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Editorial Board Member of *World Journal of Gastroenterology*, Paola De Nardi, MD, FASCRS, Doctor, Surgeon, Surgical Oncologist, Division of Gastrointestinal Surgery, IRCCS San Raffaele Scientific Institute, via Olgettina 60, Milan 20132, Italy. denardi.paola@hsr.it

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OPINION REVIEW

Approach to medical therapy in perianal Crohn's disease

Abhinav Vasudevan, David H Bruining, Edward V Loftus Jr, William Faubion, Eric C Ehman, Laura Raffals

ORCID number: Abhinav Vasudevan 0000-0001-5026-9014; David H Bruining 0000-0003-4320-8429; Edward V Loftus Jr 0000-0001-7199-6851; William Faubion 0000-0003-1291-5745; Eric C Ehman 0000-0003-4613-7101; Laura Raffals 0000-0002-5267-2592.

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Abhinav Vasudevan, David H Bruining, Edward V Loftus Jr, William Faubion, Laura Raffals, Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN 55905, United States

Eric C Ehman, Department of Radiology, Mayo Clinic, Rochester, MN 55905, United States

Corresponding author: Abhinav Vasudevan, BMed, FRACP, MPH, PhD, Doctor, Division of Gastroenterology and Hepatology, Mayo Clinic, 200 1st St SW, Rochester, MN 55905, United States. vasudevan.abhinav@mayo.edu

Abstract

Perianal Crohn's disease remains a challenging condition to treat and can have a substantial negative impact on quality of life. It often requires combined surgical and medical interventions. Anti-tumor necrosis factor (anti-TNF) therapy, including infliximab and adalimumab, remain preferred medical therapies for perianal Crohn's disease. Infliximab has been shown to be efficacious in improving fistula closure rates in randomized controlled trials. Clinicians can be faced with a number of questions relating to the optimal use of anti-TNF therapy in perianal Crohn's disease. Specific issues include evaluation for the presence of perianal sepsis, the treatment target of therapy, the ideal time to commence treatment, whether additional medical therapy should be used in conjunction with anti-TNF therapy, and the duration of treatment. This article will discuss key studies which can assist clinicians in addressing these matters when they are considering or have already commenced anti-TNF therapy for the treatment of perianal Crohn's disease. It will also discuss current evidence regarding the use of vedolizumab and ustekinumab in patients who are failing to achieve a response to anti-TNF therapy for perianal Crohn's disease. Lastly, new therapies such as local injection of mesenchymal stem cell therapy will be discussed.

Key Words: Fistula; Biologics; Inflammatory bowel disease; Surgery; Stem cells; Infliximab; Ustekinumab

Core Tip: Early commencement of anti-tumor necrosis factor (anti-TNF) therapy in perianal Crohn's disease is preferred over delaying treatment, although perianal sepsis should be treated first. Symptomatic remission remains the treatment goal, with

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radiographic healing an evolving target. Concomitant antibiotic therapy while initiating anti-TNF therapy is efficacious. Therapeutic drug monitoring and dose adjustment of anti-TNF therapy, targeting a higher trough level than what is routinely used for luminal disease, may improve treatment response. Ustekinumab may be efficacious in anti-TNF refractory individuals, although more studies are needed. Mesenchymal stem cell injection can be used in individuals who are refractory to anti-TNF therapy.

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INTRODUCTION

Perianal Crohn's disease is a debilitating complication of Crohn's disease, occurring in up to 30% of individuals with luminal disease[1-3]. It remains a difficult condition to treat, with relapses occurring frequently, and it is associated with a higher rate of hospitalization and surgery compared to individuals with Crohn's disease without perianal disease[4-6]. Perianal Crohn's disease is also associated with a significant burden on quality of life with far reaching consequences on the well-being of an individual, including affecting social relationships and work-related opportunities[7]. Treatment often requires multimodal therapy with surgical treatment and systemic medical therapy used in combination, and this has been shown to be more efficacious than either strategy alone[8,9]. Significant advancements have been made in recent times with surgical and local therapy for perianal disease, with local injections of mesenchymal stem cell therapy showing remarkable promise in randomized studies [10,11]. Despite the advancements in surgical techniques and local therapy in perianal disease and the plethora of medical therapies that are being explored for the treatment of luminal Crohn's disease, systemic medical therapy in perianal Crohn's disease has been dominated by anti-tumor necrosis factor (TNF) therapy, which was shown to be efficacious over two decades ago[12]. Infliximab is the only biologic treatment that has been proven efficacious in a dedicated randomized placebo-controlled trial in individuals with symptomatic perianal Crohn's disease.

While the efficacy of anti-TNF therapy has been established, there is a wide variation in the management of perianal Crohn's disease amongst treating clinicians [13]. This may partially relate to the fact that there are a number of situations that the treating clinician may encounter that are not addressed by randomized studies and where evidence is either insufficient or evolving. These include the timing and duration of anti-TNF therapy, the need for concomitant or increased dose of therapy and what alternative biologics can be used in an individual who is failing anti-TNF therapy. While conclusive data may not be available in many of these situations, there are studies that have been conducted that may provide further insight into decision making in these situations. The aim of this paper, therefore, is to address these potential questions that may assist treating clinicians who are considering medical therapy or have commenced anti-TNF therapy for the treatment of perianal Crohn's disease.

WHAT MODALITIES SHOULD I USE TO EVALUATE PERIANAL CROHN'S DISEASE PRIOR TO COMMENCING ANTI-TNF THERAPY?

An important component of medical therapy when treating perianal Crohn's disease is to control underlying infection prior to commencing immunosuppressive therapy. There are several modalities that can be utilized to evaluate for the presence of a perianal abscess or deep infection, including examination under anesthesia (EUA), magnetic resonance imaging (MRI) pelvis and transrectal ultrasound (TRUS). All modalities have been shown to correctly classify Crohn's perianal fistulae in over 85% of patients and when two modalities are used, the accuracy approaches 100%[14].

Transperineal ultrasound, which is a non-invasive and relatively cheaper modality of imaging, has also been shown to have similar accuracy to TRUS and MRI pelvis in diagnosing perianal fistulae[15]. If available, pelvic MRI provides the most comprehensive evaluation of the perianal region (Figure 1). Small field of view images accurately depict even the most complex fistulous tracts as well as the size, number and location of an abscess cavities including disease extending above the levator ani musculature. Associated features, such as rectal inflammation and lymphadenopathy, can also be seen. Use of one or more of these forms of diagnostic imaging or an EUA should be performed prior to commencing medical therapy with an anti-TNF for the treatment of perianal Crohn's disease.

WHEN IS THE BEST TIME TO COMMENCE ANTI-TNF THERAPY IN PERIANAL CROHN'S DISEASE?

A retrospective claims-based study evaluated the role of early use of immunosuppressive therapies in reducing the risk of developing future perianal disease amongst indi-viduals with a new diagnosis of Crohn's disease. In this study, individuals with a new diagnosis of Crohn's disease without perianal disease exposed to at least 90 days of immunosuppressants or anti-TNF therapy had a 59% reduction in risk, compared to those not using these agents, of developing symptomatic perianal disease over 2 years [16]. While these results require further validation, this study raises the possibility that individuals who are identified as having perianal disease may benefit from earlier medical therapy with immunosuppressive or anti-TNF therapy even if they are minimally symptomatic.

In contrast to these findings, data would suggest that there is often a significant delay in the commencement of biologic therapy in patients with perianal Crohn's disease, with a median of over 6 months between the initial diagnosis of perianal Crohn's disease and the commencement of anti-TNF therapy[17]. The concern for worsening perianal infection may partially explain this delay of immunosuppressive treatment. A retrospective study compared fistula recurrence rates amongst 76 individuals with perianal Crohn's disease who were either started on infliximab late (median, 250 days) or early (median, 12 days) after seton insertion for perianal Crohn's disease[18]. Recurrence rates were low in both groups of this study (8% overall), and no difference was found between early and late commencement of infliximab and no reports of abscesses or perianal sepsis in either group.

A multicenter randomized study assigned patients with newly diagnosed and recurrent perianal Crohn's disease with a seton inserted and fistula drainage to one of three groups: chronic fistula drainage for 12 months, anti-TNF therapy (adalimumab or infliximab) for 12 months with removal of seton at 6 weeks, or initial anti-TNF therapy followed by surgical closure of the fistula within 8 weeks to 12 weeks[19]. The study was initially designed to show superiority of seton insertion in reducing future surgical procedures, but was stopped early due to futility, as the chronic seton group showed a significantly higher rate of fistula re-intervention compared to both the anti-TNF therapy and the surgical closure after anti-TNF therapy groups (74% *vs* 42% *vs* 23%, respectively, P = 0.02). Serious adverse events were numerically higher in the anti-TNF group than the chronic seton group (36% *vs* 21%), but the nature of the events and the rate of infection are not described. Overall, these would suggest that early anti-TNF therapy following seton insertion can be utilized for perianal Crohn's disease.

WHAT SHOULD BE THE TARGET OF TREATMENT?

There has been work to establish a consensus on the most appropriate treatment targets in inflammatory bowel disease, given the variability in treatment outcomes that has been reported in clinical trials, and the fact that treatment goals have evolved and become more stringent with the availability of an increasing number of efficacious therapies. A core outcome set in perianal disease was developed by consensus collaboration between key stakeholders in the United Kingdom following a systematic review of the literature and patient interview by a three-round Delphi process[20]. This identified 3 patient-reported outcomes (incontinence, quality of life and patient priorities) and five clinical-reported outcomes of perianal disease (activity, new abscesses or sepsis, new or recurrent fistula, unplanned surgery and fecal diversion).

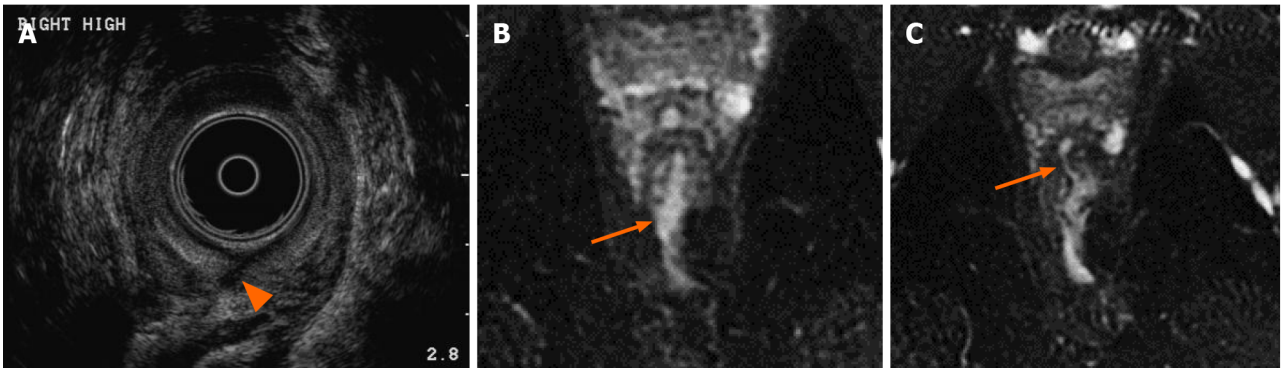

Figure 1 A 37-year-old-man with history of Crohn's disease. A: Transrectal ultrasound identifies a defect in the internal anal sphincter posteriorly (arrowhead); B: Axial T2 weighted, fat saturated image of the anal canal obtained 6 d later more clearly show a transsphincteric perianal fistula arising at 6 o'clock with a fluid filled sinus tract extending posteriorly to exit at the skin surface; C: Magnetic resonance imaging (MRI) image also demonstrates an additional transsphincteric fistula which arises at the 1 o'clock position extending anteriorly and communicating with a complex branching tract which also exited to the skin surface (not shown). MRI provides comprehensive multiplanar imaging of fistulizing disease, allowing visualization of the full extent of disease including supralevator disease and more complete classification of branching or complex fistulas. Additionally, post contrast sequences allow differentiation between fluid containing tracts from granulation tissue seen following healing.

Fistula response on MRI pelvis during follow-up was considered an optional outcome, partially related to issues with the cost and accessibility of MRI. This contrasts with the STRIDE guidelines for luminal inflammatory bowel disease, where endoscopic remission is the agreed target of medical therapy in both Crohn's disease and ulcerative colitis[21,22]. MRI provides a potential advantage over clinical evaluation for tract closure, as it has been shown that internal fistula tracts can persist despite closure of the external opening[23].

Studies evaluating MRI healing and clinical symptoms suggest that MRI healing can lag significantly behind clinical remission, with radiographic findings persisting for up to 12 months after clinical remission[24]. Additionally, many studies of long-term outcomes following treatment with infliximab based on MRI findings have not identified any MRI parameters that accurately distinguish individuals with clinical features of active disease compared to those who achieved clinical remission[25-27]. One observational study suggested that the disappearance of T2 hyperenhancement and absence of enhancement strongly correlated with clinical remission in patients who had an MRI at 12 months after treatment[28]. Many of these studies have used 1.5T MRI machines, which may provide a lower quality image than more modern machines, and there is a lack of study into newer machines, such as 3T machines and evaluation of MRI parameters. Finally, interpretation of perianal protocol MRI is experience-dependent, and outside large centers, there may not be familiarity with the correct imaging protocol and interpretation of these examinations.

Overall, this would suggest that clinical remission remains the main target in treatment of anti-TNF therapy in perianal disease; however, assessment with an MRI pelvis at baseline and during follow-up may provide further information to help guide treatment.

WHAT CAN BE DONE TO OPTIMIZE RESPONSE WHILE ON ANTI-TNF THERAPY FOR PERIANAL CROHN'S DISEASE?

Concomitant medications

Antibiotics: Three trials have evaluated the use of antibiotic therapy, primarily with ciprofloxacin, in combination with anti-TNF treatment for perianal Crohn's disease. All have shown a trend toward improved fistula response rates at 18 weeks and 24 weeks, although none of these results were statistically significant by the end of the study period[29-31]. One of these trials comparing adalimumab with ciprofloxacin to adalimumab with placebo showed an initial significant difference at week 12, with 65% achieving closure of all perianal fistulas on adalimumab with ciprofloxacin compared to 33% on adalimumab with placebo, but this difference was not sustained by the end of follow-up, despite remaining 15% higher in the adalimumab with ciprofloxacin group (62% *vs* 47%, respectively). These studies may have been underpowered to show a significant difference between the groups, and a meta-

analysis suggested that the induction of fistula response (relative risk, 1.58) and remission (relative risk, 1.94) is significantly superior with antibiotics in combination with infliximab compared to anti-TNF therapy alone[32]. The use of antibiotics in this setting, therefore, has been advised by experts, particularly during initial treatment of perianal disease with anti-TNF therapy[33,34].

Immunomodulator therapy: There have not been any dedicated randomized trials specifically comparing use of anti-TNF therapy in combination with immunomodulator therapy to anti-TNF monotherapy. A secondary analysis of two of the placebo-controlled randomized studies comparing infliximab and placebo for perianal Crohn's disease compared anti-TNF therapy in combination with immunomodulators and anti-TNF monotherapy, and did not show a difference in fistula outcomes with induction or maintenance treatment[12,35]. Similar findings were also noted in observational studies comparing combination therapy to infliximab monotherapy[36,37]. It should be noted, however, that patients in these studies had previously failed to achieve a response with immunomodulator therapy prior to initiation of anti-TNF therapy, and some observational studies have shown a significantly higher rate of fistula closure amongst patients on combination therapy compared to anti-TNF monotherapy[38]. None of the aforementioned studies evaluated the use of anti-TNF therapeutic drug monitoring, so it is not clear whether any benefit is related to the effect of the immunomodulator on anti-TNF levels or independent of this. Given the known effects of immunomodulators of reducing immunogenicity and therefore increasing levels of infliximab, and the fact that higher anti-TNF drug levels have been associated with greater response in perianal Crohn's disease (see the below section on 'Therapeutic drug monitoring'), serious consideration should be given to commencing an immunomodulator with anti-TNF therapy, particularly if proactive therapeutic drug monitoring is not being utilized.

Therapeutic drug monitoring

Multiple retrospective and observational studies have identified an association between higher infliximab drug levels and a greater rate of fistula response, healing and closure[39-43]. While the cut-off infliximab level has varied between studies, possibly related to the assay used and end points assessed, it appears that the target level is higher than what is conventionally used to control luminal Crohn's disease (Table 1). Davidov *et al*[44] performed infliximab levels during induction therapy for patients with perianal Crohn's disease and found that those who had a clinical response to treatment by week 14 had significantly higher median infliximab drug levels beginning at week 2 (20 μg/mL *vs* 5.6 μg/mL), and these remained higher than non-responders at weeks 6 (13.3 μg/mL *vs* 2.55 μg/mL) and 14 (4.1 μg/mL *vs* 0.14 μg/mL). Yarur *et al*[39] similarly found significantly higher trough infliximab levels in a cross-sectional study of individuals with perianal Crohn's disease on long-term infliximab who achieved fistula healing compared to those who did not achieve healing (median, 15.8 mcg/mL *vs* 4.4 mcg/mL, $P < 0.001$). This study also found that the odds of achieving fistula healing were over 8 times greater in individuals who underwent dose escalation of infliximab. Intuitively this would suggest that monitoring anti-TNF drug levels proactively and performing dose adjustments in individuals with lower levels may improve fistula healing rates, although this has not been prospectively evaluated.

Duration of anti-TNF therapy and discontinuation

De-escalation of medical therapy in inflammatory bowel disease involves the deliberate reduction in dose or cessation of a treatment once the treatment target has been achieved. A key component of de-escalation of therapy is establishing a treatment target that has good correlation with better long-term outcomes. In luminal Crohn's disease, it has been established that endoscopic remission correlates better with the long-term clinical course of inflammatory bowel disease than symptomatic remission [45]. However, given that treatment of perianal disease remains primarily targeted at symptom-based remission, de-escalation of therapy may be more difficult to complete successfully.

The ACCENT II study evaluated the need for ongoing infliximab therapy in patients with perianal Crohn's disease by comparing the proportion of individuals who lost response to maintenance therapy with either placebo or ongoing infliximab in all individuals who achieved a clinical response to infliximab induction therapy[35]. After 52 wk, 42% of patients had lost response in the infliximab maintenance group compared to 62% in the placebo group ($P < 0.001$). Other retrospective studies have also found that over half of patients who cease anti-TNF therapy will have a clinical

Table 1 Table of trials evaluating ideal cut off value for infliximab and adalimumab concentrations

Ref.	Population (adults or children)	Number	Study design	Follow up or time on therapy	Outcomes	Median level	Median level
Trial: Infliximab							
Plevris *et al* [43]	Adult	29	Retrospective single center cross sectional	2.6 yr	Fistula healing; Fistula closure	8.1; 8.2	3.2; 3.2
Strik *et al*[40]	Adult	47	Retrospective single centercross sectional	3.5 yr	Fistula closure	6.0	2.3
Davidov *et al* [44]	Adult	36	Retrospective observational two centers	Week 2; Week 6; Week 14	Decrease or cessation of fistula drainage at week 14	20; 13.3; 4.1	5.6; 2.55; 0.14
Zhu *et al*[69]	Adult	157	Retrospective single center	Week 30; Week 78; Week 116	Radiological remission (absence of high-signal tracks on fat-saturated T2-weighted sequences)	3.5; 2.85; 2.84	1.9; 1.63; 0.7
El-Matary *et al*[42]	Pediatric	85	Prospective observational 12 centers	Week 14	Clinical fistula healing at week 24	12.7	5.4
Yarur *et al* [39]	Adult	117	Retrospective, cross sectional, 2 centers	29 wk	Fistula healing	15.8	4.4
Trial: Adalimumab							
Plevris *et al* [43]	Adult	35	Retrospective single center cross sectional	1.7 yr	Fistula healing; Fistula closure	14.8; 12.6	5.7; 2.7
Strik *et al*[40]	Adult	19	Retrospective single center cross sectional	3.5 yr	Fistula closure	7.4	4.8
Ruemmele *et al*[41]	Pediatric	36	Randomized control trial	Week 16; Week 52	Clinical fistula closure	7.4; 7.5	6.4; 5.6

relapse with long-term follow-up, so stopping treatment either due to clinical remission or for other reasons is associated with a high risk of relapse[46-48].

Radiological healing is a more stringent treatment target in perianal Crohn's disease, and whether anti-TNF therapy can be stopped after this end point is achieved has not been well studied. A prospective observational study identified 12 patients who were treated with infliximab alone or in combination with a thiopurine who achieved radiological healing[24]. Amongst this group, 5 remained on infliximab therapy and 7 stopped treatments either due to intolerance, treatment de-escalation or switched therapies. There was a trend toward greater rates of radiological healing amongst individuals who remained on infliximab as all maintained radiological remission, while 3/7 in the group that stopped infliximab maintained radiological remission (P = 0.08). Another observational study found that 6/9 (67%) of patients who had achieved radiological healing and stopped maintenance anti-TNF therapy had fistula recurrence[49]. This data would support the use of ongoing anti-TNF therapy in patients who achieve clinical remission of perianal fistulae.

WHAT OTHER THERAPIES CAN BE USED FOLLOWING ANTI-TNF FAILURE?

Vedolizumab

No dedicated randomized controlled trials have assessed the efficacy of vedolizumab for perianal Crohn's disease, and clinical data have shown limited benefit. A post hoc analysis of the GEMINI 2 study compared the efficacy of maintenance vedolizumab to placebo in achieving fistula closure at weeks 14 and 52 following therapy with vedolizumab amongst patients with at least 1 externally draining fistula related to Crohn's disease[50]. Over 40% of patients in each group had previously been treated with anti-TNF therapy. The study identified a numerical difference in fistula closure rates in favor of vedolizumab at week 14 (28% *vs* 11%) and 52 (31% *vs* 11%), although these did not reach statistical significance, and no difference was noted between maintenance therapy given every 4 wk or 8 wk. The ENTERPRISE study was a

randomized trial comparing two intravenous regimens of vedolizumab for treatment of perianal Crohn's disease, with 78% of patients having previously failed anti-TNF therapy[51]. Patients either received standard induction vedolizumab of 300 mg at weeks 0, 2 and 6 then at weeks 14 and 22, while the other group received the same regimen plus a week 10 dose. There was no placebo group in the study and 92% of patients had a seton inserted at baseline. They found that by week 30, 54% of the group had a clinical response to treatment, while 43% had complete closure of fistulae and there was no significant difference between the groups. A nationwide cohort study of 151 patients with perianal Crohn's disease (99% of patients having previously been treated with at least one anti-TNF) who were treated with vedolizumab showed that 23% of patients achieved clinical success (no draining fistula at clinical examination) after 6 mo of treatment, while 67% of patients stopped vedolizumab by 30 wk of treatment due to uncontrolled perianal or luminal disease[52]. Additionally, 31% of patients with perianal disease who had no clinical symptoms at the time of initiation of vedolizumab developed symptoms after commencing therapy. Concomitant immunomodulator therapy was used in 44% of patients in this cohort and this was not a predictor of the success of therapy. Based on these data it appears that there is insufficient evidence to support the widespread use of vedolizumab following anti-TNF failure in perianal Crohn's disease, and further dedicated studies are needed.

Ustekinumab

Available data have provided some support for the use of ustekinumab for perianal disease amongst patients who have trialed or failed anti-TNF therapy. A post hoc analysis of the combined results on fistula healing from the large placebo-controlled trials for ustekinumab in Crohn's disease found that there was a trend toward higher rates of resolution of fistula symptoms by week 8 of ustekinumab therapy compared to placebo (25% *vs* 14%) which had increased to 80% *vs* 46% by week 44[53]. Numbers in the latter analysis were small, with just 26 patients in both groups, and the results were not statistically significant. These trials included a combination of anti-TNF-naïve and anti-TNF-experienced patients, and the results do not distinguish between the groups. A Dutch Nationwide study in which 99% of patients had prior exposure to at least one anti-TNF therapy found that 36% of individuals with perianal fistula at initiation of ustekinumab achieved complete clinical resolution by 24 wk[54]. Further retrospective studies into the use of ustekinumab for perianal Crohn's disease were combined with the aforementioned results in a meta-analysis which found a 56% response rate and 17% remission rate after 52 wk of therapy[55]. Moderate heterogeneity was noted. It appeared that the efficacy of ustekinumab increased between week 8 and week 52. For individuals who are not responding to initial ustekinumab, the use of dose intensification of therapy can result in a clinical response in perianal disease, with one observational study noting 12/24 patients (50%) when escalated to four or six weekly therapy[56]. The limited data available on the use of ustekinumab levels in perianal Crohn's disease do not show an association between serum levels during induction nor maintenance therapy and fistula response at 44 wk[57]. A major limitation of these observational studies is the lack of a comparator being utilized, so while these results suggest ustekinumab may be beneficial in individuals who do not respond to anti-TNF therapy, the size of this effect is not known.

Mesenchymal stem cell therapy

While the exact mechanism that leads to the efficacy of mesenchymal stem cells in the treatment of perianal Crohn's disease is not known, it likely relates to their ability control the local inflammatory response and allow for fistula healing[58]. A placebo-controlled randomized trial found a single dose (120 million cells) of intralesional injection of adipose derived allogeneic cells to the fistula tract in patients with perianal Crohn's disease who were refractory to medical therapy (79% treated with anti-TNF therapy in past 6 mo) achieved combined clinical and radiographic remission in 56% of patients compared to 39% in the placebo group after 12 mo[10,59]. Further studies evaluating the use of both autologous adipose-derived mesenchymal stem cells and bone marrow-derived mesenchymal stem cells have also shown efficacy of these therapies[11,60-62]. While questions remain regarding the optimal dosing and treatment protocol to use, the results suggest treatment to be efficacious and safe even amongst individuals who are not responding to anti-TNF therapy, so this will likely remain an important therapeutic option to consider when it widely available.

Temporary fecal diversion

Reports of the efficacy of a temporary diverting stoma in the pre-biologic era

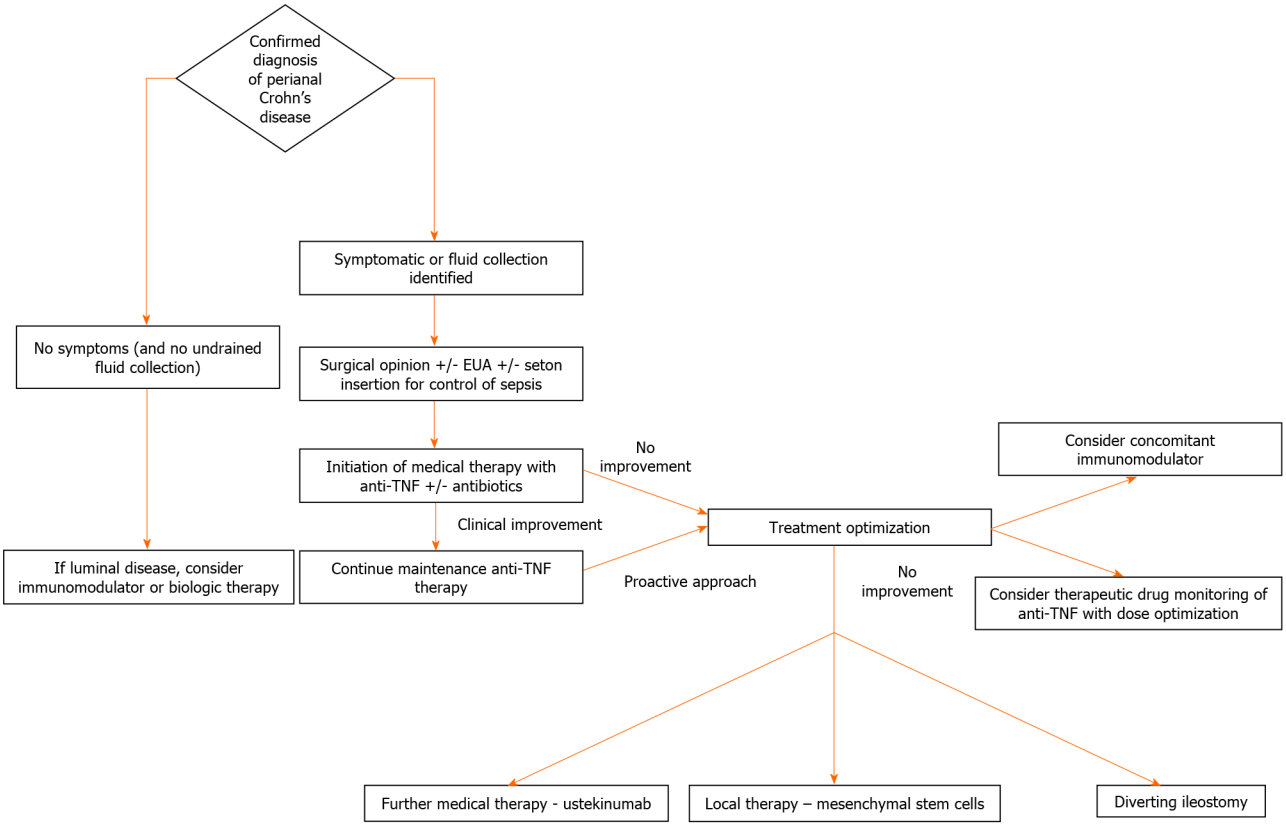

Figure 2 A suggested approach to treatment in perianal Crohn's disease. Anti-TNF: Anti-tumor necrosis factor; EUA: Examination under anesthesia.

suggested it was highly efficacious, with response rates reported to occur in over 80% of individuals in the short term[63,64] and a meta-analysis found response rates in 3 mo to 6 mo in 64% of individuals[65]. However, reports of the use of defunctioning stoma in individuals after failing biologic therapy has shown a lower response rate, with cohort studies suggesting response rates of 46%[66,67]. Rates of successful restoration of bowel continuity also remain low at 18%, and the use of biologic therapy following diverting therapy does not appear to improve the rate of stomal reversal[65,68]. This suggests that while defunctioning surgery remains an option following failure of anti-TNF therapy, the chances of restoration of bowel continuity remain low.

CONCLUSION

Anti-TNF therapy remains the most established therapy for the treatment of perianal Crohn's disease. Emerging evidence would support its early use in treatment, in combination with surgical intervention, to try to minimize complications from perianal disease (Figure 2).

Antibiotics should be considered during induction anti-TNF therapy, but their benefits beyond 24 weeks are not known. While on anti-TNF therapy the use of therapeutic drug monitoring and optimization of anti-TNF levels may improve fistula response rates, but it is likely that higher anti-TNF drug levels are required than are used for the treatment of luminal Crohn's disease. The use of combination therapy with an immunomodulator may be beneficial, particularly in individuals who have not previously failed immunomodulator therapy, although the additional benefit of immunomodulator therapy beyond improving immunogenicity of anti-TNF therapy is not known.

In individuals who fail to respond to anti-TNF therapy for perianal Crohn's disease, ustekinumab may allow for healing of fistula and dose interval shortening of therapy may be of benefit in individuals who are not responding to 8-weekly therapy.

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REVIEW

Incorporating mucosal-associated invariant T cells into the pathogenesis of chronic liver disease

Albert J Czaja

ORCID number: Albert J Czaja 0000-0002-5024-3065.

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Albert J Czaja, Department of Medicine, Mayo Clinic College of Medicine and Science, Rochester, MN 55905, United States

Corresponding author: Albert J Czaja, FAASLD, AGAF, FACG, FACP, MD, Emeritus Professor, Department of Medicine, Mayo Clinic College of Medicine and Science, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, United States. czaja.albert@mayo.edu

Abstract

Mucosal-associated invariant T (MAIT) cells have been described in liver and non-liver diseases, and they have been ascribed antimicrobial, immune regulatory, protective, and pathogenic roles. The goals of this review are to describe their biological properties, indicate their involvement in chronic liver disease, and encourage investigations that clarify their actions and therapeutic implications. English abstracts were identified in PubMed by multiple search terms, and bibliographies were developed. MAIT cells are activated by restricted non-peptides of limited diversity and by multiple inflammatory cytokines. Diverse pro-inflammatory, anti-inflammatory, and immune regulatory cytokines are released; infected cells are eliminated; and memory cells emerge. Circulating MAIT cells are hyper-activated, immune exhausted, dysfunctional, and depleted in chronic liver disease. This phenotype lacks disease-specificity, and it does not predict the biological effects. MAIT cells have presumed protective actions in chronic viral hepatitis, alcoholic hepatitis, non-alcoholic fatty liver disease, primary sclerosing cholangitis, and decompensated cirrhosis. They have pathogenic and pro-fibrotic actions in autoimmune hepatitis and mixed actions in primary biliary cholangitis. Local factors in the hepatic microenvironment (cytokines, bile acids, gut-derived bacterial antigens, and metabolic by-products) may modulate their response in individual diseases. Investigational manipulations of function are warranted to establish an association with disease severity and outcome. In conclusion, MAIT cells constitute a disease-nonspecific, immune response to chronic liver inflammation and infection. Their pathological role has been deduced from their deficiencies during active liver disease, and future investigations must clarify this role, link it to outcome, and explore therapeutic interventions.

Key Words: Innate-like lymphocytes; Antimicrobial; Immune regulatory; Pathogenic; Mucosal-associated invariant T cell

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Core Tip: Circulating mucosal-associated invariant T cells are depleted in chronic liver disease, and they have a disease-nonspecific, hyper-activated, immune exhausted, and dysfunctional phenotype. Antimicrobial, immune regulatory, pro-inflammatory, and anti-inflammatory actions are established biological functions of these innate-like lymphocytes, and each function has been invoked to understand the pathogenesis of chronic hepatitis and cholestatic liver disease. Future investigations must establish their pathological role in each form of chronic liver disease, determine the factors that direct function in the hepatic microenvironment, associate deficient functionality with disease severity and outcome, and explore therapeutic manipulations.

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INTRODUCTION

Mucosal-associated invariant T (MAIT) cells are a subset of lymphocytes that engage in innate and adaptive immune responses[1]. MAIT cells react rapidly to pathogens. They can be activated by cytokine stimulation in an antigen-independent manner, and they can eliminate infected or altered cells by releasing pro-apoptotic granzyme B and perforin[2-4]. These features of an innate immune response are complemented by features of an adaptive immune response[1,5]. MAIT cells express a semi-invariant T cell antigen receptor (TCR) that is specific for a limited, but enlarging, array of antigens[6-10]. Furthermore, they can rapidly develop as effector memory cells[11,12].

MAIT cells can recognize riboflavin metabolites or other non-peptide antigens. These antigens are presented by the major histocompatibility complex (MHC) class I-related protein, MR1, which is expressed on antigen-presenting cells (APCs)[13-16]. Antigen-dependent MAIT cell activation can result in chemokine-directed tissue infiltration and release of diverse pro- and anti-inflammatory cytokines[11,12,15,17]. The complex functional phenotype of MAIT cells justifies their inclusion in the family of innate-like lymphocytes[1]. This family includes gamma delta cells (γδ cells)[18-20], natural killer T cells (NKT cells)[21-25], and innate-like B cells (B1 cells, marginal zone B cells, and regulatory B cells)[26-29].

MAIT cells can enhance the adaptive immune response to microbial antigens[2,3]. Bacteria and fungi engaged in riboflavin biosynthesis can trigger the release of pro-inflammatory cytokines and pro-apoptotic molecules that eliminate infected cells[13,14]. Furthermore, the engagement of highly selected microbial antigens with the semi-invariant TCR of the MAIT cells can generate a cytotoxic $CD8^+$ T cell response[2,30]. MAIT cell activation has been ascribed a protective antimicrobial role in several bacterial (*Salmonella typhimurim*, *Mycobacterium tuberculosis*, *Escherichia coli*, and *Klebsiella pneumoniae*)[2,3,31] and viral [dengue virus, influenza virus, and hepatitis C virus (HCV)] infections[32-34].

MAIT cells have now been assessed in chronic hepatitis B[35-39], chronic hepatitis C [40-44], chronic hepatitis D[45], alcoholic liver disease (ALD)[46-48], non-alcoholic fatty liver disease (NAFLD)[49-51], autoimmune hepatitis[52,53], primary biliary cholangitis (PBC)[54,55], primary sclerosing cholangitis (PSC)[56,57], and decompensated cirrhosis[58]. The pathological role of MAIT cells in these diverse forms of chronic liver disease remains unclear. They may be disease-nonspecific perpetrators or facilitators of inflammatory activity[59-62]. MAIT cells may stimulate hepatic fibrosis [52,63] and protect against microbial infection in decompensated cirrhosis[58]. They may modulate the cellular immune response[17,64-66] or limit the immune stimulatory effects of bacterial antigens from the intestinal microbiome[46,67-70].

MAIT cells are emerging as pivotal mediators of chronic liver disease[61,62], and clarification of their pathological role may lead to improved management strategies [71]. The goals of this review are to describe the biological properties of MAIT cells, present evidence of their critical involvement in chronic liver disease, and identify investigational opportunities to clarify their disease-associated roles and therapeutic implications.

METHODOLOGY

English abstracts were identified in PubMed using the primary search words, "MAIT cells", "MAIT cells and chronic liver disease", "MAIT cells and chronic viral hepatitis", MAIT cells and chronic alcoholic liver disease", "MAIT cells and fatty liver disease", and "MAIT cells and autoimmune liver disease". Abstracts judged pertinent to the review were identified; key aspects were recorded; and full-length articles were selected from relevant abstracts. A secondary bibliography was developed from the references cited in the selected full-length articles, and additional PubMed searches were performed to expand the concepts developed in these articles. The discovery process was repeated, and a tertiary bibliography was developed after reviewing selected articles from the secondary bibliography. Eight hundred and fifty-three abstracts and 134 full length articles were reviewed through November 2020.

CANONICAL MAIT CELLS

Defining features

MAIT cells are defined by a semi-variant α-chain within the TCR that is encoded in humans as Vα7.2-Jα33 by the TRAV1-2/TRAJ33 gene[3,7,72,73] (Table 1). The TCR α-chain associates with a constrained number of TCR β-chains. Vβ6 and Vβ20[61,73] are the principal β-chains associated with the TCR of MAIT cells, and they are encoded by the TRBV6 and TRBV20-1 genes in humans[6,8,74] (Figure 1). Together, the α- and β-chains form a TCR that can accommodate a limited number of chemical structures[8,10]. Human MAIT cell TCRs have hypervariable complementarity-determining regions (CDRs) in the TCRα-[75] and TCRβ-[8] chains that have restricted lengths. CDR3β of the TCR Vβ chain is stable between individuals, has a length of 11-14 amino acids, and accommodates 80% of the TCRβ repertoire of MAIT cell antigens[8].

Antigen-presentation to MAIT cells is limited to the class 1b antigen-presenting molecule, MR1[2,7,76]. This restriction of antigen-activation is another defining aspect of MAIT cells (Table 1). The high surface expression of the C-type lectin, CD161[77-79], cytokine receptors for interleukin (IL)-7, IL-12, IL-18, and IL-23[62,77,80,81], and the chemokine receptors, CCR5, CCR6, CCR9, and CXCR6 are other phenotypic features[59,62,77,82] (Figure 1).

Key attributes

Most MAIT cells express the multidrug resistance protein 1 (also called the multidrug *ABCB1* transporter)[11,62,83]. They also possess the nuclear receptor transcription factors, promyelocytic leukemia zinc finger (PLZF) (also known as ZBTB16)[61,84,85], retinoic acid-related orphan receptor gamma t (RORγt)[86], and T-box transcription factor (T-bet)[87-91] (Figure 1). The ATP binding cassette sub-family B member 1 gene (*ABCB1*) renders MAIT cells more resistant to cytotoxic drugs, chemotherapeutic agents, and gut-derived xenobiotics than other lymphocytes[11]. The *ABCB1* transporter does not protect MAIT cells from immunosuppressive drugs such as tacrolimus and mycophenolic acid which are used in the treatment of autoimmune hepatitis[83,92].

The diverse nuclear transcription factors, PLZF, RORγt, and T-bet, are involved in the lineage development of T helper 1 (Th1) cells[87,88,93], memory cells[91], Th17 cells[86], NKT cells[84], and MAIT cells[84,85] (Table 1). PLZP controls the phenotype and functionality of MAIT cells, and it directs the development of an effector memory phenotype[85,94-96]. These memory cells typically have the molecular signature, CD44hiCD95hiCD45RO+CD62Llo[11,12,15,94,97]. They can be activated without prior clonal expansion in response to IL-7[82,98].

PLZP also controls the fate of MAIT cells by activating intracellular caspases and rendering MAIT cells sensitive to apoptotic stimuli[99]. This sensitivity to apoptosis can be counterbalanced by the X-linked inhibitor of apoptosis protein (XIAP)[100]. The counterbalancing effects of PLZP and XIAP may account in part for the variable numbers of MAIT cells detected in the circulation and tissue sites during active inflammation[99].

Activated MAIT cells rapidly secrete interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), IL-17A, and IL-22[11,17,81,101,102]. These cytokines have pro-inflammatory and antiviral effects (Figure 1). Stimulation of human MAIT cells in culture for 7-10 d can induce the robust release of the anti-inflammatory cytokine, IL-13[64,66]. IL-4 and IL-5 are also released but to a lesser extent[65,66]. IL-4 and IL-13 can induce an immunosuppressive phenotype in macrophages[66,103-105], and their

Table 1 Mucosal-associated invariant T cell characteristics and clinical implications

Feature	Characteristics	Clinical implications
Semi-invariant TCR	Semi-invariant α-chain in the TCR[3,7,72]; Canonical Vα7.2-Jα33 α-chain[3,7]; *TRAV1-2/TRAJ33* encodes Vα7.2-Jα33[3]; Vβ6 and Vβ20 most common β-chains [61]; *TRBV6, TRBV20-1* encode Vβ6, Vβ20[8,74]; Restricted length of CDRs[8, 75]; CDR3β key to antigen recognition[10]	Limited number of antigens recognized[8,10]; Antigen diversity still possible[10]
MR1-restricted antigens	Class 1b antigen-presenting molecule[2,7]; Expressed on surface of APC[3]	MR1 limits antigens presented by APCs[10,114]
CD161	High surface expression[77-79]	Shared phenotypic marker with other T cells[78]
Cytokine receptors	IL-7, IL-12, IL-18, IL-23 receptors[80]	Multiple cytokines can activate MAIT cells[80,81]
Chemokine receptors	CCR5, CCR6, CCR9, CXCR6[59,62,77,82]	Chemokine-directed tissue migration[77]
Nuclear transcription factors	PLZF (also known as ZBTB16)[61,84,85]; RORγt[86]; T-bet[87,88,90,91]; ABCB1 [11,62,83]	Control phenotype and functionality[84-88]; Direct development of memory phenotype[91]; Activate caspases and induce apoptosis[99]; Increase resistance to drugs, xenobiotics[11]
Cytokine production	IFN-γ, TNF-α, IL-17A, IL-22[11,17,81,102]; IL-13, IL-4, IL-5 (anti-inflammatory) [64,66]; IL-10 (mainly in adipose tissue)[106]	Pro-inflammatory and antiviral effects[17,81,102]; Anti-inflammatory effects[66,106]; Cross regulation of immune responses[64,65]
Effector phenotype	Granzyme B[4,107,108]; Perforin[4,74,108]	Antimicrobial and pro-apoptotic actions[4]; Eliminates infected or altered cells[4,107,108]
Subsets	Mostly CD8αα cells in liver and blood[101]	More IFN-γ and TNF-α than CD8αβ subset[101]

ABCB1: Multidrug resistance protein 1; APCs: Antigen presenting cells; CDRs: Complementarity-determining regions; IL: Interleukin; IFN-γ: Interferon-gamma; MAIT: Mucosal-associated invariant T cell; MR1: Major histocompatibility complex I-related molecule; PLZF: Promyelocytic leukemia zinc finger; T-bet: T-box transcription factor; TCR: T cells antigen receptor; t RORγt: Retinoic acid-related orphan receptor gamma t; TNF-α: Tumor necrosis factor-alpha.

production in inflammatory liver disease may cross-regulate the pro-inflammatory type 1 immune response[64,65] (Table 1). Little or no IL-10 is produced by activated human MAIT cells in the circulation, but 14% of resident MAIT cells in human adipose tissue produce IL-10[17,106]. Activated MAIT cells also secrete granzyme B and perforin which can have an antimicrobial effect by eliminating infected or altered cells [4,107,108] (Figure 1).

MAIT cell subsets

Most MAIT cells in the liver and peripheral circulation are CD8+ T cells[61,109]. They can be further characterized by the expression of the cell surface glycoproteins CD8α and CD8β[110,111] (Table 1). CD8α can exist as a disulfide-linked homodimer (CD8αα) or as a heterodimer (CD8αβ) on the MAIT cell surface[111]. Most MAIT cells in humans are CD8αα-positive, and most CD8αα-positive T cells in humans are MAIT cells[101] (Figure 1). Other subsets account for less than 10% of the MAIT cell population[101], and they exist as CD8αβ-positive, CD8-CD4- double-negative, and CD4-positive MAIT cells[61,112].

The CD8αα-positive subset produces more IFN-γ and TNF-α than the CD8αβ subset, and it may be more active in inflammatory and immune-mediated diseases [101]. Expansion and maturation of the CD8αα-positive subset of MAIT cells occur after exiting the thymus. The CD8αα-positive MAIT cells are probably driven by antigens encountered in the periphery, including the intestinal microbiome[101,113].

NON-CANONICAL MAIT CELLS AND MAIT-LIKE CELLS

T cell populations other than the typical Vα7.2-Jα33 MAIT cells have a semi-invariant TCR, reactivity to non-peptide antigens presented by a restricted MHC class I-like molecule, and responses that have innate and adaptive immune features. These populations include MAIT cell variants[8,10,74,114], MR1-restricted non-MAIT cells (MR1T cells)[114], and invariant NKT (iNKT) cells[115-119].

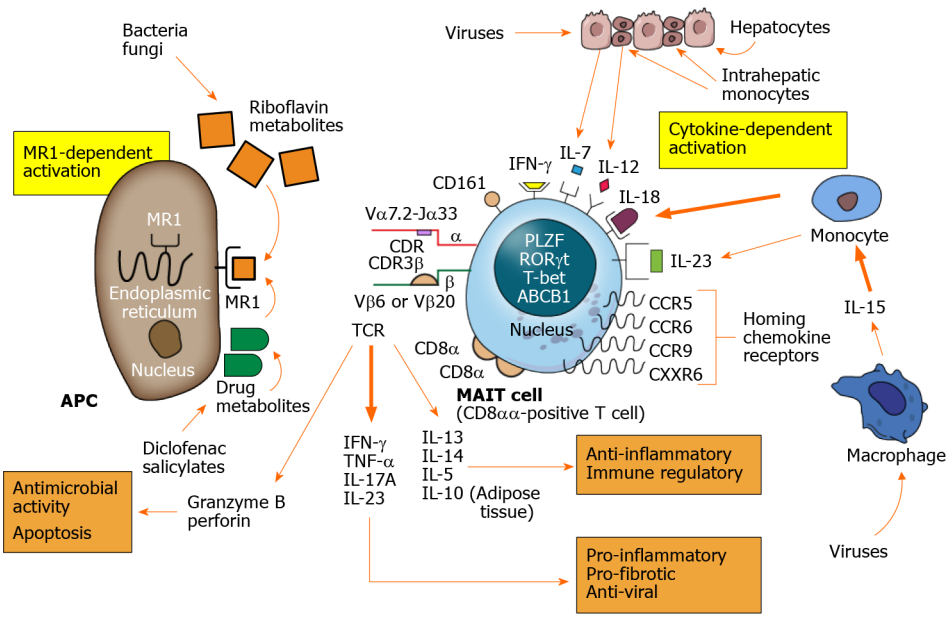

Figure 1 Mucosal-associated invariant T cell activation and actions. Mucosal-associated invariant T (MAIT) cells are activated by MR1-dependent and cytokine-dependent mechanisms. The major histocompatibility complex class I-related protein, MR1, is expressed on the surface of the antigen presenting cell after stimulation. Riboflavin (vitamin B) metabolites synthesized by bacteria and fungi are presented by the MR1 molecule as are select drug metabolites. The T cell antigen receptor of the MAIT cell consists of a semi-invariant alpha (α) chain and restricted beta (β) chain with antigen selectivity influenced by short length complementarity-determining regions. MR1-dependent activation results in MAIT cell production of multiple cytokines as well as granzyme B and perforin. The cytokines can have pro-inflammatory, pro-fibrotic and antiviral effects (lower right panel) and anti-inflammatory and immune regulatory effects (lower right panel). The granzyme B and perforin can have antimicrobial activity and eliminate infected cells by apoptosis (lower left panel). Cytokine-dependent stimulation is activated by phagocytic macrophages and monocytes resident in the liver or circulation and by injured hepatocytes. Multiple cytokines can activate MAIT cells, especially interleukin 18, after virus infection. Chemokine receptors help direct the activated MAIT cells to the site of inflammation, and CXCR6 is the principal chemokine that directs MAIT cells to the liver. MAIT cells contain diverse nuclear transcription factors that influence phenotype and function, especially promyelocytic leukemia zinc finger, retinoic acid- related orphan receptor gamma t, and T-bet. The nucleus also contains the *ABCB1* that affects resistance to gut-derived xenobiotics and certain drugs. The principal subset of activated MAIT cells consists of CD8αα-positive T cells. APC: Antigen presenting cell; TCR: T cell antigen receptor; CDR: Complementarity-determining region; IL: Interleukin; IFN-γ: Interferon-gamma; TNF-α: Tumor necrosis factor-alpha; MAIT: Mucosal-associated invariant T.

MAIT cell variants

MAIT cell variants have semi-invariant TCR α-chains that are encoded by non-classical genes or paired with different TCR β-chains. MAIT cells can have TCR α-chains encoded as Vα7.2-Jα12 by the TRAV1-2/TRAJ12 gene or Vα7.2-Jα20 encoded by the TRAV1-2/TRAJ20 gene[8,73]. These MAIT cell variants have fundamental traits that are identical to the Vα7.2-Jα33 MAIT cell population. They are activated by riboflavin metabolites in a MR1-dependent manner and by cytokine production. They differ only in homing characteristics. The Vα7.2-Jα12 MAIT cells seem to predominate in solid tissues[8]. A MAIT cell variant that is TRAV1-2neg retains the fundamental properties of MAIT cells and warrants inclusion in this variant category[74].

MR1-restricted non-MAIT cells

MR1-restricted T cells (MR1T cells) lack the fundamental characteristics of MAIT cells [114]. MR1T cells have diverse TCRα and TCRβ chains, and they fail to respond to microbial vitamin B metabolites. They produce a wide range of cytokines, and they recognize monocyte-derived dendritic cells as APCs[114]. MR1T cells have specificity for cell-derived antigens that can polarize their function after antigen stimulation [114]. They may thereby influence adaptive immune responses and immune tolerance of self-antigens. MR1T cells represent 1:2500-1:5000 of circulating T cells in healthy individuals, and they can be activated by a wide array of antigens presented in an MR1-binding groove. This MR1-restricted binding groove is larger and less restrictive than the MR1 of MAIT cells[114].

iNKT cells

iNKT cells are similar to MAIT cells in that they have a semi-invariant TCR and react to non-peptide antigens presented by an MHC class I-like molecule[5,81,120]. They express the natural killer (NK) antigen, NK1.1, which is known as CD161 on MAIT cells, and they secrete pro-inflammatory cytokines, including IFN-γ, TNF-α, IL-17, and IL-22[120-122]. iNKT cells differ from MAIT cells in that their TCR α-chain is encoded

as Vα24-Jα18 in humans and paired mainly with the TCR β-chain, Vβ11[120,123]. The antigen-restricted molecule that activates iNKT cells is CD1d (cluster of differentiation 1d) rather than MR1, and the activating antigens are lipid-based, including glycosylceramides (primarily, α-galactosylceramide), glycosphingolipids, and phospholipids [120,124-127]. Furthermore, iNKT cells are rare in the peripheral circulation (0.01%-0.1%)[120,122,128] and liver (0.5%)[120,122,129]. Unlike circulating MAIT cells[17], iNKT cells in the peripheral circulation secrete the anti-inflammatory cytokine, IL-10 [130], and participate in the differentiation of regulatory T cells (Tregs)[23].

MAIT CELL DEMOGRAPHICS

Frequency

MAIT cells constitute 0.1%-10% of the circulating $CD3^+$ T lymphocytes in healthy individuals[61,131], and they are a resident population in the intestine (2%-20%), lung (1%-10%), and liver (10%-40%)[11,61,81,132] (Table 2). Their predominance in the liver [11,43,82,133] and paucity in lymphoid tissue (< 1%)[11] suggest that they are positioned to react with microbial antigens in the portal circulation or metabolic by-products within the liver or biliary circulation[133].

Intrahepatic distribution

MAIT cells are localized mainly in the intrahepatic bile ducts, portal tracts, and sinusoids, and the nature of the liver disease may affect their distribution[55,133] (Table 2). MAIT cells have the ability to migrate based on the expression of tissue-homing chemokine receptors and the location of transmembrane adhesion molecules (integrins)[77]. MAIT cells can be directed to the bile ducts by CCR6, CXCR6, and integrin αEβ7 or to the hepatic sinusoids by CXCR3 and the integrins, LFA-1 (lymphocyte function-associated 1 protein) and VLA-4 (very late antigen 4)[133,134].

Age-related changes

The number of circulating MAIT cells increases from birth to adulthood (ages, 20-40 years) in healthy individuals[135]. Maximum circulating levels are in the third and fourth decades[136] (Table 2). The number of circulating MAIT cells declines after the age of 60 years in association with a gradual increase in MAIT cell apoptosis[135]. It is ten-fold less than in young adulthood after the age of 80 years[136]. The annual decline in the circulating level has been estimated as 3.2% in men and 1.8% in women[131].

Gender differences in the frequency of MAIT cells [131,136] and age-related differences in the phenotype and function of MAIT cells have been described but not established[131,135-137] (Table 2). Advancing age has been associated with an increasing proportion of $CD4^+$ MAIT cells and decreasing proportion of $CD8^+$ MAIT cells in some[131,136,137], but not all[135], ethnic cohorts. The pattern of cytokine production by MAIT cells has also changed to a less inflammatory profile with advancing age in one Asian cohort[131] but not in another[135]. The inconsistent phenotypic and functional age-related alterations may reflect ethnic and environmental factors[135], and they have yet to be ascribed an impact on specific pathological states[136].

MAIT cells are absent in germ-free animals (unlike NKT cells)[7], and their numbers can be reconstituted by intestinal colonization with various microbial organisms[30]. Diverse antigenic encounters during aging may explain the increasing numbers of MAIT cells that are detected in individuals as they enter adulthood[131,135,136].

MAIT CELL ACTIVATION

MAIT cells are activated by MR1-dependent mechanisms that are antigen-mediated and by MR1-independent mechanisms that are mainly cytokine-mediated[71]. Superantigens produced by certain bacteria (mainly, Staphylococcus aureus) can also activate MAIT cells without involvement of MR1[138,139].

MR1-dependent activation

The MR1 molecule is sequestered in the endoplasmic reticulum within the APC[140], and it may be undetectable on the APC surface until antigen exposure[141-146] (Figure 1). The antigen binding groove of the MR1 molecule has two pockets, and it can only bind small molecules[13,147]. The development of murine MAIT cells in the thymus requires exposure to microbial riboflavin metabolites[113]. Most bacteria and

Table 2 Mucosal-associated invariant T cell demographics and clinical implications

Feature	Demographics	Clinical implications
Frequency (based on percentage of $CD3^+$ T cells)	Circulation, 0.1%-10%[11,61,81,131]; Intestine, 2%-20%[11,61,102,132]; Lung, 1%-10%[11,15,61,81]; Liver, 10%-40%[11,43,61,82,133]; Lymph nodes, < 1%[11,61]	Liver is most MAIT cell enriched tissue[61]; MAIT cells can react with microbial antigens and metabolites in portal circulation and in bile[133]
Hepatic distribution	Present in bile ducts, portal tracts, sinusoids[55,133]; Chemokine-directed migrations[77]; CCR6, CXCR6, integrin αEβ7 to bile ducts[77,133]; CXCR3, LFA-1, VLA-4 to sinusoids[77,133]	Nature of the liver disease may direct MAIT cell migration to key site of inflammation[77,133,134]
Age-related changes	Numbers in blood increase up to age 40 yr[135]; Numbers in blood decline after age 60 yr[135]; MAIT cell apoptosis increases with age[135]; Depletion nadir after age 80 yr[136]; Depletion may be faster in men than women[131]; Shift from $CD8^+$ to $CD4^+$ cells with aging[131,137]; May be less pro-inflammatory with aging[131]	Ethnic and environmental factors possible[135]; Uncertain effect on severity and outcome[136]; Consider in design of clinical investigations

LFA-1: Lymphocyte function-associated-1 protein; MAIT: Mucosal-associated invariant T cell; VLA-4: Very late antigen 4.

fungi synthesize riboflavin and generate riboflavin metabolites that can bind to MR1 and activate MAIT cells[30,148].

The principal ligands of MR1 are riboflavin (vitamin B2)-based metabolites designated as ribityllumazines[13] (Table 3). These ligands are characterized by a ribityl tail that can dock with MR1[13,14,74,149]. They are derived from the biosynthetic pathway for riboflavin which is present in most bacteria and yeast[13] (Figure 1). Folic acid (vitamin B9) derivatives, including 6-formylpterin (6-FP), can also be captured by MR1[13], but they lack the ribityl component and are unrecognizable by MAIT cells[74,143]. The synthetic folate derivative, acetyl-6-formylpterin, inhibits MAIT cell function by competing with other bacterial products for MR1 ligation. Its therapeutic value in modulating MAIT cell activity warrants further investigation[143,150].

Antigen diversity: The antigenic repertoire that binds to MR1 and activates MAIT cells has expanded beyond the ribityllumazines. Transitory neo-antigens have been described that are generated by the interaction of 5-amino-6-d-ribitylaminouracil, an early intermediate in the bacterial synthesis of riboflavin[151], with the metabolic by-products, glyoxal and methylglyoxal[152] (Table 3). Chemically unstable pyrimidine intermediates, 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-d-ribitalaminouracil (5-OE-RU), are formed, and these lumazine precursors can activate MAIT cells[9]. The unstable 5-OP-RU and 5-OE-RU molecules have diverse bacterial origins, and they are trapped in the antigen binding groove of the MR1 molecule by a reversible covalent Schiff base[9].

The antigen-binding groove of the MR1 molecule can also accommodate the structurally distinct compounds associated with the metabolism of drugs[153] (Figure 1). Salicylates and diclofenac can generate small molecules that bind with MR1 and exert a stimulatory (salicylates) or inhibitory (diclofenac) effect (Table 3). The findings indicate that diverse ligands outside the riboflavin and folic acid metabolites can be recognized by MAIT cells. They support the prospect that additional antigens will be discovered that modulate MAIT cell function[10,74].

Modulation of MAIT cell response: The MAIT cell response reflects mainly ligand-specific MR1 dependencies and TCR β-chain biases for a particular antigen[148] (Table 3). Different microbial species may produce different riboflavin metabolites and generate a selective MAIT cell response[3] or trigger the memory of a previous microbial exposure[154]. Conformational changes within the CDR3β segment may alter TCR flexibility and modulate antigen recognition within individual MAIT cell populations[143].

MAIT cell activation may also be modulated by the cytokine milieu created by the cells at the site of inflammation (Table 3). IL-7 produced by hepatocytes during inflammation can up-regulate MAIT cell production of Th1 cytokines and IL-17A[82]. Factors that regulate the cell surface expression of MR1 could also influence MAIT cell activation[144]. The non-microbial ligand, 3-([2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl]formamido) propanoic acid, down-regulates cell surface expression of MR1 and prevents antigen recognition[155].

The generation of MAIT cell antigens from bacterial and host-derived metabolic by-products constitute a rapidly responsive mechanism by which to target particular microbial pathogens under prescribed circumstances[9].

Table 3 Mucosal-associated invariant T cell activation and clinical implications

MAIT cell activation	Features	Clinical implications
MR1-dependent stimulation	Adaptive immune response[1,5]; Antigen-triggered MAIT cell activation[8,10,114]; MR1 undetectable before antigen exposure[140,144]; MR1 binds only small non-peptide molecules[147]; Riboflavin metabolites are main MR1 ligands[13]; Ribityllumazines are main riboflavin metabolites[13]; Bacterial and metabolic by-products can activate[9]; Drugs and drug metabolites can bind to MR1[153]	Antigens for presentation restricted[8,10]; Most microbes metabolize riboflavin[148]; Neo-antigens diversify MR1 repertoire[9]; Can develop effector memory cells[11]; Drugs can modulate MR1 signaling[153]; MR1 expression can be inhibited [155]
Modulation of MAIT cell response	Response biased by ligand and TCR β-chain[148]; Riboflavin metabolites differ among microbes[3]; CDR3β rearrangements alter antigen recognition[143]; IL-7 and non-microbial molecules can regulate[155]	Response differs among microbes[154]; TCR plasticity can affect response[143]; Local milieu modulates response[82,155]
Cytokine-dependent stimulation	Innate immune response[1,5]; Activates MAIT cells without TCR ligation[156]; Receptors for IL-7, IL-12, IL-18, IL-23, IFN-γ[81]; IL-18 is main MAIT cell activator[157,158]; IL-18 usually with other mediators[82,157,158]; IL-7, IL-18 produced by hepatocytes[81,158]; IL-1β, IL-18, IL-23 produced by monocytes[81,158]; IL-15 acts on MAIT cells directly and indirectly[158]; Bacteria elicit TLR8-induced cytokines[160]	Initiates rapid antimicrobial response[156]; Response affected by local mediators[81]; Effective against viral infections[32,33,159]; Anti-bacterial monocyte response[160]
Superantigen stimulation	Rapid powerful response to severe infection[138,163]; Bacterial exotoxins activate T cell populations[161]; Foregoes MR1 antigen activation[138,161]; Direct activation by binding to TCR Vβ[71,138,165]; Indirect activation by released IL-12, IL-18[71,138]; Generates robust release of cytokines[138]	MAIT cells are major responders[138]; May result in toxic shock[162]; Causes immune exhaustion[138,139,163]; May exacerbate autoimmune disease[168]; Induces pathogenic autoantibodies[166]

CDR3β: Complementarity-determining region 3-beta; IFN-γ: Interferon-gamma; IL: Interleukin; MAIT: Mucosal-associated invariant T; MR1: Major histocompatibility complex I-related molecule; TCR: T cell antigen receptor; TLR8: Toll-like receptor 8.

Cytokine-dependent activation

MAIT cells can be activated directly by cytokine stimulation in the absence of TCR ligation[156] (Table 3). MAIT cells express cytokine receptors for IL-7, IL-12, IL-18, and IFN-γ, and they can express the receptor for IL-23 after activation[81] (Figure 1). Cytokine activation of MAIT cells is mainly dependent on IL-18 in association with other inflammatory mediators (IL-1β, IL-7, IL-12, IL-15, and the type 1 interferons)[32,33,82,157,158]. These inflammatory mediators are derived from different cell types at the site of inflammation, and they can modulate the MAIT cell reaction in different combinations[81].

Hepatic inflammation can release IL-7 and IL-18 from hepatocytes, and activated monocytes can produce IL-1β, IL-23, and IL-18 after stimulation with IL-15[81,158] (Figure 1). Combinations of IL-1β, IL-7, IL-12, and IL-23 can affect MAIT cell production of IFN-γ and IL-17A[11,81,82]. IL-15 can directly stimulate MAIT cell production of IFN-γ or indirectly induce MAIT activation by stimulating monocyte production of IL-18[81,158].

MAIT cell activation in viral infections (dengue virus, influenza virus, and HCV infections) is dependent on IL-18 production[32]. The antimicrobial protection afforded by MAIT cells against influenza infection is based on the anti-viral activity of IFN-γ. MAIT cells release IFN-γ after stimulation with IL-18 alone[159] or in synergy with IL-12[33,157,160]. Intrahepatic monocytes are activated to produce IL-12 and IL-18 after stimulation of Toll-like receptor 8 (TLR8)[160]. The cytokine milieu at the site of inflammation is a key modulator of MAIT cell activity, and it can tailor or finely tune the MAIT cell response to the disease process.

Superantigen activation

Superantigens are bacterial exotoxins that activate large numbers of T cells without undergoing the conventional route of activation by antigen-presenting MHC class I or class II molecules[70,138,161-163]. The superantigens bind to the lateral surfaces of class II MHC molecules expressed on APCs[164] or to Vβ regions within the TCR of T lymphocytes[70,165]. The massive simultaneous activation of exposed T cells can generate a robust release of cytokines, precipitate a toxic shock syndrome, and result in immune cell exhaustion and anergy[162,166]. Staphlococcus aureus, Streptococcus pyogenes, gram negative bacteria, mycoplasma, and viruses are key producers of superantigens[163].

MAIT cells are major responders to microbial infection and superantigens[138] (Table 3). MAIT cells can be activated directly by binding superantigens to their TCR Vβ region[71,138,139,165]. They can also be activated indirectly by the release of IL-12 and IL-18 from superantigen-activated T cells[71,138]. MAIT cell activation may in

turn induce T cell exhaustion and immunosuppression that prevent adequate control of infection[138].

The impact of MAIT cell activation by superantigens on the occurrence and course of chronic inflammatory or immune-mediated disease is unclear. By activating large numbers of T cells, bacterial superantigens may enhance the proliferation of autoreactive T cells as well as MAIT cells[167,168]. Superantigens may also facilitate production of pathogenic autoantibodies by previously primed B cells[166,169,170]. These consequences could exacerbate an autoimmune disease. Alternatively, superantigens could promote immunosuppression by T cell exhaustion and prevent or ameliorate immune-mediated disease[168]. The actual impact may reflect the timing and intensity of MAIT cell activation[168].

MAIT CELLS IN CHRONIC NON-HEPATIC INFLAMMATORY DISEASES

MAIT cells have been evaluated in diverse chronic non-hepatic inflammatory diseases [15], including systemic lupus erythematosus (SLE)[171], rheumatoid arthritis[171,172], multiple sclerosis[120,173-176], inflammatory bowel disease[132,177,178], celiac disease[179], and infection with the human immunodeficiency virus (HIV)[109,180]. The findings have commonly demonstrated reduced frequencies of circulating MAIT cells[171,176,178-180], increased numbers of MAIT cells infiltrating the involved tissue (synovium, central nervous system, and ileum)[132,171,173,176], and uncertainties about the pathological role of the infiltration[15,120,181,182]. Furthermore, the studies have been complicated by disparities in the methodology to detect MAIT cells[12,73,183,184] and difficulties in separating treatment-associated from disease-related findings[15,177,185].

MAIT cell depletion in the circulation has been attributed to tissue migration during inflammation[132,176], apoptosis[99], activation-induced cell death (AICD)[109], exhaustion[186], and concurrent therapy with glucocorticoids[15,177,185]. MAIT cell determinations based on cell surface expression of CD161 have been faulted since chronic inflammation can down-regulate CD161 expression[12,73], and the preferred assay for MAIT cell recognition based on antigen-loaded MR1 tetramers has been used inconsistently[12,73,184,187]. The clinical investigations of MAIT cells in chronic non-hepatic inflammatory diseases provide insights, justifications, comparisons, and caveats that can strengthen and extend the investigations of MAIT cells in chronic liver disease.

MAIT CELLS IN CHRONIC HEPATITIS

The investigations of MAIT cell involvement in chronic hepatitis have been broad, mainly descriptive, and generally consistent with findings demonstrated in other chronic inflammatory diseases. They have positioned MAIT cells at the interface of inflammation and tissue injury, and they have confirmed their lack of disease-specificity. They have also expanded their possible roles as pathogenic, protective, and immune regulatory agents. Furthermore, they have indicated that in some liver diseases MAIT cells have contradictory effects.

MAIT cells may represent a primary, albeit defective, host-directed anti-viral response in chronic viral hepatitis[32,43,45,188]. They may constitute a hyperactive, immune exhausted, and depleted antibacterial defense in alcoholic hepatitis[46,47]. They may be a source of regulatory cytokines that reduce liver injury in NAFLD[51], and they may promote pro-inflammatory responses that enhance tissue injury and hepatic fibrosis in autoimmune hepatitis[52,53]. The MAIT cell investigations in chronic hepatitis provide foundational knowledge that should incentivize further studies and impact on future management strategies.

MAIT cells and chronic viral hepatitis

The number of MAIT cells has been assessed in the blood and liver of patients with chronic hepatitis B[35-39] and chronic hepatitis C[40-44], and the functionality of circulating MAIT cells has been determined in both patient populations[36,39-42].

Reduced frequency of circulating MAIT cells: Five of six studies that have evaluated the frequency of circulating MAIT cells in patients with chronic hepatitis B have found reduced numbers compared to healthy individuals[35-39,45] (Figure 2). In one of these studies, patients with concurrent infection with hepatitis delta virus had more severe

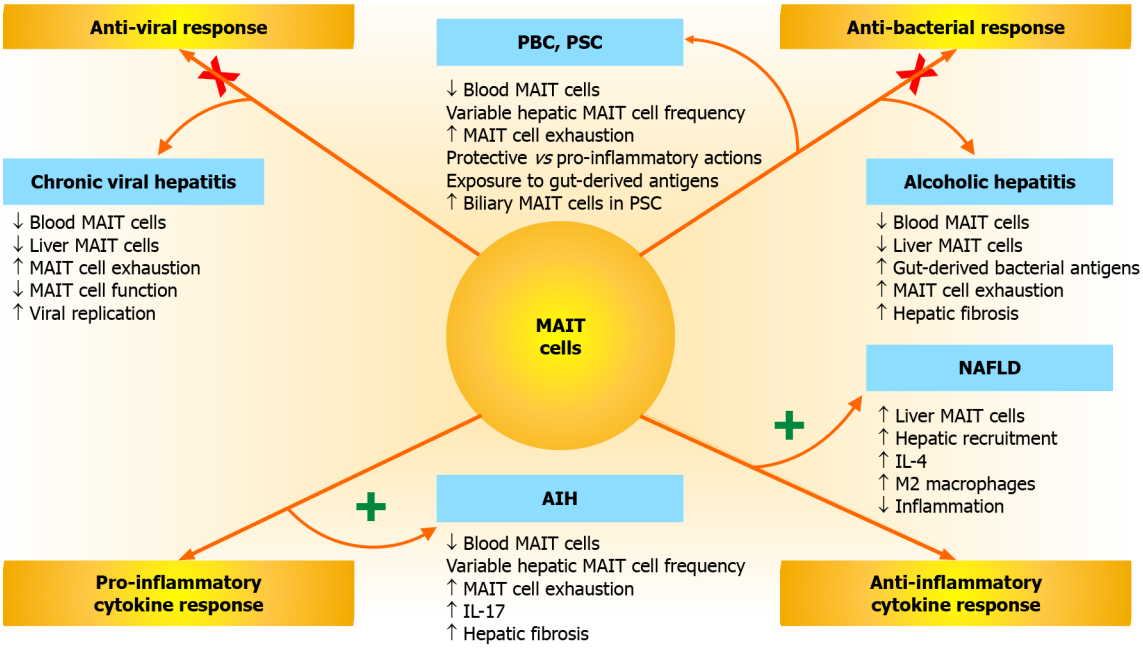

Figure 2 Mucosal-associated invariant T cell responses and associations with chronic liver disease. Mucosal-associated invariant T (MAIT) cells have anti-viral, anti-bacterial, anti-inflammatory, and pro-inflammatory responses that can affect the occurrence, severity, and outcome of diverse chronic liver diseases, including chronic viral hepatitis, alcoholic hepatitis, non-alcoholic fatty liver disease (NAFLD), autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC). Impairments in the anti-viral and anti-bacterial responses of MAIT cells (indicated by an "X" across each activation pathway) may promote chronic viral hepatitis, PBC, PSC, and alcoholic hepatitis. Hyperactivity of the anti-inflammatory and pro-inflammatory cytokine responses of MAIT cells (indicated by a "+" next to each activation pathway) may affect NAFLD and AIH. Increased release of interleukin 4 (IL-4) and IL-17 may contribute to the modulation of these responses. MAIT: Mucosal-associated invariant T; NAFLD: Non-alcoholic fatty liver disease; AIH: Autoimmune hepatitis; PBC: Primary biliary cholangitis; PSC: Primary sclerosing cholangitis; IL: Interleukin.

depletion than patients with mono-infection[45] (Table 4). Similar findings of a reduced number of circulating MAIT cells relative to other T cells have been reported in 6 studies that have evaluated this issue in patients with chronic hepatitis C[32,40-44].

Reduced frequency of intrahepatic MAIT cells: The number of intrahepatic MAIT cells was reduced in three of four studies in chronic hepatitis B[35,38,39,45] (Figure 2). Of two studies evaluating the number of intrahepatic MAIT cells in chronic hepatitis C, one disclosed reduced numbers[43] and another failed to associate the marked depletion in blood with an increased accumulation in liver[44] (Table 4). The lack of hepatic accumulation weakens the possibility that hepatic migration of MAIT cells explains the reduced peripheral count. The liver-homing chemokine, CXCR6[189], was also down-regulated on circulating MAIT cells in patients with chronic hepatitis B[39].

Impaired functionality of MAIT cells: Circulating MAIT cells in patients with chronic hepatitis B and chronic hepatitis C have impaired function (Figure 2). This impairment has been manifested mainly by reduced production of granzyme B[36,39,188], IFN-γ [36,39,188], and IFN-α[38] in patients with chronic hepatitis B and by reduced TCR-dependent antigen activation and IFN-γ production in patients with chronic hepatitis C[40,43] (Table 4). In patients with chronic hepatitis B, IFN-γ production has been reduced mainly in patients with serum conjugated bilirubin levels > 10-fold the upper limit of the normal range (ULN). This deficiency has been corrected by TCR-mediated stimulation[39].

In patients with chronic hepatitis C, receptor-mediated, but not cytokine-mediated, activation has been impaired[40,43]. Despite this deficiency, MAIT cells from the liver have had higher levels of activation and cytotoxicity than MAIT cells from the peripheral circulation ($P < 0.0001$). Furthermore, the frequency of MAIT cells in the liver has correlated inversely with histological inflammation ($r = -0.5437$, $P = 0.0006$) and fibrosis ($r = -0.5829$, $P = 0.0002$)[43]. The findings suggest that deficiencies in the number and function of intrahepatic MAIT cells in chronic hepatitis C contribute to the pathological process or are consequences of it.

Table 4 Mucosal-associated invariant T cells in chronic hepatitis and clinical implications

Liver disease	MAIT cell features	Clinical implications
Chronic hepatitis B	Reduced frequency circulating MAIT cells[37,38]; Depletion increased by delta infection[45]; Depleted intrahepatic MAIT cells[38,39,45]; Less granzyme B, IFN-γ, IFN-α release[36,188]; Conjugated bilirubin linked to dysfunction [39]; Increased PD-1 and CTLA-4 on MAIT cells[36]; Exhaustion correlates with HBV DNA level[36]	Chronic activation and exhaustion[36,39]; Less antiviral action[36,39,188]; Increased MAIT cell death[99]; Presumed defective protective role[36]
Chronic hepatitis C	Reduced frequency circulating MAIT cells[41,42]; Depleted intrahepatic MAIT cell[43]; Increased histologic indices reflect depletion[43]; Less TCR-activation and IFN-γ production[40,43]; Increased PD-1 and CTLA-4 on MAIT cells[41]	Hyper-activation and exhaustion[40,41,43]; Increased MAIT cell death[43,61,99]; Antiviral therapy not restorative[40,42,43]; Presumed defective protective role [43]
Alcoholic hepatitis	Reduced frequency in blood and liver[46,47,133]; Decreased granzyme B and IL-17 production[46]; Circulating bacterial products[46,47]; Increased percentage PD-1$^+$ MAIT cells[47]; Abundant circulating stimulatory cytokines [47]; Myofibroblasts stimulated and pro-fibrotic[63]	Hyper-activation and dysfunctional[46,47]; Immune exhaustion[46,47]; Impaired intestinal mucosal barrier [46,47]; Increased MAIT cell death[47]; Diminished anti-bacterial function[133]; Presumed defective protective role[46,47]
NAFLD	Circulating MAIT cell frequency decreased[51]; Circulating cells express PD-1 and CD69[51]; Increased intrahepatic MAIT cell frequency[51]; Frequency correlates with NAFLD score[51]; Decreased IFN-γ and TNF-α production [51]; IL-4 induced polarization to M2 macrophages[51]	Activated and immune exhausted[51]; Increased hepatic migration[51]; Recruited by inflammatory activity[51]; Reduced functionality[51]; Promotes anti-inflammatory milieu[51]; Presumed defective protective role[51]
Autoimmune hepatitis	Circulating MAIT cell frequency decreased[52,53]; Reduced granzyme B and IFN-γ secretion[52,53]; Variable intrahepatic frequency[52,53]; Increased IL-17A and HSC stimulation[52]; Increased expression of PD-1 and TIM-3[52]	Activated and immune exhausted[52,53]; Reduced functionality[52,53]; Pro-inflammatory cytokine milieu [52]; Progressive fibrosis[52]; Presumed active pathogenic role[52]

CTLA-4: Cytotoxic T lymphocyte antigen 4; HBV: Hepatitis B virus; HSC: Hepatic stellate cell; IFN-α: Interferon-alpha; IFN-γ: Interferon-gamma; MAIT: Mucosal-associated invariant T; NAFLD: Non-alcoholic fatty liver disease; PD-1: Programmed cell death 1; PBC: Primary biliary cholangitis; PSC: Primary sclerosing cholangitis; TIM-3: T cell immunoglobulin and mucin domain 3; TNF-α: Tumor necrosis factor-alpha.

MAIT cell exhaustion: Circulating MAIT cells in patients with chronic hepatitis B[36,39] and chronic hepatitis C[41] have expressed surface markers indicative of immune exhaustion, especially programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), CD39, T cell immunoglobulin mucin protein 3 (TIM-3), CD57, CD38, CD69, and HLA-DR (Table 4). The expression of PD-1 and CTLA-4 and the percentage of MAIT cells expressing these markers have been higher in patients with chronic hepatitis B and viremia than in patients without viremia[36]. Furthermore, the expression of PD-1 and CTLA-4 on MAIT cells has correlated positively with the level of viremia as assessed by plasma levels of hepatitis B virus deoxyribonucleic acid (HBV DNA)[36].

Similar findings have been described in patients with chronic hepatitis C (Table 4). Circulating MAIT cells in patients with chronic hepatitis C have expressed higher levels of surface markers for immune exhaustion (HLA-DR, CD38, PD-1 TIM-3, and CTLA-4) and immunosenescence (CD57) than healthy controls[41]. Chronic immune stimulation and exhaustion could account for MAIT cell dysfunction in chronic viral hepatitis[39] (Figure 2).

Consequences of MAIT cell exhaustion and dysfunction: The consequences of MAIT cell exhaustion and dysfunction in chronic viral hepatitis may include depletion of circulating and intrahepatic MAIT cells and reduced suppression of virus replication [39] (Figure 2). An inverse correlation between the percentage of circulating MAIT cells and the expression of HLA-DR rather than viral load suggests that MAIT cell depletion is a consequence of chronic immune stimulation and exhaustion in chronic hepatitis B[36] (Table 4). MAIT cells have a pro-apoptotic propensity that may contribute to their peripheral depletion in the exhausted state and reduce their accumulation in the liver[43,99].

The positive association of exhausted MAIT cells with plasma HBV-DNA levels in chronic hepatitis B[36] attest to the potential value of MAIT cells as protective agents in chronic viral hepatitis[43]. This association is also supported by the inverse relationship between the number of intrahepatic MAIT cells and the severity of liver inflammation and fibrosis in patients with chronic hepatitis C[43]. The reduced number of intrahepatic MAIT cells in chronic viral hepatitis differs from experiences in non-hepatic chronic inflammatory diseases. In inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis, MAIT cell numbers are increased in the inflamed tissue consistent with active recruitment[132,171,173,176]. In chronic viral hepatitis,

hepatic recruitment may be less or their intrahepatic destruction more robust[43,61].

Successful antiviral therapy with direct-acting agents does improve the number of intrahepatic MAIT cells[43], but it does not restore the number and function of circulating MAIT cells in patients with chronic hepatitis C[40,42,43] (Table 4). Immune exhaustion is a reversible state in most instances[190-192]. Failure to normalize the circulating MAIT cell population after virus eradication may reflect the low frequency of circulating MAIT cells in healthy individuals, the high density of other immune cells in the circulation, and the long lag time before full restoration[43]. Normalization of the serum conjugated bilirubin level and administration of the mitogen, IL-2, have expanded MAIT cells under experimental conditions in chronic hepatitis B[39]. This observation has not been investigated further.

Conjugated hyperbilirubinemia: The frequencies of circulating and intrahepatic MAIT cells have correlated inversely with the serum conjugated and total bilirubin levels in patients with chronic hepatitis B[39]. Serum conjugated bilirubin levels > 10-fold ULN in chronic hepatitis B have been associated with a reduced number of circulating MAIT cells and a high rate of apoptosis. Surface markers for activation and exhaustion have been present; cytokine production has been variable; and MAIT cell proliferation and expansion have been impaired[39] (Table 4).

Concentrations of conjugated bilirubin equivalent to serum levels > 10-fold ULN have induced apoptosis in MAIT cells obtained from healthy donors, but other direct effects of conjugated bilirubin on MAIT cell function remain unclear[39]. Therapeutic strategies directed at reducing the serum concentration of conjugated bilirubin may be effective because of overall improvement in liver inflammation and function rather than elimination of a prime pathogenic factor[39].

MAIT cells and alcoholic hepatitis

The frequencies of circulating and intrahepatic MAIT cells have been decreased in most studies evaluating patients with alcoholic hepatitis[46-48,63,133] (Figure 2). Furthermore, the residual MAIT cells have been hyper-activated and dysfunctional in a manner similar to patients with chronic viral hepatitis[46,47] (Table 4). MAIT cell expression of transcription factors, RORγt, PLZF, T-bet, and eomesodermin (eomes), has been weak and associated with peripheral MAIT cell depletion and reduced secretion of granzyme B and IL-17[46]. Bacterial products, including endotoxin, D-lactate, and lipopolysaccharide (LPS), have been present in the circulation. Furthermore, macrocyte/monocyte activation by bacterial LPS has been implied by the presence of the soluble receptor for LPS (sCD14)[46,47]. These findings have suggested that bacterial translocation from the intestine is a basis for chronic MAIT cell stimulation in alcoholic hepatitis.

Gut-derived MAIT cell stimulation: Chronic MAIT cell stimulation can impair the expression of nuclear transcription factors that are critical for maintaining antibacterial activity[107]. In alcoholic hepatitis, chronic exposure to gut-derived bacterial products may be a basis for impairing the antibacterial function of MAIT cells and creating a detrimental feedback loop[46] (Table 4). Moreover, fecal extracts from the stools of patients with alcohol-related liver disease have induced MAIT cell depletion in the absence of marked apoptosis or immune exhaustion[46] in a manner suggestive of AICD[46,193].

The presence of gut-derived bacterial markers in the peripheral circulation has also been demonstrated in another study of patients with alcoholic hepatitis[47]. An increased frequency of PD-1-positive MAIT cells has indicated immune exhaustion, and the frequency of exhausted MAIT cells has correlated inversely with the percentage of circulating MAIT cells[47]. Furthermore, there has been a shift in the MAIT cell phenotype from $CD8^+$ to $CD4^+$. The findings suggest that chronic bacterial stimulation contributes to MAIT cell hyper-activation, immune exhaustion, and depletion[47].

Cytokine-mediated MAIT cell stimulation: The hyper-activation of MAIT cells in alcoholic hepatitis could also be ascribed to increased circulating levels of cytokines associated with MAIT cell activation, including IL-7, IL-15, IL-17, IL-18, IL-23, IFN-γ, and TNF-α[47] (Table 4). Most of these cytokines have subsided after 6 mo of alcohol abstinence, but circulating levels of IL-18, IL-23, and IFN-γ have remained elevated for at least 12 mo[47]. Persistence of these cytokines could continue to stimulate and exhaust the MAIT cells. Future investigations must evaluate factors contributing to this sluggish improvement in MAIT cell numbers and function during alcohol abstinence. Persistent permeability of the intestinal mucosal barrier and protracted gut-derived bacterial translocation to the peripheral circulation are key aspects that

require further evaluation.

MAIT cells and hepatic fibrosis: Activated human MAIT cells stimulate the proliferation of hepatic myofibroblasts in co-cultures[63] (Figure 2). The direct contact of activated MAIT cells with myofibroblasts obtained from patients with alcohol-related cirrhosis promotes their proliferation in an MR1-dependent manner (Table 4). The activated MAIT cells release TNF-α which can in turn increase the production of IL-6 and IL-8 by the hepatic myofibroblasts[63]. Under these circumstances, mitigation of MAIT cell activity could have a beneficial effect by reducing the release pro-inflammatory cytokines and the propensity for hepatic fibrosis.

Principal MAIT cell role in alcoholic hepatitis: The principal role of MAIT cells in alcoholic hepatitis appears to be protective. IL-17, which is a product of activated MAIT cells, is pivotal in initiating a cascade of antibacterial responses[194], and activated MAIT cells can eliminate MR1-positive infected cells[133]. MAIT cell dysfunction in alcoholic hepatitis may weaken these defense mechanisms and contribute to a high frequency of bacterial infection (49%)[195] and sepsis (13%)[196]. It may also account for the increased risk of mortality (hazard ratio, 2.33, $P < 0.001$) in severe alcoholic hepatitis[195].

The pathogenic association between MAIT cell dysfunction and adverse outcomes in alcoholic hepatitis remains conjectural, and it awaits studies that establish the protective value of enhanced MAIT cell function. The major therapeutic challenge of investigational MAIT cell manipulation is to promote the antibacterial properties of MAIT cells over their potential pro-inflammatory and pro-fibrotic effects.

MAIT cells and NAFLD

The frequency of circulating MAIT cells is also decreased in patients with NAFLD, and the frequency is inversely correlated with serum concentrations of gamma glutamyl transferase (γ-GGT) and triglycerides[51] (Table 4). Unlike patients with chronic viral hepatitis or alcoholic hepatitis, patients with NAFLD have an increased number of intrahepatic MAIT cells, and this number correlates directly with the NAFLD activity score[51] (Figure 2). Furthermore, the expression of the liver-homing chemokine, CXCR6[189], is increased as is the expression of CCR5 in the circulating MAIT cells [51]. CCR5 promotes MAIT cell migration to areas of hepatic steatosis by interacting with CCL5 (also known as RANTES)[197]. In patients with NAFLD, MAIT cells congregate around degenerating fat-laden hepatocytes in sinusoidal areas[51]. These observations suggest that MAIT cells are actively recruited from the circulation to the liver in response to inflammatory stimuli and hepatic steatosis.

As in chronic viral hepatitis and alcoholic hepatitis, the circulating MAIT cells are activated and immune exhausted. CD69 and PD-1 are expressed on the circulating MAIT cells, and the MAIT cells are functionally altered[51] (Table 4). The production of IFN-γ and TNF-α is decreased, and the release of IL-4 is increased (Figure 2). IL-4 has anti-inflammatory properties which can be manifested in NAFLD as a polarization of Kupffer cells into an M2 phenotype[198].

Macrophages can have a pro-inflammatory M1 phenotype in response to IFN-γ[199] or an anti-inflammatory M2 phenotype stimulated by IL-4[198,199]. A high percentage of monocytes/macrophages co-cultured with activated MAIT cells from patients with NAFLD display the M2 phenotype, and the ratio of M2:M1 macrophages is increased [51] (Figure 2). M2 macrophages can induce the apoptosis of M1 macrophages by a mechanism mediated by IL-10 produced by the M2 macrophages[198]. M2 polarization is a pathway that could reduce inflammatory activity in NAFLD, and it may be influenced by MAIT cells[51].

