; BC: B cells; Pla: Plasmocytes; Mye: Myeloid cells; EC: Endothelial cells.

pathways, immune cell recruitment, tissue fibrosis, and angiogenesis collectively reflect the body's response to inflammation. Concurrently, the downregulation of metabolic pathways and changes in cellular state suggest a functional decline within the affected tissues. These differential gene transcription profiles provide a valuable landscape for assisting in the identification of potential therapeutic targets for UC.

Compromised endothelial mobility during UC progression

Prior research has emphasized the regulatory role of ECs in immune responses, encompassing functions like filtration, endocytosis, antigen presentation, and leukocyte recruitment[24,25]. Aberrant crosstalk between ECs and immune cells were established in injury tissues[26,27]. Furthermore, ECs undergo significant alterations themselves, contributing to inflammation[28]. These findings not only underscore the intricate interplay between ECs and the inflammatory microenvironment but also accentuate the potential of targeting EC-specific mechanisms as a promising avenue for UC therapy.

To gain a deeper understanding of the specific cellular changes that ECs undergo during UC, we conducted pseudotime analysis to elucidate the progressive changes in EC states. Notably, we identified a distinct differentiation trajectory from healthy to UC-associated ECs. Healthy ECs were predominantly situated at the front end of the differentiation trajectory, while UC-associated subpopulations were concentrated toward the middle and end, providing compelling evidence that ECs undergo a series of alterations in their cellular states during UC (Figure 3A and B).

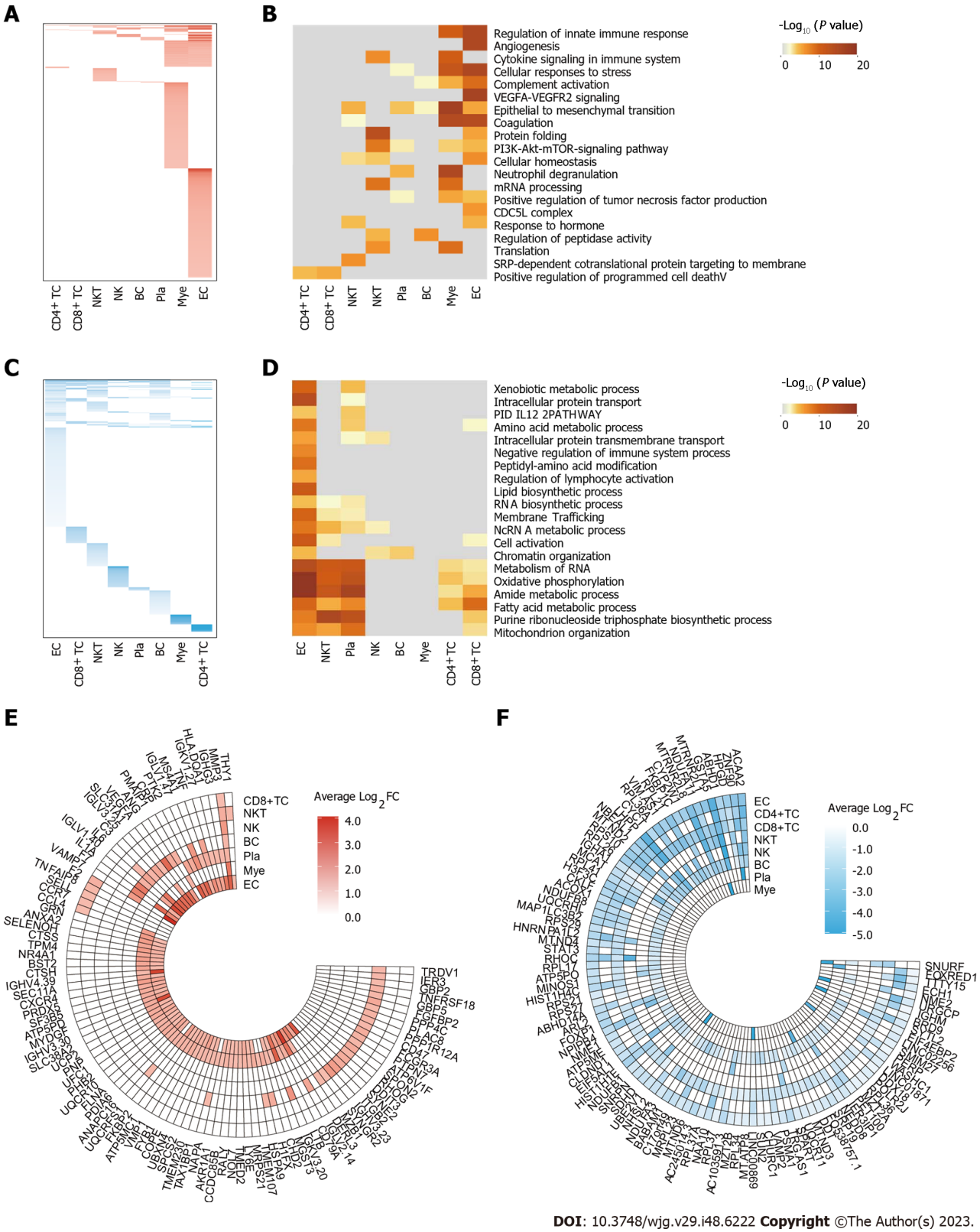
We then focused on the transcriptomic profiles alterations during UC by conducting gene expression patterns that gradually evolved along the pseudotime trajectory. These genes were then categorized into two distinct groups based on their expression trends: the cluster 1 displayed increasing expression as pseudotime advanced, while the cluster 2 exhibited decreasing expression (Figure 3C).

