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Contents

Weekly Volume 30 Number 8 February 28, 2024

EDITORIAL

779 Immunotherapy of gastric cancer: Present status and future perspectives

Triantafillidis JK, Konstadoulakis MM, Papalois AE

794 Immune signature of small bowel adenocarcinoma and the role of tumor microenvironment

Christodoulidis G, Kouliou MN, Koumarelas KE

799 Management of autoimmune hepatitis induced by hepatitis delta virus

Gigi E, Lagopoulos V, Liakos A

806 Adjuvant therapy for hepatocellular carcinoma: Dilemmas at the start of a new era

Zhong JH

OPINION REVIEW

811 Nonsteroidal anti-inflammatory drugs before endoscopic ultrasound guided tissue acquisition to reduce the incidence of post procedural pancreatitis

de Jong M, van Delft F, Roozen C, van Geenen EJ, Bisseling T, Siersema P, Bruno M

REVIEW

817 Autoimmune pancreatitis: Cornerstones and future perspectives

Gallo C, Dispinzieri G, Zucchini N, Invernizzi P, Massironi S

MINIREVIEWS

833 Fecal microbiota transplantation for treatment of non-alcoholic fatty liver disease: Mechanism, clinical evidence, and prospect

Qiu XX, Cheng SL, Liu YH, Li Y, Zhang R, Li NN, Li Z

ORIGINAL ARTICLE

Retrospective Study

843 Transcatheter arterial chemoembolization combined with PD-1 inhibitors and Lenvatinib for hepatocellular carcinoma with portal vein tumor thrombus

Wu HX, Ding XY, Xu YW, Yu MH, Li XM, Deng N, Chen JL

855 Immunoglobulin G-mediated food intolerance and metabolic syndrome influence the occurrence of reflux esophagitis in *Helicobacter pylori*-infected patients

Wang LH, Su BB, Wang SS, Sun GC, Lv KM, Li Y, Shi H, Chen QQ

863 Evaluating the influence of sarcopenia and myosteatosis on clinical outcomes in gastric cancer patients undergoing immune checkpoint inhibitor

Deng GM, Song HB, Du ZZ, Xue YW, Song HJ, Li YZ

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Weekly Volume 30 Number 8 February 28, 2024

Contents

Observational Study

881 Mitochondrial dysfunction affects hepatic immune and metabolic remodeling in patients with hepatitis B virus-related acute-on-chronic liver failure

Zhang Y, Tian XL, Li JQ, Wu DS, Li Q, Chen B

Basic Study

901 Metadherin promotes stem cell phenotypes and correlated with immune infiltration in hepatocellular carcinoma

Wang YY, Shen MM, Gao J

919 Lipid metabolism-related long noncoding RNA RP11-817I4.1 promotes fatty acid synthesis and tumor progression in hepatocellular carcinoma

Wang RY, Yang JL, Xu N, Xu J, Yang SH, Liang DM, Li JZ, Zhu H

SYSTEMATIC REVIEWS

943 Quality of life after pancreatic surgery

Li SZ, Zhen TT, Wu Y, Wang M, Qin TT, Zhang H, Qin RY

META-ANALYSIS

956 Prevalence and clinical impact of sarcopenia in liver transplant recipients: A meta-analysis

Jiang MJ, Wu MC, Duan ZH, Wu J, Xu XT, Li J, Meng QH

SCIENTOMETRICS

969 Bibliometrics analysis based on the Web of Science: Current trends and perspective of gastric organoid during 2010-2023

Jiang KL, Jia YB, Liu XJ, Jia QL, Guo LK, Wang XX, Yang KM, Wu CH, Liang BB, Ling JH