Free fatty acids increase the surface expression of MR1 in monocyte-derived macrophages[51], and they can also activate macrophages by signaling through TLR4 on the macrophage surface[200]. Free fatty acids could thereby increase macrophage activity and stimulate Kupffer cell expression of MR1. They could also increase TCR-mediated activation of MAIT cells and alter MAIT cell function by immune exhaustion. The net pathological consequence would depend on the balance between MAIT cell production of IL-4, the level of Kupffer cell polarization to the M2 phenotype, the pro-inflammatory effects of free fatty acids, and the extent of MAIT cell exhaustion. Clarification of the sequence and pivotal interactions of these pathological events could identify key targets for therapeutic intervention.

MAIT cells in obesity and diabetes: Obesity and diabetes are conditions that may accompany NAFLD, and MAIT cells can affect these co-morbidities. The frequency of circulating MAIT cells is decreased in obese patients and in obese and non-obese

patients with type 2 diabetes[49] (Table 4). The decreased frequency is accompanied by increased activation (high expression of CD69) of the residual circulating MAIT cells and impaired production of IFN-γ and TNF-α[49]. The frequency of circulating MAIT cells that produce IL-17 is increased with obesity, and the expression of PD-1 is up-regulated, suggesting functional exhaustion[106]. Similar changes have been described in children with type 1 diabetes[50].

Adipose tissue from the omentum of obese patients has a higher frequency of MAIT cells than the peripheral blood (0.59% *vs* 0.06%) consistent with active recruitment, and the percentage of omental MAIT cells producing IL-17 is greater than the percentage in lean persons (26.9% *vs* 7.5%, P = 0.0009)[49]. IL-17 induces insulin resistance in adipocytes and hepatocytes[201,202], and the dysfunctional MAIT cells in adipose tissue and peripheral circulation may be a link between obesity and insulin resistance [106].

MAIT cells in human adipose tissue also produce IL-10[106] and IL-4[49]. MAIT cells from obese individuals produce less IL-10 and more IL-17 than MAIT cells from non-obese individuals, and the counterbalance between IL-10 and IL-17 production may modulate insulin resistance[106]. MAIT cells from obese individuals also express less of the anti-apoptotic molecule, B cell lymphoma 2 (Bcl-2)[49]. A shortened survival within the MAIT cell population may be another factor that alters the cytokine milieu.

In a murine model of obesity, MAIT cell frequency has been reduced in blood, epididymal adipose tissue, and ileum compared to lean mice[203]. The MAIT cells in the epididymal adipose tissue of obese mice have produced more IL-17A and TNF-α than in the lean mice, and the MAIT cells in the ileum have produced more IL-17A. The pro-inflammatory cytokine milieu has polarized the macrophages to an M1 phenotype in the epididymal adipose tissue of the obese mice, and it has been associated with an intestinal dysbiosis[203]. Treatment with the folic acid metabolite and TCR-blocking ligand, acetyl-6-formylpterin, decreased IL-17A production in the adipose tissue and ileum, prevented dysbiosis, and improved insulin sensitivity and glucose intolerance[203]. This mouse model of obesity has supported the clinical observations in obese individuals by implicating pro-inflammatory MAIT cells in adipose tissue as a potentially treatable factor contributing to insulin resistance and diabetes risk.

MAIT cell role in NAFLD, obesity, and diabetes: MAIT cells may have a protective role in patients with NAFLD by creating an anti-inflammatory cytokine milieu that polarizes M2 macrophages[51] (Figure 2). This protective role has been supported by animal studies that have demonstrated the development of severe hepatic steatosis[51] or exacerbated diabetes[50] in MAIT cell-deficient mice. MAIT cells may also have a pro-inflammatory effect that contributes to insulin resistance. This possibility has been supported by studies in obese patients[106], diabetic patients[50], and murine models of obesity[203] and diabetes[50]. The clinical and experimental studies in NAFLD, obesity, and diabetes underscore the diversity of MAIT cell activities and the need to better understand the factors that influence these activities within the involved tissue. This understanding is essential before considering targeted therapeutic manipulations.

MAIT cell deficiencies do improve as obesity and diabetes are successfully treated [49,50]. However, the relationship between correction of the MAIT cell deficiency and improvement of the metabolic disease remains conjectural. Bariatric surgery and subsequent weight loss have not normalized MAIT cell frequency or decreased IL-17 production[49]. Furthermore, insulin treatment of type 1 diabetes has not normalized the MAIT cell phenotype in children[50]. The possibility of a genetic predisposition for MAIT cell deficiency or a disease-acquired impairment that persists long-term has not been excluded. Future investigations of MAIT cells in NAFLD and its metabolic co-morbidities will need to define more fully the disease response as a function of MAIT cell activity.

MAIT cells and autoimmune hepatitis

The MAIT cell abnormalities described in untreated autoimmune hepatitis mirror those recognized in other forms of chronic hepatitis. Circulating MAIT cells are activated but depleted[52,53], and they are functionally altered in a manner consistent with immune exhaustion (reduced production of granzyme B and IFN-γ)[52,53] (Figure 2). The number of intrahepatic MAIT cells varies from low[52] to high[53], and the bases for this variation remain uncertain (Table 4). The key insight has been the recognition of MAIT cells as pivotal pro-inflammatory and pro-fibrotic agents.

The frequency of circulating, activated MAIT cells has been significantly higher (P = 0.009) in patients with autoimmune hepatitis and advanced hepatic fibrosis (fibrosis

score, F3-F4) than in patients with little or no fibrosis (fibrosis score, F0-F2)[53]. Furthermore, blood levels of IL-17 have been increased in patients with autoimmune hepatitis[204]. IL-17A, which is a product of activated MAIT cells, is a recognized pro-inflammatory and pro-fibrotic cytokine[11,101]. This production has been robust despite features of MAIT cell exhaustion in autoimmune hepatitis[52].

MAIT cells stimulate proliferation of hepatic stellate cells and increase the expression of genes that regulate the production of pro-fibrotic (collagen 1, lysyl oxidase, and tissue inhibitor of metalloproteinase 1) and pro-inflammatory (IL-1β, IL-6, IL-8 and CCL2) molecules[52]. These deleterious actions could help explain progressive hepatic fibrosis in autoimmune hepatitis. They have also been implicated in ALD[63].

MAIT cells have been associated mainly with detrimental pro-inflammatory and pro-fibrotic effects in autoimmune hepatitis[52,53] (Figure 2). Glucocorticoids have decreased the frequency of circulating MAIT cells by 23% in asthmatic patients[177], and they have altered MAIT cell populations in other autoimmune diseases[15,177,185]. Studies that explore the effects of glucocorticoids on MAIT cell numbers and function in patients with autoimmune hepatitis could clarify their pathogenic role and further validate or improve current corticosteroid-based management strategies[92].

MAIT CELLS IN CHRONIC CHOLESTATIC LIVER DISEASE AND DECOMPENSATED CIRRHOSIS

MAIT cells have been evaluated in patients with chronic cholestatic liver disease[54-57] and patients with decompensated cirrhosis[58] (Figure 2). The findings strengthen perceptions already developed in the various forms of chronic hepatitis. MAIT cells are common components of the host response to liver injury independent of etiology or clinical phenotype, and they may be protective, pathogenic, or both protective and pathogenic. The modifiable factors that direct these actions remain unclear and warrant further investigation.

MAIT cells and PBC

MAIT cells in PBC have abnormalities reminiscent of the findings in chronic hepatitis. Circulating MAIT cells are decreased in frequency compared to control subjects[54,55]; intrahepatic MAIT cells are reduced[54] or increased[55] in number; expression of the liver-homing chemokines, CXCR6 and CCR6, are up-regulated; and cytokine production is altered[54] (Table 5). Immune exhaustion has been invoked as a basis for aberrant MAIT cell function[54,55]. Apoptosis, attributable to AICD, has been proposed as a reason for MAIT cell depletion[54,55]. The key insight has been the recognition of MAIT cells as a pivotal immune regulatory population with complex and possibly contradictory actions.

MAIT cells may have a protective effect in PBC. The frequency of circulating MAIT cells has correlated negatively with the serum alkaline phosphatase level, suggesting that the loss of a protective MAIT cell function may permit worsening cholestasis[55] (Table 5). MAIT cell production of IL-17 is increased, and the production of IFN-γ is reduced[55]. IFN-γ is a cytokine that can impair hepatic stellate cell activation[205-207], and the reduced production of IFN-γ by MAIT cells could favor the pro-fibrotic actions of IL-17 in PBC[55,207]. MAIT cells preferentially infiltrate the portal tracts in PBC, and the expression of MR1 is up-regulated in hepatocytes, injured biliary epithelial cells, and inflammatory cells[55]. MR1-mediated stimulation of MAIT cells, possibly by bacterial ligands derived from the intestinal microbiome, could be a protective antimicrobial mechanism in PBC[55].

MAIT cells may also have a pro-inflammatory effect in PBC. The association of MAIT cell activation with elevated serum alanine aminotransferase levels suggests that the MAIT cell response could enhance inflammatory activity[54]. Furthermore, IL-7 stimulates the production of inflammatory cytokines and granzyme B by MAIT cells [55], and IL-7 is increased in the plasma and liver tissue of patients with PBC[55] (Table 5). The bile acid, cholic acid, up-regulates the expression of IL-7 in hepatocyte lines, and the intrahepatic MAIT cells in PBC may develop a pro-inflammatory phenotype[55]. Circulating MAIT cells have reduced expression of receptors for IL-7 (IL-7R) and IL-18 (IL-18R) in PBC, possibly because of immune exhaustion, and it is unclear how this observation might affect the intrahepatic MAIT cell response to IL-7 [54].

Table 5 Mucosal-associated invariant T cells in cholestatic liver disease and decompensated cirrhosis

Liver disease	MAIT cell features	Clinical implications
PBC	Circulating MAIT cells decreased[54,55]; Intrahepatic MAIT cells variable [54,55]; Upregulated liver-homing CXCR6, CCR6[54]; Aberrant MAIT cell function[55]; Depletion associated with increased AP[55]; Low IFN-γ unable to impair HSC activation[55]; Preferential portal tract distribution [55]; Activation associated with increased ALT[54]; Cholic acid-induced hepatocyte IL-7[55]; IL-7-induced pro-inflammatory cytokines[55]; Limited expression of IL-7R and IL-18R[54]	Immune exhaustion[54,55]; Apoptosis-based depletion (AICD)[54,55]; Unable to prevent cholestasis[54,55]; Unable to inhibit hepatic fibrosis[55]; Defective barrier to gut-derived ligands[55]; Pro-inflammatory cytokine milieu [54,55]; UDCA improves but not restorative[54,55]; Presumed defective protective role[54,55]; Presumed active pathogenic role[54,55]
PSC	Circulating MAIT cell frequency reduced[57]; Intrahepatic MAIT cell frequency less[56]; CD69, CD56, PD-1, and CD39 expressed[57]; Impaired response to bacteria[57]; Abundant extrahepatic bile duct MAIT cells[57]	Activated and immune exhausted[57]; Depleted in circulation and liver tissue[56,57]; Less anti-bacterial protection[57]; Abundant migration to bile ducts[57]; Presumed defective protective role[57]
Decompensated cirrhosis	Circulating MAIT cell frequency reduced[58]; High expression of activation markers[58]; MAIT cell frequency increased in ascites[58]; Increased cytokines from peritoneal cells[58]; Increased granzyme B from peritoneal cells[58]; Increased frequency in SBP ascites[58]; Homing chemokine CXCR3 on MAIT cells[58]; Abundant CXCL10 ligand in ascites [58]	Activated and recruited to ascites[58]; Anti-microbial protective response[58]; Protective role of uncertain efficacy[58]

AICD: Activation-induced cell death; ALT: Serum alanine aminotransferase level; AP: Serum alkaline phosphatase level; HSC: Hepatic stellate cells; IFN-γ: Interferon-gamma; IL-7: Interleukin 7; IL-7R: Interleukin 7 receptor; IL-18R: Interleukin 18 receptor; MAIT: Mucosal-associated invariant T; PBC: Primary biliary cholangitis; PD-1: Programmed cell death 1; PSC: Primary sclerosing cholangitis; SBP: Spontaneous bacterial peritonitis; UDCA: Ursodeoxycholic acid.

Therapy with ursodeoxycholic acid (UDCA) for at least 6 mo in 24 patients with PBC has increased the frequency and absolute number of circulating MAIT cells in those patients whose liver tests had improved[55]. The expression of the activation marker, CD25, has decreased; the percentage of apoptotic MAIT cells has diminished; and the frequency of MAIT cells producing IL-17A and granzyme B has decreased. Similar improvements in the frequency and absolute number of MAIT cells have also been demonstrated in 7 patients who had normalized liver enzymes after 6 mo of UDCA therapy[54]. In this series, the frequency of MAIT cells and the expression of IL-7R and IL-18R did not fully recover.

MAIT cells and PSC

Studies of MAIT cells in PSC have been limited, and the findings have been familiar (Figure 2). The frequency of circulating MAIT cells has been dramatically reduced in PSC, and the reduction has been similar to that encountered in patients with inflammatory bowel disease or PBC[57] (Table 5). The MAIT cell depletion has not been associated with particular clinical characteristics, and the MAIT cells have expressed markers of activation (CD69 and CD56) and immune exhaustion (PD-1 and CD39)[57]. The MAIT cell response to bacteria has been reduced, and the cytokine-dependent response has also been impaired[57]. MAIT cells have localized mainly to fibrotic areas in liver samples, and the overall intrahepatic population has been less than in donor or resected liver specimens[56].

The key finding in these studies has been the demonstration of abundant MAIT cells within the biliary tract by brushings obtained at endoscopic retrograde cholangiography[57] (Table 5). The proportion of MAIT cells in the biliary brush samples has been four-fold greater than in matched peripheral blood samples, and the MAIT cell accumulation at the site of inflammation has been consistent with a chronic protective response against intestinal pathogens in the biliary mucosa[57]. Patients with PSC have a high frequency of bacteria in bile cultures, especially with dominant strictures [208-210], manifest intestinal dysbiosis[211,212], and improve clinically after antibiotic therapy[213-216]. These observations have supported the concept that bacterial by-products from the intestinal microbiome stimulates immune-mediated damage to the biliary tree and liver and that MAIT cells have a protective, antimicrobial function in PSC[67,210].

MAIT cells and decompensated cirrhosis

The protective, anti-microbial role of MAIT cells in chronic liver disease has been supported by studies of MAIT cell frequency and function in patients with cirrhosis and hepatic decompensation (Table 5). The frequency of circulating MAIT cells has been reduced in these patients, and the MAIT cells have had an activated phenotype (high levels of HLA-DR, CD25, CD38, CD56)[58]. The frequency of MAIT cells has

been increased in the ascites, and the highly activated peritoneal MAIT cells have produced higher levels of IFN-γ and granzyme B than the MAIT cells in matched blood samples[58].

MAIT cell frequency and total number have also been significantly increased in the ascites of patients with spontaneous bacterial peritonitis compared to patients with uninfected ascites[58] (Table 5). The peritoneal MAIT cells have lacked markers of tissue residency, and they have not been actively proliferating[58]. These features have suggested that the MAIT cells have migrated to the ascites from the circulation. The expression of the homing chemokine, CXCR3, by the peritoneal MAIT cells and the presence of high levels of CXCL10 in the ascites have also supported this conjecture.

MAIT cells in decompensated cirrhosis may have a protective function in uninfected ascites and a critical antimicrobial function in infected ascites. Studies that evaluate interventions to expand and strengthen the MAIT cell population in ascites might improve the control of bacterial infection in patients with decompensated cirrhosis. Investigations in a murine model have already demonstrated that IL-23 combined with the MR1-ligand, 5-OP-RU, can protect against pulmonary infection with Legionella [217], and they encourage similar evaluations in models of advanced liver disease.

INCORPORATING MAIT CELLS INTO THE PATHOGENESIS OF CHRONIC LIVER DISEASE

The incorporation of MAIT cells into the pathogenesis of chronic liver disease requires clarification of the role of MAIT cells in each type of chronic liver disease and demonstration that MAIT cell manipulation can affect disease severity and outcome.

Clarification of the disease-related role of MAIT cells

MAIT cells have been proposed as the new guardians of the liver[17], and patients with chronic viral hepatitis, alcoholic hepatitis, PSC, and decompensated cirrhosis could be protected by the antimicrobial actions of fully functioning MAIT cells[2-4,30,218]. Similarly, patients with NAFLD might be protected by the anti-inflammatory cytokines (IL-4, IL-5, IL-10, and IL-13) generated by MAIT cells in the liver[51] and in adipose tissue[49,106]. Conversely, patients with autoimmune hepatitis could be disadvantaged by abundant MAIT cell production of pro-inflammatory and pro-fibrotic cytokines[52,53], and patients with PBC could be either disadvantaged by MAIT cell production of pro-inflammatory cytokines[55] or benefited by antibacterial actions against products from the intestinal microbiome[55].

The variability of the presumed consequences of MAIT cell depletion or dysfunction in the different liver diseases suggests that local factors in the hepatic microenvironment modulate the nature of the MAIT cell response. These factors may include local inflammatory cues (cytokine milieu)[219], bile acids (cholic acid)[55], gut-derived bacterial products (LSP, endotoxin, antigens)[46,47,69], and metabolic by-products (riboflavin metabolites, glyoxal, methylglyoxal)[9,13,14,151,152].

MAIT cells have different activation thresholds which can modulate their response to non-commensal antigens. Inflammatory signals derived from IL-12, IL-15, or IL-18 elicit robust antiviral (IFN-γ) and antibacterial (granzyme B) responses from MAIT cells[219], whereas direct TCR stimulation induces a brief, less robust release of IFN-γ and TNF-α and a less vigorous effector response[219]. The most potent effector function is elicited when both the inflammatory (cytokine-mediated) and TCR (antigen-mediated) signals are delivered concurrently to the MAIT cells[219]. Conditions at the site of inflammation could modify the activation thresholds for various MAIT cell functions and elicit antimicrobial, anti-inflammatory, or pro-inflammatory actions that are situation-specific[219,220]. Clarification of the conditions that drive the MAIT cell response toward a protective or pathogenic nature are needed to develop targeted, disease-appropriate, therapeutic interventions[220].

Manipulating MAIT cells to assess effects on disease severity and outcome

MAIT cell manipulations are necessary to establish the impact of MAIT cells on disease severity and outcome. Animal studies have demonstrated that genetically manipulated mice without MR-1 and MAIT cells exacerbate experimental NAFLD [51], whereas studies that demonstrate the effect of restoring the MAIT cell population are lacking. These studies would validate a critical pathogenic relationship that is necessary before considering therapeutic manipulations of MAIT cells in clinical trials.

MAIT cells can be up-regulated and down-regulated by several interventions that could help determine their pathogenic role in animal models. MAIT cells are immune exhausted in chronic liver disease, and they may be less responsive to MR1-dependent stimulation[82]. Cytokine-mediated stimulation may be the preferred method of restoring immune exhausted MAIT cells, and recombinant IL-7 has the potential to improve MAIT cell proliferation, cytokine production, effector (granzyme B) function, and MR1-mediated activation[82,107]. Furthermore, the administration of recombinant IL-7 to patients with chronic HIV infection has restored the integrity of the intestinal mucosal barrier[221]. Protection from gut-derived bacterial products by the administration of recombinant IL-7 might have a particular advantage in experimental models of alcoholic hepatitis[46,47], PBC[55], or PSC[57]. IL-15, IL-1β and IL-23 are other cytokines that can activate MAIT cells, but they have less potency than IL-7[82,158,222]. Cytokines of less potency or in various combinations may achieve the desired result without generating opposite or adverse effects.

Drugs and drug-like molecules can also modulate the function of MAIT cells as ligands that bind to MR-1[153]. Diclofenac metabolites can promote MAIT cell activity, and salicylates can inhibit this activity. Drugs have the promise of achieving the MAIT cell response appropriate for the individual disease as an agonist or antagonist of MAIT cell activity. Other therapeutic possibilities are *ex vivo* re-programming of MAIT cell function with select cytokines[82,107], vaccination with IL-23 in combination with 5-OP-RU[217], and re-programming and re-differentiating MAIT cells using induced pluripotent stem cells derived from MAIT cells[223]. Glucocorticoids reduce MAIT cell frequency in certain immune-mediated diseases[15,177,185], and they also may have a role in modulating MAIT cell activity.

Specific molecular interventions that could down-regulate MAIT cell activity are 3-([2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl]formamido) propanoic acid which can impair cell surface expression of MR1 and prevent antigen recognition[155] and acetyl-6-formylpterin, which can inhibit MAIT cell function by competing with other bacterial products for MR1 ligation[143,150]. These molecular interventions have the potential to clarify mechanisms of MR1 expression and function and possibly emerge as experimental agents by which to modulate MAIT cell activity.

MAIT cell manipulation will be an important experimental method to establish the protective and pathogenic importance of MAIT cells in chronic liver disease. This laboratory experience will be essential before considering MAIT cell intervention as a therapeutic strategy in patients.

CONCLUSION

MAIT cells are innate-like T lymphocytes with antimicrobial and immune regulatory properties that are activated, immune exhausted, and dysfunctional in the different types of chronic liver disease. The similarity of findings in diverse liver and non-liver diseases of an infectious or non-infectious nature suggests that MAIT cells are a disease-nonspecific, T cell response to chronic inflammation.

MAIT cells may have a protective function in chronic viral hepatitis, alcoholic hepatitis, NAFLD, and PSC, exert pro-inflammatory and pro-fibrotic actions in autoimmune hepatitis, and have protective and pathogenic effects in PBC. The variable effects of MAIT cells despite their similar clinical phenotype suggests that disease-related factors in the hepatic microenvironment (inflammatory cues, bile acids, gut-derived bacterial products, and metabolic by-products) influence MAIT cell function.

Future investigations must establish the role of MAIT cells as critical determinants of disease severity and outcome in chronic liver disease, identify the local disease-related factors that drive their particular functions, and evaluate interventions that modulate their protective and pathogenic properties in a disease-appropriate manner.

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REVIEW

Artificial intelligence in small intestinal diseases: Application and prospects

Yu Yang, Yu-Xuan Li, Ren-Qi Yao, Xiao-Hui Du, Chao Ren

ORCID number: Yu Yang 0000-0002-0549-8755; Yu-Xuan Li 0000-0003-2568-6347; Ren-Qi Yao 0000-0002-3173-4301; Xiao-Hui Du 0000-0002-8713-1358; Chao Ren 0000-0003-0655-0209.

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Yu Yang, Yu-Xuan Li, Xiao-Hui Du, Department of General Surgery, Chinese People's Liberation Army General Hospital, Beijing 100853, China

Ren-Qi Yao, Chao Ren, Trauma Research Center, The Fourth Medical Center and Medical Innovation Research Division of the Chinese People's Liberation Army General Hospital, Beijing 100048, China

Ren-Qi Yao, Department of Burn Surgery, Changhai Hospital, Naval Medical University, Shanghai 200433, China

Corresponding author: Chao Ren, MD, PhD, Trauma Research Center, Fourth Medical Center and Medical Innovation Research Division of the Chinese People's Liberation Army General Hospital, No. 51 Fucheng Road, Beijing 100048, China. rc198@sina.com

Abstract

The small intestine is located in the middle of the gastrointestinal tract, so small intestinal diseases are more difficult to diagnose than other gastrointestinal diseases. However, with the extensive application of artificial intelligence in the field of small intestinal diseases, with its efficient learning capacities and computational power, artificial intelligence plays an important role in the auxiliary diagnosis and prognosis prediction based on the capsule endoscopy and other examination methods, which improves the accuracy of diagnosis and prediction and reduces the workload of doctors. In this review, a comprehensive retrieval was performed on articles published up to October 2020 from PubMed and other databases. Thereby the application status of artificial intelligence in small intestinal diseases was systematically introduced, and the challenges and prospects in this field were also analyzed.

Key Words: Artificial intelligence; Machine learning; Deep learning; Prognosis prediction; Small intestinal diseases

Core Tip: Artificial intelligence has been widely used in the management of small intestinal diseases, which has greatly improved the diagnostic efficiency of capsule

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endoscopy and other examination methods, and at the same time, beneficial progression has also been obtained in the prognosis prediction of small intestinal diseases. Although AI still faces risks such as overfitting and black box effects, its stability and efficiency give it great potential in the management of small intestinal diseases. This article reviews the current application status of AI in small intestinal diseases. In addition, challenges and prospects in this field are discussed.

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INTRODUCTION

The small intestine is located in the middle of the gastrointestinal digestive system, with a total length of 5 m to 7 m, including the duodenum, jejunum and ileum, and is the longest organ of the digestive system. Small intestinal diseases (SIDs) mainly include celiac disease (CD), small intestinal Crohn's disease (SICD), primary small intestinal tumor (PSIT), obscure gastrointestinal bleeding and so on. The traditional examination methods include X-ray barium enterography, computed tomography (CT), magnetic resonance imaging (MRI), balloon-assisted enteroscopy, deep enteroscopy and so on. In recent years, the emergence of capsule endoscopy (CE) has brought a revolutionary breakthrough for the diagnosis of SIDs. However, because of the special anatomical position of the small intestine (far away from the oral cavity and anus, overlap and peristalsis), there are still many problems in the diagnosis of SIDs, such as high technical requirements, low positive rate of diagnosis, inaccurate qualitative location of the disease, patient intolerance and so on. In addition, the onset of SIDs is insidious, the specificity of clinical symptoms is low, and the lesion site is not easy to explore, so the clinical diagnosis of SIDs has always been a difficult problem. With the emergence of artificial intelligence (AI) and its wide application in the medical field, it also provides new methods for the whole management process of SIDs and greatly improves the efficiency of SIDs management.

AI is a concept put forward in the 1950s. It is a frontier cross discipline developed on the basis of computer science, neuropsychology, philosophy, linguistics, cybernetics, information theory and so on[1]. The research fields of AI include expert system, machine learning (ML), fuzzy logic, natural language processing and so on. Research methods are also developing continuously, from ML to deep learning and then to convolutional neural network (CNN), promoting the rapid development of research in various fields. The research of AI in the medical field is mainly focused on auxiliary diagnosis. This series of methods of AI has become a hot implementation tool in the field of medical imaging and digestive endoscope[2,3]. Taking the experiment of the breast cancer AI detection system established by Google as an example, the computer-aided diagnosis system based on AI can help doctors reduce the misdiagnosis rate of breast cancer by 5.7%[4]. Researchers at Houston Methodist Hospital also said in their study that they have developed AI software that parses breast X-ray images 30 times faster than ordinary doctors, with an accuracy of 99%[5]. AI is widely used in the study of digestive fields such as gastric cancer[6], colorectal cancer[7], esophageal cancer[8] and so on. AI has also been extensively researched in the field of SIDs, which will be introduced in this paper.

This study used the keywords of "artificial intelligence" and "small intestine" to search the relevant literature in the databases of PubMed, Embase, Web of Science and Cochrane Library up to October 2020. Studies included in our review were required to meet the following inclusion criteria: (1) full-text paper available in English; and (2) studies that associated AI with the small intestinal diseases. We excluded descriptive papers without validation of methods. The application status of AI in SIDs was summarized, and the challenges and prospects in this field were discussed.

AI IN SMALL INTESTINE ANATOMY

Organ segmentation of the small intestine

With the advent of AI, it is possible to perform computer-assisted organ segmentation in CT, MRI, endoscopy and other examination methods and has shown good application potential in the fields like assisted localization of radiotherapy. The following will introduce the research progress of this aspect in the field of the small intestine especially the duodenum (Table 1).

CT: Some studies had used the CNN method to automatically segment duodenum and other abdominal organs from CT images, with clinically acceptable accuracy and efficiency[9,10]. Tong *et al*[11] proposed an end-to-end segmentation network for improving multiorgan segmentation performance using the ML method. The dice similarity coefficient and average surface distance were quantitatively evaluated, and the results confirmed this network had good accuracy and timeliness in the anatomical segmentation of abdominal organs including the duodenum.

MRI: Fu *et al*[12] conducted a retrospective analysis on 3D MR images of 120 patients and proposed a CNN model, which has been verified to accurately segment the abdominal organs including the duodenum and expedite the contouring process for MRI-guided adaptive radiotherapy. Chen *et al*[13] also conducted a similar study, and in their study, the inference process was completed within 1 min, indicating an obvious advantage of timeliness. The length of the small intestine is an important factor in the management of patients with short bowel syndrome. Some scholars designed a special software algorithm to calculate the length of small intestine based on magnetic resonance enterography images in mice. Compared with the measured results of anatomical specimens, the mean absolute difference between the two methods was 1.8 ± 3.8 cm (P = 0.24), and the mean percentage difference was 9.4% ± 6.0%[14].

Endoscopy: In a Japanese study based on GoogLeNet architecture, a CNN diagnostic program was constructed, using 27335 esophagogastroduodenoscopy (EGD) images for the training set and using 17081 EGD images for the independent validation set. The results showed that the CNN has a good effect to classify the anatomical location of EGD images for stomach and duodenum images, with an area under the curve of 0.99[15]. Igarashi *et al*[16] used AlexNet (a deep learning framework) to retrospectively analyze 85246 original images of EGD images in 441 patients with gastric cancer and developed an anatomical organ classifier. The accuracy rates of the training and validation sets were 0.993 and 0.965, respectively.

Diagnosis of small intestinal mucosal lesions

With the emergence of CE in 2000[17], it has revolutionized our understanding of small intestinal mucosa[18-20], enabling doctors to detect small intestinal mucosal erosion, ulcers, vascular disease, bleeding, polyps, parasite and other lesions more efficiently. However, reliable and rapid reading of video is still a challenge, but more and more studies have shown that the combination of AI and CE can greatly improve the efficiency of our evaluation of small intestinal mucosal lesions; the detection accuracy was above 90% in most studies[21-27].

Ulcer: Previous studies have confirmed that applying a CNN system of deep learning to the reading process of CE can reduce the reading time without decreasing the detection rate of erosion and ulcer lesions[28-32].

Angioectasias and bleeding: Intestinal angioectasias cause more than 8% of all gastrointestinal bleeding episodes[33]. Different studies have applied ML, CNN and computer algorithms to the differential diagnosis of intestinal angioectasias and have achieved high sensitivity and specificity[34-39]. AI is also applied to the direct examination of intestinal mucosal bleeding by CE, which can directly calculate the blood content in the digestive tract and infer whether there is active bleeding in the small intestinal mucosa[40-44].

Protruding lesions: There are a variety of small intestinal mucosal protruding lesions. CNN can help doctors describe their shape features, help analyze their nature and distinguish polyps, epithelial tumors, submucosal tumors, *etc.*[34,45,46].

Villous atrophy: Villous atrophy is a defining symptom of some digestive tract diseases such as CD. Some scholars combined AI methods with CE for the detection

Table 1 Applications of artificial intelligence in organ segmentation of the small intestine

Ref.	Diagnostic method	AI technology	Training set	Validating set	Outcomes
Tong *et al*[11]	CT	ML	90 images	-	DSC of duodenum: 69.26%
Kim *et al*[9]	CT	CNN	80 images	40 images	DSC of duodenum: 0.595
Peng *et al*[10]	CT	CNN	43 images	-	DSC of duodenum: 0.61
Fu *et al*[12]	MRI	CNN	100 images	20 images	Dice coefficient of duodenum: 65.50% ± 8.90%
					Dice coefficient of bowel: 86.60% ± 2.69%
Chen *et al*[13]	MRI	DL	66 images	36 images	DSC of duodenum: 0.80
Takiyama *et al*[15]	EGD	CNN	27335 images	17081 images	AUCs: 0.99
Igarashi *et al*[16]	EGD	ML	49174 images	36072 images	Accuracy (Ts: 0.993, Vs: 0.965)

AI: Artificial intelligence; AUCs: Area under the curves; CNN: Convolutional neural network; CT: Computed Tomography; DL: Deep learning; DSC: Dice similarity coefficient; EGD: Esophagogastroduodenoscopy; ML: Machine learning; MRI: Magnetic resonance imaging; Ts: Training set; Vs: Validating set.

and measurement of villous atrophy and successfully mapped the extent of the diseased small intestine[47].

AI is also used in risk prediction and clinical treatment decisions of small intestinal mucosal lesions. For example, one study used CNN for the risk prediction of acute intestinal bleeding[48], and another study applied CNN to risk prediction and therapeutic tactics selection for duodenal ulcers[49]. Wong *et al*[50] built a ML model, based on data from 22854 patients with gastroduodenal ulcer including six clinical parameters to identify patients at high risk for recurrent ulcer bleeding within 1 year. Gastrointestinal bleeding is a common complication of left ventricular assist device treatment. Axelrad *et al*[51] developed an endoscopic algorithm. Compared with conventional cohorts, the implementation of the algorithm increased endoscopic diagnostic efficiency by 68%, treatment efficiency by 113%, the number of procedures per patient decreased by 27%, the length of hospital stay decreased by 33%, and the estimated cost decreased by 18%.

In addition, the interference of intestinal contents to CE can also be reduced by AI. Combined with support vector machine, Bashar *et al*[52] designed a classifier for separating useless frames that are highly contaminated by turbid fluids, fecal materials and/or residual foods. The accuracy of this classifier was more than 80%. Pietri *et al*[53] developed a computer algorithm to automatically evaluate the demeanor of small intestinal bubbles in CE images. The specificity of this algorithm was 95.79%, the sensitivity was 95.19%, and the calculation time was 0.037 s per frame. It can be used to reduce the interference of bubbles in CE images. Klein *et al*[54] created a computed algorithm based on the pixels in the color bar to score and classify the preparation of the small intestine for CE, and this automatic scoring method has a concordance rate of more than 90% with the assessment of clinicians.

AI IN COMMON SMALL INTESTINAL DISEASES

AI in celiac disease

CD is a complex autoimmune disease. Patients who ingest foods containing gluten will develop an autoimmune response that causes damage to the small intestine. CD is one of the most common chronic digestive diseases, with a prevalence rate of 1% worldwide[55]. Duodenal biopsy is the gold standard for diagnosis[56]. Noninvasive methods such as endoscopy and clinical features analysis are also widely used in diagnosis, but the diagnostic rate of CD is only 15%–20% through current strategies [57]. However, with the increasing application of AI in the diagnosis of CD, the accuracy and efficiency of diagnosis are greatly improved[58] (Table 2).

Previous studies have confirmed that AI-assisted duodenoscope images analysis can greatly improve the diagnostic efficacy of CD, with the accuracy between 80% and 100% and specificity and sensitivity over 80%[59-61]. In the diagnosis of CD, the combination of AI and CE is closer, which can improve the accuracy of diagnosis and

Table 2 Applications of artificial intelligence in celiac disease

Ref.	Diagnostic method	AI technology	Training set	Testing set	Outcomes
Chetcuti *et al*[62]	CE	ML	81 patients	-	Accuracy: 75.3%
Li *et al*[63]	CE	Computer-assisted recognition	Ep: 240, Cp: 220	-	Accuracy: 93.9%
Vicnesh *et al*[64]	CE	Computerized algorithm	21 patients	-	Accuracy: 89.82%
Zhou *et al*[65]	CE	CNN	Ep: 6, Cp: 5	Ep: 5, Cp: 5	Accuracy: 100%
Gadermayr *et al* [59]	EGD	Computer-assisted	290 patients (2835 images)	-	Accuracy: 94%-100%
Das *et al*[67]	Mucosal biopsies	Computer-assisted	Ep: 124, Cp: 137	Ep: 120, Cp: 105	Sen: 90.3%, Spe: 93.5%, AUCs: 96.2%
Wei *et al*[66]	Mucosal biopsies	DL	212 images	-	Accuracy: 95.3%, AUCs > 0.95
Pastore *et al*[70]	Clinical data	Computer-assisted	100 patients	-	Reliability: 0.813
Tenório *et al*[60]	Clinical data	Decision trees, Bayesian inference, k-nearest neighbor algorithm, support vector machines, artificial neural networks	178 patients	38 patients	Accuracy: 80.0%, Sen: 0.78, Spe: 0.80, AUCs: 0.84
Virta *et al*[68]	Micro-CT	Computer-assisted point cloud analysis	81 patients	-	Accuracy: 100%
Sangineto *et al*[69]	Gene expression in PBMCs	ML, random forest algorithm	Ep: 17, Cp: 20	-	Accuracy: 100%

AI: Artificial intelligence; AUCs: Area under the curves; CE: Capsule endoscopy; CNN: Convolutional neural network; Cp: Control group; DL: Deep learning; EGD: Esophagogastroduodenoscopy; Ep: Experimental group; ML: Machine learning; micro-CT: X-ray microtomography; PBMCs: Peripheral blood mononuclear cells; Sen: Sensitivity; Spe: Specificity.

significantly save the diagnosis time[62-65]. AI was also used in the analysis of duodenal mucosa biopsy, which can help with qualitative analysis and play an important role in quantitative analysis[66,67]. At the same time, the application of AI with X-ray images[68], peripheral blood mononuclear cells[69] and clinical features[60,70] in the diagnosis and classification of CD have also achieved progress.

AI in small intestinal Crohn's disease

Crohn's disease is a chronic nonspecific inflammatory bowel disease that affects the entire gastrointestinal tract, in which 30% of patients are confined to the small intestine, commonly known as small intestinal Crohn's disease[71]. SICD most often involves the distal ileum as well as the jejunum and the digestive tract above and has a higher incidence of intestinal strictures than colonic Crohn's disease[72,73]. The application of AI in the management of SICD is comprehensive, including diagnosis, risk prediction, extra-intestinal manifestation (EIM) prediction and so on (Table 3).

Diagnosis: Lamash *et al*[74,75] used CNN to analyze MRI images and construct an assessment model for SICD. Their model could effectively distinguish active and inactive inflammatory segments, distinguish segments with strictures and segments without strictures and could be used to measure the length of intestinal strictures. Parfenov *et al*[76] used a software diagnostic algorithm to analyze the CE images of 25 SICD patients, preliminarily confirming that CE could be used to diagnose early SICD with intestinal mucosal inflammation. Klang *et al*[77] performed automatic analysis of CE images of 49 SICD patients using a CNN method, achieving diagnostic accuracy of more than 95% and significantly reducing reading time. Yang *et al*[78] also attempted to combine CNN with a microultrasound system for early diagnosis of SICD in mice and achieved good effectiveness in the identification of early inflammation.

Risk prediction of SICD: Taylor *et al*[79] used ML classifiers (elastic network and random forest) to classify small intestine inflammation in asymptomatic first-degree relatives of patients with SICD. They found that genetic variants associated with SICD, family history and fecal calprotectin together identified individuals with presymptomatic intestinal inflammation who are therefore at risk for SICD. Shen *et al*[80] developed a web-based SICD hazard stratification tool. Predicting high-risk populations for SICD based on altered bowel habit, abdominal pain, white blood cell

Table 3 Applications of artificial intelligence in small intestinal Crohn's disease

Ref.	Diagnostic method	AI technology	Training set	Testing set	Outcomes
Yang *et al*[78]	Microultrasound	CNN	43 mice	-	AUCs: 0.8831
Shen *et al*[80]	Clinical data	Computerized algorithm	Ep1: 61, Cp1: 78	Ep2:42, Cp2: 57; Ep3:84, Cp3: 495	AUCs: 0.92
Bottigliengo *et al* [81]	Clinical data	BMLTs (NB, BN, BART)	152 patients	-	AUCs without genetic variables (NB: 0.71, BN: 0.50, BART: 0.76), AUCs with genetic variables (NB: 0.75, BN: 0.67, BART: 0.78)
Taylor *et al*[79]	Clinical data	ML (elastic net and random forest)	480 first-degree relatives	-	AUCs (elastic net): 0.80, AUCs (random forest): 0.87
Menti *et al*[82]	Clinical data	BMLTs	152 patients	-	Accuracy without genetic variables: 82%, accuracy with genetic variables: 89%
Klang *et al*[77]	CE	DL	49 patients (17640 images)	-	AUCs: 0.94-0.99, accuracy: 95.4%-96.7%
Parfenov *et al*[76]	CE	Computerized algorithm	25 patients	-	44% patients confirmed only with the help of AI
Lamash *et al*[74, 75]	MRI	CNN	15 patients	8 patients	Dice coefficients: 75%-97%

AI: Artificial intelligence; AUCs: Area under the curves; BART: Bayes additive return trees; BMLTs: Bayesian machine learning techniques; BN: Bayesian network; CE: Capsule endoscopy; DL: Deep learning; CNN: Convolutional neural network; Cp: Control group; Ep: Experimental group; ML: Machine learning; MRI: Magnetic resonance imaging; NB: Naive Bayes.

count, albumin and platelet count abnormalities allowed clinicians to identify potential SICD earlier.

Risk prediction of EIMs: AI is controversial in the evaluation of the EIMs of SICD. In the study of Bottigliengo *et al*[81], based on Bayesian machine learning technology evaluation combined with genetic factors to predict the occurrence of EIMs in Crohn's disease, it has no advantage over traditional statistical tools. Whereas Menti *et al*[82] used Bayesian machine learning technology to predict the risk of occurrence of EIMs in Crohn's disease, and the prediction accuracy was 82% when considering only clinical factor and 89% combined with genetic factors, which was outperforming other prediction techniques.

AI in primary small intestinal tumor

The incidence of PSIT is about 5% of gastrointestinal tumors and 0.2% of all kinds of tumors[83,84]. The main site of PSIT is the duodenum, followed by the jejunum and ileum[85]. There are a variety of pathological types of malignant PSIT. Adenocarcinoma is the most common pathological type, up to 40%, followed by neuroendocrine tumors (25%), malignant lymphomas (10%-15%) and malignant stromal tumors (9%) [86]. PSIT lacks specific manifestations in the early stage, and they are faced with many problems in the clinic, such as difficult diagnosis, high misdiagnosis rate, nonstandard treatment and so on[87]. AI has been applied in the field of auxiliary diagnosis and prognostic analysis of PSIT, and has an important impact on the management (Table 4).

Diagnosis: Inoue *et al*[88] used CNN to analyze EGD images for the diagnosis of superficial nonampullary duodenal epithelial tumors. The overall diagnosis accuracy of CNN was 94.7%, including 94% for adenomas and 100% for high-grade dysplasias, and it only took 12-31 s for analysis. The method of support vector machine was applied to the automatic analysis of CE images, which greatly improved the accuracy and efficiency of diagnosis[89-92]. In addition, Barbosa *et al*[93] used the CNN to automatically analyze CE images for the diagnosis of PSIT, which also had high sensitivity and specificity, reaching 98.7% and 96.6%, respectively.

Risk stratification and prognosis prediction: In different studies, ML was used to analyze the pathological tissue samples, plasma protein multibiomarker and miRNA markers of patients with small intestinal neuroendocrine tumors[94-96]. Their studies provided some new and effective methods for early diagnosis, treatment strategy selection, prognosis prediction and recurrence risk prediction of small intestinal

Table 4 Applications of artificial intelligence in primary small intestinal tumor

Ref.	Diagnostic method	AI technology	Training set	Testing set	Outcomes
Inoue *et al*[88]	EGD	CNN	531 images	1080 images	Accuracy: 94.7%-100%
Liu *et al*[90]	CE	SVM	89 patients	-	Sen: 97.8%, Spe: 96.7%
Vieira *et al*[89,91]	CE	SVM	29 patients (936 images)	-	This SVM outperforms others by more than 5%
Barbosa *et al*[93]	CE	CNN	Ep: 104, Cp: 100	Ep: 92, Cp: 100	Sen: 98.7%, Spe: 96.6%
Panarelli *et al*[94]	MicroRNA sequencing	ML	84 samples	-	Accuracy (Ts: 98.5%, Vs: 94.4%)
Drozdov *et al*[95]	Gene expression profiling	ML	73 samples	-	Differentiated from normal cells (Sen: 100%, Spe: 92%), metastases prediction (Sen: 100%, Spe: 100%)
Kjellman *et al*[96]	Plasma protein multibiomarker	Random forestmodel	Ep:135, Cp: 143	-	AUCs: 0.97
Yan *et al*[97]	CT	Random forestmodel	213 patients	-	AUCs: 0.943

AI: Artificial intelligence; AUCs: Area under the curves; CE: Capsule endoscopy; CNN: Convolutional neural network; CT: Computed tomography; Cp: Control group; EGD: esophagogastroduodenoscopy; Ep: Experimental group; ML: Machine learning; SVM: Support vector machine; Sen: Sensitivity; Spe: Specificity; Ts: Training set; Vs: Validating set.

neuroendocrine tumors. In the study of Yan *et al*[97], random forest models were performed to evaluate the correlation of risk stratification for small intestinal stromal tumors. Their study suggested multidetector CT texture analysis may become an important comprehensive tool for preoperative risk stratification of small intestinal stromal tumors.

AI in other small intestinal diseases

Small intestinal obstruction: Cheng *et al*[98,99] used CNN to analyze abdominal radiographs to assist in the diagnosis of small intestinal obstruction (SIO). The sensitivity and specificity of the CNN diagnostic system were 83.8% and 68.1%, respectively, based on the training set of 2210 abdominal radiographs. When the training set was expanded to 7768 abdominal radiographs, the diagnostic sensitivity and specificity were increased to 91.4% and 91.9%, respectively. Their study suggests that the accuracy of detection of SIO by CNN improves significantly with the increasing number of training radiographs. Lucas *et al*[100] explored the development of an ML tool for SIO detection based on CT images. They evaluated the accuracy of eye tracking in image centerline annotation of the small intestine as the first step in the development of an ML tool for SIO. Their results showed that the eye tracking-based annotation was accurate and precise enough for application in ML-based small intestinal centerline annotation.

Small intestinal motor dysfunction: Small bowel intestinal dysfunction (SIMD) can occur during the development of many diseases, so the evaluation of small intestinal motor function is an important means for the auxiliary diagnosis and severity evaluation of these diseases. AI is widely used to evaluate small intestinal motor function through CE images. Using intraintestinal manometry as the gold standard for the diagnosis of SIMD, Malagelada *et al*[101] proved that an ML model was reliable to evaluate CE images for the diagnosis of SIMD. Applying this model, they found that 29% of patients with functional intestinal disorders had SIMD, significantly higher than that of the healthy population (3%), confirming the pathophysiological changes in the intestine of functional intestinal disorders[102]. Furthermore, a classification method for classifying functional intestinal disorders according to small intestinal motor function was also proposed[103].

De Iorio *et al*[104] also demonstrated that ML can reliably detect reduced intestinal muscle activity and motion by CE images through the method of injecting intestinal muscle inhibition (glucagon) into healthy subjects. Moreover, the study of Seguí *et al*[105] suggested that CNN was also reliable in the description and classification of small intestine motor characteristics with the classification accuracy reaching 96%. Malagelada *et al*[106] also used ML to analyze CE and abdominal MRI images of patients with cystic fibrosis and confirmed that the delay in small bowel and colonic transit times in patients with cystic fibrosis is associated with known endocrine

dysfunction and with SIMD.

Small intestinal ischemia-reperfusion injury: Intraoperative evaluation of intestinal viability in patients with acute intestinal ischemia is a critical factor for surgical decision making. In the pig jejunum experiment, Strand-Amundsen *et al*[107] attempted to apply ML to the analysis of multivariate time-series of bioimpedance sensor data to analyze intestinal viability after intestinal ischemia-reperfusion. The results suggested that the measurement should be made before the onset of reperfusion, and the prediction effect was better when the measurement was repeated continuously during ischemia and reperfusion. The detection accuracy of irreversible damage may be close to 100%.

Enteropathies associated with undernutrition: There is a significant histopathological overlap in duodenal biopsies of enteropathies associated with undernutrition such as environmental enteropathy and CD. Syed *et al*[108] used CNN to establish a histopathological analysis model, which can effectively distinguish environmental enteropathy, CD and normal intestinal mucosa. The detection accuracy was 93.4%, and the false-negative rate was 2.4%.

CHALLANGES AND PROSPECTS

At present, in order to promote the application of AI in the field of SIDs, we still need to solve some problems and challenges: (1) Insufficiency of training sample size. The incidence of most SIDs is not high. For example, the incidence of PSIT is much lower than that of gastric tumors and colorectal tumors, which affects the amount of data in the training set. Measurement errors are easy to occur when the sample size is small [109], and as suggested by the continuity study of Cheng *et al*[98,99], expanding the sample size of the training set can significantly improve the inspection accuracy of AI model; (2) Lack of prospective data. Most of the studies used retrospective data, which have been artificially screened before AI model training and lack prospective studies; (3) Single source of data. Most of the training sets and verification sets used in the study come from single-center data, so it is still necessary to further improve the repeatability and stability of the model through multicenter data. As in Alzheimer's disease research, each single center should be encouraged to share data in anticipation of establishing large-scale and open databases[110]; (4) The interpretation of the results. Due to the inevitable problems of AI, like overfitting of training set data[111] and the "black box" characteristic of the algorithm[112], the accuracy and interpretation of the AI model are inconsistent, which may have a negative impact on clinical application[113]. More intensive basic research and extensive verification are needed to improve this deficiency; (5) Ethical and legal issues. Can we trust the results of AI? Once the AI diagnosis and treatment prediction fails, it will give rise to a series of social, ethical and legal problems[114,115]. It is necessary to combine human supervision with AI tools more reliably; and (6) AI has been widely researched in various fields. We should attach importance to learning experience from different research fields and try to carry out related research in the field of SIDs, so as to promote the continuous progress of AI research in the field of SIDs.

CONCLUSION

The advantages of AI in the diagnosis and prognosis analysis of SIDs have been increasingly recognized, and the high accuracy and efficiency of AI detection greatly reduce the workload of doctors. Although there are still various challenges in the application of AI, the potential of AI in improving the management efficiency of diseases cannot be ignored. Clinicians should work together with experts in various fields to promote the development of AI in SIDs.

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REVIEW

Impact of the COVID-19 pandemic on inflammatory bowel disease patients: A review of the current evidence

Marko Kumric, Tina Ticinovic Kurir, Dinko Martinovic, Piero Marin Zivkovic, Josko Bozic

ORCID number: Marko Kumric 0000-0002-9696-3359; Tina Ticinovic Kurir 0000-0003-1695-9235; Dinko Martinovic 0000-0003-2060-5130; Piero Marin Zivkovic 0000-0001-5649-698X; Josko Bozic 0000-0003-1634-0635.

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Marko Kumric, Tina Ticinovic Kurir, Dinko Martinovic, Josko Bozic, Department of Pathophysiology, University of Split School of Medicine, Split 21000, Croatia

Tina Ticinovic Kurir, Department of Endocrinology, University Hospital of Split, Split 21000, Croatia

Piero Marin Zivkovic, Department of Gastroenterology, University Hospital of Split, Split 21000, Croatia

Corresponding author: Josko Bozic, MD, PhD, Associate Professor, Department of Pathophysiology, University of Split School of Medicine, Soltanska 2, Split 21000, Croatia. josko.bozic@mefst.hr

Abstract

Since the initial coronavirus disease 2019 (COVID-19) outbreak in China in December 2019, the infection has now become the biggest medical issue of modern medicine. Two major contributors that amplified the impact of the disease and subsequently increased the burden on health care systems were high mortality among patients with multiple co-morbidities and overcapacity of intensive care units. Within the gastroenterology-related community, particular concern was raised with respect to patients with inflammatory bowel disease (IBD), as those patients are prone to opportunistic infections mainly owing to their immunosuppressive-based therapies. Hence, we sought to summarize current knowledge regarding COVID-19 infection in patients with IBD. Overall, it seems that IBD is not a comorbidity that poses an increased risk for COVID-19 acquisition, except in patients treated with 5-aminosalicylates. Furthermore, outcomes of the infected patients are largely dependent on therapeutic modality by which they are treated, as some worsen the clinical course of COVID-19 infection, whereas others seem to dampen the detrimental effects of COVID-19. Finally, we discussed the present and the future impact of COVID-19 pandemic and concomitantly increased health care burden on IBD-management.

Key Words: COVID-19; SARS-CoV-2; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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Core Tip: Coronavirus disease 2019 (COVID-19) is the biggest medical issue of the 21st century so far. Within the gastroenterology-related community, COVID-19 is a concern in patients with inflammatory bowel disease (IBD), as those patients are prone to opportunistic infections owing to their immunosuppressive-based therapies. Hence, in this review, we summarized currently available data and concluded that patients with IBD are not at a higher risk for COVID-19 development, unless treated with 5-aminosalicylates, and that the outcomes of infected patients depend on their respective therapeutic modalities. Finally, we discussed the impact of the COVID-19 pandemic and the concomitantly increased health care burden on IBD-management.

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INTRODUCTION

With the immense burden that coronavirus disease 2019 (COVID-19) posited on health care systems and the global economy in general, the disease is unequivocally the biggest medical concern of the 21st century so far[1]. Globally, by January 2021, there have been over 84 million confirmed cases of COVID-19, with more than 1.8 million deaths reported by the World Health Organization (WHO)[2]. The causative agent of the pandemic, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), belongs to the family of *Coronaviridae*, a group of viruses which have already been associated with epidemics in the early 2000s, *i.e.*, severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS)[3,4]. Similar to its related viruses, SARS-CoV-2 primary pathologic manifestations occur in the respiratory system[5,6]. Initial clinical presentation is usually characterized by fever, cough, shortness of breath and specific loss of smell and taste, whereas in a smaller, yet significant amount of patients, the disease progresses to severe lung injury, resulting in the need for intensive care support and concomitant tertiary care equipment[7,8]. This raises two principal issues surrounding COVID-19 and explains why it has caused such a huge global impact. The issue first is high mortality in those patients and the other is the overcapacity of intensive care units (ICU), subsequently burdening health care systems. In the early phases of the pandemic, based on initial observations and knowledge about communicable diseases, particular concern was raised with respect to patients who were at high risk of acquiring severe illness. High risk patients mainly consist of the elderly, obese and patients with pre-existing comorbidities, especially those who are immunocompromised and immunosuppressed[9-11].

Therefore, within the gastroenterology-related community consisting of both medical staff and patients, inflammatory bowel disease (IBD) emerged as an important concern, mainly owing to the IBD therapeutic approach as opposed to the disease itself. Although IBD pathophysiology includes immune dysregulation, the available data does not support the notion that patients with IBD are at a higher risk of acquiring communicable diseases[12-14]. Nevertheless, the IBD therapeutic approach is mainly based on a palette of immunosuppressants, medications whose role in promoting opportunistic infections has been well-established[15-18]. Hence, major organizations instantly provided recommendations for the management of patients with IBD[19-21]. However, knowledge regarding SARS-CoV-2 evolves on a daily basis, resulting in updates to recommendations in order to reach an optimal approach for patients with IBD. In this review, we sought to address the main concerns regarding the relationship between of IBD and COVID-19. Specifically, we summarized the current data and tried to elucidate whether IBD is associated with a higher risk of COVID-19 infection, whether infected patients have worse outcomes than the general population and finally, we discussed how the COVID-19 pandemic and concomitantly increased health care burden influenced IBD-management.

THE RISK OF COVID-19 INFECTION AMONG PATIENTS WITH IBD

Early in the pandemic, a major concern among gastroenterologists was about the occurrence of COVID-19 among patients with IBD for several reasons. SARS-CoV-2 binds to targeted cells *via* angiotensin-converting enzyme 2 (ACE2), a protein constitutively expressed by epithelial cells of the blood vessels, lung, kidney and especially the intestines, where ACE2 expression is among the highest in humans[22-24]. Moreover, as shown by proteomic tissue analysis, ACE2 gastrointestinal expression is increased in IBD patients, especially among the Crohn's disease (CD) subgroup, where expression is markedly higher than in ulcerative colitis (UC)[25]. ACE2 has been also implicated in the pathophysiology of IBD, having a dual-role: aggravation of colitis *via* the classical renin/angiotensin II/aldosterone pathway and amelioration of colitis *via* the ACE2/MAS-1 receptor pathway[26-29]. Except for ACE2, SARS-CoV-2 pathogenesis depends on the specific "spike" glycoprotein that mediates fusion of the coronavirus envelope with the host cell membrane[30]. This protein is activated through the trypsin-like protease, the transmembrane protease serine 2 (TMPRSS2), the activity of which has been shown to be up-regulated in IBD[31]. Furthermore, in up to 50% of COVID-19 patients, fecal samples were positive for SARS-CoV-2 virus, with more than one-fifth of the samples testing positive even after subjects tested negative from respiratory samples, implicating the fecal route of SARS-CoV-2 transmission[32,33]. This could be even more important, as patients with IBD are more frequently assessed with invasive gastrointestinal procedures such as esophagogastroduodenoscopy and ileocolonoscopy compared with the non-IBD population, subsequently exposing both the patient and the examiner to a higher risk of infection[34]. Finally, the use of IBD immunosuppressive therapies has been associated with an increased risk of infections[13,14]. All of these findings suggest that patients with IBD should be the "perfect" host for SARS-CoV-2 viral infection (Figure 1). However, results from a recent systematic review that comprised 13 cohort studies and 5 single case reports from all around the world suggest that patients with IBD do not seem to have a higher risk of COVID-19 infection with respect to the general population, not even in IBD patients treated with immunosuppressive drugs [35]. Another systematic review and meta-analysis by Singh *et al*[36] concluded similarly and additionally determined that there was no difference in COVID-19 occurrence between IBD subgroups, *i.e.*, between CD and UC. Regarding the IBD therapeutic strategies, Singh *et al*[36] demonstrated that no use of therapeutics was associated with an increased risk of COVID-19 acquisition aside from the use of 5-aminosalicylic acid (5-ASA). In fact, Taxonera *et al*[37] evaluated the age-standardized incidence of COVID-19 in IBD patients, and suggested that COVID-19 incidence might be overestimated in the IBD population. Unfortunately, larger studies did not conduct a comparable evaluation.

Multiple authors struggled to explain the discrepancy between the expected and evidence-based COVID-19 incidence among populations with IBD[36,38]. Firstly, a major determinant to reduced COVID-19 incidence could be the tighter containment of patients with IBD, since people suffering from chronic diseases, especially patients treated with immunosuppressants, were warned by experts to follow strict social distancing measures, known as shielding, from the beginning of the pandemic. Furthermore, ACE2, the above-noted protein that is up-regulated in IBD, has two distinct functional forms. The full-length form of ACE2 possesses an extracellular domain that binds to the SARS-CoV-2 virus and transmembrane domain, which anchors the first domain to the plasma membrane and aids viral entry into the cell[39]. Conversely, the soluble form of ACE2 lacks a transmembrane domain and it is therefore a sort of a decoy receptor for SARS-CoV-2 virus in the blood[40,41]. Notably, the latter form is up-regulated in IBD patients, as a consequence of ACE2 membrane cleavage into the soluble form, in a process regulated by the tumor necrosis factor-alpha (TNF-α) convertase ADAM17 (a disintegrin and metalloproteinase 17), the protease is up-regulated in patients with active IBD[42,43]. Furthermore, although SARS-CoV-2 is detectable in fecal samples and active viral replication in the enterocytes of the small intestine has been reported[33,44], to this day there is no firm evidence to imply that increased SARS-CoV-2 replication in intestines is proportional to intestinal ACE2 expression. This is substantiated by the fact that SARS coronavirus, a SARS-CoV-2 close relative, spreads through the upper respiratory tract (URT) very effectively despite only modest ACE2 expression in the URT[24]. Overall, it seems that SARS-CoV-2 also needs the presence of a co-receptor for host cell infection, similarly to HIV infection[45]. However, the hypothesized co-receptor that synergistically with ACE2 leads to SARS-CoV-2 infection has yet to be determined in future studies. The reasons for the increased risk of COVID-19 acquisition with 5-ASA are still unclear

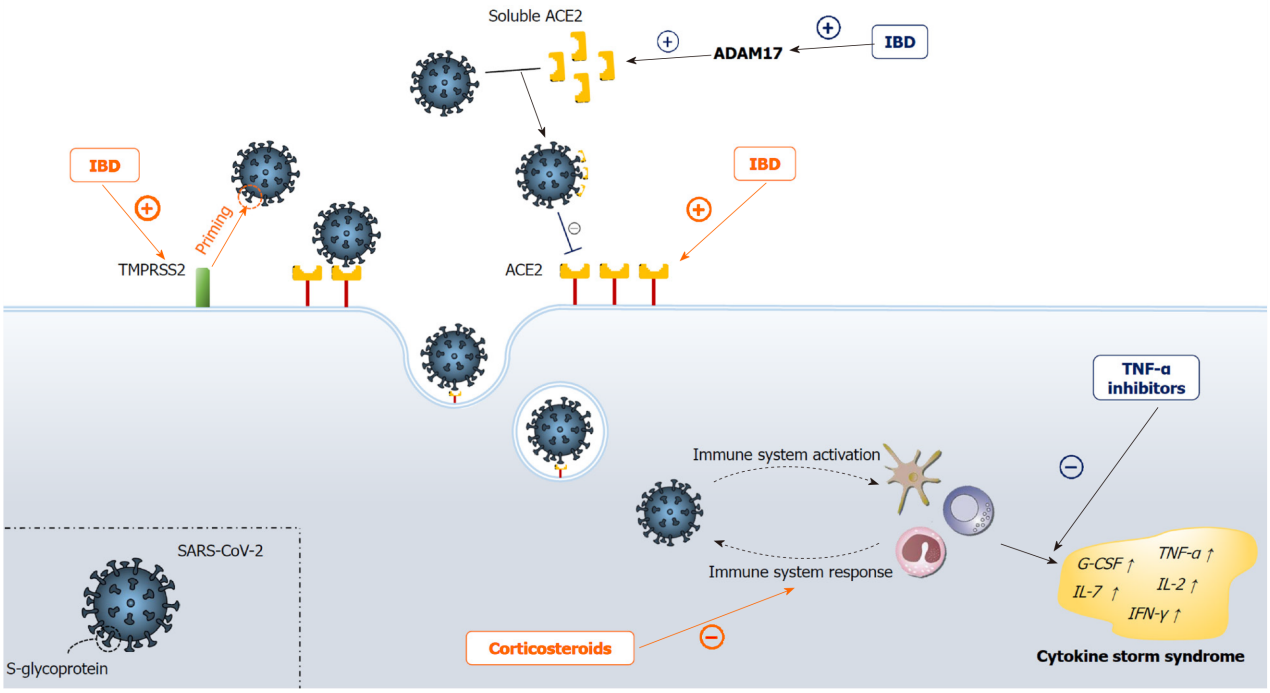

Figure 1 Pathogenetic pathway of intestinal severe acute respiratory syndrome coronavirus 2 infection with proposed inflammatory bowel disease effects. The blue lines represent inflammatory bowel disease (IBD)/IBD approach-mediated reduction in either coronavirus disease 2019 (COVID-19) acquisition or poor outcomes of it, whereas the red lines represent IBD/IBD approach-mediated increase in risk for COVID-19 acquisition or poor outcomes. IBD: Inflammatory bowel disease; ADAM17: A disintegrin and metalloproteinase 17; TNF-α: Tumor necrosis factor-alpha; ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane protease serine 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; IL: Interleukin; G-CSF: Granulocyte-colony stimulating factor; IFN-γ: Interferon-γ.

but, as discussed by Singh *et al*[36], the observed increase could be related to the fact that 5-ASA use may be a proxy for underlying UC in these circumstances. Namely, patients with UC have higher ACE2 levels (although, as we discussed, this is not a reliable indicator) and a population with UC tends to be older than a CD population [46], hence they are more prone to get tested, as Singh *et al*[36] argue. Despite the sensitivity of the fecal reverse transcription polymerase chain reaction (RT-PCR) test for the diagnosis of COVID-19, its diagnostic power still needs to be elucidated. D'Amico *et al*[47] hypothesized that fecal RT-PCR testing may be useful in IBD patients to distinguish disease re-exacerbation from SARS-CoV-2 superinfection, allowing better patient management and targeted therapy.

CLINICAL COURSE OF COVID-19 INFECTION IN PATIENTS WITH IBD

Since the detrimental effects of immunosuppressive agents on the host-cell defense against pathogens have been well-established, particular concern was raised with respect to the clinical course of COVID-19 in patients on various immunosuppressive therapies and ubiquitous therapeutic strategies for patients with IBD[15-17,48]. Furthermore, active IBD itself might worsen COVID-19 outcomes, as those patients are markedly frailer and more prone to adverse outcomes by virtually any infection[49,50]. Finally, non-IBD patients with COVID-19 have high fecal calprotectin even after diarrhea resolves and COVID-19 patients with ongoing diarrhea have even higher levels in comparison with COVID-19 patients without diarrhea[51,52], suggesting that the presence of SARS-CoV-2 in the gastrointestinal tract is associated with greater intestinal inflammation. This indicates that COVID-19 could exacerbate inflammation and, subsequently, symptoms in IBD patients. However, it is very challenging to assign a symptom to the underlying disease, its exacerbation, or the concomitant infection, making these characteristics difficult to interpret. Of note, in the aforementioned studies[51,52], patients with diarrhea exhibited higher serum interleukin 6 concentrations, raising the possibility of more severe systemic inflammation in this group of patients[53].

Fortunately, an abundance of clinical studies demonstrated that most of the hypothesized adverse outcomes were not observed in IBD patients that acquired COVID-19. Nonetheless, in some cases this comorbidity seems to even dampen the deleterious effects of COVID-19. Considering the differences in the initial clinical presentation, although in concordance with the non-IBD population, fever and cough were the most common clinical findings. Further, COVID-19 positive IBD patients presented with diarrhea significantly more often than the general population[54-57]. This disparity could be associated with the influence of the underlying disease on the number of evacuations, justifying the greater percentage of diarrhea in IBD patients than in the general population. In contrast, the observed difference could also be due to the aforementioned exacerbation of IBD as a result of COVID-19 infection. The risk of severe COVID-19 outcomes, *i.e.*, the need for hospitalization, admission to the ICU, mechanical ventilation or death, were not higher among IBD patients in comparison to the general population, as demonstrated in multiple systematic reviews and meta-analyses[35,36,47]. These results are also in accordance with the latest data from the Surveillance Epidemiology of Coronavirus Under Research Exclusion for Inflammatory Bowel Disease (SECURE-IBD), an international web-based database where physicians are encouraged to report all cases of COVID-19 in patients with IBD[58]. A total of 4038 IBD patients with confirmed COVID-19 were reported in the SECURE-IBD database as of January 6, 2021 with 19% of patients in need of hospitalization, 3% admitted to the ICU, and 3% in need of mechanical ventilation, where the case fatality rate was 2%. Of note, in a study by Lukin *et al*[59], the authors included a control group consisting of non-IBD patients with COVID-19. Rather interestingly, death and ICU admission were numerically lower in the IBD group than in the control group. Although these results should be taken with caution, it is possible that certain IBD medications led to the blunting of the cytokine release syndrome and subsequently to more favorable outcomes.

The therapeutic choice emerged as a major determinant for COVID-19 prognosis in patients with IBD. Accumulating data implies that the use of systemic corticosteroids is associated with the highest risk of severe COVID-19 outcomes[35,36,47,58]. It is well known that corticosteroids affect the immune system *via* multiple mechanisms, including the inhibition of adhesion molecules, decreasing the expression of inflammatory cytokines and inducing apoptosis of activated lymphocytes. Moreover, in studies that tested the use of corticosteroids on MERS and SARS patients, authors have demonstrated a delayed viral clearance in patients receiving high-dose corticosteroids [60]. However, the effects of corticosteroid use on COVID-19 adverse outcomes in IBD is not as clear as it may seem. In a report from the large RECOVERY trial, in which the effects of dexamethasone on hospitalized COVID-19 patients were assessed, authors concluded that the use of dexamethasone resulted in lower 28-day mortality among those who were receiving either invasive mechanical ventilation or oxygen alone at the time of randomization but not among those receiving no respiratory support[61]. Furthermore, in a recently published meta-analysis by van Paassen *et al*[62], which included the largest number of studies and COVID-19 patients, the authors demonstrated the beneficial effects of corticosteroids use on short-term mortality and a reduction in the need for mechanical ventilation. Notably, the authors also found a signal of delayed viral clearance, but data in the studies were too uncertain to reach any firm conclusions. Three other meta-analyses that were conducted on this topic have rather conflicting reports. The WHO Rapid Evidence Appraisal for COVID-19 Therapies Working Group[63] concluded similarly to van Paassen *et al*[62], reporting that the administration of systemic corticosteroids was associated with lower 28-day all-cause mortality in comparison to placebo or usual care. Tlayjeh *et al*[64] found no difference in mortality or the necessity for mechanical ventilation, yet similarly to van Paassen *et al*[62], they observed a prolonged viral clearance time. Sarkar *et al*[65] demonstrated that in patients with COVID-19, corticosteroids may be associated with a twofold increase in mortality, yet their analysis was based on low-quality evidence with high variability. Considering the beneficial effects of corticosteroid use in COVID-19 patients, doubt was raised with respect to poor outcomes of their use in COVID-19 patients with concomitant IBD. It is possible that the corticosteroid use in these circumstances is merely an indicator from the subset of patients with active IBD who are predisposed to adverse outcomes. In fact, in a retrospective cohort study, Singh *et al*[66] reported that IBD patients who received corticosteroids up to 3 months before the diagnosis of COVID-19 had a higher risk of severe COVID-19 in comparison to patients who did not receive corticosteroids. Although the authors conducted an unadjusted analysis, because corticosteroid use in IBD is associated to worsening of the disease, these results could imply that in this setting corticosteroids were not the cause, but an indicator of higher risk for a severe COVID-19 clinical course[67].

Apart from corticosteroid use, as shown by multiple studies, the use of 5-ASA has been also associated with more severe COVID-19 outcomes[35,36,47,58,68]. This finding persisted even after controlling for confounding factors such as age, comorbidities, IBD disease characteristics and corticosteroid use. Since mechanisms by which 5-ASA exerts its anti-inflammatory effect are rather disperse and include peroxisome proliferator-activated receptor-γ up-regulation, cyclooxygenase 2/prostaglandin E2 down-regulation, reactive oxygen species scavenging and many more[69], we could only hypothesize which of these pathways is responsible for more severe COVID-19 outcomes. Primarily, large, well-designed studies should be conducted to confirm that an increased risk really exists in the first place.

Immunomodulators, a group of medications used in IBD treatment that includes azathioprine, 6-mercaptopurine, and methotrexate have been known to inhibit the immune response to viral infections by multiple mechanisms[70,71]. However, the data regarding the role of immunomodulators in COVID-19 is quite reassuring, as conducted studies do not seem to demonstrate any difference in severe outcomes in comparison to the general population[35,36,47,58,68]. Since the effects of immunomodulators and corticosteroids on the suppression of the immune system are in part overlapping[72,73], we hypothesize that this provides further evidence toward the notion that the use of corticosteroids itself does not result in a more severe form of COVID-19. In fact, different results between the use of corticosteroids and immunomodulators from the above-noted studies could be due to the different clinical profiles of patients between the two groups, with corticosteroid use implying poor IBD control.

The most ambiguous results that emerged from observational studies is in relation to the use of biological agents, an immunosuppressive medication group used in the management of IBD which includes infliximab, adalimumab, golimumab, certolizumab pegol, ustekinumab and vedolizumab[74]. Biological agents, particularly TNF-α inhibitors, have been known to mitigate the host immune system response against infectious organisms, especially intracellular pathogens, such as mycobacterial, fungal and viral infections[75,76]. However, aside from the systematic review by Macaluso and Orlando[35] which showed no difference as opposed to the general population, available data suggests that patients treated with biological agents are significantly less prone to develop severe forms of COVID-19, distinctly in terms of mortality[36,58,68]. Accumulating evidence implies that COVID-19 severity is associated with a cytokine storm syndrome, an immune-mediated process characterized by hyperactivation of T cells and massive production of TNF-α, interleukin 2, interleukin 7, granulocyte-colony stimulating factor, interferon-γ (IFN-γ), IFN-γ–inducible protein 10, monocyte chemoattractant protein 1, and macrophage inflammatory protein 1-a [77]. Based on these findings, it is legitimate to hypothesize that the use of biological drugs which selectively inhibit specific cytokines or small molecules that simultaneously block multiple cellular pathways may play a role in the treatment of these patients. Interestingly, in two case reports, the remission of COVID-19 symptoms in IBD patients was achieved after treatment with biological agents[78,79]. This evidence is certainly insufficient to recommend the use of these drugs in COVID-19 management, yet they are reassuring for IBD patients, and shed light on biological agents as feasible therapeutic agents in COVID-19 treatment. In fact, there are several ongoing clinical trials that assess the efficacy and safety of these drugs in this setting (NCT-04344249 and NCT04425538).

Between the IBD subgroups, in all of the aforementioned reports[35,36,47,58,68], patients with UC had markedly worse outcomes than patients with CD. Singh *et al*[36] attribute this risk incrementation to the fact that patients with UC are more likely to be older and undergo different therapeutic modalities between the two groups[36,80]. However, since Singh *et al*[36] did not provide the age-adjusted comparison of outcomes, whether the poor outcomes of UC in contrast to CD are related to old age remains unclear. Regarding the difference in therapeutic modalities, patients with UC are more likely to be treated with 5-ASA, a treatment shown to pose a risk for more severe outcomes, whereas patients with CD are more likely to be treated with biological agents, treatment that seem to have a protective role in COVID-19 infection [81,82]. In addition, it is unclear if the observed disparity is due to the pathobiological differences between the two types of IBD, including the variability in expression of ACE2 and TMPRSS2.

THE IMPACT OF THE COVID-19 PANDEMIC ON IBD MANAGEMENT: PRESENT AND FUTURE PERSPECTIVES

Current guidelines for IBD treatment with respect to COVID-19

Two major organizations that cover the issues regarding IBD, the International Organization for the Study of Inflammatory Bowel Disease (IOIBD) and European Crohn's and Colitis Organization (ECCO) have partnered and provided a set of guidelines regarding the management of IBD in relation to COVID-19[83]. However, there are two important notions to accentuate regarding the present guidelines. Firstly, as currently there is no adequate evidence-based data, the recommendations are based on a consensus between a group of international IBD and infectious disease experts. Secondly, since the speed of publishing is slower than the amount of emerging data, organizations urged physicians to continue to check the IOIBD or the ECCO websites for the most up-to-date information.

The most important question that was raised in the guidelines concerns the IBD immunosuppressive therapies, *i.e.*, whether the infected patients should discontinue these therapies and if should, for how long. The current consensus is that recommencing these therapies should be influenced by the clinical severity of both IBD and COVID-19. Conceptually speaking, the greater the severity of IBD and the lesser the severity of COVID-19, the discontinuation of therapy should be shorter and vice versa. Experts suggest that for most patients, a symptom-based strategy is suitable [84]. According to this strategy, COVID-19 resolution is evaluated according to symptom onset (≥ 10 d) and clinical improvement. Current expert recommendations regarding immunosuppressive therapies are summarized in Table 1. Other aspects regarding recommendations, such as care for patients with IBD requiring hospitalization, priority for endoscopy, guidance for the infusion centers, management of pregnant IBD patients and a very practical set of ten "Do's" and "Don'ts" for IBD management during the COVID-19 outbreak were further discussed in the aforementioned IOIBD/ECCO guidelines[85-89]. Following the approval of several COVID-19 vaccines, the IOIBD experts have recently issued a statement regarding vaccination [90]. The expert group advised vaccinating all patients with IBD as soon as they are able to receive a vaccine, regardless of their immune-modifying therapies.

Impact of COVID-19 and lockdown on the management of IBD patients

There is a scarcity of data regarding the post-lockdown phase in terms of health-care procedures[91,92], let alone in the IBD population[93-96]. Early into the pandemic, hospitals were urged to restructure their daily activities to meet the needs of health care practitioners and to provide the facilities to treat COVID-19 patients. The restructuring of the health care system did not circumvent IBD management. Consequently, the risks of secondary harm emerged, as the latter resulted in reduced access to diagnostic endoscopy, lack of face-to-face clinics, difficulties in continuing day-case infusions, issues in performing routine blood and/or stool monitoring as well as patients' fears which may have reduced their attendance in hospitals[93]. Particular problems emerged in the pediatric population, since a delay in diagnosis and delayed treatment has the potential to result in serious repercussions, such as an impact on children's growth[97,98]. Recent Italian and Spanish surveys both demonstrated that the management of urgent activities and administration of biological therapies in both the lockdown and post-lockdown periods substantially maintained the pre-pandemic standards of care[93,94]. However, the surveys also highlighted that the reduction in number of visits, endoscopies and gastrointestinal ultrasounds observed in the lockdown but also in the post-lockdown phase could result in worse long-term outcomes[93,94]. A study in the pediatric population had similar conclusions regarding the quality of care[93]. This study accentuated concerns with respect to newly diagnosed IBD patients as this subgroup was diagnosed without a histological confirmation of the disease, which is a controversial exception that had to be adopted given the present special circumstances[93]. These patients were diagnosed *via* a combination of blood tests, radiological imaging, fecal calprotectin and exclusion of infectious causes, followed by multidisciplinary discussion. The commencement of the systemic immunosuppression in children without endoscopic or histological diagnosis was an additional concern, yet physicians could adapt in the beginning exclusive enteral nutrition as a first-line therapy as an induction strategy with multiple benefits [99]. Although a delay in exposing patients to systemic immunosuppression for 4-8 weeks is beneficial, after this time patients enter a period where immunomodulatory and biological therapy should commence, while simultaneously, the ability to conduct a full disease assessment beforehand may continue to be limited. Overall, experts

Table 1 Latest International Organization for the Study of Inflammatory Bowel Disease/European Crohn's and Colitis Organization recommendations for coronavirus disease 2019 infected inflammatory bowel disease-patients[101]

COVID-19 symptomatology	SARS-CoV-2 status	Recommendation
Asymptomatic	Non-tested	Do not withhold therapy (reduce corticosteroid use if possible)
Asymptomatic	Positive	Withhold therapy for 10 days[1]
Symptomatic	Positive	Withhold therapy until all of the following is fulfilled[1]: (1) At least 10 days has passed since symptoms onset; (2) Improvement in respiratory symptoms; and (3) Days without fever (without the use of antipyretics)

[1]If clinical presentations imply that therapy should not be postponed, despite meeting the clinical criteria stated above, guidelines recommend moving ahead with the scheduled treatment. COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

agree that the implementation of a telemedicine approach has played an important role in maintaining the standards of quality of care in IBD management during the pandemic[93,94,100].

Future perspectives of IBD management

Even though COVID-19 had a range of detrimental effects on health care systems globally, it also opened a space for improvements in clinical practice, which could be used long after the COVID-19 pandemic resolves. One of those is the use of telemedicine, *i.e.*, the implementation of virtual technologies in routine clinical practice. An expansion in technological solutions and loosening restrictions on how telemedicine can be deployed and reimbursed have opened the way for telemedicine to become an integral part of clinical practice both now and in the future. In the IBD population, virtual appointments, multidisciplinary discussions, and improvement of networks by remote collaboration all provide the opportunity for better care within specific situations, with the simultaneous reduction of the transmission of infectious diseases. Today it is COVID-19, but in the future, it could be some other virus, especially as we are now more than ever aware of our susceptibility to a viral pandemic. IOIBD/ECCO issued a summary of the best strategies for IBD management *via* telemedicine, carefully covering every aspect of the patient-physician relationship [101]. Another very important aspect of telemedicine is its inexpensiveness. As a global economic crisis is imminent, this will actually become the critical reason for widespread implementation of telemedicine. Regarding the disease itself, although for now a lot of data substantiates the fact that COVID-19 does not influence the short-term prognosis for IBD patients, the long-term effects are quite unknown. We believe that poorer long-term outcomes will be mainly due to delayed diagnostic (especially endoscopy) therapeutic procedures and not COVID-19 itself. However, Gower-Rousseau *et al*[102] argue that most of the researchers too hastily concluded that the COVID-19 pandemic is relatively safe for IBD patients. They highlighted that the recently published, underpowered studies cannot provide answers for patients with IBD, or other infrequent diseases for that matter. Further, Gower-Rousseau *et al*. asserted that low-quality studies might even prompt misguided and harmful treatment decisions. On the contrary, they argue that well-grounded answers to these questions require complex epidemiologic risk and benefit analyses with an *a priori* sample size calculation and a removal of unwanted biases.

CONCLUSION

The initial fear of COVID-19 infection among patients in the IBD community that was based on available knowledge, now seems unnecessary. Accumulating data suggests that IBD is not a comorbidity that poses an increased risk for COVID-19 acquisition, except in patients treated with 5-ASA. Furthermore, although the outcomes of infected patients are largely dependent on the therapeutic modality by which they are treated, overall, IBD patients seem to have COVID-19 outcomes similar to the general population. This is in contrast to those on corticosteroids, as they currently seem to have a less favorable prognosis. Biological agents even dampen the detrimental effects of COVID-19 by inhibiting cytokine storm syndrome, according to the available data. However, preliminary data must be interpreted with caution, as the long-term effects

of both COVID-19 and IBD management during the COVID-19 outbreak are quite unknown. Finally, the COVID-19 outbreak could also change the future of IBD management, and management of the diseases in general, as telemedicine could dethrone face-to-face examinations in the following years.

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REVIEW

Management of hepatitis B virus infection in patients with inflammatory bowel disease under immunosuppressive treatment

Georgios Axiaris, Evanthia Zampeli, Spyridon Michopoulos, Giorgos Bamias

ORCID number: Georgios Axiaris 0000-0001-7115-4291; Evanthia Zampeli 0000-0003-4246-1350; Spyridon Michopoulos 0000-0001-7371-8601; Giorgos Bamias 0000-0002-5492-5717.

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Georgios Axiaris, Evanthia Zampeli, Spyridon Michopoulos, Gastroenterology Department, "Alexandra" Hospital, Athens 11528, Greece

Giorgos Bamias, GI Unit, 3rd Academic Department of Internal Medicine, National and Kapodistrian University of Athens, Sotiria Hospital, Athens 11526, Greece

Corresponding author: Giorgos Bamias, MD, PhD, Associate Professor, GI Unit, 3rd Academic Department of Internal Medicine, National and Kapodistrian University of Athens, Sotiria Hospital, 44 Kifisias Avenue, Athens 11526, Greece. gbamias@gmail.com

Abstract

Hepatitis B remains a significant global clinical problem, despite the implementation of safe and effective vaccination programs. The prevalence of hepatitis B virus (HBV) in patients with inflammatory bowel disease (IBD) largely follows the regional epidemiologic status. Serological screening with hepatitis B surface antigen (HBsAg), and antibodies to hepatitis B surface (anti-HBs) and core (anti-HBc) proteins is a key element in the management of IBD patients and, ideally, should be performed at IBD diagnosis. Stratification of individual cases should be done according to the serologic profile and the IBD-specific treatment, with particular emphasis in patients receiving immunosuppressive regimens. In patients who have not contracted HBV, vaccination is indicated to accomplish protective immunity. Vaccination in immunosuppressed patients, however, is a challenging issue and several strategies for primary and revaccination have been proposed. The risk of HBV reactivation in patients with IBD should be considered in both HBsAg-positive and HBsAg-negative/anti-HBc-positive patients, when immunosuppressive therapies are administered. HBV reactivation is preventable *via* the administration of prophylactic nucleot(s)ide analogues and should be the standard approach in HBsAg-positive patients. HBsAg-negative/anti-HBc-positive patients represent a non-homogeneous group and bear a significantly lower risk of HBV reactivation. Biochemical, serological and molecular monitoring is currently the recommended approach for anti-HBc patients. Acute HBV infection is rarely reported in IBD patients. In the present review, we outline the problems associated with HBV infection in patients with IBD and present updated evidence for their management.

Key Words: Hepatitis B virus; Inflammatory bowel disease; Reactivation; Immunosuppression; Vaccination; Prophylaxis

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Core Tip: The management of hepatitis B virus (HBV) infection poses significant challenges for patients with inflammatory bowel disease (IBD). Lower rates of vaccination for HBV have been reported in this population and immunization programs should be encouraged and intensively implemented. In addition, patients who receive immune-modifying therapies may develop suboptimal responses to vaccination. In the presence of present or past HBV infection, immunosuppressive therapies may increase the risk for reactivation of the virus with adverse clinical outcomes. Close surveillance and/or prophylactic anti-viral treatment may be employed depending on the status of HBV infection and the IBD-specific therapy.

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INTRODUCTION

Hepatitis B is among the most common infections worldwide and represents a major global health problem due to its potential for considerable morbidity and mortality. According to a World Health Organization report, in 2015, 257 million people were living with chronic hepatitis B infection[1]. In fact, it has been estimated that approximately one-third of the world's population has been exposed to hepatitis B virus (HBV). HBV infection is a frequent cause of both acute and chronic hepatitis. The latter may result in a variety of outcomes, which range from asymptomatic infection to end-stage liver disease with cirrhosis, hepatocellular cancer and death. Accordingly, it has been reported that HBV infection accounts for 5%-10% of liver transplantations[2]. On the other hand, nowadays, HBV infection is a preventable disease, due to the development and universal application of highly effective and safe vaccines against HBV.

Crohn's disease (CD) and ulcerative colitis (UC), collectively referred to as the inflammatory bowel diseases (IBDs), are immune-mediated diseases that manifest with chronic relapsing inflammation of the gastrointestinal tract. HBV infection may be of particular significance for patients with IBD, due to several factors. Firstly, although initially described as diseases of the West, since the second half of last century, IBDs have displayed accelerating incidence and prevalence rates in developing areas of the world[3-5]. As those regions include countries where HBV is highly prevalent, such trends translate to a constantly increasing number of patients with IBD that may be exposed to and infected with HBV. Secondly, there are reports indicating that patients with IBD may have increased rates of HBV positivity, while, at the same time, vaccination rates may be considerably low in this population. Finally, and more importantly, the mainstay of IBD treatment has been immunomodulatory drugs. Despite the shift from generalized immunosuppression with steroids or thiopurines to selective, targeted immunomodulation *via* biologics or small molecules, various degrees of immune response compromise are expected in IBD patients under treatment[6,7]. This state of acquired immunodeficiency may render patients with IBD more vulnerable to acquisition and to a more severe course of various infectious diseases, which include HBV infection. This also encompasses the danger for reactivating a previously latent HBV infection.

In this review, we will present the current status regarding the complex relationship between HBV and IBD, including updated epidemiological trends, screening and vaccination guidelines, and the associations between immunomodulatory treatment and the various clinical scenarios of anti-HBV immunity.

NATURAL HISTORY OF HBV INFECTION

HBV is a hepatotropic DNA virus that belongs to the Hepadnaviridae family. It is

transmitted sexually, parenterally (by contact with infected body fluids or blood) and perinatally, from a hepatitis B surface antigen (HBsAg)-positive mother to her child (vertical transmission). Following transmission, the HBV virion enters the hepatocyte using a cellular receptor, the sodium taurocholate co-transporting polypeptide (known as NTCP)[8]. The first step in HBV replication is the formation of covalently closed circular DNA (cccDNA) in the cell nucleus, a mini chromosome, which is a key structure for the longevity of the virus[9]. It is also known that fragments of HBV DNA integrate in the human DNA, but this integration is not vital for viral replication. After contact with the virus, the risk of chronic infection largely depends on the age of the subject, being 90% for infants, 25%-50% for toddlers, and 1%-5% for adults[10].

Chronic HBV infection consists of different phases with distinct serologic profiles, as have recently been described[11]. Phase I is the HBeAg-positive chronic HBV infection, formerly known as the immune tolerance phase, which is characterized by the presence of HBsAg and hepatitis B e antigen (HBeAg), very high HBV DNA levels and persistently normal transaminases, with no or minimal necroinflammation in the liver. The second phase is the HBeAg-positive chronic HBV hepatitis, which is characterized by high HBV DNA levels, increased transaminases and moderate to severe necroinflammatory activity and fibrosis in the liver tissue. This phase leads to seroconversion in the majority of cases, with loss of the HBeAg and appearance of antibodies to hepatitis B surface protein (anti-HBs). Phase III is the HBeAg-negative chronic HBV infection, previously known as the inactive carrier state, which is characterized by the presence of antibodies to hepatitis B e protein (anti-HBe), undetectable or low levels of HBV DNA, normal alanine aminotransferase (ALT) levels and HBsAg levels usually below 100 IU/mL. Phase IV is the HBeAg- chronic hepatitis, which is characterized by a moderate/severe necroinflammatory process in the liver, high HBV DNA levels and fluctuating liver enzymes. Phase V is the HBsAg-negative phase, also known as occult HBV infection, which is characterized by the absence of the HBsAg, positivity for antibodies to hepatitis B core protein (anti-HBc), with or without anti-HBs antibodies, usually normal ALT, and not necessarily measurable HBV DNA in the serum but detectable in the liver tissue.