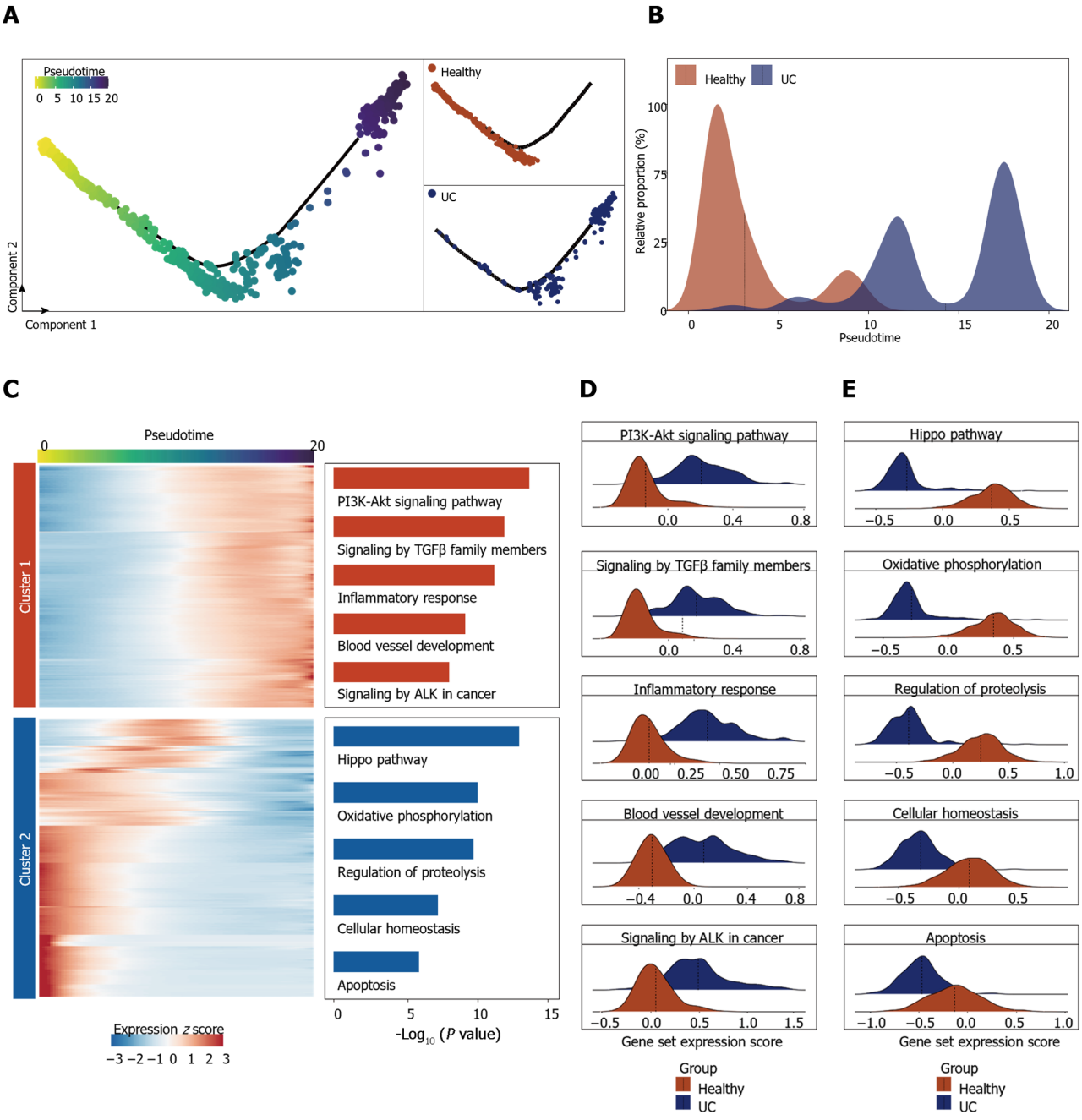
The upregulated genes offer insights into the transcriptional alterations occurring in ECs during the inflammatory process. Notably, they were primarily associated with critical signaling pathways, including the phosphatidylinositol-3-kinase-protein kinase B signaling pathway, known as a key regulator in multiple inflammations. the TGF-beta pathway, known for its role in cellular proliferation and differentiation, as well as tumor genesis. The blood vessel development pathway, indicated ECs' active engagement in inflammation-related angiogenesis processes. Additionally, the anaplastic lymphoma kinase pathway in cancer pathway, suggested that ECs possibly implicating them in a potential predisposition towards carcinogenesis (Figure 3C and D). Intriguingly, these observations underscored the active involvement of ECs in angiogenesis and their responsiveness to various signals driving inflammation. Moreover, it hints at a proclivity of ECs themselves towards malignant transformation. Conversely, the downregulated genes were predominantly linked to pathways such as the Hippo signaling pathway, which plays a pivotal role in regulating cell proliferation and organ size. The apoptosis pathway, responsible for programmed cell death and damaged cell clearance. Furthermore, cellular homeostasis pathways, indicated the disorder of cell state (Figure 3C and E). Collectively, the suppression of these pathways suggests that ECs might undergo functional changes, contributing to their altered roles during inflammation progression.