CASE REPORT

984 Cronkhite-Canada syndrome with esophagus involvement and six-year follow-up: A case report

Tang YC

LETTER TO THE EDITOR

991 Monitoring of hepatocellular carcinoma

Akkari I, Jaziri H

ABOUT COVER

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EDITORIAL

Immunotherapy of gastric cancer: Present status and future perspectives

John K Triantafillidis, Manousos M Konstadoulakis, Apostolos E Papalois

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Abstract

In this editorial, we comment on the article entitled "Advances and key focus areas in gastric cancer immunotherapy: A comprehensive scientometric and clinical trial review (1999-2023)," which was published in the recent issue of the *World Journal of Gastroenterology*. We focused on the results of the authors' bibliometric analysis concerning gastric cancer immunotherapy, which they analyzed in depth by compiling the relevant publications of the last 20 years. Before that, we briefly describe the most recent data concerning the epidemiological parameters of gastric cancer (GC) in different countries, attempting to give an interpretation based on the etiological factors involved in the etiopathogenesis of the neoplasm. We then briefly discuss the conservative treatment (chemotherapy) of the various forms of this malignant neoplasm. We describe the treatment of resectable tumors, locally advanced neoplasms, and unresectable (advanced) cases. Special attention is given to modern therapeutic approaches with emphasis on immunotherapy, which seems to be the future of GC treatment, especially in combination with chemotherapy. There is also a thorough analysis of the results of the study under review in terms of the number of scientific publications, the countries in which the studies were conducted, the authors, and the scientific centers of origin, as well as the clinical studies in progress. Finally, an attempt is made to draw some conclusions and to point out possible future directions.

Key Words: Gastric cancer; Chemotherapy; Immunotherapy; Immune checkpoint inhibitors; Bibliometrics; Scientometrics

Core Tip: Gastric cancer (GC) remains a major cause of morbidity and mortality worldwide. Conservative treatment in the form of chemotherapy has recently made remarkable advances, particularly in the field of immunotherapy. Immunotherapy of GC represents one of the most important fields of research worldwide today, particularly in China, the United States, and Japan, as well as in some Western European countries. Several treatment regimens have been approved and are being implemented with satisfactory results. Also, several treatment regimens are currently under investigation, which are expected to improve the disappointing prognosis of this malignant neoplasm.

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INTRODUCTION

In this editorial, we comment on the article by Li *et al*[1] entitled "Advances and key focus areas in gastric cancer immunotherapy: A comprehensive scientometric and clinical trial review (1999-2023)" published in the recent issue of the *World Journal of Gastroenterology*[1]. Given the very interesting conclusions of the study regarding the number and type of publications, journals, centers, and countries of origin of the papers, the most recent data on the epidemiological parameters of gastric cancer (GC) and chemotherapy of the various types of this malignant neoplasm will be briefly described. Special attention is given to modern therapeutic approaches with particular emphasis on immunotherapy, which seems to be the future of GC treatment, mainly in combination with chemotherapy.

GC is a major cause of morbidity and mortality worldwide particularly in South and Central Asian countries. GC is a complex and heterogeneous disease whose etiopathogenesis includes many risk factors. Despite the recent trend of decreasing incidence, prevalence, and mortality in several countries, GC is one of the most common human malignancies with high mortality. This is particularly true for some Asian countries such as China in which it represents the third most common malignancy. The spatial differences in GC incidence and mortality are very evident, especially in developing regions of the world where the adoption of effective prevention and early diagnosis strategies is evident[2]. Its etiology is multifactorial including environmental, genetic, and infectious factors. The prognosis even today is still disappointing with an overall 5-year survival of less than 5%.

Incidence-prevalence

GC remains one of the most important malignant causes of morbidity and mortality worldwide. The prevalence and incidence of the disease change over time. Thus, while in some countries and demographic groups there is a significant decrease, in other regions of the world epidemiological parameters remain stable or even increase such as in Asia. Changes have also been observed in GC incidence rates in the two main sites of occurrence (non-cardia and cardia), in countries where the rates of the disease are increasing[3]. The reasons why a decrease in GC incidence has been observed are primarily related to the introduction of the electric refrigerator through which good food preservation and subsequent inhibition of microbial proliferation, along with other factors such as the reduction in the consumption of smoked, salted, and canned foods, and an increase in the consumption of fresh fruits and vegetables, have reduced the dietary factors that promote gastric carcinogenesis, alongside with the reduction in smoking in Western countries. On the other hand, excessive consumption of antibiotics reduced *Helicobacter pylori (H. pylori)* infection, a major contributor to gastric carcinogenesis. Nevertheless, GC is not only caused only by *H. pylori* as only 2%-5% of individuals with *H. pylori* infection develop GC[4].