The natural history of HBV depends on the phase of the infection; in patients with chronic hepatitis B, progression to cirrhosis is observed at an annual rate of 2%-2.5%, whereas HBeAg-negative patients exhibit a faster progression, at a rate of 8%-20% per year[12]. Thus, signs and symptoms of portal hypertension should be regularly sought for in this population, as should the degree of liver fibrosis *via* imaging. Furthermore, HBV is strongly associated with hepatocellular carcinoma and patients should be stratified according to the risk for malignancy *via* simple scores like the PAGE-B, which incorporates parameters like age, gender, and platelets[13]. Screening for hepatocellular carcinoma is based on abdominal ultrasounds at 6-mo intervals, with or without alpha-fetoprotein measurement.

HBV REACTIVATION

Reactivation of HBV is a distinct event in the natural history of the infection, that is signified by the recurrence of viral replication in patients with a quiescent disease status. According to the American Association for the Study of Liver Diseases[14], in HBsAg-positive patients, reactivation is defined as either at least 2 Log (or 100-fold) increase in HBV DNA compared with the baseline level or HBV DNA at least 3 Log (or 1000) IU/mL in a patient with previously undetectable HBV DNA or HBV DNA at least 4 Log (or 10000) IU/mL if the baseline level is not available. In HBsAg-negative, anti-HBc-positive patients, reactivation is considered as reappearance of the S antigen or the detection of HBV DNA. It is worth mentioning that there are patients who remain HBsAg-negative upon reactivation. This is genetically determined and related to the presence of additional N-linked glycosylation sites in the major hydrophilic region of the S antigen[15] and with the emergence of viral strains with mutated HBsAg[16]. HBV reactivation in patients under immunosuppression may have a deleterious effect. HBV reactivation presents with a wide range of manifestations, from asymptomatic, mild hepatitis to acute liver failure and death. In some cases, HBV reactivation precipitates the induction of chronic hepatitis B, potentially leading to liver cirrhosis and even hepatocellular carcinoma. Severe flares resulting in hepatic decompensation with an unfavorable outcome, commonly present with jaundice. A flare of hepatitis is defined as an ALT level above 100 U/L and at least 3 times higher than baseline. Patients with acute liver failure as a result of HBV reactivation present with higher HBV viral loads and lower IgM anti-HBc titers as compared with patients

with new onset HBV-related acute liver failure[17].

The major precipitating factor for HBV reactivation is the induction of an immunodeficient state in the host, more commonly *via* the administration of immunosuppressive therapies. This is due to the fact that HBV is controlled through the immunological system by specific T and B cells[18]. It follows that, in the presence of immunosuppression, the virus may regain its ability to proliferate and replicate[14]. This explains why HBV reactivation is of particular significance for patients with IBD, as the latter are often treated with therapies that modify the function of the immune system. It should be noted, however, that the large majority of the data originates from studies in oncology, hematology and rheumatology and that extrapolation of these data to IBD patients should be done with caution[19]. Nevertheless, immunosuppression does take place in treated patients and may be of relevance. As an example, tumor necrosis factor (TNF), which is the target of pivotal treatments for patients with IBD, is known to enhance virus clearance; hence, inhibition of TNF signaling enhances HBV replication[11]. Moreover, HBV has a glucocorticoid responsive element in its genome that is stimulated by the use of steroids, another frequently used therapy during flares of IBD[20].

Besides immunosuppression, HBV reactivation may also depend on both host and viral factors. Male sex has been associated with HBV reactivation in oncology patients [21]. HBsAg-positive patients carry higher risk for reactivation as compared to HBsAg-negative, anti-HBc-positive patients. Furthermore, higher HBV DNA levels before the start of immunosuppression confer an elevated risk of reactivation[22]. Finally, among HBsAg-negative patients, those who are anti-HBc-positive with detectable HBV DNA and undetectable anti-HBs before immunosuppression are more susceptible to reactivation[23].

After immunosuppression is imposed, HBV reactivation develops through sequential distinct stages[14,24]. At first, immunosuppression leads to an increase in HBV DNA, whilst the patient remains asymptomatic and transaminases are normal. Subsequently, hepatitis ensues with increased transaminases, with or without symptoms. In some cases, liver damage at this stage may lead to liver failure and even death. Once immunosuppression is either discontinued or reduced or/and antiviral treatment is initiated, a progressive decline of the HBV DNA is observed and the hepatitis flare resolves. In a small percentage of patients, despite such therapeutic measures, progressive deterioration of liver function may still be observed. Moreover, acute liver failure in patients under immunosuppression is associated with poor short-term prognosis and reduced 21-d overall survival compared with immunocompetent patients[25].

HBV PREVALENCE IN PATIENTS WITH IBD

HBV prevalence exhibits a geographic variability around the world, with areas of low (< 2%), medium (2%-7%) and high (> 8%) endemicity[11]. Highly endemic areas include Southeast Asia, the Pacific (excluding Japan and Australia), sub-Saharan Africa and some Eastern European countries. Areas with intermediate endemicity include South, Central and Southwest Asia, Israel, Japan, Eastern and Southern Europe, Russia and most of Central and South America. Low-endemic areas include North America, Western and Northern Europe, Australia, and parts of South America. The worldwide prevalence of HBV has changed over the last 30 years, due to immigration, improvement of the socioeconomic level and the implementation of mandatory vaccination.

The prevalence of hepatitis B in IBD patients has been investigated in several studies from Europe, Asia and the Americas (Table 1). Older studies from European countries showed higher incidence of hepatitis B core antibody (HBcAb) positivity among IBD patients[26,27]. Nevertheless, in more recent reports from Italy[28] and France[29], the prevalence of HBV infection among IBD patients was not different from that in the general population. Similarly, results of two Greek studies also reported prevalence of 2.3%-5% among IBD patients, which was in accordance to what was expected[30,31]. In addition, a study from Poland, a country with intermediate endemicity, also showed that the prevalence of HBV infection among IBD patients was comparable to that of the general population[32]. The most recent study by Losurdo *et al*[33], published in 2020, evaluated the burden of viral hepatitis in IBD and demonstrated that HCV was more frequent than HBV infection in IBD patients but with low overall prevalence.

Table 1 Studies investigating hepatitis B virus prevalence in inflammatory bowel disease patients

Ref.	Type of study; Country	Patients	HBsAg-positive	Anti-HBc-positive	Anti-HBs-positive	Comments
Losurdo *et al*[33], 2020	Single-center cohort; Italy	807 IBD	0.9%	7.7%		Similar to regional prevalence
		438 CD				
		369 UC				
Fousekis *et al*[31], 2019	Retrospective single-center; Greece	602 IBD	5.3%	13.4%	32.4%	Similar to regional prevalence
Silva *et al*[94], 2019	Cross-sectional; Brazil	306 IBD	0.7%			Similar to regional prevalence
		165 UC				
		141CD				
Chou *et al*[95], 2019	Retrospective; Taiwan	190 IBD	13.3%			Higher prevalence of HBsAg in IBD
		80 CD				
		110 UC				
Yeo *et al*[96], 2018	Prospective cohort; Korea	210 IBD	3.8%	26.2%		Treatment-naïve similar prevalence
		109 UC				
		101 CD				
Chen *et al*[35], 2017	Retrospective; China	980 IBD	7.9%	41.2%	46.6%	Higher prevalence of HBsAg in IBD
		334 UC	8.1%	52.7%	48.8%	
		646 CD	7.7%	35.3%	45.5%	
Harsh *et al*[34], 2017	Retrospective; India	908 IBD	2.4%			Similar to regional prevalence
		581 UC	2.2%			
		327CD	2.8%			
Waszczuk *et al* [32], 2016	Prospective cross-sectional; Poland	147 IBD		14.3%		Similar to regional prevalence
Chan *et al*[97], 2016	Retrospective cohort; China	406 IBD	5.7%			Similar to regional prevalence
He *et al*[98], 2015	Retrospective; China	449 CD	13.6%	25.4%	31.2%	Similar to regional prevalence
		226 UC	16.8%	30.1%	24.3%	
Ben Musa *et al* [47], 2014	Retrospective observational; United States	500 IBD	1.8%	3.2%		Similar to regional prevalence (screening rate 51%)
Huang *et al*[99], 2014	Retrospective; China	714 IBD	5.5%	40.6%	21.6%	Higher prevalence of HBsAg in IBD patients
Kim *et al*[37], 2014	Observational; Korea	513 IBD	3.7%			Similar to regional prevalence
		241 CD	4.1%			
		272 UC	3.3%			
Papa *et al*[28], 2013	Prospective; Italy	301 IBD	0.3%	7.3%		Similar to regional prevalence
Park *et al*[93], 2012	Retrospective; Korea	4153 IBD	4.1%			Similar to regional prevalence
		1521 CD	3.6%			
		1728 UC	4.6%			
Katsanos *et al*[30], 2010	Retrospective; Greece	482 IBD	2.3%			Similar to regional prevalence
Chevaux *et al* [100], 2010	Hospital-based; France	315 IBD			48.9%	Similar to regional prevalence
		252 CD	0.8%	2.8%		
		63 UC	1.6%	1.6%		
Loras *et al*[101],	Multicenter Hospital-based;	2076 IBD				Similar to regional prevalence

2009	Spain	1128 CD	0.6%	7.1%	17%	
		928 UC	0.8%	8%	14.9%	
		20 IC	0	5.3%	17.6%	
Tolentino *et al*[27], 2008	Hospital-based; Brazil	102 CD	0	43.3%		Higher prevalence of anti-HBc patients
		74 UC	2.3%	56.7%		
Esteve *et al*[102], 2004	Multicenter; Spain	80 CD	7.5%			Screening prior to anti-TNF treatment
Biancone *et al*[26], 2001	Multicenter; Italy	332 CD	2.1%	10.9%	14.4%	Higher prevalence of HBsAg in IBD
		162 UC	0.6%	11.5%	15.8%	

CD: Crohn's disease; HBc: Hepatitis B core protein; HBs: Hepatitis B surface protein; HBsAg: Hepatitis B surface antigen; IBD: Inflammatory bowel disease; TNF: Tumor necrosis factor; UC: Ulcerative colitis.

Studies from parts of the world with high HBV endemicity have also been published. In a report from India, the prevalence of HBV among 908 CD and UC patients was 2.8% and 2.2% respectively, both being comparable to the national prevalence of HBV[28]. Interestingly, HBV prevalence was higher in patients with intestinal tuberculosis than in IBD patients[34]. Data from China are contradictory; the prevalence of ongoing HBV infection in IBD patients paralleled that in the general population (7.86% and 7.3%)[29]. However, when assessing the prevalence of both present and past infection (HBsAg-positive and HBsAg-negative, anti-HBc positive) prevalence was significantly greater in IBD patients than in healthy individuals[35]. Additionally, in this group of patients, age above 30 years, UC and previous surgery were found to be the main risk factors[35]. Two studies from Korea, a highly endemic area for HBV with predominantly vertical/perinatal mode of transmission, demonstrated higher prevalence of HBV infection among IBD patients than in the western countries, but similar to the Korean general population[36,37]. It is noteworthy that, in the above studies, HBV DNA was not determined in anti-HBc-positive patients, and therefore the true frequency of occult hepatitis B was not determined.

Taken together, the majority of available evidence support the hypothesis that the cumulative prevalence of HBV in IBD patients parallels the national trends for HBV infection in each country. This is particularly true for European countries and, overall, indicates that IBD alone does not seem to constitute a risk factor for hepatitis B.

MANAGEMENT OF HBV INFECTION IN PATIENTS WITH IBD

All patients who have been diagnosed with IBD should be screened for their immunological status regarding exposure to HBV, preferably at the time of diagnosis. Unfortunately, surveys conducted by the European Liver Patients Association (commonly known as the ELPA) suggest that up to 90% of HBV-infected people in Europe are unaware of their condition[38]. It is, therefore, of great importance that patients be screened upon diagnosis and that the screening be performed in a pre-defined manner *via* the implementation of checklists provided by International societies[7,39,40]. It is obvious that such practice is of far greater significance for those patients who are scheduled to commence immunosuppressive therapy[7].

Initial screening should include testing for HBsAg, as well as anti-HBc and anti-HBs [7]. Based on the results from these tests, the immunization status may fall into one of three categories that should be then managed appropriately (Table 2).

HBsAg-negative, anti-HBs-negative, anti-HBc-negative patients

Patients who test negative for all three serological markers are susceptible to HBV infection upon contact with the virus. Currently, vaccination is recommended to all HBsAg-negative patients who are also negative for both anti-HBc and anti-HBs[7].

Encouraging immunization is very important. Treating gastroenterologists should explain the advantages of vaccination, while reassuring patients and providing them with vaccine-related information, as this will lead to better compliance and increased participation in vaccination programs[41,42]. Constant training of the physicians and participation in educational activities is similarly important, because gaps in the knowledge of gastroenterologists regarding vaccinations have been reported[43].

Table 2 Serologic profiles after initial screening

	HBsAg	Anti-HBc	Anti-HBs
Susceptible	Negative	Negative	Negative
Immune due to vaccination	Negative	Negative	Positive
HBV infection	Positive	Positive	Negative
Resolved HBV infection or occult HBV infection	Negative	Positive	Positive or negative

HBc: Hepatitis B core protein; HBs: Hepatitis B surface protein; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

Implementation of a thorough guidance regarding vaccines has been shown to improve the overall adherence to vaccination guidelines[44].

Overall, vaccination rates in IBD patients have been reported to be low[45]. Memled *et al*[46] reported an overall vaccination rate of 28%, which reflects the fact that immunization history is often omitted. Even in tertiary centers, only half of IBD patients may have been screened for HBV[47]. Nevertheless, vaccination attitudes may be changing, nowadays, as improvements in HBV immunization practices for IBD patients have been documented during the last decades. According to Shah *et al*[48], only 8.1% of patients were vaccinated in 2003, *vs* 43.2% in 2011[48]. In this study, between 2003-2011, an overall HBV screening rate of 23.7% in a population of IBD patients under anti-TNF treatment was recorded. Another study from the Netherlands, spanning from 2000 to 2010, showed that the screening rates increased from 36% to 49% during the last 2 years of the study[49]. According to a more recent study of 1834 anti-TNF-naïve patients, HBV screening rates significantly improved between 2010 and 2019 (64% and 87.4% respectively)[50].

Available vaccines for HBV constitute the first- (plasma-derived), second- (yeast- or mammalian-derived recombinant major S antigen) and third-generation (major S and pre-S1 and -S2 proteins) vaccines (Table 3). Vaccines are further differentiated into single antigen vaccines, such as Engerix-B® (GlaxoSmithKline Biologicals, Rixensart, Belgium), HBVaxPRO® (Sanofi-Pasteur, Lyon, France), Recombivax® (Merck & Co, Inc, Kenilworth, NJ, United States), Heplisav-B® (HepB-CpG; Dynavax Technologies, Emeryville, CA, United States), Fendrix® (GlaxoSmithKline Biologicals), Sci-B-Vac® (VBI Vaccines, Cambridge, MA, United States) and combination vaccines like Twinrix® (GlaxoSmithKline Biologicals) (HBV + hepatitis A virus) and Pediarix® (GlaxoSmithKline Biologicals) (HBV + diphtheria/tetanus/whooping cough (pertussis) + polio).

The standard vaccination schedule in IBD patients is the same as that with the general population, which consists of three standard doses of rHBAg (20 μg) at months 0, 1 and 6, although deviations do occur in practice. For example, Indian guidelines recommend a double-dose, accelerated three-dosing scheme of 40 IU/mL in 0-1-2 mo[51]. Furthermore, in 2018, a novel recombinant vaccine was approved (Heplisav-B® - HepB-CpG), which bears a unique adjuvant sequence and is administered in two doses, 1 mo apart. This two-dose vaccine has shown promising results in individuals with risk factors for hyporesponsiveness (see below) but has not yet been tested in IBD patients specifically[52].

After completion of the dosing schedule, titers of anti-HBs should be checked within 1-3 mo to confirm the establishment of adequate anti-HBV immunological status, which is signified by detection of anti-HBs antibodies in the serum. In healthy individuals, HBV vaccination confers > 90% protective immunity. In contrast, it has been demonstrated that response rates after HBV vaccination are reduced compared with healthy individuals[53]. Indeed, it was shown that patients with future diagnosis of IBD had suboptimal vaccination response even before the clinical manifestation of IBD[54]. Based on a meta-analysis by Jiang *et al*[53], which included 13 studies with 1688 patients, the pooled response rate to vaccination for HBV among IBD patients was 61%. Rates were not affected by the specific diagnosis of CD or UC[55]. Not only are the rates of anti-HBs positivity decreased in patients with IBD but the titers of antibodies are also lower. In various studies, the reported average anti-HBs titers in healthy individuals after successful immunization were 720[56] and 822[57] IU/mL. In comparison, IBD patients under anti-TNF therapy had reported average values around 245 IU/mL[56]. This was also confirmed by another study by Belle *et al*[58], wherein median anti-HBs levels after vaccination were significantly lower in immunosuppressed patients (253 IU/mL *vs* 497 IU/mL). The significance of such differences is further exemplified by the fact that post-vaccination targets for anti-HBs titers may

Table 3 Available vaccines

	Company	Antigen - Adjuvant	Posology	Dosing
Engerix-B®	GlaxoSmithKline	Rec. major S Ag (yeast) Aluminum	1 mL	3- or 4-dose
			20 mcg	0-1-6 mo
				0-7 d-21 d-12 mo
HBVaxPRO®	Sanofi-Pasteur MSD	Rec. major S Ag (yeast) Aluminum	1 mL	3- or 4-dose
			5 mcg	0-1-6 mo
			10 mcg	0-1-2-12 mo
			40 mcg	
Fendrix®	GlaxoSmithKline	Rec. major S Ag (yeast) Aluminum + 3-O-desacyl-4 - monophosphoryl lipid A	0.5 mL	4-dose
			20 mcg	0-1-2-6 mo
Heplisav-B®	Dynavax	Rec. major S Ag (yeast) HepB-CpG ligand	0.5 mL	2-dose
			20 mcg	0-1 mo
Twinrix®	GlaxoSmithKline	In.HAV + Rec. major S Ag (yeast) Aluminum	1 mL	3-dose
			720/20 mcg	0-7-21 d
				0-1-6 mo
Sci-B-Vac®	VBI Vaccines	Major S Ag, minor pre-S1 + pre-S2 Ag (mammalian cell) Aluminum	1 mL	3-dose
			10 mcg	0-1-6 mo
Pediarix®	GlaxoSmithKline	DTaP + inactivated poliovirus + Rec.S (yeast) Aluminum	0.5 Ml	3-dose
			10 mcg	(6-8 wk interval)

Ag: Antigen; DTaP: Diphtheria-tetanus-whooping cough (pertussis); In.HAV: Inactivated hepatitis A virus; Rec.: Recombinant.

differ between IBD patients and healthy controls. In immunocompetent individuals, a titer above 10 IU/mL is considered adequate; whereas, in immunosuppressed patients, a higher titer, of 100 IU/mL, is considered protective[51]. Indeed, according to Loras *et al*[59], a distinction is made based on the quantity of the antibodies in the serum between seroprotection for titers > 10 IU/mL and effective vaccination for titers > 100 IU/mL.

Factors that have been associated with an inadequate immune response to HBV vaccine have included older age, immunosuppressive therapy, and incomplete dosing (< 3 doses administered)[53,60]. In addition, Altunoz *et al*[55] noted a negative correlation between disease activity and adequate antibody response both for CD and UC. In another study, it was found that ileal disease correlated with lower responses to Engerix-B®. From an interesting perspective, gut microbiota was recently implicated as a regulator of immune response to vaccination. Experimental data from germ-free mice showed reduced response rates after vaccination, which were ameliorated after establishing normal microbiome[61]. As patients with IBD demonstrate intestinal dysbiosis, it could be hypothesized that the latter may negatively affect immunization in a similar manner. Nonetheless, such a concept has yet to be proven.

The major factor, however, that has been studied in relation to the efficacy of HBV vaccination in IBD patients has been the administration of immunomodulatory therapies[53]; hence, this has been the subject of research and discussion, given the fact that it affects the majority of people with CD or UC. Therefore, it is generally recommended to proceed with HBV vaccination ideally at the time of IBD diagnosis and preferably before initiating treatment. Among all immunosuppressive therapies anti-TNF treatment is a major negative influencer of HBV vaccination, as stated in the meta-analysis of Jiang *et al*[53]. The effect of anti-TNF treatment on vaccine efficacy in patients with IBD was assessed by Gisbert *et al*[62], who reported a response rate of 46%. On the other hand, in a European study, the response rates after primary vaccination with Engerix-B® in IBD patients, despite being lower in comparison with healthy individuals, were not adversely associated with the use of biologics and immunomodulators[58]. In the study of Loras *et al*[59], total response rates of 59% after first and second vaccination attempts were observed in patients treated with anti-TNF

monotherapy; whereas, in patients under combination therapy, only a 38% seroprotection rate was observed, highlighting the negative effect of combinatorial immunosuppression. Among the various anti-TNF biologics, the use of infliximab correlated with lower antibody response rate (16.7%) compared with adalimumab (48.4%)[56]. In the same study, ustekinumab exhibited 72% antibody response after vaccination. Treatment with vedolizumab does not seem to influence immune response after vaccination based on anti-HBs titers that were determined in the context of a randomized double-blind placebo control trial[63]. These findings are confirmed by recent data from Harrington *et al*[64], whereby 62.5% of IBD patients treated with vedolizumab achieved an anti-HBs level above 10 mIU/mL after a three-dose standard vaccination scheme with Engerix-B®, which is comparable to the response of immunocompetent patients. Data regarding the effect of treatment with tofacitinib on the vaccination efficacy in IBD patients are scarce. Studies in rheumatology show that it is associated with diminished response to pneumococcal but not influenza vaccination[65]. In a small retrospective study of patients with rheumatoid arthritis on tofacitinib, only 2 HBsAg-positive patients, who did not receive prophylaxis, developed reactivation[66].

Based on the aforementioned results, it appears that patients with IBD who are treated with immune-modifying agents may exert suboptimal immunization status after HBV vaccination. Consequently, strategies to improve efficacy have been implemented with various results (see next paragraph). Such approaches include accelerated, repeated or increased dosing, whereas different vaccines may also display diverse efficacies. Nevertheless, the most valuable approach may be the selection of the appropriate timepoint for vaccination. In fact, early vaccination of young patients who have not received any immunosuppressants is the best strategy for optimal immune response.

Gisbert *et al*[67] tested a protocol combining both increased dose and shorter intervals of vaccination using double-dose of Engerix-B® given in 0-1-2 mo. This accelerated scheme achieved an improvement in immunization rates from 41% to 75% compared with the standard protocol. Loras *et al*[59] used the same scheme for the vaccination of IBD patients under anti-TNF treatment and found a 57% response rate. A study by Chaparro *et al*[68] compared the double-dose Engerix-B® vaccine given in a four-dose schedule (0-1-2-6 mo intervals) with the single-dose Fendrix® given in the same intervals. The two vaccines demonstrated equivalent effectiveness rates but the four-dose protocol exhibited enhanced efficacy (range: 68%-75%) in achieving anti-HBs titers above 100 IU/mL. In another recent study by Haykir *et al*[56], comprising a standard schedule high-dose immunization program for a mixed population of IBD and rheumatology patients under immunosuppression, did not significantly improve the immune response. Similarly, use of third-generation vaccines (*e.g.*, Sci-B-Vac®) did not show any additional benefit compared with second-generation vaccines (*e.g.*, Engerix-B®)[69], despite elucidating higher response rates in immunocompetent healthy individuals. Taken together, these studies show that intensified protocols may accomplish higher rates of effective immunization. The variety of such approaches indicates that each IBD center should test and propose its own protocols, based on local experience, efficacy rates and available options.

Patients who have been effectively immunized should be monitored at least every 2 years, by assessing anti-HBs titers. It has been shown that 18% of patients lose their antibodies on a yearly basis[69,70]. In the case that the first immunization attempt is unsuccessful, several revaccination strategies have been proposed (Table 4). The most common approach in this setting entails the repetition of a standard three-dose scheme. Indeed, a three-dose revaccination schedule yielded better response rates (62.9%) than a single-dose or 2 additional doses (40.2%), when evaluated according to patient ability to mount an anti-HBs titer above 10 IU/mL, emphasizing the need of completion of the three-dose schedule[71]. The same three-dose revaccination approach in the study of Cossio-Gil *et al*[60] achieved a response rate of 52.8%. However, in these studies, an anti-HBs threshold of 10 IU/mL, instead of 100 IU/mL, was used. Levels of anti-HBs between 10-100 IU/mL after first vaccination are correlated with effective protective immunity characterized by obtaining anti-HBs > 100 IU/L after revaccination[59]. The response rate for patients older than 35 years who initially developed anti-HBs titer < 10 IU/mL was only 25% after repeat vaccination[59].

The Turkish society of gastroenterology recommends a response-guided approach based on anti-HBs titers after primary vaccination[72]. In patients with undetectable and/or < 10 IU/mL anti-HBs levels a double-dose, 0-1-6 mo complete revaccination scheme is proposed; whereas, in patients with anti-HBs levels between 10-100 IU/mL, a single double-dose booster dose is administered[72]. In healthy individuals without

Table 4 Revaccination studies for hepatitis B virus in inflammatory bowel disease

Ref.	Study	Patients, *n*	Strategies	Response rate for anti-HBs
Pratt *et al*[71], 2019	Retrospective cohort	149	3-dose schedule *vs* 1 or 2 doses	62.9%
				40.2% (> 10)
Cossio-Gil *et al*[60], 2015	Retrospective cohort	53	3-dose schedule	52.8%
Loras *et al*[59], 2014	Prospective	389	Double-dose 0-1-2	31.3% (> 100)
				44.4% (10-100)

HBs: Hepatitis B surface protein.

adequate immune response after primary vaccination with Engerix-B®, the use of a different vaccine (*i.e.*, Fendrix® or HBVaxPRO®) for revaccination resulted in improved response rates[73]. However, this practice has not been evaluated in IBD patients. At present, the optimal revaccination protocol in this population is unknown and further studies are needed. Vaccination for hepatitis B is generally considered safe. Injection site reactions and mild systemic adverse events are the most commonly reported problems[1]. HBV vaccination does not influence the course of IBD.

HBsAg-positive patients

Patients who test positive for HBsAg have ongoing HBV infection and are at the highest risk for reactivation if their disease is quiescent at baseline. Those patients should be initially tested with complete blood count and for levels of ALT, aspartate aminotransferase, albumin, and HBV DNA, as well as undergoing liver stiffness measurement and tests for other hepatitis virus (*e.g.*, hepatitis D virus). Results from those tests will help assess HBV status and evaluate the need for antiviral treatment and monitoring[7,11]. Patients who have HBsAg-positive and HBeAg-positive or -negative chronic hepatitis B [HBV DNA > 2000 IU/mL, ALT > upper limit of normal and/or at least moderate liver necroinflammation or fibrosis] need to receive antiviral treatment, irrespective of type of immunosuppression[11]. HBsAg-positive patients with IBD who will commence treatment with immune-modifying medications are at risk for reactivation of HBV. Factors that relate to HBV reactivation in IBD patients include infection status, level of immunosuppression and duration of immunosuppressive therapy.

Various guidelines have been offered as to the optimal course of action regarding the management of HBsAg-positive patients with IBD[7,11]. As a general rule, all HBsAg-positive patients with either chronic infection or hepatitis should receive prophylactic antiviral treatment before starting any type of immunosuppressive treatment[7,11,51]. The only exception is the statement from the American Gastroenterological Association (AGA)[74], whereby guidelines are diversified according to the estimated risk for HBV reactivation into high (> 10%), moderate (1%-10%) and low (< 1%) risk. In IBD patients, the therapy conferring high risk of reactivation is prednisone ≥ 10 mg daily for ≥ 4 wk. Therapies with moderate risk include TNF-α inhibitors, cytokines or integrin inhibitors and prednisone < 10 mg daily for ≥ 4 wk. Low risk for reactivation is conferred by treatment with traditional immunosuppressive agents (azathioprine, methotrexate) and any dose of oral steroids for ≤ 1 wk or low dose (< 10 mg daily) for ≥ 4 wk. Accordingly, high and moderate risk individuals should receive prophylactic nucleos(t)ide therapy, whereas patients at moderate risk are given the option for monitoring. The AGA recommends that antiviral treatment should be continued for at least 6 mo after discontinuation of the immunosuppressants[74]. In patients at low risk for reactivation, monitoring is sufficient. In addition, combination of immunomodulatory medications increases the risk of reactivation. In a multicenter Spanish study, Loras *et al*[59] demonstrated a pronounced risk of HBV reactivation in HBsAg-positive patients receiving ≥ 2 immunosuppressants without antiviral prophylaxis.

It should be noted that these recommendations are primarily based on data derived from oncology, rheumatology and hematology studies[75-77]. The extension of these conclusions in IBD patients is precarious, since differences do exist. In particular, the duration of treatment differs considerably between the two groups. Chemotherapy is usually offered for a finite number of treatment cycles. In contrast, IBD treatments may last life-long. In relevance to this, it should be remembered that HBV is not a direct

cytopathogenic virus and that liver cell damage in chronic hepatitis B is the result of immune system activation against the infected cells. As a result, a hepatitis flare is not observed during the time of maximal immunosuppression but at a later time point. Thus, anti-viral therapy should be continued for at least 12 mo after the cessation of any immunosuppressive therapy in patients with immune-mediated diseases, including IBD[78].

Nucleos(t)ide analogs are the preferred antiviral therapy in patients with IBD who will receive immunosuppression, as long-term treatment is an effective and safe strategy. In contrast, the use of pegylated-interferon is discouraged. The third-generation antivirals of tenofovir, entecavir and tenofovir alafenamide are recommended, due to their high antiviral activity with practically no resistance (antivirals with high resistance barrier). Entecavir or tenofovir alafenamide should be preferred over tenofovir in patients above 60 years of age, in patients with a history of bone disease (*i.e.*, fracture, osteoporosis, chronic steroid use), and in patients with renal dysfunction (with estimated glomerular filtration rate < 60 mL/min/1.1, albuminuria > 30 mg/24 h, or low phosphate < 2.5 mg/dL)[11]. Lamivudine is a good antiviral for short-term use, as the rate of 1-year and 2-year resistance is 20% and 30%, respectively. Given that immunosuppressives for IBD patients are long-term therapies, the use of lamivudine is discouraged. In fact, in a small published study, it was shown that 6 out of 8 IBD patients who received lamivudine prophylaxis required a change to newer antivirals[30].

Antiviral treatment should ideally start 2 wk prior to the commencement of immunosuppression and should be continued for at least 12 mo after treatment cessation, provided that the underlying HBV infection is quiescent.

According to a series of patients with chronic HBV infection and IBD with a long-term follow up (20 years), the natural history of HBV is not affected in this group of patients[36]. Nevertheless, an association between chronic viral hepatitis and non-alcoholic fatty liver disease has been shown in patients with IBD. In a recent study by Losurdo *et al*[33], IBD patients with concurrent chronic viral hepatitis present more frequently with diabetes, wide waist circumference and increased liver stiffness. In addition, the frequent use of steroids, is further considered a risk factor for NAFLD and the combination of liver steatosis and viral hepatitis may sensitize the liver and render it more vulnerable to developing liver-associated complications. These facts further emphasize the need for prophylaxis and treatment strategies for HBV in patients with IBD.

HBsAg-negative/anti-HBc-positive patients

Patients with isolated anti-HBc antibodies on serological testing represent a non-homogenous population. This serologic profile reflects either a false positive result particularly in regions of low endemicity or corresponds to the 'window' period before the appearance of anti-HBs, as in the case of resolved HBV infection. Moreover, isolated anti-HBc antibodies in the presence of undetectable anti-HBs may be found in a patient with resolved HBV infection, due to waning immunity after many years or to treatment with immunomodulating therapy. The latter scenario is of potential significance for the IBD patient, as it carries risk for HBV reactivation. This risk is lower than in HBsAg-positive patients, and is estimated to be between 4%-5%, based primarily on findings from oncological studies. In a meta-analysis by Cholongitas *et al* [79], the risk of HBV reactivation in anti-HBc-positive patients with non-hematological diseases was 3.6%. Quantification of anti-HBc antibodies can help distinguish occult hepatitis B infection from a past HBV infection, with a cutoff of 6.6 IU/mL[80]. Detection of anti-HBc antibodies serves as a surrogate marker of occult HBV infection, which is defined as the detection of HBV DNA in the liver tissue (gold standard) or in the blood[23].

The prevalence of occult HBV infection in this particular subgroup of patients varies, depending on the HBV DNA threshold used (200 U/mL) and ranges between 11%-89%[81]. More recent data indicate detectable HBV DNA in 0-27% of patients with exclusive anti-Hbc positivity[82]. When IBD populations were exclusively examined, the rates of anti-HBc positivity were highly dependent on the HBV endemicity. Data from low endemicity areas such as Italy and France range from 7.7% [33] to 0.6%[29] respectively, whereas data from eastern Europe (intermediate endemicity region) indicate a prevalence of 12%. In an HBV endemic area, anti-HBc positivity was 41.2%[35]. The risk of HBV reactivation depends also on the specific state of immunodeficiency, including the particular immunosuppressive therapy, being significantly higher in patients with malignant hematological diseases and especially with the use of the anti-CD20 agent rituximab.

The first case of HBV reactivation in an IBD patient with isolated anti-HBc positivity was reported in 2006, involving a woman with CD who was treated with infliximab [83]. Ever since, studies assessing the reactivation probability in patients under anti-TNF treatment have been performed, ultimately confirming a low risk of reactivation. Indeed, the reported reactivation rates vary between 3.13%[84] and 7%[59]. It has been reported that the risk of reactivation may be higher with the use of infliximab than with the other anti-TNFs[85].

When an anti-HBc-positive patient is scheduled to start therapy with immune-modifying medications, he should have an evaluation for HBV DNA presence in the blood. In the case of detectable HBV DNA, the patient is treated as being HBsAg-positive. The management of patients showing negativity for HBV DNA is not equally straightforward, particularly in patients with IBD, as literature is scarce. According to the AGA guidelines, anti–HBc-positive, HBV DNA-negative IBD patients who receive corticosteroids in high dose (> 20 mg prednisone daily) or moderate dose (10-20 mg prednisone daily for ≥ 4 wk) and/or anti-TNFs should receive antiviral prophylaxis over monitoring[74]. Antivirals should be continued for at least 6 mo after stopping immunosuppressants. It should be noted, however, that the level of evidence is "weak recommendation/moderate quality evidence" and, thus, the alternative for monitoring is also an acceptable option. According, to the European Association for the Study of the Liver (commonly referred to as the EASL) and the European Crohn's and Colitis Organisation (commonly referred to as the ECCO) guidelines, surveillance is strongly indicated and includes monitoring of transaminases, HBsAg, anti-HBs antibodies and HBV DNA, in the first month of treatment and every 3 mo thereafter. Pre-emptive antiviral therapy is commenced in the case of HBsAg seroconversion or HBV DNA detection. If monitoring cannot be ensured, it would be prudent to adopt the approach of the AGA and start prophylactic antiviral therapy. Accessibility to regular testing and follow-up as well as patient preferences should also be incorporated in decision-making. Treatment with azathioprine, 6-mercaptopurine or methotrexate in anti-HBc-positive patients carries a low risk of reactivation.

The frequency of anti-HBs in anti-HBc patients is approximately 75%. Emerging evidence suggests that HBV reactivation is most likely in those with low or undetectable anti-HBs levels[86]. The protective role of anti-HBs was illustrated in a meta-analysis by Paul *et al*[86], where patients with detectable anti-HBs had 79% lower odds of reactivation. Nevertheless, in the same meta-analysis, it is noted that HBV reactivation still occurs in patients with both anti-HBc positivity and anti-HBs positivity. In lymphoma patients, undetectable anti-HBs or titers of 10-100 mIU/mL at baseline are independent predictive factors for reactivation compared with anti-HBs titers of ≥ 100 mIU/mL[87]. Furthermore, in lymphoma patients treated with chemotherapy, quantification of anti-HBc and anti-HBs might help to predict HBV reactivation. High anti-HBc (6.41 IU/mL) and low anti-HBs (< 56.48 mIU/mL) at baseline are associated with a higher risk of reactivation (hazard ratio: 517.29; $P < 0.001$)[88]. However, this approach has not been evaluated in IBD patients. The proposed strategy according to ECCO guidelines for patients with anti-HBc and/or anti-HBs positivity is monitoring[7].

Acute HBV infection

Acute HBV infection during the course of IBD is a rare occurrence. Limited data exist in the literature and only few clinical cases have been described. Data from these case reports are inconsistent, underlining the variable evolution of acute HBV infection in immunocompromised IBD patients. In the case of a patient who developed acute hepatitis B, infliximab was temporarily discontinued, until antiviral treatment was commenced without further complications[48]. In contrast, a young CD patient on infliximab developed acute HBV infection that rapidly evolved to fatal liver failure [89].

In healthy adult individuals, acute HBV infection leads to resolution in approximately 90% of cases; whereas, in immunosuppressed patients, spontaneous recovery rates are reduced[90]. The diagnosis of acute hepatitis B infection is based on the detection of IgM anti-HBc. Yet, anti-HBc IgM are also detected during exacerbations of chronic hepatitis B. Quantitative measurement of anti-HBc IgM may be helpful in differentiating acute HBV infection from a flare of a chronic infection, as lower levels are detected in the latter[91]. Specific threshold values depend on the assay used. For instance, when applying chemiluminescent immunoassay (also commonly known as CLIA), levels > 10 show 100% sensitivity and 99% specificity[92] and levels > 8 show sensitivity of 96.2% and specificity of 89.7%[93]. Similarly, for HBV DNA, lower levels are observed in acute *vs* a flare of chronic HBV infection[91].

Kinetics of HBsAg have been used to predict the likelihood of chronicity, as a 50% reduction within 4 wk is predictive of resolution of HBV infection[91]. Prevention of liver failure is the therapeutic goal in acute hepatitis B in immunocompetent adults. This is why only patients with a protracted course or coagulopathy are candidates for antiviral treatment. In immunosuppressed patients, however, timely initiation of nucleos(t)ide analogues is suggested in order to avert the progression to liver failure.

CONCLUSION

Despite the implementation of mandatory vaccination of newborns against HBV, hepatitis B remains a significant global clinical problem. As the prevalence of IBD is also growing around the world, a substantial number of CD and UC patients with concomitant HBV infection remains and new cases are expected to occur. Screening of IBD patients with HBV serology is of paramount significance and should be actively communicated among patients and ideally implemented at diagnosis on IBD. The initial vaccination approach for IBD patients is largely similar to the recommendations for the general population, despite a lower reported vaccination efficacy, with considerable variation in revaccination strategies. It should be kept in mind that immunosuppressive treatment increases the risk of HBV reactivation; thus, close monitoring of patients at-risk is required. In the case of HBsAg positivity, antiviral prophylaxis is offered to IBD patients regardless of type of immunosuppression. On the other hand, HBsAg-negative, anti-HBc-positive patients represent a low reactivation risk group, for whom monitoring according to international and local guidelines is indicated.

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MINIREVIEWS

Worldwide management of hepatocellular carcinoma during the COVID-19 pandemic

Riccardo Inchingolo, Fabrizio Acquafredda, Michele Tedeschi, Letizia Laera, Gianmarco Surico, Alessia Surgo, Alba Fiorentino, Stavros Spiliopoulos, Nicola de'Angelis, Riccardo Memeo

ORCID number: Riccardo Inchingolo 0000-0002-0253-5936; Fabrizio Acquafredda 0000-0002-8601-7537; Michele Tedeschi 0000-0002-2101-5418; Letizia Laera 0000-0003-2183-8817; Gianmarco Surico 0000-0002-8329-0204; Alessia Surgo 0000-0003-4502-9635; Alba Fiorentino 0000-0003-4201-5852; Stavros Spiliopoulos 0000-0003-1860-0568; Nicola de'Angelis 0000-0002-1211-4916; Riccardo Memeo 0000-0002-1668-932X.

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Riccardo Inchingolo, Fabrizio Acquafredda, Department of Interventional Radiology Unit, "F. Miulli" General Regional Hospital, Acquaviva delle Fonti 70021, Bari, Italy

Michele Tedeschi, Riccardo Memeo, Unit of Hepato-Pancreatic-Biliary Surgery, "F. Miulli" General Regional Hospital, Acquaviva delle Fonti 70124, Bari, Italy

Letizia Laera, Gianmarco Surico, Department of Oncology, "F. Miulli" General Regional Hospital, Acquaviva delle Fonti 70021, Bari, Italy

Alessia Surgo, Alba Fiorentino, Department of Radiation Oncology, "F. Miulli" General Regional Hospital, Acquaviva delle Fonti 70021, Bari, Italy

Stavros Spiliopoulos, Department of 2nd Radiology, National and Kapodistrian University of Athens, Athens 12461, Greece

Nicola de'Angelis, Unit of Minimally Invasive and Robotic Digestive Surgery, "F. Miulli" General Regional Hospital, Acquaviva delle Fonti 70021, Bari, Italy

Corresponding author: Riccardo Inchingolo, MD, Chief Doctor, Director, Department of Interventional Radiology Unit, "F. Miulli" General Regional Hospital, Strada Prov. 127 Acquaviva–Santeramo, Acquaviva delle Fonti 70021, Bari, Italy. riccardoin@hotmail.it

Abstract

The coronavirus disease 2019 (COVID-19) pandemic has impacted hospital organization, with the necessity to quickly react to face the pandemic. The management of the oncological patient has been modified by necessity due to different allocation of nurses and doctors, requiring new strategies to guarantee the correct assistance to the patients. Hepatocellular carcinoma, considered as one of the most aggressive types of liver cancer, has also required a different management during this period in order to optimize the management of patients at risk for and with this cancer. The aim of this document is to review recommendations on hepatocellular carcinoma surveillance and management, including surgery, liver transplantation, interventional radiology, oncology, and radiotherapy. Publications and guidelines from the main scientific societies worldwide regarding the management of hepatocellular carcinoma during the COVID-19 pandemic were reviewed.

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Core Tip: Hepatocellular carcinoma is one of the most common cancers in the world. The aim of this review is to focus on the impact of the coronavirus disease 2019 pandemic on the management of patients with hepatocellular carcinoma and to verify how multidisciplinary management has changed to face the necessity of hospital reorganization.

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INTRODUCTION

In December 2019, the world experienced a new coronavirus from China, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is responsible for a respiratory disease with the characteristics of a worsening interstitial pneumonia, later called coronavirus disease 2019 (COVID-19). This virus spread quickly across all continents generating a global pandemic, officially declared on March 11, 2020, which has put hospitals around the world under pressure as they were unprepared to face a very high number of hospitalizations in intensive care units and medical wards[1]. This affected not only intensive care, but clinical services of cancer patients as well. In fact, the recruitment of a large part of medical staff in the aforementioned departments in order to contain and fight this new health emergency caused serious problems in the management of all other wards with consequent delays in the care and surveillance of patients with other pathologies and, in particular, of those with oncological disease[2,3]. Moreover, cancer patients are at major risk to contract COVID-19 infection both from nosocomial exposure and due to their immunocompromised states [4].

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world, which accounts for approximately 6% of all cancer incidences[5] and is nowadays considered a global health problem[6]. Compared to other neoplasms, HCC patients are more susceptible to the effects of the COVID-19 pandemic because the hepatic injury[7] caused by SARS-CoV-2 could complicate the pre-existing hepatitis virus infection and cirrhosis in most HCC patients.

Due to the major treatment limitations and exposure risk created by the pandemic for patients with HCC, a general consensus amongst clinicians acknowledged that deviations from the standard of care are necessary, and specific international guidelines on HCC management have been drafted by the American Association for the Study of Liver Disease[8], the European Association for the Study of the Liver[9], the International Liver Cancer Association[10], the Asian Pacific Association for the study of the Liver[11], and the São Paulo Clínicas Liver Cancer Group Multidisciplinary Consensus Statement[12] (Table 1).

The aim of this review is to compare and summarize all these recommendations and guidelines, particularly in the diagnostic and therapeutic process of patients affected by HCC to provide clinicians with the best measures to be adopted in the framework of this ongoing pandemic.

SURVEILLANCE

As recommended by the American Association for the Study of Liver Disease and the European Association for the Study of the Liver, deferring HCC surveillance by 2–3 mo during the COVID-19 pandemic because of reduced radiologic capacity is likely

Table 1 Recommendations from main international liver society

	APASL	HC-FMUSP	EASL-ESCMID	ILCA	AASLD
Liver resection	Generally, liver resection with curative intent should not be delayed. However, in cases of high risk of decompensation or comorbidities, surgical intervention should be postponed or alternative therapy such as ablation should be adapted	The indications for surgical resection are the same as those during the prepandemic period and are as follows (18-21): In patients without cirrhosis: Solitary or oligonodular HCCs; in patients with chronic liver disease: Solitary tumors (regardless of size), preserved liver function (Child–Pugh A), and absent or mild portal hypertension (small caliber esophageal varices and platelets 100000/ mm^3)			Can consider bridging locoregional therapy (TACE/TARE/SBRT), systemic therapy, or active monitoring if necessary to delay surgery
LT	LT for patients with poor short-term prognosis should not be delayed. Elective living donor transplantation may be suspended. In patients with complete response to bridging therapy on transplant list, transplantation may be suspended	LT is the treatment of choice for patients with early HCC (BCLC-A) and impaired liver function (Child–Pugh B/C), clinically significant portal hypertension, and those with early HCC who are not candidates for resection	Patients on the LT waiting list with decompensated cirrhosis are at high risk of severe COVID-19 and death following SARS-CoV-2 infection. We therefore recommend that LT centers aim to restore transplantation services following the peak of the COVID-19 epidemic wherever possible. In centers with ongoing resource limitations, LT should be prioritized for patients with poor short-term prognosis including those with acute liver failure, ACLF, high MELD score (including exceptional MELD points), and HCC at the upper limits of the Milan criteria	Unique considerations of COVID-donor derived infection and immunosuppression post-transplant. Consider cessation of LDLT (lower MELD) and delaying transplant in those with complete response	Limit the number of patients coming to clinic for transplant evaluations. Consider evaluating only patients with HCC or those patients with severe disease and high MELD scores who are likely to benefit from immediate liver transplant listing
Ablation	Ablation with curative intent should not be delayed. Ablation is an acceptable alternative to resection for cases of three or fewer tumors, each 3 cm or smaller, and of Child–Pugh class A or B liver dysfunction	Radiofrequency ablation can be performed in patients with very early (BCLC-0) or early (BCLC-A) HCC and have solitary nodules < 3 cm in size			Reserve for those with best chance of response (size < 3 cm) and can consider SBRT
Vascular intervention	Vascular interventions may be postponed because they are used as cytoreductive treatments in most cases. Vascular interventions should be suspended in cases of risk of decompensation or comorbidities that increase the risk of severe COVID-19	TACE/TAE: Can be performed in patients with solitary nodules > 3 cm in size as local disease control or as a "bridge treatment" to surgery			Consider TACE for single or multifocal HCC. Consider TAE, DEB-TACE or TARE instead of TACE and perhaps systemic therapy in some patients with large tumor burden
Radiation therapy	Radiation therapy for cases of symptom control or at low risk of progression may be postponed. However, radiation therapy for function- or life-threatening situations have to be treated without delay. The course of radiation	SBRT: Can be considered in patients who have contraindications to RFA or TACE/TAE			

	should be shortened when appropriate	
Systemic therapy	Oral tyrosine kinase inhibitors would be better than infusion regimens during the pandemic. The impact of immunotherapy on the course of COVID-19 is not known	Systemic therapy: May be used as a "bridge therapy" for surgery in patients with contraindications to other treatments

ILCA: International Liver Cancer Association; AASLD: American Association for the Study of Liver Disease; SBRT: Stereotactic body radiation therapy; COVID-19: Coronavirus disease 2019; TACE/TAE: Transarterial chemoembolization/embolization; LT: Liver transplantation; HCC: Hepatocellular carcinoma; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; LDLT: Living donor liver transplantation; MELD: Model for end-stage liver disease; ACLF: Acute-on-chronic liver failure; EASL-ESCMID: European Association for the Study of the Liver-European Society of Clinical Microbiology and Infectious Diseases; APASL: Asian-Pacific Association for the Study of the Liver society; RFA: Radio frequency ablation; TARE: Transarterial radioembolization; BCLC: Barcelona Clinic Liver Cancer; DEB-TACE: Drug-eluting bead transarterial chemoembolization; HC-FMUSP: São Paulo Clínicas Liver Cancer Group Multidisciplinary Consensus Statement.

safe. In patients with active COVID-19 infection, HCC surveillance should be postponed until they recover[13].

Although surveillance is important for early detection of HCC, risk varies between patients; in an environment with limited resources, risk stratification models[14-16] may be used even if they have not been fully clinically validated. This can be useful to identify patients who need to be prioritized for surveillance.

If ultrasound-based surveillance is not possible for extended periods of time because of lack of social distancing between operator and patient, blood-based biomarkers may be considered as an alternative strategy. Given the insufficient sensitivity and specificity of alpha-fetoprotein used alone[17], different biomarker panels have been proposed. The best evaluated is GALAD, which combines gender, age, and three biomarkers (alpha-fetoprotein, alpha-fetoprotein-lectin-reactive, and des-gamma carboxyprothrombin)[18].

HCC surveillance after curative therapy (surgery and ablation) is recommended and should be performed using cross-sectional imaging with multiphase abdominal computed tomography or contrast enhanced magnetic resonance imaging, the latter associated with noncontrast chest computed tomography[19].

A principle of maximizing the risk-benefit ratio should be taken for the surveillance of HCC and monitoring of patients who have received therapies for HCC. Prioritizing imaging resources to those patients at highest risk of incident HCC and recurrence following therapy, while prioritizing those patients who are eligible for an imminent liver transplantation (LT) is a judicious strategy to risk stratifying these patients.

INTERVENTIONAL RADIOLOGY

During the outbreak of the COVID-19 pandemic in early 2020, serious restrictions were applied in interventional radiology (IR) departments regarding the management of elective procedures. Resources and facilities were diverted to treat the large number of COVID patients, allowing limited opportunities for non-COVID-19 related procedures. Primarily urgent/emergency and carefully selected elective cases were performed in IR departments. This largely affected IR cancer care worldwide[20]. More specifically, for patients with HCC where meticulous coordination between different specialties and sequential various IR treatments (mainly percutaneous ablation and different types of transarterial embolotherapy) followed by strict imaging follow-up protocols are required to maintain an efficient therapy. The pandemic caused significant disturbances and negatively affected patient care[7].

There is consensus that HCC patients should continue to receive locoregional therapy during the COVID-19 pandemic, with choice of therapy discussed in a multidisciplinary format[8]. There is concern for serious COVID-19 infection in those receiving conventional transarterial chemoembolization (TACE) (with cytotoxic agents) because of systemic absorption with increased myelosuppression, and therefore the International Liver Cancer Association recommends other forms of locoregional therapy over conventional TACE (*e.g.*, bland embolization, drug-eluting bead-TACE, Y-90)[10]. Finally, consideration for earlier transition to systemic therapy could be considered in locally advanced HCC patients[21].

Several authors investigated the effect of the pandemic in HCC patients. In a study published by an Italian center, the authors reported that clinical practice adapted to minimize HCC patient exposure to the virus. As a result, a delay of treatment (transarterial procedures, thermal ablation, and systemic treatment) of ≥ 2 mo was noted in 26% of the patients during the COVID-19 outbreak compared with the same period in 2019[22]. Similarly, in a French multicentric study investigating patients from six referral centers of the metropolitan area of Paris (an area highly impacted by the COVID-19 pandemic), 21.5% of patients experienced a treatment delay > 1 mo in 2020 compared to 9.5% in 2019 (P < 0.001), although a significant difference in the modification in treatment strategy between the two periods was not noted[23]. Nevertheless, the impact of the COVID-19 pandemic on IR treatment outcomes in patients with HCC remains to be determined.

Generally, experts recommend avoiding treatment delays in HCC patients in order to decrease the risk of tumor progression, while patients with the highest chance for definite cure and those with the lower risk of developing severe complications should be prioritized for treatment. In an elegant review of current evidence, Chan *et al*[7] stratified the modifications of treatment required for HCC patients according to the stage of the disease[7]. The authors summarized that all patients should undergo multidisciplinary team evaluation using stricter selective criteria in order to identify those who would benefit the most from IR therapy. Optimally, patients could be referred in centers where IR services were less affected by the pandemic. In early-stage HCC, ablation was advised over surgical resection due to the limited availability of operating rooms, intensive care unit beds, and anesthetic facilities. In intermediate-stage HCC, TACE patient selection to identify patients that will benefit more could be performed using specific stratification scores, such as the Barcelona Clinic Liver Cancer (BCLC) stage B subclassification, the hepatoma arterial-embolization prognostic score, and the "beyond-up-to-seven" criteria. A personalized decision should be then taken regarding the number and frequency of TACE sessions, while in cases in which TACE therapy could not be appropriately followed due to unacceptable delays, systemic treatment or regular surveillance could be alternatively used.

SURGERY

Several guidelines have been proposed to clarify how to maintain standard of care of HCC in this pandemic era.

According to the European Association for the Study of the Liver- European Society of Clinical Microbiology and Infectious Diseases, listing for transplantation should be restricted to patients with poor short-term prognosis including those with acute/acute-on-chronic liver failure, high model for end-stage liver disease score (including exceptional model for end-stage liver disease scores), and HCC at the upper limits of the Milan criteria because transplantation activities/organ donations will likely be reduced in many countries and areas.

For patients already listed, SARS-CoV-2 routine testing should be performed before LT in both donors and recipients. Living-donor transplantations should be considered on a case-by-case basis. After LT, guidelines should be followed as usual trying to avoid as much contact as possible with medical staff.

In HCC patients, care should be maintained according to guidelines, including treatment and evaluation for LT[9].

A paper from the Italian Association of the Study of the Liver stated that the management of HCC has been negatively affected by the pandemic with, unfortunately, a reduction of surveillance and follow-up. Particularly, surgical procedures had been reduced or suspended in 44% of the centers. COVID-19 has also affected the liver transplant activity with a reduction in the number of performed transplants in 23% of the centers and interruption of the activity in less than 1%[24].

A multidisciplinary evaluation has been proposed by Santambrogio *et al*[25] with a treatment strategy adapted to the tumor size. Particularly, HCC less than 2 cm should be followed up monthly, those between 2 cm and 3 cm should be preferentially treated by local ablation (percutaneous or laparoscopic), while HCC > 3 cm require a surgical approach, preferably avoiding major hepatectomies and using the laparoscopic approach[25]. The Asian-Pacific Association for the Study of the Liver Society proposes that liver resection with curative intent should not be delayed. However, in cases of high risk of decompensation or comorbidities that increase risk of severe COVID-19, surgical intervention should be postponed or alternative therapy such as ablation should be adapted. Laparoscopic or robotic surgery during the pandemic may

contribute to decreased length of stay as compared with open surgery as well as minimizing the need for medical treatments. On the other hand, pneumoperitoneum, which is inevitable in laparoscopic or robotic surgery, may bring a higher risk of aerosol exposure to the surgeons and staff.

Transplantation should be decided on case-by-case basis. LT for patients with poor short-term prognosis, such as with high model for end-stage liver disease score and HCC at the upper limits of the Milan criteria are in high priority and should not be delayed. Those with compensated liver disease and within the lower limits of Milan criteria have medium priority and may be suspended to minimize the risk of the donor and the recipient. In patients with complete response to bridging therapy on the transplant list, transplantation may also be suspended until it can be performed safely with sufficient resources[11].

The International Liver Cancer Association suggests that surgical treatment should be offered to patients with low risk of decompensation and without comorbidities that increase the risk of severe COVID-19. If surgery is not possible, then alternative strategies should be performed (local ablation, TACE)[10].

The policy formulated by The Working Group Report of the Japan Association of Molecular Targeted Therapy for HCC recommends that patients not requiring emergency surgery, based on the macroscopic classification, degree of differentiation, and staging of the tumor, should be advised to avoid hospital admission by postponing surgery. The results of the surgery *vs* radiofrequency ablation trial indicate that radio frequency ablation, the less invasive option, should be proactively considered if there are ≤ three nodules each measuring ≤ 3 cm, which would shorten hospital stay[26]. If postponing surgical resection is considered, tumor growth should be suppressed using alternative outpatient therapy, such as bridging systemic therapy, with surgery rescheduled after carefully evaluating the risks and benefits of hospital admission in light of the COVID-19 pandemic[27].

The São Paulo Clínicas Liver Cancer Group Multidisciplinary guidelines state that surgery should be considered for patients with chronic liver disease and solitary tumors > 3 cm in size, to preserve liver function, with no clinically significant portal hypertension or other comorbidities, who are young, and with favorable locations for resection. This multidisciplinary group agrees with others that the living donor liver transplant program should be temporary suspended in order to preserve both donors and recipients.

They also consider delaying LT in patients with a complete response to "bridge therapies" and maintain close monitoring with imaging examinations to detect any recurrence. Patients with HCC who have significant liver dysfunction and/or viable tumors and have a high risk of losing eligibility for transplantation, especially those who do not respond to "bridge therapies" or present with tumor progression, should remain on the list for LT[12].

ONCOLOGY

During the COVID-19 pandemic, the medical community is experiencing a crisis due to lack of resources. But cancer patients represent a heterogeneous group that differ in prognosis, progression, and treatment. Oncologists have an important role to play for their patients in regard to resource reallocation[28]. In this era, the oncological approach to HCC treatment has been modified. Since February 2020, the first analysis related to SARS-CoV-2 infection patients demonstrated a higher risk of mortality between patients with cancer. The risk is due to the cancer but also to age, smoking history, and comorbidities[29]. In addition, patients with liver cancer appear to be more vulnerable because of underlying liver disease that can alternate both innate and adaptive immune responses[30].

The systemic treatment in advanced HCC (BCLC-C patients/Child Pugh-A liver function) is generally with multitargeted tyrosine kinase inhibitors characterized by daily oral administration (*e.g.*, lenvatinib, sorafenib). Not all countries have approved the combination of immune check point inhibitors and the antiangiogenic agent bevacizumab[31] with an intravenous administration. Oral tyrosine kinase inhibitors should be preferred over an infusion regimen during the pandemic to protect both patients and medical staff. The role of immunotherapy in SARS-CoV-2 infection is unknown. The use should be considered on a case-by-case basis[11].

In accord to the international and national guidelines during the COVID-19 pandemic, the telemedicine approach must be preferred[9,10]. Some recommendations suggest to rank in person patient visits as high, medium, and low priority. For

example, high priority patients are taking first and following line therapies, patients showing moderate or severe side effects to treatment, or with decompensated liver disease. In any case, a phone assessment is preferred[32].

If systemic therapy is ongoing, the use of telephone-based consultations should be preferred, enabling careful monitoring of patients without the need for frequent hospital visits over a short period of time[27]. The recommendation is to reduce the access in the hospital for a visit and improve telephone-based assessment with clinical evaluation and blood tests. In the case of in person visits, a phone call the day before the appointment should be made to consider the patients' general condition and the presence of any suspected symptoms (for example fever or rhinorrhea). In addition, at the hospital entrance temperature should be measured, hands should be disinfected, and the patients should be alone. Related to less complicated toxicities, such as hypertension, dermatological problems, and diarrhea, the management can be performed by community doctors after coordination with the patient's team[7].

For liver cancer clinical trials, regular visits should be maintained[33].

These modifications for clinical daily practice should be taken to reduce the risk of SARS-CoV-2 infection in patients with advanced HCC undergoing systemic treatment.

RADIOTHERAPY

During the COVID-19 pandemic, new guidelines have been introduced for the management of oncological treatments in radiotherapy (RT) departments[34,35].

Cancer patients are known to have a higher degree of fragility and therefore are inclined to complications of COVID-19 infection. Therefore, an appropriate risk-benefit assessment of each procedure should be considered as part of the treatment strategies for cancer patients during the pandemic[36].

RT is one of the cancer weapons, reporting a benefit in terms of overall survival and disease-free survival. By the introduction of advances in RT, providing high precision delivery while sparing at-risk organs, it is generally well tolerated with a median overall treatment time of 20-30 daily fraction. Furthermore, the use of hypofractionation or stereotactic RT treatments (SBRT) with few fractions (about 1-15) is preferred during the pandemic to reduce access times in hospitals.

In this context, SBRT treatment has a role as a noninvasive and effective therapy, for patients with HCC from the early stages to the most advanced stages. In general, the total dose on the tumor was 30-60 Gy in 3-5 fractions[34].

In early stage HCC, local therapy can be offered as a bridging treatment if surgery would be postponed or as a definitive therapy in the context of locoregional treatments, in the case of the inability to perform surgery[12,34].

In the case of intermediate stage HCC, SBRT has been proposed as an alternative option, particularly after an incomplete response to the previous TACE or for patients unfit for TACE[37]. Finally, in the case of locally advanced HCC with vascular invasion, RT with TACE has been shown to improve survival compared to TACE alone in patients with HCC[38]. In patients with advanced disease, the use of SBRT can reduce the time of suspension of systemic therapy if the patient requires palliative therapies to control symptoms derived from the disease[11,12,34].

In addition, the collaboration between 19 multidisciplinary liver specialists from high-volume liver malignancy academic centers in seven countries and five continents has resulted in a series of recommendations regarding the management of HCC patients in the era of COVID-19. According to the BCLC classification system, it is evident that RT and SBRT treatments are actively included in every stage of HCC[39].

When standard therapies are not available these recommendations can be considered: (1) BCLC-0 or BCLC-A: if a liver transplant or surgical resection is unavailable, consider bridging with locoregional therapies or surveillance. In this case SBRT 30–60 Gy/3–5 fractions; (2) BCLC-B: consider locoregional therapies, such as TACE, transarterial embolization, transarterial radioembolization, or SBRT 30-60 Gy/3-5 fractions or surveillance; and (3) BCLC-C: if patient has portal vein thrombosis and no extrahepatic disease, consider systemic therapy or a combination of TACE and radiotherapy (45 Gy in 15 fractions), SBRT in 3-5 fractions, or transarterial radioembolization; if patient has extrahepatic disease, use systemic therapy and/or palliative radiotherapy in a single 8 Gy fraction for symptomatic disease[12].

Using SBRT hospital access times are reduced compared to using standard fractionated or hypofractionated treatments (a max of six treatments for SBRT compared to fifteen or more in the other cases). Furthermore, the recommendations during the pandemic require the use of noninvasive immobilization systems, such as

active breathing and motion control management (breath-hold techniques, respiratory gating, abdominal compression) or free breathing treatment, according to the possibilities of each radiotherapy department, to mitigate respiratory movements avoiding the surgical implantation of liver fiducial markers during radiotherapy simulation phase and treatment.

It is fundamental for accurate triage to identify positive or suspected cases before and during treatment, and if the patient should test positive for SARS-CoV-2 during radiation therapy, then they may consider treatment suspension or the continuation of the same in a protected environment both in consideration of the patient's clinical condition and treatment purpose and in relation to the situation of each center to ensure a safe radiation treatment[36].

It is known, despite numerous phase II prospective and retrospective studies demonstrating SBRT safety and efficacy[40,41], that most guidelines in the management of patients with HCC limit the use of RT to patients who are not suitable or refractory to other locoregional treatments due to the lack of phase III trials. However, as discussed in the literature[42], it is anticipated that with more widespread clinical use of SBRT during the COVID-19 pandemic and the result of ongoing trials, there may be increased evidence to support the effectiveness of SBRT for HCC treatment.

CONCLUSION

The COVID-19 pandemic has strongly impacted the management of the oncological patient due to the reduction of inpatient beds and reallocation of nurses and doctors to the COVID departments that were rapidly developed in each hospital to face the pandemic. Despite this unexpected reorganization of hospitals, the necessity to continue to manage patients with HCC has required the continuation of multidisciplinary management while reducing the risk of COVID-19 negatively affecting the short and long-term oncological outcome. The main goals should be to optimize the risk/benefit balance and focus on patients with a more aggressive tumor. Flexibility has become mandatory in order to adapt to the different phases of the pandemic. The aim of these guidelines is to prepare physicians to manage the second and third wave of the COVID-19 pandemic.

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MINIREVIEWS

Human immune repertoire in hepatitis B virus infection

Qiao Zhan, Jing-Hang Xu, Yan-Yan Yu, Emily Lo KK, Felicianna, Hani El-Nezami, Zheng Zeng

ORCID number: Qiao Zhan 0000-0001-7785-2373; Jing-Hang Xu 0000-0001-8848-3876; Yan-Yan Yu 0000-0002-7557-1305; Emily Lo KK 0000-0001-9258-0716; Felicianna - 0000-0003-1252-5667; Hani El-Nezami 0000-0001-6700-4346; Zheng Zeng 0000-0002-4997-4223.

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Qiao Zhan, Jing-Hang Xu, Yan-Yan Yu, Zheng Zeng, Department of Infectious Diseases, Peking University First Hospital, Beijing 100034, China

Emily Lo KK, Felicianna, Hani El-Nezami, School of Biological Sciences, University of Hong Kong, Hong Kong, China

Hani El-Nezami, Institute of Public Health and Clinical Nutrition, School of Medicine, University of Eastern Finland, Kuopio FI-70211, Finland

Corresponding author: Zheng Zeng, MD, PhD, Chief Physician, Department of Infectious Diseases, Peking University First Hospital, No. 8 Xishiku Street, Xicheng District, Beijing 100034, China. zeng@bjmu.edu.cn

Abstract

Hepatitis B virus (HBV) infection is a public health threat that affects 257 million people worldwide and can progress to liver cirrhosis, liver failure, and hepatocellular carcinoma. The HBV antigen- induced adaptive immune response plays an important role in HBV clearance. Immune repertoire sequencing (IRS) has been used to investigate the molecular mechanisms behind the immune system, find novel ways to treat HBV infection, and evaluate the genetic responses and immune characteristics of individuals infected by HBV or immunized by HBV vaccine. This review summarizes the human immune repertoire analysis methodology, and the application of the IRS in the prediction of HBV infection progression, treatment, and vaccination.

Key Words: Immune repertoire; T-cell receptor; B-cell receptor; Hepatitis B virus; Chronic viral infection; High-throughput sequencing

Core Tip: A Hepatitis B virus (HBV) cure depends on activation of the anti-HBV adaptive immune system. Immune repertoire sequencing is a novel method to investigate all aspects of the human adaptive immune system. However, the immune repertoire in HBV infection is still not clear. We review the immune repertoire analysis methodology and provide a new insight into outcomes of HBV infection and vaccination.

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INTRODUCTION

Hepatitis B virus (HBV) is a hepatotropic, small, enveloped DNA virus that belongs to the Hepadnaviridae family and causes an acute or chronic infection in humans. The duration of infection is variable, ranging from 8 weeks to over 6 months. Chronic hepatitis B (CHB) infection is more common clinically in patients whose immune system fail to fight against HBV, and is characterized by a high HBV DNA load and HBsAg seropositivity. It can lead to advanced liver cirrhosis, liver failure, and hepatocellular carcinoma (HCC). CHB cannot be completely cured currently because of the existence of covalently closed circular DNA (cccDNA) in the cell nucleus, but it can be controlled by nucleos(t)ide analogs (NUCs) and interferon (IFN).

The immune response against HBV is the key to a cure, which involves both innate and adaptive immune responses. During acute infection, hepatocytes produce type 1 IFN, which inhibits viral packaging. The clearance of HBV is accompanied by asymptomatic or flulike symptoms[1]. Dysfunction and exhaustion of HBV-specific CD4+ and CD8+ T cells, decreased numbers and dysfunction of dendritic cells (DCs), nature killer (NK), and NKT cells are immune responses seen during CHB infection, as well as upregulated expression of immune molecules, including programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4), and mucin domain -3 (Tim^3)[2-6]. Innate immune response are also downregulated during CHB infection, with dysfunction of toll-like receptors (TLRs)[7], which contribute to inducing a robust IFN response and suppressing HBV replication. In this review, we summarize the current state of knowledge of the immune repertoire and the relevance to understanding HBV infection (Table 1).

The immune repertoire includes the human T-cell receptors (TCRs) and B-cell receptors (BCRs) of the adaptive immune system. The generation of diverse TCRs expressed on the cell membrane of T lymphocytes and BCRs expressed by B lymphocytes maintain the balance of adaptive immunity and recognize the amounts of antigens. There are two types of T cells, αβT cells and γδT cells, according to their TCR genes. In αβT cells, the TCRs are composed of one alpha and one beta chain. In γδT cells, the TCRs consist of one gamma and one delta chain. The variable region of each chain consists of three complementarity-determining regions (CDRs), which are variable and determine the antigen specificity, and four frame regions (FRs). CDR1 and CDR2 are coded by variable (V) genes, and CDR3 is generated by random selection and recombination of variable (V), diversity (D), and joining (J) genes[8,9]. In all, the generation of diversity in TCR consists of a vast number of TCR gene segments recombinants, junction diversity, and alpha/beta or delta/gamma chain pairing during the development of T cells[10-12]. As for immunoglobulin (Ig) antibodies, BCR induces a Y-shaped protein composed of two large heavy chains encoded by the immunoglobulin gene heavy locus and two small light chains encoded by the immunoglobulin lambda locus (IGL) or immunoglobulin kappa locus (IGK). The heavy chain variable region is encoded by V, D, J gene families like those of T cells. The light chain is encoded by only V and J gene families. Before antigen stimulation, V(D)J recombination is mediated by several enzyme-like recombination-activating genes (RAG1/RAG2). Random additions and deletions of V-D and D-J (V-J for the light chain) joining regions contribute to the diversity of Ig[13,14]. The post antigen-stimulation response of Ig is more complicated, and includes somatic hypermutation, which is a programmed mutation process whereby changes are introduced to the nucleotide sequence of the immunoglobulin gene DNA during development[15]. The response also includes gene conversion, the asymmetrical segregation of genes during replication, which leads to the production of nonreciprocal recombinant strands and the apparent conversion of one allele into another[15]. Finally, class-switch recombination, B-lymphocyte gene rearrangement that results in substitution of the type of heavy chain constant region that is expressed and allows a change of the effector response while the antigen-binding specificity (*i.e.* the variable region) remains the same[15]. These three responses resulting in the functional differences of IgG, IgE, IgA, IgD, or IgM[16].

The ability of T cells and B cells to recognize and respond to their specific antigen is central to adaptive immunity. The fully functional antigen receptor complex with

Table 1 Immune repertoire investigation of hepatitis B virus infection

Number	Ref.	Patients	Amounts of samples	Repertoire	Sequencing reads	Tools
1	Deulofeut *et al*[59], 1997	2 volunteers	2	TCR repertoire	-	PRISM
2	Höhn *et al* [60], 2002	Healthy individuals immunized with recombinant hepatitis B surface antigen	7	TCR-VA and -VB repertoire in CD8+ T cells	-	FITC/PE
3	Soroosh *et al*[69], 2003	Nine healthy adult responders and six nonresponders	30	TCR beta chain variable gene		FRAGMENT MANAGER' software program
4	Yang *et al* [41], 2011	Forty-two patients with CSHB	42	TCR CDR3	-	SPSS 16.0 software
5	Xiong *et al* [44], 2014	18 patients with CSHB and 8 controls	26	TCR Vβ repertoire	-	DNAMAN software version 1.0
6	Han *et al* [52], 2015	Liver cancers and healthy adults	160	TCR CDR3	495708702	BLAST
7	Galson *et al* [62], 2015	Naïve group: $n = 9$; vaccine group: $n = 9$	108	B-cell repertoire	365863	R
8	Qu *et al* [37], 2016	4 CHB patients before and after HBeAg seroconversion	8	TCR Vβ repertoire	370210 to 685596	Homemade Perl script
9	Yang *et al* [55], 2016	12 HBeAg SC patients and 20 no HBeAg SC patients	96	TCRBV families	-	SPSS software version 19.0
10	Galson *et al* [63], 2016	9 healthy subjects	63	B-cell repertoire		R
11	Chang *et al* [70], 2016	4 pairs of HBeAg positive carrier and HBsAg negative non-carrier siblings	16	IgG immune repertoire	2.2 million	PEAR, Python, Numpy and SciPy, Ipython
12	Ma *et al* [64], 2017	3 healthy volunteers	6	IgM and IgG H chain CDR3 repertoires		IMGT/High V-Quest
13	Jiang *et al* [36], 2018	3 pairs of healthy identical twins and 7 pairs of chronic hepatitis B patients	20	CD8+ T-cell receptor beta (TCRβ) chains	50 million	MIXCR
14	Yan *et al* [49], 2019	6 patients with HBV-related ACLF and 6 controls	12	BCR CDR3 region	An average number of 12243860.30 in the control group and an average number of 1229965.30 in the ACLF group	GraphPad Prism software
15	Miyasaka *et al*[61], 2019	5 volunteers	30	T-cell and B-cell receptor repertoire	TCR β chain repertoire was 153151 before HB vaccination, 180093 after the second HB vaccination, and 129044 the third HB vaccination; BCR IgG heavy (H) chain repertoires were 106664 before HB vaccination, 126237 after the second HB vaccination, and 135663 the third HB vaccination	SPSS software package, Easy R (EZR) version 1.37, GraphPad software package
16	Shen *et al* [48], 2020	5 HBV-ACLF	20	TCR repertoire	163259321	MIXCR
17	Lian *et al* [57], 2020	20 CHB patients undergoing 1-yr ETV treatment (10 HBeAg SC patients and 10 no HBeAg SC patients)	60	T-cell repertoire	-	MIXCR

ACLF: Acute-on-chronic liver failure; BCR: B-cell receptor; CDR3: Complementary determining region 3; CSHB: Chronic severe hepatitis B; ETV: Entecavir; HBV: Hepatitis B virus; Ig: Immunoglobulin. SC: Seroconversion; TCR: T-cell receptor.

coreceptors and costimulatory receptors on T cells and B cells has not only exquisite

antigen specificity but also different signaling pathways (Figure 1). The T-cell receptor complex consists of an antigen-binding TCR α and TCR β heterodimer associated with CD3 that has four signaling chains (two ε, one δ, and one γ), as well as a homodimer of ζ chains. Each CD3 chain has one tyrosine-based immunoreceptor activation motif (ITAM); each ζ chain has three. After a T cell has detected its specific antigen, phosphorylation of tyrosine residues in the ITAMs of the TCR enables binding of the cytosolic tyrosine kinase-zeta chain of the TCR-associated protein kinase 70 (ZAP-70), followed by the CD4 and CD8 coreceptors, which bind to major histocompatibility complex (MHC) class 2 molecules and class 1 molecules. Activated ZAP-70 leads to membrane recruitment of phospholipase C-γ (PLC-γ), which initiates three important signaling pathways that involve activation of nuclear factor of activated T cells (NFAT), nuclear factor kappa B (NF-κB), and activator protein-1 (AP-1). Antigen detection thus results in the differentiation and proliferation that characterize the immune response[17,18]. The BCR complex includes cell-surface immunoglobulin with one each of the invariant signaling proteins, Igα and Igβ, each of which has a single ITAM in their cytosolic tails that enables signal initiation after the BCR binds to an antigen. The logic of BCR signaling is similar to that of TCR signaling, but some of the signaling components are specific to B cells. When BCRs have bound a multivalent antigen, which cross-links them, three protein tyrosine kinases of the Src-family, Fyn, Blk, and Lyn, are activated and phosphorylate the ITAM tyrosine residues, which creates binding sites for the cytosolic protein, kinase spleen-associated tyrosine kinase (Syk). Syk then phosphorylates and activate the enzyme PLC-γ, which then initiates signaling pathways just as occurs with TCRs[19,20].

IMMUNE REPERTOIRE ANALYSIS METHODOLOGY

High-throughput sequencing (HTS) has increased the range, complexity, sensitivity, and accuracy with a great increase in the scale of operation, the number of nucleotides, and the number of copies of each nucleotide sequenced[15]. Investigation of the immune repertoire has thus become more effective, convenient, and less costly. The depth and amount of sequencing data obtained from of disease-specific TCR or BCR clones provide investigators with a great chance to identify individualized and common clonality during HBV infection. There are five phases of immune repertoire analysis starting with cell isolation or tissue collection (Figure 2). After collecting patient samples (cells and/or clots), DNA or mRNA is extracted, purified, and sequenced. DNA, is more suitable for calculating the proportions of antigen-specific T or B cells and for studying the functional/phenotypic evolution of specific TCR/BCR clonotypes. Therefore, it is recommended to choose mRNA to study cell function and activation[21].

It is important to complete the library preparation and amplification as that affects the accuracy of sequencing data. Two next-generation sequencing (NGS)-based amplification methods are currently applied. Multiplex PCR is the most convenient and straightforward approach for DNA samples. Because the V segment is highly variable, multiplex PCR incorporates V and J gene [constant (C) gene for mRNA] specific multiplex primers to amplify the full recombined variable region or CDR3 region of TCR/BCR gene[22,23]. PCR with the 5′ RACE method for rapid amplification of cDNA ends, which only works with mRNA, but can theoretically avoid V gene bias. RNA is reverse transcribed with a gene-specific primer targeting a known 3′ end sequence. The 5′ end of an unknown sequence is amplified with a synthetic oligonucleotide[24,25]. Other methods like anchored multiplex PCR (AMP) introduced by Zheng *et al*[26] and TCR ligation-anchored-magnetically captured PCR (TCR-LA-MC PCR) introduced by Ruggiero *et al*[27] are designed to avoid amplification bias and sequencing sensitivity.

The fourth phase of immune repertoire is sequencing with HTS, which has been used since 2009. The most widely used HTS platforms are the Roche 454 sequencing system and the Illumina HiSeq platform. The Roche 454 sequencing system was the first HTS platform. It provides an average 500 bp read length per run and sequences millions of molecules per repertoire[28]. The Illumina HiSeq platform provides a shorter read length but significantly higher read throughput and a lower cost per read compared with the Roche 454, which cater to the demand of deep sequence of the complex immune repertoire[20,29].

The fifth phase is analyzing the TCR and BCR sequence data with appropriate bioinformatic tools. Many computational tools are available to analyze HTS data including IMGT/High V-QUEST[30], new IgBLAST[31], Decombinator[32], pRESTO

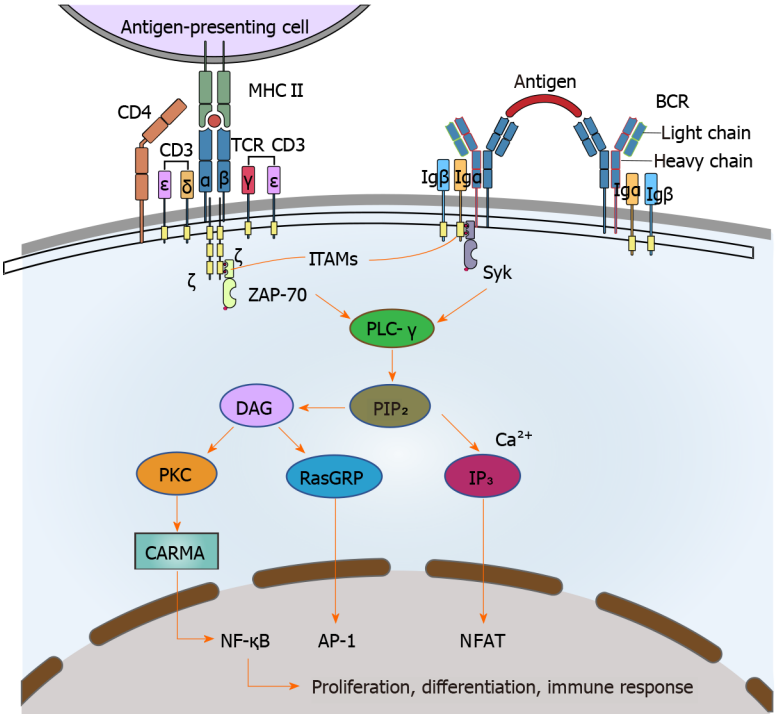

Figure 1 T-cell receptor and B-cell receptor structure and signaling pathways. T-cell receptor and B-cell receptor complexes include both variable antigen-recognition proteins and invariant signaling proteins. Phosphorylation of the ITAMs in CD3 ε, γ, δ, and the ζ chain enables them to bind the cytosolic tyrosine kinase ZAP-70, which in turn recruits and activates PLC-γ. Activated PLC-γ cleaves PIP_2 to yield DAG and IP_3. IP_3 increases intracellular Ca^{2+} concentration, activating calcineurin, a phosphatase that then activates an NFAT transcription factor. DAG recruits PKC to activate CARMA, which leads to activation of NF-κB and recruits RasGRP, which activates AP-1. These three important signaling pathways activate transcription factors in the nucleus, including NF-κB, NFAT, and AP-1, which result in cell differentiation, proliferation, and immune response. AP-1: Activator protein-1; CARMA: Caspase recruitment domain family, member 14 protein; DAG: Diacylglycerol; IP_3: Inositol trisphosphate; ITAM: Immunoreceptor tyrosine-based activation motif; NFAT: Nuclear factor of activated T cells; NF-κB: Nuclear factor kappa B; PIP_2: Phosphatidylinositol bisphosphate; PKC: Protein kinase C; PLC-γ: Phospholipase C-γ; RasGRP: RAS guanyl releasing protein; Syk: Spleen-associated tyrosine kinase; ZAP-70: Zeta chain of T-cell receptor-associated protein kinase 70.

[33], and MiXCR[34]. These bioinformatic tools can be used for VDJ gene assignment, CDR and FR annotation, CDR3 length identification, insertion and deletion analysis, and mutation spectrum analysis. What's more, the international immunogenetics information system (ImMunoGeneTics database) also provides important information about specific BCR and TCR V, D, J, and C genes[35].

CHARACTERISTICS OF THE IMMUNE REPERTOIRE IN HBV INFECTION PROGRESSION

Chronic HBV infections can persist with an asymptomatic carrier status or as chronic hepatitis B (CHB) that can progress to chronic severe hepatitis B (CSHB), cirrhosis, HBV-related acute-on-chronic liver failure (HBV-ACLF), and HCC. The progression of HBV infection is associated with the immune response, especially the adaptive immune response. The characteristics of the immune repertoire in HBV infection progression involve both TCRs and BCRs. Hepatitis B e antigen (HBeAg) seroconversion is an important step toward achieving a CHB cure, which appears to be dependent on an immune response to clear the virus. Jiang *et al*[36] analyzed $CD8^+$ T-cell receptor beta (TCRβ) chains in seven pairs of monozygotic twins with CHB and three healthy control pairs by HTS. Six pairs were infected with HBV during childhood; four of the six pairs had the same clinical outcomes. A high level of similarity in the TCR repertoire of each pair was found in average TCR Vβ segment expression and the frequency of the CDR3 pattern and skewed or oligoclonal clonotypes. Notably, the detailed CDR3 pattern and frequency were related to disease prognosis. There was an increased abundance of immunodominant clonotypes in patients with HBV antigen seroconversion[35]. Analysis of the TCR β chain repertoire in PBMCs from four CHB patients with HBeAg seroconversion demonstrated that TRBVβ12-4, Vβ28, Jβ2-1, V7-2-01-J2-1, V12-4-J1-1, and V28-1-J1-5 were associated with the development and treatment of CHB. No significant changes were observed following seroconversion

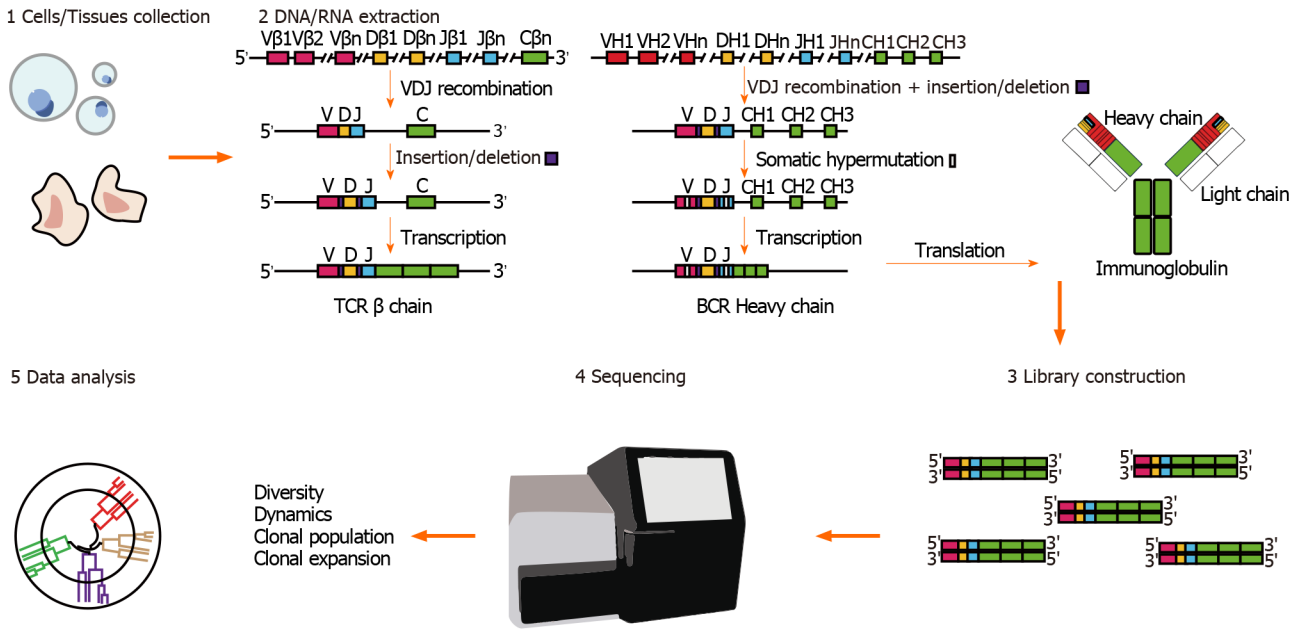

Figure 2 Workflow for immune repertoire sequencing. There are mainly five sections in immune repertoire analysis. 1: Cell/tissue collection. 2: DNA/RNA extraction. The figure illustrates the gene recombination and transcription of T-cell receptors (TCRs) and B-cell receptors (BCRs). The V, D, and J gene segments of TCRs and BCRs undergo somatic rearrangement and gene insertion/deletion before transcription to generate highly variable CDR3 regions, which are then translated as TCRs and immunoglobulins. 3: Library construction by next-generation sequencing methods are used to complete the library preparation and amplification. 4: Data sequencing. 5: Data analysis. BCR: B-cell receptor; CDR3: Complementary determining region 3; TCR: T-cell receptor.

[37].

CHB patients who develop CSHB have more than 50% mortality, and the rate is related to significant increases in the levels of CD8+ and nonspecific T cells[38,39]. Analysis of TCR Vβ diversity in peripheral CD4+ and CD8+ T lymphocytes obtained from 18 patients with CSHB found that CD8+ T cells play a major role in the pathogenesis of CSHB[40]. CDR3 spectratype analysis showed predominant expression of TCR Vβ5, Vβ7, Vβ9, Vβ12, and Vβ18 families in the CD8+ T cell subsets of CSHB patients. In addition, JB1S1 and JB2S7 region genes were present at a high frequency. Furthermore, three conserved amino acid motifs were identified, including GSF, LF and GS, which may be involved in binding to HBV-specific antigens[40]. In contrast, TCR Vβ7 and Vβ11 were more frequently expressed in the PBMCs and CD8+ subsets of CSHB patients, possibly because of differences in the human leukocyte antigen (HLA) types and HBV genotype variants. Interestingly, the two CDR3 amino acid sequences had conserved BV11, AGEL or VYNEQ and BV7, QDSVTTGAQ motifs, and the CSHB patients who expressed the "AGEL" TCRBV11 CDR3 amino acid sequence had better short-term responses than patients expressing the "VYNEQ" TCRBV11 CDR3 sequence[41]. Immunostaining of liver biopsies from untreated CHB patients found the portal-periportal CD4+ and intralobular CD8+ cell frequencies were increased in severe hepatitis, which indicated that the CD4+ T cells were also active in the liver micro-immune environment[42].