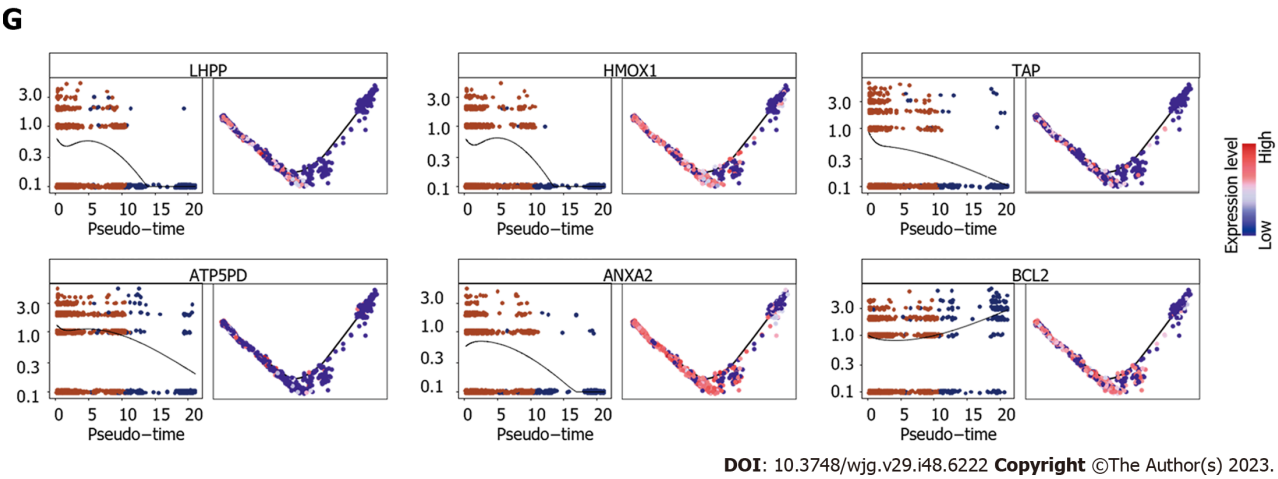
Among genes whose expression exhibited the most significant changes along the pseudotime trajectory, *TGFB3*, a member of the transforming growth factor-beta family, is well-known for its role in regulating cell growth, differentiation, and immune responses. Its upregulation in ECs implies its potential involvement in the activation of signaling pathways critical for inflammation-related angiogenesis and microenvironment remodeling. Similarly, the upregulation of *EGFR* and *FGFR2* suggests the heightened responsiveness of ECs to growth factor signaling, possibly fueling their angiogenic activities and interaction with inflammation microenvironment. The increased expression of *EPHB4*, a receptor involved in cell-cell communication and tissue patterning, hints at its role in EC differentiation and response to inflammation. Moreover, the upregulation of *ILA* and *COL1A2* highlights the active participation of ECs in immune responses and extracellular matrix remodeling within the UC microenvironment (Figure 3F). Conversely, we observed a downregulation of LHPP, a phosphatase implicated in cell cycle regulation, may suggest a reduced control over EC proliferation and differentiation during UC progression. *HMOX1*, a stress-responsive enzyme, exhibited decreased expression, possibly indicating altered oxidative stress responses in ECs within the UC microenvironment. The decreased expression of *ATP5PD* may implicate alterations in energy metabolism within ECs during inflammation. The downregulation of *ANXA2*, a protein involved in cellular processes such as membrane trafficking and cell adhesion, suggests potential changes in cell-cell interactions. The downregulation of *BCL2* may indicate alterations in apoptosis regulation in ECs within the UC pathological process (Figure 3G).