Regarding the epidemiological parameters, it is estimated that about one million patients are diagnosed with GC every year worldwide[5]. It is the 7th most common cancer and the 6th most commonly diagnosed cancer in the world[5]. The countries with the highest GC incidence rates (more than 15 new cases/100000 population) are Mongolia, Japan, Korea, Tajikistan, and Kyrgyzstan. Countries with higher than average rates include Eastern European countries, Turkey, Mali, Portugal, Peru, Ecuador, and Colombia. Lower rates (less than 5 new cases/100000 population) are observed in Western countries such as France, United States, Canada, United Kingdom, Norway, Sweden, Norway, Sweden, Australia, and Central African countries, although this could partly be attributed to low diagnostic capacity. The disease mainly affects males. In total, 66% of new GC cases diagnosed in 2020 were male. The highest cumulative GC risk is observed in East Asia (2.64%) and the lowest in Southern Africa (0.42%)[6]. East Asia has the highest incidence of GC, followed by Eastern and Central Europe. Enteric type of GC is more common in Caucasians, while GC located in the gastric cardia is less frequent in Africa and Latin America. Tumor protein p53 (*TP53*), low-density lipoprotein receptor-related protein 1B, and AT-rich interactive domain-containing protein 1A (*ARID1A*) are the genes that are most frequently altered in all population groups. Finally, African patients are younger, and the proportion of women is higher than men[7].

In China, 396500 new cases of GC were diagnosed (276300 men, 120200 women) in 2016, corresponding to 1086 newly diagnosed cases every day[8]. In recent years, it has become apparent that both the incidence and mortality of GC in

China have shown a downward trend, probably due to the implementation of effective governmental prevention strategies and changes in many aspects of individual life adopted by the Chinese people. As is the case in most countries of the world, *H. pylori* infection, poor dietary habits, smoking, and family history of GC are the main risk factors for GC in China[9]. The overall crude and age-standardized rates of incidence of GC by the standard Chinese population are 28.7 per 100000 and 17.6 per 100000, respectively. The incidence in males (25.1 per 100000) is 2.5 times higher than that in females (10.3 per 100000 population). The incidence of GC varies in different parts of this huge country as a result of different living conditions and environmental factors. For example, the age-standardized rate of GC is higher in Northwest China, lower in East and Central China, and even lower in South China. Apparently due to environmental factors the incidence of the disease was higher in rural compared to urban areas[10]. It would be of interest to compare the epidemiological parameters of two great Asian countries, namely Japan and China, where half of the world's GC cases are diagnosed. Epidemiological data suggest that there are divergent trends in GC incidence in the two countries, although without obvious or adequate explanation. It appears that the trends in age-standardized incidence rates for GC for both sexes decreased significantly but the decrease was greater in Japan. In both countries, the risk of GC increases with age. The two countries had divergent trends over the study period, with the risk of GC decreasing in Japanese men but increasing among Chinese men[11].

Mortality-survival

GC is the fourth most common cause of cancer death worldwide, with 783793 deaths in 2020, of which 502788 were in men. Asia has the highest mortality (575206), followed by Europe (96997), Latin America, and the Caribbean (53392 deaths). The annual percentage change in GC mortality decreased between 1980 and 2005 at a rate of 3%-4% in Europe, Korea, Japan, and Australia[12]. In Latin America, the annual percentage change is lower but stable: (Brazil and Chile-1.6%, Argentina and Mexico-2.3%). Currently, the only country showing an increasing trend in male mortality is Thailand (annual percentage change + 3.92). In the United States, the 5-year survival rate for GC is 31% since the majority of patients already have metastases at the time of diagnosis. However, when there are no metastases at the time of diagnosis, the survival rate increases to 67%[13]. Disease prognosis and survival are better in Asian patients compared to Caucasians[14].

ETIOPATHOGENESIS

Genetics

It is known that the tumor suppressor gene *TP53* is the gene that shows the highest number of mutations in GC cases. Furthermore, significant mutations have been observed in the kirsten rats sarcomaviral oncogene homolog, beta-catenin, and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha oncogenes as well as in the tumor suppressor genes mothers against decapentaplegic homolog 4, and adenomatous polyposis coli. Somatic copy number alterations activate oncogenes and inactivate tumor suppressor genes in GC patients. Alterations of RTK/RAS/mitogen-activated protein kinase signaling pathways including human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), mesenchymal epithelial transition, and fibroblast growth factor receptor (FGFR2) şi RAS are observed in 30%-40% of GC cases. Amplification of cell cycle regulatory genes *CCND1*, cyclin E1, and cyclin-dependent kinase 6 has also been observed[15].