CHB can progress to HBV-related HBV-ACLF, which is the most common type of liver failure. It is characterized by rapid deterioration of liver function, coagulopathy, and subsequent multiple organ failure with a 28-d mortality in the Asia-Pacific and African regions[43,44]. Recent studies indicated that patients with HBV-ACLF had more IL-17-producing CD8+ T (Tc17) cells than patients with CHB, a decrease in CD4+ and CD8+ T cells, or an increase in regulatory T cells (Tregs) in HBV-ACLF patients [45-47]. Shen *et al*[48] studied the dynamic changes of TCR repertoires in patients with HBV-ACLF and demonstrated that there was a significant decrease in the diversity of CD8 T-cell repertoire that was positively correlated with a reduction of the Model for End-Stage Liver Disease score. CD8 TCRβ repertoire diversity may be a potential predictive marker of disease outcome[48]. Yan *et al*[49] analyzed BCR heavy chain CDR3 sequences from patients with HBV-ACLF and control subjects and found that clonal expansion was more extensive in the ACLF patients and the distribution ratios of the V, D, J and VJ combinations revealed differential expression, with six upregulated genes and 19 downregulated genes in the ACLF patients. ACLFspecific BCR CDR3 sequences hold future therapeutic promise for HBVACLF[49].

Table 2 Specific TRB family and clinical significance

TRB family	Clinical significance
TRBVβ2	Development and treatment of CHB
TRBVβ3	HBeAg seroconversion
TRBVβ5	Severity of CHB
TRBVβ7	Development and treatment of CHB, severity of CHB
TRBVβ9	Severity of CHB
TRBVβ11	HBeAg seroconversion, severity of CHB
TRBVβ12	Development and treatment of CHB, HBeAg seroconversion, severity of CHB
TRBVβ14	HBeAg seroconversion
TRBVβ18	Severity of CHB
TRBVβ20	HBeAg seroconversion
TRBVβ24	HBeAg seroconversion
TRBVβ28	Development and treatment of CHB

CHB: Chronic hepatitis B; HBeAg: Hepatitis B e antigen.

HBV is the most common cause of HCC, with an estimated prevalence of 44%-55% of HCC cases worldwide[50]. The mechanism underlying HBV-related HCC and other HBV-related liver cancers is not clear. Some studies have shown that the HBV x gene promotes cell cycle progression, inactivates negative growth regulators, and inhibits the expression of the p53 tumor-suppressor gene[51]. The adaptive immune system also plays an important role in carcinogenesis. An investigation of TRB V usage in HBV-associated HCC found that the T-cell repertoires of HCC, intrahepatic cholangiocarcinoma, and mixed hepatocellular and cholangiocellular carcinoma tumors and adjacent tissues were significantly different ($P < 0.01$). The highly expanded clone ratio in blood samples from liver cancer patients differed significantly from those in the blood of healthy adults and hepatitis patients. The results suggest that comparison of the T-cell repertoires of tissue and blood could be used to distinguish liver cancer patients from healthy adults and from hepatitis patients[52].

APPLICATION OF IMMUNE REPERTOIRE ANALYSIS IN HBV THERAPY

Current treatment of CHB is limited to nucleos(t)ide analogs, such as entecavir (ETV), tenofovir disoproxil fumarate (TDF), tenofovir alafenamide (TAF), and others, which block DNA synthesis; and interferon-α, which direct suppresses viral RNA and protein production in infected cells and inhibits HBV DNA replication. It also activates NK cells and T cells that target HBV-infected cells[2]. Studies that evaluated HBV treatment found differences in the antiviral effect that were associated with the adaptive immune response. Yan *et al*[53] reported that telbivudine reduced HBV DNA levels and downregulated the proportion of circulating Tregs. A reduction in the Treg proportion was observed in CHB patients during TDF treatment[54].

A clinical trial including 12 patients with HBeAg seroconversion and 20 without seroconversion who were treated with 300 mg TDF daily for 96 wk found a T-cell receptor beta-chain variable (TCRBV) family that was associated with HBV DNA levels in the seroconversion group[55]. Six TCRBV families (BV3, BV11, BV12, BV14, BV20, and BV24) were more prevalent than the other TCRBVs in the seroconversion group. BV12, BV15, and BV22 were predominant in the patients who had not seroconverted. In addition to the Treg frequency and alanine aminotransferase (ALT) level, the Treg profile and TRBVs were associated with HBeAg seroconversion and may be potential predictors of HBeAg seroconversion and treatment outcome in CHB patients [55,56]. A recent TCR repertoire study by Lian *et al*[57] in 20 CHB patients undergoing 1-year ETV treatment found that the diversity of TCRβ repertoires was decreased in the 10 HBeAg- seroconverted patients but increased in the 10 patients without HBeAg seroconversion. The number of unique TCRβ clonotypes was positively correlated with the ALT or HBV DNA level in seroconverted patients, which indicated that the

TCR repertoire along with ALT or HBV DNA level may be a biomarker to predict HBeAg seroconversion during and after antiviral treatment[57]. The TRB family specific to HBV infection and its clinical significance are shown in Table 2.

APPLICATION OF IMMUNE REPERTOIRE ANALYSIS IN HBV VACCINATION

The immune repertoire plays an important role in HBV vaccine response. The HBV vaccine consists of recombinant HBsAg, an aluminum hydroxide adjuvant, and/or a virus-like particle that stimulates T-helper (Th2) cells to produce IL-4 to promote the production of anti-HBsAg antibodies[58]. The human TCR repertoire response to HBsAg is oligoclonal, involves multiple TCRBV families, and has individual specificity [59]. Analysis of the TCR-VA and -VB repertoire in CD8+ T cells from individuals immunized with recombinant HBsAg detected monoclonal TCR transcripts exclusively in CD8+, but not in CD4+ T cells[60]. TCR β chain CDR3 repertoire diversity increased, and the BCR IgG H chain CDR3 repertoire diversity decreased, indicating that diversity changes may be associated with a better response to the HBV vaccine [61]. An investigation of the BCR repertoire by Galson *et al*[62,63] found that vaccine-specific BCR sequence clusters expanded after each of three sequential vaccine doses. Additionally, many vaccine-specific BCR clusters appeared to largely derive from previously activated cross-reactive B cells that had low affinity for the vaccine antigen, and subsequent doses were required to yield higher affinity B cells[62,63]. Analysis of the IgG and IgM heavy chain CDR3 repertoire before and after immuni-zation with recombinant HBV vaccine, found the diversity of IgG heavy chain CDR3 repertoires was 1/6 of IgM on average[64]. Moreover, the mechanism of high frequency CDR3 generation was associated with the maturation of IgG affinity[64].

About 5% to 10% of healthy adults fail to produce protective levels of anti-hepatitis B surface antibodies after vaccination with recombinant anti-HBsAg vaccines[58,65]. In "nonresponders," the mechanisms that contribute to the lack of humoral immune response to HBsAg include defective in Th1- and Th2-specific responses, dysfunction of antigen-presenting cells, immunologic tolerance, and too few HBsAg-specific B cells and T cells[66-68]. Study of the immune repertoire could provide a new perspective to overcome obstacles by understanding the adaptive immune profile to HBV vaccination. Differences in the BV5S2-3 gene family in CD4+ T cells and several TCR BV genes, such as BV12 in the CD4+ population and BV24 in the CD8+ population, may explain the unresponsiveness to recombinant HBsAg vaccine[69].

CONCLUSION

IRS has demonstrated its potential in advancing our understanding of the progression of HBV infection, antiviral treatment, and vaccination. Ongoing study of immune repertoires in the field of HBV infection, overcoming technical problems, and increased sharing of sequencing data, the reporting of interesting clinical discoveries will increase.

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MINIREVIEWS

Emerging applications of radiomics in rectal cancer: State of the art and future perspectives

Min Hou, Ji-Hong Sun

ORCID number: Min Hou 0000-0002-2445-140X; Ji-Hong Sun 0000-0001-5362-2450.

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Min Hou, Ji-Hong Sun, Department of Radiology, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou 310016, Zhejiang Province, China

Corresponding author: Ji-Hong Sun, MD, PhD, Attending Doctor, Professor, Department of Radiology, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, No. 3 East Qingchun Road, Hangzhou 310016, Zhejiang Province, China. sunjihong@zju.edu.cn

Abstract

Rectal cancer (RC) is the third most commonly diagnosed cancer and has a high risk of mortality, although overall survival rates have improved. Preoperative assessments and predictions, including risk stratification, responses to therapy, long-term clinical outcomes, and gene mutation status, are crucial to guide the optimization of personalized treatment strategies. Radiomics is a novel approach that enables the evaluation of the heterogeneity and biological behavior of tumors by quantitative extraction of features from medical imaging. As these extracted features cannot be captured by visual inspection, the field holds significant promise. Recent studies have proved the rapid development of radiomics and validated its diagnostic and predictive efficacy. Nonetheless, existing radiomics research on RC is highly heterogeneous due to challenges in workflow standardization and limitations of objective cohort conditions. Here, we present a summary of existing research based on computed tomography and magnetic resonance imaging. We highlight the most salient issues in the field of radiomics and analyze the most urgent problems that require resolution. Our review provides a cutting-edge view of the use of radiomics to detect and evaluate RC, and will benefit researchers dedicated to using this state-of-the-art technology in the era of precision medicine.

Key Words: Computed tomography; Magnetic resonance imaging; Radiomics; Rectal cancer; Clinical applications; Overall survival

Core Tip: Radiomics has exhibited significant potential for risk stratification of rectal cancer and has yielded excellent performance in response assessment of neoadjuvant radiochemotherapy. While the past 3 years has witnessed an exponential growth of the

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field, research on radiomics remains in its infancy and is constantly evolving. More rigorous analyses are emerging, and improvements in bias reduction techniques accompanied with multicentric studies will hopefully enable more robust and generalizable models. Here, we review recent updates on the use of radiomics based on computed tomography and magnetic resonance imaging in the detection and evaluation of rectal cancer.

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INTRODUCTION

Colorectal cancer (CRC) is ranked third among the most common cancers worldwide and accounts for nearly one-tenth of cancer-related deaths globally[1,2]. Rectal cancer (RC) comprises 27%-58% of all CRCs[3]. The development of treatment strategies and the revision of multidisciplinary treatment approaches such as local excision, total mesorectal excision (TME), and neo-adjuvant radiochemotherapy (nCRT) have decreased local recurrence and distant metastasis rates in recent decades[4,5]. However, accurate pretreatment tumor staging by imaging remains essential for precise decision-making[6].

Traditional medical imaging is routinely used in initial diagnosis of RC and has played a critical role as a non-invasive tool during follow-up[7]. According to the 2018 European Society of Medical Oncology (ESMO) Guidelines, high-resolution magnetic resonance imaging (MRI) is the standard staging modality for RC and has exhibited superior performance for tumor staging when compared with digital rectal examination, computed tomography (CT), and endoscopic ultrasound[8]. CT is mainly used for local lesions due to its inherent traits of low soft-tissue contrast, which limits the accurate approximation of T stage. Even in T4 lesions with gross invasion of adjacent organs, false-positive cases may occur. Therefore, CT is typically employed primarily for the detection of metastases. CT has faster acquisition time and is more practical than MRI, as it is more widely available[9]. However, both CT and MRI have restricted resolution in clinical applications.

Radiomics has emerged with consistently developing methodology and promising results[10-12]. Radiomics is a method that enables quantitative extraction of radiomics features that cannot be captured by visual inspection from available radiological images[13]. In the past few years, an increasing number of studies have evaluated abdominal radiomics models in different oncological scenarios and reported impressive performance for evaluating tumor biological behaviors, prognosis, and prediction of therapeutic responses[14-16]. Radiomics has been validated as a novel approach for improved characterization of tumor subtypes and the lesion microenvironment[17,18]. By making use of the medical images and clinical data, radiomics models have the potential to provide more detailed information to tailor individualized treatment scheme and patient management[19-21].

The purpose of this review is to describe and summarize the recent advances in clinical applications of radiomics based on CT and MRI, to highlight the potential role of radiomics in disease evaluation and clinical decision-making of RC, and to discuss the current limitations and possible optimization directions in the future.

RADIOMICS WORKFLOW AND METHODOLOGICAL ADVANCES IN RC

The concept of radiomics was first developed by Lambin *et al*[22] in 2012. Radiomics is defined as a research method that includes quantitative data extraction from multimodality medical images, analysis, and modeling of high-dimensional medical image features to explore relationships with clinical outcomes[11,13]. Related research can be divided into five stages: Data acquisition and analysis, medical imaging segmentation, feature extraction and selection, clinical target-oriented modeling, and research quality evaluation. The workflow is presented in Figure 1.

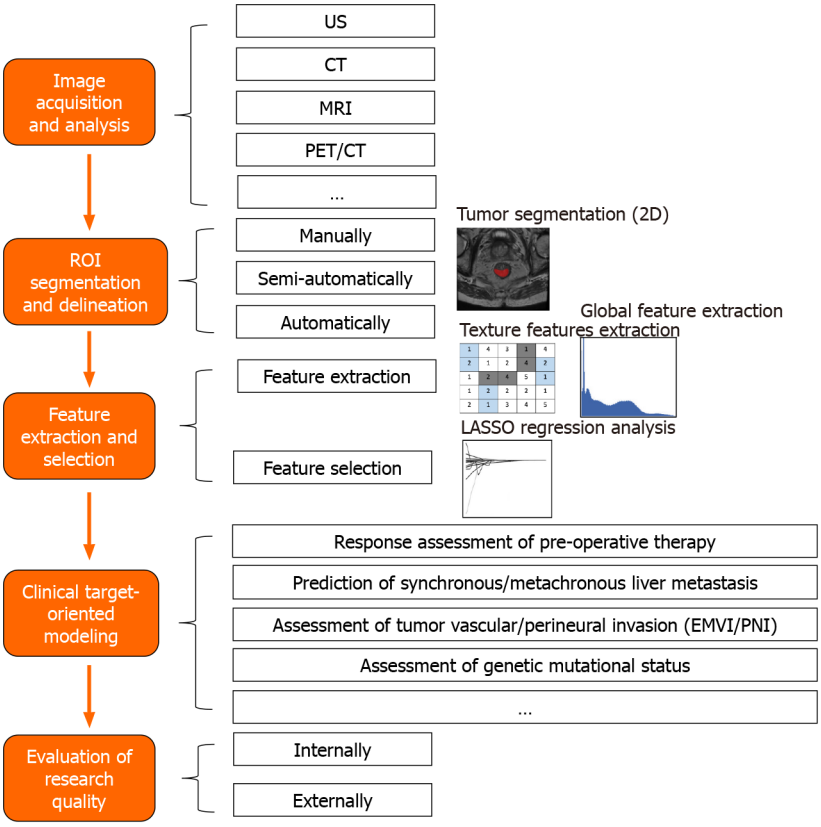

Figure 1 Workflow of radiomics applied in rectal cancer. US: Ultrasonography; CT: Computed tomography; MRI: Magnetic resonance imaging; PET: Positron emission tomography; ROI: Region of interest; EMVI: Extramural venous invasion; PNI: Perineural invasion.

Data acquisition and analysis

The radiomics workflow begins with the determination of the region of interest (ROI), which depends on the specific clinical problem that requires resolution. Data used in radiomics studies may be retrospective or prospective and single-center or multi-center. ROIs are determined by specific targeted research; therefore, researchers should first define the clinical problems to solve. Here, we consider RC as an example, whereby primary tumors are analyzed and related to existing treatment outcomes such as survival or recurrence. The analysis of lesions and normal tissues may affect the treatment strategies. *Via* the establishment of a large image database, a large body of imaging data is stored to generate an integrated medical and health care network. Radiologists should be cognizant that imaging protocols may not always be standardized, and variability exists among medical images and institutions. In this regard, the recommendations of the Image Biomarker Standardization Initiative may help to reduce the variability in image pre-processing prior to analysis[23].

ROI segmentation and delineation

Normalizing the original image is essential because data results may be affected by different machines or different parameters during collection. The process of ROI segmentation in radiomics can be performed either manually, semi-automatically, or automatically with software. Each approach has advantages and disadvantages. Manual segmentation is more precise in some occasions (*e.g.*, delineating the RC bed after nCRT) but has lower repeatability. Automatic segmentation depends on algorithms, which are efficient and may help to eliminate subjective errors[24]. To date, a mature automatic segmentation algorithm for RC is lacking. According to our PubMed search results, most radiomics studies on RC applied manual segmentation in which the segmentation is performed by radiologists to annotate the location and precise boundary of the ROI. Itk-snap software (www.itksnap.org) is used extensively for segmentation. Figure 2 shows the segmentation of a rectal tumor using Itk-snap software. Given that subjective bias may occur, segmentation may be inconsistent. At present, several steps are enacted to minimize bias, including the involvement of different medical professionals, multiple segmentation in different respiratory cycles, and adding noise to segmentation[25].

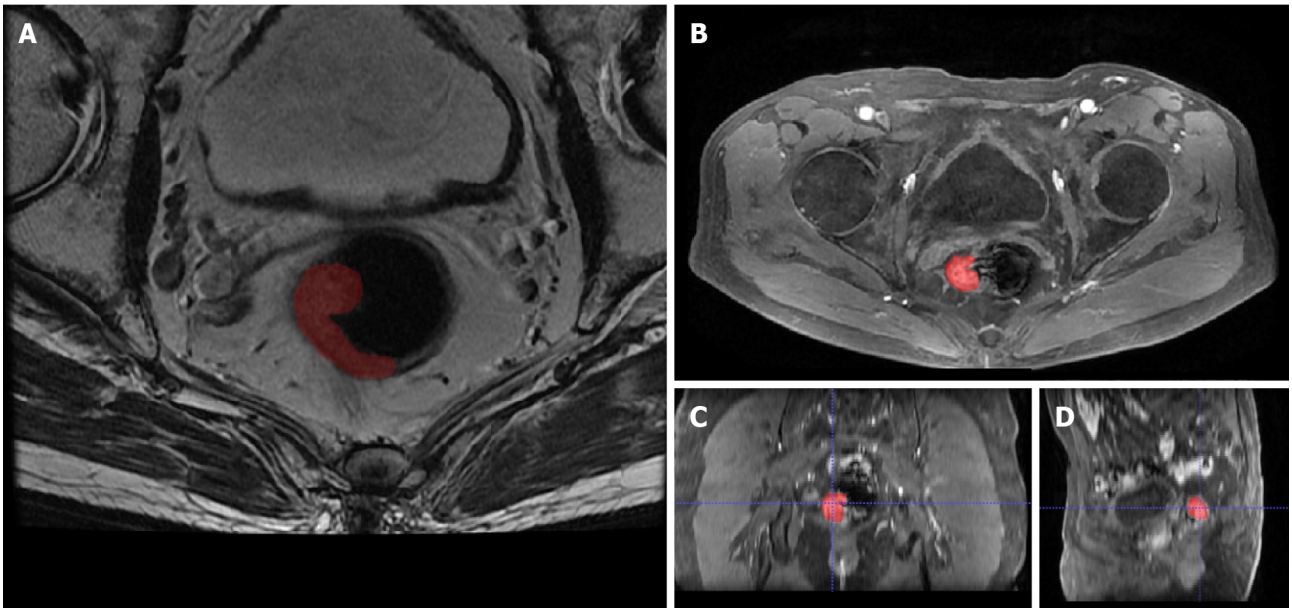

Figure 2 Segmentation of a rectal tumor with Itk-snap software. A: Example of tumor segmentation using Itk-snap software (www.itksnap.org) on axial plain magnetic resonance image; B-D: Axial (B), reconstructed coronal (C), and sagittal (D) contrast-enhanced magnetic resonance images in the venous phase in a 72-year-old man with rectal cancer.

Feature extraction and selection

Following the evaluation of the consistency of feature calculations across researchers, the initial selection of high-level feature sets is performed, and feature values that are sensitive to subjective factors are ultimately retained. Parmar *et al*[26] used the "IRR" (evaluator reliability) package in R software to evaluate the consistency of features extracted from ROIs, whereby features with a consistency coefficient less than 0.8 would be deleted.

The key to radiomics is the extraction of high throughput features that are difficult to depict by observers using mathematical algorithms from ROI segmentation directly. These features of essential value can either be directly extracted from original medical images or after applying a transformation or filter method. The process can be performed using different tools (*e.g.*, PyRadiomics, Texrad, MaZda, and others) which are readily available as open-source software. Image filtration enhances the edge qualities and removes interference by noise, thus permitting the collection of more information on spatial locations of features. At present, the main extraction methods are improved based on the method published by Aerts *et al*[27] in 2014, according to which radiomics features are divided into machine-learning-based features and deep learning features. There are several commonly used subgroups of the former, such as shape features (describing the geometric attributes of the ROIs), histogram-based features (capturing the first-order statistical characteristics of rectal lesions), and texture features (describing the granular textural pattern of the ROIs). Commonly used engineered features according to "order" are presented in Table 1. Recently, a set of 169 standardized radiomics features was published, which enabled the verification and calibration of different radiomics software[23].

Given that the size of the data sample is relatively small compared with features, this may result in over-fitting and it may be time-consuming to include all features in the classification. Feature selection is a necessary step to obtain features that are closely related to the target results in radiomics analyses. Typically, features extracted by radiomics are high-dimensional in a dataset, and a large proportion of features may not be useful for the task; hence, unstable features should be excluded to retain the most important features and prevent over-fitting. The main methods applied in feature selection can be divided into univariate or multivariate, based on whether the association among features can be considered to contribute towards a specific outcome. In machine-learning, the most commonly used feature selection algorithms include least absolute shrink and selection operator regression, minimum redundancy maximum correlation, recursive feature elimination, and principal component analysis [28].

Table 1 Commonly-used radiomics feature categories according to "order"

"Order"	Paraphrase	Categories	Description
First-order	Based on an intensity histogram of pixel values, providing no information on neighboring interactions or the spatial distribution	Mean	Average intensity of the pixels
		Skewness	Asymmetry of histogram
		Kurtosis	Magnitude of pixel distribution
		Entropy	Irregularity of the structure
Second-order	Considers the spatial relationship between 2 pixels	Grey level co-occurrence matrix (GLCM)	Frequency of specific gray values along a distance or direction
		Grey level run Length Matrix (GLRLM)	Length of consecutive pixels or voxels with the same grey values in a specific direction
		Grey level size zone matrix (GLSZM)	Length of consecutive pixels or voxels with the same grey values in all directions
Superior-order	Describes the neighborhood gray difference matrices and the relationship between 3 or more pixels	Neighboring gray tone difference matrix (NGTDM)	Describes the sum and average grey levels of discretized voxels in planes
		Gray level dependence matrix (GLDM)	Describes the coarseness of overall texture by evaluating the grey levels between a voxel and the neighborhood in 3 dimensions

In reference to "order", it is defined as the number of stages required to obtain the quantitative information in a model.

Clinical target-oriented modeling

Radiomics feature analysis and modeling involve the establishment of a prediction model based on selected imaging characteristics. The model generally encompasses clinical, biological, and occasionally genetic information. Commonly used models include logistic regression, support vector machine (SVM), and random forest[13]. In radiomics analysis, machine-learning algorithms are typically required to establish a classification or prediction model to obtain reliable results instead of relying solely on single factor analysis. The Cox proportional risk model is usually employed as a survival analysis model. Each modeling method has its own limitations. For example, feature independence, feature discretization, and network configuration dependence should be considered in logistic regression, Bayesian networks, and deep learning, respectively.

In the process of model-building, researchers can employ different software tools. R language contains software packages for data-mining and modeling. Clinicians and radiologists can operate various components *via* a graphical interface, compare different modeling algorithms, and identify the most effective way to resolve clinical issues. SPSS modeler is a commercial data-mining software tool with functions encompassing almost all data-mining procedures. This software possesses a graphical operation interface and automatic modeling function, which permits management of large amounts of data and provision of steady data-mining models. Other software for modeling, data-mining, and analysis, such as Weka (based on JAVA) and B11 (used in combination with Mazda), may also be employed. These software tools contain various modeling algorithms, including artificial neural network, k-nearest neighbor, K-means, hierarchical clustering, and similarity-based clustering methods.

Evaluation of research quality

For a high-quality radiomics study, it is essential to try different types of modeling methods and to choose the best algorithms. Moreover, all the models should be validated internally and externally (multicenter validation would be better if permitted)[29]. Validation is an indispensable part of complete radiomics analysis, because the value of the unverified evaluation model is limited. Except for validation of the model, quality assessment should also be conducted to ensure reproducibility in a radiomics study. A prediction model is appropriate for clinical decision-making only when a standardized evaluation of its performance is accomplished.

Radiomics based on deep learning

Deep learning is defined as a deep neural network structure based on a broad spectrum of algorithms, and the frameworks permit machines to learn highly complex mathematical models for data representation and can subsequently be used to perform

accurate data analysis. Radiomics algorithms based on deep learning have developed substantially in recent years[30,31]. Three types of deep learning models are commonly used in medical imaging: Convolutional neural networks (CNNs), generative adversarial networks, and sparse auto encoders. Deep learning-based radiomics performs learning procedures *via* convolutional operations and CNN structures. Compared with traditional radiomics, convolution operation has strong feature extraction abilities. The neural network structure can flexibly extract different task-related features by changing the convolution kernel and modifying the structure, thereby enabling a more targeted approach. Different features can be obtained by adaptively changing the convolution kernel. For example, convolution and Laplacian kernels can extract high-frequency features, while the Gaussian kernel can propose low-frequency features. For this reason, the deep learning approach exhibits superior feature extraction by extracting and selecting supplementary high-dimensional features through an automatic-learning neural network, and this enables more comprehensive mining of image information. To date, there are six radiomics studies conducted based on deep learning structure in RC. More novel neural network structures are warranted in this field.

RADIOMICS APPLICATIONS IN RC

We searched PubMed (December 17, 2020) for 83 radiomics studies on RC using the terms ("rectal cancer" AND "radiomics"), and identified 78 clinical target-oriented published works. The volume of radiomics-based articles on RC published in medical journals since 2018 has witnessed a steep rise, indicating a trend of growing interest in artificial intelligence-based approaches in the field.

The literature search revealed an exponential growth in RC radiomics studies in the past 3 years. Of the 78 studies, most (61 out of 78) were based on MRI, eight employed CT modality, and nine were based on positron emission tomography/CT or ultrasound images. As of December 2020, most existing studies were performed in a single center with a retrospective cohort, while only seven studies were multi-center. Validation of radiomics models in independent cohorts was performed in all of the studies. However, few studies included external validation cohorts (n = 7). The number of included RC patients ranged widely from 13 to 700. In addition, almost a half of current studies focus on locally advanced RC (LARC) (38 out of 78) or tumor response to nCRT. We suggest that this is mainly because LARC is a major sticking point in the management of RC. Figure 3 shows the distribution of current focus of attention. Here, we discuss recent advances in radiomics applied to CT and MRI for the evaluation of RC.

Response assessment of pre-operative therapy and long-term prognosis prediction in LARC

LARC is of significant concern due to the potential for deterioration. Over the past decade, both disease-free survival and overall survival of LARC have been prolonged, owing to the growing practice of multi-disciplinary treatment and routinization of management, including TME, nCRT, and immunotherapy[3,32]. However, patients tend to present heterogeneous long-term outcomes in clinical practice due to individual responses to preoperative therapy such as short-course preoperative radiotherapy and nCRT[32,33]. Thus, those who are sensitive to chemoradiotherapy may achieve a clinical complete response and employ a watch-and-wait strategy, whereas patients who are resistant to nCRT need more radical measures[34]. Tailoring treatment schemes to a particular patient based on individual probability for achieving a good response is thus essential. The current pre-treatment response prediction approach to nCRT remains indecisive and fails to adequately estimate a patient's response to a specific therapy. Traditional medical imaging methods including CT, quantified MRI, and functional imaging have limitations in response prediction. In contrast, radiomics with high-dimensional feature extraction applied in RC may facilitate the *a priori* evaluation of treatment efficacy and selection of optimal therapy.

Almost 60% of the current research focuses on tumor response assessment to preoperative therapies or prediction of long-term prognosis (n = 49), mainly in LARC (38 out of 49). Most studies to date have evaluated T2-weighted imaging (T2WI) and diffusion weighted MRI. Heterogeneous results have been reported in different prediction models, referring to entropy, energy, and kurtosis. Two well-conducted studies reported that none of the T2WI radiomics features were significant predictors of response[35,36]. SVM, RF, and Naïve Bayesian network based on T2WI yielded

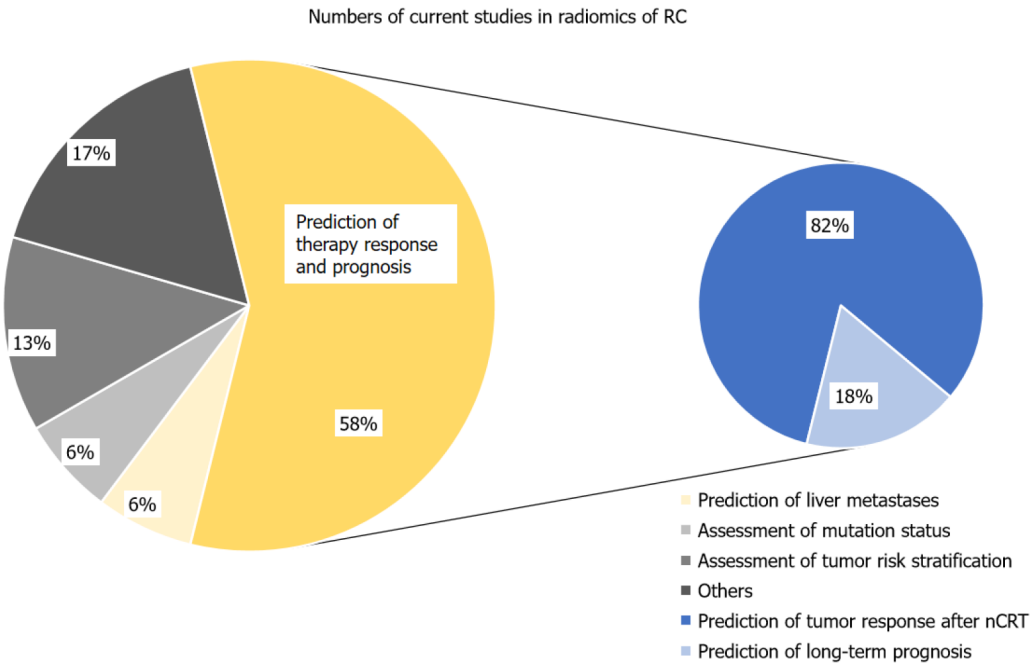

Figure 3 Distribution of the current focus in the industry. Currently 58% of research focuses on tumor response assessment to preoperative neo-adjuvant radiochemotherapy (nCRT) therapies or prediction of the long-term prognosis, of which most studies are about prediction of tumor response after nCRT. RC: Rectal cancer; nCRT: Neo-adjuvant radiochemotherapy.

promising results for complete response (CR) prediction [area under the receiver operating characteristic curve (AUC): 0.71-0.87][35,37,38]. In contrast, apparent diffusion coefficient and intravoxel incoherent motion (IVIM) histogram features did not exhibit predictive value for CR[39]. Similar discrepancies were identified in gray-level co-occurrence matrix (GLCM) dissimilarities for preoperative evaluation of neoadjuvant chemoradiotherapy responses. In this regard, Liu *et al*[39] proposed that GLCM IVIM parameters independently predicted CR in multivariate analysis. Both the random forest model of Yang *et al*[40] and logistic regression model of van Griethuysen *et al*[41] may assist in distinguishing non-insensitive responders to chemoradiotherapy.

Assessment of tumor vascular/perineural invasion (extramural venous invasion/perineural invasion)

Extramural venous invasion (EMVI), which presents in one-third of all patients with RC, is a major factor for higher risk of recurrence and an independent indicator of poorer prognosis[42]. MRI is more suitable to identify the patients with obvious blood vessel invasion beyond the muscularis propria[43]. The verdict of conventional MRI may be affected by surrounding inflammation, edema, and fibrosis caused by nCRT, let alone heterogeneity with image quality, methods, and diagnostic accuracy[44,45]. To obtain more accurate preoperative risk stratification, Yu *et al*[46] constructed and validated several radiomics models and compared them with quantitative models. All radiomics models outperformed quantitative models for predictive performance in identifying EMVI. Notably, the team compared these models with a perfusion parameter-based model from dynamic contrast-enhanced-MRI and demonstrated that the latter had a weaker predictive value.

The positive perineural invasion (PNI) status at resection can independently predict the local recurrence of RC or progression, indicating that the tumor may be of a more aggressive phenotype. According to the latest ESMO guidelines, PNI is a key factor for determining whether patients with stage II RC would potentially benefit from nCRT and postoperative adjuvant chemotherapy[8]. Unlike EMVI, PNI can only be confirmed in post-resection pathological tests, whereas conventional MRI is inoperative for assessing PNI status, as it is unable to visualize peripheral nerves[47]. Therefore, developing a radiomics prediction model to preoperatively identify PNI and to assist in the selection of patients for adjuvant therapy is crucial. Chen *et al*[48] developed a MRI-based radiomics predictive model, which yielded favorable performance for individualized PNI prediction in patients with RC. However, this was a single-center retrospective study based only on T2WI sequences. Future studies

should explore this issue further with heterogeneous and multi-sequence data.

Prediction of synchronous/metachronous liver metastasis

Approximately 15%-20% of patients with RC have liver metastases at the time of diagnosis, which is defined as synchronous liver metastasis (SLM)[49]. At present, only two studies have explored the use of a radiomics nomogram for predicting SLM derived from primary RC lesions[50,51]. Shu *et al*[50] extracted a total of 328 radiomics features from the T2WI images and developed a radiomics model composing T-stage and radiomics signatures. Quantified analysis revealed that the Rad score of patients with primary CRC and liver metastasis was significantly higher than that of patients without liver metastasis. Receiver operating characteristic curves based on all 194 patients with RC were plotted for a radiomics nomogram, and the AUC was 0.932. Liu *et al*[51] subsequently constructed a novel radiomics nomogram and improved the AUC to 0.944, with a sensitivity of 95.83% and specificity of 88.89%, indicating that the radiomics model exhibited good predictive performance for the diagnosis of SLM in patients with RC and may assist physicians in clinical decision-making. The heterogeneity of primary tumors often determines tumor relapse and progression; in contrast, the biological behavior of tumor metastasis and recurrence is closely related to the pathological heterogeneity of the primary tumor. In addition, the radiomics features derived from the primary tumor itself are often more stable in patients with RC. Therefore, radiomics studies examining the prediction of liver metastasis based on primary RC lesions are warranted.

Metachronous liver metastasis (MLM) is defined as the absence of evidence of metastatic disease at initial diagnosis but the presence of liver metastasis after baseline staging and treatment[52,53]. MLM is thought to evolve from occult and micro metastases[54]. Approximately 26.5% of patients with RC develop MLM within 5 years of follow-up[55]. Based on machine-learning algorithms and imaging sequences, noninvasive radiomics models constructed on baseline rectal MRI presented good potential for MLM prediction in patients with RC. The most optimal model employed a logistic regression algorithm, incorporating both T2WI and venous phase radiomics features[56]. To date, reports on MLM prediction based on primary rectal tumors are lacking.

Assessment of genetic mutational status

With the development of targeted therapies such as epidermal growth factor receptor (EGFR)-targeted monoclonal antibody treatment, genetic profiling for mutations is recommended when metastases are diagnosed in patients with RC[8,57]. Previous studies have demonstrated that the mutation statuses of rat sarcoma viral oncogene homolog and v-raf murine sarcoma viral oncogene homolog B1 are critical biomarkers in the prognosis of RC, especially for patients with suspected or proven metastases [58]. Kirsten rat sarcoma (KRAS) mutations, which occur in approximately 27%-43% of patients with RC, have been identified as a critical factor, as they indicate a lack of response to EGFR-targeted therapy[59]. Pathologic tests are the current gold standard of genotyping diagnosis in clinical practice, although results can only be obtained after invasive procedures and may not always be available or reliable. Thus, personalized treatment strategies are warranted to develop a non-invasive and more feasible, timely, and cost-effective surrogate biomarker to evaluate mutation status. With the advent of artificial intelligence (AI) approaches, non-invasive prediction of genetic status and efficacy of CRT using radiomics analysis has become a highlight in the field.

We identified a limited number of studies that used modality-based radiomics models to predict KRAS mutation status or microsatellite instability in RC[60], or to evaluate the relative diagnostic potential of radiomics features for predicting mutational status[59,61-64]. Relationships between radiomics features extracted from CT or multiparametric MRI and KRAS mutations have been confirmed in several studies. Meng *et al*[60] and Xu *et al*[61] reported that radiomics features based on MRI predicted KRAS mutations. Oh *et al*[62] reported that MR-based texture analysis differentiated KRAS mutation status with an accuracy of 81.7%. Furthermore, Cui *et al*[63] used a two-center cohort to predict KRAS mutations in RC and observed that the model obtained with the SVM classifier exhibited the best predictive value. Notably, most of these MRI-derived radiomics models demonstrated moderate predictive ability for KRAS mutations. Further, they were underscored by small sample sizes and lack of independent/external validation. As such, further research is warranted.

FUTURE CHALLENGES AND OPPORTUNITIES

Extant literature has highlighted the potential of radiomics analysis for prediction of nCRT response and prognosis, tumor risk stratification, and evaluation of gene mutation status. Nevertheless, some limitations of radiomics applied for RC should be acknowledged. Current issues arise due to the reliance of results on high-quality data sets, which may bias comparisons of efficacy among radiomics studies due to differences in scanning parameters, feature extraction, software, and vendors/modalities. Hence, there is an urgent need to establish unified data acquisition and processing standards. In addition, radiomics is sensitive to accurate ROI segmentation in the acquisition process. Currently, the process is predominantly conducted manually by radiologists or physicians. Manual segmentation is laborious, and the results may be affected by the observer's subjectivity. The use of semi-automatic or fully automatic methods for segmentation may resolve these issues and should be pursued in future studies. In this regard, accurate and automatic labeling procedures should be developed and promoted to address current technical limitations, and there is still a long way to go. In addition, most existing radiomics studies are retrospective in nature and lack independent external validation across races or populations from various geographical sources, which may limit the reliability and applicability of results. Therefore, further multi-center and prospective studies with standardized acquisition, segmentation, and imaging postprocessing are required to ensure the generalizability and validation of radiomics findings. With regard to mechanistic investigations of biological substrates and their relationships with pathological underpinnings, AI algorithms with improved accuracy and interpretability will facilitate broader translation and clinical adoption. Moreover, radiogenomics for RC is burgeoning, which will bridge the gap between AI-aided prognostics and precision medicine.

As we have discussed above, radiomics models based on deep learning have proven superior. Nevertheless, several issues remain to be optimized; although more parameters exist in the whole connection layer, the gradient of the input end can easily dissipate during network training. A possible solution is to reduce the complexity of the model by reducing network parameters and optimizing the gradient flow, such as VGg, concept, residual network, and dense net.

CONCLUSION

Radiomics is an emerging quantitative technique that has witnessed exponential growth in application of RC management. In this review, we discuss the current utility of radiomics in RC research and describe its potential applications for precision diagnostics and cancer treatment. Thus far, radiomics has shown promise in the diagnosis, treatment evaluation, and prediction of prognosis in RC. Nevertheless, further multi-center and prospective validation studies are required to validate its clinical utility, especially in prognosis-related targets. The purpose of this review is to help radiologists, endoscopists, and oncologists better understand and harness radiomics for tailoring personalized treatments and to encourage their collaboration with AI scientists to promote the translation of research into clinical practice.

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MINIREVIEWS

Advances in paediatric nonalcoholic fatty liver disease: Role of lipidomics

Anna Di Sessa, Simona Riccio, Emilia Pirozzi, Martina Verde, Antonio Paride Passaro, Giuseppina Rosaria Umano, Stefano Guarino, Emanuele Miraglia del Giudice, Pierluigi Marzuillo

ORCID number: Anna Di Sessa 0000-0002-5877-3757; Simona Riccio 0000-0003-3644-9259; Emilia Pirozzi 0000-0002-0988-0687; Martina Verde 0000-002-7596-1434; Antonio Paride Passaro 0000-0002-4054-7903; Giuseppina Rosaria Umano 0000-0002-5570-6620; Stefano Guarino 0000-0002-0551-5236; Emanuele Miraglia del Giudice 0000-0002-1492-076X; Pierluigi Marzuillo 0000-0003-4682-0170.

Author contributions: Di Sessa A wrote the manuscript; Miraglia del Giudice E, Di Sessa A, and Marzuillo P conceived the manuscript; Guarino S, Miraglia del Giudice E, and Marzuillo P supervised the manuscript drafting; Riccio S, Passaro AP, Verde M, Pirozzi E, and Umano GR reviewed the literature data; Riccio S prepared the tables; all author contributed important intellectual content during manuscript drafting or revision.

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Anna Di Sessa, Simona Riccio, Emilia Pirozzi, Martina Verde, Antonio Paride Passaro, Giuseppina Rosaria Umano, Stefano Guarino, Emanuele Miraglia del Giudice, Pierluigi Marzuillo, Department of Woman, Child, and General and Specialized Surgery, University of Campania Luigi Vanvitelli, Naples 80138, Italy

Corresponding author: Pierluigi Marzuillo, MD, PhD, Associate Professor, Department of Woman, Child, and General and Specialized Surgery, University of Campania Luigi Vanvitelli, Via de Crecchio 4, Naples 80138, Italy. pierluigi.marzuillo@gmail.com

Abstract

Due its close relationship with obesity, nonalcoholic fatty liver disease (NAFLD) has become a major worldwide health issue even in childhood. The most accepted pathophysiological hypothesis is represented by the "multiple hits" theory, in which both hepatic intracellular lipid accumulation and insulin resistance mainly contribute to liver injury through several factors. Among these, lipotoxicity has gained particular attention. In this view, the pathogenic role of different lipid classes in NAFLD (*e.g.*, sphingolipids, fatty acids, ceramides, *etc.*) has been highlighted in recent lipidomics studies. Although there is some contrast between plasma and liver findings, lipidomic profile in the NAFLD context provides novel insights by expanding knowledge in the intricate field of NAFLD pathophysiology as well as by suggesting innovative therapeutic approaches in order to improve both NAFLD prevention and treatment strategies. Selective changes of distinct lipid species might be an attractive therapeutic target for treating NAFLD. Herein the most recent evidence in this attractive field has been summarized to provide a comprehensive overview of the lipidomic scenario in paediatric NAFLD.

Key Words: Fatty; Liver; Lipidomics; Children; Nonalcoholic fatty liver disease

Core Tip: Insightful data from lipidomics studies have recently expanded knowledge about nonalcoholic fatty liver disease pathophysiology. In fact, different lipids have been found to exert specific pathogenic roles in liver injury through several pathways

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(in particular by impairing insulin signalling). Given the cardiometabolic burden of nonalcoholic fatty liver disease even in childhood, lipidomics findings might improve strategies for nonalcoholic fatty liver disease treatment by providing novel therapeutic targets.

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INTRODUCTION

An increasing prevalence of nonalcoholic fatty liver disease (NAFLD) up to 25% has been found in adults, but alarming paediatric data have also been reported[1-3]. Due to the obesity epidemic, NAFLD has become the most prevalent liver chronic disease in childhood, affecting 3%-10% of the general paediatric population and up to 50% of obese children with a relevant cardiometabolic burden[2,4]. It includes different degree of hepatic steatosis ranging from simple fat accumulation to steatohepatitis and fibrosis, but its pathogenesis remains to be fully elucidated[1].

To date, "multiple hits" have been recognized in the NAFLD pathophysiology, with a pivotal role of the hepatic intracellular lipid accumulation and insulin resistance favouring liver damage through several factors such as lipotoxicity, oxidative stress, inflammation, genetics, gut axis, metabolic, and dietary factors[3,5]. Among these pathogenic factors, lipotoxicity has gained particular remarkable attention[6]. Lipid induced oxidative stress, inflammation, and cell death have been largely studied as major players of this process, and their interplay represent a critical step in both NAFLD development and progression[6-8]. Of note, it must be considered that the chronic inflammation closely related to lipotoxicity represents one of the most important features of several metabolic diseases such as obesity and type 2 diabetes, resulting in a dangerous "vicious circle" with dramatic clinical implications[6]. In fact, lipotoxicity affects a broad range of tissues such as adipose tissue, heart, brain, pancreatic islets, and skeletal muscle with a complex interrelation favouring the development of metabolic syndrome[6].

Although intrahepatic fat accumulation has been widely accepted as the hallmark of NAFLD, overwhelming evidence showed that both quantity and quality of accumulated hepatic lipids play a pathogenic role in NAFLD[7,9-12]. In particular, specific lipid classes such as sphingosine, diacylglycerols, and ceramides act as liver damaging agents through multiple pathways[6,7,9,13,14].

To date, the growing interest in lipidomic studies has provided meaningful data regarding lipid profiles involved in the pathogenesis of liver injury and its modulation as a potential therapeutic target[8,11,12-14]. In view of the clinical relevance of these findings in the NAFLD treatment scenario, evidence is currently available in adults and in children[15-19] (Tables 1 and 2).

We aimed to summarize the most recent findings regarding lipidomic studies in paediatric NAFLD by providing an overview of the different lipid class and their potential therapeutic implications.

LIPIDS IN NAFLD

Triacylglycerols (TAG) are the most representative lipidic class accumulated in the liver of NAFLD patients. Nevertheless, they would seem to be protective against lipotoxicity due to lipid overload. Lipotoxicity is mainly caused by increasing levels of saturated fatty acids (SFAs), free cholesterol, glycerophospholipids, sphingolipids, and deficient levels of phospholipids, u-3 polyunsaturated fatty acids (PUFAs), or PUFA-derived specialized proresolving mediators[20,21]. Monounsaturated fatty acids (MUFAs), lysophosphatidylcholine (LPC), and ceramide are also increased while phosphatidylcholine (PC) is reduced in nonalcoholic steatohepatitis (NASH)[22,23].

Table 1 Comparison between adult and paediatric lipidomic findings in nonalcoholic fatty liver disease

Lipid class	Changes in adult NAFLD patients	Changes in paediatric NAFLD patients
SFAs	Increased in liver[31] and in plasma[24]	Increased in liver and in plasma[32]
MUFAs	Increased in liver[35,36] and in plasma[25]	Increased in liver and in plasma[32]
PUFAs	Increased total PUFAs in liver[31] and n-6 LCPUFA in liver phospholipids[35]. Decreased LCPUFA of the n-6 and n-3 series in liver TAG, n-3 LCPUFA in phospholipids, total PUFA[35,36], n-3 PUFA[35], n-6 PUFA[32]	Increased in liver and in plasma[32]
PUFAs derived	Increased 5-HETE, 8-HETE, 11-HETE, and 15-HETE in NAFLD and NASH patients[27]. Increased 11,12-diHETrE, dhk PGD2, 20-COOH AA in NASH patients[26]	Increased EDPs, EEQs, EETs with progression of steatosis; reduced with progression of fibrosis[32]
TAG	Increased in liver[31] and in plasma[25]	Increased (TG[O]); TG (O-52:0), TG (O-52:1), TG (O-52:2), TG (O-52:3), TG (O-54:1), TG (O-54:2), TG (O-56:1) and TG (O-56:2) in serum[19]
DAG	Increased in liver[31] and in plasma[25]	No available data
FC	Increased in liver[31]	No available data
PC	Reduced in the liver[31], conflicting data for changes in serum [28,29]	Reduced serum alkyl/alkenyl-phosphatidylcholine (PC[O]) levels[19]
LPC	No statistically significant changes in plasma and serum[28, 29]	No available data
PE	Decreased in liver[22] and increased in serum of NASH patients[28]	Increased PE in serum[19]
LPE	Decreased in serum of patients with NAFLD and NASH[28]	Increased LPE (20:3) and LPE (22:5); decreased [LPE(O)]) in serum[19]
PS	Reduced in liver[22], increased in plasma[29]	No available data
PI	Reduced in liver[22], increased in plasma[29]	No available data
PL	No change in liver[56]; decreased in plasma of NASH patients [25,57]	No available data
SM	Conflicting results in NAFLD and NASH patients[22,28,29,31, 37,51]	Increased SM (d39:0), SM (d41:0) in serum[19]
CE	Increased in liver and in plasma[51,64,65]	Increased in serum[20]

20-COOH AA: 20-carboxy arachidonic acid; CE: Ceramides; DAG: Diacylglycerols; dhk PGD2: 13,14-dihydro-15-keto prostaglandin D2; diHETrE: Dihydroxy-eicosatrienoic acid; EDP: Epoxyeicosapentaenoic acid; EET: Epoxyeicosatrienoic acid; EEQ: Epoxyeicosatetraenoic acid; FC: Free cholesterol; HETE: Hydroxyeicosatetraenoic acid; LCPUFA: Long chain polyunsaturated fatty acid; LPC: Lysophosphatidylocholine; LPE: Lysophosphatidylethanolamine; LPE(O): Alkyl/alkenyl-lysophosphatidylethanolamine; MUFAs: Monounsaturated fatty acids; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PI: Phosphatidylinositol; PL: Plasmalogens; PS: Phosphatidylserine; PUFAs: Polyunsaturated fatty acids; SFAs: Saturated fatty acids; SM: Sphingomyelin; TAG: Triacylglycerols; TG(O): Alkyl-diacylglycerols.

Of interest, lipidomic studies conducted in both plasma and serum of NAFLD patients reported overlapping results with those found in the liver. In addition, increased levels of total SFA in phospholipids[24], metabolites of lipoxygenase, 5-hydroxyeicosatetraenoic acid (HETE), 8-HETE[25], 15-HETE, 5,6 dihydroxy- eicosatrienoic acid[26], palmitoleic acid in cholesteryl ester[27], PC and sphingomyelin[28], phosphatidylserine, and phosphatidylinositol[29] were found in plasma and serum of NAFLD patients. On the other hand, decreased levels of eicosanoic acid (C20: 0), cis-11-octadecenoic acid (C18: 1n-7), docosahexaenoic acid in phospholipids[24], 12,13-dihydroxy-9-octadecenoic acid[26], and lysophospatidylethanolamine[28] have been described.

Fatty acids

The increased share of free fatty acids reaching the liver in NAFLD has been related to three main mechanisms: lipolysis of adipose tissue, *de novo* lipogenesis, and diet[30].

Both hepatic[31] and plasma[24] findings from adults and children[32] with NAFLD showed an increased content of SFAs. Of concern, SFAs seem to be one of the major players involved in lipotoxicity. In fact, evidence linked inhibition (genetic or pharmacological) of the enzyme converting SFAs to MUFAs, namely stearoyl-CoA desaturase-

Table 2 Main findings of lipidomic studies in paediatric nonalcoholic fatty liver disease

Ref.	Study design and methods	Population (*n*)	Main findings
Wasilewska *et al*[20], 2018	Prospective study	80 children at median age 12 (7-17 yr)	Higher total serum CE concentration in NAFLD patients, compared to the controls and of certain CEs (C14:0, C16:0, C16:1, C18:0, C18:1, C22:0, C24:0). Total CE concentration was positively correlated with HOMA-IR and insulin levels
Draijer *et al* [8], 2020	Case-control study	21 children with obesity and steatosis and 21 with only obesity. Mean age of NAFLD patients: 14.8 yr; mean age of non-NAFLD patients 14.7 yr	Statistically significant alterations in 5 major lipid classes [TG(O), PE, PE(O), LPE(O), PC(O)] and 12 individual lipid species
Kalveram *et al* [32], 2021	Prospective study	40 children with biopsy-proven NAFLD. Mean age 14.2 ± 2.3 yr	Hepatic epoxyeicosanoids levels increased with higher degrees of steatosis. CYP epoxygenase activity increased, protein level, and activity of sEH decreased. In contrast, hepatic epoxyeicosanoids decreased with higher stages of fibrosis, with a decrease of CYP epoxygenase activity and protein expression

CE: Ceramides; CYP: Cytochrome P450; HOMA-IR: Homeostasis model assessment; NAFLD: Nonalcoholic fatty liver disease; LPE(O): Alkyl/alkenyl-lysophosphatidylethanolamine; PC(O): Alkyl/alkenyl-phosphatidylcholine; PE: Phosphatidylethanolamine; PE(O): Alkyl/alkenyl-phosphatidylethanolamine; sEH: Soluble epoxide hydrolase; TG(O): Alkyl-diacylglycerols.

1, to different processes such as hepatocyte apoptosis, lipotoxicity, and development of steatohepatitis[33]. Consequently, balancing between MUFAs and SFAs might represent a central player in the progression from isolated hepatic steatosis to progressive steatohepatitis and fibrosis[34].

MUFAs were also found to be increased in liver and plasma of both adult and paediatric patients with NAFLD[25,32,35-37]. This lipid class has been considered as less lipotoxic than SFAs because channelling free fatty acids into MUFAs allow their incorporation into triglycerides and storage in lipid droplets[34].

The long chain PUFAs such as eicosapentaenoic, docosahexanoic, and arachidonic acids were decreased in liver and plasma of patients with NAFLD[29,31,35,36]. This reduction could be due to impairments in dietary intake or the biosynthesis process. A pivotal pathogenic role in NAFLD progression (from simple steatosis to NASH) has been attributed to decreased activity of fatty acid desaturase 1, an enzyme involved in the PUFAs metabolism[6].

Through the activity of hepatic cytochrome P450 enzymes, PUFAs derived from monoepoxides are collectively termed epoxyeicosanoids[38-40]. Those deriving from arachidonic acid (epoxyeicosatrienoic acid), eicosapentaenoic acid (epoxyeicosatetraenoic acid), and docosahexaenoic acid (epoxyeicosapentaenoic acid) have anti-inflammatory, antisteatotic, and antifibrotic properties[41]. In a recent paediatric study [32], 40 youths with biopsy-proven NAFLD underwent lipidomic evaluations by analysing liver tissue and blood samples. Upregulated hepatic epoxyeicosatrienoic acid, epoxyeicosatetraenoic acid, and epoxyeicosapentaenoic acid levels were found in children with steatosis. This might be due to reduced activity and protein expression of soluble epoxide hydrolase, metabolizing epoxyeicosanoids to vicinal diols. On the contrary, at the stage of fibrosis the aforementioned epoxyeicosanoids were found to be decreased in liver and plasma because of a potential reduction of cytochrome P450 epoxygenase expression. Therefore, the cytochrome P450 epoxygenase/soluble epoxide hydrolase pathway seem to represent a potential innovative pharmacologic target for NAFLD treatment.

Proinflammatory molecules are also derived from PUFAs. Puri *et al*[25] found increased plasma levels of arachidonic acid (5-HETE, 8-HETE, 11-HETE, and 15-HETE) lipoxygenase metabolites in NAFLD and NASH patients compared to lean normal controls. Moreover, plasma arachidonic acid-derived metabolites 11,12-dihydroxyeicosatrienoic acid, 13,14-dihydro-15-keto prostaglandin D2, and 20-carboxy arachidonic acid levels were found to be significantly increased by Loomba *et al*[26] in subjects with NASH than those with NAFLD.

With respect to the wide cardiometabolic burden of NAFLD, changes in FA metabolism have also been linked to its related comorbidities including obesity, diabetes, and cardiovascular risk[20,28,29].

Neutral lipids

The hallmark of NAFLD is the accumulation of lipid droplets in the hepatocytes containing TAG[7]. TAGs were found to be increased in both plasma and liver of

patients with NAFLD compared to healthy controls[25,31]. They represent a less toxic form of storing lipids. The inhibition of diacylglycerol acyltransferase 2, the enzyme catalysing the final step in the assembly of TAG, reduced steatosis but at the same time increased hepatic free fatty acids, lipid peroxidation, oxidative stress, necroinflammation, and fibrosis[42]. In mice defective for perilipin-5, a protein binding lipid droplet and regulating TAG storage, the reduction in the size of lipid droplets caused increased lipolysis and lipotoxicity[43].

Diacylglycerols (DAG) were also increased in plasma and liver of patients with NAFLD[25,31], and the ratio of TAG/diacylglycerols seemed to increase in the evolution from NAFLD to NASH[31].

In a paediatric study, Draijer *et al*[8] performed lipidomic analyses in plasma samples of 21 children with obesity and proton magnetic resonance spectroscopy-detected hepatic steatosis compared to the lipidome of 21 samples of nonsteatotic subjects with obesity. The authors found an overall significant increase in NAFLD patients of serum alkyl-diacylglycerols [TG(O)], in particular 8 TG(O) species (TG(O-52:0), (TG(O-52:1) TG(O-52:2), TG(O-54:1), TG(O-54:2), TG(O-52:3), TG(O-56:1) and TG(O-56:2)].

Finally, it should also be noted that the amount of hepatic free cholesterol increases with the progression to NASH, without an increase in cholesterol esters[31]. It is considered a cytotoxic lipid disrupting membrane integrity and inducing oxidative stress, mitochondrial dysfunction, and apoptosis[44].

Glycerophospholipids

Glycerophospholipids represent a significant lipidic fraction of the cell membrane. Reduced hepatic PC levels were observed in both NAFLD and NASH subjects[22,31]. However, conflicting data about alterations in serum were reported[6]. Low hepatic levels of PC influenced circulating very low-density lipoproteins, which are therefore reduced with consequent hepatic accumulation of TAG[45,46]. Low levels also increased de novo lipogenesis and formation of lipid droplets in hepatocytes by activation of sterol regulatory element-binding protein 1[47].

Liver phosphatidylethanolamine (PE) content was found to be decreased among subjects with NASH[22], while serum PE increased[28]. The enzyme phosphatidylethanolamine n-methyltransferase catalyses the reaction converting PE to PC. Studies reported that a loss-of-function polymorphism in the phosphatidylethanolamine n-methyltransferase gene predisposed to NAFLD susceptibility[48]. Lower hepatic PC/PE ratio was also reported in NAFLD individuals. Interestingly, a reduced PC/PE ratio in red blood cell membranes has been found to enhance predisposition to NAFLD[49]. As a consequence, loss of membrane integrity and higher permeability to proinflammatory factors were observed[50].

Paediatric data reported significantly increased PE serum levels and reduced specific etherphospholipid classes such as alkyl/alkenyl-phosphatidylethanolamine, alkyl/alkenyl-lysophosphatidylethanolamine [LPE(O)], and alkyl/alkenyl-phosphatidylcholine in subjects with NAFLD. When looking at individual lipid species, two LPE species such as LPE (20:3) and LPE (22:5) were found to be increased[19].

Phosphatidylserine and phosphatidylinositol were decreased in the liver[22] but increased in the plasma[29] of NAFLD patients. However, these results are conflicting in other studies[25,28,51].

LPC was increased in the liver of NASH patients[6], while no statistically significant changes in plasma and serum of LPC content were reported in patients with NAFLD or NASH[28,29]. LPC derived from PC by the action of lipoprotein associated phospholipase A2 at the intracellular level, whereas in the extracellular milieu by lecithincholesterol acyltransferase activity. In patients with NAFLD, phospholipase A2 levels were found to be decreased, while those of lecithincholesterol acyltransferase increased[52,53]. LPC downregulates genes involved in fatty acid oxidation, upregulates genes involved in cholesterol biosynthesis, and promotes apoptosis of hepatocytes[54]. Inhibitors of phospholipase A2 decreased palmitate-induced lipotoxicity and cell apoptosis[54,55].

In the liver of NAFLD patients no change in plasmalogen content was reported[56], while this class was decreased in the plasma of NASH patients[25,57]. Animal data demonstrated that a specific mechanism (involving peroxisome proliferator-activated receptor-alpha) sustained by endogenous hepatic plasmalogens may prevent liver steatosis and NASH[58].

Circulating plasmalogen levels, particularly 16:0 and 18:1, were found to be reduced in NAFLD individuals with the GG-genotype of patatin-like phospholipase domain-containing 3 (*PNPLA3*) compared to those with the C or CG allele[28]. The *PNPLA3* gene is highly expressed in hepatic stellate cells of the liver and adipose tissue and

encodes adiponutrin, a protein exerting both lipase and acyltransferase activity[59]. Adiponutrin variant p.I148M [rs738409 (G)] enhanced PUFA content of TAGs and diacylglycerols and negatively affected both PC synthesis and lipid droplet hydrolysis [60].

An elegant paediatric study examining NAFLD genetic factors demonstrated that *PNPLA3* rs738409 (G) represented the strongest determinant of the presence of NAFLD as compared to healthy controls and conferred the highest risk of severity of steatosis. Interestingly, a specific steatosis pattern (including an increased percentage of portal inflammation) was reported in homozygous *PNPLA3* rs738409 (G) patients [61].

In addition to NAFLD, a significant association of these compounds has been found in a larger cardiometabolic-related context such as obesity and cardiovascular disease [51,57].

Sphingolipids

Sphingolipids are structural components of cellular membranes and signalling molecules in mammalian cells. Conflicting results were found about sphingomyelin (SM) trends in NAFLD and NASH patients[22,28,29,37,51]. Barr *et al*[62] found an increase in serum levels of certain sphingomyelin species, such as SM (36:3), (d18:2/16:0), (d18:2/14:0), (d18:1/18:0), (d18:1/16:0), (d18:1/12:0), and (d18:0/16:0) in NAFLD individuals compared to controls. Instead, reduced circulating levels of SM (d18:1/24:1), SM (d18:1/16:0), SM (d18:1/22:0), SM (d18:1/24:0), SM (d18:1/18:0), SM (d18:1/20:0), SM (d18:1/23:0), SM (d18:0/16:0), and SM (d18:0/20:4) were observed by Zhou *et al*[63] in NASH adult subjects compared to controls. Moreover, increased serum levels of two SM species such as SM(d39:0) and SM (d41:0) were found in the serum of NAFLD paediatric patients[19].

Higher ceramide levels were found in plasma and liver biopsies of NAFLD subjects [51,64,65]. These lipids decreased insulin sensitivity in skeletal muscle and hepatocytes [66] and enhanced several unfavourable biological processes such as oxidative stress, mitochondrial dysfunction, and cell apoptosis[66,67]. Moreover, they seem to regulate the synthesis of high-density lipoproteins. Animal data reported that myriocin, acting through ceramide biosynthesis inhibition–promoted insulin receptor and steatosis and enhanced apolipoprotein AI production rate, resulting in an increased high-density lipoprotein production rate[68].

In a prospective study[20] including 80 obese children, total ceramide concentration was significantly increased in the serum of obese and NAFLD patients than in the reference group. In addition, increased levels of distinct fatty acid ceramides, such as myristic, palmitic, palmitoleic, stearic, oleic, behenic, and lignoceric were observed in children with NAFLD compared to controls. Furthermore, a significant positive association of total ceramide levels with homeostasis model assessment and insulin levels was reported[20].

Taken together, these findings might pave the way for a wider risk assessment for these patients, as suggested by paediatric evidence indicating a significant association of distinct sphingolipids with NAFLD and with its cardiometabolic burden including obesity, cardiovascular disease, and metabolic derangements[20,69-71].

CONCLUSION

Lipidomic studies have added novelty by allowing an accurate characterization of lipidomic profile of both plasma and liver tissues in NAFLD[7,12,17]. Besides experimental data providing additional insights about the pathophysiology of NAFLD and its progression, there is a growing body of evidence from human studies[8,14,20]. In particular, a clear effect for specific ceramides in impairing insulin signalling pathways has been found[10,13,15].

Interestingly, different lipid classes have been demonstrated to exert pathogenic distinct roles in NAFLD and in other metabolic diseases such as obesity, metabolic syndrome, and type 2 diabetes[14,15]. Thus, manipulation of the expression of certain lipids (*e.g.*, selective lowering of specific ceramides) might represent a novel target for both prevention and treatment of these diseases. In fact, this attractive therapeutic approach might pave the way for novel strategies to counteract the increasing NAFLD-related cardiometabolic burden even in childhood.

Further research is needed to validate these findings and to provide a more comprehensive assessment of the exact pathogenic role of specific lipids in the NAFLD context.

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MINIREVIEWS

Autoimmune pancreatitis and pancreatic cancer: Epidemiological aspects and immunological considerations

Dimitri Poddighe

ORCID number: Dimitri Poddighe 0000-0001-6431-9334.

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Dimitri Poddighe, Department of Medicine, School of Medicine, Nazarbayev University, Nur-Sultan 010000, Kazakhstan

Dimitri Poddighe, Department of Pediatrics, National Research Institute for Maternal and Child Health (NRCMCH), University Medical Center (UMC) Nur-Sultan 010000, Kazakhstan

Corresponding author: Dimitri Poddighe, MD, MSc, PhD-Eq., Associate Professor, Department of Medicine, School of Medicine, Nazarbayev University, Kerei-Zhanibek Str. 5/1, Nur-Sultan 010000, Kazakhstan. dimitri.poddighe@nu.edu.kz

Abstract

Ordinary chronic pancreatitis is a well-known risk factor for pancreatic cancer, whereas such an association with autoimmune pancreatitis (AIP) is widely debated. Due to the rarity of the latter disorder, there are few specific clinical and epidemiological studies investigating the relation between AIP and pancreatic cancer, which do not seem to support it. However, these studies are affected by several limitations and, therefore, a link between AIP (and, specifically, type 1 AIP) and pancreatic cancer cannot be ruled out definitively on this basis. Moreover, several immunopathological aspects of type 1 AIP and, in general, immunoglobulin G4-related disease can create an immunological context that may impair the tumoral immunosurveillance and promote the pancreatic carcinogenesis and its progression. In detail, Th2 immunological dominance, type 2 macrophage polarization and basophil infiltration observed in type 1 AIP, may play a permissive role in creating a favorable immunological environment for pancreatic carcinogenesis, in addition to the immunosuppressive therapies that can be used in these patients.

Key Words: Autoimmune pancreatitis; Chronic pancreatitis; Pancreatic cancer; Immunoglobulin G4-related disease; Epidemiology; Immunology; Basophils; Macrophages; Th2 cells; Systemic lupus erythematosus

Core Tip: This mini-review discusses the debated issue of autoimmune pancreatitis (type 1) as a potential risk factor for pancreatic cancer. After summarizing the few

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available (low-quality) epidemiological evidence that does not clearly support this role, the immunopathological characteristics of type 1 autoimmune pancreatitis (including Th2 immunological dominance, type 2 macrophage polarization and basophil infiltration) are discussed as potential factors that may actually create a tolerogenic immunological environment favorable to pancreatic carcinogenesis and/or tumor progression.

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INTRODUCTION

Autoimmune pancreatitis: Definition, pathology, and epidemiology

Chronic pancreatitis (CP) is a persistent inflammatory disease of the exocrine pancreas leading to progressive fibrotic tissue damage. CP prevalence varies between 13.5-98.7 cases per 100000 people with an incidence of around 4-5 new cases per 100000 people every year. Most cases of CP (also defined as "ordinary" or "generic") are triggered by the repeated and/or persistent activation of intrapancreatic digestive enzymes, due to the variable combination of some environmental factors (*e.g.*, excessive alcohol consumption, regular tobacco use, hypertriglyceridemia, *etc.*) and/or genetic factors (*e.g.*, Chymotrypsin C mutations)[1].

Autoimmune Pancreatitis (AIP), which represents < 5%-10% of pancreatitis cases and has an estimated prevalence of approximately 1-2/100000 people, is much rarer[1,2]. However, the real incidence of AIP is currently unknown: indeed, it is diagnosed in around 2% of patients who have undergone pancreas surgical resection for presumed pancreatic cancer and, therefore, this condition may be underdiagnosed[3].

The term AIP was originally introduced by Yoshida *et al*[4] in 1995 to describe a patient diagnosed with pancreatitis who "had hyperglobulinemia, was autoantibody-positive, and responded to steroid therapy". Currently, AIP diagnosis must be supported by well-defined and specific clinical, radiological, serological and histopathological criteria[3]. Importantly, based on histopathological aspects (according to the Honolulu classification), AIP is classified into two main subtypes that are mainly distinguished by the absence (type 1 AIP) or presence (type 2 AIP) of one peculiar finding, called "granulocytic epithelial lesion"(GEL), consisting of the neutrophilic infiltration of medium/small-sized ducts and often acini[5]. Thus, type 1 AIP is characterized by a substantial lymphoplasmacytic infiltration, where eosinophils may be relatively frequent and neutrophils are rare. Indeed, it was originally described as lymphoplasmacytic sclerosing pancreatitis[5,6]. Moreover, it is much more frequent than type 2 AIP, which accounts for 10%-20% and no more than 5% of all AIP cases in Western and Eastern countries, respectively. Importantly, type 1 AIP is now accepted as a clinical-pathological aspect of immunoglobulin G4 (IgG4)-related disease (IgG4-RD)[6,7].

IgG4-RD is a multisystem immune-mediated fibroinflammatory condition where several organs can be involved and, most frequently, pancreas, bile ducts, salivary glands, lacrimal glands, kidneys, retroperitoneum, and lungs. Pancreas is involved in at least 45% of IgG4-RD patients as type 1 AIP[8]. Therefore, unlike ordinary CP, type 1 AIP is usually the pancreatic manifestation of a systemic immunological disorder, in which several alterations of the innate and acquired immune system contribute to the lympho-plasmocytic inflammatory infiltration of the pancreas[9].

IgG4-RD pathogenesis is complex and involves both the innate and adaptive immune system; as regards the latter one, Th2 (specifically, follicular Th2 cells, Tfh2) and Treg lymphocytes (and related cytokines) have been clearly and mainly implicated in this pathologic process characterized by altered B cell and plasma cell activation, enhancement of IgG4 class switch recombination and, finally, development of ectopic germinal centers and induction of fibrosis[9,10].

Type 2 AIP pathogenesis is more elusive and, indeed, is also called idiopathic duct-centric pancreatitis. It is not characterized by IgG4 increase and other specific extra-pancreatic manifestations, except for an association with inflammatory bowel diseases

in 20%-30% cases[6,7].

Type 1 and type 2 AIP cannot be certainly differentiated by radiological imaging and share similar clinical presentations. However, the diagnostic differentiation is very important since the prognosis is quite different. Indeed, type 1 AIP is usually more aggressive, showing a much higher rate of relapse after steroid therapy, and is characterized by synchronous or metachronous extra-pancreatic organs involvement, as previously mentioned[11].

Both AIP types are almost exclusively adulthood diseases with a median age at diagnosis between 40-50 years, even though patients with type 1 AIP are 10-15 years older on average. AIP has been rarely described in the pediatric population, where the few reported cases were consistent with type 2 AIP in most cases[7,12].

PANCREATIC CANCER AND AIP: EPIDEMIOLOGICAL ASPECTS

Pancreatic cancer is represented by adenocarcinoma in > 85% of cases. Nowadays the overall incidence of pancreatic cancer is estimated to be around 11.0 per 100,000 people per year in the United States, where it accounts for 3% of all diagnosed malignancies. These numbers make it be the twelfth and eleventh most common cancer in men and women, respectively. Pancreatic cancer remains a highly fatal malignancy: Worldwide, it is the seventh leading cause of cancer death in both genders, with a 5-year survival rate of 10% or less. Like most malignancies, incidence rates for pancreatic cancer are age-related: In general, it is rare before the age of 50 years. The main risk factors for pancreatic cancer are modifiable and include tobacco smoking, obesity, physical inactivity and high-calorie/fat diets; these lifestyle-related risk factors clearly contributed to the increase of the disease incidence in the last three decades, especially in developed countries, where diagnostic improvements and increased life expectancy have played a role as well[12,13].

CP (and, in detail, ordinary CP) was shown to be a clear risk factor for pancreatic cancer. In 1993 Lowenfels *et al*[14] published the results of a large multicenter and international cohort study including 2,015 patients with CP, who showed an increased risk of pancreatic cancer, independent from gender, country and type of pancreatitis. It was estimated that approximately 5% of CP patients receive a diagnosis of pancreatic cancer within 2 decades after that diagnosis[12,14].

The potential association between pancreatic cancer and AIP is currently debated. Even though AIP is a rare form of CP, this issue is raised especially in patients affected with type 1 AIP that, compared to type 2 AIP, is more common and characterized by high percentages of disease relapse after steroid therapy[15,16].

Moreover, as summarized and discussed below, all reported cases of AIP-related pancreatic cancer were described in patients affected with type 1 AIP; actually, some concerns were raised in terms of potential association with malignancies in general. Most studies on IgG4-RD and/or type 1 AIP are from Japan: Yamamoto *et al*[17] and Shiokawa *et al*[18] first reported an overall increase of malignancy incidence in their patients, even though they actually described no cases of pancreatic cancer.

A larger multi-centric and international study by Hart *et al*[15] (including 1,064 patients with AIP and, exactly, 978 with type 1 and 86 with type 2) reported 5 diagnoses of pancreatic cancer in type 1 AIP patients (no one affected with type 2 AIP developed this complication). However, as discussed by the authors themselves, even though they found a small number of pancreatic cancers (compared to the total cumulative frequency of malignancies, n = 57), the limited follow-up and lack of a control population may have limited the clinical significance of their analysis. Moreover, this study (and also another one authored by the same research group) were not focused on pancreatic cancer[15,19], as well as the following studies. Hirano *et al*[20] described their case series of 113 IgG4-RD patients (type 1 AIP: n = 95) and reported 2 patients who developed pancreatic cancer; in general, their conclusion was that the cumulative incidence of any kind of malignancies in IgG4-RD patients was similar to that observed in the general population. Shimizu *et al*[21] also made the same conclusion in their 84 type 1 AIP patients; in detail, among 9 patients diagnosed with cancer, only one developed it at the pancreas. Buijs *et al*[22] analyzed a Dutch case series of 107 patients affected with AIP (type 1: 90%, type 2: 10%), and reported no apparent difference in malignancy incidence compared to an ethnic-, age-, and sex-matched reference population (in detail, no patients developed pancreatic cancer).

However, although several clinical studies tried to address this issue, the prevalence and risk assessment of pancreatic cancer in the pathological setting of AIP have been greatly hampered by the relatively small number of participants, variable and/or

relatively short follow-up period, retrospective study design and the lack of appropriate control groups in most of these studies[23].

Therefore, some authors proposed that patients affected with AIP should be regularly monitored to reveal any potential cancerous changes and/or malignancy onset, anyway[24]. For instance, unlike the aforementioned studies, Huggett *et al*[16] reported a statistically significant odd ratio increase (odds ratio = 2.2) for all malignancy risk in type 1 AIP patients: 13 malignancy cases (out of 115 type 1 AIP and/or IgG4-RD and, 106 patients with pancreatic disease) were diagnosed, and all those tumors arose in systemic IgG4-RD patients, who represented only 56% of the whole cohort. However, only one case was affected with pancreatic adenocarcinoma.

Additional and interesting research articles, which were more focused on pancreatic complications and carcinogenesis in AIP patients, were published in the last few years. A clinical research by Gupta *et al*[25] investigated the pancreatic carcinogenesis in patients affected with AIP. These authors reviewed a case series of 84 AIP patients, and they compared the prevalence of pancreatic intraepithelial neoplasia (PanIN) in 28 cases of AIP and 30 cases of CP not otherwise specified. Overall, 82% AIP patients showed PanIN with variable histologic grade, which resulted to be more frequent than in non-autoimmune CP (63%), though such a difference was not statistically significant. Therefore, the prevalence of PanIN in AIP resulted to be comparable to ordinary CP; moreover, in the same article the authors also described their clinical experience with 84 AIP patients, and the only 2 cases of pancreatic cancer were diagnosed in the context of type 1 AIP, which was diagnosed 6 and 10 years before the detection of the malignancy, respectively. Therefore, this study raised some specific concerns as regards the risk of pancreatic malignancy in type 1 AIP patients[25]. Similarly, Ikeura *et al*[26] diagnosed 3 cases of pancreatic cancer (4.8%) in their 63 type 1 AIP patients, which was similar to what observed in the comparison group, consisting of 41 patients affected with ordinary (alcoholic and hereditary) CP, characterized by only one patient (2.1%) with a diagnosis of pancreatic malignancy.

Starting from a different perspective, Ngwa *et al*[27] described 548 patients diagnosed with pancreatic cancer and 99 different patients affected with type 1 AIP. In this study, whose main aim was to compare the IgG4 serum profiles between those two groups of patients (rather than investigating an epidemiological and/or causal link), the authors suggested no relationship between AIP and pancreatic cancer; however, a minority of histological specimens (only 30 pancreatic tissue specimens from patients with pancreatic cancer and 6 patients with "high-risk features" AIP) was reviewed. Indeed, a recent retrospective study by Xiang *et al*[28], including 74 patients with type 1 AIP, revived the debate by reporting that 5 of them (6.7%) were concomitantly diagnosed with a pancreatic tumor (pancreatic ductal adenocarcinoma, n = 3; solitary extramedullary plasmacytoma, n = 1; cholangiocarcinoma, n = 1). Moreover, an interesting observation was reported by Hedayat *et al*[29], who re-examined 21 pancreas specimens of previously diagnoses of intraductal papillary-mucinous neoplasm, which is a cystic and usually benign neoplasm, but with the potential for progression to pancreatic cancer. Interestingly, 4 of them (19%) showed infiltrates of IgG4-positive plasma cells, consistent with a "peri-tumoral" type 1 AIP reaction[29,30].

Conversely, two very recent retrospective studies were not supportive in this sense. Tang *et al*[31] described 17 neoplastic cases in a large cohort of 587 Chinese patients diagnosed with IgG4-RD; among those, 11 were also affected with AIP, but all developed extra-pancreatic malignancies only. The study by Ishikawa *et al*[32], including 123 type 1 AIP patients, identified only 2 patients diagnosed with pancreatic cancer (1.6%) and concluded that "AIP is unlikely to be a precancerous condition of the pancreas", but at the same time they stated that "because of the small number of cases, the characteristic findings of pancreatic cancers that develop in AIP patients are not clear".

Therefore, as summarized by these two final statements, this short and schematic overview of clinical studies provides conflicting evidence and conclusions regarding the association between AIP (and, in detail, type 1) and pancreatic cancer. Table 1 summarizes all the aforementioned clinical studies investigating the association between AIP and pancreatic cancer, by using a chronological order and focusing on the main study features and findings.

Even though one may conclude that most of the available studies do not support this association, there are some clinical and pathological observations coming from studies more focused on pancreatic cancer[25,26,28] which should keep high the attention on this issue before making final conclusions; however, this will require larger, prospective, and longer (in terms of follow-up period) studies. Unfortunately, the clinical research on this topic is undoubtedly hampered by the low prevalence and incidence of AIP, in general and in the landscape of CP, in addition to the challenges of

Table 1 Schematic overview of the clinical studies investigating the association between autoimmune pancreatitis and pancreatic cancer.

Ref.	Study population (disease)	Total patients (*n*)	AIP type 1 (*n*)	Cancer (overall) (*n*)	Pancreatic cancer (*n*)	Median follow-up (yr)	Additional specifications
Yamamoto *et al* [17] (Japan, 2012)	IgG4-RD	106	10	2	0	NA	-
Shiokawa *et al* [18] (Japan, 2013)	AIP	108	104	18	0	3.3	These 18 malignancies were diagnosed in 15 patients.
Hart *et al*[15] (International, 2013)	AIP	1064	978	57	5	NA	No patients with AIP type 2 developed any malignancies.
Gupta *et al*[25] (United States, 2013)	CP	58	11	NA	7 (PanIN)	N/A	Retrospective analysis of patients with CP. There was no statistically significant difference in the frequency of PanIN between ordinary CP and AIP: in the latter group, no difference between type 1 and type 2. In general, the only case of PanIN3 was detected in one AIP type 1 specimen.
Gupta *et al*[25] (United States, 2013)	AIP	84	NA	NA	2	4.1	These 2 cases of pancreatic cancer were diagnosed in type 1 AIP patients.
Hart *et al*[19] (United States, 2014)	AIP	116	116	23	1	3.6	-
Hirano *et al*[20] (Japan, 2014)	IgG4-RD	113	95	14	2	6	2 IgG4-RD patients diagnosed with malignancy (out of 14) were not affected with AIP.
Huggett *et al*[16] (United Kingdom, 2014)	IgG4-RD	115	106	13	3	2.7	Of these 3 cases of pancreatic malignancies, 2 were cholangiocarcinoma cases and 1 was a pancreatic adenocarcinoma.
Ikeura *et al*[26] (Japan, 2014)	AIP	63	63	NA	3	5.2	-
Shimizu *et al*[21] (Japan, 2015)	AIP	84	84	9	1	4.5	-
Buijs *et al*[22] (The Netherlands, 2015)	AIP	107	96	8	0	6.25	-
Ngwa *et al*[27] (United States, 2015)	AIP	99	99	NA	0	NA	The aim of this study was to evaluate the clinical significance of elevated sgG4 levels in patients with AIP and pancreatic cancer and potential prognostic implications of those in patients with pancreatic cancer.
Xiang *et al*[28] (China, 2019)	AIP	74	74	NA	4	NA	3 patients were diagnosed with pancreatic ductal adenocarcinoma and 1 with cholangiocarcinoma. There was also 1 case of solitary extramedullary plasmacytoma.
Tang *et al*[31] (China, 2020)	IgG4-RD	587	NA	17	0	5.1	-
Ishikawa *et al*[32] (Japan, 2020)	AIP	123	123	NA	2	4.6	-

AIP: Autoimmune pancreatitis; CP: Chronic pancreatitis; IgG4-RD: Immunoglobulin G 4-related disease; N/A: Not applicable; NA: Not available; PanIN: Pancreatic intra-epithelial neoplasia.

pancreatic diagnostics.

Nonetheless, it is established that chronic inflammatory processes represent a risk factor for pancreatic cancer and, as such, type 1 AIP, especially if relapsing and/or persistent, should be considered in the same way in principle. Moreover, concomitant immunosuppressive therapies (including steroids, rituximab, azathioprine., *etc.*) may represent additional predisposing factors to the cancer development[11]. Finally, some specific immunological aspects and considerations (also related to IgG4-RD pathogenesis) might support these concerns, as discussed in the next sections.

TYPE 1 AIP: GENERAL IMMUNOPATHOGENESIS

IgG4-RD is the clinical expression of a systemic immunological dysregulation leading to chronic inflammation and lymphocyte infiltration in several organs (including pancreas), which might have some implications in terms of pancreatic and extra-pancreatic carcinogenesis[8]. However, as discussed, the available clinical evidence does not support this hypothesis, but the small number of studies, which are also characterized by several and important limitations, likewise do not allow to rule out it either.

As said, type 1 AIP is the prevalent form of AIP and all cases of AIP-related pancreatic malignancies were described in this pathological setting. Therefore, the following immunological considerations on AIP immunologic pathogenesis and its potential role in pancreatic carcinogenesis, will refer to type 1 AIP and/or IgG4-RD specifically.

Even though the pathogenesis of IgG4-RD is complex and relies on both innate and acquired immune system mechanisms, the final result is an immunological environment characterized by a Th2 dominant immune response, which is indeed associated with increased level of serum IgG4 (systemically) and IgG4 switched lymphocytes and plasma cells (in the pancreas and other affected organs). Therefore, IgG4-RD and type 1 AIP are characterized by a cytokine profile whereby interleukin (IL)-4, IL-5, IL-13, IL-10 and transforming growth factor (TGF)-β are overexpressed [33].

Recent studies evidenced the central role that is played by the T follicular helper cells (Tfh cells), which can be found in the extra-nodal ectopic germinal centers and are involved in the generation of long-lasting humoral responses by B cells and plasma cells[9]. Increased levels of Tfh cells and, in detail, Th2-polarized cells (Tfh2 cells), have been described in IgG4-RD patients, in whom those cells correlate with serum IgG4 and IL-4 levels[34]. Moreover, Tfh2 cells, but not Tfh1 or Tfh17 cells, were demonstrated to induce the differentiation of naïve B cells into plasma blasts (with enhanced production of IgG4) and, importantly, the activation of Tfh2 cells resulted to correlate with the disease activity[35].

However, in addition to the probable intrinsic dysregulation of the adaptive immune system, the origin of these Tfh2 expansion and activation (and, more in general, Th2 predominance) must be sought in the innate immune system as well[9]. Through the use of an experimental murine model of AIP (namely, MRL/Mp mice treated with polyinosinic-polycytidylic acid), Arai *et al*[36] described the pancreatic accumulation of plasmocytoid dendritic cells (pDCs) producing IFN-α. They also showed that pDCs from human patients with type 1 AIP had an increased production of IFN-α, which was able to promote the B cells switch toward IgG4 production in a T-cell independent manner, probably by the enhanced production of BAFF (B cell-activating factor belonging to the tumor necrosis factor family). Actually, BAFF production upon Toll-like-receptor (TLR) activation was described in other innate immune cells, including basophils, through the stimulation of TLR2 and TLR4[37]. This finding is particularly interesting because the presence of basophils has been recently described in the pancreatic tissue of most patients with type 1 AIP[38].

Basophils have been demonstrated to be more than simple effector cells in several pathological contexts (including asthma and other allergic diseases) and, in detail, were proposed as one of the main sources of early IL-4, being able to drive and/or support the Th2 polarization of activated CD4+ T cells[39-41]. Overall, these observations may support the fact that basophils are activated in a IgE- (and, thus, B-cell) independent manner and, indeed, TLR-activated basophils can contribute to drive the Th2 response in type 1 AIP[38].

Interestingly, in a recent review article, Watanabe *et al*[9] highlighted several immunological similarities between IgG4-RD and Systemic Lupus Erythematosus (SLE), which is also characterized by an increased type I IFN production through pDCs, a Th2 immunological dominance and a substantial dysregulation of humoral immunity. Moreover, some human studies and murine experimental models suggested a pathogenic role for basophils in SLE, indeed[42].

However, in the landscape of all the innate immune cells implicated in the pathogenesis of type 1 AIP, the role of pDCs appears to be prominent. In addition to IFN-α, these cells resulted to be the main source of another important cytokine that has been implicated in type 1 AIP immunopathogenesis, namely IL-33. Importantly, IL-33 was also recognized to be an important inflammatory mediator in ordinary CP, whereby actually the main cell source is represented by the pancreatic acinar cells[9, 43]. IL-33 is able to activate Th2 cells and also group 2 innate lymphoid cells, which can further stimulate the production of IL-4, IL-5, and IL-13[44,45].

Finally, to complete the landscape of the innate immunity involvement in type 1 AIP, it is worth to mention M2-polarized macrophages, which were described in the pancreatic tissues of type 1 AIP patients affected with IgG4-RD[46]. Compared to classically activated or M1 macrophages (which are clearly pro-inflammatory, are polarized by lipopolysaccharide and/or Th1 cytokines, and mainly produce IL-1β, IL-6, IL-12, IL-23 and tumor necrosis factor-α), alternatively activated or M2 macrophages are polarized by Th2 cytokines and produce anti-inflammatory cytokines, such as IL-10 and TGF-β. Therefore, M2 macrophages have been implicated in angiogenesis and tissue repair, and are considered to exert an anti-inflammatory (and, for some aspects, tolerogenic effect) also in IgG4-RD and type 1 AIP[47,48].

Indeed, IL-10 and TGF-β are the main cytokines secreted by the antigen-induced or adaptive Tregs. In detail, IL-10 regulates the functions of many different immune cells, including macrophages themselves, dendritic cells, and both T and B lymphocytes. In detail, IL-10 is a potent inhibitor of antigen presentation by reducing the expression of major histocompatibility complex molecules and costimulatory molecules CD80 and CD86. Moreover, it inhibits the differentiation of dendritic cells (DCs) themselves from monocyte precursors and their further maturation. Finally, upon antigen presentation, IL-10 inhibits macrophage production of pro-inflammatory cytokines (IL-1, IL-6, IL-12, tumor necrosis factor-α), even though it does not seem to directly affect the Th1/Th2 balance[49,50].

These immune-pathogenic aspects of type 1 AIP and IgG4-RD may create a favorable immunological background in the affected pancreas for carcinogenesis promotion, as discussed in the next section.

PANCREATIC CANCER AND TYPE 1 AIP: IMMUNOLOGICAL CONSIDERATIONS

As already mentioned, the cumulative risk of pancreatic cancer in subjects with ordinary CP was reported to be 1.8% after 10 years and 4.0% after 20 years, and it is estimated to be more than 10 times higher in these patients than in healthy subjects[14,51].