LHPP was identified as a core regulator for UC

To deepen our insight of the correlation among dysregulated genes in the UC transcriptome profile and uncover key regulator into the molecular mechanisms underlying UC pathogenesis, we conducted an integrated analysis of UC by combining data from disease databases and scRNA-seq DEGs. We identified 13 commonly upregulated genes, including *CFB*, *SPINK4*, *FOXP1*, *TIMP1*, *CSF3R*, *STAT3*, *DUOXA2* and *MMP1*, and 7 downregulated genes (*LHPPP*, *CK1*, *CLDN8*, *ABCG2*, *FMO5*, *CKB*, *GUCA2A*) across these datasets (Figure 4A). Notably, apart from pathways associated with inflam-


A
CD4+ TC
CD8+ TC
NKT
NK
BC
Pla
Mye
EC
B
Regulation of innate immune response
Angiogenesis
Cytokine signaling in immune system
Cellular responses to stress
Complement activation
VEGFA-VEGFR2 signaling
Epithelial to mesenchymal transition
Coagulation
Protein folding
PI3K-Akt-mTOR-signaling pathway
Cellular homeostasis
Neutrophil degranulation
mRNA processing
Positive regulation of tumor necrosis factor production
CDC5L complex
Response to hormone
Regulation of peptidase activity
Translation
SRP-dependent cotranslational protein targeting to membrane
Positive regulation of programmed cell deathV
-Log10 (P value)
0
10
20
C
D
Xenobiotic metabolic process
Intracellular protein transport
PID IL12 2PATHWAY
Amino acid metabolic process
Intracellular protein transmembrane transport
Negative regulation of immune system process
Peptidyl-amino acid modification
Regulation of lymphocyte activation
Lipid biosynthetic process
RNA biosynthetic process
Membrane Trafficking
NcRN A metabolic process
Cell activation
Chromatin organization
Metabolism of RNA
Oxidative phosphorylation
Amide metabolic process
Fatty acid metabolic process
Purine ribonucleoside triphosphate biosynthetic process
Mitochondrion organization
E
F
Average Log2FC
4.0
3.0
2.0
1.0
0.0
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
TRDV1
IFI3
GBP2
TNFRSF18
FGFBP2
PPP4C
PPP4C6
CD247
IL12A
CCR3A
SNURF
FOXRED1
TTY15
ECH1
TME2
CRGCP
BCHM
CD9
TTC2
UCHL5IP
ZNF256
ZNF121
ZNF671
ZNF621
ZNF621
ZNF621


A
Pseudotime
0 5 10 15 20
Healthy
UC
Component 2
Component 1
B
Healthy
UC
Relative proportion (%)
100
75
25
0
0 5 10 15 20
Pseudotime
C
Pseudotime
0
20
Cluster 1
Cluster 2
PI3K-Akt signaling pathway
Signaling by TGFβ family members
Inflammatory response
Blood vessel development
Signaling by ALK in cancer
Hippo pathway
Oxidative phosphorylation
Regulation of proteolysis
Cellular homeostasis
Apoptosis
0 5 10 15
-Log10 (P value)
Expression z score
−3 −2 −1 0 1 2 3
D
PI3K-Akt signaling pathway
0.0 0.4 0.8
Signaling by TGFβ family members
0.0 0.4 0.8
Inflammatory response
0.00 0.25 0.50 0.75
Blood vessel development
−0.4 0.0 0.4 0.8
Signaling by ALK in cancer
−0.5 0.0 0.5 1.0 1.5
Gene set expression score
Group
Healthy
UC
E
Hippo pathway
−0.5 0.0 0.5
Oxidative phosphorylation
−0.5 0.0 0.5
Regulation of proteolysis
−0.5 0.0 0.5 1.0
Cellular homeostasis
−0.5 0.0 0.5
Apoptosis
−1.0 −0.5 0.0 0.5 1.0
Gene set expression score
Group
Healthy
UC
F
TGFB3
EGFR
FGFR2
EPHB4
IL1A
COL1A2
10.0
1.0
0.1
0 5 10 15 20
Pseudo−time
Expression level
High
Low