Mutations in the *cadherin 1* (*CDH1*) gene encoding the cell adhesion protein E-cadherin are currently considered to be the cause of hereditary diffuse-type GC (HDGC), through disruption of cell-to-cell adhesion and activation of oncogenic signaling pathways. In carriers of this mutated gene with a positive history of HDGC, prophylactic gastrectomy is recommended, although the use of systematic protocols with multiple biopsies may be an alternative approach to the problem. Through animal models, molecular drivers have been established in the gastric epithelium of experimental animals with loss of E-cadherin. Based on the above, it is possible to adopt new prevention strategies as well as strategies for targeted treatment in patients with HDGC[16]. DNA methylation is another pathogenic factor that can cause genetic alterations. Methylation of CDH1, runt-related transcription factor 3, p16, and human mutL homolog 1 has been described in GC patients[17]. Finally, mutations in the tumor suppressor gene *ARID1A*, which encodes the SW1/SNF chromatin remodeling complex, have been described. Mutations in interleukin 17 (IL-17) and IL-10 have been described in Asian populations.

Pre-existing conditions

Atrophic gastritis and intestinal metaplasia: They are the most important precancerous conditions. Chronic inflammation induces the transcription factor nuclear factor kappa B, one of the most important mediators of inflammation. The inflammatory process promotes oxidative stress and the production of reactive oxygen species and nitrosamines by leukocytes and macrophages[18].

Ménétrier's disease: It is a hypertrophic gastropathy, characterized by the huge growth of mucus cells in the gastric mucosa. The risk of malignant potential exceeds 10%[19].

Gastric remnant: GC in the gastric remnant is defined as GC occurring at least 5 years after partial gastrectomy. The mechanism of carcinogenesis is related to postoperative hypochlorhydria, which results in bacterial overgrowth with the production of nitroso enzymes. Endoscopic monitoring is the best strategy for prevention and early diagnosis[20].

Gastric polyps: Gastric polyps are usually an incidental finding during gastroscopy. Polyps are removed endoscopically and tested histologically to exclude malignancy. Hyperplastic polyps may undergo carcinomatous transformation through the dysplasia/carcinoma sequence. The risk of malignancy is increased in sessile polyps and advanced polyps (polyps larger than 1 cm). It is estimated that 8%-59% of gastric adenomas occur concomitantly with GC[21].

Microbes

Gastric microbiota: During the evolutionary course of GC, changes in the microbiota of the stomach are observed. Advances in next-generation sequencing and metagenomics have shown that the stomach microbiota is diverse and includes five major phyla. It appears that the positivity of *H. pylori* influences other bacterial communities in terms of richness and evenness. *H. pylori* is a fundamental risk factor for GC development, especially strains positive for Cag pathogenicity island and the CagA oncoprotein. Existing data support that there are differences in the microbiota of patients with GC and patients with precancerous conditions with a decrease in microbial diversity and an increase in the presence of microbes that can produce nitrite. Interestingly, the data support that in GC patients there is an increase in the oral microbiota. All of these data suggest that the gastric microbiota in addition to *H. pylori* plays a role in the latter stages of gastric carcinogenesis[22]. Furthermore, data derived from experiments in transgenic mouse models with insulin-gastrin transplantation and human gastric microbiome support the view linking the gastric microbiota to GC development[19,23]. Finally, it appears that the colonic microbiota favors GC development through various metabolites. Various antimicrobial therapies, probiotics, phages, dietary elements, and fecal transplantation are being studied regarding their role in GC[24].

***H. pylori* infection:** The mechanisms of carcinogenesis associated with *H. pylori* infection are based, on the one hand, on the existence and maintenance of chronic inflammation and, on the other hand, on the presence of *H. pylori*-specific infectious agents, that have the potential to damage gastric epithelial cell DNA and promote genomic instability[25]. Neutrophil migration is triggered by *H. pylori*. Neutrophils induce nitric oxide synthase as well as reactive oxygen species which in turn lead to DNA damage[26]. DNA damage triggers the process of apoptosis, which manifests as gastric atrophy. Eradication of the infection normalizes the apoptosis rate.