Whereas carcinogenesis in the context of ordinary CP is then well established epidemiologically, this methodological approach has not clearly supported the association between AIP (in detail, type 1 AIP) and pancreatic cancer, but the numerous study limitations should prevent from ruling it out definitively, as it was previously discussed.

To start with, a few initial molecular findings should be considered. Some specific molecular alterations of pancreatic carcinogenesis may be functional to this discussion, such as K-ras mutations, which resulted to be present in > 90% of pancreatic cancers and, importantly, may occur at an early stage of this neoplastic process, namely at the PanIN stage[52]. For instance, Kamisawa *et al*[53] found that codon-12 mutation of K-ras was significantly more frequent in the pancreato-biliary regions of patients with AIP than in those affected with chronic alcoholic pancreatitis.

More recently, Kinugawa *et al*[54] also described some methylation abnormalities of tumor suppressor genes in AIP patients. Promoter region hyper-methylation with consequent gene silencing was reported for several genes in pancreatic cancer. In detail, among the 6 genes that these authors have investigated (because those were previously reported as methylated in pancreatic cancer), they found a statistically significant difference in terms of the TFPI2 (tissue factor pathway inhibitor 2) methylation ratio between specimens taken from AIP patients and those from pancreas resected for non-tumoral diseases. TFPI2 was recognized as a tumor suppressor gene, and a previous study described methylation abnormalities for this gene in 73% of pancreatic carcinomas, whereas those were completely absent in normal pancreas specimens[55].

However, further theoretical support and biological plausibility supporting a role for type 1 AIP as potential risk factors for pancreatic cancer, may derive from some immunological considerations.

Indeed, the role of the immune system and, in detail, the impairment of the immunological surveillance in the development and progression of cancer, is well supported. Both the innate and the adaptive immune system play an active role in this regard. Generally speaking, cytotoxic CD8+ T cells, Th1 cells, mature DCs, classically activated pro-inflammatory macrophages M1 and natural killer cells are variably described as the main effectors of this anti-cancer immunological surveillance[56,57]. Therefore, several aspects of the immunological environment created by and during

type 1 AIP (and, systemically, by IgG4-RD) may be favorable to cancer development in the pancreas.

First, the Th2 signature dominating these diseases, does not support cytotoxic CD8+ T cell responses[56]. Tassi *et al*[58] showed that patients affected with pancreatic cancer have a Th2 skewed immune response, which can impair the specific CD4+ T cells response against a tumor-associated antigen, namely CEA; moreover, this polarization was suggested to negatively affect the T CD8+ cell compartment as well.

Second, in type 1 AIP macrophages tend to switch to a M2 phenotype, characterized by the production of IL-10 and TGF-β, which can create a tolerogenic immunological environment in the pancreas. By the way, Th2 cells themselves produce IL-10, in addition to their hallmark cytokines, such as IL-4, IL-5 and IL-13[47]. As explained, these M2 macrophages are also characterized by poor antigen presenting ability rather than supporting lymphocyte activation, proliferation and cytotoxicity. Moreover, they are committed to promote tissue remodeling and, importantly, neo-angiogenesis. Finally, M2 macrophages also express inhibitory ligands, such as PD-L1, which is known to induce peripheral T cell tolerance. All these properties suggested an important role of M2 macrophage in reducing the anti-tumor immunity for several malignancies, including pancreatic cancer. Indeed, they represent the prevalent macrophage phenotype among so-called tumor-associated macrophages and have been thus implicated in the processes of tumoral growth and cell migration/metastasis [59-62]. Accordingly, the degree and type of M2 macrophages infiltration into the tumoral stroma resulted to be an independent prognostic factor in patients with pancreatic cancer[63].

Third, in addition to M2 macrophages, basophils were recently suggested to play a role in the pathophysiology of type 1 AIP, upon activation *via* TLR signaling[38,64]. As previously explained, these cells can strongly shift the immunological balance toward a more tumor-tolerogenic Th2-polarized tissue microenvironment. Therefore, in addition to some initial evidence that basophils may be directly implicated in type 1 AIP pathogenesis, this way basophils might indirectly promote the pancreatic tumorigenesis as well. Recently, De Monte *et al*[65] investigated the presence of basophils in in tumor-draining lymph nodes of patients affected with pancreatic cancer, which resulted to correlate with both Th2 inflammation and reduced patients' survival. In this study, the role of basophils in tumor develop-ment/progression was also supported through some observations in basophil-deficient murine experimental models. Therefore, basophils may favor the tumorigenesis and progression of pancreatic cancer by promoting both Th2 and M2 polariza-tion[66].

Interestingly, these novel findings might open to additional important associations between pancreatic cancer and autoimmune diseases other than type 1 AIP and IgG4-RD. As mentioned, Watanabe *et al*[9] provided an interesting immunopathological parallelism between IgG4-RD and SLE. Therefore, it is very interesting that a very recent meta-analysis by Seo *et al*[67] evidenced that SLE was associated with increased risk for pancreatic cancer. These authors speculated several mechanisms that may potentially explain this association (*e.g.*, chronic inflammation, excess autoantibody effect, metabolic alterations); however, based on the present discussion, basophils activation and, more in general, Th2-driven inflammation described in active SLE might be added to these speculations on the hypothetical pathophysiologic permissive mechanisms[68,69].

CONCLUSION

Currently, the association between (type 1) AIP and pancreatic cancer does not find any clear epidemiological support, even though it cannot be ruled out definitively due to the small number of participants in the available clinical studies and the numerous study limitations. Some immunological aspects characterizing type 1 AIP, such as Th2 dominance, M2 macrophage polarization and basophil implication, may lead to an immunological environment favorable to pancreatic carcinogenesis and/or tumor progression.

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MINIREVIEWS

Gut microbiota in obesity

Bing-Nan Liu, Xiao-Tong Liu, Zi-Han Liang, Ji-Hui Wang

ORCID number: Bing-Nan Liu 0000-0001-6850-8466; Xiao-Tong Liu 0000-0002-7236-6226; Zi-Han Liang 0000-0001-6902-0358; Ji-Hui Wang 0000-0002-6363-0769.

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Bing-Nan Liu, Xiao-Tong Liu, Zi-Han Liang, Ji-Hui Wang, School of Bioengineering, Dalian Polytechnic University, Dalian 116034, Liaoning Province, China

Ji-Hui Wang, Engineering Research Center of Health Food Design & Nutrition Regulation, Dongguan University of Technology, Dongguan 523808, Guangdong Province, China

Corresponding author: Ji-Hui Wang, PhD, Professor, School of Bioengineering, Dalian Polytechnic University, No. 1 Qinggongyuan, Dalian 116034, Liaoning Province, China. wangjh_dlpu@163.com

Abstract

Obesity is a major global health problem determined by heredity and environment, and its incidence is increasing yearly. In recent years, increasing evidence linking obesity to the gut microbiota has been reported. Gut microbiota management has become a new method of obesity treatment. However, the complex interactions among genetics, environment, the gut microbiota, and obesity remain poorly understood. In this review, we summarize the characteristics of the gut microbiota in obesity, the mechanism of obesity induced by the gut microbiota, and the influence of genetic and environmental factors on the gut microbiota and obesity to provide support for understanding the complex relationship between obesity and microbiota. At the same time, the prospect of obesity research related to the gut microbiota is proposed.

Key Words: Gut microbiota; Obesity; Dysbiosis; Genetics; Ecology

Core Tip: Obesity is closely related to the gut microbiota. The study of the gut microbiome provides a basis for the reconstruction of the gut microbiota of obese patients. Here, we discuss the characteristics of the gut microbiota in obesity, the mechanism by which the gut microbiota induces obesity, and the relationships between genetic and environmental factors and the gut microbiota in obesity.

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INTRODUCTION

Obesity is a complex metabolic disorder caused by a variety of genetic and nongenetic factors (such as environmental factors). The World Health Organization defines obesity as having a body mass index (BMI) greater than 30, but the definition varies from country to country. In China, for example, a BMI of 28 or greater is considered obese. A comprehensive analysis shows that approximately one-third of the world's population are overweight, and approximately 10% are obese[1]. It is predicted that by 2030, the number of obese people worldwide will reach 1.12 billion[2]. The health risk of obesity has caused widespread concern and has become an important global health problem. Obesity not only manifests as changes in appearance but is also associated with lipid and glucose metabolism disorders, chronic inflammation, oxidative stress, and an increased risk of a variety of diseases, most notably cardiovascular disease, diabetes, and cancer[3,4]. In recent years, increasing evidence has shown that an imbalance in the gut microbiota may be a factor leading to obesity[5,6].

Up to 100 trillion symbiotic microbes live in the gut, called the gut microbiota, which comprises 10 times the number of cells in the body itself[7]. The gut microbiota relies on food residues that the human body does not digest, mucus secreted by the gut, and dead cells that are shed as nutrients to maintain its high population levels[8]. The active gut microbiota will produce a large number of physiologically active substances, including short-chain fatty acids, vitamins, and health-beneficial products such as anti-inflammatory, analgesic, and antioxidant products, along with potentially harmful products such as neurotoxins, carcinogens, and immunotoxins[9,10]. These products can enter the blood, directly regulate the expression of genes, and affect human immune and metabolic processes. Therefore, a healthy gut microbiota is essential for maintaining the body's metabolism and energy balance. An imbalance in the gut microbiota can cause metabolic disorders and increase central appetite, leading to obesity. This article reviews the research progress of the relationship between the gut microbiota and obesity.

LITERATURE SEARCH

PubMed (https://pubmed.ncbi.nlm.nih.gov/) was used for search with the following keywords: Obesity, gut microbiota, dysbiosis, energy absorption, appetite, fat storage, chronic inflammation, and circadian rhythm. More than 4000 published papers including 178 clinical trial related to gut microbiota in obesity from 2000 through 2021 have been searched.

RESULTS

After the search, we summarize some characteristics of the gut microbiota of obese patients. Many results suggest that an increased Firmicutes/Bacteroidetes ratio at the phylum level is an important feature of the gut microbiota in obesity. The family Christensenellaceae and the genera *Methanobacteriales*, *Lactobacillus*, *Bifidobacteria*, and *Akkermansia* are usually considered as probiotics, and their relative abundance is often inversely associated with obesity. Gut microbiota regulates obesity by regulating energy absorption, central appetite, fat storage, chronic inflammation, and circadian rhythms. Finally, the effects of genetic and environmental factors on gut microbiota in obesity are discussed.

THE HEALTHY GUT MICROBIOTA

The development of the Human Microbiome Project has promoted the in-depth study of the gut microbiota[11,12]. With the application of metagenomic and 16S ribosomal RNA gene sequencing, the diversity of the gut ecosystem has been widely studied, which enables us to accurately understand the composition and function of the gut microbiota[13]. So far, the gut microbes of 6457 taxa have been collected in the gutMEGA database[14]. The normal gut microbiota of the human body is mainly composed of *Firmicutes*, *Bacteroides*, *Proteus*, *Actinomycetes*, *Fusobacteria*, and *Verrucomicrobia*, among which *Firmicutes* and *Bacteroides* dominate[15]. The core functions of a healthy gut microbiota include the biodegradation of polysaccharides, the production

of short-chain fatty acids, the enrichment of specific lipopolysaccharides, and the production of vitamins and essential amino acids[16]. A healthy gut microbiota is generally highly diverse; conversely, the relative lack of diversity in the gut microbiota leads to diseases such as obesity[17]. Another sign of a healthy gut microbiota is dynamic equilibrium, which refers to its ability to resist perturbation and return to a healthy state, such as with antibiotic treatment[18]. Therefore, changes in the structure and metabolism of the gut microbiota will affect the body's physiological processes, such as nutrient absorption and energy metabolism.

ASSOCIATION OF THE GUT MICROBIOTA WITH OBESITY

The hypothesis that the gut microbiota may be a relevant environmental factor in obesity has led to the study of the gut microbiomes of obese individuals. The first evidence for a link between the gut microbiota and obesity was from studies of germ-free mice. Transplanting gut microbes from conventionally raised mice into germ-free mice increased the transplants' fat content and insulin resistance levels even with reduced food intake, which proved that gut microbes can increase the accumulation of adipose tissue in the host[19]. Furthermore, 16S rRNA gene sequencing showed that obesity may be associated with two dominant bacterial phyla: Firmicutes and Bacteroidetes. The gut microbiota of obese mice showed a 50% decrease in the abundance of Bacteroidetes and a proportional increase in Firmicutes[20]. Turnbaugh *et al*[21] further confirmed that the Firmicutes/Bacteroidetes ratio increased significantly in obese mice and proved that the capacity of the microbiota in obese mice to harvest energy from the diet was stronger. Similar phenomena occur in humans; for example, the proportion of Firmicutes in the gut of obese children was increased, and the proportion of Bacteroidetes was decreased[22]. A study of the Ukrainian population found that the Firmicutes/Bacteroidetes ratio increased with increasing BMI [23]. However, some other studies have found opposite results. Zhang *et al*[24] found that the difference in the abundance of *Bacteroidetes* was not significant between obese and normal people. A study comparing the gut microbiota of obese and healthy people based on the public database of the intestinal program in the United States analyzed the gut microbiota of 1655 healthy and 898 obese adults and found that the ratio of Firmicutes/Bacteroidetes in obese people was relatively low[25]. Therefore, more research is needed to explore the relationship between the gut microbiota and obesity.

Other works have associated obesity with specific bacteria, such as the family Christensenellaceae and the genera *Methanobacteriales*, *Lactobacillus*, *Bifidobacteria*, and *Akkermansia*. Recently, the family Christensenellaceae was found to be associated with weight loss, and its relative abundance was inversely related to host BMI[26]. *Akkermansia muciniphila* is a key bacterium for weight loss. Supplementation with *A. muciniphila* improves metabolic parameters in overweight and obese subjects[27]. *Lactobacillus* and *Bifidobacterium* are traditional probiotics that play an important role in the balance of the human intestinal microecology. Crovesy *et al*[28] summarized the effect of *Lactobacillus* on body weight in overweight subjects and found that the beneficial effects were species specific. The abundance of *Lactobacillus paracasei* (*L. paracasei*) was negatively correlated with obesity, while the abundances of *L. reuteri* and *L. gasseri* were significantly correlated with obesity. At present, evidence of *Bifidobacterium* resistance to obesity has been obtained from animal experiments. Following administration of *Bifidobacterium* in animal models of diet-induced obesity, it also showed a strain-dependent effect on obesity[29]. However, reduced *Bifidobacterium* abundance in the gut is associated with obesity[30]. Million *et al*[31] found that *M. smithii* and *B. animalis* were associated with normal weight, while *L. reuteri* was associated with obesity. These findings suggest that obesity-related microorganisms are species specific and that bacteria in the same genus may have opposite effects, which may be related to the complex metabolic mechanism of obesity.

The persons with obesity can be divided into two different types: Subcutaneous obesity and visceral obesity. As mentioned above, an elevated Firmicutes/Bacteroidetes ratio is a biomarker for obesity. However, the relative abundance of Firmutes in morbid obesity is positively correlated with brown adipocytes markers in subcutaneous adipose tissue, but not in visceral adipose tissue[32]. The browning of the white adipose tissue is conducive to maintaining a relatively healthy obesity phenotype, suggesting that the higher relative abundance of Firmutes may be beneficial for subcutaneous obesity. In an experiment of rapid weight loss through a very low calorie diet in obese postmenopausal women, a decrease abundance of *Roseburia* and increased *Christensenellaceae* (unknown genus) were found to be

positively correlated with several gene expression pathways, such as protein-amino acid N-glycation, in subcutaneous adipose tissue[33]. Probiotics play a positive role in visceral obesity. Increased *Akkermansia* population reduced body weight and visceral obesity in mice fed a high-fat/high-sugar diet[34]. The probiotic mixture including *L. acidophilus*, *L. rhamnosus*, *L. paracasei*, *Pediococcus pentosaceus*, *B. lactis*, and *B. breve* significantly reduced BMI and intrahepatic fat fraction in patients with nonalcoholic fatty liver disease after 12 wk of administration. Except *L. paracasei*, the abundance of the other five probiotics increased in the gut of the probiotic mixture group[35]. Tavella *et al*[36] proposed that the relevance of large numbers of *Christensenellaceae*, *Porphyromonadaceae* and *Rikenellaceae* in the elderly gut microbiota contributed to the reduction of visceral adipose tissue. Due to the lack of studies on gut microbiota specifically targeting subcutaneous obesity and visceral obesity, further studies are needed to determine whether there are significant differences in gut microbiota characteristics between patients with subcutaneous obesity and visceral obesity.

From the above analysis, the role of microorganisms in obesity is strain specific, as there are both beneficial and harmful bacteria within the same taxon. Therefore, it is difficult to classify obesity-related bacterial communities according to their taxonomic relationships. Recent studies have introduced the concept of a 'guild' into the study of the gut microbiota, defining a group of microorganisms that utilize similar resources or perform the same biological function as the same guild to identify potential clusters associated with specific disease phenotypes and identify candidate gut microbes that may contribute to human health and disease[37]. Using a clinical study of genetically obese children, the researchers found that the abundance of one strain of *E. coli* increased sharply after 30 d of eating a diet rich in nondigested carbohydrates, while the abundance levels of the other four strains decreased[37]. Therefore, guilds, as an important form of intermember organization in ecology, can better reflect the changes in specific responses of strains.

The diversity of the gut microbiota is another important factor related to obesity. Most studies have shown that the diversity and richness of the gut microbiome are reduced in obese subjects[38-40]. Wu *et al*[25] found that the α-diversity of obese people was significantly lower than that of healthy people, but there was no significant difference in the structure of the gut microbiota (β-diversity) between them. However, some studies believed that there was not necessarily a correlation between the diversity of the gut microbiota and diseases[41]. Strict statistical analysis showed that only in approximately 1/3 of the cases was there a significant relationship between bacterial diversity and "microbiota-related diseases". Since bacterial ecosystems may possess a considerable degree of stability (including resistance to disease), the impression seems to be that bacterial diversity is closely related to disease occurrence and progression[41]. Obesity, as a flora-related disease without specific pathogens, may be directly related to the dysbiosis of the bacterial ecosystem. Therefore, in the face of the dilemma of diversity research, ecological theory is needed for guidance. The association between gut microbiomes and obesity has been summarized in Table 1.

OBESITY MECHANISMS INDUCED BY THE GUT MICROBIOTA

Energy absorption

Studies have found that genetically obese mice consume more carbohydrates and protein through the gut microbiota to provide energy for the host[42,43]. In animal experiments, it was found that in the case of no differences in diet or weight of the mice, the total body fat of the germ-free mice colonized by the 'obese microbiota' increased significantly compared with those colonized by the 'lean microbiota'[21]. This finding indicates that the obese-type gut microbiota has an increased ability to absorb energy from the diet. Multiomics analysis showed increased lipid absorption in obese hosts. Colonization of *Clostridia* in germ-free mice downregulated the genes that control lipid absorption[44]. Therefore, the gut microbiota of obese patients can promote the absorption of energy, resulting in excessive accumulation of energy and increased weight gain.

The gut microbiota ferments hard-to-digest carbohydrates into short-chain fatty acids (SCFAs), which are either absorbed by the gut or excreted in feces. SCFAs are crucial for energy homeostasis regulation[45]. SCFAs are composed mainly of acetate, propionate, and butyrate. Acetate can produce beneficial effects on the energy metabolism of the host by secreting glucagon-like peptide-1, peptide YY, and other intestinal hormones, reduce systemic lipolysis and proinflammatory cytokine levels, and increase energy consumption and lipid oxidation[46]. Propionate promotes

Table 1 Association between gut microbiomes and obesity

Microbiota characteristics in obesity	Preclinical or clinical	Study subjects	Ref.
Firmicutes/Bacteroidetes ratio increased	Preclinical	Mice	Ley *et al*[20], Turnbaugh *et al*[21]
	Clinical	Childhood	Indiani *et al*[22]
	Clinical	Adult ukrainian population	Koliada *et al*[23]
The relative abundance of Christensenellaceae was inversely related to host BMI	Clinical	Human	Waters *et al*[26]
	Clinical	Postmenopausal women	Alemán *et al*[33]
	Clinical	Italian elderly	Tavella *et al*[36]
Increased *Akkermansia* population reduced body weight	Clinical	Human	Depommier *et al*[27]
	Preclinical	Mice	Anhê*et al*[34]
Lactobacillus paracasei decreased, while *Lactobacillus reuteri* and *Lactobacillus gasseri* increased	Clinical	Human	Crovesy *et al*[28], Million *et al*[31]
Bifidobacteria reduced	Preclinical	Rats	Waldram *et al*[30]
Methanobacteriales smithii and *Bifidobacterium* were associated with normal weight	Clinical	Human	Million *et al*[31]

BMI: Body mass index.

intestinal lipolysis and energy homeostasis in mice through the AMPK/LSD1 pathway [47]. Butyrate is the colon's main energy source, and intestinal epithelial cells derive most of their energy from the oxidation of butyrate. The increase in butyrate-producing bacteria in the gut microbiota increases the production of butyrate, thereby improving lipid metabolism through the butyrate-SESN2/CRTC2 pathway[48]. However, excessive butyrate may reduce the proportion of probiotics and reverse the metabolic effects[48]. In recent years, SCFAs have attracted special attention due to their beneficial effects on intestinal homeostasis and energy metabolism, but their role in obesity remains controversial. Higher concentrations of fecal SCFAs are associated with gut permeability, metabolic disorder markers, obesity, and hypertension[49]. Teixeira *et al*[50] found that fecal SCFAs in women were positively correlated with obesity, waist circumference, and other indicators of metabolic syndrome. Overall, SCFAs seem to be a double-edged sword. Although they can protect the host from diet-induced obesity, excessive SCFAs provide extra energy for the host, thus promoting obesity.

Central appetite

In recent years, the microbiota has emerged as one of the key regulators of gut-brain function. This gut-brain axis has received increasing attention in the study of the biological and physiological bases of obesity and its related diseases. The microbiota and the brain communicate with each other through a variety of pathways, including endocrine, immune, and neural pathways[51]. The gut microbiota affects the host's central nervous system through the gut-brain axis. The central nervous system can also affect the composition and structure of the gut microbiota. The gut microbiota influences food intake by regulating brain function in a number of ways, such as by contributing to the production of neuromodulators, such as serotonin, which plays an important role in regulating gastrointestinal function[52]. Lactate produced by *Lactobacillus* and *Bifidobacterium*, which acts as a substrate for neuron cells, can prolong satiety after a meal[53]. The gut microbiota also participates in the gut-brain axis by regulating gut hormones secreted by enteroendocrine cells. Peptide YY, pancreatic polypeptide, and glucagon-like peptide-1 (GLP-1) are gut-brain peptides that play important roles in information communication of the gut-brain axis. Peptide YY and pancreatic polypeptide are anorexia hormones secreted by the gut. GLP-1 lowers glucagon levels, slows gastric emptying, stimulates insulin synthesis, and reduces food intake [54,55]. Studies have found that the levels of peptide YY and GLP-1 in obese patients are significantly decreased. However, after fat reduction surgery, the levels of peptide YY and GLP-1 gradually increase[56]. Schéle *et al*[57] compared the gene expression

levels of food intake-regulating neuropeptides in germ-free and conventionally raised mice and found that the gut microbiota decreased the expression of two genes encoding the body fat-inhibiting neuropeptides Gcg and Bdnf, which may contribute to the induction of fat mass by the gut microbiota. The gut microbiota and its metabolites have been shown to stimulate gut satiety. This short-term regulation of gut satiety, which is associated with bacterial growth, can be combined with long-term appetite regulation controlled by neuropeptide energy circuits in the hypothalamus[58].

Fat storage

In 2004, researchers reported the ability of the gut microbiota to regulate fat storage [19]. The gut microbiota increases the absorption of glucose in the host's intestine and the content of glucose in the serum, thereby increasing the expression levels of two basic transcription factors, ChREBP (carbohydrate response element binding protein) and SREBP-1 (sterol regulatory element binding protein), which induce fat synthesis in the liver. Lipoprotein lipase (LPL) helps triglycerides enter the circulatory system from the liver, where they are absorbed by fat cells. Intestinal epithelial cells can produce Fiaf, an inhibitor of LPL. Fiaf is selectively inhibited in normal mouse intestinal epithelial cells, thereby increasing the host's energy storage. Aronsson *et al*[59] found that *L. paracasei* regulated ANGPTL4, a central player in fat storage regulation, using a mouse model with high-fat diet-induced obesity. *L. paracasei* could induce the expression of ANGPTL4, partly through the peroxisomal proliferator-activated receptors α and γ. ANGPTL4 inhibited LPL, leading to decreased fat storage. *L. paracasei*-colonized mice resisted high-fat diet-induced obesity[60]. *In vitro*, *L. paracasei* inhibits the Akt/mTOR pathway, indicating that several regulatory pathways are involved in different intracellular lipid accumulations mediated by *L. paracasei*.

Chronic inflammation

Chronic inflammation is one of the characteristics of metabolic disorders such as obesity[61]. Evidence shows that these disorders are characterized by the gut microbiota and its metabolites crossing the intestinal barrier, affecting various metabolic organs, such as the liver and adipose tissue, leading to chronic inflammation[62]. Through fecal microbiota transplantation, the microbiota of normal mice was transplanted to chronic colitis mice. Fecal microbiota transplantation could reduce colitis in mice with chronic intestinal inflammation by regulating the expression of proinflammatory genes, antimicrobial peptides and mucin[63]. Lipopolysaccharide (LPS), an endotoxin, has been shown to be expressed at elevated levels in obesity and adipose tissue inflammation[64]. LPS binds to Toll-like receptor 4 on immune cells, thereby activating a proinflammatory cascade in the gut[65]. Studies have shown that increased levels of butyrate-producing Ruminococcaceae and Lachnospiraceae lead to reduced levels of members of the LPS family S247, thereby reducing chronic low-grade inflammation[66]. The gut microbiota can also help prevent high-fat diet-induced intestinal barrier dysfunction by inhibiting cannabinoid receptor type 1[66]. The endocannabinoid system is a major regulator of fat synthesis in the gut and fat cells, and its activation can increase appetite and food intake. In addition, the effects of the consumption of antibiotics on the microbiota are sufficient to block the obesity protection phenotype, further indicating the roles of the microbiota in chronic inflammation and obesity.

SCFAs play a key role in the interaction between the gut microbiota and the activation or inhibition of the inflammatory cascade. Butyrate is an anti-inflammatory metabolite known to inhibit pathways leading to the production of pro-inflammatory cytokines[67]. Through epigenetic interactions, butyrate stimulates adipoliolysis and mitochondrial oxidative phosphorylation, thereby achieving greater energy consumption and preventing obesity[68]. As stated above, butyrate has also been shown to reduce LPS in the gut, thereby reducing LPS-related effects. Another SCFA, acetate, has a more controversial role in chronic inflammation and obesity. On the one hand, acetate can be used as a substrate for cholesterol synthesis, thus helping to raise serum cholesterol levels, which can increase the risk of obesity[69]. On the other hand, acetate has been reported to suppress appetite and reduce the risk of obesity[70]. Acetate is an important metabolite, and its role in the regulation of the gut microbiota and obesity is quite complicated. Therefore, further research is needed to clarify the exact mechanisms of the interactions between SCFAs and chronic inflammation, microbiota composition, and obesity.

Circadian rhythm

The gut microbiota has been shown to influence host circadian rhythms in a diet-

dependent manner[71]. The interruption of the circadian rhythm may lead to an increase in the incidence of obesity[72]. Microorganisms regulate lipid uptake and storage by regulating the circadian transcription factor NFIL3. The study also found that the ILC3-STAT3 signaling pathway is a key molecular pathway for the interaction between the microbiota and the circadian clock. A recent study has shown that the gut microbiota programs rhythmic histone acetylation through HDAC3 (histone deacetylase 3) expression in intestinal epithelial cells so that *Cd36* (lipid transporter gene) transcription becomes rhythmic, which promotes lipid absorption and obesity[73]. The recruitment of histone modifiers to chromatin is a key mechanism for generating rhythm. Therefore, the gut microbiota regulates the circadian rhythm through HDCA3. Feeding rhythms are considered to be a potential regulator of circadian rhythms and the gut microbiota. Studies have shown that time-restricted feeding reduces the harmful effects of a high-fat diet by regulating the circadian rhythm of the gut microbiota[74,75]. The feces of individuals experiencing jet lag and individuals with regular schedules were transplanted into germ-free mice. As a result, the transplanted mice that experienced jet lag developed obesity and insulin resistance. Due to impaired feeding rhythms, jet lag induces microbiota dysbiosis, promoting glucose intolerance and obesity[76].

Metabolites of the gut microbiota can also affect the host rhythm system. Bile metabolism is an example of the rhythmic interaction between host and gut microbes. Microbial bile salt hydrolases are related to the regulation of the circadian clock and lipid metabolism-related genes[77]. Gut bacteria such as Lachnospiraceae, Clostridiaceae, Ruminococcaceae, *Lactobacillus*, *Bacteroides*, and *Bifidobacterium* play a role in the biotransformation of bile salts[78]. SCFAs directly regulate the expression of clock genes in liver cells. Treatment with acetate or butyrate regulated the expression of the clock genes *Per2* and *Bmal1* in hepatic cells synchronized with serum shock[71]. Another study suggested that SCFAs induce indirect regulation of the internal circadian clock because no phase change in the peripheral circadian clock was detected in cultured fibroblasts or cultured liver slices[79].

The mechanism of obesity induced by the gut microbiota is summarized in Table 2. Dysbiosis of the microbiota causes metabolic disorders and promotes the occurrence and development of obesity through the direct interaction between the microbiota and local tissues or the indirect interaction between metabolites and remote organs (Figure 1). Although we can often detect specific different microorganisms between obese and normal individuals and verify the role of the bacteria in obesity through germ-free mouse experiments, more attention should be paid to ecological theory applications in relation to the gut microbiota as a group. The gut microbiota affects appetite, energy absorption, fat storage, circadian rhythm, and chronic inflammation, leading to obesity. Therefore, targeted reconstruction of the gut microbiota structure, such as through fecal bacterial transplantation, is one of the means of treating obesity.

MICROBIOTA IN OBESITY: LINKS WITH GENETICS AND TRANSMISSION

Obesity is the result of interactions between genetic and environmental factors. It is not surprising that host genetics shapes the gut microbiota. In fact, several genetic variations explain differences in the composition and diversity of the gut microbiota in obese people. Whole-genome association was used to determine the relationship between the genetic variation of twins and different species of bacteria. More than 12 health-related gut microorganisms were identified[80]. These microorganisms are environment-acquired, but genes also have a certain impact on them, such as the associations between *Bifidobacterium* and the lactase gene locus[80] and AMY1-CN (CN variation of the AMY1 Locus, which encodes salivary amylase) as a genetic factor related to the composition and function of the microbiome[81]. The abundance of resistant starch-degrading microbes in the gut microbiota of high-AMY1-CN subjects increased, which resulted in a higher incidence of obesity after the microbes were transferred to germ-free mice[81]. In another study, host kinship indicated the structural similarity of the gut microbiota in wild house mice. The exon sequencing results of host genes were compared with the microbiota structure, and 20 host genes were found to be related to the diversity or abundance of the gut microbiota, including a cytokine IL12A gene with three nonconsensus mutations. Among the 20 related genes, there are a large number of homologous genes of human-microbiota interactions, including genes related to immune regulation and obesity[82]. Furthermore, a human genome-wide association analysis revealed that there was an association between an obesity-related genus (*Akkermansia*) and a variant near the phospholipase

Table 2 Mechanism of obesity induced by gut microbiota

Effect	Microbiota characteristics	Mechanism	Ref.
Increased energy absorption	Expansion of *Desulfovibrio* and loss of Clostridia	Elevated the expression of genes that control lipid absorption such as CD36	Petersen *et al* [44]
Extra energy for the host	The inverse association between fecal SCFAs and gut microbiota diversity; Faecalibacterium prausnitzii, Roseburia faecis, and other Clostridiales increased; Akkermansia muciniphila, Alistipes finegoldii, Bacteroides, Christensenellaceae, Methanobrevibacter, and Oscillospira decreased	Excessive SCFAs	de la Cuesta-Zuluaga *et al* [49]
Increased appetite	A community dominated by members of the Clostridial clusters XIVa and IV	The levels of peptide YY and GLP-1 in obese patients decrease significantly	Wu *et al*[54], Salehi *et al* [55], Federico *et al*[56]
Decreased Fat storage	Germ free mice colonized with *Lactobacillus paracasei*	Increase the expression of ANGPTL4, and inhibit LPL, leading to decreased fat storage	Aronsson *et al* [59], Tazi *et al* [60]
Increased fat storage	Transplanting gut microbes from conventionally raised mice into germ-free mice	Increasing the expression of ChREBP and SREBP-1, Fiaf is inhibited, activate LPL, help triglycerides enter the circulatory system from the liver	Bäckhed *et al* [19]
Decreased chronic inflammation	Increase levels in the butyrate-producing bacteria such as Ruminococcaceae and Lachnospiraceae	Inhibit pathways leading to the production of pro-inflammatory cytokines; Stimulate adipoliolysis and mitochondrial oxidative phosphorylation, thereby achieving greater energy consumption; Reduce LPS, thereby reducing chronic low-grade inflammation	Kang *et al*[66], Lührs *et al*[67], Jia *et al*[68]
Interruption of circadian rhythm	Bile salts biotransformation bacteria such as Lachnospiraceae, Clostridiaceae, Ruminococcaceae, Lactobacillus, Bacteroides, and Bifidobacterium	Regulate transcription of key genes involved in circadian rhythm (*Dbp, Per1/2*) and lipid metabolism (*Pparγ, Angptl4*)	Joyce *et al*[77], Parkar *et al*[78]

SCFAs: Short-chain fatty acids; LPL: Lipoprotein lipase; LPS: Lipopolysaccharide.

D1 gene, which is related to BMI[83].

In addition, the gut microbiota can be transmitted from mother to child. In a passage experiment with germ-free mice, it was found that the gut microbiota of these mice was very stable. These microbiota types accounted for the majority of the gut microbiota of the mice in the normal environment, which proved that most of the gut microbiota of mice came from vertical transmission from the mother[84]. Studies have shown that the microbiota exists in the placenta, amniotic fluid, umbilical cord blood, and meconium, and maternal microorganisms may play an important role in the establishment of the child's microbiota[85]. Therefore, maternal obesity during pregnancy is accompanied by dysbiosis of the gut microbiota and metabolic disorders, and the maternal microbiota is passed on to the child, which can lead to metabolic disorders in the child. Obesity-related bacteria are present in the placenta and amniotic fluid in obese mothers. The fetus swallows these bacteria, resulting in gut microbiota colonization in the uterus. Studies have confirmed that maternal obesity is related to dysbiosis of the gut microbiota in offspring[86]. Therefore, the increased risk of obesity in children with obese mothers can be partly explained by the spread of gut microbes from obese mothers to their offspring.

MICROBIOTA IN OBESITY: LINKS WITH ENVIRONMENTAL FACTORS

Although genes play important roles in the gut microbiota, environmental factors have a more significant impact. A study of 1000 Israelis from all over the world who have very similar eating habits and lifestyles found that their ancestry was not related to the microbiome. The overall heritability of the microbiome may be less than 2%, and more than 20% of the variation in the microbiome is related to diet, drugs, and anthropometric measurements[87].

Diet is one of the most important factors inducing obesity. The eating habits of developed countries and regions have gradually become characterized by high fat and high sugar consumption, which has contributed to the gradual increase in obesity. Gut microbes depend on the host diet to survive and harvest energy, and dietary changes

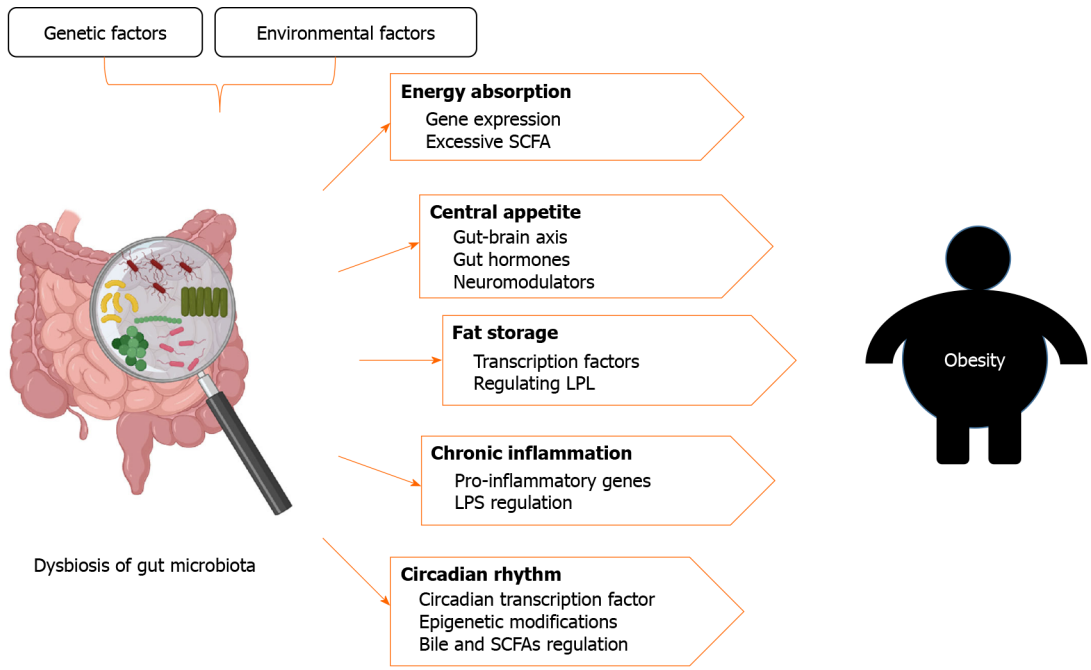

Figure 1 Gut microbiota and obesity. Both genetic and environmental factors can cause dysbiosis of the gut microbiota. Dysbiosis can increase energy absorption through changes in gene expression and excessive accumulation of short-chain fatty acids (SCFAs); improve central appetite through gut-brain axis, gut hormones, and neuromodulators; regulate fat storage through transcription factors and lipoprotein lipase; cause chronic inflammation through regulation of inflammatory gene expression and lipopolysaccharide; and disrupt the circadian rhythum by affecting the circadian transcription factors, epigenetic modifications, and the synthesis of bile and SCFAs. These factors appear to increase susceptibility to obesity. The figure was created with BioRender.com. SCFA: Short-chain fatty acids; LPL: Lipoprotein lipase; LPS: Lipopolysaccharide.

have a great impact on the gut microbiota. For example, the abundance of *Bacteroidetes* decreased while that of *Firmicutes* and *Proteobacteria* increased in mice fed a high-fat diet. These changes were observed in mice resistant to weight gain, suggesting that dietary fat has a direct effect on the microbiome[88,89]. Sleep disturbance is another cause of obesity. Lack of sleep leads to disrupted circadian rhythms, which can affect the gut microbiome and contribute to obesity. Chronic sleep fragmentation resulted in increased food intake and reversible changes in the gut microbiota, with increases in the abundance levels of Lactobacillaceae and *Ruminococcus* and a decrease in the abundance of Lactobacillaceae. These factors lead to systemic and visceral white adipose tissue inflammation and changes in insulin sensitivity[90]. Stress activates genes that affect metabolism and promotes the consumption of sweet and fatty foods, thus increasing appetite and contributing to obesity[91]. Stress significantly affects the microbiota-gut-brain axis at all stages of life[51]. During stress, independent of diet, the α-diversity of the gut microbiota increased, 50% of identified genera changed, and the abundance of Bacteroides decreased, while the abundance levels of less dominant taxa increased[92]. In addition, unhealthy lifestyle choices (sedentary, lack of exercise), emotional disorders, and drugs can also contribute to obesity. In short, as a "migrant", the gut microbiota itself is obtained from the environment; thus, environmental factors have a greater impact on it and can affect the occurrence and development of obesity.

STUDY LIMITATIONS

Although there are many factors contributing to obesity, the link between gut microbiota and obesity is widely accepted. In this review, the relationship between gut microbiota and obesity and the mechanism of gut microbiota inducing obesity are provided. However, the researches on gut microbiota in obesity are limited by the underrepresentation of individuals. This drawback limits the generality of these findings. Because of the specificity of the strains, microbes from the same genus may have opposite effects on obesity, which partly explains some of the contradictory results. In addition, this review only discusses the occurrence and development of obesity from the perspective of gut microbiota, and does not make further analysis of the relevant treatment plan.

CONCLUSION

Dysbiosis of the gut microbiota has been shown to be closely linked to obesity. Many gut microorganisms have been identified to be related to obesity. They induce the occurrence and development of obesity by increasing host energy absorption, increasing central appetite, enhancing fat storage, contributing to chronic inflammation, and regulating circadian rhythms. Due to the complexity and diversity of the gut microbiota, the mechanism by which the gut microbiota induces obesity still needs to be further studied. Obesity is the result of a combination of genetic and environmental factors. Data analysis based on larger samples to clarify the mechanism of the association between the gut microbiota and obesity, functional group studies of ecological significance to identify potential pathogenic members of the gut microbiota associated with obesity, and specific microbiota management for obese individuals will be the focus of future research.

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ORIGINAL ARTICLE

Basic Study

Zinc oxide nanoparticles reduce the chemoresistance of gastric cancer by inhibiting autophagy

You-Han Miao, Li-Ping Mao, Xiao-Juan Cai, Xiao-Ying Mo, Qi-Qi Zhu, Fei-Tong Yang, Mei-Hua Wang

ORCID number: You-Han Miao 0000-0003-4001-7715; Li-Ping Mao 0000-0002-9219-3887; Xiao-Juan Cai 0000-0002-1345-2140; Xiao-Ying Mo 0000-0001-6635-6489; Qi-Qi Zhu 0000-0001-9525-2072; Fei-Tong Yang 0000-0003-0474-160X; Mei-Hua Wang 0000-0003-3939-6551.

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You-Han Miao, Li-Ping Mao, Xiao-Juan Cai, Xiao-Ying Mo, Qi-Qi Zhu, Fei-Tong Yang, Mei-Hua Wang, Department of Infectious Disease, Nantong Third People's Hospital, Nantong 226006, Jiangsu Province, China

Corresponding author: Mei-Hua Wang, PhD, Chief Physician, Department of Infectious Disease, Nantong Third People's Hospital, No. 60 Qingnian Middle Road, Nantong 226006, Jiangsu Province, China. huatsbfyy3617@163.com

Abstract

BACKGROUND

Gastric cancer (GC) is a common malignancy that results in a high rate of cancer-related mortality. Cisplatin (DDP)-based chemotherapy is the first-line clinical treatment for GC therapy, but chemotherapy resistance remains a severe clinical challenge. Zinc oxide nanoparticle (ZnO-NP) has been identified as a promising anti-cancer agent, but the function of ZnO-NP in GC development is still unclear.

AIM

To explore the effect of ZnO-NP on chemotherapy resistance during GC progression.

METHODS

ZnO-NP was synthesized, and the effect and underlying mechanisms of ZnO-NP on the malignant progression and chemotherapy resistance of GC cells were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, colony formation assays, transwell assays, wound healing assays, flow cytometry, and Western blot analysis in GC cells and DDP-resistant GC cells, and by tumorigenicity analyses in nude mice.

RESULTS

Our data revealed that ZnO-NP was able to inhibit proliferation, migration, and invasion and induce apoptosis of GC cells. Meanwhile, ZnO-NP significantly reduced the half maximal inhibitory concentration (IC_{50}) of DDP for the inhibition of cell proliferation of DDP-resistant SGC7901/DDP cell lines. Autophagy was increased in DDP-resistant GC cells, as demonstrated by elevated light chain 3-like protein 2 (LC3II)/LC3I and Beclin-1 expression and repressed p62 expression in SGC7901/DDP cells compared to SGC7901 cells. Mechanically, ZnO-NP inhibited autophagy in GC cells and treatment with DDP induced autophagy, which was reversed by ZnO-NP. Functionally, ZnO-NP attenuated the tumor

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growth of DDP-resistant GC cells *in vivo*.

CONCLUSION

We conclude that ZnO-NP alleviates the chemoresistance of GC cells by inhibiting autophagy. Our findings present novel insights into the mechanism by which ZnO-NP regulates the chemotherapy resistance of GC. ZnO-NP may serve as a potential therapeutic candidate for GC treatment. The potential role of ZnO-NP in the clinical treatment of GC needs clarification in future investigations.

Key Words: Gastric cancer; Progression; Chemoresistance; Zinc oxide nanoparticle; Autophagy; MTT assays

Core Tip: We show that zinc oxide nanoparticle (ZnO-NP) reduces the chemoresistance of gastric cancer (GC) cells by inhibiting autophagy. Our findings provide innovative insights into the scenario in which ZnO-NP mediates chemotherapy resistance in GC. ZnO-NP may serve as a potential therapeutic candidate for GC treatment.

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INTRODUCTION

Gastric cancer (GC) is the second most common cause of cancer-related mortality globally[1]. Chemotherapy is the preferred treatment for advanced-stage GC patients, in which oxaliplatin, 5-fluorouracil (5-FU), cisplatin (DDP) are first-line therapies[2-4]. Although advancements have been made in chemotherapy effectiveness, the survival rate remains unsatisfactory due to chemotherapy resistance[5,6], which significantly limits the efficiency of GC treatments[7]. The molecular mechanisms underlying the regulation of GC chemotherapy resistance are complicated and remain poorly understood[8]. Accordingly, therapeutic strategies for the attenuation of chemotherapy resistance are urgently needed.

Autophagy is a process in which cellular contents, such as dysfunctional organelles and large protein groups, are transported to lysosomes for degradation and reuse[9]. Autophagy sustains cellular homeostasis and limits cellular damage under multiple stresses[10]. Autophagy has dual roles in cancer progression[11]. In some cases, autophagy induces cancer cell survival by recovering intracellular contents and increasing energy generation to reach the high metabolic requirements of cancer cells. In other contexts, autophagy inhibits cell imbalance and damage to attenuate tumorigenesis[12]. Autophagy is closely associated with the chemotherapy resistance of GC cells, and inhibition of autophagy relieves chemoresistance[13,14]. Autophagy is correlated with cell differentiation and tumor development in GC[15]. Thus, autophagy-related factors may be promising prognostic indicators of advanced GC[16].

Nanoparticles (NPs) are currently applied in multiple biomedical fields including bone regeneration, wound healing, bio-imaging, and targeted-drug transmission systems[17-21]. The conversion of material to nanoscale regularly leads to changes in chemical, physical (electric and magnetic), morphological, and structural properties [19,21]. These modifications permit NPs to cooperate with different biomolecules to affect certain responses[19,21]. Due to the unique surface and size properties, NPs have numerous benefits that enable them to serve as potential anti-tumor therapeutics [22]. Among them, metal zinc oxide NPs (ZnO-NP) have various properties and are widely used as critical components of many biomedical and cosmetic applications including sunscreen, foot care, and ointments[23]. ZnO-NPs exhibit antibacterial activities[24,25], and are also extensively used in drug targeting due to their biocompatibility[26]. However, the effect of ZnO-NP on the chemotherapy resistance of GC

cells remains unknown.

In this study, we focused on the impact of ZnO-NP on chemotherapy resistance of GC cells. We revealed the innovative role of ZnO-NP in repressing chemoresistance and reducing GC progression *via* inhibition of autophagy.

MATERIALS AND METHODS

Cell culture and treatment

The SGC7901, BGC823, and SGC7901/DDP cell lines were maintained in the lab. The cells were incubated in an incubator of 5% CO_2 and 37 °C in RPMI 1640 medium (Hyclone, Logan, UT, United States) with fetal bovine serum (10%; Hyclone), streptomycin (0.1 mg/mL; Hyclone) and penicillin (100 units/mL; Hyclone). DDP was obtained (Sigma, St. Louis, MO, United States) and used at the indicated doses.

ZnO-NP synthesis

A total of 0.5 mol/L zinc nitrate was plated to 1 mol/L sodium hydroxide solution, followed by continuous stirring (15 min). The white precipitate formed was washed and centrifuged, followed by repeated distilling of H_2O. The collected white powder [Zn(OH)2] was dried in a hot air oven at 60 °C. During drying, Zn2[24] was entirely converted to ZnO. Then, the dried ZnO powder was annealed at 500 °C for 3 h to convert to ZnO-NP. To analyze the effect of ZnO-NP on the malignant progression and chemotherapy resistance, the GC cells were treated with ZnO-NP at a dose of 5 μg/mL.

MTT assay

Cell viability was assessed by MTT assays at the indicated times in 6-well dishes. Briefly, the MTT solution (Solarbio, Beijing, China) was added to the cells and cultured at 5% CO_2 and 37 °C for 4 h. Next, dimethyl sulfoxide (100 μL, 10 min; Sigma) was used to terminate the reaction. Cell viability was analyzed at an absorbance of 490 nm with a microplate reader (Thermo Fisher Scientific, Waltham, MA, United States).

Colony formation assays

About 1×10^3 cells were plated in 6-well dishes and cultured in RPMI 1640 medium at 5% CO_2 and 37 °C. After 2 wk, cells were washed with phosphate-buffered saline (PBS) for about 30 min and dyed with 1% crystal violet dye, after which the number of colonies was calculated.

Transwell assays

Transwell assays were conducted to analyze the invasion and migration of melanoma cells by using a Transwell plate (Corning, New York, NY, United States) according to the manufacturer's instructions. Briefly, the upper chambers were plated with about 1×10^5 cells. Then cells were fixed in 4% paraformaldehyde and dyed with crystal violet. The invaded and migrated cells were recorded and calculated.

Wound healing assay

Cells were plated in a 24-well plate at a density of 3×10^5 cells/well and cultured overnight to reach full confluence as a monolayer. A 20 μL pipette tip was applied to slowly cut a straight line across the well. Then the well was washed three times with PBS, and changed to serum-free medium, followed by continued culture. The wound healing percentage was calculated.

Analysis of cell apoptosis

Cell apoptosis was measured using the Annexin-V-Fluorescein Isothiocyanate Apoptosis kit (BD Biosciences, San Jose, CA, United States) based on flow cytometry analysis using the FACSCalibur flow cytometer, followed by quantification with FlowJo software.

Western blot analysis

Total proteins were extracted from the cells using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA, United States) and quantified using the BCA Protein Quantification Kit (Abbkine Scientific Co., Ltd., Palo Alto, CA, United States). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene fluoride membranes (Millipore,

Burlington, MA, United States), followed by incubation with 5% milk and with primary antibodies at 4 °C overnight. The membranes were incubated with the corresponding secondary antibodies (Boster Biotechnology, Wuhan, China) for 1 h at room temperature, followed by protein detection by chemiluminescence (Beyotime Biotechnology, Shanghai, China). The primary antibodies used in this study were against light chain 3B (LC3B), p62, Beclin-1, and β-actin (all from Abcam, Cambridge, MA, United States).

Analysis of tumorigenicity in nude mice

The tumor growth of GC cells *in vivo* was evaluated in Balb/c nude mice (4-week-old, male, n = 5). About 1 × 10^7 SGC7901/DDP cells were subcutaneously injected in the mice. After 5 d, we measured tumor growth every 5 d. We sacrificed the mice after 30 d, and tumors were scaled. Tumor volume (V) was determined by estimating the length (L) and width (W) with calipers and measured with the formula (L × W^2) × 0.5. Animal care and methods were authorized by the Animal Ethics Committee of Nantong Third People's Hospital (Jiangsu, China).

Statistical analyses

Data are presented as mean ± SD, and statistical analyses were performed with GraphPad Prism 7. The unpaired Student's t-test was applied for comparing two groups, and one-way analysis of variance was applied for comparing among multiple groups. $^aP < 0.05$ was considered statistically significant.

RESULTS

ZnO-NP inhibits proliferation and induces the apoptosis of GC cells

To investigate the effect of ZnO-NP on GC cells, SGC7901 and BGC823 cells were treated with ZnO-NP or an equal volume of saline. The cell viability was significantly inhibited by ZnO-NP treatment of the cells (Figure 1A and B). Consistently, ZnO-NP markedly reduced the colony numbers of SGC7901 and BGC823 cells (Figure 1C and D). Moreover, the apoptosis of SGC7901 and BGC823 cells was enhanced by treatment with ZnO-NP (Figure 1E and F), suggesting that ZnO-NP is able to inhibit proliferation and induce apoptosis of GC cells.

ZnO-NP reduces the invasion and migration of GC cells

Next, the role of ZnO-NP in regulating the invasion and migration was evaluated. Transwell assays revealed that the invasion and migration of BGC823 and SGC7901 cells were significantly attenuated upon treatment with ZnO-NP (Figure 2A and B). In addition, wound healing assays demonstrated that ZnO-NP markedly enhanced the wound proportion in SGC7901 and BGC823 cells (Figure 2C and D), indicating that ZnO-NP alleviates the migration and invasion of GC cells.

ZnO-NP attenuates the chemotherapy drug resistance of GC cells

We further explored the impact of ZnO-NP on the DDP of GC cells. Significantly, treatment with ZnO-NP notably reduced the IC_{50} value of DDP for inhibition of cell proliferation in DDP-resistant SGC7901/DDP cell lines (Figure 3A). Furthermore, DDP enhanced the apoptosis of SGC7901/DDP cells, which was markedly reinforced by ZnO-NP treatment (Figure 3B and C), suggesting that ZnO-NP attenuates the DDP resistance of GC cells.

Autophagy is increased in chemotherapy-resistant GC cells

Next, we were interested in the correlation of autophagy with the DDP resistance of GC cells. For this purpose, we analyzed the expression of autophagy markers including LC3B-II, LC3B-I, Beclin-1, and p62 in SGC7901 and SGC7901/DDP cell lines. Our data showed that the expression ratio of LC3II/LC3I and levels of Beclin-1 were elevated while p62 expression was inhibited in SGC7901/DDP cells compared with those in SGC7901 cells (Figure 4), indicating that autophagy is increased in DDP-resistant GC cells.

ZnO-NP inhibits autophagy in GC cells

We investigated the effect of ZnO-NP on autophagy in GC cells. We found that the treatment of ZnO-NP inhibited LC3II/LC3I and Beclin-1 levels but promoted p62 expression in SGC7901 and BGC823 cells (Figure 5A-H). Moreover, our data revealed

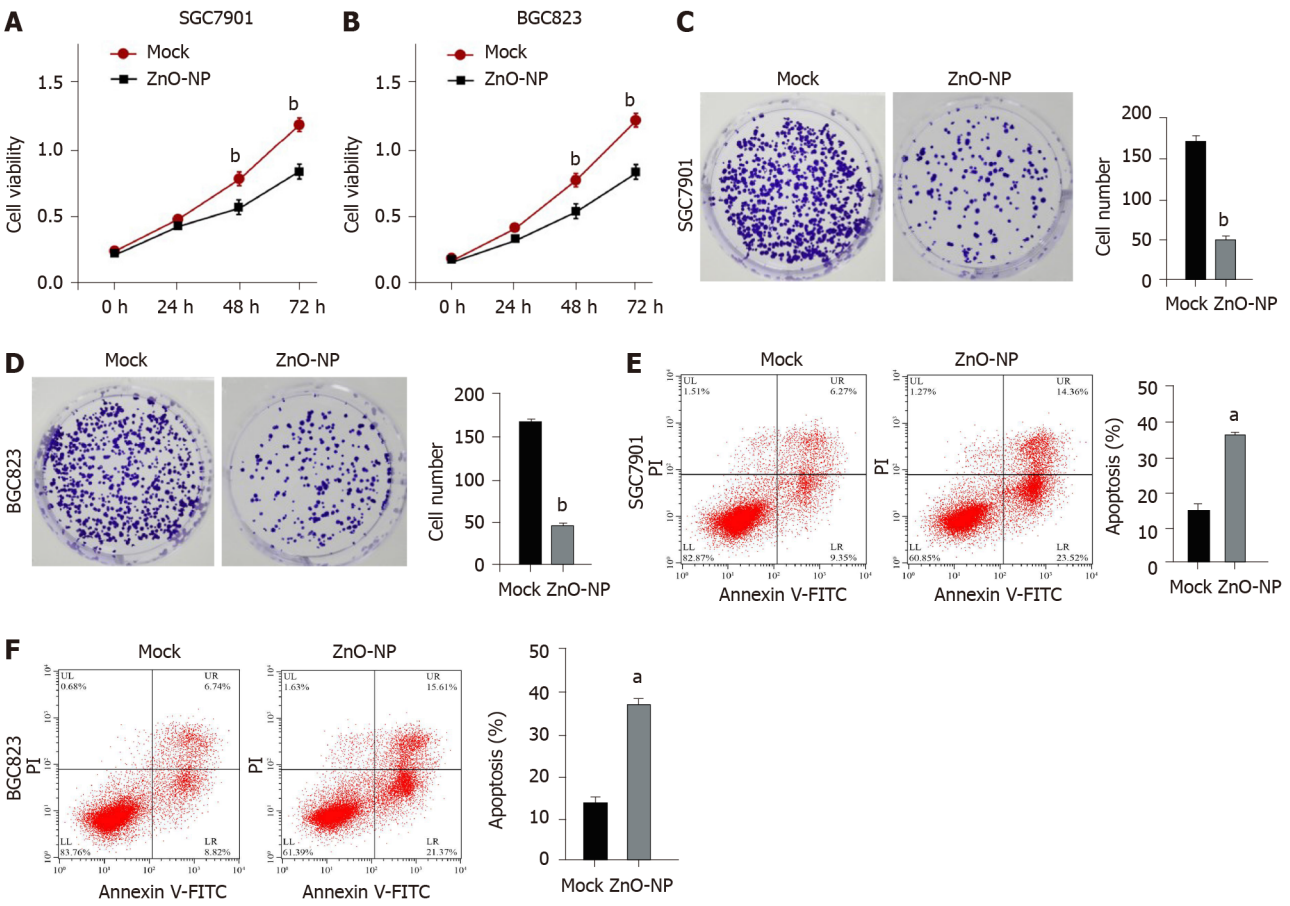


Figure 1 Zinc oxide nanoparticle inhibits proliferation and induces apoptosis of gastric cancer cells. SGC7901 and BGC823 cells were treated with the zinc oxide nanoparticle (ZnO-NP, 5 μg/mL) or an equal volume of saline. A and B: Cell viability was analyzed by the MTT assay; C and D: Cell proliferation was assessed by the colony formation assay; E and F: Cell apoptosis was measured by flow cytometry. Data are presented as the mean ± SD. Statistically significant differences are indicated: $^{a}P < 0.05$; $^{b}P < 0.01$. FITC: Fluorescein isothiocyanate.

that treatment with DDP induced the expression ratio of LC3II/LC3I and the levels of Beclin-1 and decreased the p62 expression in the cells; treatment with ZnO-NP reversed this effect (Figure 5I and J), indicating that ZnO-NP can inhibit autophagy in GC cells.

ZnO-NP attenuates chemotherapy drug resistance by inhibiting the autophagy of GC cells

We explored whether ZnO-NP modulated the DDP resistance of GC cells by regulating autophagy. Treatment with DDP reduced the viability of SGC7901 and BGC823 cells, while ZnO-NP or the autophagy inhibitor 3-methyladenine (3-MA) was able to further inhibit the phenotype (Figure 6A and B). Moreover, the cell apoptosis of SGC7901 and BGC823 cell lines was induced by DDP treatment, in which the treatment of ZnO-NP or 3-MA could reverse this effect in the cells (Figure 6C and D), suggesting that ZnO-NP attenuates chemotherapy drug resistance by inhibiting autophagy of GC cells.

ZnO-NP reduces the tumor growth of chemoresistant GC cells in vivo

Next, the effect of ZnO-NP on DDP-resistant GC cell growth *in vivo* was assessed by tumorigenicity analysis. The tumor growth of SGC7901/DDP cells was attenuated by ZnO-NP treatment of nude mice (Figure 7), indicating that ZnO-NP is able to reduce the tumor growth of chemoresistant GC cells *in vivo*.

DISCUSSION

The chemotherapy resistance of GC patients serves as a severe clinical challenge[1]. ZnO-NP has potential anti-tumor activities, but its role in modulating the chemo-

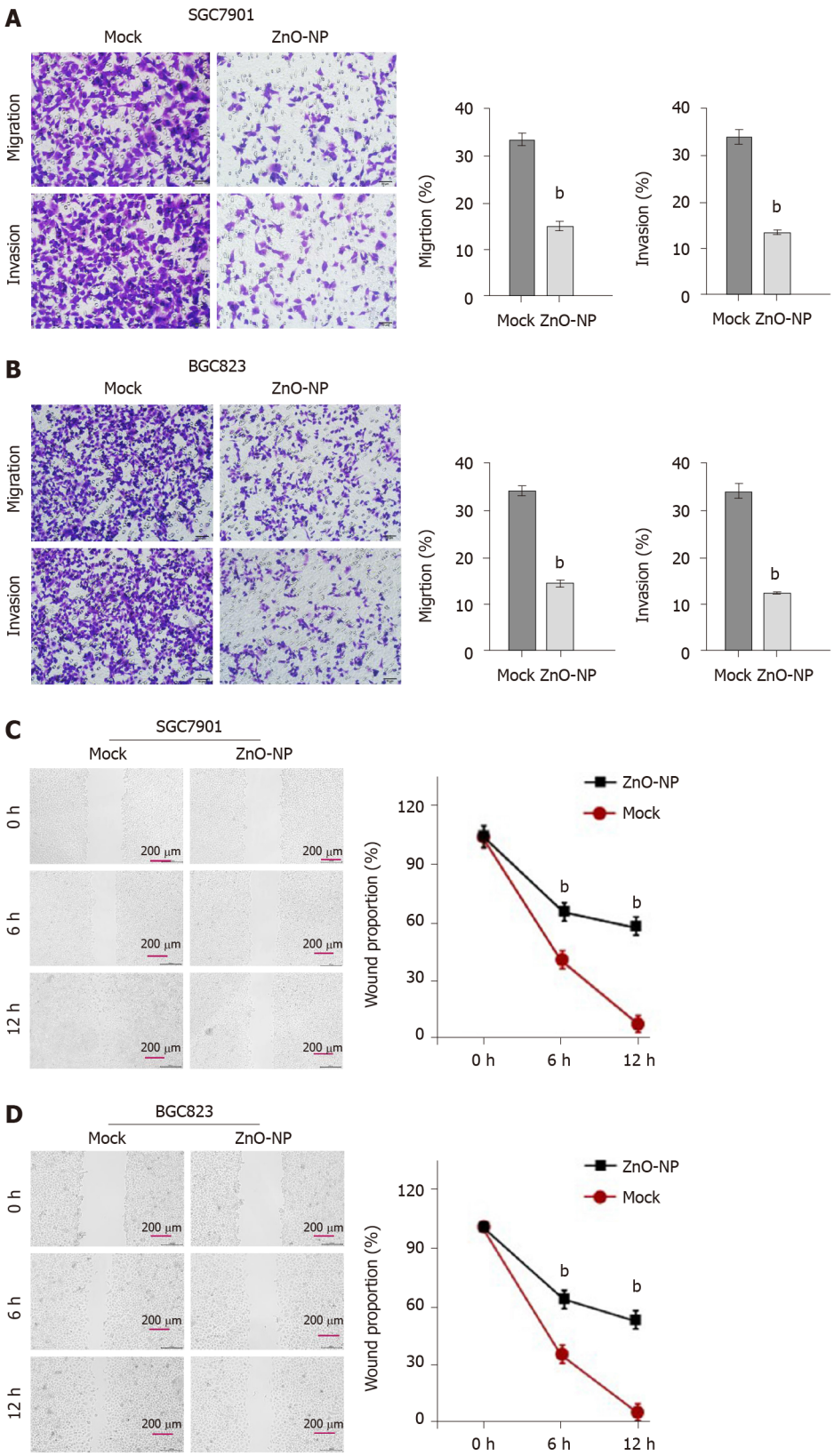
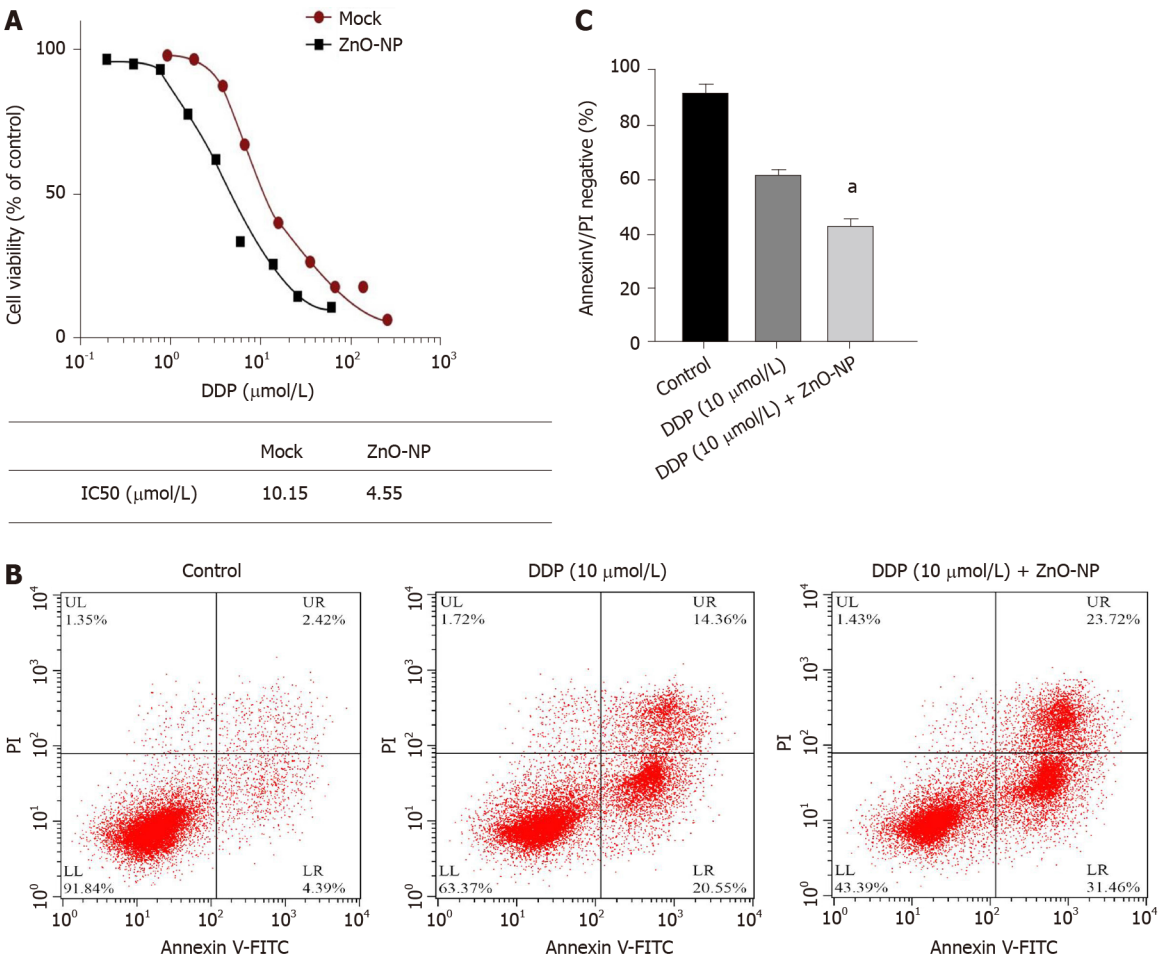


Figure 2 Zinc oxide nanoparticle reduces the invasion and migration of gastric cancer cells. SGC7901 and BGC823 cells were treated with zinc oxide nanoparticle (5 μg/mL, ZnO-NP) or an equal volume of saline. A and B: Cell migration and invasion were determined by transwell assays; C and D: Migration and invasion were examined by wound healing assays. The wound healing proportion is shown. Data are presented as the mean ± SD. Statistically significant differences are indicated: $^{b}P < 0.01$.

therapy resistance of GC cells is unclear. In this study, we found that ZnO-NP attenuated the chemoresistance of GC cells by inhibiting autophagy.



	Mock	ZnO-NP
IC50 (μmol/L)	10.15	4.55

Figure 3 Zinc oxide nanoparticle attenuates the chemotherapy drug resistance of gastric cancer cells. A: SGC7901/cisplatin (DDP) cells were treated with DDP at the indicated doses and treated with zinc oxide nanoparticle (ZnO-NP, 5 μg/mL) or an equal volume of saline. Cell viability was measured by the MTT assay; B and C: SGC7901/DDP cells were treated with DDP or co-treated with DDP and ZnO-NP (5 μg/mL). Cell apoptosis was assessed by flow cytometry. Data are presented as the mean ± SD. Statistically significant differences are indicated: ns, no significance, $^{a}P < 0.05$. FITC: Fluorescein isothiocyanate.

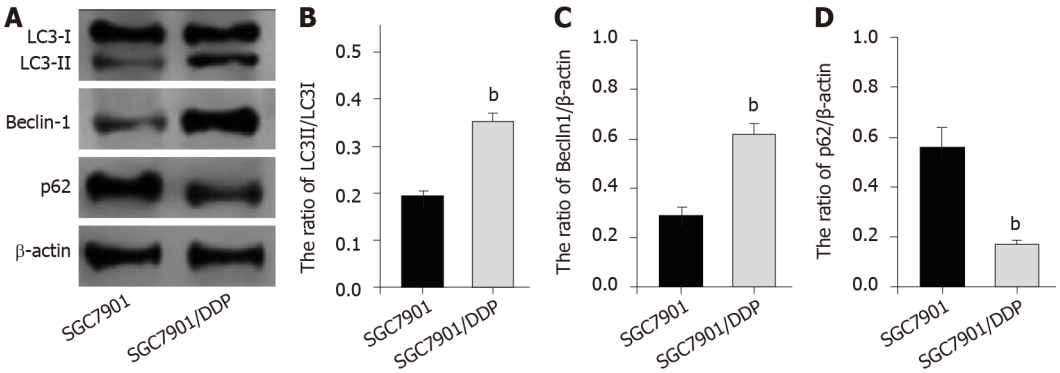


Figure 4 Autophagy is increased in chemotherapy-resistant gastric cancer cells. A: Expression of light chain 3B-II (LC3B-II), LC3B-I, Beclin-1, p62, and β-actin was measured by Western blot analysis in SGC7901 and SGC7901/cisplatin (DDP) cells; B: Ratio of LC3II/LC3I; C: Ratio of Beclin-1/β-actin; D: Ratio of p62/β-actin. The results of Western blot analysis were quantified by ImageJ software. Data are presented as the mean ± SD. Statistically significant differences are indicated: $^{b}P < 0.01$.

Previous studies have identified the cancer-inhibitory effect of ZnO-NP. ZnO-NP promotes proteotoxic and oxidative stress and induces the apoptosis of ovarian cancer cells in a p53-mutation-dependent manner[27]. ZnO-NP enhances the cell death of multiple human myelomas by regulating reactive oxygen species and cytochrome c/apoptotic protease activating factor 1/caspase-9 signaling[28]. ZnO-NP increases the apoptosis of human ovarian cancer cells[29]. Frizzled-7-targeted delivery of ZnO-NP

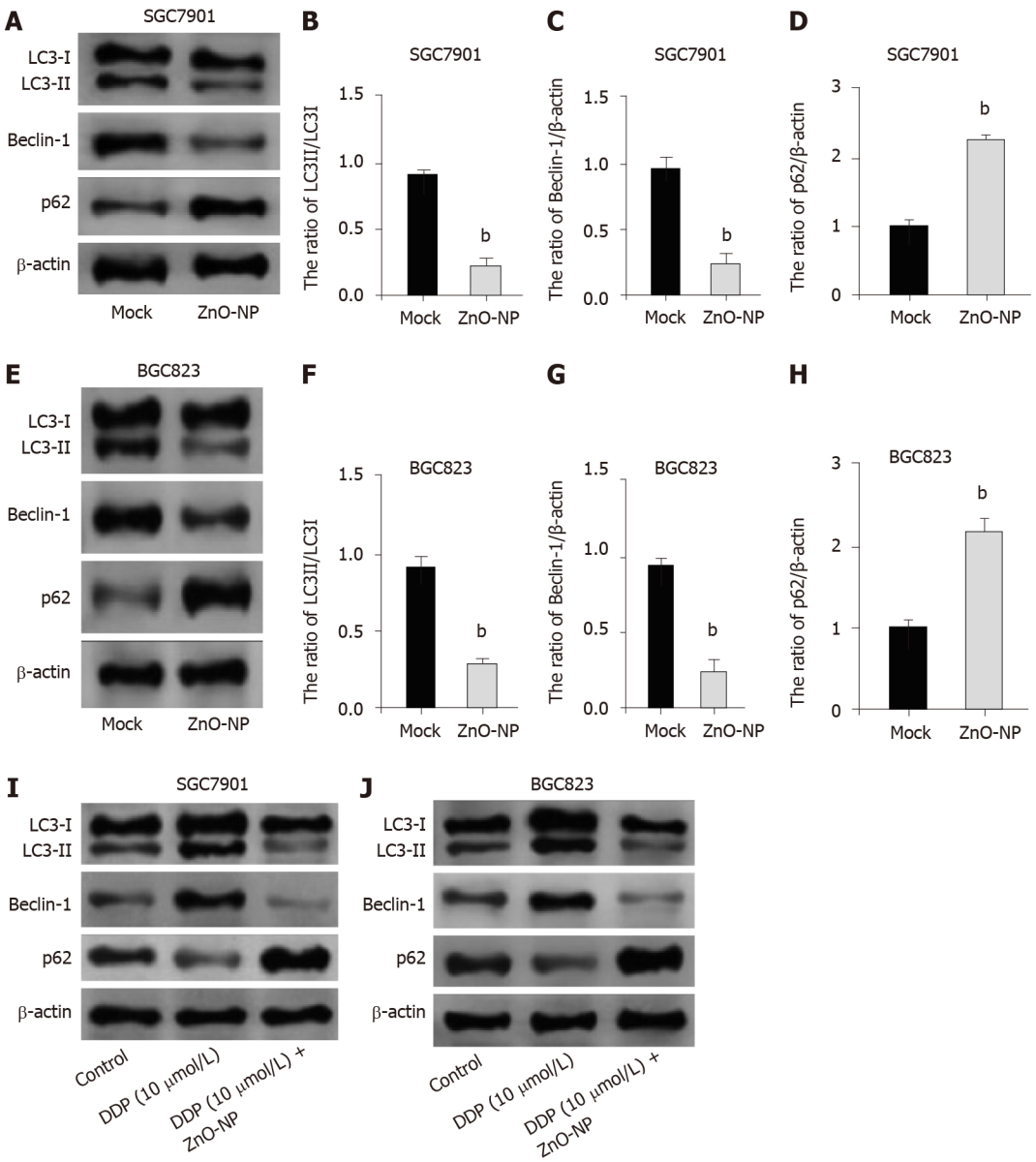


Figure 5 Zinc oxide nanoparticle inhibits autophagy in gastric cancer cells. A-H: SGC7901 and BGC823 cells were treated with zinc oxide nanoparticle (ZnO-NP, 5 μg/mL) or an equal volume of saline. The expression of light chain 3B-II (LC3B-II), LC3B-I, Beclin-1, p62, and β-actin was measured by Western blot analysis. The results of Western blot analysis were quantified by ImageJ software; I and J: SGC7901 and BGC823 cells were treated with cisplatin (DDP, 10 μmol/L) or co-treated with DDP (10 μmol/L) and ZnO-NP (5 μg/mL). The expression of LC3B-II, LC3B-I, Beclin-1, p62, and β-actin was analyzed by Western blot analysis. Data are presented as the mean ± SD. Statistically significant differences are indicated: $^{b}P < 0.01$.

induces inhibitory effects on the drug resistance of breast cancer cells[30]. Moreover, iron NPs reverse the chemotherapy resistance of GC cells[31]. In this study, we found that ZnO-NP inhibited proliferation, migration, and invasion and induced apoptosis of GC cells. Meanwhile, ZnO-NP attenuated the DDP resistance of GC cells. Moreover, ZnO-NP was able to repress the tumor growth of chemoresistance GC cells *in vivo*. Our findings indicate the innovative effect of ZnO-NP on the chemotherapy drug resistance of GC cells, demonstrating critical evidence of metal oxide NPs in the regulation of cancer development.

Furthermore, previous studies have identified that autophagy is clearly correlated with chemotherapy drug resistance and the development of GC, and targeting autophagy is involved in the modulation of chemoresistance GC cells. Long noncoding RNA MALAT1 modulates autophagy-related chemoresistance by targeting miR-23b-3p in GC[14]. Autophagy contributes to the chemoresistance of GC stem cells by regulating Notch signaling[32]. Tripartite motif containing 14 induces autophagy and chemotherapy resistance of GC cells *via* modulating adenosine monophosphate-activated protein kinase/mechanistic target of rapamycin (mTOR) signaling[33]. Cluster of differentiation 133 (CD133) inhibition reduces DDP resistance by repressing

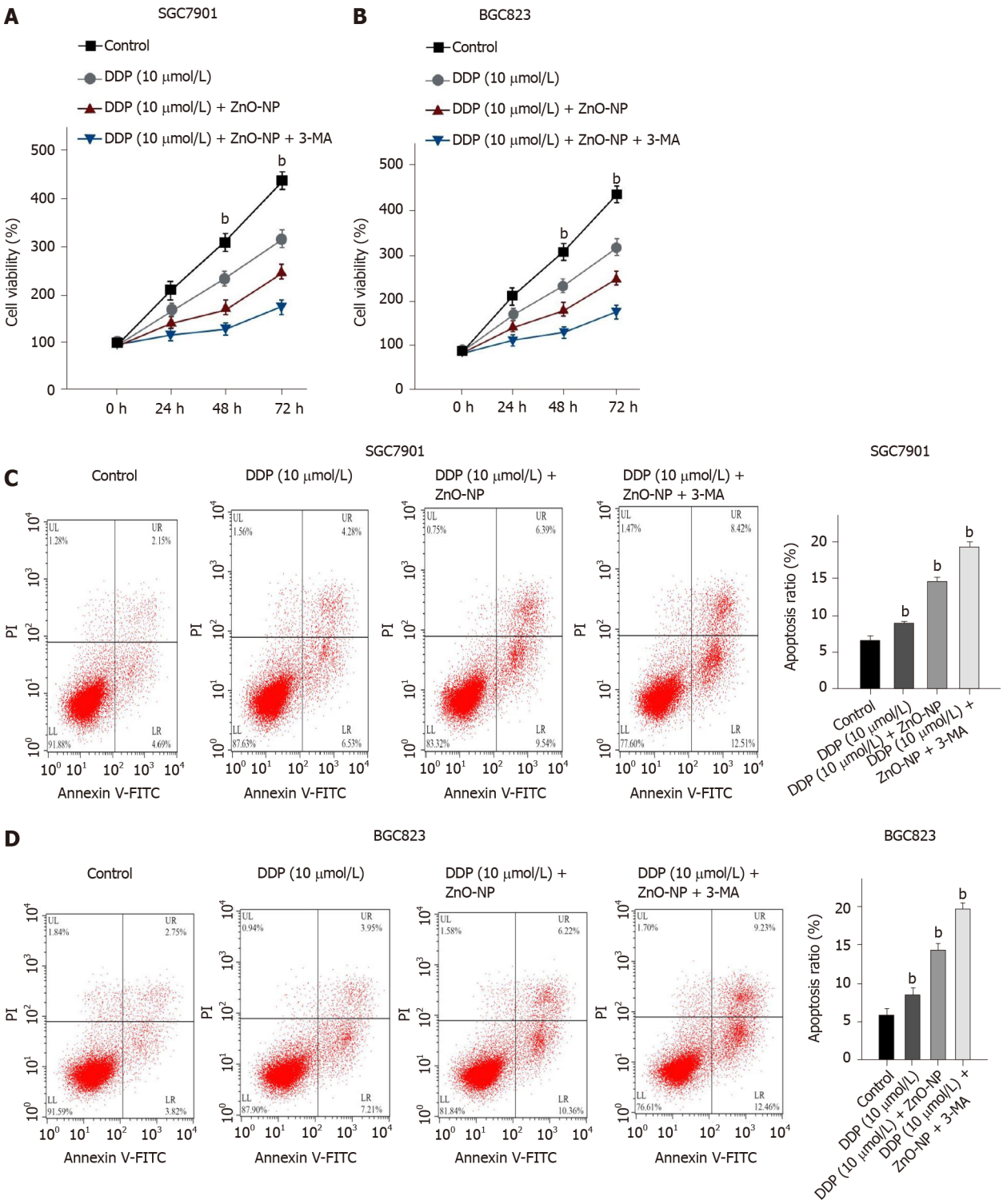


Figure 6 Zinc oxide nanoparticle attenuates chemotherapy drug resistance by inhibiting the autophagy of gastric cancer cells. SGC7901 and BGC823 cells were treated with cisplatin (DDP), DDP and zinc oxide nanoparticle (ZnO-NP, 5 μg/mL), co-treated with DDP, ZnO-NP (5 μg/mL) and 3-methyladenine (3-MA, 5 mmol/L). A and B: Cell viability was determined by the MTT assay; C and D: Cell apoptosis was analyzed by flow cytometry. Data are presented as the mean ± SD. Statistically significant differences are indicated: $^{b}P < 0.01$. FITC: Fluorescein isothiocyanate.

autophagy and phosphatidylinositol 3-kinase/AKT/mTOR signaling in CD133-positive GC cells[34].

CDGSH iron sulfur domain 2 improves the chemosensitivity of GC cells by enhancing 5-FU-promoted apoptosis and inhibiting autophagy through AKT/mTOR signaling[35]. In this study, our data showed that autophagy was increased in chemotherapy-resistant GC cells. Treatment with DDP induced autophagy in the cells, which was reversed with treatment of ZnO-NP. ZnO-NP attenuated chemotherapy drug resistance by inhibiting the autophagy of GC cells[36]. These data reveal an

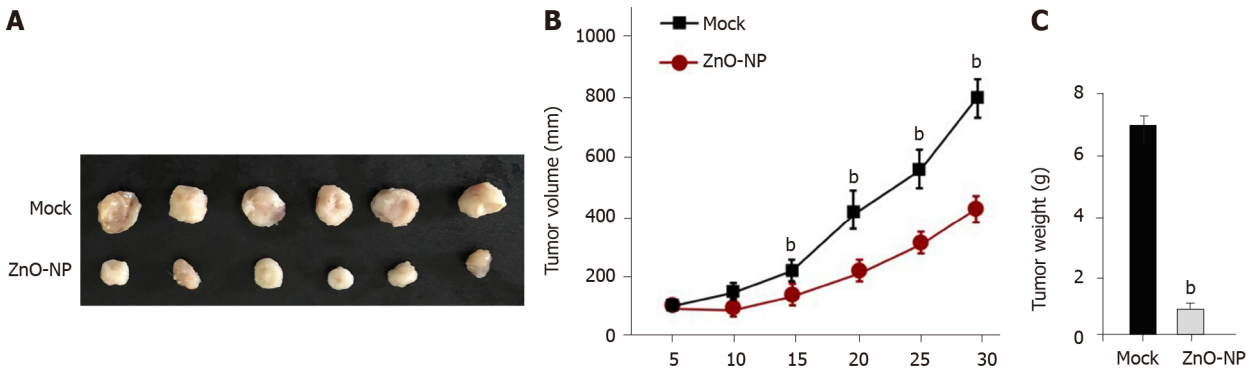


Figure 7 Zinc oxide nanoparticle reduces the tumor growth of chemoresistant gastric cancer cells *in vivo*. The impact of zinc oxide nanoparticle (ZnO-NP) on tumor growth of cisplatin (DDP)-resistant gastric cells *in vivo* was analyzed by the nude mice tumorigenicity assay (n = 5). SGC7901/DDP cells were treated with ZnO-NP (5 μg/mL) or an equal volume of saline. A: Representative images of dissected tumors from nude mice are shown; B: The average tumor volume was calculated; C: The average tumor weight was calculated. Data are presented as the mean ± SD. Statistic significant differences are indicated: $^{b}P < 0.01$.

unreported mechanism involving autophagy underlying ZnO-NP-induced anti-tumor function and inhibition of chemotherapy drug resistance of GC cells, demonstrating the association of ZnO-NP with autophagy in cancer cells.

CONCLUSION

In summary, we conclude that ZnO-NP reduces the chemoresistance of GC cells by inhibiting autophagy. Our findings provide innovative insights into the scenario in which ZnO-NP mediates the chemotherapy resistance of GC. ZnO-NP may serve as a potential therapeutic candidate for GC treatment. The potential role of ZnO-NP in the clinical treatment of GC needs to be clarified in future investigations.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is a common cancer and results in a high rate of tumor-related mortality. Cisplatin (DDP)-based chemotherapy is the first-line treatment of GC, but chemoresistance remains a severe clinical problem. Zinc oxide nanoparticle (ZnO-NP) has been identified as a promising anti-cancer agent, but its role in GC development is still unclear.

Research motivation

To identify the role of ZnO-NP in the regulation of GC progression.

Research objectives

This study explored the effect of ZnO-NP on chemotherapy resistance during GC progression.

Research methods

ZnO-NP was synthesized, and the effect and underlying mechanism on the malignant progression and chemotherapy resistance of GC cells were assessed by tumorigenicity in nude mice and evaluated by Western blotting, flow cytometry analysis, wound healing assays, transwell assays, colony formation assays, and MTT assays in GC cells and DDP-resistant GC cells.

Research results

ZnO-NP inhibited proliferation, migration, and invasion and induced apoptosis of GC cells. Meanwhile, ZnO-NP significantly reduced the IC_{50} value of DDP for the inhibition of cell proliferation of DDP-resistant SGC7901/DDP cell lines. Autophagy was increased in the chemotherapy-resistant GC cells, as demonstrated by elevated LC3II/LC3I and Beclin-1 expression and repressed p62 expression in the SGC7901/

DDP compared with that in SGC7901 cells. Mechanically, ZnO-NP inhibited autophagy in GC cells, and treatment with DDP induced autophagy in the cells, which was reversed by ZnO-NP. Functionally, ZnO-NP attenuated the tumor growth of chemoresistant GC cells *in vivo*.

Research conclusions

ZnO-NP alleviates the chemoresistance of GC cells by inhibiting autophagy. Our findings provide innovative insights into the scenario in which ZnO-NP mediates chemotherapy resistance in GC.

Research perspectives

ZnO-NP may serve as a potential therapeutic candidate for GC treatment. The potential role of ZnO-NP in the clinical treatment of GC needs to be clarified in future investigations.

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ORIGINAL ARTICLE

Basic Study

PPARGC1A rs8192678 G>A polymorphism affects the severity of hepatic histological features and nonalcoholic steatohepatitis in patients with nonalcoholic fatty liver disease

Rui-Nan Zhang, Feng Shen, Qin Pan, Hai-Xia Cao, Guang-Yu Chen, Jian-Gao Fan

ORCID number: Rui-Nan Zhang 0000-0001-9049-3010; Feng Shen 0000-0001-7782-2211; Qin Pan 0000-0001-5855-4952; Hai-Xia Cao 0000-0002-8265-9460; Guang-Yu Chen 0000-0002-9564-8971; Jian-Gao Fan 0000-0001-7443-5056.

Author contributions: Zhang RN and Shen F contributed equally to this work; Zhang RN, Pan Q, Shen F, Chen GY, and Cao HX enrolled the cohorts and collected blood samples; Zhang RN, Pan Q, and Shen F performed genotyping; Zhang RN, Pan Q interpreted the data and were involved in the manuscript preparation; Fan JG designed and supervised the study; Zhang RN and Fan JG wrote the manuscript.