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Figure 3 Cellular and molecular dynamics of endothelial cells during ulcerative colitis progression. A: Pseudotime trajectory analysis of endothelial cell (EC). Left, pseudotime scores of EC. Top right, the distribution of ECs in healthy group. Bottom right, the distribution of ECs in ulcerative colitis group; B: Ridge plot showing the cell number distribution of EC in healthy and ulcerative colitis groups along pseudotime trajectory of Figure 3A; C: Heatmap showing the time-related gene expression profiles during ulcerative colitis, with gene function annotation on the right; D: Ridge plots showing the expression score of gene set from cluster 1 in Figure 3C of healthy and ulcerative colitis groups; E: Ridge plots showing the expression score of gene set from cluster 2 in Figure 3C of healthy and ulcerative colitis groups; F: Scatter plots and trajectory plots showing the expression level of top genes in cluster 1 of Figure 3C; G: Scatter plots and trajectory plots showing the expression level of top genes in cluster 2 of Figure 3C.

mation, we observed the upregulation of pivotal transcription factors such as *STAT3* and *FOXP1*. Correlation analysis of common regulatory factors revealed a negative association between LHPP and the expression of *STAT3* and *FOXP1*. LHPP, known for its role in cell proliferation and tumor suppression, displayed a significant negative correlation with *STAT3* and a less significant correlation with *FOXP1* (Figure 4B and C).

To further substantiate our findings, we recruited a cohort of 15 UC patients and 12 healthy controls, from whom we obtained intestinal tissue samples. Histological assessment (HE staining) of these tissue specimens indicated a significantly elevated presence of chronic inflammation in UC patients ($P < 0.05$; Figure 4D). Subsequent qPCR analysis of these tissue specimens demonstrated elevated mRNA expression levels of inflammation-related genes, including *IL6*, *IL1A*, *CRP*, consistent with our preceding analyses (Figures 2A and C). Notably, in comparison to the healthy control group, we observed a significant reduction in LHPP mRNA expression and an increase in *STAT3* expression within the UC patient group ($P < 0.05$), although the increase in *FOXP1* expression did not reach statistical significances (Figure 4E). Furthermore, western blot analysis of the enrolled individuals provided further confirmation, showing a substantial decrease in LHPP expression and an increase in STAT3 expression among UC patients ($P < 0.05$; Figure 4F and G). These findings suggest a potential transcriptional inhibitory role of STAT3 on LHPP expression.