Epstein-Barr virus infection: It is well established that a proportion of GC is etiologically related to Epstein-Barr virus (EBV). In a meta-analysis of 220 studies by Hirabayashi *et al*[27] that included more than 68000 GC cases, it was found that the frequency of virus detection in cancer cells exceeded 7.5% and was higher in males than females in the diffuse compared to the intestinal type and in the proximal compared to the distal region. Furthermore, EBV prevalence reached the level of 75.9% among lymphoepitheliomatous GC and 26.3% among GC cases in gastric remnant. Assuming that a causal relationship between EBV and GC does indeed exist and based on GLOBOCAN 2020 data, the authors estimated that primary prevention, with the development of an effective EBV vaccine, could prevent 81000 EBV-associated GC cases worldwide annually[27]. It has also been confirmed that EBV induces GC through DNA methylation[28]. EBV-associated GC cells are derived from an EBV-infected monocytic clone. In addition, the vast majority of GC patients have a history of *H. pylori* infection. It appears that *H. pylori* infection may influence the development of EBV-associated GC, a subtype of GC. Therefore, it remains unclear whether *H. pylori* infection is a cofactor for EBV-induced gastric carcinogenesis or whether *H. pylori* and EBV act independently in the development of GC. The possibilities are either that EBV infection participates in *H. pylori*-induced GC tumorigenesis, or that *H. pylori* infection accelerates EBV-initiated carcinogenesis[29].

Other risk factors

Diet: Excessive salt in the daily diet has a direct carcinogenic effect[30]. There seems to be a synergistic effect of salt consumption and *H. pylori* infection in GC patients. The decrease in the incidence of GC has been attributed to efficient food preservation over the last 50 years, as the refrigerator replaced salt as the main method of preservation[31]. Nitrates are chemical compounds containing the NO group. These chemical compounds are not only ingested from food and smoking but also from endogenous sources. Processed meats and dairy products are rich in nitrates. After absorption, nitrates react with amines, amides, or amino acids to form N-nitro compounds. High levels of N-Nitro in the stomach have been associated with the presence of advanced precancerous lesions[32]. In 2015, the World Health Organization classified processed meats as a Group 1 carcinogen. It even appears that fried foods are etiopathogenetically associated with GC. In a meta-analysis of 18 studies, Zhang *et al*[33] compared the effects of fried food intake in GC patients (n = 5739) and healthy adults (n = 70933). They found a significant positive association between GC risk and fried food intake in both non-East Asians and East Asians[33].

Smoking: The association between smoking and GC has been demonstrated in several epidemiological and metanalysis studies. The European EPIC study showed an increased risk of GC, but this risk decreases after 10 years of smoking cessation. It is estimated that 18% of GC cases are associated with smoking[34].

Non-steroidal anti-inflammatory drugs: These have been shown to reduce the risk of GC[35]. The effect is even more favorable in *H. pylori*-positive patients[36].

PREVENTIVE STRATEGIES

One of the most important primary prevention strategies for GC involves early detection and elimination of *H. pylori*

infection through endoscopic and radiological screening of the asymptomatic population in countries with a high incidence of the disease. In countries with a low prevalence of infection, upper digestive endoscopy with biopsies for any evidence of precancerous lesions is recommended[37]. Therefore, *H. pylori* eradication and endoscopic surveillance are the most important methods aimed at reducing the incidence and prevalence of GC[38].

H. pylori eradication has been shown to actually reduce the risk of GC. A meta-analysis of 27 studies, with 48606 *H. pylori*-positive patients, of whom 715 developed GC, showed that patients cured of the infection had a lower incidence of GC[39]. Of note, a recent prospective, randomized, placebo-controlled study with 26.5 years of follow-up demonstrated that *H. pylori* eradication therapy can provide long-term protection against GC in high-risk populations, particularly those without advanced gastric lesions[40]. However, there is still no consensus on population control. Some guidelines recommend that it is beneficial in populations at increased risk for GC.

TREATMENT MODALITIES

From the outset, it is emphasized that a multidisciplinary approach is the appropriate way to treat patients with GC to increase the survival rates of patients. Surgery remains the main treatment approach for cases of surgical GC, while chemotherapy serves as the basis of treatment for metastatic GC. Until recently, the two treatment modalities mentioned above along with radiotherapy and targeted therapy were the main available means of treating GC.