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Rui-Nan Zhang, Feng Shen, Qin Pan, Hai-Xia Cao, Center for Fatty Liver, Department of Gastroenterology, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

Guang-Yu Chen, Clinical Epidemiology Research Center, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

Jian-Gao Fan, Center for Fatty Liver, Department of Gastroenterology, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai Key Lab of Pediatric Gastroenterology and Nutrition, Shanghai 200092, China

Corresponding author: Jian-Gao Fan, MD, PhD, Director, Professor, Center for Fatty Liver, Department of Gastroenterology, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai Key Lab of Pediatric Gastroenterology and Nutrition, No. 1665 Kongjiang Road, Yangpu District, Shanghai 200092, China. fanjiangao@xinhuamed.com.cn

Abstract

BACKGROUND

The association between PPARGC1A rs8192678 and nonalcoholic fatty liver disease (NAFLD) requires further confirmation. In addition, it is still unknown whether PPARGC1A rs8192678 is associated with hepatic histological features in NAFLD in the Chinese population.

AIM

To investigate the interaction between PPARGC1A rs8192678 and nonalcoholic steatohepatitis (NASH), and whether this polymorphism is associated with hepatic histological features.

METHODS

Fifty-nine patients with liver biopsy-proven NAFLD and 93 healthy controls were recruited to a cohort representing the Chinese Han population. The SAF (steatosis, activity, and fibrosis) scoring system was used for hepatic histopathological evaluation. The polymorphisms of PPARGC1A rs8192678 and patatin-like phospholipase domain-containing protein 3 (PNPLA3) rs738409 were genotyped. The intrahepatic mRNA expression of PPARGC1A was evaluated by real-time polymerase chain reaction.

approved by the institutional review board of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

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RESULTS

Thirty-seven patients with NAFLD had NASH, of which 12 were nonobese. The PPARGC1A rs8192678 risk A allele (carrying GA and AA genotypes) had the lowest *P* value in the dominant model; the odds ratio (OR) for NAFLD was 2.321 [95% confidence interval (CI): 1.121-4.806]. After adjusting for age, sex, and the PNPLA3 rs738409 risk G allele, the PPARGC1A rs8192678 A allele was a risk factor for NAFLD (OR 2.202, 95%CI: 1.030-4.705, P = 0.042). The genetic analysis showed that patients with NAFLD, moderate-to-severe steatosis (S2-3), and Activity 2-4 (A ≥ 2) were more likely to carry A in PPARGC1A rs8192678 (OR 5.000, 95%CI: 1.343-18.620, P = 0.012; and OR 4.071, 95%CI: 1.076-15.402, P = 0.031). The multivariate logistic regression analysis showed that PPARGC1A rs8192678 risk A allele was also independently associated with S2-3, A ≥ 2, and NASH (OR 6.190, 95%CI: 1.508-25.410, P = 0.011; OR 4.506, 95%CI 1.070-18.978, P = 0.040; and OR 6.337, 95%CI: 1.135-35.392, P = 0.035, respectively) after adjusting for age, sex, body mass index, and PNPLA3 rs738409 risk G allele. The results also showed that this polymorphism was associated with nonobese NASH (OR 22.000, 95%CI: 1.540-314.292, P = 0.021). The intrahepatic expression of PPARGC1A mRNA was significantly lower in the group of patients who carried the risk A allele (P = 0.014).

CONCLUSION

The PPARGC1A rs8192678 risk A allele is associated with NAFLD, and with S2-3, A ≥ 2 and NASH in NAFLD patients, independent of PNPLA3 rs738409, and may be associated with nonobese NASH.

Key Words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; PPARGC1A rs8192678 polymorphism; Steatosis; Activity

Core Tip: The PPARGC1A rs8192678 A allele was found to be a risk factor for liver biopsy-proven nonalcoholic fatty liver disease (NAFLD) after adjusting for age, sex, and patatin-like phospholipase domain-containing protein 3 rs738409 polymorphism. The multivariate logistic regression analysis showed that the PPARGC1A rs8192678 risk A allele was also independently associated with the severity of hepatic histological features (S2-3 and A ≥ 2) and nonalcoholic steatohepatitis (NASH), and might also be associated with nonobese NASH, indicating that the PPARGC1A rs8192678 risk A allele was associated with NAFLD in the Chinese Han adult population. Also, the intrahepatic expression of PPARGC1A mRNA was significantly lower in the patients who carried the risk A allele, implying that it might be a genetic contribution to the pathogenesis of NAFLD.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has become one of the most common forms of chronic liver diseases worldwide. The overall global prevalence of NAFLD is about 25%. The clinical spectrum of NAFLD ranges from nonalcoholic fatty liver (NAFL), nonalcoholic steatohepatitis (NASH), liver fibrosis and cirrhosis, and finally hepatocellular carcinoma. NAFLD also has high risk and is associated with multisystem diseases, such as obesity, type 2 diabetes mellitus (T2DM), cardiovascular diseases, chronic kidney disease, and malignancy[1,2].

The pathogenesis of NAFLD is multifactorial and strongly associated with obesity, insulin resistance, T2DM, and metabolic syndrome. Although NAFLD has a high prevalence in obese patients, nonobese patients and even lean patients, were recently found to have NAFLD as well. The anthropometric data on body mass index (BMI) was used to define nonobese or lean. The BMI values of 25 and 30 kg/m^2 are the thresholds to define overweight and obese participants, respectively, as recommended by the World Health Organization. In Asia, BMI < 25 kg/m^2 is defined as nonobese, whereas BMI < 23 kg/m^2 is defined as lean[3]. A meta-analysis was carried out to estimate the overall prevalence of NAFLD in nonobese and lean populations (15.7% and 10.2%, respectively)[4].

The differences in prevalence, clinical profile, hepatic histology severity, and outcomes of NAFLD among different ethnic groups suggested that both environmental and genetic factors influenced susceptibility to NAFLD in individuals[5]. Genetic factors have been implicated in the occurrence and development of NAFLD; the gene of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), encoded by PPARGC1A, is also a susceptibility candidate gene for NAFLD[6-8]. PGC-1α is a transcriptional coactivator with a crucial impact on multiple aspects of cellular energy metabolism including mitochondrial biogenesis and cellular respiration, regulation of adaptive thermogenesis, adipocyte cell development, and lipid and glucose metabolism. A number of genetic variants have been identified in PPARGC1A, and the Gly482Ser polymorphism (a G-to-A transition that predicted a glycine (G) to serine (S) substitution at amino acid position 482 in exon 8, rs8192678) is the most common variant, which was associated with T2DM, obesity, and hypertension in several studies[9-13]. However, the results were controversial in the Chinese Han adult population, which require further confirmation[14,15]. Whether this polymorphism is associated with the severity of hepatic histological features [steatosis, activity (hepatocyte ballooning and lobular inflammation), and fibrosis] and with NASH or nonobese NASH needs further investigation.

The present study aimed to determine the relationship between polymorphism rs8192678 of *PPARGC1A* gene and the risk of NAFLD, especially in NASH, in a Chinese Han population, and to investigate the association between PPARGC1A rs8192678 G>A polymorphism and the severity of hepatic histological features.

MATERIALS AND METHODS

Study populations

A total of 152 unrelated adult participants (18-70 years old) were recruited between March 2012 and March 2013. Patients with NAFLD were enrolled from Xinhua Hospital, Shanghai, China. All participants were Han Chinese in origin. Each patient had undergone an ultrasound-guided percutaneous liver biopsy and met the diagnostic criteria for NAFLD. The liver stiffness measurement (LSM) and controlled attenuation parameter (CAP) values were tested using FibroScan 502 (Echosens, Paris, France) by previously described methods.

The exclusion criteria were as follows: (1) excessive alcohol consumption (> 30 g/d for men and > 20 g/d for women); other diseases that led to fatty liver, such as chronic hepatitis C, drug-induced liver injury, Wilson's disease, total parenteral nutrition, and autoimmune hepatitis; (2) previous liver transplantation; (3) other end-stage diseases or malignancies; and (4) contraindications to FibroScan examination (*e.g.*, ascites, implanted pacemakers, nonhealing wounds in the upper-right quadrant of the abdomen, and pregnancy) or unreliable measurement of LSM and CAP values.

All control participants were confirmed to be free of liver diseases by both B-mode ultrasound and FibroScan 502 examination (CAP ≤ 240 dB/m and LSM values < 7.0 kPa)[16]; they all had normal liver function test results and lacked evidence of etiologies of liver injury. The study protocol was approved by the Ethics Committees of Xinhua Hospital, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Clinical and laboratory evaluation

Demographic information, such as age and sex, was collected. Venous blood samples were obtained from the participants after overnight fasting (12 h) to measure the levels of alanine transaminase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), total bilirubin, directed bilirubin, fasting blood glucose (FBG), total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and uric acid (UA). All laboratory biochemical

parameters were measured in a conventional automated analyzer (Hitachi 7600, Tokyo, Japan).

Hepatic histopathologic assessment

Liver biopsy specimens were fixed with 10% buffered formalin, embedded in paraffin, sliced, and stained with hematoxylin & eosin (HE), reticulin, and Masson trichrome. A minimum 15-mm biopsy specimen or the presence of at least 10 complete portal tracts was required. For each liver biopsy, the steatosis, activity, and fibrosis (SAF) score summarizing the main histological lesions was defined. The SAF score was designed by the fatty liver inhibition of progression Pathology Consortium. The steatosis (0-3) was categorized as follows: S0: < 5%; S1: 5%-33%; S2: 34%-66 %; and S3: ≥ 67%. The grade of hepatic inflammatory activity (A, from 0 to 4) was calculated by adding together the grades for hepatocyte ballooning (0-2) and lobular inflammation (0-2). Liver fibrosis was staged as follows: F0, none; F1, perisinusoidal or portal fibrosis; F2, perisinusoidal and periportal fibrosis without bridging; F3, bridging fibrosis; and F4, cirrhosis. A case presenting with at least grade 1 of each of the three features (steatosis, ballooning, and lobular inflammation) was classified as NASH; other cases were diagnosed as NAFL.

DNA preparation and single-nucleotide polymorphism genotyping

Polymorphism genotyping of genomic DNA was prepared from each blood sample using the QiAamp DNA Mini Kit (Qiagen, Hilden, Germany). The custom Ion AmpliSeq panel (Life Technologies, MA, United States) of PPARGC1A and patatin-like phospholipase domain-containing protein 3 (PNPLA3) was designed. The polymerase chain reaction (PCR) of the template was performed using the Ion OneTouch 2 System (Life Technologies, MA, United States) following the manufacturer's instructions. PPARGC1A and PNPLA3 variants were genotyped by DNA sequencing using the Ion 318 Chip (Life Technologies, United States) following the Ion PGM 200 Sequencing kit protocol. Negative controls were introduced for each run to ensure genotyping quality. Samples giving discordant results were reanalyzed.

Real-time PCR for measuring PPARGCA1 mRNA expression

Total RNA was isolated from samples of liver biopsy specimens using the TRIzol reagent (Takara, Dalian, China) and reverse transcribed using the PrimeScript RT Reagent Kit (Takara, Dalian, China). The mRNA expression of PPARGC1A and β-actin was measured by real-time PCR (RT-PCR) using the SYBR Premix Ex Taq Kit (Takara, Dalian, China) and an ABI 7500 RT-PCR System (Applied Biosystems, United States). Target mRNA levels were normalized to β-actin expression levels. The following primers were used: PPARGC1A (F: 5'-GAGTGACATCGAGTGTGCTG-3'; R: 5'-GGGCAATCCGTCTTCATCCA-3'); β-actin (F: 5'-TCCTTCCTGGGCATGGAGT-3'; R: 5'-CAGGAGGAGCAATGATCTTGAT-3'). A duplicate of this experiment was performed for the same reaction. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of each gene. Thermal cycling was performed as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 34 s.

Statistical analysis

Continuous variables were expressed as mean ± SD, median and interquartile range for those with a skewed distribution and categorical variables as the frequency or percentage. The *t* test and chi-squared test were used where appropriate. Multiple logistic regression models were used to assess the factors independently associated with the severity of histological parameters. In the dominant model, the frequencies of the participants with the allele 1 (allele 1 homozygote and heterozygote) were compared with the participants homozygous for the allele 2 using a 2 × 2 contingency table. The odds ratio (OR) and the 95% confidence interval (CI) were calculated using Woolf's method. In all analyses, GA and AA for PPARGC1A rs8192678 was considered when choosing a dominant model of inheritance. Analyses were performed using SPSS 23.0 (SPSS Inc., IL, United States). A two-sided $P < 0.05$ indicated a statistically significant difference.

RESULTS

Anthropometric and clinical data

The clinical characteristics of 152 participants (59 patients with NAFLD and 93

controls) are listed in Table 1. Age, BMI, ALT, GGT, TC, TG, LDL-C, UA, CAP, and LSM values were significantly higher in the NAFLD group. No differences in sex, ALP, HDL-C, and FBG were observed between the two groups. Among the patients with NAFLD, 37 (62.71%) had NASH, and 22 (37.29%) had NAFL. Furthermore, 69.49% (41/59) were obese, and 30.51% (18/59) were nonobese. Patients with NAFLD also showed a significantly higher frequency of hypertension and T2DM. The ALT, GGT, CAP, and LSM levels were significantly higher in patients with NASH compared to patients with NAFL, with no significant difference in the frequency of obesity, hypertension, and T2DM (Tables 1 and 2).

PPARGC1A rs8192678 polymorphism genotypes and allele frequencies and the risk of NAFLD between different groups

The percentage of PPARGC1A rs8192678 GA genotype was significantly higher in the NAFLD group than in the control group (62.71% *vs* 39.78 %), suggesting that carriers of this genotype had a significantly increased risk of NAFLD (OR 2.786, 95%CI: 1.300-5.962, P = 0.008) (Table 3). The carriers of the homozygous AA genotype had no significantly increased risk of NAFLD compared to participants with GG homozygous alleles (P = 0.609). The close association was further revealed in the dominant model (combined GA and AA genotypes, GA + AA *vs* GG, OR 2.321, 95%CI: 1.121-4.806, P = 0.022) (Tables 3 and 4).

The percentage of the PNPLA3 rs738409 GG genotype was also significantly higher in patients with NAFLD than in controls (33.90% *vs* 16.13%, P = 0.004). The OR of NAFLD was 3.889-fold (95%CI: 1.534-9.926, P = 0.004) higher in participants with the GG genotype than in those with the CC genotype; and the participants carrying G allele also had a high risk of NAFLD (GG + GC *vs* CC, OR 2.364, 95%CI: 1.105-5.055, P = 0.025) (Table 3). The multiple logistic regression analysis revealed that the PPARGC1A rs8192678 A allele was a risk factor for NAFLD (OR 2.202, 95%CI: 1.030-4.705, P = 0.042) independent of age, sex, and PNPLA3 rs738409 polymorphism (Table 5).

Association between PPARGC1A rs8192678 polymorphism and hepatic histological features

The hepatic histological features, including steatosis, activity (lobular inflammation and ballooning), and fibrosis, were assessed in patients with NAFLD. The frequencies of PPARGC1A rs8192678 GA + AA genotypes were remarkably higher in patients with moderate-to-severe steatosis (S2-3) and activity 2-4 (A ≥ 2) compared with those with mild steatosis (S1) and activity 0-1 (A < 2). Also, the carriers with risk A allele (carrying GA and AA genotypes) had a significantly increased risk in NAFLD patients with S2-3 and A ≥ 2 (OR 5.000, 95%CI: 1.343-18.620, P = 0.012 and OR 4.071, 95%CI: 1.076-15.402, P = 0.031). The multivariate analysis showed that PPARGC1A rs8192678 A allele was also significantly associated with S2-3 and A ≥ 2 (OR 6.190, 95%CI: 1.508-25.410, P = 0.011 and OR 4.506, 95%CI: 1.070-18.978, P = 0.040) after adjusting for age, sex, BMI, and PNPLA3 rs738409 risk G allele. However, no significant differences were found in significant fibrosis 2-4 (F2-4) and advanced fibrosis 3-4 (F3-4) compared with F0-1 and F0-2 (P = 0.186 and P = 0.426) (Figure 1A-E and Table 6).

The patients with NASH carrying PPARGC1A rs8192678 GA + AA genotypes had an OR of 4.431 (95%CI: 1.245-15.763, P = 0.017) when compared with NAFL (Table 4). The multivariate analysis also showed that PPARGC1A rs8192678 risk A allele was independently associated with NASH after adjusting for age, sex, BMI, and PNPLA3 rs738409 risk G allele in patients with NAFLD (OR 6.337, 95%CI: 1.135-35.392, P = 0.035) (Table 6).

PPARGC1A rs8192678 genotype is associated with nonobese NASH patients

The GA + AA genotypes were not associated with obese NAFLD, obese NASH, and obese NAFL in the dominant model compared with nonobese NAFLD, nonobese NASH, and nonobese NAFL, respectively. Genetic analysis also showed no significant difference in the patients carrying A allele in PPARGC1A rs8192678 between obese NASH and obese NAFL groups (Table 4).

The remaining anthropometric values, biochemical test values, and PNPLA3 polymorphism did not show any significant differences between the two groups, apart from a trend in a higher level of GGT (P = 0.068) and CAP (P = 0.056) in nonobese NASH. However, genetic analysis showed that the proportion of PPARGC1A rs8192678 GA + AA genotypes was significantly higher in the patients with nonobese NASH compared with patients with nonobese NAFL, which was significantly associated with nonobese NASH (OR 22.000, 95%CI: 1.540-341.292, P = 0.021) (Tables 4

Table 1 Clinical characteristics of patients with nonalcoholic fatty liver disease and controls

Variables	NAFLD (*n* = 59)	Controls (*n* = 93)	*P* value
Sex (M/F)	43/16	59/34	0.227
Age (year)	38.20 ± 13.78	42.20 ± 11.05	0.039
BMI (kg/m^2)	27.31 ± 3.31	23.06 ± 2.74	< 0.0001
ALT (U/L)	70.56 ± 47.6	24.98 ± 16.07	< 0.0001
GGT (U/L)	72.75 ± 55.22	25.94 ± 18.17	< 0.0001
ALP (U/L)	97.07 ± 44.73	96.50 ± 32.54	0.347
TC (mmol/L)	4.86 ± 0.86	4.39 ± 0.89	0.001
TG (mmol/L)	1.99 ± 1.25	1.14 ± 0.74	< 0.0001
HDL-C (mmol/L)	1.20 ± 0.28	1.23 ± 0.28	0.177
LDL-C (mmol/L)	2.83 ± 0.7	2.24 ± 0.50	< 0.0001
FBG (mmol/L)	5.93 ± 2.62	5.31 ± 0.79	0.941
UA (μmol/L)	361.32 ± 105.78	274.25 ± 76.53	< 0.0001
CAP (dB/m)	313 (277-351)	212 (172-232)	< 0.0001
LSM (kPa)	7.7 (5.6-12)	5.25 (4.1-7.0)	< 0.0001
Hypertension, *n* (%)	22 (37.29%)	15 (16.13%)	0.003
T2DM, *n* (%)	7 (11.86%)	1 (1.08%)	0.005
Obesity, *n* (%)	41 (69.49%)	19 (20.43%)	< 0.0001
Steatosis (S, *n*)			
S1/S2/S3	25/25/9	/	/
Activity (A, *n*)			
A0/A1/A2/A3/A4	1/12/16/22/8	/	/
Fibrosis (F, *n*)			
F0/F1/F2/F3/F4	11/29/8/7/4	/	/

The data are expressed as mean ± SD, or as *n* (%) for normally distributed variables and as median (interquartile range) when distribution of the variable was skewed. NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; TC: Total cholesterol; TG: Triglycerides; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; FBG: Fasting blood glucose; UA: Uric acid; LSM: Liver stiffness measurement; CAP: Controlled attenuation parameter; T2DM: Type 2 diabetes mellitus.

and 7).

mRNA expression of PPARGC1A in the liver of patients with NAFLD

The expression of PPARGC1A mRNA in the liver biopsy specimens of patients with NAFLD was evaluated by RT-PCR. The results obtained by RT-PCR in the GA or AA group (n = 5) were compared with the results in the GG group (n = 5) at PPARGC1A rs8192678. As shown in Figure 1F, the expression of PPARGC1A mRNA transcripts in the liver was significantly lower in the GA or AA group than in the GG group (P = 0.014).

DISCUSSION

Genetic factors may be important in the development of NAFLD, in either obese or nonobese patients. The present study further indicated that PPARGC1A rs8192678 risk A allele was associated with liver biopsy-proven NAFLD susceptibility in a Chinese Han adult population, and it was especially associated with NASH compared with NAFL. The PPARGC1A rs8192678 risk A allele was also associated with nonobese NASH in patients with nonobese NAFLD. In addition, this polymorphism was

Table 2 Clinical characteristics of patients with nonalcoholic steatohepatitis and nonalcoholic fatty liver among patients with nonalcoholic fatty liver disease

Variable	NAFL (*n* = 22)	NASH (*n* = 37)	*P* value
Sex (M/F)	17/5	26/11	0.559
Age (year)	37.23 ± 13.64	38.78 ± 14.01	0.605
BMI (kg/m^2)	27.07 ± 3.69	27.38 ± 3.17	0.675
ALP (U/L)	102.96 ± 52.34	93.56 ± 39.91	0.982
GGT (U/L)	38.00 (23.80-69.03)	69.70 (39.00-85.50)	0.023
ALT (U/L)	46.00 (30.33-64.95)	71.40 (46.9-111.70)	0.006
TC (mmol/L)	5.03 ± 1.04	4.76 ± 0.72	0.801
TG (mmol/L)	1.91 ± 0.74	2.04 ± 1.48	0.532
HDL-C (mmol/L)	1.21 ± 0.28	1.19 ± 0.28	0.903
LDL-C (mmol/L)	2.97 ± 0.91	2.72 ± 0.49	0.444
FBG (mmol/L)	6.43 ± 3.59	5.62 ± 1.77	0.318
UA (μmol/L)	360.91 ± 107.80	361.62 ± 106.22	0.482
CAP (dB/m)	295.50 (249.25-345.00)	333.00 (296.25-356.25)	0.038
LSM (kPa)	6.20 (4.55-9.95)	9.60 (6.50-12.85)	0.008
Obesity, *n* (%)	16 (72.73)	25 (60.97)	0.677
Hypertension, *n* (%)	8 (36.36)	14 (37.84)	0.910
T2DM, *n* (%)	3 (13.64)	4 (10.81)	0.746

The data are expressed as mean ± SD, or as *n* (%) for normally distributed variables and as median (interquartile range) when distribution of the variable was skewed. NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; BMI: Body mass index; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; ALT: Alanine aminotransferase; TC: Total cholesterol; TG: Triglycerides; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; FBG: Fasting blood glucose; UA: Uric acid; LSM: Liver stiffness measurement; CAP: Controlled attenuation parameter; T2DM: Type 2 diabetes mellitus.

Table 3 Genotype distribution of PPARGC1A rs8192678 and patatin-like phospholipase domain-containing protein 3 rs738409 in patients with nonalcoholic fatty liver disease and controls

Genotype	NAFLD (*n* = 59)	Controls (*n* = 93)	χ^2	*P* value	OR (95%CI)
PPARGC1A rs8192678					
GG	14 (23.73%)	39 (41.94%)			
GA	37 (62.71%)	37 (39.78%)	7.148	0.008	2.786 (1.300-5.962)
AA	8 (13.56%)	17 (18.28%)	0.262	0.609	1.311 (0.464-3.704)
GA+AA	45 (76.27%)	54 (58.06%)	5.269	0.022	2.321 (1.121-4.806)
PNPLA3 rs738409					
CC	12 (20.34%)	35 (37.63%)			
CG	27 (45.76%)	43 (46.24%)	2.152	0.142	1.831 (0.812-4.131)
GG	20 (33.90%)	15 (16.13%)	8.423	0.004	3.889 (1.524-9.926)
CG + GG	47 (79.56%)	58 (62.37%)	5.055	0.025	2.364 (1.105-5.055)

OR: Odds ratio; CI: Confidence interval; PNPLA3: Patatin-like phospholipase domain-containing protein 3; NAFLD: Nonalcoholic fatty liver disease.

associated with moderate-to-severe steatosis and higher histological activity, but not with significant and advanced fibrosis.

PPARGC1A/PGC-1α is a coactivator for a number of transcription factors, including the peroxisome proliferator-activated receptor. PGC-1α is a critical regulator

Table 4 Association tests of PPARGC1A rs8192678 polymorphism between different groups

PPARGC1A rs8192678	Case			Control			Dominant model		
	GG	GA	AA	GG	GA	AA	χ^2	*P* value	OR (95%CI)
NAFLD *vs* Control	14	37	8	39	37	17	5.269	0.022	2.321 (1.121-4.806)
NASH *vs* Control	5	28	4	39	37	17	9.550	0.002	4.622 (1.653-12.929)
NASH *vs* NAFL	5	28	4	9	9	4	5.721	0.017	4.431 (1.245-15.763)
Obese *vs* nonobese participants	17	33	10	36	41	15	1.864	0.172	1.626 (0.807-3.276)
Obese NAFLD *vs* nonobese NAFLD	9	27	5	5	10	3	0.235	0.628	1.368 (0.384-4.865)
Obese NASH *vs* nonobese NASH	4	19	2	1	9	2	0.408	0.348	0.477 (0.047-4.806)
Obese NAFL *vs* nonobese NAFL	5	8	3	4	1	1	2.264	0.132	4.400 (0.596-32.501)
Obese NASH *vs* obese NAFL	4	19	2	5	8	3	1.324	0.250	2.386 (0.531-10.734)
Nonobese NASH *vs* nonobese NAFL	1	9	2	4	1	1	6.785	0.021	22.000 (1.540-314.292)

NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease; OR: Odds ratio; CI: Confidence interval.

Table 5 Multiple logistic regression analysis of predictive factors for nonalcoholic fatty liver disease

Variables	B	S.E.	Wald	OR (95%CI)	*P* value
Age (year)	-0.031	0.015	4.236	0.970 (0.942-0.999)	0.040
Sex (Male)	-0.233	0.385	0.365	0.793 (0.373-1.686)	0.546
PPARGC1A rs8192678 A allele	0.789	0.388	4.148	2.202 (1.030-4.705)	0.042
PNPLA3 rs738409 G allele	0.857	0.403	4.524	2.356 (1.070-5.191)	0.033

OR: Odds ratio; CI: Confidence interval; PNPLA3: Patatin-like phospholipase domain-containing protein 3.

Table 6 Association of the PPARGC1A genotypes with steatosis, activity, and fibrosis in patients with nonalcoholic fatty liver disease

Histological features		Genotypes		Dominant model		Multivariate analysis[1]	
		GG (*n* = 14)	GA + AA (*n* = 45)	*P* value	OR (95%CI)	*P* value	OR (95%CI)
Steatosis (S)	S1	10 (71.43%)	15 (33.33%)				
	S2-3	4 (28.57%)	30 (66.67%)	0.012	5.000 (1.343-18.620)	0.011	6.190 (1.508-25.410)
Activity (A)	A < 2	6 (42.86%)	7 (15.56%)				
	A ≥ 2	8 (57.14%)	38 (84.44%)	0.031	4.071 (1.076-15.402)	0.040	4.506 (1.070-18.978)
Significant fibrosis	F0-1	12 (85.71%)	28 (62.22%)				
	F2-4	2 (14.29%)	17 (37.78%)	0.100	3.643 (0.726-18.292)	0.186	3.449 (0.551-21.598)
Advanced fibrosis	F0-2	13 (92.86%)	35 (77.78%)				
	F3-4	1 (7.14%)	10 (22.22%)	0.206	3.714 (0.432-31.949)	0.426	2.549 (0.255-25.490)
NASH	NAFL	9 (64.28%)	13 (28.89%)				
	NASH	5 (35.71%)	32 (71.11%)	0.017	4.431 (1.245-15.763)	0.035	6.337 (1.135-35.392)

[1]Multivariate analysis adjusted for age, sex, body mass index, and patatin-like phospholipase domain-containing protein 3 rs738409 risk G allele. NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; OR: Odds ratio; CI: Confidence interval.

of adaptive thermogenesis, cellular respiration, and energy metabolism[17]. Recent studies suggested that PGC-1α also regulates lipid metabolism, PGC-1α stimulates the expression of farnesoid X receptor (FXR) target genes in hepatic cells and reduces TG secretion by enhancing FXR activity[18]. In addition, PGC-1α can transactivate the

Table 7 Biochemical and genetic characteristics of patients with nonobese nonalcoholic fatty liver disease

Variables	NAFL (*n* = 6)	NASH (*n* = 12)	*P* value
Sex (M/F)	4/2	10/2	0.569
Age (year)	35.33 ± 18.44	39.67 ± 4.80	0.452
BMI (kg/m^2)	22.83 ± 1.61	23.69 ± 0.90	0.122
ALP (U/L)	123.32 ± 77.62	84.25 ± 23.28	0.426
GGT (U/L)	28.50 (21.18-85.00)	73.25 (54.75-99.25)	0.068
ALT (U/L)	42.00 (21.78-78.50)	71.15 (41.10-115.25)	0.325
TC (mmol/L)	5.11 ± 0.55	4.96 ± 1.02	0.606
TG (mmol/L)	2.00 ± 0.43	1.77 ± 0.95	0.371
HDL-C (mmol/L)	1.10 ± 0.16	1.24 ± 0.44	0.943
LDL-C (mmol/L)	3.16 ± 0.46	2.82 ± 0.80	0.524
FBG (mmol/L)	5.03 ± 0.94	5.24 ± 0.85	0.587
UA (μmol/L)	360.86 ± 167.34	354.17 ± 67.43	0.684
CAP (dB/m)	257.00 ± 57.03	316.73 ± 43.48	0.056
LSM (kPa)	6.95 ± 4.07	6.79 ± 2.06	0.421
PPARGC1A rs8162678			
GG, *n* (%)	4 (66.67)	1 (8.33)	
GA + AA, *n* (%)	2 (33.33)	11 (91.67)	0.021
PNPLA3 rs738409			
CC, *n* (%)	2 (33.33)	1 (8.33)	
CG + GG, *n* (%)	4 (66.67)	11 (91.67)	0.245

The data are expressed as mean ± SD, or as *n* (%) for normally distributed variables and as median (interquartile range) when distribution of the variable was skewed. NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; BMI: Body mass index; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; TC: Total cholesterol; TG: Total triglycerides; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; FBG: Fasting blood glucose; UA: Uric acid; LSM: Liver stiffness measurement; CAP: Controlled attenuation parameter; PNPLA3: Patatin-like phospholipase domain-containing protein 3.

hepatocyte nuclear factor 4α-dependent human hepatic lipase gene promoter to increase the production of hepatic lipase. The PGC-1α is also highly expressed in the liver and coordinates the induction of fatty acid oxidation when fasting. Multiple studies showed that decreased PGC-1α expression might be the mechanism underlying metabolic diseases. Tissue-specific reduction of PGC-1α expression in adipose, liver, and muscle reduced glucose tolerance and insulin sensitivity in mice[19, 20], supporting the hypothesis that decreased PGC-1α expression in insulin-sensitive tissues might contribute to a higher risk of T2DM. PPARGC1A mRNA expression decreased in muscle and adipose tissue of human participants with T2DM[21], and a correlation existed between adipose PGC-1α protein levels and decreased insulin sensitivity[22]. T2DM was also a risk factor for NAFLD. Another reported PPARGC1A polymorphism rs2290602 also decreased the expression of PPARGC1A in patients with NAFLD[6]. Therefore, genetic factors that alter the expression of PGC-1α might participate in the pathogenesis of NAFLD. The present study also indicated that the expression of PPARGC1A was significantly lower in patients who carried the A allele, which supported the hypothesis that decreased PGC-1α expression in steatosis liver tissues might contribute to a higher risk of NAFLD.

Recently, the association between different single nucleotide polymorphisms (SNPs) and metabolic diseases, such as NAFLD, was reported[23]. Therefore, different polymorphisms of PPARGC1A associated with different metabolic diseases were investigated. Iglseder *et al*[24] showed that the -3974T/C (rs2970865) polymorphism of the *PPARGC1A* gene was associated with the severity of carotid atherosclerosis. The variations in rs2290602 in the *PPARGC1A* gene was expected to affect lipid and glucose metabolism and result in the development of NAFLD and NASH[6]. Another

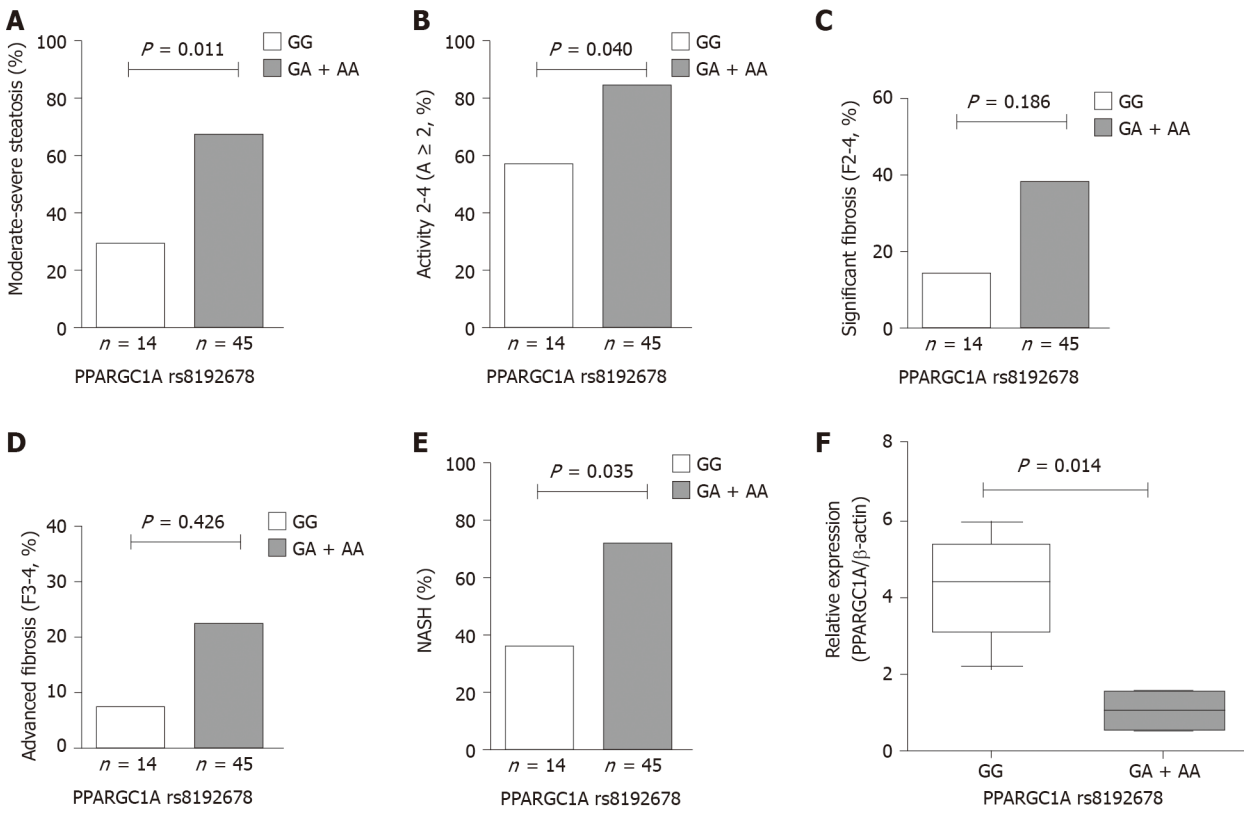

Figure 1 Comparison of various quantitative phenotypes (hepatic histological features and mRNA expression) in patients with nonalcoholic fatty liver disease at the PPARGC1A rs8192678 polymorphism. A-E: Prevalence of moderate-severe steatosis (S2-3), activity 2-4 (A ≥ 2), significant fibrosis (F2-4), advanced fibrosis (F3-4), and nonalcoholic steatohepatitis according to the PPARGC1A rs8192678 polymorphism in 59 patients with nonalcoholic fatty liver disease. The reference genotype for the PPARGC1A rs8192678 polymorphism was GG, and a mode of dominant inheritance was used; F: The intrahepatic mRNA expression of PPARGC1A between the GG group (n = 5) and the GA or AA group (n = 5) at PPARGC1A rs8192678. NASH: Nonalcoholic steatohepatitis.

common PPARGC1A rs8192678 polymorphism might contribute to the risk of coronary artery disease in the Chinese population[9,24], and was also associated with obesity, hypertension, and T2DM. This polymorphism in PPARGC1A was also reported to be related to the susceptibility of NAFLD in different populations and ethnicities. The present study showed that the variation of PPARGC1A rs8192678 GA genotype was significantly higher in patients with NAFLD, but PPARGC1A rs8192678 AA genotype showed no difference between NAFLD and control groups, and the percentage of AA genotype was only 13.56% (8/59) in NAFLD patients, which was consistent with the findings of Lin *et al*[7] in obese children in Taiwan. The explanation of this finding in Lin *et al*[7] concluded two probable reasons. First, the number with PPARGC1A rs8192678 AA genotype was relatively small, because of the sample variation and inadequate statistical power, the role of AA genotype in NAFLD was not presented. Second, the cellular effects of PGC-1α were complicated, and the mechanism by which PGC-1α influenced the pathogenesis of NAFLD was still unclear [7]. However, a comparison between GA + AA and GG genotypes revealed that the participants who carried the PPARGC1A rs8192678 A allele were found to be significantly associated with not only liver biopsy-proven NAFLD but also NASH, independent of age, sex, BMI, and PNPLA3 rs738409 risk G allele in the dominant model. No study analyzed the association between the PPARGC1A rs8192678 A allele and hepatic histological features in NAFLD. Significant associations of PPARGC1A rs8192678 risk A allele with moderate-to-severe steatosis and higher histological activity in patients with NAFLD were found in this study. However, significant fibrosis and advanced fibrosis were not associated with PPARGC1A rs8192678 risk A allele. This suggested that PPARGC1A rs8192678 risk A allele was associated with steatosis and liver damage, contributing to the occurrence of NASH.

Several studies reported that PPARGC1A rs8192678 was significantly associated with obesity; this polymorphism conferred a higher risk of obesity in certain populations[25,26]. The patients who carried PPARGC1A rs8192678 AA genotype benefited more from interventions aimed at weight loss, including caloric restriction,

bariatric surgery, and acarbose treatment[27,28]. The prevalence of lean NAFLD and nonobese NAFLD has increased in eastern and western countries in recent years. The genetic, metabolic, microbiome, metabolomics, and inflammatory factors may also be the causes of NAFLD in lean or nonobese individuals. Genetic factors may also be important in the occurrence and development of nonobese NAFLD. IFNL3 rs12979860 polymorphism has been shown to be associated with hepatic inflammation and fibrosis in patients with nonobese NAFLD[29]. Two SNPs rs12447924 and rs12597002 of the cholesteryl ester transfer protein gene were reported to be associated with a risk of steatosis in nonobese individuals[30]. Wang *et al*[31] found a significantly higher prevalence of advanced fibrosis (F ≥ 3) and LSM values in patients with nonobese NASH compared with obese NASH; also, a higher prevalence of TM6SF2 T allele was observed in patients with nonobese NASH, indicating that patients with nonobese NASH had more severe hepatic histological changes and genetic susceptibility in the Chinese population[31]. The present study found that the PPARGC1A rs8192678 A allele was not significantly closely associated with obese participants, patients with obese NAFLD, and patients with obese NASH. However, the PPARGC1A rs8192678 A allele was relatively more common in patients with nonobese NASH compared with patients with obese NASH. The results also showed that the PPARGC1A rs8192678 GA + AA genotypes were remarkably associated with nonobese NASH, indicating that the PPARGC1A rs8192678 A allele was associated with a high risk of NASH. This, especially, increased the risk of nonobese NASH, and the PPARGC1A rs8192678 polymorphism might be the pathogenesis of nonobese NASH. Despite no significant difference in TC, TG, ALT, GGT, FBG, UA, and CAP values and PNAPL3 rs738409 between the nonobese NAFL and NASH groups, the levels of GGT (P = 0.068) and CAP (P = 0.056) followed an increasing trend in nonobese NASH. This suggested that genetic background might also be a risk factor for nonobese NASH. The influence of metabolic factors, such as lipid and glucose, needs further exploration using a large sample.

The association between PPARGC1A rs8192678 and NAFLD in the Asian population is controversial. Hui *et al*[15] and Zhou *et al*[14] reported no association between the PPARGC1A rs8192678 variant and ultrasound-defined NAFLD in Chinese adults[14,15]. However, Tai *et al*[8] reported that the PPARGC1A variant rs8192678 was associated with liver biopsy-proven NASH in severely obese Taiwanese patients[8]. Saremi *et al*[32] also indicated that the AA genotype and A allele of PPARGC1A increased in Iranian patients with liver biopsy-proven NAFLD[32]. The frequency of the PPARGC1A rs8192678 A allele was 0.453 in Han Chinese, 0.350 in Europeans, and 0.040 in Africans according to the National Center for Biotechnology Information human SNP database, which inferred that the PPARGC1A rs8192678 risk A allele conferred a higher genetic susceptibility to NAFLD in Asians than in Europeans and Africans[7]. Also, the PPARGC1A rs8192678 was associated with NAFLD, especially NASH, in the Chinese Han adult population with liver biopsy-proven NAFLD. This contradictory conclusion might be due to the different criteria used for the definition of NAFLD; ultrasonography or liver biopsy was carried out in different studies. In the present study, the gold diagnostic standard for NAFLD, liver histopathology, was used to avoid the potential image bias in Chinese Han patients with NAFLD.

However, this study also had limitations. First, obtaining liver biopsies was difficult, and hence patients with NAFLD were relatively few, especially those with nonobese NAFLD. Consequently, no statistically significant differences were identified. Second, the risk factors such as hypertension and diabetes, which might influence obese/nonobese NAFLD, were not mentioned in this study. Therefore, a large number of patients with liver biopsy-proven NAFLD should be included in further studies, and well-designed case-control studies should be performed to confirm and support these findings.

CONCLUSION

In conclusion, the present study showed that PPARGC1A rs8192678 risk A allele was associated with liver biopsy-proven NAFLD/NASH, which also had an effect on the severity of hepatic histological features (S2-3 and A ≥ 2), and might also be associated with nonobese NASH in the Chinese Han adult population. These findings also suggested that PPARGC1A polymorphism rs8192678 risk A allele and lower expression of PPARGC1A mRNA might contribute to the etiology of NAFLD. Further studies with large samples are required to confirm these findings.

ARTICLE HIGHLIGHTS

Research background

Nonalcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease and is a significant global burden worldwide. It is also common in obese and nonobese populations. Although the mechanism of NAFLD is unclear, genetic susceptibility plays a vital role in NAFLD. The association between PPARGC1A rs8192678 polymorphism and NAFLD has been reported in several studies, but it is controversial in the Asian population. Whether PPARGC1A rs8192678 is associated with hepatic histological features in NAFLD in the Chinese population is unknown.

Research motivation

The association between PPARGC1A rs8192678 polymorphism and NAFLD requires further investigation, and the association with hepatic histological features and nonobese NAFLD in the Chinese population is unknown.

Research objectives

The aim was to investigate the association between PPARGC1A rs8192678 polymorphism and nonalcoholic steatohepatitis (NASH) and to determine whether PPARGC1A rs8192678 is associated with the hepatic histological features of NASH.

Research methods

Patients with NAFLD and healthy controls were recruited to a cohort representing the Chinese Han population. Patients with NAFLD were proven by liver biopsy. The SAF (Steatosis, activity, and fibrosis) scoring system was used for histopathological evaluation. The polymorphisms of PPARGC1A rs8192678 and patatin-like phospholipase domain-containing protein 3 (PNPLA3) rs738409 were genotyped. The intrahepatic mRNA expression of PPARGC1A was evaluated by real-time polymerase chain reaction.

Research results

In NAFLD patients, 37 patients had NASH, of which 12 were nonobese NASH. The PPARGC1A rs8192678 risk A allele (carrying GA and AA genotypes) increased the risk of NAFLD in the dominant model. The PPARGC1A rs8192678 A allele was also found to be a risk factor for NAFLD after adjusting for age, sex, and PNPLA3 rs738409 polymorphism. In the hepatic histological features of NAFLD patients, moderate-to-severe steatosis (S2-3), and Activity 2-4 (A ≥ 2) were more likely to carry PPARGC1A rs8192678 risk A allele. After adjusting for age, sex, body mass index, and PNPLA3 rs738409 risk G allele, the PPARGC1A rs8192678 risk A allele was also independently associated with S2-3, A ≥ 2, and NASH. The results also showed that this polymorphism was associated with nonobese NASH. In the group of patients who carried A allele (GA or AA genotypes), the intrahepatic expression of PPARGC1A mRNA was significantly lower than that in patients with GG genotype.

Research conclusions

The PPARGC1A rs8192678 A allele is a risk factor for NAFLD, and is associated with the severity of hepatic histological features (S2-3 and A ≥ 2) and NASH in NAFLD patients, independent of PNPLA3 rs738409, and might be associated with nonobese NASH.

Research perspectives

The result that PPARGC1A rs8192678 was associated with liver biopsy-proven NASH and had an additive effect on the severity of hepatic histological features was further confirmed in the Chinese Han adult population. PPARGC1A rs8192678 might contribute to the etiology of NAFLD. PPARGC1A rs8192678 might be a useful tool for diagnosing NASH and predicting the severity of the hepatic histological features of NAFLD.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Does endoscopic intervention prevent subsequent gastrointestinal bleeding in patients with left ventricular assist devices? A retrospective study

Sonali Palchaudhuri, Ishita Dhawan, Afshin Parsikia, Edo Y Birati, Joyce Wald, Shazia Mehmood Siddique, Laurel R Fisher

ORCID number: Sonali Palchaudhuri 0000-0002-2689-0553; Ishita Dhawan 0000-0002-6871-3512; Afshin Parsikia 0000-0002-0092-4882; Edo Y Birati 0000-0002-4787-0893; Joyce Wald 0000-0003-0276-3709; Shazia Mehmood Siddique 0000-0002-8608-3374; Laurel Fisher 0000-0002-0519-6597.

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Sonali Palchaudhuri, Ishita Dhawan, Afshin Parsikia, Shazia Mehmood Siddique, Laurel R Fisher, Division of Gastroenterology and Hepatology, University of Pennsylvania, Philadelphia, PA 19104, United States

Edo Y Birati, Joyce Wald, Division of Cardiovascular Medicine, University of Pennsylvania, Philadelphia, PA 19104, United States

Corresponding author: Sonali Palchaudhuri, MD, Attending Doctor, Division of Gastroenterology and Hepatology, University of Pennsylvania, 3400 Civic Center Boulevard South Pavilion, 4th Floor, Philadelphia, PA 19104, United States.
sonali@post.harvard.edu

Abstract

BACKGROUND

Patients with left ventricular assist devices (LVADs) are at increased risk for recurrent gastrointestinal bleeding (GIB) and repeat endoscopic procedures. We assessed the frequency of endoscopy for GIB in patients with LVADs and the impact of endoscopic intervention on preventing a subsequent GIB.

AIM

To evaluate for an association between endoscopic intervention and subsequent GIB. Secondary aims were to assess the frequency of GIB in our cohort, describe GIB presentations and sources identified, and determine risk factors for recurrent GIB.

METHODS

We conducted a retrospective cohort study of all patients at a large academic institution who underwent LVAD implantation from January 2011 – December 2018 and assessed all hospital encounters for GIB through December 2019. We performed a descriptive analysis of the GIB burden and the outcome of endoscopic procedures performed. We performed multivariate logistic regression to evaluate the association between endoscopic intervention and subsequent GIB.

RESULTS

In the cohort of 295 patients, 97 (32.9%) had at least one GIB hospital encounter.

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There were 238 hospital encounters, with 55.4% (132/238) within the first year of LVAD implantation. GIB resolved on its own by discharge in 69.8% (164/235) encounters. Recurrent GIB occurred in 55.5% (54/97) of patients, accounting for 59.2% (141/238) of all encounters. Of the 85.7% (204/238) of encounters that included at least one endoscopic evaluation, an endoscopic intervention was performed in 34.8% (71/204). The adjusted odds ratio for subsequent GIB if an endoscopic intervention was performed during a GIB encounter was not significant (odds ratio 1.18, $P = 0.58$).

CONCLUSION

Patients implanted with LVADs whom experience recurrent GIB frequently undergo repeat admissions and endoscopic procedures. In this retrospective cohort study, adherence to endoscopic guidelines for performing endoscopic interventions did not significantly decrease the odds of subsequent GIB, thus suggesting the uniqueness of the LVAD population. A prospective study is needed to identify patients with LVAD at risk of recurrent GIB and determine more effective management strategies.

Key Words: Gastrointestinal bleeding; Left ventricular-assist device; Endoscopic intervention, Inpatient care; Hospital readmissions; Recurrent bleeding

Core Tip: Patients implanted with left ventricular assist devices (LVADs) whom experience recurrent gastrointestinal bleeding (GIB) frequently undergo repeat admissions and endoscopic procedures. In this retrospective cohort study, a majority of GIB resolved by discharge without intervention and adherence to endoscopic guidelines for performing endoscopic interventions did not significantly decrease the odds of subsequent GIB, thus questioning the role of endoscopy in this population. A prospective study is needed to identify patients with LVAD patients at risk of recurrent GIB and determine more effective management strategies.

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INTRODUCTION

In the last decade, there has been an overall increase in the implantation of continuous-flow left ventricular assist devices (LVADs), a treatment modality for end-stage heart failure as a bridge to transplant, bridge to recovery, or destination therapy (DT). An overall unchanged rate of heart transplantation has resulted in growing cohort of patients with LVADs[1]. Gastrointestinal bleeding (GIB) is one of the most common adverse event in patients with LVADs[2], cited as affecting 21%-36% of patients[2,3], suggested to be due to chronic anticoagulation (AC) and continuous-flow states[4]. The incidence increases with length of time exposed to LVAD.

Prior cohort studies at tertiary care centers, including one at our institution, describe the GIB sources and outcomes of endoscopic evaluations, suggesting that endoscopic interventions are successful in short-term resolution of GI bleeding[3,5]. Meanwhile, multiple studies have shown that up to 30%-60% of patients experience recurrent bleeding (defined as 2 or more episodes) regardless of intervention[3,4], and a large portion of patients require repeat interventions for recurrent bleeding[6]. There are limited data on whether endoscopic intervention reduces recurrent bleeding, bringing into question its utility in managing this chronic issue.

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The primary aim of this study was to evaluate whether endoscopic intervention could prevent a subsequent GIB in patients with LVADs. Secondary aims were to assess the frequency of GIB in our cohort, describe GIB presentations and sources identified, and determine risk factors for recurrent GIB.

MATERIALS AND METHODS

Study population

We performed a retrospective cohort study of all patients ≥ 18 years old who underwent LVAD implantation between January 1, 2011 and December 31, 2018 at our large academic institution. For these 319 patients, we reviewed the electronic medical record (EMR) for demographics, and the date, purpose, and type of LVAD implantation. We excluded patients with temporary devices implanted (CentriMag, Thor BiVAD, Total Artificial Heart), resulting in a total of 295 patients. Data were collected from time of LVAD implantation until death, heart transplant, LVAD explantation, or last contact through the EMR, defined as the number of days followed. This chart review was conducted by two clinical physicians and a medical doctorate-trained research assistant.

For each patient, we reviewed the EMR for hospital encounters from January 1, 2011 through December 31, 2019 to identify instances of GIB on admission or during hospitalization, as indicated in discharge summaries, GI consult notes, and/or endoscopic procedure notes. We included encounters as a GIB if there was overt bleeding reported or documented, or the cardiology team documented suspicion for a GIB based on a drop in hemoglobin or other clinical factors with the lack of other explanation. All encounters with procedures for non-bleeding related indications, like colon cancer screening, or iatrogenic bleeding specifically from prior endoscopic procedures were excluded from analysis. Per standard practice by the cardiology team, patients with concern for GIB are managed in the inpatient setting, so there are no outpatient endoscopic procedures.

For each GIB encounter, we recorded laboratory data, blood transfusion requirements, endoscopic data including video capsule endoscopy findings, and relevant patient medications on admission and discharge (AC, antiplatelet, and octreotide). For encounters when GIB was present on admission, we recorded the length of stay. We classified the GIB presentation as overt *vs* occult, where overt indicated bloody output from the GI tract (*i.e.* hematemesis, hematochezia, melena, coffee-ground emesis), and occult indicated no bloody output visualized but the presence of a hemoglobin drop with no other known etiology.

During a GIB encounter, the primary admitting cardiology team would consult the GI service to determine whether to perform an endoscopic procedure. Medical management regarding acid suppression therapy and octreotide was at the discretion of the cardiology team; standard of care was to continue or initiate acid suppression therapy if concerned for upper GI source and only octreotide if concerned for variceal source. Radiologic studies like computed tomography were performed at the discretion of the cardiology team. All endoscopic procedures were performed with GI endoscopists in the inpatient endoscopy operating room under monitored anesthesia care and the presence of an LVAD coordinator. For patients with elevated international normalized ratio (INR)s, endoscopic procedures aside from video capsule endoscopy (VCE) were performed after an INR normalized to 1.5 or below. The decision to proceed with planned endoscopy based on clinical status of the patient and performance of an endoscopic intervention was at the discretion of the GI endoscopist.

This project was reviewed and determined to qualify as Quality Improvement by the University of Pennsylvania's Institutional Review Board.

Data collection

We reviewed each GIB encounter for endoscopic procedures, including upper endoscopy (EGD) push enteroscopy, single balloon enteroscopy, double balloon enteroscopy, colonoscopy, and VCE. Data were extracted from procedure reports for the presence of a bleeding source and the occurrence of an endoscopic intervention. Endoscopic interventions were defined as epinephrine injection, clip placement, argon plasma coagulation (APC), and bipolar coagulation. For VCE studies, we recorded whether a source was identified and whether an endoscopic procedure occurred afterwards with or without endoscopic intervention.

Study data were collected and managed using Research Electronic Data Capture (REDCap) electronic data capture tools hosted at the University of Pennsylvania[7,8]. REDCap is a secure, web-based software platform designed to support data capture for research studies, providing: (1) An intuitive interface for validated data capture; (2) Audit trails for tracking data manipulation and export procedures; (3) Automated export procedures for seamless data downloads to common statistical packages; and (4) Procedures for data integration and interoperability with external sources.

Power calculation

Based on pilot data suggesting that endoscopic interventions occurred in about a third of encounters, we used a 2:1 allocation ratio. We assumed that endoscopic intervention would decrease the probability of a subsequent GIB from 30% to 50% based on pilot data and what we deemed clinically significant. In order to reject the null hypothesis that endoscopic intervention did not affect subsequent bleeding with a probability (power) of 80% and a type I error probability of 0.05, we needed to study a total of 200 GIB encounters.

Independent (exposure) and dependent (outcome) variables

For the primary aim, the primary outcome variable was a subsequent encounter for GIB. Independent variables included: GIB presentation (overt *vs* occult as defined above), change in AC or antiplatelet therapy, defined as increasing or decreasing doses during the hospitalization or switching agents; days from hospital presentation to endoscopy; source identification during endoscopy or VCE; and endoscopic intervention.

For the secondary aims, the outcome variable was a first GIB. Independent variables included demographics, type of LVAD, purpose of LVAD, and days of exposure to LVAD, defined as the time since LVAD implantation.

Statistical analysis

To evaluate the demographic and clinical differences between those who never had a GIB *vs* those who had at least one, we used a paired *t* test and Wilcoxon rank-sum test for continuous variables that are normally distributed or skewed respectively, and Fisher's exact test for binary and categorical variables. For medians reported, we assessed interquartile range (IQR). We used logistic regression to determine the risk-adjusted impact of endoscopic intervention on subsequent GIB encounters. Risk adjustment variables included age, sex, race, AC status on admission, changes in AC during the admission, source of bleed if applicable, and clinical presentation of GIB. All analyses were performed using STATA version 13.0 (College Station, TX) and reviewed by a biomedical statistician.

RESULTS

Frequency of GIB in patients with LVADs

There was a total of 295 patients who had undergone LVAD implantation during the study period. The devices used were HeartMate2 (57.3%), Heartmate3 (11.2 %), and HeartWare (31.5%). 82.3% were male and median age at time of implant was 58.5 years. Patients were followed for a median of 601 d (IQR 165-1138); there were 120 patients who were followed for less than 1 year due to death, device explantation, or heart transplant.

Of the 295 patients, 97 (32.9%) patients presented for at least one GIB encounter. There was a total of 238 bleed encounters, of which 132 (55.4%) were within the first year of LVAD implantation. Time to index GIB was a median of 132 days (IQR 29-338). 87.4% (208/238) of the GIB encounters were in patients on active AC with either warfarin or a direct oral anticoagulant. Aspirin dose was 81mg daily and 325 mg daily in 25.6% (61/238) and 38.7% (92/238) encounters respectively. The most common presentation of GIB was melena (52.9%), followed by other overt GIB (total 21.4%; hematochezia 11.5%, hematemesis 3.0%, coffee-ground emesis 0.4%, not further characterized 6.5%) and occult bleeding (24.0%). Hemoglobin on presentation was a mean of 7.8 g/dL (IQR 6.3-8.8).

Patient characteristics are described in Table 1 comparing those who had at least one GIB *vs* those without one. Those with GIB were more likely to be older at the time of LVAD implant (age 60.8 *vs* 56.9; P = 0.01) and have a higher number of LVAD exposure days (348 *vs* 895, P < 0.01). They were also more likely to have the LVAD

Table 1 Comparative characteristics of patients with left ventricular assistant devices with gastrointestinal bleeding *vs* no gastrointestinal bleeding, *n* (%)

Factor		No GIB	At least 1 GIB	*P* value
n		198	97	
Age implant, median (IQR)		56.9 (46.9, 67.0)	60.8 (52.7, 69.8)	0.014
Sex (male)		160 (81.2)	82 (84.5)	0.52
Race	White	113 (57.1)	58 (59.8)	0.067
	Black	54 (27.3)	31 (32.0)	
	Asian	0 (0.0)	1 (1.0)	
	Other	12 (6.1)	5 (5.2)	
	Unknown	19 (9.6)	2 (2.1)	
Type of LVAD	Heartware	66 (33.3)	27 (27.8)	0.22
	HeartMate 2	114 (57.6)	55 (56.7)	
	HeartMate3	18 (9.1)	15 (15.5)	
LVAD purpose	Destination (DT)	113 (57.1)	70 (72.2)	0.030
	bridge to transplant (BTT)	80 (40.4)	25 (25.8%)	
	Bridge to Recovery (BTR)	5 (2.5)	2 (2.1)	
LVAD exposure (d) (IQR)		348 (103, 947)	895 (520, 1433)	< 0.001

The following statistical tests were utilized: Wilcoxon rank-sum test [age, left ventricular-assist device(LVAD)], and Fisher's exact test (sex, race, type of LVAD, LVAD purpose). GIB: gastrointestinal bleeding; IQR: Interquartile range; LVAD: Left ventricular-assist device.

placed as DT (72.2 % *vs* 57.4 %; $P = 0.03$), though in a logistic regression adjusted for the number of days followed, this was no longer significant ($P = 0.14$). Three patients died during active GIB; in two encounters, the patient had a LVAD thrombosis while AC was held, while in the third, the family declined further evaluation of the GIB.

Characteristics of endoscopic procedures and interventions

Of the 238 GIB encounters, 204 (85.7%) included at least one endoscopic evaluation including VCE. After excluding 13 encounters with only VCE evaluation, 191 (80.3%) had an invasive procedure. The median number of endoscopic procedures done per encounter was 2 (range 0 to 8). A source was identified in 130/238 (54.6%) encounters; when identified, the source was in the stomach (41.5%), deep small bowel (jejunum and ileum) (30.8%), colon (13.1%), duodenum (13.1%), and esophagus (1.5%). Of 115 encounters where the first procedure was an EGD, 9 (7.8%) included a push enteroscopy later in the same encounter.

An endoscopic intervention was performed in 34.8% (71/204) of encounters with endoscopic evaluation. The most common lesion intervened upon was angioectasias (Table 2). The second most common lesion intervened upon was non-specific oozing, referring to scenarios where there was no identifiable ulcer, angioectasia, or vessel. Other/uncharacterized category includes cases where the documentation did not express the source in the categorical terms. The most common type of intervention was APC and injection was always in conjunction with another intervention.

Among patients who presented to the hospital for a GIB, 45% of encounters with an endoscopic procedure performed within four days resulted in an endoscopic intervention during the hospitalization, compared to 25% if performed on days 5-7 from presentation, and 0 if performed later. Thus, days to the first endoscopic study impacts whether an endoscopic intervention was performed during the encounter [unadjusted odds ratio (OR) 0.80, confidence interval (CI) 0.67-0.96, $P = 0.018$].

Other predictors of endoscopic intervention included overt GIB compared to occult (OR 2.41, CI 1.14-5.10, $P = 0.022$) and transfusion with packed red blood cells (OR 10.0, CI 1.31-76.47, $P = 0.027$).

In encounters for occult bleeding, a culprit lesion was found in 42.1% of cases (24/57). An endoscopic intervention was performed in 10 of 24 (41.6%) and the attributed source was identified as an angioectasia in eight encounters and as oozing

Table 2 Lesion types and interventions used in endoscopic procedures with interventions, *n* (%).

Category		*n* (%)
Type of intervention	APC	37 (51.4)
	Hemoclip	31 (43.1)
	Injection	23 (31.9)
	Bipolar	16 (22.2)
Culprit lesion	Ulcer	8 (11.1)
	Angioectasia	34 (47.2)
	Dieulafoy	3 (4.2)
	Non-specific oozing	14 (19.4)
	Other/uncharacterized	13 (18.1)

Sum of percentages is greater than 100% as some procedures involved multiple interventions. One encounter had two lesions. APC: Argon plasma coagulation.

without a discrete lesion in the other two encounters. Of 12 encounters where a source was identified in the deep small bowel, six were addressed with endoscopic intervention. Of gastric occult sources, described as erosions, gastropathy, or gastritis, only one (1) underwent intervention.

Excluding the three encounters resulting in death before resolution of GIB, a source was not identified in 46.0% of encounters (108/235), and an intervention was not performed in 69.8% (164/235) encounters, in all of which the GIB resolved on its own by discharge.

Subsequent bleeding: Impact of source identification and endoscopic interventions

Patients who experienced a GIB had a mean of 2.2 encounters for GIB; 55.7% (54/97) of patients with GIB had 2 or more encounters for GIB and 59.2% (141/238) of all encounters were for recurrent GIB (2nd or greater episode). Of all patients with an LVAD, 11.9% (35/295) had 3 or more GIB encounters, resulting in 157 hospitalizations over the span of 7 years (mean 22.4 per year, max 52 in the year 2018).

Table 3 compares the encounter characteristics of GIB encounters with a subsequent GIB *vs* those without, where multiple encounters regarding the same patient are represented individually. There was no statistical difference in age, race, or sex. There was also no statistical difference whether there was a change in AC, if the GIB was overt *vs* occult, or if a source was identified. Endoscopic intervention during an encounter did not significantly impact the odds of a subsequent GIB (adjusted OR 1.18, $P = 0.58$). The median number of days to a subsequent GIB was 78 d (IQR 21-212) and not statistically different between encounters with endoscopic intervention and those without (Table 4). The proportion of encounters with subsequent GIB within 30 d was 29.5% in those with endoscopic intervention and 34.0% for those without, which was also not statistically different ($P = 0.37$). For those with GIB on admission, length of stay was median 12 days (IQR 8-21 d) and not statistically different between encounters with endoscopic intervention and those without ($P = 0.58$).

For subsequent bleeds when a prior source was not identified, a source on the current admission was identified in 20 of 45 (44%). Among the 51 encounters in which a source was identified in both the current GIB and the prior GIB, the source was in the same described area in 36 (70.6%) encounters. Of 22 cases of recurrent bleeding when the prior GIB source was deep small bowel, the current source was also in the deep small bowel in 18; the other 4 encounters sourced the bleed in the duodenum. Of the 28 patients who were found to have a small bowel bleed on at least one encounter, 11 (39.28%) patients had at least one subsequent bleed with a source identified in the small bowel. Otherwise, there was no significant association between the location of bleed identified and the presence of a subsequent bleed.

DISCUSSION

GIB is one of the most common complications in LVAD patients after implantation

Table 3 Comparative characteristics of gastrointestinal bleeding encounters with a subsequent gastrointestinal bleeding *vs* no subsequent gastrointestinal bleeding, *n* (%)

Factor		No subsequent GIB	Had a subsequent GIB	*P* value
n		97	141	
Change in anticoagulation		56 (64.4)	87 (65.4)	0.89
Overt bleed		75 (78.9)	102 (73.4)	0.36
Hemoglobin, median (IQR)		7.8 (6.8, 9.1)	7.5 (6.2, 8.4)	0.043
Source identified		51 (52.6)	79 (56.0)	0.69
Culprit lesion	Ulcer	3 (11)	5 (11)	1.00
	Angioectasia	13 (48)	21 (48)	
	Dieulafoy	1 (4)	2 (5)	
	Non-specific oozing	5 (19)	9 (20)	
	Other	5 (19)	7 (16)	
Culprit lesion location	Esophagus	2 (2.2)	0 (0.0)	0.38
	Stomach	24 (25.8)	30 (22.6)	
	Duodenum	6 (6.5)	11 (8.3)	
	Deep small bowel	12 (12.9)	28 (21.1)	
	Colon	7 (7.5)	10 (7.5)	
	Not identified	42 (45.2)	54 (40.6)	
Endoscopic intervention performed		27 (27.8)	44 (31.2)	0.58
Days to first endoscopic study (mean ± SD)		3.4 ± 7.1	2.9 ± 3.6	0.52

Deep small bowel refers to jejunum and ileum. The following statistical tests were utilized: paired *t* test (days to first endoscopic study), Wilcoxon rank-sum test (age, hemoglobin), and Fisher's exact test (change in anticoagulation, overt bleed, culprit lesion, culprit lesion location, endoscopic intervention performed). GIB: Gastrointestinal bleeding; IQR: Interquartile range; SD: Standard deviation.

Table 4 Outcomes for gastrointestinal bleeding encounters with endoscopic intervention *vs* none gastrointestinal bleeding

Factor	Endoscopic intervention	No endoscopic intervention	*P* value
Median number of days to subsequent GIB (IQR)	113 (15-302)	72 (24-178)	0.51
	n = 44	*n* = 97	
Proportion with subsequent GIB within 30 days	29.5%	34.0%	0.37
	n = 44	*n* = 97	
Median length of stay in days for those with GIB on admission (IQR)	12 (10-23)	12 (8-21)	0.58
	n = 31	*n* = 86	

The following statistical tests were utilized: Wilcoxon rank-sum test [days to subsequent gastrointestinal bleeding (GIB), length of stay], and Fisher's exact test (proportion with subsequent GIB within 30 d). GIB: Gastrointestinal bleeding; IQR: Interquartile range.

and has become a frequent cause of hospitalization for this population. Patients may have multiple bleeding episodes and nearly 10% of LVAD patients will have 3 or more encounters for GIB. This is the first study to our knowledge that is powered to evaluate whether endoscopic intervention reduces the risk for subsequent GIB. Our results confirm that a high proportion of GIB in our LVAD population clinically stops without endoscopic therapy and that endoscopic intervention does not prevent subsequent bleeding.

Our cohort size is within the wide range of sizes of studied cohorts at other tertiary care centers in terms of the numbers of patients with LVAD implantations and of the encounters for GIB bleeding[3,9,10]. Median time to bleed varies in the prior literature from 55 d to 197 d[3,9,10], where our composite median time of 129 d may reflect a higher proportion of late and recurrent GIB and fewer early GIBs. We found similar factors that correlated with GIB and findings: age correlated with having a GIB[9,11], while overt presentation and need for transfusion support correlated with performing endoscopic interventions[11]. The highest diagnostic yield was confirmed for upper procedures including EGD and push enteroscopy[9,11]. We also found a high burden of GIB caused by angioectasias[5,9]. While Dakik *et al*[6] and Axelrad *et al*[9] found that hemostatic therapy during an index examination was a statistically significant risk factor for a subsequent GIB, this was not the case in our cohort that had more GIB encounters and was followed for a longer period of time. Several factors may have had an impact on this disparity, including cohort size, efficacy of operator specific treatment techniques, selection of significant lesions, nuances in timing, or subtleties in patient demographics.

There are several areas that still warrant further investigation. First, it is unclear if the suboptimal initial diagnostic yield is attributable to delay in endoscopy, impediments imposed by anticipation of INR normalization or completion of bowel preparation, or intermittent visibility and bleeding of lesions such as angioectasias. These variabilities may explain why subsequent GIB encounters are able to isolate a source in some encounters. This low rate of identifying a source is consistent with other studies [9]. While source identification or endoscopic intervention on a visualized lesion did not result in reducing subsequent GIB or readmission rates, other benefits to endoscopy such as shorter length of stay may exist, although we did not find this in our cohort. Addressing these issues may be important in improving the success of endoscopic intervention.

Our findings indicate that minimizing endoscopic utilization may be beneficial for patients and healthcare utilization. There are a few proposed solutions to reduce the burden of low-yield procedures and reduce delay to source identification. Axelrad *et al* [12] proposed an endoscopic algorithm that consisted of push enteroscopy, instead of EGD, or colonoscopy for overt signs of bleeding, along with conservative management without endoscopic evaluation for occult bleeding, and found in a retrospective analysis that this method would improve resource utilization and limit lower-yield procedures. While VCE is often a second line study for persistent bleeding after negative upper and lower endoscopic evaluation[3], Marya *et al*[13] determined that early VCE compared to standard approaches to endoscopy in patients presenting with non-hematemesis GIB increased source localization, with no difference in direct costs of hospitalization. We propose that the VCE be performed urgently in the acute setting while awaiting normalization of the INR prior to possible endoscopy, especially when an endoscopic evaluation has been performed on a prior encounter. This proposal may obviate unnecessary endoscopies should the VCE exclude a targetable lesion, or help identify the most appropriate and high-yield procedure (EGD, push enteroscopy, or balloon enteroscopy) for treatment of an accessible lesion or indicative presence of blood. The yield and cost effectiveness of this strategy deserves further study.

Alternatively, intervention with endoscopic techniques may simply be an inappropriate long-term approach to treatment of certain common hemorrhagic lesions such as angioectasias, which likely represent a systemic process rather than a cluster of focal endoscopic lesions. Designing a randomized trial which withheld endoscopic intervention from some patients with GIB may be impractical given the unclear criteria by which to exclude higher risk patients. However, the creation of a prognostic risk score for patients with LVAD-related GIB could help triage low and high risk patients to different care pathways.

More importantly, recognizing that endoscopy may be only a temporizing measure, there is an urgent need for utilization of medical management protocols to prevent recurrent bleeding. Angioectasia recidivism after endoscopic therapy is common, and endoscopy is rarely a long term solution. Early data suggest that blockade of the angiotensin II receptor activation with angiotensin-converting enzyme inhibitors (ACE inhibitors) or angiotensin receptor blockers may reduce GIB episodes by reducing angioectasia formation[14]. Yin *et al*[10] built a model to predict who may have recurrent bleeding based on age and comorbidities while Welden *et al*[15] found better INR control and early endoscopy within 48 h of admission as clinical predictors of reduced recurrent GIB[15]. AC regimens need better study and standardization to best balance the risks of bleeding and thrombosis[16].

Collaboration amongst gastroenterologists and cardiologists is paramount for achieving optimal patient care, including the management of anti-platelet and AC therapies, LVAD pump speed adjustments, and endoscopic guidance. Alternative pharmaceutical strategies for reducing GIB in this population include the use of octreotide, thalidomide in restricted populations, and desmopressin (which increases the risk for thrombosis); ultimately, cardiac transplantation significantly reduces future GIB, when pulsatile flow and other parameters have been restored[17].

There are several limitations to our study. Our cohort was adequately powered to evaluate for a significant relationship between endoscopic intervention and subsequent GIB, but a larger patient cohort may be able to find statistical significance for a subpopulation, such as those with overt *vs* occult bleeding or with specific sources of bleeding. The retrospective and observational nature of our study limited the ability to show an impact of other parameters like change in AC or change in acid suppression therapy upon admission. Data abstraction was limited for some earlier encounters, including comorbidities and general health condition, due to an interim transition in EMR. There are other factors that contribute to the decisions for GIB management by the individual cardiologists and gastroenterologists that may not have been captured on chart review. We were able to evaluate for GIB encounters within our hospital system and may have missed other GIB admissions, though we think this to be an infrequent scenario as these patients are closely followed by our cardiology colleagues.

CONCLUSION

Patients with LVAD implantation are at high risk for recurrent GIBs. A majority of episodes of GIB resolve without endoscopic intervention. While endoscopic intervention for GIB is the established practice for the general population, our data do not support this position for patients with LVAD. The percentage of patients who had recurrent bleeding after endoscopic intervention was not statistically different than in those who had no intervention (P = 0.92). The rational and focused use of endoscopy, as part of an algorithm for GIB tailored specifically to patients with LVAD, is paramount for providing optimal patient care, limiting risks, and using resources most effectively. We advocate for more prospective evidence which will help support the creation of an evidence-based protocol to manage recurrent GIB and prevent future occurrences.

ARTICLE HIGHLIGHTS

Research background

Patients with left ventricular assist devices (LVADs) are at increased risk for recurrent gastrointestinal bleeding (GIB) and repeat endoscopic procedures.

Research motivation

There are limited data on whether endoscopic intervention reduces recurrent bleeding, bringing into question its utility in managing this chronic issue.

Research objectives

Our primary aim was to evaluate for an association between endoscopic intervention and subsequent GIB. Secondary aims were to assess the frequency of GIB in our cohort, describe GIB presentations and sources identified, and determine risk factors for recurrent GIB.

Research methods

We conducted a retrospective cohort study of all patients at a large academic institution who underwent LVAD implantation from January 2011 – December 2018 and assessed all hospital encounters for GIB through December 2019. We performed a descriptive analysis of the GIB burden and the outcome of endoscopic procedures performed. We performed multivariate logistic regression to evaluate the association between endoscopic intervention and subsequent GIB.

Research results

In the cohort of 295 patients, 97 (32.9%) had at least one GIB hospital encounter and recurrent GIB occurred in 55.5% (54/97) of patients. There were 238 hospital encounters, and GIB resolved on its own by discharge in 69.8% encounters. Of the 85.7% (204/238) of encounters that included at least one endoscopic evaluation, an endoscopic intervention was performed in 34.8% (71/204). The adjusted odds ratio for subsequent GIB if an endoscopic intervention was performed during a GIB encounter was not significant (odds ratio 1.18, $P = 0.58$).

Research conclusions

In this retrospective cohort study, adherence to endoscopic guidelines for performing endoscopic interventions did not significantly decrease the odds of subsequent GIB, thus suggesting the uniqueness of the LVAD population.

Research perspectives

A prospective study is needed to identify patients with LVAD at risk of recurrent GIB and determine more effective management strategies.

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ORIGINAL ARTICLE

Retrospective Study

Diverse expression patterns of mucin 2 in colorectal cancer indicates its mechanism related to the intestinal mucosal barrier

Guo-Lian Gan, Hua-Tao Wu, Wen-Jia Chen, Chun-Lan Li, Qian-Qian Ye, Yi-Feng Zheng, Jing Liu

ORCID number: Guo-Lian Gan 0000-0001-8512-1730; Hua-Tao Wu 0000-0002-1640-6094; Wen-Jia Chen 0000-0001-7157-3242; Chun-Lan Li 0000-0002-1649-9059; Qian-Qian Ye 0000-0003-3752-3238; Yi-Feng Zheng 0000-0002-5366-2388; Jing Liu 0000-0002-7483-4572.

Author contributions: Liu J and Zheng YF contributed to the conception and design of the study; Gan GL and Wu HT performed most of the experiments, analyzed the data, and drafted and revised the manuscript carefully; Chen WJ, Li CL, Ye QQ, and Zheng YF performed some of the experiments and interpreted the data; Wu HT, Zheng YF, and Liu J provided essential reagents; Liu J assisted with the experimental design and data analysis, and revised the original manuscript critically; all authors contributed to manuscript revision, and read and approved the submitted version.

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Guo-Lian Gan, Hua-Tao Wu, Yi-Feng Zheng, Department of General Surgery, The First Affiliated Hospital of Shantou University Medical College, Shantou 515041, Guangdong Province, China

Wen-Jia Chen, Chun-Lan Li, Qian-Qian Ye, Jing Liu, Changjiang Scholar's Laboratory, Shantou University Medical College, Shantou 515041, Guangdong Province, China

Wen-Jia Chen, Chun-Lan Li, Qian-Qian Ye, Jing Liu, Guangdong Provincial Key Laboratory for Diagnosis and Treatment of Breast Cancer, Shantou University Medical College, Shantou 515041, Guangdong Province, China

Wen-Jia Chen, Chun-Lan Li, Qian-Qian Ye, Jing Liu, Department of Physiology, Shantou University Medical College, Shantou 515041, Guangdong Province, China

Corresponding author: Jing Liu, MD, PhD, Associate Professor, Changjiang Scholar's Laboratory, Shantou University Medical College, No. 22 Xinling Road, Shantou 515041, Guangdong Province, China. jliu12@stu.edu.cn

Abstract

BACKGROUND

Abnormal expression patterns of mucin 2 (MUC2) have been reported in a variety of malignant tumors and precancerous lesions. Reduced MUC2 expression in the intestinal mucosa, caused by various pathogenic factors, is related to mechanical dysfunction of the intestinal mucosa barrier and increased intestinal mucosal permeability. However, the relationship between MUC2 and the intestinal mucosal barrier in patients with colorectal cancer (CRC) is not clear.

AIM

To explore the relationship between MUC2 and intestinal mucosal barrier by characterizing the multiple expression patterns of MUC2 in CRC.

METHODS

Immunohistochemical staining was performed on intestinal tissue specimens from 100 CRC patients, including both cancer tissues and adjacent normal tissues. Enzyme-linked immunosorbent assays were performed on preoperative sera from 66 CRC patients and 20 normal sera to detect the serum levels of MUC2, diamine oxide (DAO), and D-lactate (D-LAC). The relationship between MUC2 expression and clinical parameters was calculated by the χ^2 test or Fisher's exact test. Prognostic value of MUC2 was evaluated by Kaplan-Meier curve and log-rank

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tests.

RESULTS

Immunohistochemical staining of 100 CRC tissues showed that the expression of MUC2 in cancer tissues was lower than that in normal tissues (54% *vs* 79%, $P < 0.05$), and it was correlated with tumor-node-metastasis (TNM) stage and lymph node metastasis in CRC patients ($P < 0.05$). However, the serum level of MUC2 in CRC patients was higher than that in normal controls, and was positively associated with serum levels of human DAO ($\chi^2 = 3.957$, $P < 0.05$) and D-LAC ($\chi^2 = 7.236$, $P < 0.05$), which are the biomarkers of the functional status of the intestinal mucosal barrier. And the serum level of MUC2 was correlated with TNM stage, tumor type, and distant metastasis in CRC patients ($P < 0.05$). Kaplan-Meier curves showed that decreased MUC2 expression in CRC tissues predicted a poor survival.

CONCLUSION

MUC2 in tissues may play a protective role by participating in the intestinal mucosal barrier and can be used as an indicator to evaluate the prognosis of CRC patients.

Key Words: Colorectal cancer; Mucin 2; Mucin; Expression; Intestinal mucosal barrier; Prognosis

Core Tip: This study found that mucin 2 (MUC2) in intestinal tissues may play a protective role on the intestine and can be used as one of the indicators to evaluate the prognosis of patients with colorectal cancer (CRC). When the intestinal mucosal barrier function of patients with CRC is impaired, the serum level of MUC2 can reflect the severity of the damage. Therefore, in CRC patients with impaired intestinal mucosal barrier function, the serum level of MUC2 could reflect the severity of the damage, providing a potential mechanism for the development of therapeutic strategies for CRC patients.

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INTRODUCTION

The incidence of colorectal cancer (CRC) ranks third in the world among the common malignant tumors, and the mortality ranks second[1]. Due to large changes in lifestyle and dietary habits, the incidence and mortality of CRC will continue to rise. In China, there are 300000 new CRC cases each year, with an annual average increase of 4.2%[2]. There are obvious gender and regional differences in the incidence and mortality rate of CRC cases, with the overall distribution being more in males than in females, and more in urban areas than in rural areas[3]. Therefore, CRC is one of the major diseases that seriously threatens human life and health, and the situation is quite serious.

The etiological mechanism of CRC tumorigenesis and development is extremely complex, involving a variety of genetic and environmental factors[4]. Among them, the impairment of intestinal barrier structure and function leads to a series of pathophysiological changes in the intestinal mucosa, which eventually evolves into tumor malignancy. Mucins (MUCs) are the main components of the mucus layer, which provides the first-line defense against infection and participates in the process of intercellular adhesion, intercellular communication, and immune regulation[5].

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Among 27 reported MUC proteins, MUC2 is a secretory mucin involved in the formation of mucus, and is mainly secreted by goblet cells[6]. Reduced MUC2 expression in the intestinal mucosa, caused by various pathogenic microorganisms and/or toxic substances, induces apoptosis of intestinal mucosal epithelial cells and destroys the mechanical barrier of the intestinal mucosa, ultimately leading to increased intestinal mucosal permeability[7]. Interestingly, an abnormal expression pattern of MUC2 has been reported in a variety of malignant tumors and precancerous lesions, suggesting an important role for MUC2 in the occurrence and development of CRC[8, 9]. However, the expression of MUC2 is tissue- and cell-specific[10]. Kasprzak *et al*[8] reported a distinct expression pattern of MUC2 in mucinous *vs* non-mucinous colorectal adenocarcinoma[8], suggesting that diverse and specific mechanisms are involved in different types of CRC.

To verify the diverse expression patterns of MUC2 in CRC patients, the current study enrolled CRC patients and investigated MUC2 levels in both malignant tissue and serum to provide an underlying mechanism of MUC2 related to intestinal barrier function, and lay a foundation for further revealing the molecular mechanism of MUC2 involvement in the malignant biological behavior of CRC.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Ethics Committee of the First Affiliated Hospital of Shantou University Medical College. All patients or their guardians signed written informed consent before the study. All experiments were conducted following the guidelines of the Ethics Review Committee.

Patient information

Cancer tissues and adjacent normal intestinal mucosal tissues (> 5 cm away from the tumor) were collected from 100 CRC patients who underwent radical resection at the Department of Gastrointestinal Surgery of the First Affiliated Hospital of Shantou University Medical College from January 2015 to December 2016. The inclusion criteria included: (1) Age from 18 to 90; (2) No medical history of blood, cardiovascular, or immune disease, or inflammatory bowel disease; (3) Pathological diagnosis of CRC; (4) Tumor-node-metastasis (TNM) stages I to III; and (5) Patients who underwent radical surgery. The exclusion criteria included: (1) Preoperative history of neoadjuvant chemotherapy or radiotherapy; (2) Binary or multivariate cancer; (3) Preoperative complications, such as malignant intestinal obstruction, perforation, or bleeding; (4) No standardized chemotherapy after the radical surgery; and (5) Incomplete clinicopathological data.

Peripheral blood was collected before surgery from 66 CRC patients who were diagnosed with CRC at the Department of Gastrointestinal Surgery of the First Affiliated Hospital of Medical College of Shantou University from January 2018 to December 2019. The inclusion criteria were almost the same as above, except that patients at stage IV were also recruited. The exclusion criteria were the same. For comparison, 20 normal subjects in the same period were recruited and their sera were collected in the Health Management Center of the First Affiliated Hospital of Shantou University Medical College.

Histology and immunohistochemistry

Formalin-fixed and paraffin-embedded CRC tissues were cut into 4-μm sections and stained with hematoxylin and eosin. Histopathological differentiation was made by two pathologists based on the World Health Organization criteria. TNM pathological staging was determined according to the staging manual of the American Joint Committee on Cancer[11].

Immunohistochemistry (IHC) was conducted as described before[12]. CRC and adjacent normal tissues were dewaxed in xylene, hydrated in a series of graded alcohols, and placed in a citric acid buffer for epitope retrieval. After immersion in 3% H_2O_2 solution to block endogenous peroxidase, the slides were incubated with anti-MUC2 monoclonal antibody (ab118964, Abcam, United Kingdom) at 4 °C overnight. Negative controls were treated with PBS instead of primary antibody. Haematoxylin was used for counterstaining.

Sections were visualized under a bright-field microscope (CKX41, Olympus, Japan) and evaluated independently by two investigators with no prior knowledge of the patient information. For tissue expression of MUC2, staining intensity was recorded as

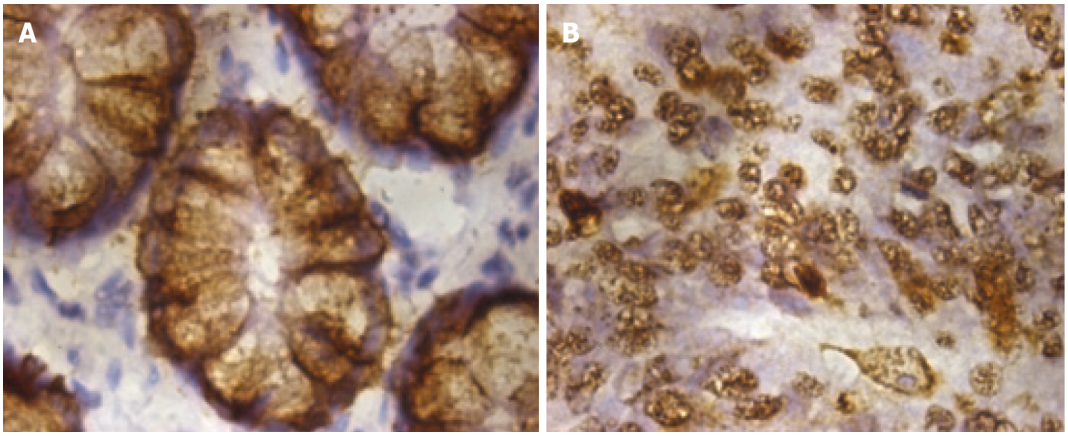

Figure 1 Mucin 2 is mainly located in the cytoplasm. A: Normal tissue; B: Colorectal cancer tissue. Magnification: × 1000.

0, 1, 2 and 3 for colorless, light yellow, brown yellow, and dark brown, respectively, and the percentage of positive cells < 5%, 6%-30%, 31%-60%, and 61%-100% was scored as 0, 1, 2, and 3 points, respectively. To evaluate the expression level of MUC2, the scores of staining intensity and the percentage of positive cells were added and defined as low expression (0-3) and high expression (4-6).

Enzyme-linked immunosorbent assay

The collected peripheral blood samples were centrifuged at 2000-3000 rpm for 15 min, and the supernatant serum was collected carefully and stored in -80 ℃. Human MUC2, human diamine oxide (DAO), and human D-lactate (D-LAC) enzyme-linked immunosorbent assay detection kits were purchased from Nanjing Senberga Biotechnology Co., Ltd., China. Experiments were carried out as previously described[13]. The standard curve was determined by the standard concentration and their corresponding absorbance (OD value). The actual concentrations of MUC2, DAO, and D-LAC were calculated based on the standard curves.

Follow-up and statistical analysis

Overall survival (OS) time was calculated in months from the date of diagnosis to the date of death of the patient or the last follow-up visit. Disease-free survival (DFS) time was determined by the date of relapse.

SPSS 21.0 statistical software was used to analyze the data. Enumerated data are expressed by the number of cases (N), and the measurement data that conformed to a normal distribution are expressed as the mean ± SD. The relationship between MUC2 and the patient's clinicopathological data was tested by χ^2 and Fisher's exact probability tests. The relationship between serum MUC2, DAO, and D-LAC was analyzed by the χ^2 test. The Kaplan-Meier survival curve and log-rank test were used to evaluate the association of MUC2 expression with prognosis. The difference between two groups was statistically significant at $P < 0.05$.

RESULTS

Expression of MUC2 is decreased in cancer tissues compared with normal tissues in CRC patients

To detect the location of MUC2 in intestinal tissues, IHC was performed and revealed that MUC2-positive staining was mainly located in the cytoplasm, both in normal tissues and CRC tissues (Figure 1). In normal tissues, the cytoplasm was diffusely and homogeneously positive, and the nucleus was not stained, while in cancer tissues, cells lost their normal morphology, adenoid structures were destroyed or had even disappeared, and heterotypic cancer cells could be detected.