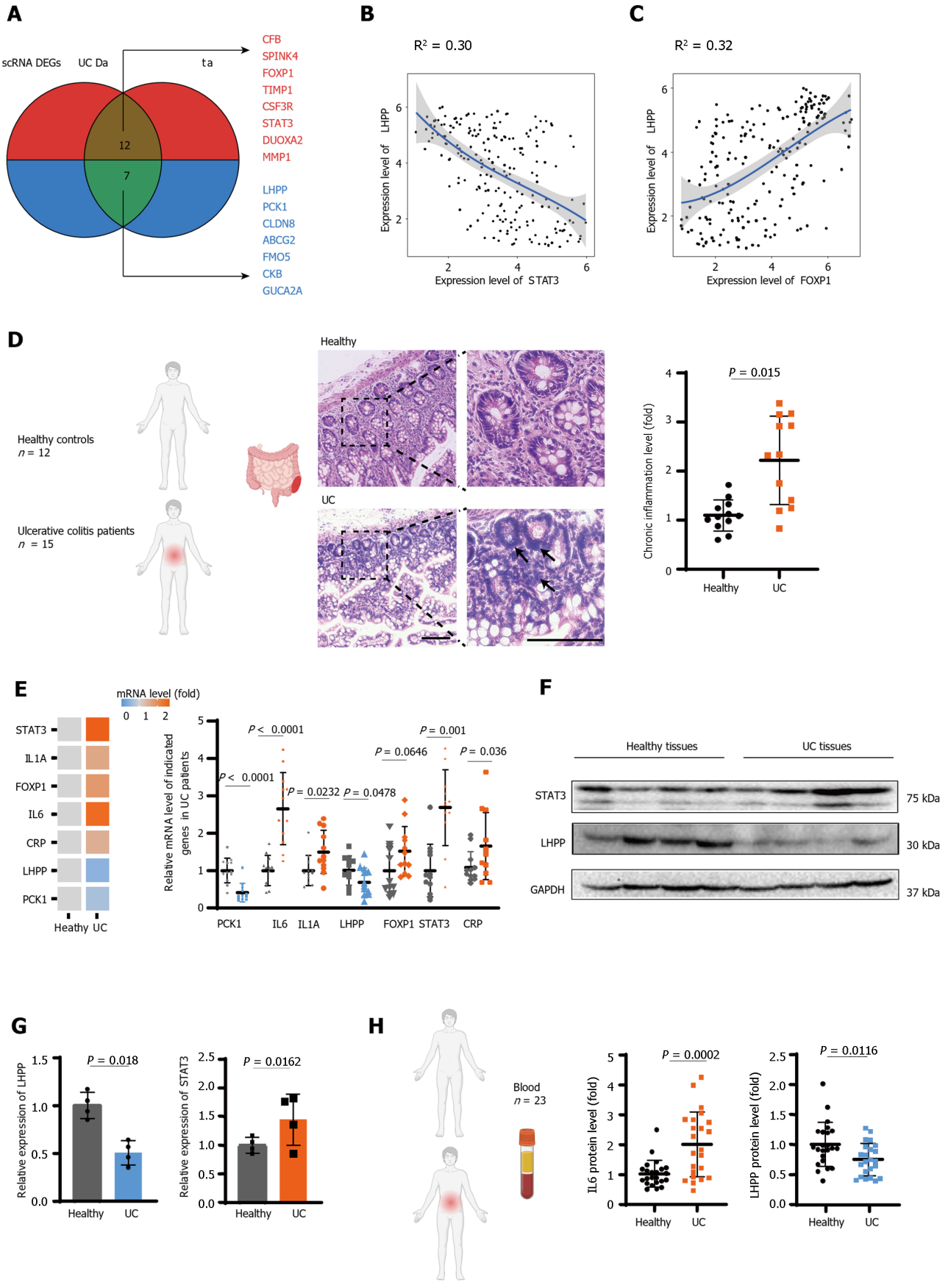
Finally, we conducted ELISA testing on blood samples collected from 23 matched pairs of healthy individuals and UC patients. Our analysis revealed a noteworthy elevation in IL6 levels among UC patients, underscoring the systemic inflammatory effects associated with UC ($P < 0.01$; Figure 4H). Furthermore, the diminished levels of plasma LHPP in UC patients hint at its potential as a predictive biomarker for UC ($P < 0.05$; Figure 4H). These findings emphasize the systemic impact of UC-related inflammation and point towards the promising prospect of utilizing LHPP as a potential predictive marker for this condition.

DISCUSSION

UC is a complex and chronic IBD that predominantly affects the colon and rectum. Despite significant progress in understanding the pathogenesis of UC, there remains a need for more precise and targeted therapeutic interventions. Our study leveraged scRNA-seq technology to delve into the intricate cellular and molecular landscape of UC. We observed a notable upregulation of inflammatory response pathways during UC progression, indicating an active immune response against inflamed tissues. The increased presence of cytotoxic $CD8^+$ T cells and NKT cells suggests an encouraging antitumor response, but the declining proportions of $CD4^+$ T cells and B cells raise concerns about potential immune evasion strategies employed by UC cells.

A particularly intriguing finding in our study was the sustained downregulation of LHPP in UC, coupled with its negative correlation with *STAT3*. Our experimental data supported the notion that *STAT3* may transcriptionally inhibit LHPP expression, both in intestinal tissue and plasma samples of UC patients. LHPP's multifaceted roles in tumor suppression, apoptosis regulation, and immune response modulation make it a promising candidate for further investigation in the context of UC pathogenesis.

Moreover, the heightened angiogenesis observed in UC underscores the importance of vascularization in disease progression. Enhanced vascularization provides UC cells with access to vital nutrients and oxygen, facilitating their survival and proliferation. This finding may open avenues for targeted therapies aimed at disrupting angiogenic



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Figure 4 Phospholysine phosphohistidine inorganic pyrophosphate phosphatase was identified as a core regulator for ulcerative colitis.

A: Venn plot showing the overlap between differential expression genes and public ulcerative colitis data; B: Scatter plot showing the correlation of expression levels between phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP) and signal transducer and activator of transcription 3 (*STAT3*); C: Scatter

plot showing the correlation of expression levels between LHPP and FOXP1; D: HE stained colon biopsy samples from healthy individuals and patients diagnosed with ulcerative colitis (UC). Representative images were captured at × 20 magnification. Black arrows: Increased chronic inflammatory infiltrate; E: The relative mRNA level of *STAT3*, *FOXP1*, *IL6*, *IL1A*, *CRP*, LHPP, and *PCK1* was detected in 12 paired heathy and UC patient tissues; F: Western blot verifying differential STAT3 and LHHP expression in heathy and UC patient tissues; G: The relative expression analysis of STAT3 and LHHP in heathy and UC patient tissues; H: Enzyme-linked immunosorbent assay analysis of IL6 and LHPP levels in serum of heathy and UC patient.

processes in UC.