Representative images of MUC2 staining in adjacent and cancer tissues are shown in Figure 2. Interestingly, the percentage of tissues with high MUC2 expression was significantly decreased from 79% in adjacent normal tissues to 54% in CRC tissues (Figure 3). The difference in MUC2 expression between normal and cancer tissues was statistically significant ($P < 0.01$; Table 1)

Table 1 Comparison of mucin 2 expression in normal and cancer tissues of patients with colorectal cancer (cases)

Group	MUC2 expression		χ^2	*P* value
	Low	High		
Normal tissues	21	79	14.028	< 0.01[1]
Cancer tissues	46	54		

[1]Indicates that the difference was statistically significant, which confirmed that the expression difference of mucin 2 (MUC2) in normal tissues and cancer tissues was statistically significant, and the high expression rate of MUC2 in cancer tissues was lower than that in normal tissues. MUC2: Mucin 2.

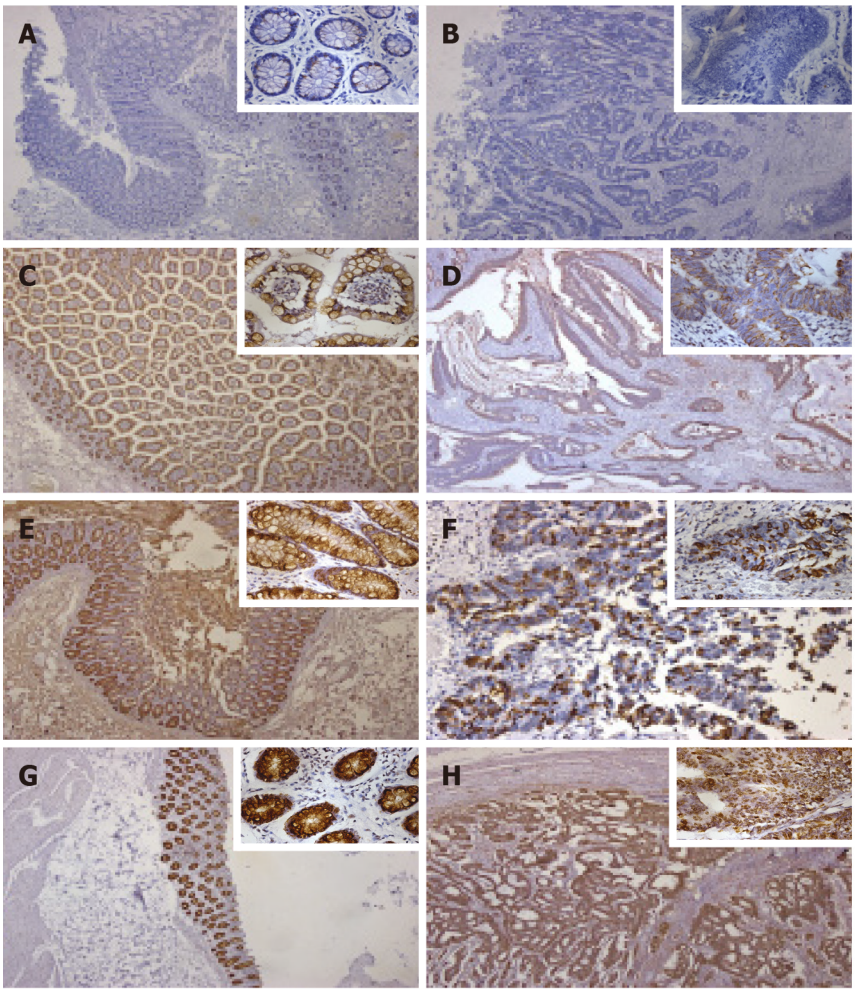

Figure 2 Representative pictures of immunohistochemical staining for mucin 2 in normal and cancer tissues of patients with colorectal cancer. A and B: Negative expression; C and D: Weak expression; E and F: Moderate expression; G and H: Strong expression. A, C, E, and G: Normal tissue; B, D, F, and H: Cancer tissue. Magnification: × 100 and × 400 (located in the upper right corner of each image).

Tissue MUC2 level is negatively associated with TNM stage and lymph node status in patients with CRC

Clinicopathological analysis showed that tissue MUC2 expression (low *vs* high) was not significantly associated with the age at diagnosis, gender, tumor location, tumor size, depth of invasion, degree of differentiation, or tumor type (Table 2). In comparison with tumors at stage III, tumors at stages I and II showed significantly more tissue MUC2 expression (χ^2 = 13.963, P < 0.05). Importantly, low expression of tissue MUC2 was more frequently observed in patients with lymph node metastasis (χ^2 = 12.538, P < 0.05) (Table 2).

Table 2 Relationship between expression of mucin 2 and clinicopathological parameters in patients with colorectal cancer, *n* (%)

Clinicopathological parameter	*n* (%)	MUC2 expression		χ^2	*P* value
		Low, *n* (%)	High, *n* (%)		
Age, yr					
≤ 60	48	22 (45.8)	26 (54.2)	0.001	0.974
> 60	52	24 (46.2)	28 (53.8)		
Gender					
Male	52	23 (44.2)	29 (55.8)	0.137	0.712
Female	48	23 (47.9)	25 (52.1)		
Tumor location					
Rectum and anus	48	21 (43.8)	27 (56.3)	0.188	0.664
Colon	52	25 (48.1)	27 (51.9)		
TNM stage					
I-II	57	17 (29.8)	40 (70.2)	13.963	< 0.01
III	43	29 (67.4)	14 (32.6)		
Maximum tumor diameter					
< 5 cm	49	23 (46.9)	26 (53.1)	0.034	0.854
≥ 5 cm	51	23 (45.1)	28 (54.9)		
Depth of invasion					
Non-immersed serosa	22	8 (36.4)	14 (63.6)	1.054	0.304
Immersed serosa	78	38 (48.7)	40 (51.3)		
Degree of differentiation					
High-moderate	93	42 (45.2)	51 (54.8)	F[1]	0.700
Low	7	4 (57.1)	3 (42.9)		
Tumor type					
Mucinous adenocarcinoma	13	5 (38.5)	8 (61.5)	0.342	0.559
Non-mucinous adenocarcinoma	87	41 (47.1)	46 (52.9)		
Lymph node metastasis					
No	56	17 (30.4)	39 (69.6)	12.538	< 0.01
Yes	44	29 (65.9)	15 (34.1)		

[1]Fisher exact test was used when the expected frequency was less than 1. Due to the small number of patients with stage I, in order to reduce bias, we combined the stage I with stage II patients for analysis. Similarly, there was only one patient with highly differentiated colorectal cancer, so the patient was combined with patients with moderately differentiated disease. It can be seen from the above table that the expression of mucin 2 is correlated with tumor-node-metastasis stage and lymph node metastasis in colorectal cancer patients. MUC2: Mucin 2; TNM: Tumor-node-metastasis.

Serum levels of MUC2 are elevated in CRC patients, and positively associated with the levels of DAO and D-LAC in serum

Serum DAO and D-LAC are indicators of intestinal mucosal barrier permeability and integrity[14,15]. As expected, in patients with CRC, the serum levels of DAO and D-LAC were significantly increased compared with those in normal controls (Figure 4A and B), indicating impaired intestinal mucosal barrier function and increased intestinal permeability in CRC patients (Table 3). Interestingly, serum levels of MUC2 were also higher than those in normal controls (Figure 4C) and closely related to the serum levels of DAO and D-LAC (Table 4).

Table 3 Comparison of serum levels of diamine oxide, D-lactate, and mucin 2 between colorectal cancer patients and normal control (mean ± SD)

Group	*n* (%)	DAO (pg/mL)	D-LAC (μg/L)	MUC2 (ng/L)
Normal control	20	158.21 ± 15.98	973.69 ± 128.08	305.98 ± 31.50
CRC	66	185.40 ± 25.49	1216.93 ± 204.20	364.58 ± 48.30
P value		< 0.01	< 0.01	< 0.01

There were 66 cases of colorectal cancer (CRC) and 20 cases of normal controls. Serum levels of mucin 2, diamine oxide, D-lactate in CRC patients were higher than those in the normal control group. MUC2: Mucin 2; DAO: Diamine oxide; D-LAC: D-lactate; CRC: Colorectal cancer.

Table 4 Relationship between serum levels of mucin 2, diamine oxide, and D-lactate in patients with colorectal cancer (cases)

MUC2	DAO		D-LAC	
	Low	High	Low	High
Low	15	8	16	7
High	17	26	15	28
χ^2	3.957		7.236	
P value	0.047		0.007	

The measured mucin 2 (MUC2) level of P95 in the normal population was taken as the normal reference range, and those beyond P95 were regarded as increased, otherwise as decreased. Based on this, colorectal cancer patients were divided into groups with high and low levels of MUC2. Similarly, the number of cases in diamine oxide (DAO) and D-lactate (D-LAC) groups with high and low levels could be obtained. Serum MUC2 levels were positively correlated with DAO and D-LAC levels. Low: Low serum level; High: High serum level; MUC2: Mucin 2; DAO: Diamine oxide; D-LAC: D-lactate.

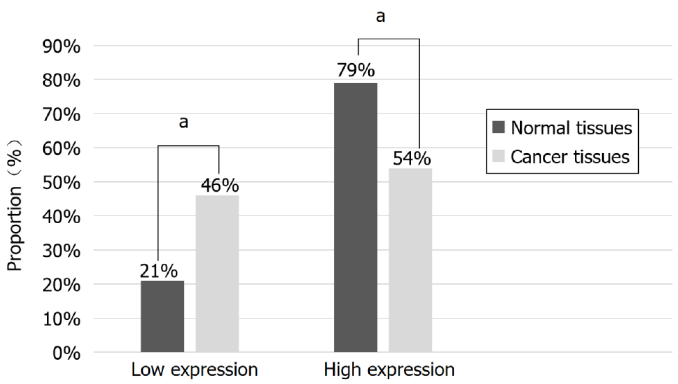

Figure 3 Histogram of mucin 2 expression in cancer and normal tissues of patients with colorectal cancer. According to the comprehensive score of staining intensity and positive cell percentage, 0-3 was classified as low expression and 4-6 as high expression. [a]*P* < 0.05.

Serum MUC2 is positively associated with TNM stage and distant metastasis in patients with CRC

As shown in Table 5, the higher the TNM stage in CRC patients, the higher the serum MUC2 level (P = 0.033). Importantly, the percentage of patients with high serum levels of MUC2 was dramatically increased in CRC patients with distant metastasis compared with those without (100.0% *vs* 59.6%, P = 0.022). The tumor type was also found to be related to the expression of serum MUC2 in CRC patients. CRC patients with mucinous adenocarcinoma had higher serum MUC2 levels than non-mucinous CRC patients (P < 0.01). However, serum MUC2 level was not associated with age at diagnosis, gender, tumor size, tumor location, depth of invasion, degree of differentiation, or lymph node metastasis (Table 5).

Low expression of MUC2 in cancer tissues predicts a poor survival in CRC patients

Kaplan-Meier curve analyses with log-rank test revealed that tissue MUC2 expression was significantly associated with DFS (P = 0.032) (Figure 5A) and OS (P = 0.037)

Table 5 Relationship between serum levels of mucin 2 and clinicopathological parameters in patients with colorectal cancer, *n* (%)

Clinicopathological parameter	*n* (%)	Serum MUC2 expression		χ^2	*P* value
		Low, *n* (%)	High, *n* (%)		
Age, yr					
≤ 60	30	11 (36.7)	19 (63.3)	0.080	0.777
> 60	36	12 (33.3)	24 (66.7)		
Gender					
Male	38	12 (31.6)	26 (68.4)	0.422	0.516
Female	28	11 (39.3)	17 (60.7)		
Tumor location					
Rectum and anus	24	10 (47.1)	14 (58.3)	0.772	0.380
Colon	42	13 (31.0)	29 (65.2)		
TNM stage					
I-II	36	16 (44.4)	20 (55.6)	6.687	0.033
III	21	7 (33.3)	14 (66.7)		
IV	9	0 (0.0)	9 (100.0)		
Maximum tumor diameter					
< 5 cm	33	12 (36.4)	21 (63.6)	0.067	0.796
≥ 5 cm	33	11 (33.3)	22 (66.7)		
Depth of invasion					
Non-immersed serosa	12	5 (41.7)	7 (58.3)	0.300	0.584
Immersed serosa	54	18 (33.3)	36 (66.7)		
Degree of differentiation					
High-moderate	60	21 (38.3)	39 (61.7)	F[1]	1.000
Low	6	2 (33.3)	4 (66.7)		
Tumor type					
Mucinous adenocarcinoma	14	8 (57.1)	6 (42.9)	21.241	< 0.01
Non-mucinous adenocarcinoma	52	15 (32.7)	37 (67.3)		
Lymph node metastasis					
No	36	16 (44.4)	20 (55.6)	3.212	0.073
Yes	30	7 (23.3)	23 (76.7)		
Distant metastasis					
No	57	23 (40.4)	34 (59.6)	F[1]	0.022
Yes	9	0 (0.0)	9 (100.0)		

[1]Fisher exact test was used when the expected frequency was less than 1. Due to the small number of patients with stage I, in order to reduce bias, we combined the stage I with stage II patients for analysis. Similarly, there was only one patients with highly differentiated colorectal cancer, so this patient was combined with the patients with moderately differentiated disease. Serum levels of mucin 2 was correlated with tumor-node-metastasis stage, tumor type, and distant metastasis in colorectal cancer patients. MUC2: Mucin 2; TNM: Tumor-node-metastasis.

(Figure 5B) in all CRC patients. Decreased tissue MUC2 level predicted a poor prognosis of CRC patients. During the 5-year follow-up period, the recurrence was 40.0% in patients with low expression of MUC2 and 18.5% in patients with high expression of MUC2 (χ^2 = 5.485, P < 0.05) (Figure 5C).

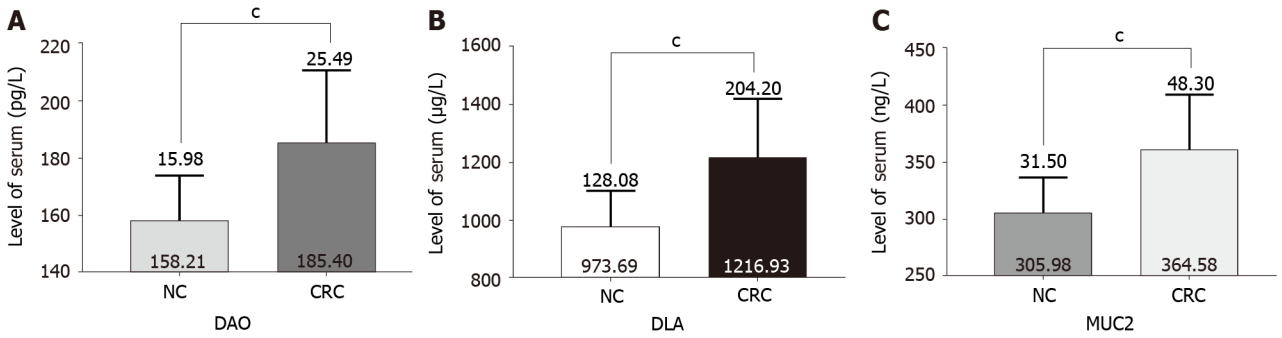

Figure 4 Comparison between the levels of serum diamine oxide, D-lactate, and mucin 2 in colorectal cancer patients and normal controls. A: The level of serum diamine oxidase in colorectal cancer (CRC) patients and normal controls (NC); B: The level of serum D-lactate in CRC patients and NC; C: The level of serum mucin 2 in CRC patients and NC. The numbers at the bottom of the bar chart represent the mean, and the numbers above the error line represent the standard deviation. °$P < 0.001$. CRC: Colorectal cancer; NC: Normal controls; DAO: Diamine oxidase; D-LAC: D-lactate; MUC2: Mucin 2.

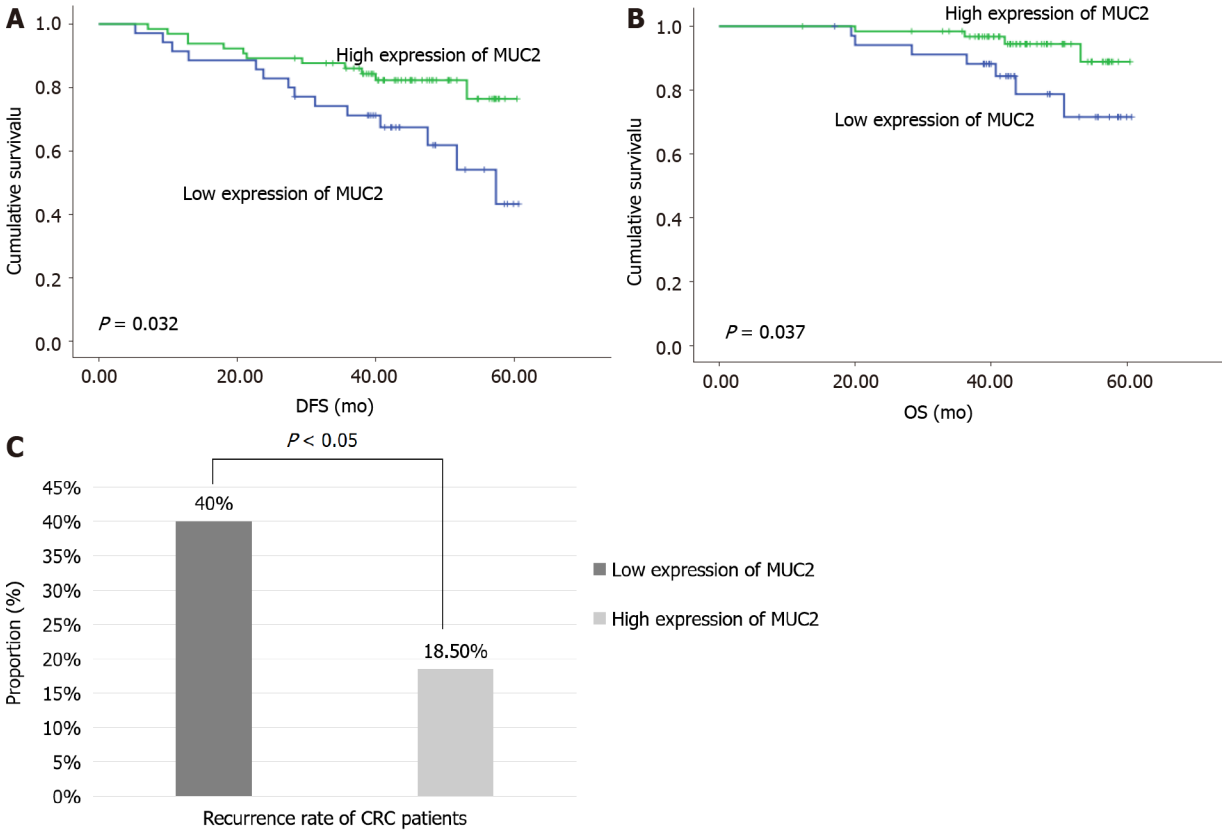

Figure 5 Prognostic value of tissue mucin 2 expression in colorectal cancer patients. A: Correlation between tissue mucin 2 (MUC2) expression and disease-free survival in colorectal cancer (CRC) patients; B: Correlation between tissue MUC2 expression and overall survival in CRC patients; C: Histogram of the recurrence rate of CRC patients with different tissue levels of MUC2 expression. DFS: Disease-free survival; OS: Overall survival. MUC2: Mucin 2; CRC: Colorectal cancer.

DISCUSSION

The protein encoded by the *MUC2* gene is the most abundant secreted mucin, covering the surface of the intestinal mucosa in the form of a gel and forming the skeleton of the mucus layer, protecting the intestine in many ways[16]. Recently, Javitt *et al*[17] presented an integrated structural analysis of the intestinal mucin MUC2, and revealed that the mucin assembly mechanism and its adaptation for hemostasis provide the foundation for rational manipulation of barrier function and coagulation[17]. On the other hand, CRC shows multiple complex pathologies based on the impaired structure and function of the intestinal mucosal barrier, and is associated with the disordered expression and dysfunction of mucins[18]. However, controversial findings of MUC2 function in the occurrence and development of CRC have required further invest-

igation to uncover the underlying mechanisms. We showed that MUC2 expression was decreased in carcinomas, compared with adjacent normal tissues, but CRC patients had higher serum levels of MUC2 compared with normal controls.

MUC2 plays an important role in maintaining the homeostasis of the intestinal environment and protecting susceptible bacteria from pathogenic microorganisms and/or toxic substances[19]. We detected decreased MUC2 in CRC tissue, compared to adjacent intestinal tissues, which may be related to the suppressed immune function in intestinal homeostasis. Importantly, decreased MUC2 expression was found to be associated with advanced CRC stage, suggesting a tumor-suppressive role in the development of CRC. In CRC patients with lymph node metastasis, the tissue MUC2 level was also lower than that in CRC patients without lymph node metastasis, further suggesting a potential protective role of MUC2 in lymph node metastasis in CRC patients. Although statistical significance was not found in the relationship between tissue MUC2 expression and tumor size, depth of invasion, or degree of differentiation, our results indicate that a more severe malignant biological behavior was more likely found in CRC tissues with low MUC2 expression, demonstrating a potential protective role of MUC2 in the development of CRC.

The expression of MUC2 in CRC tissues is reported to be related to the histopathological types of CRC. The expression of MUC2 in mucinous adenocarcinoma is increased, while that in non-mucinous adenocarcinoma is decreased[8,20]. In this study, patients with mucinous adenocarcinoma tended to have a higher proportion of patients with high expression of MUC2 than those with non-mucinous adenocarcinoma. However, the underlying roles of MUC2 in different types of CRC are still unclear and need further investigation.

Li *et al*[21] reviewed the prognostic and clinicopathological significance of MUCs in CRC, and demonstrated that upregulated MUC2 expression is associated with a better OS, while upregulated MUC1 expression is associated with a poor OS[21]. Elzagheid *et al*[22] reported that loss of MUC2 expression is associated with disease recurrence and tumor location. However, in multivariate survival analysis, MUC2 lost its power as an independent predictor of DFS and disease-specific survival[22]. To verify the prognostic value of MUC2 expression in CRC patients, we used Kaplan-Meier curve analyses and showed that low expression of tissue MUC2 was associated with a poor DFS and OS in CRC patients, which may be related to the high recurrence rate exhibited by CRC patients with low tissue MUC2 levels.

To investigate the potential function of secreted MUC2 in CRC patients, this study further analyzed the expression of serum MUC2 in CRC patients and normal controls. As serum DAO and D-LAC are biomarkers of the functional status of the intestinal mucosal barrier[23], we also monitored serum DAO and D-LAC levels. Interestingly, the serum level of MUC2 was positively related with serum DAO and D-LAC levels, which indicates dysfunction of intestinal mucosal barriers and increased intestinal permeability in CRC patients. The increased serum level of MUC2 may be associated with the progress of tumor infiltration.

The increased serum levels of MUC2 may be related to the fact that cancer cells gradually destroy the mucus layer, intestinal mucosal epithelial cells, and tight junctions, which result in the destruction of the MUC2 skeletal structure in the mucus layer, the apoptosis of intestinal mucosal epithelial cells, and abnormal expression and distribution of tight junction proteins. Decreased MUC2 in the intestinal mucosa and the damage of the intestinal barrier could result in invasion of various pathogenic microorganisms and toxic substances in the intestinal cavity to further aggravate the damage of the intestinal barrier and constitute a vicious circle, increasing intestinal mucosal permeability and promoting the translocation of MUC2 from epithelial cells to the blood.

CONCLUSION

This study found that MUC2 in intestinal tissues may play a protective role in the intestine and can be used as one of the indicators to evaluate the prognosis of patients with CRC. When the intestinal mucosal barrier function of patients with CRC is impaired, the serum level of MUC2 can reflect the severity of damage. Subsequent studies can further investigate the role of MUC2 in the malignant transformation of colorectal inflammatory diseases, cancer cell proliferation, invasion, metastasis, and the mechanism of resistance to chemotherapeutic drugs at the molecular level.

ARTICLE HIGHLIGHTS

Research background

At present, several studies have reported abnormal expression patterns of mucin 2 (MUC2) in cancerous lesions, including colorectal cancer (CRC). However, as a member of the intestinal mucosal mechanical barrier, the relationship between MUC2 and the intestinal mucosal barrier in patients with CRC remains unclear. Revealing this association will help us more fully understand the role of MUC2 in CRC.

Research motivation

Although many studies have proved that intestinal mucosal barrier function is impaired and MUC2 expression is abnormal in patients with CRC, the direct relationship between MUC2 and intestinal mucosal barrier has rarely been studied. The main problem to be solved in this study is to clarify this relationship and lay a foundation for further studies on the molecular mechanism of MUC2 involvement in CRC.

Research objectives

This study aimed to explore abnormal expression patterns of MUC2 and the relationship between MUC2 and intestinal mucosal barrier by characterizing the multiple expression patterns of MUC2 in CRC. The findings will provide a basis for further study of the pathogenesis of MUC2 in the process of intestinal mucosal barrier damage in CRC.

Research methods

Immunohistochemical staining was performed on cancer tissue and normal tissue samples from 100 patients with CRC to evaluate the expression of MUC2 in two different tissues, and these patients were followed for 12-60 mo to understand the overall survival (OS) and disease-free survival (DFS). Preoperative serum levels of MUC2, diamine oxide (DAO), and D-lactate (D-LAC) in 66 patients with CRC were detected by enzyme-linked immunosorbent assay and compared with those in 20 normal controls, so as to evaluate the damage of intestinal mucosal barrier in patients with CRC. The statistical methods involved in this study include χ^2 test, Fisher's exact test, Kaplan-Meier curve, and log-rank tests.

Research results

Immunohistochemical staining results showed that the expression of MUC2 in cancer tissues was lower than that in normal tissues (54% *vs* 79%, $P < 0.05$), and the expression of MUC2 was correlated with tumor-node-metastasis (TNM) stage and lymph node metastasis in CRC patients ($P < 0.05$), but not significantly related to the patient's age, sex, tumor location, size, depth of invasion, or degree of differentiation. The serum levels of MUC2, DAO, and D-LAC in patients with CRC were higher than those in normal people ($P < 0.05$), and were positively associated with serum levels of human DAO ($\chi^2 = 3.957$, $P < 0.05$) and D-LAC ($\chi^2 = 7.236$, $P < 0.05$), which are the biomarkers of the functional status of the intestinal mucosal barriers. It was suggested that the intestinal mucosal barrier was damaged, and MUC2 can also be used as a new evaluation index. The serum levels of MUC2 were correlated with TNM stage, tumor type, and distant metastasis in CRC patients ($P < 0.05$). It seems to be a trend that patients with higher malignancy and later stage of tumors have higher serum MUC2 levels. Survival analysis showed that decreased expression of MUC2 in CRC tissues predicted a poor survival. The expression of MUC2 in tissues was significantly correlated with DFS ($P = 0.032$) and OS ($P = 0.037$). And the recurrence rate of patients with low expression of MUC2 was higher than that of patients with high expression of MUC2 (40% *vs* 18.5%, $\chi^2 = 5.485$, $P < 0.05$).

Research conclusions

MUC2 in the intestinal tissue may play a protective role on the intestine, which can be used as an indicator to evaluate the prognosis of CRC patients. Intestinal mucosal barrier function of CRC patients is impaired, and the serum MUC2 level can reflect the severity of the damage.

Research perspectives

Future researchers can further study the molecular mechanism of MUC2 in the process of intestinal mucosal barrier damage, which may reveal the pathological mechanism of CRC from a new perspective and provide a basis for the development of new targeted

therapy drugs. In addition, related research can also be carried out in inflammatory bowel disease.

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ORIGINAL ARTICLE

Retrospective Study

Clinical characteristics of patients in their forties who underwent surgical resection for colorectal cancer in Korea

Chang Sin Lee, Se-Jin Baek, Jung-Myun Kwak, Jin Kim, Seon-Hahn Kim

ORCID number: Chang Sin Lee 0000-0002-4640-1753; Se-Jin Baek 0000-0002-3185-8777; Jung-Myun Kwak 0000-0002-2181-4279; Jin Kim 0000-0001-6479-9673; Seon-Hahn Kim 0000-0002-4526-5147.

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Chang Sin Lee, Se-Jin Baek, Jung-Myun Kwak, Jin Kim, Seon-Hahn Kim, Department of Surgery, Korea University College of Medicine, Seoul 02841, South Korea

Corresponding author: Se-Jin Baek, MD, PhD, Department of Surgery, Korea University College of Medicine, Goryeodae-ro 73, Seongbuk-gu, Seoul 02841, South Korea. xezin@korea.ac.kr

Abstract

BACKGROUND

The proportion of young patients with colorectal cancer (CRC), especially in their 40s, is increasing worldwide.

AIM

To confirm the clinical characteristics of such patients, we planned a study comparing them to patients in their 30s and 50s.

METHODS

Patients undergoing primary resection for CRC, patients in their 30s, 40s and 50s were included in the study. Patient and tumor characteristics, and perioperative and oncologic outcomes were compared.

RESULTS

Most clinical characteristics of 451 (10.5%) patients in their 40s were more similar to those of patients in their 30s than those in their 50s. On pathology data, there were more metastatic lesions (30s *vs* 40s *vs* 50s; 17.5% *vs* 21.1% *vs* 14.9%, $P = 0.012$) in patients in their 40s. There was a trend toward less frequent K-ras mutations among patients in their 40s (48.5% *vs* 33.3% *vs* 44.5%, $P = 0.064$). The proportion of patients receiving postoperative chemotherapy was also significantly greater among patients in their 40s (58.3% *vs* 63.9% *vs* 56.3%, $P = 0.032$). Five-year overall survival (OS) and disease-free survival (DFS) did not differ between the three groups (5-year OS, 92.2% *vs* 89.8% *vs* 92.2%, $P = 0.804$; 5-year total DFS, 98.6% *vs* 95.7% *vs* 96.1%, $P = 0.754$; 5-year local DFS, 98.6% *vs* 94.3% *vs* 94.9%, $P = 0.579$; 5-year systemic DFS, 86.4% *vs* 87.9 % *vs* 86.4%, $P = 0.908$).

CONCLUSION

Patients with CRC in their 40s showed significantly more numerous metastatic lesions. The oncologic outcome of stage 1-3 patients in their 40s was not inferior compared to that of those in their 30s and 50s.

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Core Tip: The age at which colorectal cancer is first diagnosed is decreasing worldwide, and colorectal cancer, especially in the 40s is very important. In our study, we found that colorectal cancer patients in their 40s had significantly more metastatic lesions and fewer K-ras mutations. Nevertheless, the oncologic outcome was never inferior compared to patients in their 30s and 50s by stages. We believe this to be a very important clinical message in the current situation.

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INTRODUCTION

Because colorectal cancer (CRC) is the third most common cancer in the world and the second most common cancer in Korea, it is a health-sociologically important cancer. The epidemiology of CRC (incidence, age distribution of CRC, *etc.*) varies greatly among different countries and races. In Korea, the proportion of CRC patients under the age of 50 is among the highest in the world. Based on the annual report of cancer statistics in Korea, of the 25881 patients diagnosed with newly developed CRC in 2019, about 10% were patients under 50[1]. Not only is the proportion of young patients high in Korea, but the rate of increase is also very rapid both in Korea and worldwide [2-7]. In the United States, data gathered over the past 40 years confirms the proportion of young CRC patients has increased gradually, mainly among patients in their 40s, and especially among African-American and Hispanic patients[8,9]. A previous study explained that the recent increase in young colorectal cancer patients is related with changes to Western style diet, in lifestyle, and an increase in environmental carcinogens[10].

Amid the global trend of increasing proportion and importance of young patients with CRC, clinical characterization of CRC patients in their 40s is somewhat unique. Patients with colorectal cancer in their 40s have the potential for both late-onset hereditary CRC and early-onset sporadic CRC. Patients in their 40s have lived long enough to be exposed to environmental carcinogens and they are in the age group where sporadic CRC can develop. However, the possibility of hereditary CRC cannot be excluded in this group, as it is relatively young compared to the average age of CRC. In hereditary CRC, genetic counseling, including somatic mutation testing, is important. However, in countries with many young CRC patients, such as Korea, genetic counseling for all patients in their 40s is not available and is not cost effective. In addition, there are secondary problems that are sometimes overlooked even in patients who need to be examined genetic testing[11].

Whatever the cause, the recent rapid increase in number of young CRC patients is a socially important issue. In particular, young people have an important socioeconomic role, so the increase in young CRC patients is directly linked to socioeconomic problems. Therefore, it is very important to understand and actively manage the characteristics of these patients. However, few studies have analyzed the characteristics of patients with CRC in their forties[12-14]. Therefore, we planned to determine the proportion of patients in their 40s with CRC and to identify their clinical characteristics, especially compared with those in their 30s and 50s, and to develop a management plan accordingly.

MATERIALS AND METHODS

Patients and study design

Among the 4326 patients with CRC who underwent primary resection in our hospital from September 2006 to July 2019, patients in their 30s, 40s, and 50s were included in the study. Only primary pathologically confirmed adenocarcinomas located in the area from the appendix to the rectum were included in the study. Patients who had only diversion without resection, or who had recurrent cancer or metastases of other cancers were excluded from the study. This study was approved by the Institutional Review Board of Korea University Anam Hospital (No. 2020AN0309) and all participants provided informed consent.

Surgical data processing

In this study, cancers of the appendix, cecum, and ascending, hepatic flexure, and transverse colon were classified as right-sided colon cancer, while cancers of the splenic flexure, descending, and sigmoid colon were classified as left-sided colon cancer, and cancers of the rectosigmoid colon were classified as rectal cancer. In our institution, most surgeries have been performed minimally invasively since 2006. In addition, D3 Lymphadenectomy is routinely performed in radical operations for all CRCs. The types of surgical procedure were so diverse that they were classified into lesional radical operations (including complete mesocolic excision or total mesorectal excision), total surgery (total abdominal colectomy or total proctocolectomy), and limited surgery. In rare cases, two or more segmental colectomies were performed simultaneously.

Perioperative management and surveillance

Our institution conducts perioperative evaluation and treatment based on the National Comprehensive Cancer Network guidelines. All patients with colon cancer had been evaluated preoperatively by physical examination, total colonoscopy, abdominopelvic computed tomography (CT), chest CT, and routine laboratory testing, which included tests for tumor markers. If necessary, additional tests, such as rectal magnetic resonance imaging (MRI), liver MRI, and positron emission tomography-CT, were performed. If necessary, stent insertion or neoadjuvant chemotherapy or radiotherapy were performed before surgery. When surgery was performed in an emergency, studies that were not evaluated before surgery were performed as soon as possible after surgery. If there were no problems after surgery, water intake was allowed the day after surgery and a soft diet started on the second day after surgery. After surgery, adjuvant treatment was performed as appropriate considering the stage of cancer, the patient's age, general condition, and socioeconomic status. After adjuvant treatment, follow-up examinations were carried out at 3-mo intervals during the first 2 years postoperatively, at 6-mo intervals until 5 years after surgery, and then annually if there was no evidence of recurrence.

Statistical analysis

Patients were divided into three groups comprising patients in their 30s, 40s, or 50s to compare patient and tumor characteristics, perioperative data, and oncologic outcomes. Descriptive results were presented as the mean or median for continuous outcomes and as frequency or percentage for categorical outcomes. For comparison between two groups, Student's *t*-tests were used to compare continuous variables, and the χ^2 test or Fisher's exact test was applied for categorical variables. ANOVA was used to compare the three groups. Five-year overall survival (OS) and disease-free survival (DFS) were analyzed using the Kaplan-Meier method. Statistical analyses was performed using SPSS® version 22.0 (IBM, Armonk, NY, United States). A P value < 0.05 was considered statistically significant.

RESULTS

Of the 4326 patients included in the study, 120 were in their 30s (2.8%), 451 were in their 40s (10.5%), and 1044 were in their 50s (24.4%). The proportion of patients in their 40s did not significantly increase or decrease over time (Figure 1). Compared to those in their 30s and 40s, the proportion of men among patients in their 50s was greater (55.0% *vs* 56.8% *vs* 65.7%, P = 0.001) (Table 1). There were significant co-morbidities among patients in their 50s, especially cardiovascular diseases (2.5% *vs* 11.8% *vs* 29.8%,

Table 1 Patient characteristics

	30s (*n* = 120)	40s (*n* = 451)	50s (*n* = 1044)	*P* value[1]	*P* value[2]	*P* value[3]	*P* value[4]
Sex, male (%)	55.0	56.8	65.7	0.730	0.001	0.020	0.001
Co-morbidity (%)	28.3	37.3	52.9	0.070	< 0.001	< 0.001	< 0.001
Endocrine	3.3	9.5	14.3	0.028	0.012	0.001	< 0.001
CVD	2.5	11.8	29.8	0.002	< 0.001	< 0.001	< 0.001
Pulmonary	5.8	3.5	6.3	0.259	0.031	0.835	0.096
Other	20.8	21.1	20.6	0.956	0.837	0.951	0.979
ASA score (%)				0.079	< 0.001	< 0.001	< 0.001
1	47.5	40.8	30.4				
2	50.8	56.3	66.3				
3	0	2.0	2.4				
4	0	0.2	0.1				
5	0	0	0.1				
Unknown	0.8	0.7	0.8				
BMI, mean (kg/m^2)	23.2	23.3	23.7	0.899	0.023	0.143	0.049
Tumor location (%)				0.975	0.061	0.268	0.126
Right-sided colon	20.0	16.0	20.9				
Left-sided colon	21.7	29.0	27.7				
Rectum	55.8	53.4	50.0				
Multiple	2.5	1.6	1.5				
CEA, median (ng/mL)	1.8	2.5	2.1	0.497	0.615	0.190	0.462
CA, median 19-9 (IU/mL)	11.1	12.6	11.3	0.215	0.527	0.887	0.781
Neoadjuvant treatment (%)	26.7	25.5	15.3	0.795	< 0.001	0.002	< 0.001
Preoperative complication (%)	14.2	17.3	12.9	0.678	0.910	0.637	0.883
Treatment	3.3	7.8	5.8	0.378	0.327	0.706	0.517

[1]30s *vs* 40s.
[2]40s *vs* 50s.
[3]30s *vs* 50s.
[4]30s *vs* 40s *vs* 50s. CVD: Cardiovascular disease; ASA: American Society of Anesthesiologists; BMI: Body mass index; CEA: Carcinoembryonic antigen.

$P < 0.001$), and endocrine disorders, including diabetes mellitus (3.3% *vs* 9.5% *vs* 14.3%, $P < 0.001$), and the American Society of Anesthesiologists score was also high ($P < 0.001$). Body mass index was also greater among patients in their 50s (23.2 *vs* 23.3 *vs* 23.7 kg/m^2, $P = 0.049$). Tumor location, preoperative carcinoembryonic antigen, carbohydrate antigen 19-9 Level, preoperative complications and treatment did not differ between the three groups, but more neoadjuvant treatments were performed in patients in their 30s and 40s (26.7% *vs* 25.5% *vs* 15.3%, $P < 0.001$).

The rate of emergency surgery tended to be higher in the younger age group, but the difference was not statistically significant (5.0% *vs* 2.4% *vs* 1.8%, $P = 0.077$; Table 2). Patients in their 50s had fewer open surgeries and more minimally invasive surgery than those in their 30s or 40s ($P = 0.043$). Operative procedures, placement of permanent colostomy, operation time, and estimated blood loss were similar in the three groups, but the combined operation rate was greater among patients in their 30s and 40s than those in their 50s (15.0% *vs* 15.7% *vs* 10.7%, $P = 0.021$). Postoperative pathology outcomes did not differ between the three groups in terms of T stage, N stage, number of positive lymph nodes (LNs), tumor size, proximal resection margin, distal resection margin, and circumferential resection margin, but the number of LNs retrieved from patients in their 30s was greater than from patients in their 40s or 50s (34 *vs* 29 *vs* 27, $P < 0.001$) (Table 3). Patients in their 40s, unlike other groups, had many

Table 2 Operative data

	30s (*n* = 120)	40s (*n* = 451)	50s (*n* = 1044)	*P* value[1]	*P* value[2]	*P* value[3]	*P* value[4]
Emergency (%)	5.0	2.4	1.8	0.143	0.434	0.023	0.077
Operation type (%)				0.931	0.023	0.155	0.043
Laparoscopy	55.8	59.2	70.1				
Robot	35.8	31.2	21.6				
Open	5.8	6.2	4.0				
Conversion	1.7	1.5	2.0				
Transanal	0.8	1.8	2.2				
Operation procedure (%)				0.847	0.914	0.897	0.983
Lesional (CME, TME)	93.3	95.6	96.1				
Total (TAC, TPC)	5.0	1.8	1.1				
Multiple	0	0.7	0.3				
Limited (Segmental, TAE, TAMIS)	1.7	2.0	2.6				
Combined operation (%)	15	15.7	10.7	0.835	0.008	0.172	0.021
Permanent colostomy (%)	3.3	4.9	3.1	0.472	0.085	0.872	0.222
Operative time, median (min)	200	205	190	0.476	0.026	0.571	0.078
EBL, mean (mL)	69.4	92.9	100.0	0.331	0.685	0.316	0.555

[1]30s *vs* 40s.
[2]40s *vs* 50s.
[3]30s *vs* 50s.
[4]30s *vs* 40s *vs* 50s. CME: Complete mesocolic excision; TME: Total mesorectal excision; TAC: Total abdominal colectomy; TPC: Total proctocolectomy; TAE: Transanal excision; TAMIS: Transanal minimally invasive surgery; EBL: Estimated blood loss.

metastatic lesions (17.5% *vs* 21.1% *vs* 14.9%, $P = 0.012$), and the TNM stage was high ($P = 0.002$). Tumor differentiation and venous/lymphatic invasion were not different among the three groups, and there were no significant differences in immunohistochemistry (IHC) or molecular pathology test results. In the younger age group, mucinous type cancers, perineural invasion, and *BRAF* positivity were more frequent, but these findings were not statistically significant (7.5% *vs* 5.1% *vs* 4.7%, $P = 0.347$; 13.3% *vs* 9.5% *vs* 7.9%, $P = 0.094$; 15.8% *vs* 4.8% *vs* 3.9%, $P = 0.076$). Similarly, a greater proportion of microsatellite instability (MSI)-high (H) was found in the younger age group (16.7% *vs* 9.0% *vs* 3.7%, $P = 0.046$). while K-ras mutation was detected less frequently in patients in their 40s (48.5% *vs* 33.3% *vs* 44.5%, $P = 0.076$).

The postoperative course was generally similar among the three groups, and there were no differences in postoperative complications or length of stay. However, the enforcement rate of postoperative chemotherapy was significantly greater in the patients in their 40s (58.3% *vs* 63.9% *vs* 56.3%, $P = 0.032$) (Table 4). When comparing survival after excluding stage 4 patients, 5-year overall survival was 92.2% among patients in their 30s, 89.8% among patients in their 40s, and 92.2% among patients in their 50s, and there were no differences between the three groups ($P = 0.804$) (Figure 2). There were also no differences in 5-year disease-free survival between the three groups (total, 98.6% *vs* 95.7% *vs* 96.1%, $P = 0.754$; local, 98.6% *vs* 94.3% *vs* 94.9%, $P = 0.579$; systemic, 86.4% *vs* 87.9% *vs* 86.4%, $P = 0.908$).

DISCUSSION

In our study, there were relatively fewer K-ras mutations in CRC samples obtained from patients in their 40s than in those of the other age groups. Patients in their 40s had aggressive CRC at a high stage and were administered active perioperative treatment. As a result, the oncologic outcome of patients in their 40s excluding stage 4 patients was not worse than that of those in their 30s and 50s.

Table 3 Pathology data

	30s (*n* = 120)	40s (*n* = 451)	50s (*n* = 1044)	*P* value[1]	*P* value[2]	*P* value[3]	*P* value[4]
pT (%)				0.251	0.986	0.214	0.446
Tis	6.7	5.8)	8.1				
T0	5.0	4.0	4.0				
T1	9.2	6.4	10.8				
T2	12.5	12.0	13.6				
T3	55.0	62.5	54.8				
T4	11.7	9.3	8.3				
Unknown	0	0	0.3				
pN (%)				0.419	0.289	0.831	0.521
N0	56.7	50.8	57.2				
N1	24.2	28.2	25.3				
N2	16.7	17.7	14.1				
Unknown	2.5	3.3	3.4				
Positive LN, mean (*n*)	1.9	1.8	1.8	0.709	0.960	0.803	0.958
Retrieved LN, mean (*n*)	34	29	27	0.030	0.015	< 0.001	< 0.001
M (%)				0.438	0.003	0.386	0.012
M0	81.7	78.3	84.8				
M1	17.5	21.1	14.9				
Unknown	0.8	0.7	0.3				
Stage (%)				0.138	< 0.001	0.613	0.002
0	11.7	8.6	11.3				
1	15.8	13.1	18.6				
2	28.3	27.1	25.7				
3	26.7	29.3	28.3				
4	17.5	21.1	14.8				
Unknown	0	0.9	1.3				
Tumor size, mean (cm)	4.9	4.7	4.3	0.797	0.079	0.207	0.125
PRM, mean (cm)	19.2	16.6	16.3	0.044	0.699	0.395	0.530
DRM, mean (cm)	6.7	6.0	6.8	0.431	0.205	0.690	0.412
CRM, mean (cm)	0.6	0.7	0.7	0.856	0.834	0.681	0.927
Differentiation (%)				0.279	0.670	0.155	0.347
Well	22.5	17.5	18.6				
Moderate	56.7	68.3	67.0				
Poor	5.0	2.9	2.4				
Mucinous	7.5	5.1	4.7				
Signet ring cell	0.8	0	0.3				
Etc.	0.8	0	0.1				
Unknown	6.7	6.2	6.9				
Venous invasion (%)	8.3	5.1	4.9	0.159	0.893	0.101	0.254
Lymphatic invasion (%)	20.0	15.3	16.8	0.194	0.432	0.365	0.415
Perineural invasion (%)	13.3	9.5	7.9	0.199	0.297	0.036	0.094

IHC (% positive)							
EGFR	53/63 (84.1)	210/274 (76.6)	506/673 (75.2)	0.197	0.636	0.113	0.275
CDX-2	23/24 (95.8)	84/87 (96.6)	236/239 (98.7)	0.869	0.194	0.268	0.338
P53	50/52 (96.2)	217/223 (97.3)	549/572 (96.0)	0.657	0.369	0.951	0.668
MLH-1	31/33 (93.9)	162/165 (98.2)	398/407 (97.8)	0.158	0.767	0.174	0.326
MSH-2	33/33 (100)	165/165 (100)	403/408 (98.8)	-	0.154	0.524	0.295
MSH-6	24/24 (100)	118/120 (98.3)	292/295 (98.9)	0.528	0.583	0.621	0.738
PMS-2	19/20 (95)	97/100 (97)	240/249 (96.4)	0.652	0.777	0.754	0.899
BRAF	3/19 (15.8)	3/63 (4.8)	7/180 (3.9)	0.108	0.765	0.024	0.076
Molecular test (% wild-type)							
K-ras	17/33 (51.5)	92/138 (66.7)	152/274 (55.5)	0.105	0.029	0.667	0.064
N-ras	20/21 (95.2)	90/95 (94.7)	193/200 (96.5)	0.926	0.475	0.770	0.768
Braf	12/12 (100)	51/53 (96.2)	93/97 (95.9)	0.502	0.917	0.478	0.778
MSI							
MSS	14/18 (77.8)	59/67 (88.1)	142/163 (87.1)	0.271	0.846	0.278	0.506
MSI-L	1/18 (5.6)	2/67 (3.0)	15/163 (9.2)	0.605	0.102	0.607	0.248
MSI-H	3/18 (16.7)	6/67 (9.0)	6/163 (3.7)	0.351	0.103	0.016	0.046

[1]30s *vs* 40s.
[2]40s *vs* 50s.
[3]30s *vs* 50s.
[4]30s *vs* 40s *vs* 50s. LN: Lymph node; PRM: Proximal resection margin; DRM: Distal resection margin; CRM: Circumferential resection margin; IHC: Immunohistochemistry; EGFR: Epidermal growth factor receptor; MSI: Microsatellite instability.

CRC in patients in their 40s may represent either late onset hereditary cancer or early onset sporadic cancer. While genetic factors are the major cause of CRC in patients under the age of 40, patients in their 40s have had relatively long periods of exposure to environmental factors, so the importance of environmental factors is relatively high. Therefore, the 40s are considered to be the age at which sporadic cancer, which occurs mainly in people over 50, begins. The distinction between hereditary and sporadic CRC has clinical significance in determining the scope of surgery, surveillance, and prevention of cancer occurrence in the family in advance [15]. Representative hereditary CRCs include familial adenomatous polyposis (FAP) and hereditary nonpolyposis CRC (HNPCC), which are characterized by onset at a young age, frequent synchronous and metachronous colorectal malignancies, and multiple extracolonic malignancies. The most important diagnostic criterion for hereditary CRC is the age of the patient; however, the appropriate cutoff age is unclear, given HNPCC (also called Lynch syndrome) typically occurs later than FAP, and it can therefore be difficult to identify sporadic CRC that developed early based only upon the patient's age. The Amsterdam criteria or Bethesda guidelines recommend that HNPCC be suspected if the patient is 50 years old or younger and has some other manifestations. However, in a country such as Korea with a large proportion of CRC patients of relatively young age, especially those in their 40s, it may be not available to conduct genetic studies in all patients in their 40s with CRC. On the other hand, if hereditary CRC is not identified where present in these patients, they may miss the additional therapeutic benefits from genetic counseling.

It should be noted that, in recent years, the age of onset of CRC is not only gradually decreasing in Korea, but also worldwide. In statistical analyses based on SEER data, Siegel *et al*[2] reported that colon cancer incidence rates increased by 1.0% to 2.4% annually since the mid-1980s in patients aged 20 to 39 years and by 0.5% to 1.3% since the mid-1990s in patients aged 40 to 54 years. Similar trends have also been identified in other countries such as Canada and Australia, and because of this, in 2018 the American Cancer Society recommended that the screening age for CRC be lowered from 50 to 45 years[16]. Thus, the dilemma regarding how to appropriately manage CRC patients in their 40s identified in Korea may be occurring in other countries. In

Table 4 Postoperative data

	30s (*n* = 120)	40s (*n* = 451)	50s (*n* = 1044)	*P* value[1]	*P* value[2]	*P* value[3]	*P* value[4]
Gas, median (d)	2	2	2	0.217	0.261	0.408	0.322
Stool, median (d)	2	2	3	0.995	0.002	0.071	0.004
Feed, median (d)	2	2	2	0.569	0.655	0.736	0.827
Postoperative hospital stays, median (d)	8	8	8	0.545	0.748	0.656	0.834
Postoperative complication (%)	27.5	25.9	23.1	0.731	0.235	0.281	0.339
Leakage	5.0	6.0	6.6	0.681	0.652	0.497	0.747
Intraabdominal abscess	3.3	2.4	1.6	0.587	0.289	0.184	0.319
Wound infection	2.5	1.3	1.2	0.361	0.893	0.264	0.532
Ileus	11.7	10.4	8.4	0.695	0.218	0.235	0.299
Bleeding	2.5	0.7	1.0	0.080	0.576	0.128	0.194
Stoma related	0	0.4	0.2	0.466	0.387	0.632	0.569
Pulmonary	0.8	1.1	0.9	0.793	0.650	0.974	0.896
Cardiovascular	0	0	0.2	-	0.353	0.632	0.579
Nephrology	0	0.4	0.2	0.466	0.387	0.632	0.569
Voiding	0.8	0.4	0.8	0.600	0.482	0.937	0.768
Chyle	3.3	3.8	4.0	0.822	0.817	0.714	0.921
Other	3.3	2.4	2.4	0.587	0.959	0.533	0.821
Reoperation (%)	1.7	2.4	3.6	0.615	0.232	0.262	0.297
Postoperative mortality (%)	0	0.4	0.3	0.466	0.632	0.557	0.722
Adjuvant treatment (%)	60.8	66.3	58.1	0.370	0.002	0.389	0.007
Chemotherapy	58.3	63.9	56.3	0.357	0.009	0.618	0.032
Radiotherapy	2.5	2.4	1.8	0.946	0.429	0.580	0.679
Recurrence (%)	15.8	20.2	16.4	0.187	0.088	0.657	0.168
Local	0.8	2.2	2.4	0.328	0.835	0.273	0.548
Systemic	6.7	6.9	6.8	0.870	0.837	0.956	0.975
Progression	8.3	11.8	7.8	0.318	0.019	0.824	0.061
Death (%)	11.7	12.4	8.7	0.824	0.027	0.286	0.074
Follow-up duration, median(mo)	26.7	25.6	24.8	0.694	0.819	0.579	0.850

[1]30s *vs* 40s.
[2]40s *vs* 50s.
[3]30s *vs* 50s.
[4]30s *vs* 40s *vs* 50s.

this regard, it is clinically important to confirm the characteristics of CRC patients in their 40s compared to those in their 30s and 50s.

In our study, patients in their 40s with CRC had independent characteristics that were distinct from those in their 30s and 50s. The proportion of women was relatively high, co-morbidity was low, and combined operations were frequent. The characteristics of patients in their 40s with CRC tended to be similar to those of patients in their 30s. However, multiple lesions were not as frequent as in those in their 30s, which led to a lower rate of total colectomy, and a significantly lower number of retrieved LNs than in patients in their 30s, similar to the characteristics of patients in their 50s. Contrary to expectation, histological characteristics were not different by age group. In younger patients, there were more cancers of the mucinous type, and greater perineural invasion, *BRAF* positivity on IHC, and rate of MSI-H, but these findings were not statistically significant.

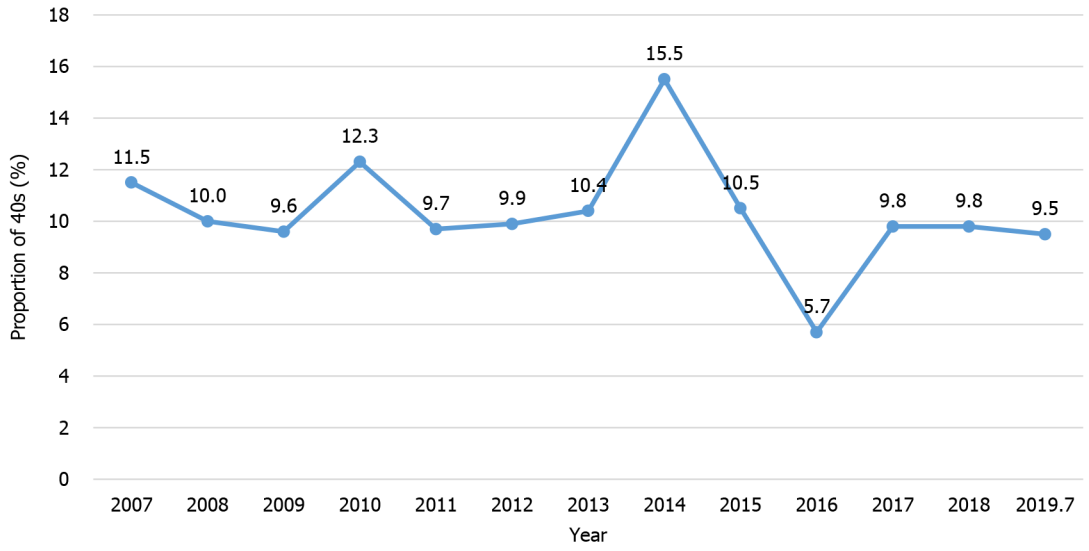

Figure 1 Changes in the proportion of colorectal cancer patients in their 40s by year.

It is noteworthy that in patients in their 40s, metastases were significantly more frequent at the time of diagnosis, as was pre-/post-surgery chemotherapy. Because many countries, including Korea, begin screening for CRC among average-risk people in their 50s, early-stage CRC in their 50s might be expected to be found relatively frequently by screening. Considering that the age is excluded from screening for CRC, it can be understood that the stage of CRC patients in their 40s is higher than those in their 50s. However, it is very surprising that the stage in the 40s is higher than even in the 30s. One hypothesis is that some of the patients with CRC in their 30s have been diagnosed with FAP or HNPCC by themselves or by their family members, and early CRC may have been detected in surveillance colonoscopy or prophylactically resected specimens. In our study, the frequency of K-ras mutation was significantly lower among patients in their 40s, but more research and consideration will be needed to determine the effect of mutation frequency on staging. Meanwhile, when assessing survival among Stage 1-3 patients, there were no differences between patients in their 40s and those in their 30s or 50s. We could find that the age of 40s itself is not a risk factor for poor survivals although other studies have been controversial about the results[12-14]. As a result, early detection of CRC patients in their 40s will be an important task. Given 54% of CRCs in patients in their 40s are rectal cancer and 28.7% are left-sided colon cancer, a new screening program in which sigmoidoscopy is recommended beginning in the 40s may be considered[17].

Another interesting finding from our study is that the proportion of CRC patients in their 40s did not change significantly throughout the study period, and has remained approximately 10% since 2007. This finding appears to conflict with those from Western countries, where the proportion of patients with CRC in their 40s has increased gradually over the last few decades[2-4]. In addition, the results of a recent analysis using the national registry of Asian countries are contrary to the reported trend in which the age at diagnosis of CRC is decreasing in several Asian countries, including Korea[5-7]. This inconsistency may have arisen because our study was conducted over a relatively short period of analysis. And, in our study, the percentage of CRC patients in their 40s was somewhat greater than in the annual report of cancer statistics in Korea, probably because our study included only patients who underwent surgery[1]. Given a significant proportion (about 10%) of all Korean CRC patients are in their 40s, an age group of major socioeconomic influence, there is an ongoing debate regarding reducing the age at which initiation of screening is recommended[16,18,19].

Our study had some limitations. First, the primary comparators were clinical variables, and immunohistochemistry tests such as epidermal growth factor receptor or CDX-2 were limited, and genetic tests such as *ras* gene or MSI were performed only in about 10%-50% of patients. In particular, in CRC cases in patients in their 30s, the likelihood of hereditary cancer is high, so it is typically necessary to perform genetic testing for somatic mutations, but there were few results. And, information on whether to diagnose FAP and HNPCC is also insufficient. The second limitation was that even in among patients in their 40s, the patient characteristics may differ between patients in their early and late 40s. Third, there were large differences in patient

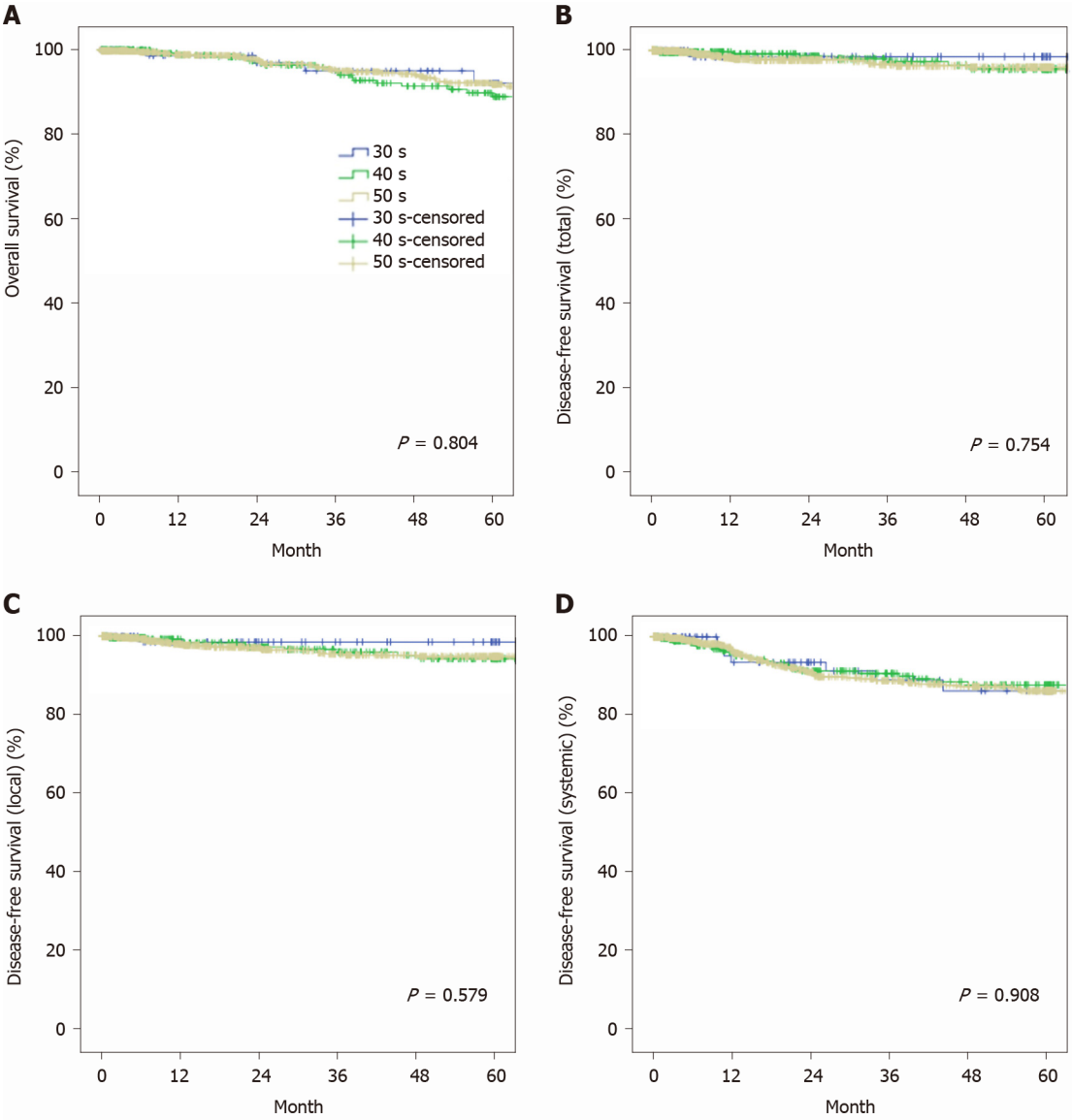

Figure 2 Comparison of 5-year overall survival, disease-free survival (total, local, and systemic) in stage 1-3 patients in their 30s, 40s and 50s with colorectal cancer. A: 5-year overall survival; B: Disease-free survival (total); C: Disease-free survival (local); D: Disease-free survival (systemic). OS: overall survival; DFS: disease-free survival.

numbers by age group, with 8-fold or more patients in their 50s than in their 30s. Despite these limitations, our findings can be meaningful in that identification of the characteristics of the growing proportion of CRC patients in their 40s may help to formulate patient management strategies.

CONCLUSION

In conclusion, patients with CRC in their 40s comprised about 10% of all CRC patients, and most of the tumor characteristics were similar to those in their 30s and 50s in our study. However, their cancers were often found at an aggressive stage compared to those found in the other age groups. Nonetheless, the oncologic outcome was not worse by stage, so it is necessary to actively perform screening tests for early detection. In addition, even in patients in their 40s, the potential for late-onset hereditary CRC must always be considered, but future studies are needed to establish new standards for conducting genetic tests for patients with CRC in their 40s.

ARTICLE HIGHLIGHTS

Research background

Globally, the proportion of young patients with colorectal cancer (CRC), especially in their 40s, is increasing, and this rate of increase is very rapid.

Research motivation

Patients with CRC in their 40s have the potential for both late-onset hereditary CRC and early-onset sporadic CRC. However, few studies have analyzed the characteristics of patients with CRC in their 40s.

Research objectives

The main aim of this study was to determine the proportion of patients with CRC in their 40s and to identify their clinical characteristics, especially compared with those in their 30s and 50s.

Research methods

We compared patient and tumor characteristics as well as perioperative outcomes of patients in their 30s, 40s, and 50s who received primary resection for CRC. In addition, the 5-year survivals were compared between the three groups, excluding stage 4.

Research results

In patients with CRC in their 40s, there were significantly more metastatic lesions and a higher TNM stage. K-ras mutations tended to be low in patients in their 40s, and postoperative chemotherapy was frequently performed in them. Excluding stage 4, the 5-year overall and disease-free survivals of CRC patients in their 40s did not differ from those in their 30s and 50s.

Research conclusions

Patients with CRC in their 40s exhibited relatively more metastatic lesions and a more advanced stage, but the oncologic outcomes of patients excluding stage 4 were not inferior compared to those in their 30s and 50s. Therefore, it is necessary to actively perform screening tests for early detection of CRC in those in their 40s.

Research perspectives

More analyses of immunohistochemistry or genetic testing results are needed to complement our results. In addition, future studies are also needed to establish new standards for conducting genetic tests for patients with CRC in their 40s.

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ORIGINAL ARTICLE

Observational Study

Effect of gastric microbiota on quadruple *Helicobacter pylori* eradication therapy containing bismuth

Zhan-Yue Niu, Si-Zhu Li, Yan-Yan Shi, Yan Xue

ORCID number: Zhan-Yue Niu 0000-0002-8017-1787; Si-Zhu Li 0000-0002-0797-5375; Yan-Yan Shi 0000-0003-0247-371X; Yan Xue 0000-0002-3955-7987.

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Zhan-Yue Niu, Si-Zhu Li, Yan Xue, Department of Gastroenterology, Peking University Third Hospital, Beijing 100191, China

Yan-Yan Shi, Research Center of Clinical Epidemiology, Peking University Third Hospital, Beijing 100191, China

Corresponding author: Yan Xue, MD, Doctor, Department of Gastroenterology, Peking University Third Hospital, No. 49 North Garden Road, Haidian District, Beijing 100191, China. xueyanby@163.com

Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) is an important pathogen that can cause a variety of diseases. Yet, full eradication of *H. pylori* remains a significant challenge in clinical practice. *H. pylori* and other microbial communities have complex interactions in the unique gastric microecological environment. However, it is not clear whether the interactions have any effect on the therapeutic effect of *H. pylori*.

AIM

The aim was to investigate the characteristics of the gastric microbiota with *H. pylori* infection and the influence on the *H. pylori* eradication treatment.

METHODS

Patients with *H. pylori* infection underwent gastroscopy and received treatment for eradication. The prescription included esomeprazole 20 mg bid, Livzon Dele 220 mg bid, amoxicillin 1000 mg bid, and clarithromycin 500 mg bid for 14 d. Patients who did not respond to treatment and failed eradication were compared with those who achieved eradication by 1:2 propensity matching. High-throughput sequencing of the gastric mucosal microbiota was performed, and the results were evaluated by alpha diversity analysis, beta diversity analysis, species correlation analysis, and metabolic pathway correlation analysis.

RESULTS

The eradication rate of all the patients was 95.5% (171/179). Twenty-four patients were enrolled in the study after propensity-matched scoring. There were eight cases in the failure group (patients who did not respond well to therapy) and 16 cases in the success group. The majority phyla in the two groups were the same, and included Proteobacteria, Bacteroides, Firmicutes, Actinomycetes, and

additional data are available.

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Fusobacteria. The microbial diversity in the failure group had a decreasing trend (P = 0.092) and the species abundance was significantly lower (P = 0.031) compared with the success group. The high rate of *H. pylori* eradication was associated with *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*, as they were significantly enriched in the successful group (P < 0.05). *Veronococcus* and *Cilium* were enriched in the mucosa of chronic atrophic gastritis patients compared with chronic superficial gastritis patients (P = 0.0466 and 0.0122, respectively). In both study groups, *H. pylori* was negatively correlated with other bacterial genera. More bacterial genera were directly related to *H. pylori* in the successful group compared with the failure group.

CONCLUSION

The effectiveness of quadruple *H. pylori* eradication therapy containing bismuth depended on gastric microbiota, and the high rate of *H. pylori* eradication was associated with the presence of *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*.

Key Words: *Helicobacter pylori*; Eradication; Quadruple therapy; Influence factors; propensity matching; Gastric microbiota

Core Tip: *Helicobacter pylori* (*H. pylori*) is an important pathogen that can cause a variety of diseases. Its eradication can be affected by many factors. In this study, we explored the effect of the gastric microbiota on quadruple *H. pylori* eradication therapy containing bismuth. The results indicated that quadruple *H. pylori* eradication therapy containing bismuth was affected by the gastric microbiota. A high rate of *H. pylori* eradication was associated with the presence of *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*. Our findings may provide the basis for clinical treatment.

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INTRODUCTION

The gastric mucosal environment was long thought to be sterile. Yet, the discovery of *Helicobacter pylori* (*H. pylori*) put an end to the traditional view of the sterile stomach [1]. Furthermore, the development of modern technology led to a deeper understanding of gastric microbiota. Numerous gastric microbiota have been discovered over the years by 16S rRNA sequencing[2]. *H. pylori* and other microbial communities have complex interactions in the unique gastric microecological environment. *H. pylori* can inhibit other microbial communities by inducing the production of cytokines and antimicrobial peptides[3]. On the contrary, other microbial communities can affect *H. pylori*. For example, *Streptococcus* can change the spiral form of *H. pylori* into a spherical shape and inhibit its growth[4].

H. pylori is an important pathogen associated with a variety of diseases[5-7] including gastric cancer[8]. About 50% of the global population is infected with *H. pylori*[9]. *H. pylori* infection is an infectious disease and a "screening-treatment" strategy has been recommended for the treatment of *H. pylori*. However, the eradication of *H. pylori* can be affected by many factors, including antibiotic resistance[10], medication compliance[11,12], virulence factors[13], and others. In this study, we investigated the characteristics of the gastric microbiota in patients with *H. pylori* infection and the effect of the gastric microbiota on the success of quadruple *H. pylori* eradication therapy containing bismuth.

MATERIALS AND METHODS

Participants

Patients diagnosed with *H. pylori* infection in the Gastroenterology Department of Peking University Third Hospital between were enrolled between July 2018 and July 2019. Patients who were (1) 18-70 years of age; (2) with *H. pylori* infection confirmed by gastroscopy and histopathology were eligible. Patients (1) with previous *H. pylori* eradication therapy; (2) using proton pump inhibitors, H_2 receptor blockers, bismuth, antibiotics, or other drugs that might affect the study results within 4 wk of inclusion; (3) with gastrointestinal tumors; (4) with a history of gastric or esophageal surgery; (5) with Zollinger-Ellison syndrome; (6) with abnormal liver or kidney function; (7) with severe cardiovascular, respiratory, blood, endocrine, neurological, or mental disease; (8) with allergy to a study drug; (9) were pregnant or lactating; and (10) or with histories of alcohol abuse or clinical conditions that might increase the risk of side effects were excluded.

Methods

Before inclusion, all patients provided informed consent for clinical sample collection. A biopsy was taken from the antrum before *H. pylori* eradication treatment. A rapid urease test (RUT) was performed during the gastroscopy, and if the result was positive, gastric mucosa biopsies from the lesser curvature of antrum and corpus were collected, placed in a cryovial, and stored −80 °C. At the same time, mucosa specimens were collected from the gastric antrum and corpus were collected for histopathological examination and Warthin-Starry (WS) staining.

Patients diagnosed with *H. pylori* infection by positive RUT results and WS staining were treated with esomeprazole 20 mg bid, amoxicillin 1000 mg bid, clarithromycin 500 mg bid, Livzon Dele bismuth potassium citrate 220 mg bid for14 d. A ^{13}C urease breath test (^{13}C-UBT) was performed 8 wk after treatment, which was considered successful if the ^{13}C-UBT was negative.

Patients were divided into failure and the success groups after their treatment was completed. Patients who did not respond well to therapy were included in the failure group. The success group was evaluated by nearest-neighbor matching, which is a type of propensity score matching and paired with patients in the failure group who had a similar propensity index. The propensity index was estimated by the model so as to equalize the covariates between the two groups. Gender, age, body-mass index (BMI kg/m^2), gastroscopy diagnosis, and background gastric mucosa were the covariables used to calculate the propensity values. Taking the sample size and matching quality, the allowable error was set to 0.1. The failure and success groups were matched at a ratio of 1:2. The gastric mucosa microbiota were assayed and compared according to the results of propensity score matching.

Microbial diversity sequencing

The total DNA of the microbiota was extracted following the instructions with E.Z.N.A.® soil DNA kits (Omega Bio-Tek, Norcross, GA, United States) following the manufacturer's instructions, and the quality of DNA extraction was assayed by 1% agarose gel electrophoresis. DNA concentration and purity were determined by a NanoDrop 2000 spectrophotometer, and 338 F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers were used for PCR amplification of the V3-V4 region of the 16S rRNA gene. The PCR products were recovered on 2% agarose gels after combining the PCR products of the same sample. Recovered products were purified with AxyPrep DNA gel extraction kits (Axygen Biosciences, Union City, CA, United States) and assayed with a Quantus™ Fluorometer (Promega, United States). The library was built with NEXTFLEX Rapid DNA-Seq Kits and sequenced with a Miseq PE300 platform (Illumina).

Sequence data analysis

Fastp software was used for quality control of the original sequence, and Flash software was used for stitching. Usearch software (version7.0, http://drive5.com/uparse/) was used to for operational taxonomic unit (OTU) clustering of sequences based on 97% similarity and elimination of chimeras. RDP classifier (http://rdp.cme.msu.edu/) was used to compare each sequence with the sequences included in Silva database (SSU132). The threshold was set to 70%, and the results of species classification annotations were obtained. The sample species composition was analyzed based on the annotation results.

The alpha diversity analysis (*i.e.* within-sample diversity) reflected species diversity, including Shannon, ace, and coverage indices. Beta diversity analysis (*i.e.* diversity between samples) or between-group differences in species composition was by principal coordinates analysis (PCoA). The Wilcoxon rank-sum test was used to compare the two groups. A P value of < 0.05 was considered significant. Correlation analysis of species was carried out by correlation heatmaps, network analysis, and metabolic pathway analysis. The relationship between the microbiota and the result of *H. pylori* eradication was preliminarily explained.

Statistical analysis

SPSS 23.0 was used for data analysis. measurement data were reported as means ± SD and compared by *t*-tests. Categorical data were compared by χ^2 tests P values of < 0.05 were considered statistically significant.

RESULTS

Basic information

Of the 179 enrolled patients, 171 responded well to therapy with successful *H. pylori* eradication. Eight patients failed treatment. The eradication therapy success rate was 95.5%. Propensity scoring resulted in matching eight failed cases with 16 successful cases. The results of gastroscopy revealed chronic gastritis in all patients; and after matching, there were no significant differences in the baseline characteristics between the two groups (Table 1).

A total of 1 204 878 reads and 1028 OTUs were obtained from 24 samples. The samples contained a mean of 50 203 reads and 191 OTUs. The reads in the failed eradication group (58 487) were significantly higher (P = 0.013) than those in the successful eradication group (46 061); the difference between the OTUs in the two groups was not significant (166 and 203, respectively, P = 0.719). The samples were randomly flattened according to the minimum number of sample sequences to avoid analysis deviation. A total of 980 OTUs were obtained, and each sample contained 30 043 reads. All samples were dominated by *H. pylori* at the genus level; *H. pylori* infection was pathologically confirmed.

Analysis of microbiota composition and differences

The proportions of bacterial species in the failure and the success groups were evaluated by Good's species coverage index, which found that the difference between the two groups was not significant (P = 0.125). The coverage index in both groups was higher than 0.99 and confirmed that the test results covered most bacterial species in the gastric mucosa. Analysis of community composition showed that the gastric mucosa microbiota mainly contained Proteobacteria, Bacteroidetes, Firmicutes, Actinomycetes, and Fusobacteria, regardless of the study group. The abundance of Proteobacteria was higher in the failure group than in the success group, and that of Actinobacteria was lower (Table 2).

Alpha and beta diversity reflect differences in the microbial composition. The results of the Shannon index, which is one an indexes of alpha diversity, showed that the diversity in the failure group was reduced compared with the success group, but the difference was not significant (P = 0.092; Figure 1A). The Ace index showed that the species abundance was significantly lower in the failure group than in the success group (P = 0.031; Figure 1B). The dominant species in both study samples was *H. pylori*, but the microbiota composition differed at the genus level (Figure 2A). PCoA analysis of beta diversity resulted in a weighted UniFrac showing that the total diversity of the first two principal coordinates was 89.96% and the difference between the two groups was significant (ANOSIM, P = 0.048; Figure 2B). According to the unweighted UniFrac, the difference between the two groups was significant (ANOSIM, P = 0.001; Figure 2C). Binary Euclidean analysis, which was used to assess the difference in species composition, showed a significant difference between the two groups (ANOSIM, P = 0.001; Figure 2D). As shown in Figure 3, *H. pylori* was more abundance in the failure than in the success group, but the difference was not significant (Wilcoxon rank-sum test, P = 0.0809). *Rhodococcus*, *Lactobacillus*, and *Sphingomonas* were significantly enriched in the success group, (Wilcoxon rank-sum test, P < 0.05).

The flora composition of gastric mucosa that were histologically different was also analyzed. The abundance of *H. pylori* was higher in the mucosa of chronic superficial gastritis but the difference was not significant (Wilcoxon rank-sum test, P = 0.1179).

Table 1 Baseline characteristics used in case matching of *Helicobacter pylori* eradication failure and success

		Failure group	Success group	*P* value
Sex	Male	7	14	1.000
	Female	1	2	
Age in yr, mean ± SD		40.13 ± 14.55	37.44 ± 6.42	0.532
BMI in kg/m^2, mean ± SD		25.01 ± 5.32	25.20 ± 2.84	0.913
Background mucosa	Chronic superficial gastritis	6	13	1.000
	Chronic atrophic gastritis	2	3	

SD: Standard deviation.

Table 2 Phylum-level differences of gastric mucosa microbiota

Phyla	Failure group, %	Success group, %	*P* value
Proteobacteria	98.26	94.60	0.0346
Bacteroidetes	0.60	1.23	0.4623
Firmicutes	0.52	1.23	0.2839
Actinomycetes	0.21	0.96	0.0016
Fusobacteria	0.11	0.27	0.6025

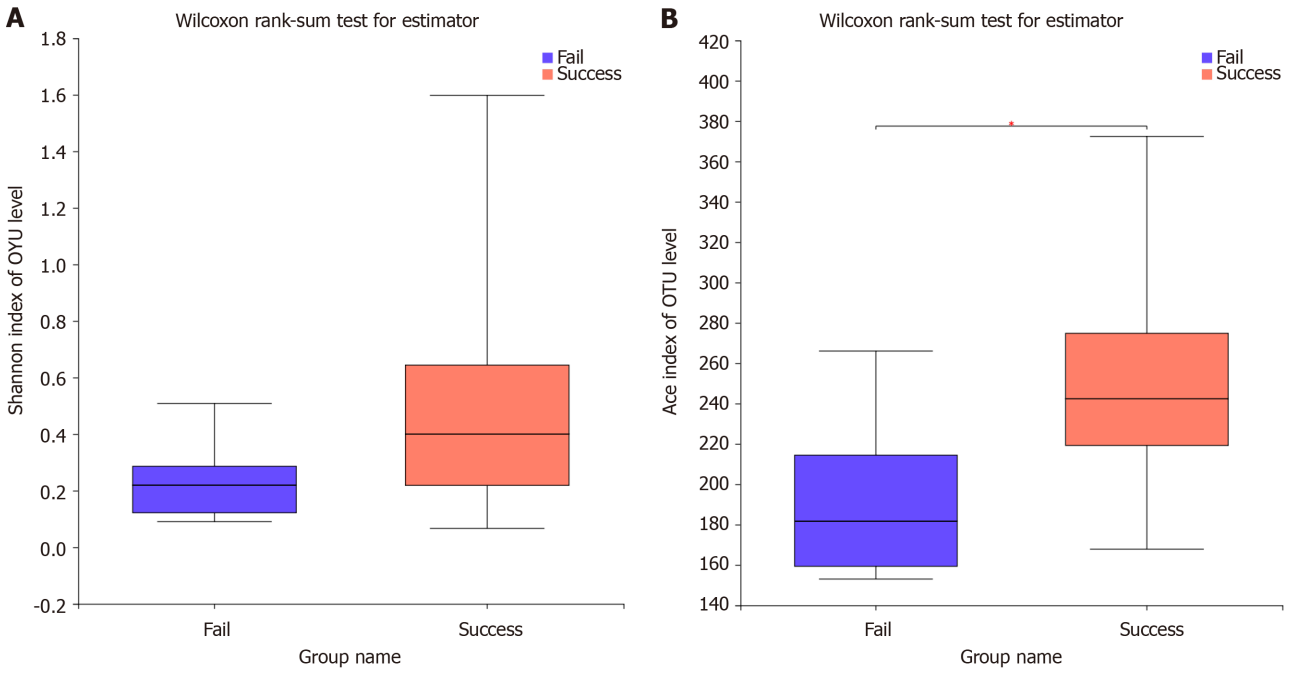

Figure 1 Alpha diversity analysis. A: Shannon index; B: Ace index.

Veronococcus and *Cilium* were more enriched in the mucosa of chronic atrophic gastritis (Wilcoxon rank-sum test, P = 0.0466 and 0.0122).

Correlation analysis of microbiota

Heatmap results of the species correlation analysis found that *H. pylori* was negatively correlated with other bacterial genera (Figure 4), *H. pylori* was negatively correlated with *Ralstonia* in the failure group (genus level) and negatively correlated with *Haemophilus, Prevotella, Streptococcus, Actinomycetes, Veillonella, Neisseria, Fusobacterium,* and *Leptotrichia* in the success group (genus level). The PICRUSt metabolic pathway

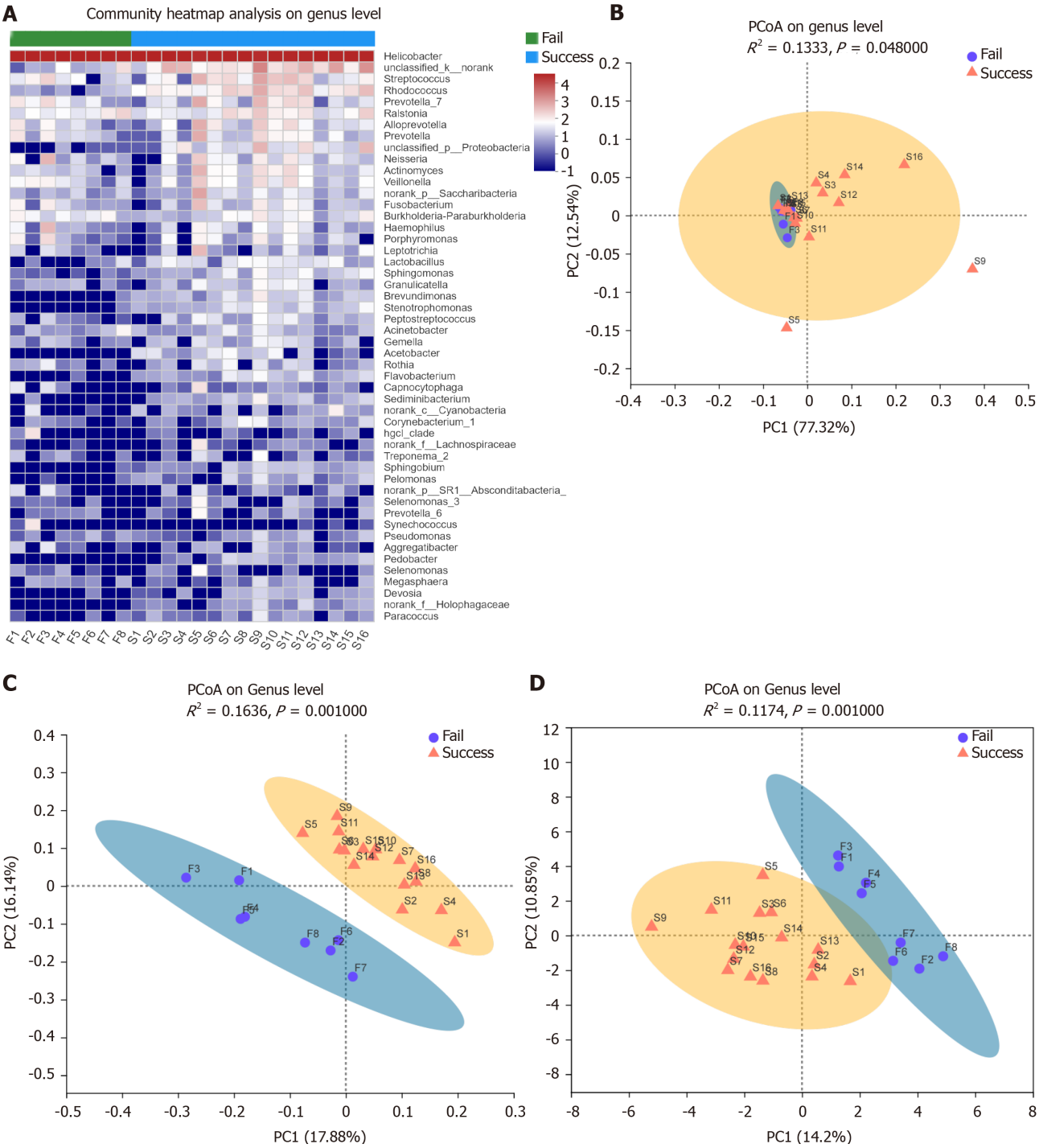

Figure 2 Beta diversity analysis. A: Heatmap; B: Weighted UniFrac; C: Unweighted UniFrac; D: Binary Euclidean. Points with different colors or shapes represent samples from different groups. The closer the two points are, the more similar the species composition is.

function prediction showed that the level two metabolic pathways in the two study groups were basically the same, mainly including carbohydrates, amino acids, energy, coenzyme factors, and vitamins.

DISCUSSION

The composition of the gastric mucosa microbiota infected by *H. pylori* mainly included Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, and Fusobacteria, which is consistent with the results reported by previous studies[14,15]. *H. pylori* infection can significantly reduce the diversity of gastric mucosal microbiota[16]. In this study, the Shannon index did not reveal a significant difference in the diversity of

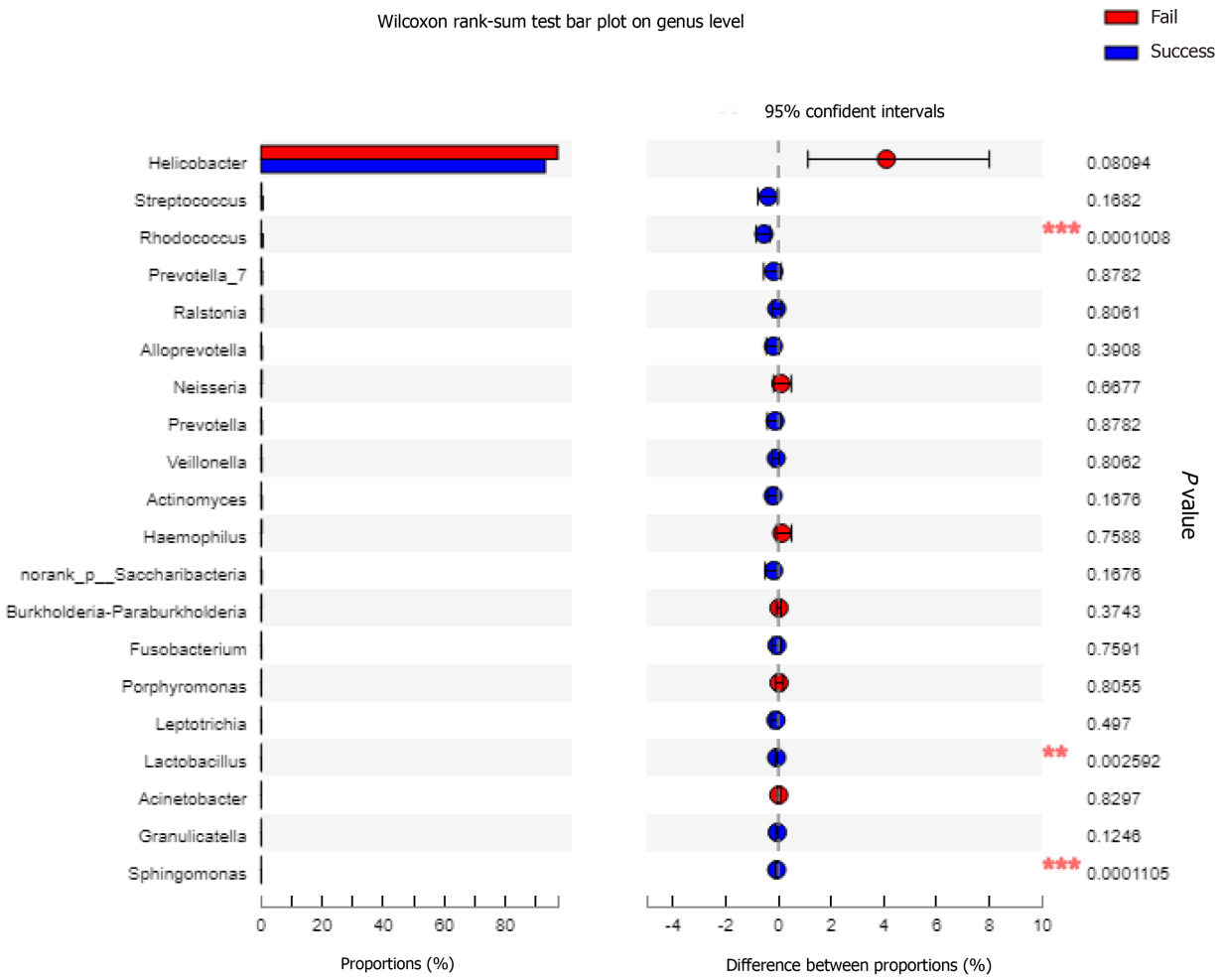

Figure 3 Genus-level differences between the success group and the failure group. The analysis includes the 20 most abundant genera.

the microbiota between the success and the failure group. However, the microbiota diversity in the failure group was lower, which suggests that eradication of *H. pylori* may be associated with the diversity of gastric mucosal microbiota.