In summary, our study sheds light on the cellular and molecular mechanisms driving UC pathogenesis. These insights provide a foundation for future research into potential therapeutic interventions, with LHPP emerging as a key player in UC regulation and the immune response. Ultimately, unraveling the complexities of UC at the single-cell level holds promise for more effective treatments and improved outcomes for patients with this challenging condition.

CONCLUSION

This study suggests that LHPP may serve as a potential therapeutic target for UC, emphasizing its importance as a potential key target in UC and unveiling its role in inflammation regulation.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC) is a chronic intestinal condition characterized by inflammation and ulceration, and it is a significant risk factor for colorectal cancer. Recent advances in single-cell RNA sequencing (scRNA-seq) technology offer a promising avenue for dissecting the complex cellular inter-actions and molecular signatures driving UC pathology.

Research motivation

A comprehensive understanding of the cellular and molecular mechanisms underlying UC development and progression remains elusive, and there is a growing need for more precise and targeted therapeutic interventions.

Research objectives

The object of this study is to utilize scRNA-seq technology to dissect the complex cellular interactions and molecular signatures that underlie UC pathology.

Research methods

We integrated and analyzed the scRNA-seq data from UC patients. Moreover, we conducted mRNA and protein level assays as well as pathology-related staining tests on clinical patient samples.

Research results

We identified the sustained upregulation of inflammatory response pathways during UC progression, characterized the features of damaged endothelial cells in colitis. Furthermore, we uncovered the downregulation of phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP) has a negative correlation with signal transducer and activator of transcription 3. Significant downregulation of LHPP in UC patient tissues and plasma suggests that LHPP may serve as a potential therapeutic target for UC. This paper highlights the importance of LHPP as a potential key target in UC and unveils its potential role in inflammation regulation.

Research conclusions

This study suggests that LHPP may serve as a potential therapeutic target for UC, emphasizing its importance as a potential key target in UC and unveiling its role in inflammation regulation.

Research perspectives

This study sheds light on the cellular and molecular mechanisms driving UC pathogenesis. These insights provide a foundation for future research into potential therapeutic interventions, with LHPP emerging as a key player in UC regulation and the immune response. Ultimately, unraveling the complexities of UC at the single-cell level holds promise for more effective treatments and improved outcomes for patients with this challenging condition.

FOOTNOTES

Co-first authors: Yan-Fei Wang and Ruo-Yu He.

Author contributions: Cao YF designed and supervised the study, and drafted the manuscript; Wang YF and He RY took the responsibility for statistical analyses and the manuscript; Xu C and Li XL performed manuscript reviewing and editing; all authors have read and approved the article. The reasons for designating Wang YF and He RY as co-first authors are twofold. Wang YF is responsible for all bioinformatics computations and analyses, including handling data. He RY collects samples and conducts experiments. They contributed efforts of equal substance throughout the research process. Second, this study was a team effort, and the co-first authorship accurately reflects how responsibilities and work were shared during the study and paper completion. This helps with effective communication and managing post-submission tasks, ultimately improving the paper's quality and reliability. Summary, we believe that designating Wang YF and He RY as co-first authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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LETTER TO THE EDITOR

Potential therapeutic targets for nonalcoholic fatty liver disease: Glucagon-like peptide 1

Yue-Hua Yin, Li-Xuan Sang, Bing Chang

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most rapidly growing contributor to liver mortality and morbidity. Hepatocellular injury in nonalcoholic steatohepatitis (NASH) is caused by an increase in metabolic substrates (glucose, fructose, and fatty acids), leading fatty acids to participate in pathways that cause cellular injury and a poor response to injury. The pathogenesis of this disease is largely associated with obesity, type 2 diabetes, and increasing age. To date, there are no Food and Drug Administration-approved treatments for NAFLD/NASH or its associated fibrosis. Since one of the pathogenic drivers of NASH is insulin resistance, therapies approved for the treatment of type 2 diabetes are being evaluated in patients with NASH. Currently, the glucagon-like peptide-1 receptor agonist (GLP-1RA) semaglutide is a safe, well-studied therapeutic for NAFLD/NASH patients. Existing research demonstrates that semaglutide can increase the resolution of NASH but not improve fibrosis. However, improving the fibrosis of NAFLD is the only way to improve the long-term prognosis of NAFLD. Given the complex pathophysiology of NASH, combining therapies with complementary mechanisms may be beneficial. Researchers have conducted trials of semaglutide in combination with antifibrotic drugs. However, the results have not fully met expectations, and it cannot be ruled out that the reason is the short trial time. We should continue to pay increasing attention to GLP-1RAs.

Key Words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Antidiabetic drugs; Glucagon-like peptide 1; Semaglutide

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Core Tip: Semaglutide is effective and safe for nonalcoholic fatty liver disease (NAFLD) but does not improve fibrosis. The treatment of NAFLD requires further combinations of drugs with different and complementary mechanisms of action.

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TO THE EDITOR

We read with great interest the work by Zhu *et al*[1], who further validated that semaglutide can improve the resolution of nonalcoholic steatohepatitis (NASH) but not fibrosis by summarizing the histological results of semaglutide in the treatment of nonalcoholic fatty liver disease[1,2].

To date, there are no Food and Drug Administration-approved treatments for nonalcoholic fatty liver disease (NAFLD)/NASH or its associated fibrosis. Fibrosis or cirrhosis has been recognized in recent guidelines as the main diagnostic and therapeutic target to halt the progression of NASH to end-stage liver disease, change the natural history of the disease, and improve the long-term prognosis of patients with NASH[3,4].

Because insulin resistance is a shared characteristic of type 2 diabetes and obesity and is a key pathogenic driver of NASH[5], pharmacologically, antidiabetic drugs with weight loss effects should be effective against NASH. Therefore, most of the antidiabetic drugs used to treat NASH focus on peroxisome proliferator-activated receptor (PPAR) agonists, glucagon-like peptide-1 receptor agonists (GLP-1RAs), or sodium-glucose transporter 2 (SGLT2) inhibitors[6,7]. Pioglitazone can improve fibrosis in NASH[7]. However, its harmful adverse effects limit its use, and its long-term benefits are not obvious, thus reducing enthusiasm for its use[8,9]. Currently, SGLT-2 is and GLP-1 RAs are gaining more attention in the treatment of NAFLD/NASH metabolic dysfunction-associated fatty liver disease.

GLP-1 RAs may represent the most promising treatment option for improving hepatic steatosis and liver enzyme levels (serum aspartate transaminase, alanine transaminase and gamma-glutamyl transferase) in patients with NAFLD[10]. As a GLP-1 RA, semaglutide appears to be more prominent in the treatment of NASH[11]. Semaglutide activates the hepatic PPAR-α, thereby reducing apolipoprotein C production and breaking down fats and triglycerides in plasma, delaying gastric emptying, prolonging satiety, and reducing waist circumference[3]. Moreover, semaglutide increases insulin production and secretion and decreases glucagon secretion[3]. Current studies have demonstrated that semaglutide is effective in reducing hepatic steatosis and inflammation but not fibrosis[2,5]. The complex pathophysiology of the disease and the multiple often redundant "escape" treatment pathways strongly suggest that combinations of therapies with different but complementary mechanisms of action are considered the best way to improve efficiency, slow disease progression, and even reverse NASH[7].

Alkhouri *et al*[12] conducted a phase 2 clinical trial to validate the potential value of combination therapy[7]. The results of this experiment showed that GLP-1 RA in combination with antifibrotic drug therapy [cilofexor (nonsteroidal FXR agonist)/firsocostat (carboxylase inhibitor)] demonstrates greater improvement in hepatic steatosis, liver biochemistry, and noninvasive fibrosis measured by the magnetic resonance imaging-estimated proton density fat fraction, despite similar weight loss (7%-10%) compared to semaglutide monotherapy[12-15]. The role of GLP-1 RAs is undeniable, and combinations of GLP-1 RAs and other incretin receptor agonists have been developed with good results [16]. Cotadutide, a GLP-1R/GcgR agonist, significantly improves glucose tolerance and decreases C3M plasma levels and P4NP7S circulating levels compared to liraglutide and obeticholic acid in biological experiments[17]. Tirzepatide, a dual agonist of GLP-1 and glucose-dependent insulinotropic polypeptide receptors, has associated therapeutic effects and can induce significant weight loss, improve glycaemic control, and improve plasma lipids in trials related to the treatment of obesity and diabetes[16,18]. In addition, the new drugs lanifibranor (a pan-PPAR agonist) and Aramchol (a partial inhibitor of hepatic stearoyl-CoA desaturase) have performed well as an antifibrotic therapy for NASH, but it is unclear whether there is an unintended effect when combined with GLP-1 RAs[7,19].

Although GLP-RA monotherapy does not perform well as an antifibrotic therapy, it is undeniable that its associated combinations have a role in NASH. In addition, GLP-1RAs have outstanding performance in fat loss, weight loss, and improvement of insulin resistance and have a potential protective effect against the complications of NASH. Thus, we should pay closer attention to GLP-1RAs.

FOOTNOTES

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