The abundance of Proteobacteria was higher in the failure group. The genus level data suggests that the higher abundance of *H. pylori* in the eradication failure group might have caused an increased abundance of Proteobacteria. Although the difference in *H. pylori* abundance between the two groups was not significant, previous studies have shown that a higher abundance of *H. pylori* may reduce the effectiveness of empirical eradication therapy[17]. It has been reported that the abundance of *H. pylori* impacted only the result of traditional triple therapy[18]. Nevertheless, our results implied that the abundance of *H. pylori* had an impact on the eradication effect of quadruple therapy containing bismuth. In addition, 16s RNA may be more accurate than the $^{13}C/^{14}C$-UBT or histological evaluation for the determination of *H. pylori* abundance.

At the genus level, the gastric mucosa microbiota species diversity was similar in the two groups. However, PCoA analysis showed that there were differences in the species composition and abundance between the two groups. The Ace index confirmed the differences in abundance of the two groups, and statistical analysis confirmed that the success group was significantly enriched in *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*. The reason for the increased abundance of Actinomycetes in the success group may be related to the enrichment of *Rhodococcus*, an opportunistic pathogen[19,20] that can be detected in the stool of healthy people. Thiamine is required for the growth of *Rhodococcus*[21] and is essential nutrient for the growth of *H. pylori*[22]. *H. pylori* is a thiamine auxotroph that lacks the gene that synthesizes thiamine[23]. Vitamin metabolism is one of the microbiota's main metabolic pathways predicted by PICRUSt. Therefore, *Rhodococcus* may inhibit the growth of *H. pylori* through the acquisition of thiamine. *Lactobacilli*, which are beneficial bacteria, effectively improve *H. pylori* eradication if added to the prescription, especially in 7-d

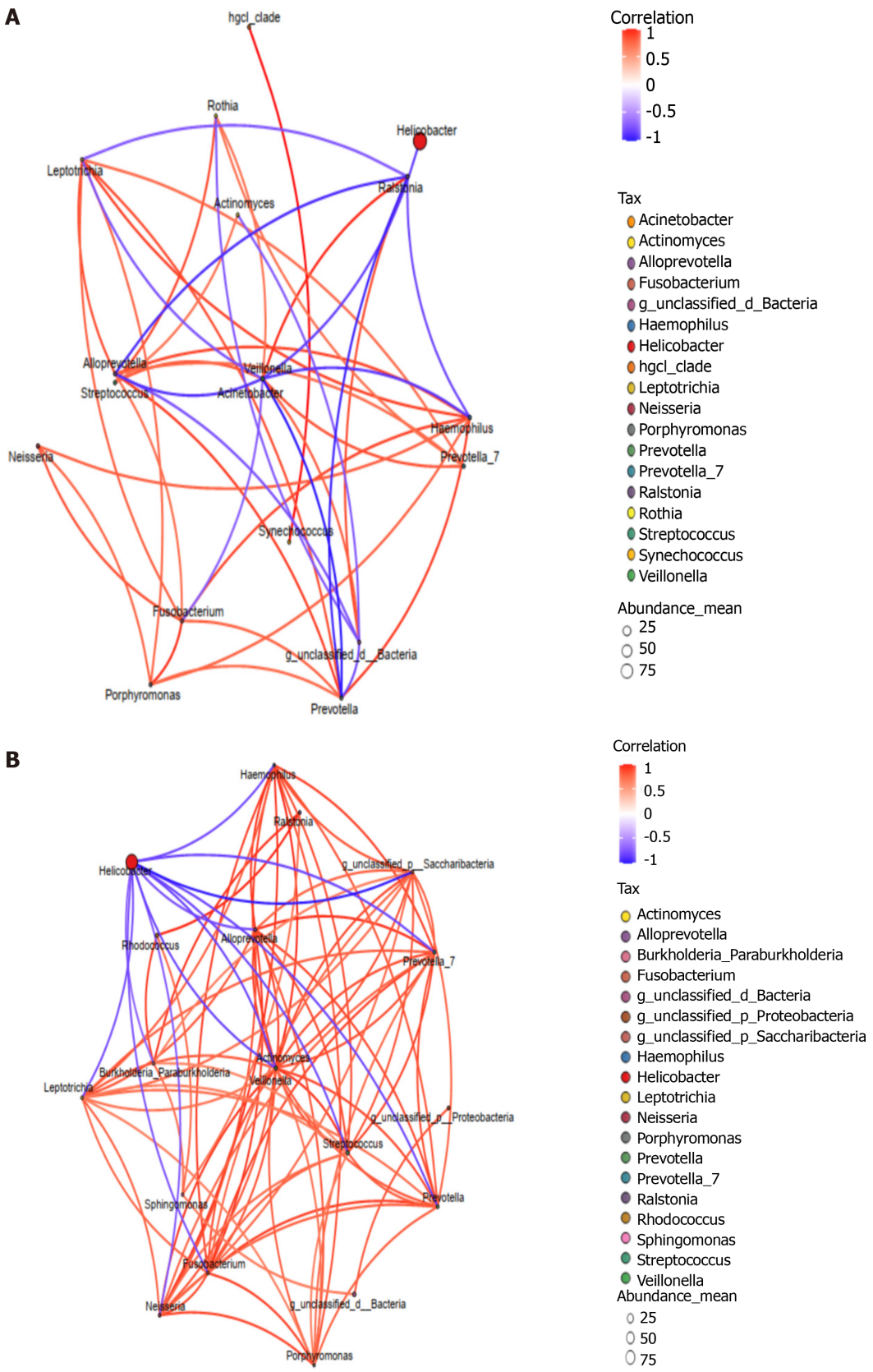

Figure 4 Single factor correlation network analysis including the 20 most abundant genera. A: Failure group; B: Success group. Each circle represents a species. Red and blue line represent positive and negative correlations. The thickness of the lines represents the correlation coefficient.

and 14-d triple therapy[24]. Previous studies have shown that *Lactobacillus* is a strong antagonist of *H. pylori* by preventing colonization and growth[25,26] and inhibiting adhesion to and invasion of gastric epithelial cells. *Lactobacillus* even has an antagonistic effect on multidrug resistant *H. pylori*[27]. In this study, the abundance of

Lactobacillus was significantly higher in the success group than in the failure group, confirming the beneficial effect of *Lactobacillus* in the eradication of *H. pylori*. Moreover, previous studies found changes in *Sphingomonas* in a variety of diseases[28,29]. In this study, it had a significant negative correlation with *H. pylori* and was more abundant in the success group than in the failure group.

Analysis of the microbiota against different gastric mucosa backgrounds showed that the genera *Veronococcus* and *Cilium* were enriched in chronic atrophic gastritis. Previous studies have shown bacterial overgrowth in the gastric mucosa of pre-gastric cancer. *Veronococcus* and *Cilium* are enriched in gastric mucosa of gastric cancer[30]. *Veronococcus* can convert nitrate to nitrite, and the increased concentration of nitrite may promote gastric cancer[31]. In addition, *Veronococcus* is enriched in patients with oral cancer, colorectal cancer, and lung cancer[32-35]. A study conducted in Colombia showed that the gastric mucosal flora of a population at high risk of gastric cancer was significantly enriched in *Veronococcus* and *Cilium* but the rate of *H. pylori* infection was not increased. Therefore, the genus *Veronococcus* and *Cilium* may be factors promoting the occurrence of gastric cancer. Our results confirmed a trend in pre-gastric cancer.

A negative correlation was found between *H. pylori* and other microbiota, which was more significant in the success group than in the failure group. The negative correlation of *H. pylori* and other microbiota was associated with a positive correlation among the other bacterial communities. Co-inhibition of *H. pylori* by a variety of mutually-promoting microbiota may improve the effectiveness of *H. pylori* eradication. There was no difference in the abundance of mutually-promoting microbiota present in the two groups. The lack of correlation between mutually-promoting microbiota and *H. pylori* in the failure group may have been related to a difference in *H. pylori* strains and needs to be confirmed by further studies.

Differences in the gastric microbiota may have contributed to *H. pylori* eradication failure, because all samples were collected before *H. pylori* eradication. This study has some limitations. It had a relatively small sample size because of the high eradication rate. The result preliminarily showed an effect of the gastric microbiota on *H. pylori* eradication, but studies that have larger sample sizes are needed to confirm these findings. This study did not determine *H. pylori* resistance, but the results provide clinically significant guidance for empirical treatment.

CONCLUSION

The effect of quadruple *H. pylori* eradication therapy containing bismuth depends on the gastric microbiota. A high rate of *H. pylori* eradication was associated with the presence of *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*. The study identified gastric microbiota beneficial to *H. pylori* eradication and laid a foundation for further research on how the gastric microbiota influences *H. pylori* eradication. In addition, the study results may help to improve the eradication rate of *H. pylori* in the future.

ARTICLE HIGHLIGHTS

Research background

There are complex interactions between *Helicobacter pylori* (*H. pylori*) and other microbial communities in the gastric microecological environment. Yet, it remains unclear whether the interactions affect the eradication of *H. pylori*.

Research motivation

The motivation was to explore the interaction between gastric microbiota and *H. pylori* and to determine the influence of gastric microbiota on the eradication of *H. pylori*.

Research objectives

To investigate the characteristics of the gastric mucosa microbiota with *H. pylori* infection and the influence on *H. pylori* eradication treatment. This may help improve the eradication rate of *H. pylori* in the future.

Research methods

Patients with *H. pylori* infection underwent gastroscopy and received treatment. Propensity matching analysis was conducted, including the number of patients who

did not respond to treatment. The gastric microbiota was assayed by high-throughput sequencing and subsequent analysis of alpha diversity, beta diversity, species correlations, and predicted metabolic pathways.

Research results

The main phyla in the two groups were the same in the eight failure group patients who did not respond well to therapy and the 16 success group patients and included Proteobacteria, Bacteroides, Firmicutes, Actinomycetes, and Fusobacteria. The high rate of *H. pylori* eradication was associated with *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*. *H. pylori* was negatively correlated with other bacterial genera, and more bacterial genera were directly related to *H. pylori* in the success group.

Research conclusions

The effectiveness of quadruple *H. pylori* eradication therapy containing bismuth depended on the gastric microbiota. The high rate of *H. pylori* eradication was associated with *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*.

Research perspectives

This study laid a foundation for further research on the mechanism of the influence of the gastric microbiota on *H. pylori* eradication, which will help to improve the eradication rate of *H. pylori* in the future.

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META-ANALYSIS

Endoscopic submucosal dissection *vs* endoscopic mucosal resection for colorectal polyps: A meta-analysis and meta-regression with single arm analysis

Xiong Chang Lim, Kameswara Rishi Yeshayahu Nistala, Cheng Han Ng, Snow Yunni Lin, Darren Jun Hao Tan, Khek-Yu Ho, Choon-Seng Chong, Mark Muthiah

ORCID number: Xiong Chang Lim 0000-0002-0812-891X; Kameswara Rishi Yeshayahu Nistala 0000-0003-1299-8712; Cheng Han Ng 0000-0002-8297-1569; Snow Yunni Lin 0000-0002-2759-2662; Darren Jun Hao Tan 0000-0001-5358-524X; Khek-Yu Ho 0000-0002-2932-1962; Choon-Seng Chong 0000-0003-0669-7307; Mark Muthiah 0000-0002-9724-4743.

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Xiong Chang Lim, Kameswara Rishi Yeshayahu Nistala, Cheng Han Ng, Snow Yunni Lin, Darren Jun Hao Tan, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore

Khek-Yu Ho, Mark Muthiah, Department of Medicine, National University Hospital, Singapore 119074, Singapore

Choon-Seng Chong, Department of Surgery, National University Hospital, Singapore 119074, Singapore

Corresponding author: Mark Muthiah, MBBS, MRCP, MMed, Assistant Professor, Department of Medicine, National University Hospital, 5 Lower Kent Ridge Road, Singapore 119074, Singapore. mdcmdm@nus.edu.sg

Abstract

BACKGROUND

Endoscopic submucosal dissection (ESD) has shown to be effective in management of colorectal neoplasm in the Asian countries, while its implementation in Western countries where endoscopic mucosal resection (EMR) is preferred is still debatable.

AIM

To compare the surgical, histological, and oncological outcomes between ESD and EMR in the treatment of colorectal polyps, with subgroup analysis comparing the efficacy of ESD and EMR between Japan and the rest of the world.

METHODS

Embase and Medline databases were searched from inception to October 2020 in accordance with PRISMA guidelines for studies comparing *en bloc*, complete resection, margin involvement, resection time, need for additional surgery, complications, and recurrence rate of ESD with EMR.

RESULTS

Of 281344 colorectal polyps from 21 studies were included. When compared to EMR, the pooled analysis revealed ESD was associated with higher *en bloc* and complete resection rate, and lower lateral margin involvement and recurrence.

ESD led to increased procedural time, need for additional surgery, and perforation risk. No significant difference in bleeding risk was found between the two groups. Meta-regression analysis suggested only right colonic polyps correlated with an increased perforation risk in ESD. Confounders including polyp size and invasion depth did not significantly influence the *en bloc* and complete resection rate, bleeding risk and recurrence. In subgroup analysis, Japan performed better than the rest of the world in both ESD and EMR with perforation risk of 4% and 0.0002%, respectively, as compared to perforation risk of 8% and 1%, respectively, in reports coming from rest of the world.

CONCLUSION

ESD resulted in better resection outcomes and lower recurrence compared to EMR. With appropriate training, ESD is preferred over EMR as the first-line therapy for resection of colorectal polyps, without restricting to lesions greater than 20 mm and those with high suspicion of submucosal invasion.

Key Words: Endoscopic mucosal resection; Endoscopy; Colonic polyps; Colorectal neoplasm; Colonoscopy

Core Tip: The present study is the most extensive meta-analysis evaluating the surgical, histological, and oncological outcomes of endoscopic submucosal dissection (ESD) in comparison to endoscopic mucosal resection (EMR) in the treatment of colorectal polyps. Our analysis also showed the increased proficiency in performing ESD and EMR in Japan as compared to the rest of the world.

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INTRODUCTION

In recent years, the incidence of colorectal polyps has increased drastically with the widespread implementation of national colorectal cancer (CRC) screening programs. CRC screening with colonoscopy provides the opportunity to identify and remove any precursor lesions[1] and polypectomy has been shown to reduce the incidence and mortality of CRC[1-3]. Currently, 2 major forms of polypectomy are performed, namely endoscopic submucosal dissection (ESD) and endoscopic mucosal resection (EMR) with the latter procedure favored by western countries.

ESD was first proposed in 1999 as an endoscopic resection technique for safe *en bloc* removal of superficial lesions in the upper gastrointestinal tract[4]. Although colorectal ESD is widely practiced in the Asian countries, the implementation of ESD in Western countries where endoscopic mucosal resection is preferred is still debatable. According to the 2015 European Society of Gastrointestinal Endoscopy (ESGE), ESD is strongly recommended when there is high suspicion of submucosal invasion as determined by morphologic factors and advanced surface pattern, especially in lesions greater than 20 mm[5]. Despite the technical difficulties faced, ESD is designed for *en bloc* resection of lesions in an attempt to achieve complete resection, reliable histopathological analysis, and reduced recurrence rate.

Previous meta-analyses and systematic reviews comparing ESD with EMR mainly focused on data reported from Asian countries with limited data coming from outside of Japan, without controlling for confounders or lesions < 20 mm[6,7]. Since then, numerous studies comparing between ESD and EMR have been reported from the West and other countries[8-16]. We sought to perform an expanded meta-analysis to include lesions ≤ 20 mm, and incorporate regression analysis to control for

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confounders.

MATERIALS AND METHODS

Search strategy and inclusion criteria

A search was conducted with reference to the PRISMA methodology on October 4, 2020 in Medline and Embase[17]. Keywords and Mesh terms were employed in the search strategy relating to the 'Colorectal', 'ESD' and 'EMR', the full list of terms included in the search can be found in the Supplementary Material 1. References were managed with Endnote X9. Two authors (XCL, KRYN) were involved in the sieving of the abstracts. Articles that compared between EMR and ESD for colorectal polyps were included in the search. Study designs included both retrospective and prospective cohorts, and case-controlled studies. Editorials, conferences abstracts, and commentaries were excluded in the review.

Data extraction

Based on the pre-determined inclusion criterion, article selection was performed by two authors with consensus from an independent third author should discrepancies arise. Information pertaining to the demographics (country, sample size, age, gender, polyp location, polyp size, polyp macroscopic type and depth of cancer invasion) and outcomes were extracted. Outcomes were limited to *en bloc* resection, complete resection, lateral and vertical margin involvement, lymphovascular invasion, mean operation time, additional surgery required, perforation, bleeding, and recurrence. *En bloc* resection was defined as the resection of the lesion in a single specimen, and complete resection was defined as the resection of the lesion with no marginal involvement of neoplastic tissue, as assessed by the pathologist. Additional surgical operations performed were considered in cases with intra-operative complications as a result of ESD or EMR or those with technical difficulties. Bleeding was considered both intra-operative and post-operative, and perforation was considered when diagnosed endoscopically during resection or radiologically by the presence of free air. Recurrence was defined as the detection of local or secondary primary tumors on interval colonoscopy. When estimates of mean and standard deviation (SD) were not available for continuous data, well established formulae were used to estimate mean and SD from median and range[18].

Statistical analysis and quality assessment

Three type of analysis were performed in this review. Firstly, a meta-analysis of proportions was used to pool results from the individual arms (ESD and EMR) using a freeman turkey double arsine transformation[19]. The freeman turkey double arsine transformation is recommended for single arm meta-analysis and necessary to stabilize the variance[20]. Next, comparative meta-analysis after a 0.5 continuity correction was to compare the difference in ESD and EMR, using risk ratios (RR) as the outcome of interest for dichotomous variables and weighted mean differences for continuous variables[21]. A 0.5 continuity correction was considered appropriate to incorporate 0 events into the meta-analysis. Regardless of heterogeneity measures (I^2, tau, Cochran Q test), all analysis was performed in random effects with the Dersimonian and Laird model[22,23]. Region (Japan *vs* rest of the world) was defined with refences to the manuscript country of origin. Publication bias was assessed with harbord test and egger regression for dichotomous and continuous variables respectively when > 10 studies were available. A univariate meta regression was also conducted with in random effects using the restricted maximum likelihood model with Kapp variance estimator to adjust for confounders in the outcomes[24]. A subgroup analysis was considered to compare between the articles originating from Japan and outside of Japan and a sensitivity analysis was performed to only include studies that have polyps size of ≥ 20 mm. All articles were graded with the Newcastle Ottawa scale for cohort studies, grading articles on the domains of study group selection, study group comparability, and ascertainment of outcomes of interest[25]. The statistical methods of this study were reviewed by Cheng Han Ng (ORCID ID: 0000-0002-8297-1569) from Yong Loo Lin School of Medicine, National University of Singapore.

RESULTS

Study selection

A systematic search of the literature using our search strategy yielded 1180 articles. After removal of duplicates, 895 were excluded based on study title and abstract, and 212 underwent full text review, of which 21 articles comparing ESD with EMR in resection of colorectal polyps were subsequently included in the meta-analysis (Figure 1)[8-16,26-37]. In total, 12 originated from Japan[26-37], five from European[10-13,16], three from South Korea[8,9,14] and one from China[15]. Of the 21 studies, there were 17 retrospective cohort studies, two prospective cohort studies and two retrospective case-control studies. In total, 281344 colorectal polyps were resected, of which 19573 underwent ESD while 261771 underwent EMR. Quality assessment of those included studies mostly scored 6-7. A summary of the study characteristics of the included articles can be found in the Supplementary Table 1.

Overall results

The summary of the results is found in Table 1. The pooled analysis showed a higher rate of *en bloc* resection (RR = 1.837; 95%CI: 1.464 to 2.305; $P < 0.001$), and a lower frequency of positive lateral margin involvement (RR = 0.292; 95%CI: 0.089 to 0.995; $P = 0.042$) in the ESD group than the EMR group. Publication bias was significant for *en bloc* resection rate ($P = 0.0025$). No significant difference in the rates of positive vertical margin involvement was observed between ESD and EMR groups (RR = 4.368; 95%CI: 0.409 to 46.710; $P = 0.223$). However, the rate of complete resection was higher in the ESD compared to EMR groups (RR = 1.504; 95%CI: 1.041 to 2.174; $P < 0.03$). Significantly, the time taken for ESD was longer than EMR (RR = 72.709; 95%CI: 54.487 to 90.931; $P < 0.001$). ESD group required more additional surgical operations relative to that of EMR group (RR = 3.139; 95%CI: 1.360 to 7.243; $P = 0.007$).

Compared to EMR, ESD shows a significant increased risk of perforation (RR = 7.597; 95%CI: 4.281 to 13.479; $P < 0.001$) (Figure 2), but no significant difference in the bleeding risk was observed between the two groups (RR = 1.277; 95%CI: 0.896 to 1.820; $P = 0.175$) (Figure 3). The rate of recurrence in the ESD group was lower than that of the EMR group (RR = 0.269; 95%CI: 0.112 to 0.648; $P = 0.003$) (Figure 4).

Meta regression

The influence of the included covariates on *en bloc* resection, complete resection, risk of perforation and bleeding, and rate of recurrence evaluated by meta-regression are summarized in Table 2. Meta-regression analysis for risk of perforation suggested that right colonic polyps ($\beta = 7.731$; 95%CI: 4.965 to 10.497; $P < 0.001$) correlated with an increased risk in perforation in ESD (Figure 5). Other factors including polyp size and depth of invasion, did not influence the *en bloc* resection rate, complete resection rate, the risk of bleeding, and recurrence rate.

Sensitivity analysis for articles ≥ 20 mm

The results of the sensitivity analysis are summarized in Supplementary Table 2 and results were largely unchanged after a sensitivity analysis. When a sensitivity analysis was performed to include ≥ 20 mm colorectal polyps only, the pooled analysis revealed higher rate of *en bloc* resection (RR = 1.932; 95%CI: 1.389 to 2.688; $P < 0.001$), longer operation time (RR = 3.247; 95%CI: 59.249 to 87.245; $P < 0.001$), higher risk of perforation (RR = 4.513; 95%CI: 2.531 to 8.046; $P < 0.001$) and lower recurrence rate (RR = 0.191; 95%CI: 0.085 to 0.431; $P < 0.001$) in ESD compared to EMR groups. Furthermore, in the two studies included, more additional surgical operations were required (RR = 3.139; 95%CI: 1.360 to 7.243; $P = 0.007$) in the ESD than in the EMR groups.

Subgroup analysis by region

The results of the subgroup analysis comparing outcomes of ESD with EMR in Japan *vs* the rest of the world are presented in Table 3.

ESD in Japan had an *en bloc* resection rate of 89% (95%CI: 0.77 to 0.97), perforation risk of 4% (95%CI: 0.01 to 0.07), bleeding risk of 2.4% (95%CI: 0.01 to 0.04) and recurrence rate of 1% (95%CI: 0.01 to 0.02) while its EMR had an *en bloc* resection rate of 53% (95%CI: 0.38 to 0.67), perforation risk of 0.0002% (95%CI: 0.00 to 0.00081), bleeding risk of 2.1% (95%CI: 0.01 to 0.03) and recurrence rate of 7% (95%CI: 0.02 to 0.15). Comparing between the two techniques in Japan, ESD had higher *en bloc* resection rate (RR = 1.658, 95%CI: 1.270 to 2.165, $P < 0.001$), perforation risk (RR = 9.586, 95%CI: 4.425 to 20.768, $P < 0.001$) and bleeding risk (RR = 1.267, 95%CI: 1.174 to

Table 1 Pooled proportions and comparative meta-analysis of endoscopic submucosal dissection and endoscopic mucosal resection

	Total papers	Sample size (ESD)	Pooled proportions	Sample size (EMR)	Pooled proportions	RR (CI)	*P* value	Publication bias
En bloc resection	11	1641	89% (0.83-0.94)	1411	47% (0.36-0.59)	1.837 (1.464-2.305)	< 0.001	0.0025
Positive lateral margin	2	123	3% (0.01-0.06)	187	14% (0.09-0.19)	0.292 (0.089-0.995)	0.042	-
Positive vertical margin	1	38	5% (0.00-0.17)	83	1% (0.00-0.07)	4.368 (0.409-46.710)	0.223	-
Complete resection	8	918	82% (0.74-0.88)	1012	56% (0.34-0.77)	1.504 (1.041-2.174)	0.03	-
Lymphovsacular invasion	1	54	6% (0.03-0.13)	23	0% (0.00-0.04)	4.352 (0.248-76.483)	0.315	-
Mean procedural time	8	1087	-	838	-	72.709 (54.487-90.931)	< 0.001	-
Additional surgery	2	99	13% (0.07-0.21)	153	5% (0.02-0.09)	3.139 (1.360-7.243)	0.007	-
Perforation	18	19470	5% (0.03-0.09)	260901	0% (0.00-0.01)	7.597 (4.281-13.479)	< 0.001	0.301
Bleeding	14	20048	3% (0.02-0.05)	257065	3% (0.02-0.04)	1.277 (0.896-1.820)	0.175	0.139
Recurrences	12	1822	2% (0.01-0.03)	37721	10% (0.04-0.17)	0.269 (0.112-0.648)	0.003	0.725

ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection; CI: Confidence interval.

1.367, $P < 0.001$) than EMR. Following sensitivity analysis, similar results were obtained, except ESD had lower recurrence rate than EMR (RR = 0.204, 95%CI: 0.097 to 0.429, $P < 0.001$), and there was no difference in bleeding risk (RR = 0.895, 95%CI: 0.438 to 1.829, $P = 0.762$) between the two techniques.

With regards to studies from the rest of the world, ESD had an *en bloc* resection rate of 90% (95%CI: 0.85 to 0.94), perforation risk of 8% (95%CI: 0.05 to 0.12), bleeding risk of 5% (95%CI: 0.02 to 0.11) and recurrence rate of 3% (95%CI: 0.00 to 0.08) while EMR had an *en bloc* resection rate of 41% (95%CI: 0.22 to 0.61), perforation risk of 1% (95%CI: 0.00 to 0.02), bleeding risk of 3% (95%CI: 0.01 to 0.06) and recurrence rate of 16% (95%CI: 0.04 to 0.32). Comparing between the two techniques, ESD had higher *en bloc* resection rate (RR = 2.201, 95%CI: 1.411 to 3.433, $P = 0.001$), perforation rate (RR = 4.602, 95%CI: 2.729 to 7.759, $P < 0.001$) and lower recurrence rate (RR = 0.245, 95%CI: 0.073 to 0.819, $P = 0.022$) than EMR. Following sensitivity analysis, similar results were obtained.

DISCUSSION

The advancements in endoscopic resection techniques have resulted in the shift from radical surgery to minimally invasive and organ-sparing endoscopic resection techniques, such as ESD and EMR, for the treatment of colorectal lesions. With reference to 2015 ESGE guidelines, ESD should be considered for colorectal lesions larger than 20 mm, with high suspicion of submucosal invasion, or those where *en bloc* resection by EMR are not feasible[5]. Previous meta-analyses comparing ESD and EMR for colorectal polyps primarily reported data from Asian countries, with 72.7% of the published studies from Japan[6]. Since then, several retrospective and prospective studies comparing ESD and EMR for the treatment of colorectal polyps have been published outside of Japan. The present study is the most extensive meta-analysis evaluating the surgical, histological, and oncological outcomes of ESD in comparison to EMR in the treatment of colorectal polyps. Nine out of 21 studies (42.9%) on colorectal polyps included in this meta-analysis were conducted in countries outside of Japan. While ESD has been known to provide significantly better resection outcomes and lower recurrence rate, our analysis found that polyp size and depth of invasion did not significantly influence the *en bloc* and complete resection rate,

Table 2 Meta-regression

	En bloc resection			Complete resection			Perforation			Bleeding			Recurrence		
	n	Beta (CI)	*P* value	*n*	Beta (CI)	*P* value	*n*	Beta (CI)	*P* value	*n*	Beta (CI)	*P* value	*n*	Beta (CI)	*P* value
Age	8	-0.017 (-0.168-0.133)	0.785	5	-0.010 (-0.426-0.406)	0.944	9	0.073 (-0.191-0.336)	0.535	8	-0.201 (-0.540-0.138)	0.197	8	0.254 (-0.039-0.547)	0.078
Gender	6	-6.023 (-23.152-11.106)	0.384	4	-20.662 (-108.758-67.434)	0.419	8	-7.868 (-22.078–6.342)	0.224	7	-4.446 (-27.915-19.022)	0.647	6	-9.096 (-62.174-43.982)	0.659
Polyp size	10	0.242 (-0.037-0.086)	0.390	6	-0.034 (-0.098-0.030)	0.217	10	-0.034 (-0.117-0.049)	0.378	9	-0.041 (-0.177-0.095)	0.498	8	0.077 (-0.118-0.272)	0.369
Lateral spreading tumor	10	1.262 (-1.072-3.596)	0.248	7	-0.481 (-4.655-3.693)	0.779	11	-2.324 (-5.536-0.888)	0.136	9	-0.680 (-5.586-4.226)	0.753	9	3.827 (-2.323-9.977)	0.185
Right colon	8	1.247 (-2.024-4.517)	0.387	5	2.373 (-0.881-5.626)	0.103	8	7.731 (4.965-10.497)	< 0.001	7	1.688 (-12.442-15.817)	0.771	6	-8.739 (-26.616-9.139)	0.246
Colon	7	-1.803 (-4.486-0.880)	0.145	4	-1.914 (-7.213-3.385)	0.26	8	0.759 (-3.184-4.701)	0.654	6	6.649 (-5.182-18.480)	0.194	7	-1.102 (-4.754-2.551)	0.473
Rectum	7	1.803 (-0.880-4.486)	0.145	4	1.914 (-3.385-7.213)	0.26	8	-0.759 (-4.701-3.184)	0.654	6	-6.649 (-18.480-5.182)	0.194	7	1.102 (-2.551-4.754)	0.473
Submucosal invasion	8	-0.004 (-0.014-0.007)	0.431	5	-0.006 (-0.020-0.008)	0.253	9	0.001 (-0.049-0.051)	0.956	8	-0.016 (-0.033-0.001)	0.061	7	-6.508 (-27.976-14.960)	0.471
Muscularis propria invasion	8	-5.870 (-17.738-5.998)	0.272	5	-0.157 (-21.715-21.401)	0.983	9	6.731 (-33.772-47.234)	0.706	8	-109.836 (-1040.943-821.272)	0.783	-	-	-

CI: Confidence interval.

bleeding and perforation risk, and recurrence rate in colorectal polyps that was not previously reported. Additionally, previous reviews were confined only to sessile lesions larger than 20 mm[6,7]. Our analysis also showed the increased proficiency in performing ESD and EMR in Japan as compared to the rest of the world.

Consistent with previous studies, ESD showed benefits in the technical, histological, and oncological outcomes. Pooled analysis showed higher rates of *en bloc* resection and complete resection in ESD than in EMR albeit significant publication bias (P = 0.0025). *En bloc* resection offers the technical advantage of removing the entire pathologic specimen, thus allowing for detailed histologic evaluation. This results in an increase in the complete resection rate which in turn reduces the recurrence rate. Therefore, *en bloc* resection with ESD is favored as it provides curative treatment without the need for surgery for lesions with significant likelihood of submucosal invasion[38]. However, the advantages of ESD come at the expense of longer procedural time, additional surgical operations, and increased perforation risk compared to EMR[6]. The high rate of additional surgical operations for ESD is presumed to be due to the aggressive selection of ESD for T1 Lesions. Although the perforation risk was higher with ESD, most perforations in the studies included were treated conservatively or endoscopically using endoclips[9,12,31,33,34,37].

Meta-regression was performed to assess the risk factors that affect surgical, histological, and oncological outcomes. Our present analysis showed that polyp size did not affect the risk of perforation, which was reported otherwise in studies by Kim

Table 3 Subgroup analysis (Japan and rest of the world) of pooled proportions and comparative meta-analysis of endoscopic submucosal dissection and endoscopic mucosal resection

	Japan papers	Pooled proportions (ESD)	Pooled proportions (EMR)	RR (CI)	*P* value	Rest of the world papers	Pooled proportions (ESD)	Pooled proportions (EMR)	RR (CI)	*P* value
Overall results										
En bloc resection	6	89% (0.77-0.97)	53% (0.38-0.67)	1.658 (1.270-2.165)	< 0.001	5	90% (0.85-0.94)	41% (0.22-0.61)	2.201 (1.411-3.433)	0.001
Positive lateral margin	2	3% (0.00-0.06)	14% (0.09-0.19)	0.292 (0.089-0.955)	0.042	-	-	-	-	-
Positive vertical margin	1	5% (0.01-0.17)	1% (0.00-0.05)	4.368 (0.409-46.710)	0.223	-	-	-	-	-
Complete resection	2	79% (0.73-0.84)	53% (0.48-0.58)	1.452 (1.303-1.618)	< 0.001	6	85% (0.78-0.91)	59% (0.27-0.88)	1.562 (0.921-2.650)	0.098
Lymphovsacular invasion	1	6% (0.03-0.13)	0% (0.00-0.04)	4.352 (0.248-76.483)	0.315	-	-	-	-	-
Mean procedural time	4	-	-	72.106 (48.831-95.382)	< 0.001	4	-	-	73.916 (36.075-111.757)	< 0.001
Additional surgery	2	13% (0.07-0.21)	5% (0.02-0.09)	3.139 (1.360-7.243)	0.007	-	-	-	-	-
Perforation	11	4% (0.01-0.07)	0.0002% (0.00-0.00081)	9.586 (4.425-20.768)	< 0.001	7	8% (0.05-0.12)	1% (0.00-0.02)	4.602 (2.729-7.759)	< 0.001
Bleeding	8	2.4% (0.01-0.04)	2.1% (0.01-0.03)	1.267 (1.174-1.367)	< 0.001	6	5% (0.02- 0.11)	3% (0.01-0.06)	1.986 (0.716-5.508)	0.188
Recurrences	7	1% (0.01-0.02)	7% (0.02-0.15)	0.274 (0.071-1.054)	0.06	5	3% (0.00-0.08)	16% (0.04-0.32)	0.245 (0.073-0.819)	0.022
Sensitivity analysis (≥ 20 mm only)										
En bloc resection	4	82% (0.72-0.91)	50% (0.33-0.67)	1.645 (1.174-2.306)	0.004	2	91% (0.88-0.93)	33% (0.28-0.37)	2.668 (1.752-4.063)	< 0.001
Positive lateral margin	2	3% (0.00-0.06)	14% (0.09-0.19)	0.292 (0.089-0.955)	0.042	-	-	-	-	-
Positive vertical margin	1	5% (0.01-0.17)	1% (0.00-0.07)	4.368 (0.409-46.710)	0.223	-	-	-	-	-
Complete resection	2	79% (0.73-0.84)	53% (0.48-0.58)	1.452 (1.303-1.618)	< 0.001	2	90% (0.87-0.93)	77% (0.73-0.82)	1.613 (0.209-12.452)	0.647
Lymphovsacular invasion	2	6% (0.03-0.13)	0% (0.00-0.04)	4.352 (0.248-76.483)	0.315	-	-	-	-	-
Mean procedural time	2	-	-	69.167 (48.446-89.888)	< 0.001	1	-	-	82.700 (67.578-97.822)	< 0.001
Additional surgery	2	13% (0.07-0.21)	5% (0.02-0.09)	3.139 (1.360-7.243)	0.007	-	-	-	-	-
Perforation	5	4% (0.01-0.07)	0% (0.00-0.01)	5.235 (2.123-12.910)	< 0.001	3	7% (0.05-0.10)	1% (0.00-0.03)	4.546 (1.674-12.346)	0.003

Bleeding	4	3% (0.00-0.07)	3% (0.01-0.05)	0.895 (0.438-1.829)	0.762	3	3% (0.00-0.08)	1% (0.00-0.04)	2.233 (0.489-10.197)	0.300
Recurrences	5	1% (0.00-0.02)	7% (0.02-0.15)	0.204 (0.097-0.429)	< 0.001	3	3% (0.00-0.10)	27% (0.10-0.20)	0.179 (0.032-0.990)	0.049

ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection; RR: Relative risk; CI: Confidence interval.

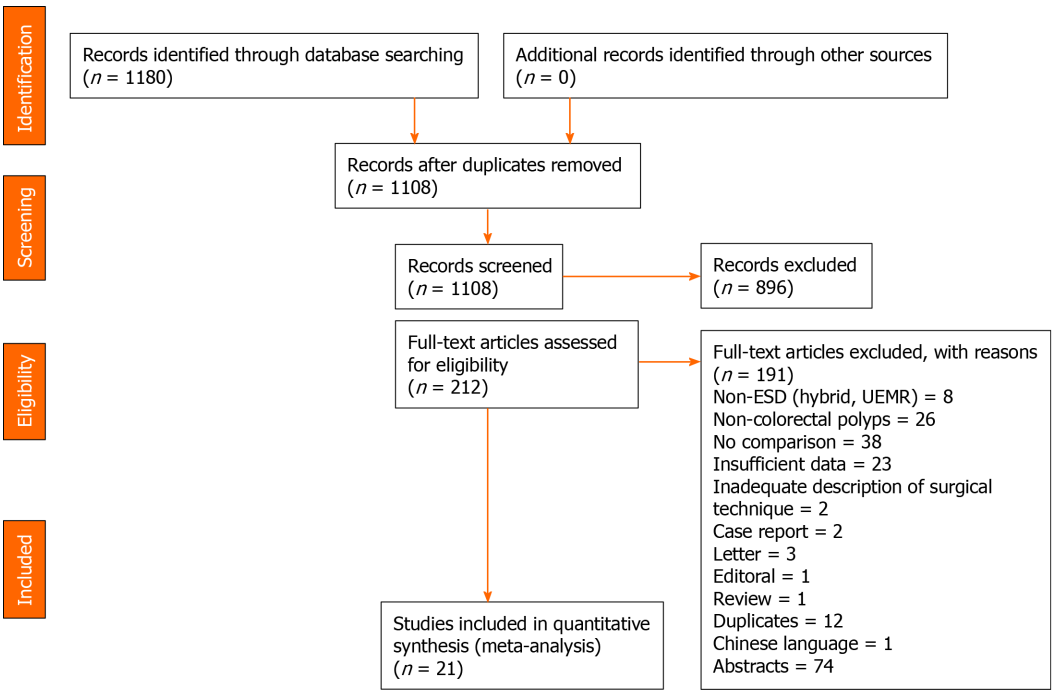

Figure 1 PRISMA flow diagram of included articles. ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection.

et al[39] and Hong *et al*[40] Polyp size and depth of invasion were also not associated with significant change in *en bloc* and complete resection rate, risk of bleeding, and recurrence between ESD and EMR. Furthermore, the *en bloc* resection rate (before RR = 1.837, 95%CI: 1.464 to 2.305, $P < 0.001$; after RR = 1.932, 95%CI: 1.389 to 2.688, $P < 0.001$) and recurrence rate (before RR = 0.269, 95%CI: 0.112 to 0.648, $P = 0.003$; after RR = 0.191, 95%CI: 0.085 to 0.431; $P < 0.001$) appeared comparable before and after the sensitivity analysis to ≥ 20 mm polyps. Instead, the risk of perforation was increased in patients with right colonic polyps and this was consistent with previous study which identified the technical difficulty and proficient endoscopic skills required to remove polyps from right colon safely[41]. As such, training should ensure endoscopists achieve procedural proficiency in left sided lesions before proceeding to attempt right sided lesions. Other factors including polyp size and depth of invasion are less important criteria when deciding between EMR and ESD in skilled tertiary centers.

Despite the advantages of ESD, the data regarding the efficacy of colorectal ESD have been inconsistent and vary between Japan and the rest of the world. One of the reasons is the limitations to the implementation of ESD in other countries, which are in part due to the lack of expertise and training centers. To date, no meta-analysis comparing ESD and EMR between Japan and the rest of the world have been performed. Our single arm meta-analysis found that Japan performed better than the rest of the world in ESD and EMR. Significantly, perforation is a major concern in ESD. The perforation risk of ESD and EMR was 4% and 0.0002%, respectively in Japan, and 8% and 1%, respectively in the rest of the world. While there is an observed increase risk of perforation from Japan only studies (RR = 9.586) compared to the rest of the world (RR = 4.602), even after sensitivity analysis, the risk of perforation for ESD was only statistically higher in Japan only studies due to the very low risk of perforation in EMR in Japan. In addition, the challenges of doing ESD in difficult could have resulted in the higher perforation rate in ESD compared with EMR in Japan. Another important factor to consider is the recurrence rate of ESD and EMR which was 1% and 7%,

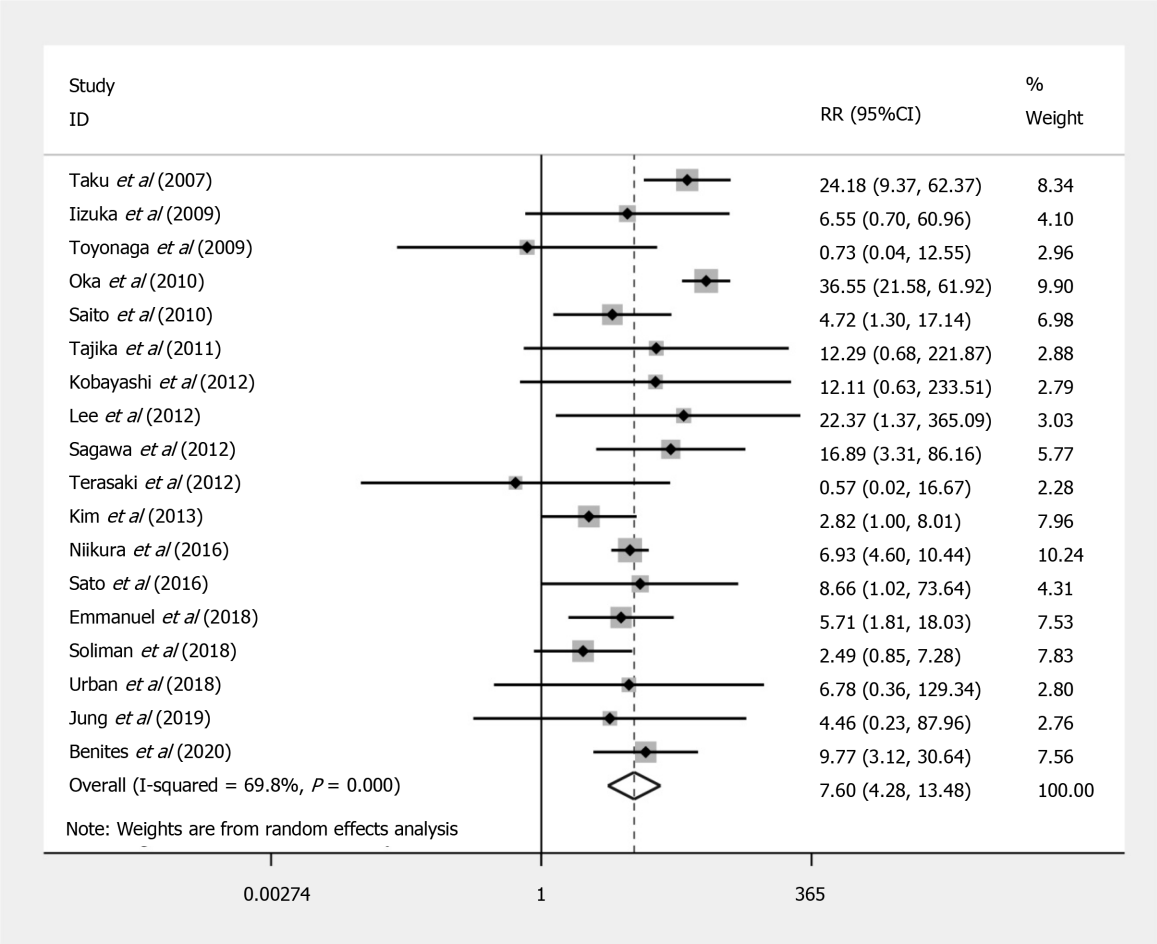

Figure 2 Forest plot for risk of perforation. RR: Relative risk; CI: Confidence interval.

respectively in Japan and 3% and 27%, respectively in the rest of the world following sensitivity analysis. The observed increase in recurrence rate from Japan only studies (RR = 0.204) compared to the rest of the world (RR = 0.179) was due to the much lower recurrence rate of EMR in Japan as compared to the rest of the world. Overall, our results seem to favor studies from Japan and are in tandem with a single arm analysis of ESD only procedures with subgroup on region efficacy[42]. However, most of the studies originating from the rest of the world should be interpreted with the understanding that these studies are mainly from tertiary centers and the results may not be generalized to non-tertiary centers.

The potential of ESD resection is limited by the difficulty in conducting the procedure as the length of procedure for ESD even when performed by experienced endoscopists can be three times longer than that for EMR[43]. However, the advancement in endoscopic resection equipment has been shown to shorten the procedure time despite a relatively short training duration[44]. Using the cumulative sum method, Miyakawa *et al*[44] recently reported the use of Stag-Beetle Knife Jr for ESD in a Japan single-center study generated good learning curve to achieve satisfactory resection speed (min/cm^2), which allowed the acquisition of proficient and safe skills within 120 cases[44]. Other alternatives to ESD do exist, such as hybrid ESD and pre-cut EMR. This hybrid approach has been shown to have lower *en bloc* resection rate (68.4% *vs* 91.0%) and complete resection rate (60.6% *vs* 82.9%) than conventional ESD[42]. Currently, underwater EMR has been thought to be a safe and effective method with higher rate of *en bloc* resection and lower rate of recurrence[45], but no head-to-head comparisons have been done between UMER and ESD.

The inclusion of 21 studies with a total of 281344 polyps based on our search strategy and inclusion criteria represents the most extensive meta-analysis on this issue. However, as no randomized controlled trials comparing the performance between EMR and ESD have yet been conducted, this highlights the need for a randomized study to better understand the efficacy and safety of these techniques in the management of colorectal polyps. The evaluation of heterogeneity allowed us to compare the significant differences in the performance of ESD and EMR between

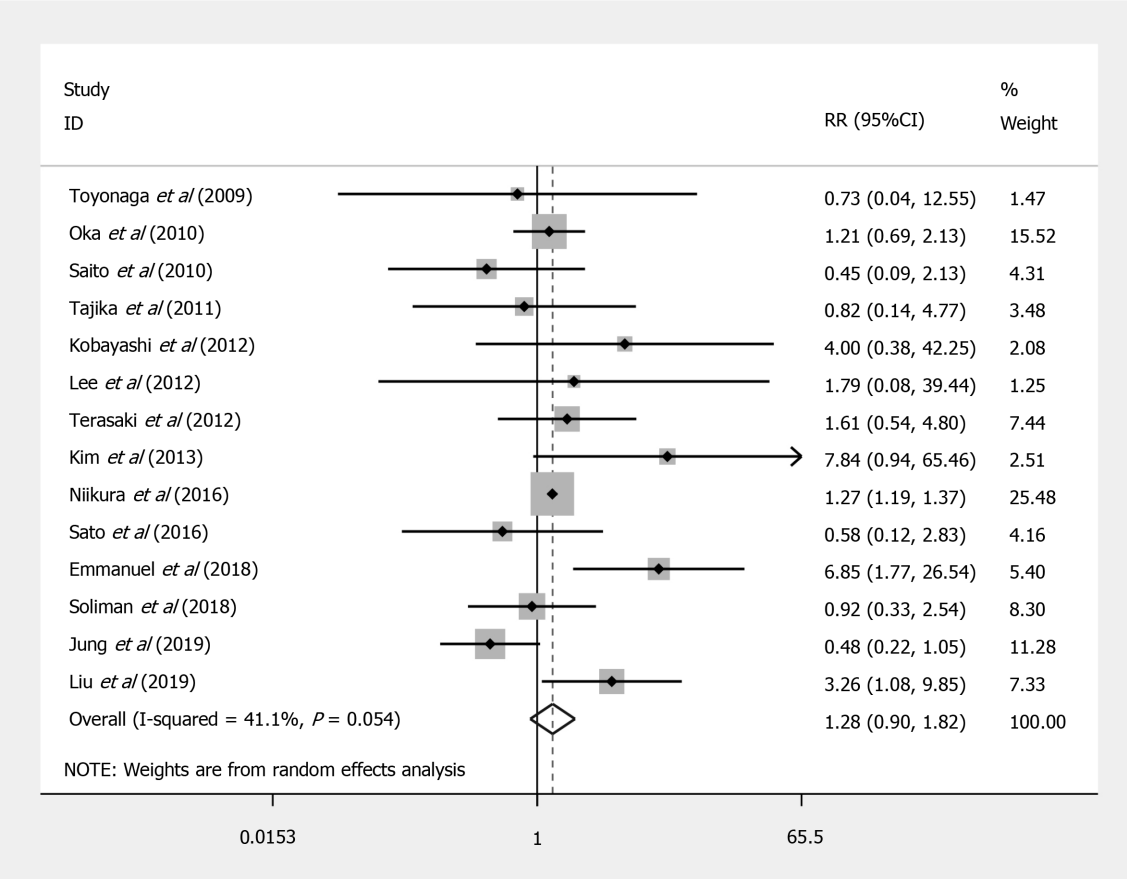

Figure 3 Forest plot for risk of bleeding. RR: Relative risk; CI: Confidence interval.

Japan and the rest of the world.

Our meta-analysis has some limitations. While we aimed to decrease heterogeneity, the included articles used a variety of EMR techniques including standard EMR, piecemeal EMR, EMR with small incision, EMR-precutting and EMR-circumferential incision. This, however, was an acceptable confounding factor in previous meta-analysis analysis. Also, a major factor that we were unable to regress for was the procedural skills of each centers. ESD and EMR are largely operator dependent and we were only able to account for it in a subgroup analysis comparing between studies conducted in Japan and the rest of the world.

CONCLUSION

Evidence from this meta-analysis suggests that with appropriate training, ESD is preferred over EMR as the first-line therapy for resection of colorectal polyps, without restricting to lesions greater than 20 mm and those with high suspicion of submucosal invasion. Our overall findings are consistent with previous meta-analyses showing ESD is associated with higher rate of *en bloc* and complete resection, and lower recurrence compared to EMR, but at the cost of increased procedural time, need for additional surgical operations and perforation risk. This is coupled with the new finding that confounders including polyp size and invasion depth did not influence the rates of *en bloc* and complete resection, bleeding risk, and recurrence.

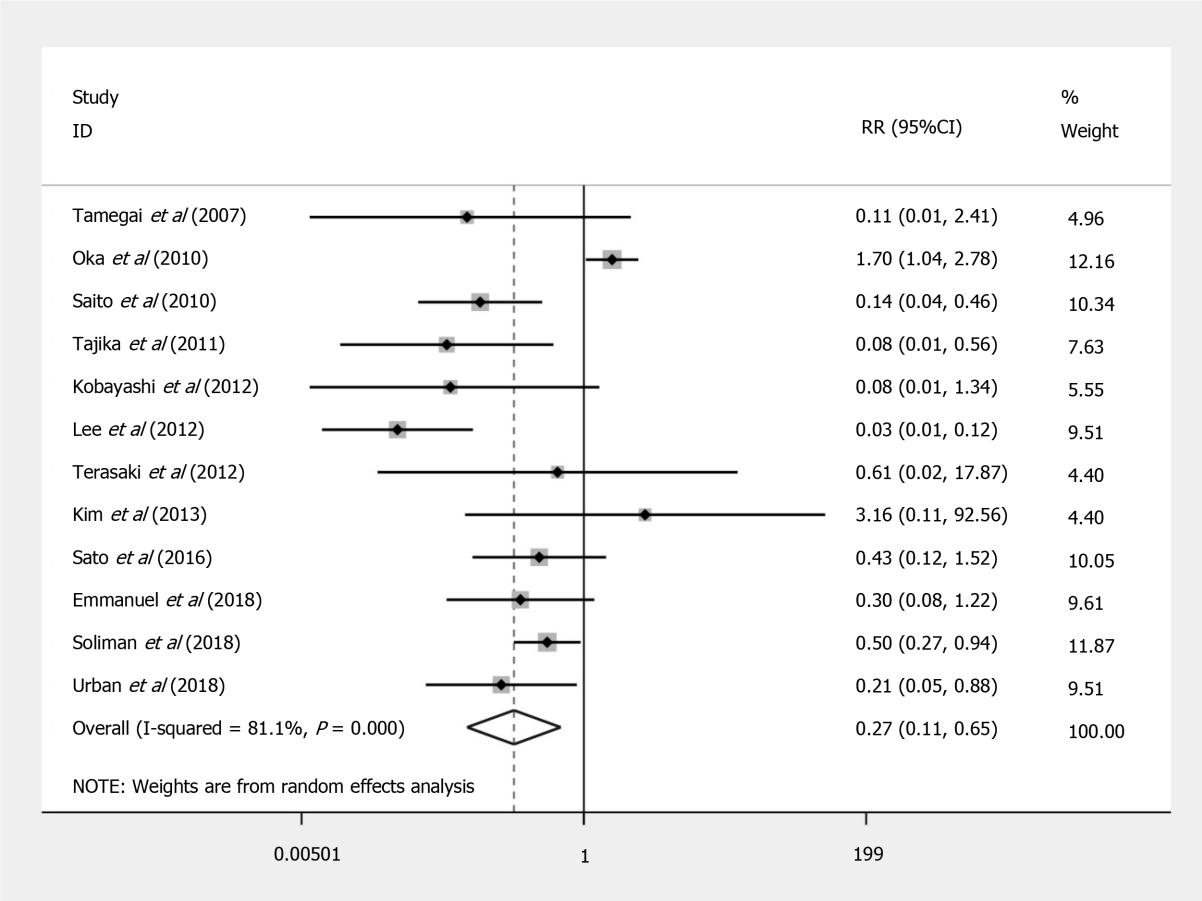

Figure 4 Forest plot for recurrence rate. RR: Relative risk; CI: Confidence interval.

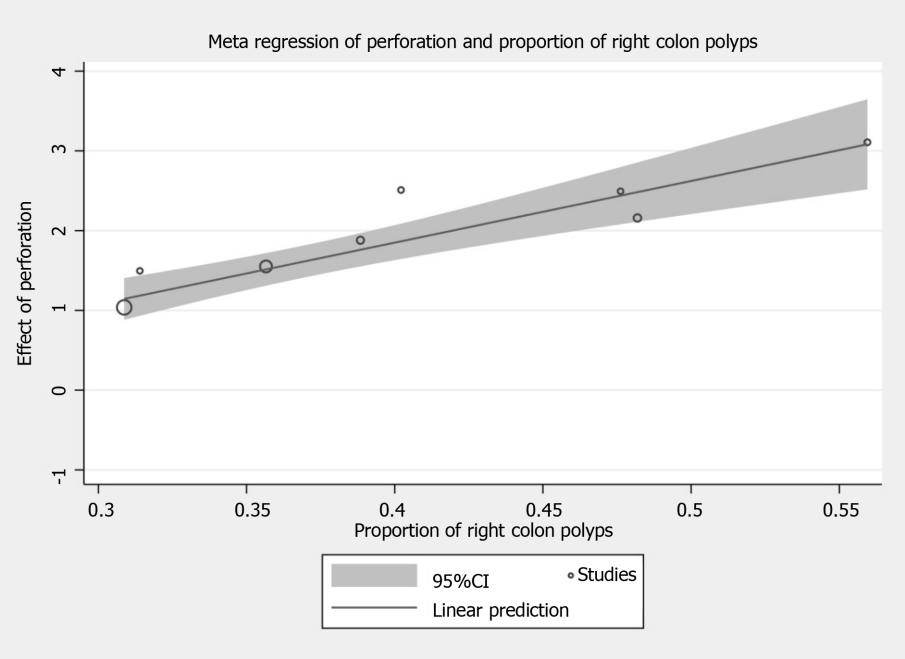

Figure 5 Meta regression of perforation and proportion of right colon polyps. CI: Confidence interval.

ARTICLE HIGHLIGHTS

Research background

Endoscopic submucosal dissection (ESD) has shown to be effective in management of colorectal neoplasm in the Asian countries, while its implementation in Western countries where endoscopic mucosal resection (EMR) is preferred is still debatable.

Research motivation

Previous meta-analyses and systematic reviews comparing ESD with EMR included studies mainly conducted in Asia, with limited data coming from outside of Japan and did not control for confounders or lesions smaller than 20 mm.

Research objectives

To compare the outcomes of ESD and EMR in the treatment of colorectal polyps, with subgroup analysis comparing the efficacy of these two techniques between Japan and the rest of the world.

Research methods

Embase and Medline databases were searched in accordance with PRISMA guidelines for studies comparing *en bloc*, complete resection, margin involvement, resection time, need for additional surgery, complications, and recurrence rate of ESD with EMR in patients with colorectal lesions.

Research results

ESD was associated with better resection outcomes and lower recurrence rate when compared to EMR. Meta-regression analysis suggested only right colonic polyps correlated with an increased perforation risk in ESD, while confounders including polyp size and invasion depth did not significantly influence the resection outcomes, bleeding risk and recurrence. Subgroup analysis showed that Japan performed better than the rest of the world in both ESD and EMR with lower perforation risk.

Research conclusions

This meta-analysis suggests that with appropriate training, ESD is preferred over EMR as the first-line therapy for resection of colorectal polyps, without restricting to lesions greater than 20 mm and those with high suspicion of submucosal invasion. Increased proficiency in performing ESD and EMR was shown in Japan as compared with the rest of the world.

Research perspectives

This highlights the need to establish adequate training programs for colorectal ESD to be performed effectively. A randomized controlled trial is necessary to better understand the efficacy and safety of these techniques in the management of colorectal polyps.

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CASE REPORT

Gastric schwannoma treated by endoscopic full-thickness resection and endoscopic purse-string suture: A case report

Zhi-Yu Lu, Dun-Yong Zhao

ORCID number: Zhi-Yu Lu 0000-0002-6701-7478; Dun-Yong Zhao 0000-0002-8877-0514.

Author contributions: Lu ZY reviewed the literature and was responsible for manuscript drafting and organization of illustrations and contributed to the interpretation of the imaging findings and endoscopic findings; Zhao DY analyzed and interpreted the pathological findings, immunohistochemical findings, and genetic mutation and was responsible for the revision of the manuscript for important intellectual content; All authors issued final approval for the version to be submitted.

Informed consent statement: Informed written consent was obtained from the patient for publication of this report and any accompanying images.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

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Zhi-Yu Lu, Dun-Yong Zhao, Departments of Gastroenterology, Institute of Digestive, Southwest Hospital, Army Military Medical University, Chongqing 400038, China

Corresponding author: Dun-Yong Zhao, MM, Associate Chief Physician, Department of Gastroenterology, Institute of Digestive, Southwest Hospital, Army Military Medical University, No. 30 Gaotanyan Main Street, Shapingba District, Chongqing 400038, China. 872083291@qq.com

Abstract

BACKGROUND

Schwannomas, also known as neurinomas, are tumors that derive from Schwann cells. Gastrointestinal schwannomas are extremely rare, but the stomach is the most common site. Gastric schwannomas are usually asymptomatic. Endoscopy and imaging modalities might offer useful preliminary diagnostic information. However, to diagnose schwannoma, the immunohistochemical positivity for S-100 protein is essential, whereas CD117, CD34, SMA, desmin, and DOG-1 are negative.

CASE SUMMARY

A 45-year-old female was found to have a gastric mass during a medical examination, which was diagnosed as a gastric schwannoma. We performed endoscopic full-thickness resection and endoscopic purse-string suture. Pathology and immunohistochemical staining confirmed the diagnosis of gastric schwannoma through the positivity of S-100 protein. Furthermore, to exclude the misdiagnosis of gastrointestinal stromal tumor, we performed a mutational detection of the *c-Kit* and *PDGFRA* genes. Postoperative follow-up revealed that the patient recovered well.

CONCLUSION

Immunohistochemical staining is essential for the diagnosis of schwannoma. Endoscopic full-thickness resection is an effective treatment method for gastric schwannoma.

Key Words: Gastric schwannoma; Endoscopic full-thickness resection; Endoscopic purse-string suture; Immunohistochemical staining; Gene mutational analysis; Case report

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Core Tip: Schwannomas can occur in any part of the digestive tract but are most common in the stomach. Gastric schwannomas are typically asymptomatic, and it is difficult to make a precise preoperative diagnosis. The final diagnosis of schwannoma is based on immunohistochemical staining. We performed endoscopic full-thickness resection and endoscopic purse-string suture. We report a case diagnosed with gastric schwannoma.

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INTRODUCTION

Schwannomas, which are also known as neurinomas, were first described in 1910 by Verocay and are rarely observed in the gastrointestinal tract[1,2]. The stomach is the site with the highest incidence of schwannomas in the gastrointestinal tract[3]. Gastric schwannomas are typically asymptomatic, and the most common symptoms are stomachache, abdominal mass, and gastrointestinal hemorrhage[4,5].

Although endoscopy and imaging modalities, such as computed tomography (CT), magnetic resonance imaging, positron emission tomography-CT, might offer useful preliminary diagnostic information, it is still difficult to achieve the precise preoperative diagnosis of gastric schwannomas. Immunohistochemical positivity of S-100 protein is essential for the final diagnosis of schwannoma, whereas CD117, CD34, SMA, desmin, and DOG-1 are negative[6,7]. Gastric schwannomas are almost always benign with no recurrence or metastasis[6,8], and the optimal treatment for gastric schwannoma is surgical resection.

In our case, the schwannoma was discovered incidentally by abdominal CT. Gastroscopy and endoscopic ultrasonography (EUS) were then performed. Endoscopic full-thickness resection and endoscopic purse-string suture were performed. Finally, the diagnosis of gastric schwannoma was confirmed by histological, immunohistochemical, and gene mutational investigations.

CASE PRESENTATION

Chief complaints

A 45-year-old female had a gastric mass during a medical examination.

History of present illness

A 45-year-old female visited a local hospital for a regular health examination without any symptoms. The patient had an abdominal CT scan, which revealed a rounded mass arising from the greater curvature of the gastric body, suggesting a gastrointestinal stromal tumor (GIST) as a likely diagnosis. For further diagnosis and treatment, she was admitted to the Department of Gastroenterology at our hospital.

History of past illness

There was no other significant medical history. The patient had no history of prior gastroenterological symptoms. There was no relevant history including past interventions and outcomes.

Personal and family history

The patient had no history of smoking or drinking alcohol. Her occupation was a housewife. There was no relevant family history.

Physical examination

All vital signs of the patient were stable, and physical examination revealed no

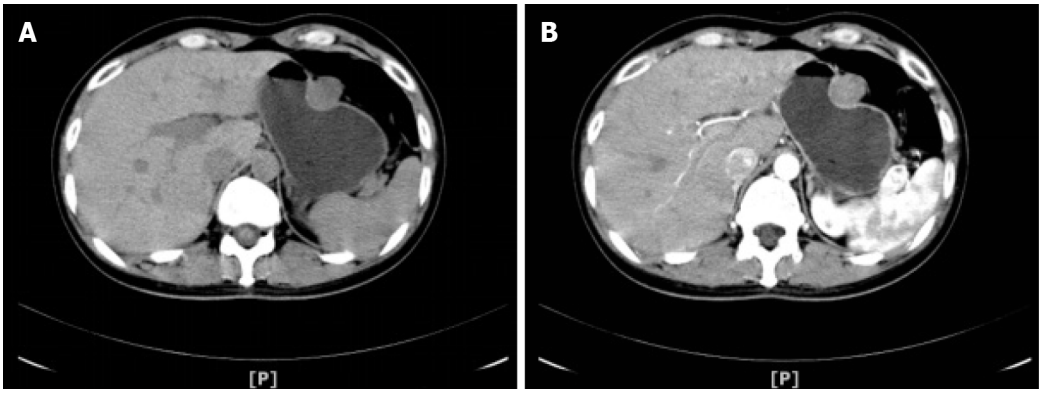

Figure 1 Abdominal computed tomography scanning. A: Computed tomography scan revealed a rounded mass arising from the greater curvature of the gastric body; B: The gastric mass exhibited slight internal contrast enhancement.

noteworthy positive sign.

Laboratory examinations

The levels of the tumor markers AFP, CA125, and CEA were in the normal range. Blood tests, fecal examinations, and coagulation function all demonstrated normal results.

Imaging examinations

Nine days before admission, the patient underwent abdominal CT scanning for a medical examination, and it revealed a 24.9 mm × 23.9 mm rounded mass arising from the greater curvature of the gastric body with slight internal contrast enhancement (Figure 1), suggesting a GIST as a likely diagnosis. No enlarged pericolic lymph nodes were observed.

Endoscopic examinations

Gastroscopy demonstrated a 2.0 cm × 1.8 cm hemispherical protrusion lesion of the gastric body, and EUS revealed hypoechoic and homogeneous echo lesions originating from the muscularis propria (Figure 2).

Pathological findings and immunohistochemical staining

Pathological analysis and immunohistochemical staining (Figure 3) confirmed the diagnosis of gastric schwannoma through positivity for S-100 protein, whereas CD117, CD34, α-SMA, desmin, and DOG-1 were negative.

Gene mutational analysis

To provide evidence for the differential diagnosis of GIST, we performed a mutational detection of the *c-Kit* and *PDGFRA* genes (Figure 4), and the results showed that no mutations were detected in the sample.

FINAL DIAGNOSIS

The final diagnosis of the presented case was gastric schwannoma.

TREATMENT

Endoscopic full-thickness resection and endoscopic purse-string suture were performed (Figure 5). Postoperatively, acid suppression, hemostasis, protection of the gastric mucosa, and nutritional support were administered.

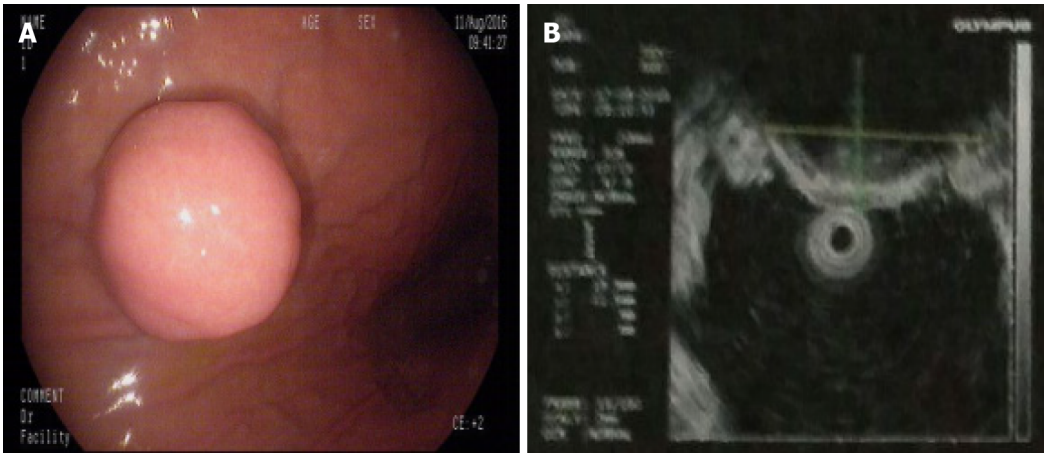

Figure 2 Gastroscopy and endoscopic ultrasonography. A: Gastroscopy demonstrated a hemispherical protrusion lesion of the gastric body; B: Endoscopic ultrasonography showed that the lesion arose from the muscularis propria.

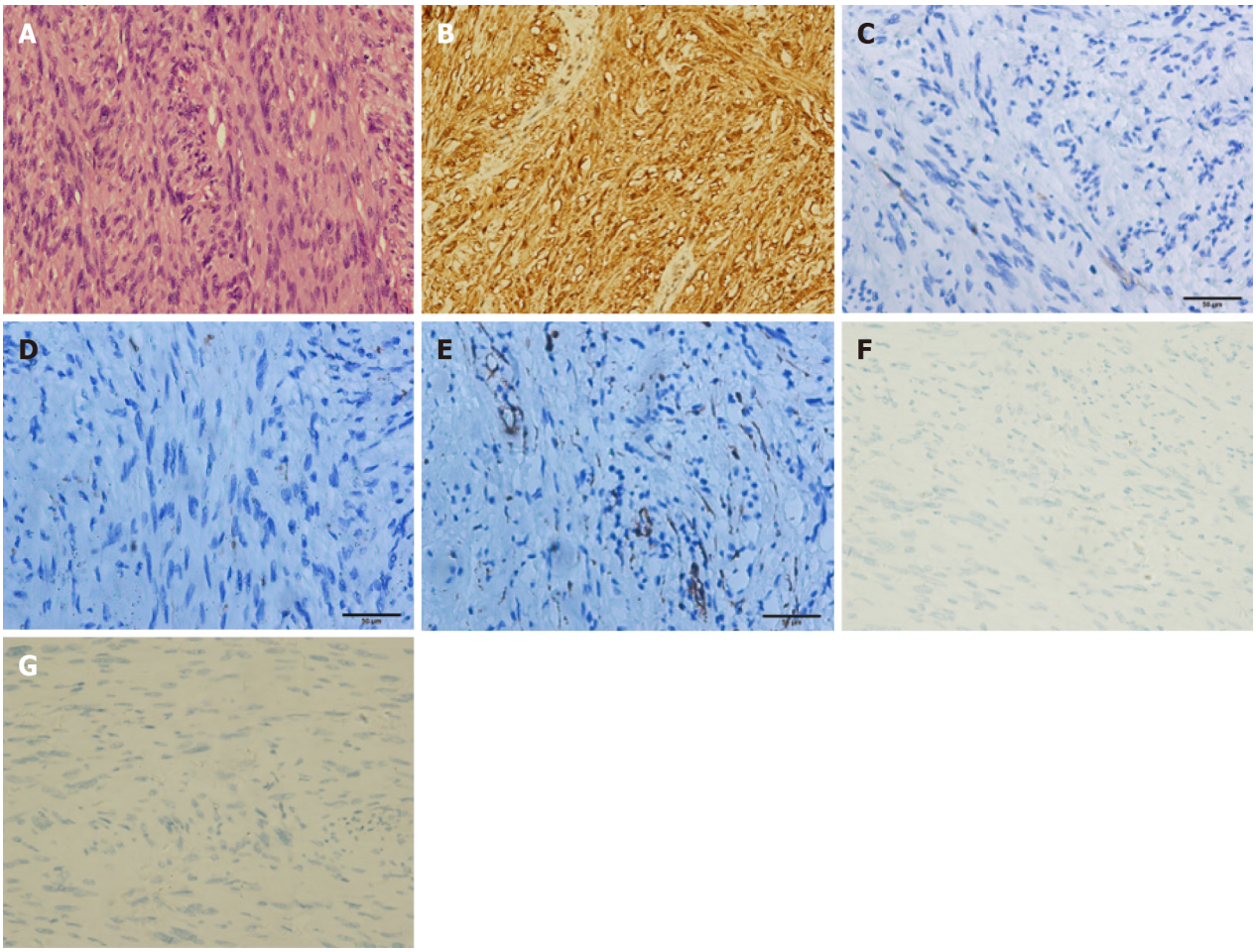

Figure 3 Pathological analysis and immunohistochemical staining. A: Hematoxylin and eosin staining revealed spindle cell tumors with mild cells, mitotic figures 1-2/50 high-power field, local inflammatory cell infiltration, and no necrosis. Combined with immunohistochemistry and gene detection results, the results were consistent with schwannoma; B-G: Immunohistochemical staining of the gastric mass confirmed a gastric schwannoma through positive staining for S-100 protein (B), whereas CD117 (C), CD34 (D), α-smooth muscle actin (E), desmin (F), and DOG1 (G) were negative.

OUTCOME AND FOLLOW-UP

The patient was well recovered and was discharged on her seventh day post operation. The patient was followed up for 16 mo after the operation. Gastroscopy was performed (Figure 6), and the results indicated that the incision recovered well.

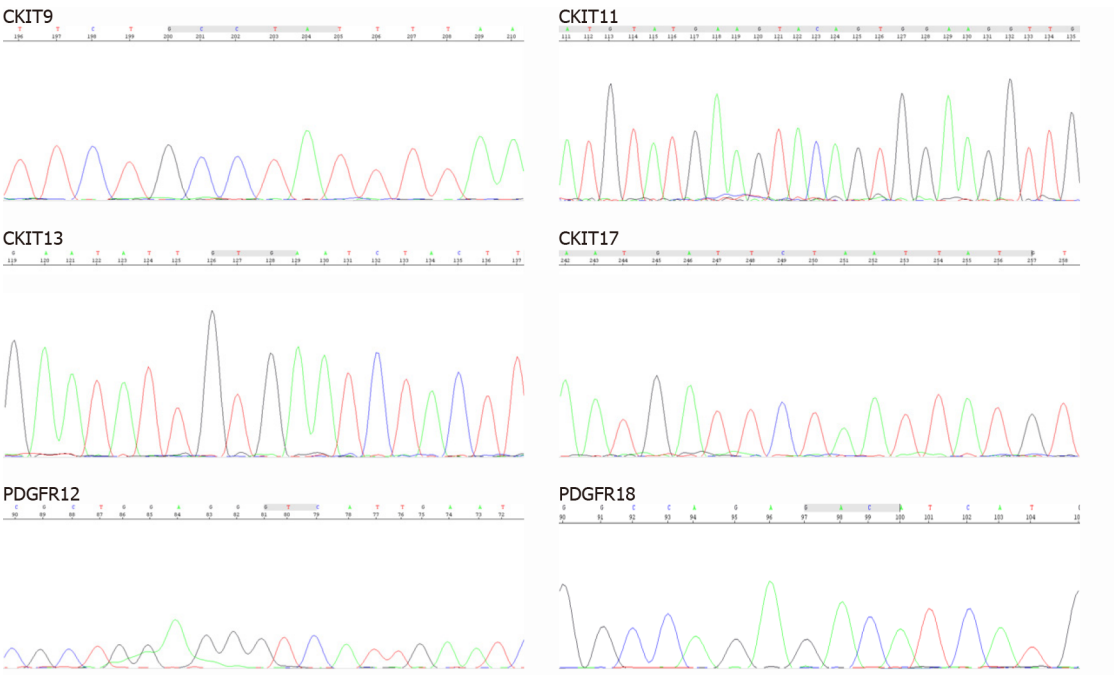

Figure 4 *c-Kit* and *PDGFRA* gene mutational analysis. DNA sequencing electropherograms revealed an absence of mutations in exons 9, 11, 13, and 17 of the *c-Kit* gene and exons 12 and 18 of the *PDGFRA* gene.

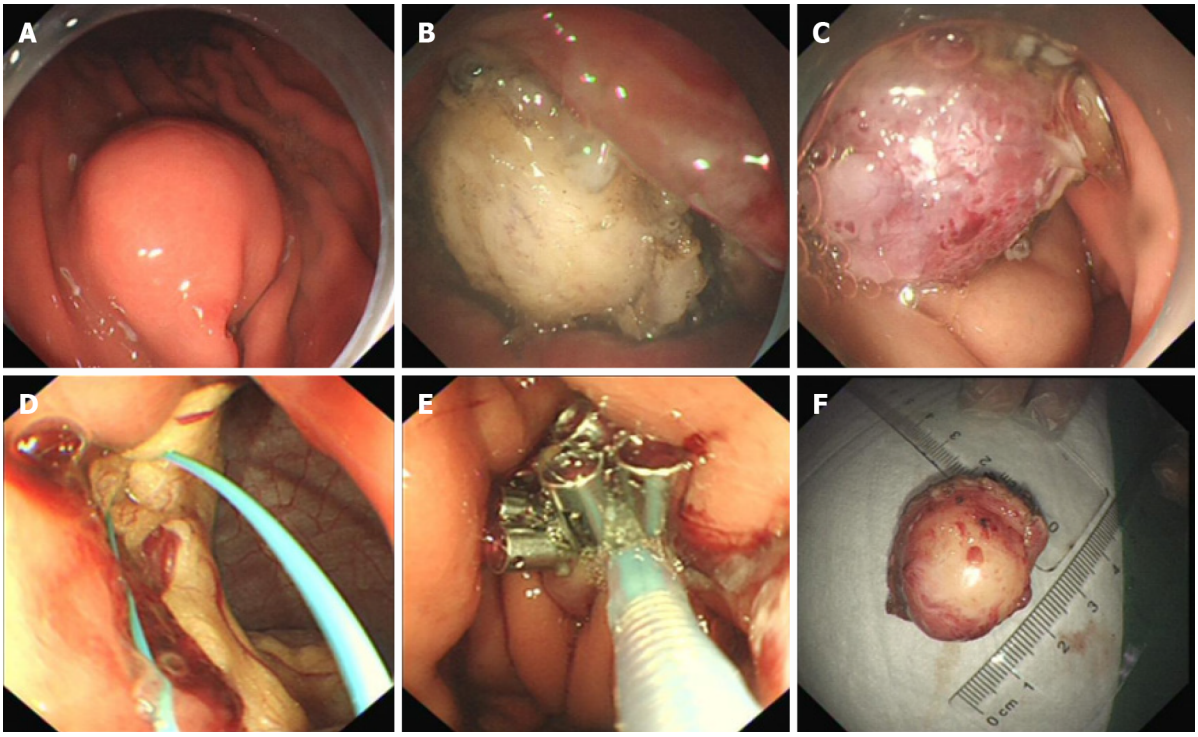

Figure 5 Endoscopic full-thickness resection operative process. A: Marked the lesion with argon plasma coagulation; B and C: Application of the insulated-tip knife to isolate the stromal tumor along its periphery; D and E: An "artificial perforation" observed after stromal tumor resection and sealed the perforation by endoscopic purse-string suture; F: The resected tumor.

DISCUSSION

Schwannomas are tumors originating from Schwann cells that usually affect the subcutaneous tissue of the distal limbs[9]. Schwannomas of the gastrointestinal tract represent approximately 3% of all mesenchymal tumors of the gastrointestinal tract [10]. In the gastrointestinal tract, the stomach is the site with the highest incidence of schwannomas followed by the colon[11]. The small intestine and esophagus are the

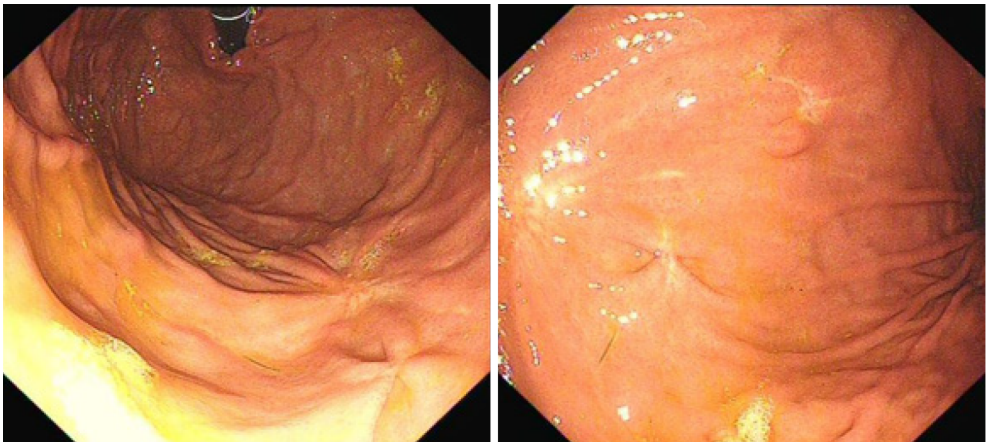

Figure 6 Gastroscopy at 16 mo after the operation revealed that the incision recovered well and that there was no recurrence.

most infrequently affected sites[12,13]. Gastric schwannomas account for 0.2% of all gastric tumors[9].

Diagnostic methods for gastric schwannomas, such as endoscopy, EUS, CT, magnetic resonance imaging, and positron emission tomography, have recently been proposed. On endoscopy, gastric schwannomas appear as elevated submucosal masses, with or without a central ulcer[14]. Endoscopic biopsy is not as effective as expected, as it can lead to false negative results[9]. On EUS evaluation, a rounded submucosal mass, a well-defined margin, heterogeneous hypoechogenicity or isoechogenicity, and deficiency of cystic change and calcification are significant for the diagnosis of gastric schwannoma[15-17].

Previous studies demonstrated that gastric schwannomas showed well-demarcated masses that are heterogeneous or homogeneous contrast enhancement on CT[14,18]. Ji *et al*[19] and Wang *et al*[20] reported that homogeneous progressive enhancement on dynamic CT was a characteristic finding of gastric schwannoma. On magnetic resonance imaging examination, the signal intensity of most gastric schwannomas is low to medium on T1-weighted images and high on T2-weighted images[21]. Recently, several cases of gastric schwannoma that were found with an increased uptake of fluorodeoxyglucose on positron emission tomography were reported[22]. Even with the above modern imaging modalities, it is still difficult to achieve the precise preoperative diagnosis of gastric schwannomas.

In our case, the schwannoma was discovered incidentally by abdominal CT, suggesting GIST as a likely diagnosis. Gastroscopy and EUS provided the same primary diagnosis. The tumor was misdiagnosed as a GIST until the immunohistochemical findings and mutational analysis were revealed.

Gastric schwannomas are almost uniformly benign without recurrence or metastasis, and no malignant variant was found in previous follow-up studies[6,8]. The optimal treatment for gastric schwannoma is surgical resection, which should follow the same principles with GISTs[23].

However, in recent years, therapies for gastric submucosal tumor resection have rapidly developed, and less invasive endoscopic techniques, such as snare polypectomy, endoscopic submucosal dissection, and endoscopic full-thickness resection (EFTR), have been considered and used more often. Zhai *et al*[24] conducted a 5-year retrospective study in consecutive patients who underwent endoscopic resection for gastric schwannoma at a large tertiary center, and the results indicated that endoscopic resection was effective and safe for patients with gastric schwannoma with favorable long-term outcomes. Jain *et al*[25] reported a systematic review of EFTR techniques for gastric tumors that originate from the muscularis propria and concluded that EFTR has a high success rate and low complication rate, which was a minimally invasive technique for gastric submucosal tumors.

In our case, the gastric schwannoma was treated by EFTR. To close the gastric perforation, endoscopic purse-string suture was performed. The patient had an uneventful recovery with no major complications.

CONCLUSION

Gastric schwannomas are relatively rare. Even with endoscopy and modern imaging modalities, the precise preoperative diagnosis of gastric schwannomas remains difficult. The final diagnosis of schwannoma is based on pathological and immunohistochemical examination. Gastric schwannomas are almost always benign, and patients with this type of tumor often have a favorable prognosis. Surgical resection is the optimal treatment for gastric schwannoma. Recently, minimally invasive techniques such as EFTR have been more widely considered and employed and are a safe and feasible treatment for gastric schwannomas.

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LETTER TO THE EDITOR

Gastrointestinal cytomegalovirus disease secondary to measles in an immunocompetent infant

Chao-Ming Hung, Po-Huang Lee, Hui-Ming Lee, Chong-Chi Chiu

ORCID number: Chao-Ming Hung 0000-0001-8348-1432; Po-Huang Lee 0000-0002-5150-1136; Hui-Ming Lee 0000-0003-3298-7957; Chong-Chi Chiu 0000-0002-1696-2648.

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Chao-Ming Hung, Hui-Ming Lee, Chong-Chi Chiu, Department of General Surgery, E-Da Cancer Hospital, Kaohsiung 82445, Taiwan

Chao-Ming Hung, Po-Huang Lee, Hui-Ming Lee, College of Medicine, I-Shou University, Kaohsiung 82445, Taiwan

Po-Huang Lee, Department of Surgery, E-Da Hospital, Kaohsiung 82445, Taiwan

Chong-Chi Chiu, School of Medicine, College of Medicine, I-Shou University, Kaohsiung 82445, Taiwan

Corresponding author: Chong-Chi Chiu, MD, Professor, Surgeon, Department of General Surgery, E-Da Cancer Hospital, No. 21 Yi-Da Road, Jiao-Su Village, Yan-Chao District, Kaohsiung 82445, Taiwan. chiuchongchi@gmail.com

Abstract

Yang *et al* reported an immunocompetent infant with gastrointestinal cytomegalovirus disease secondary to measles infection. We express our opinion about the diagnosis and treatment of this rare disease.

Key Words: Gastrointestinal cytomegalovirus disease; Measles; Diarrhea; Immunocompetent infant; Rare disease

Core Tip: We want to discuss the diagnosis and treatment issues in the rare gastrointestinal cytomegalovirus disease secondary to measles infection.

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TO THE EDITOR

We read with interest the study by Yang *et al*[1]. They highlighted the differential diagnosis and pathological features of gastrointestinal cytomegalovirus (CMV) infection in a 9-mo-old boy. In our opinion, some concepts about the diagnosis and treatment should be emphasized.

Measles leads to the morbidity of diarrhea and may cause dehydration and secondary malnutrition[2]. Its incidence is about 8%[3]. Instead, uncontrolled diarrhea caused by postnatally infected CMV in immunocompetent infants has been rarely reported. Differential diagnosis of the diarrhea cause is a great challenge to pediatric physicians, especially when most infants with neither endoscopic exam nor pathological confirmation of gastrointestinal CMV infection.

To our knowledge, most measles-infected patients only need supportive management, including fluid supply, antipyretics, and prevention of superimposed bacterial infections. There is no specific antiviral therapy. Although the efficacy in preventing and treating CMV infection has been proven in transplant recipients, Ganciclovir has not been supported effectively in treating pediatric patients[4]. It has been administered to infants with congenital infection[5] and cholestasis[6]; however, there are no controlled studies to support its effectiveness[5]. Fortunately, this 9-mo-old boy recovered completely after intravenous Ganciclovir administration with no evident side effects.

Low serum vitamin A level has been a common situation among children, even in some developed countries, *e.g.*, United States. Significant lower levels were encountered in critically ill children. Vitamin A deficit hinders the recovery course and increases measles-related complications. Besides, measles infection would further deteriorate the deficit of vitamin A serum concentration and aggravate the severity of xerophthalmia[7]. In a randomized controlled trial, lower morbidities and mortality have been found in measles-infected children after vitamin A supplement[8]. Thus, the World Health Organization recommended vitamin A administration to all acute measles-infected children[9]. We also suggest the same management to this 9-mo-old boy during the treatment course.

Vaccination is the most effective strategy to interrupt this virus transmission because it could lead to herd immunity, which must be maintained above 85% to 95% [10]. Thus, encouragement of measles vaccination is essential to avoid the occurrence of similar episodes.

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