Chemotherapy

For resectable GC, perioperative chemotherapy has become the standard treatment and ongoing investigations are exploring the potential benefits of targeted therapy or immunotherapy in the perioperative or adjuvant setting. However, differences in the extent of standard lymphadenectomy between Eastern and Western countries have led to different standard treatments: Perioperative (neoadjuvant) and adjuvant therapy[41]. Trastuzumab or pembrolizumab should be added to first-line chemotherapy but only in HER2-positive patients. The combination of immunotherapy with therapies targeting HER2 has shown synergistic effects in preclinical models, and clinical trials in locally advanced GC (AGC) and metastatic GC. In addition, disruption of antibody-drug conjugates (ADCs) and other agents targeting HER2 has resulted in numerous clinical trials with promising results[42]. Trastuzumab can be combined either with fluoropyrimidine and a platinum agent or in combination with capecitabine + oxaliplatin (XELOX)/PF[43]. In HER2-negative patients, treatment regimens include nivolumab, cindilimab, and tislelizumab in combination with first-line chemotherapy. Docetaxel, cisplatin, 5-fluorouracil (DCF), modified DCF, and paclitaxel/oxaliplatin/5-fluorouracil/leucovorin also shows satisfactory activity.

In cases of unresectable locally advanced, recurrent, or metastatic GC, combination anti-HER2 therapy with chemotherapy and possibly pembrolizumab in HER2-positive patients is preferred. HER2 is overexpressed in 10%-20% of patients with GC. The implementation of targeted anti-HER2 therapy as part of the standard of care treatment in metastatic disease improved the prognosis of patients. Of note, the addition of pembrolizumab achieves high objective response rates (ORRs). Regardless of whether or not HER2 is positive, nivolumab is recommended as part of systemic treatment regimens.

The choice of second-line regimen depends on the previous treatment and the performance status of the patients. Ramucirumab combined with paclitaxel is the preferred second-line therapy. The drugs docetaxel, paclitaxel, irinotecan, albumin-paclitaxel, pembrolizumab, nivolumab, vedicitumab, and apatinib mesylate have also been used as second-line therapies. In the large class of patients with metastatic or locally AGC, treatment is complex and involves a combination of agents. These combinations include S-1 + oxaliplatin, docetaxel + oxaliplatin + fluorouracil, docetaxel + oxaliplatin + S-1 (DOS), XELOX, and folinic acid/5-fluorouracil/oxaliplatin chemotherapy (FOLFOX). Administration of intilimab in combination with chemotherapy results in a significant degree of pathological complete response with a satisfactory safety profile. Other phase II studies showed that durvalumab combined with DOS as neoadjuvant chemotherapy gave satisfactory results. Equally satisfactory results were produced by the combination of tremelimumab and durvalumab as neoadjuvant therapy in patients with exceptional GC of high microsatellite instability (MSI).

Immunotherapy

The significant advances made in recent years in the understanding of factors in the cancer microenvironment have resulted in significant progress in immunotherapy of AGC. Immunotherapy induces the generation of immune responses that destroy cancer cells, achieving satisfactory clinical outcomes with tolerable toxicity. Therefore, this novel strategy is becoming increasingly popular[44]. In healthy individuals, to maintain normal T cell functions, the normal immune checkpoint regulates and controls the actions of ligands and receptors. T cell activation results in the expression of many receptors such as programmed cell death protein 1 (PD-1) or cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). Anti-PD-1/anti-programmed death-ligand 1 (PD-L1) agents inhibit immune checkpoints and activate T cells. The results of monotherapy in GC are disappointing. Therefore, combination therapy is a practical treatment option for metastatic GC [45].

The main classes of immunotherapy are shown in Table 1. Currently, the following strategies are used: immune checkpoint inhibitors (ICIs), tumor vaccines, adoptive immunotherapy, and nonspecific immunomodulators (Figure 1). The increasingly used strategy of administering ICIs is due to their satisfactory efficacy and low toxicity compared to traditional therapies. The combination of immunotherapy and conventional therapy has been shown to achieve more satisfactory results because most GCs are resistant to the administration of a single drug. Nevertheless, many further studies are needed due to the inherent challenges of the complexity of the immune microenvironment and the hetero-

Table 1 Food and Drug Administration-approved immunotherapy options for gastric cancer

	Target	Action	Approved for
Targeted antibodies			
Ramucirumab (Cyramza®)	VEGF/VEGFR2 pathway	Inhibits tumor blood vessel growth	Subsets of patients with advanced GC
Trastuzumab (Herceptin®)	HER2 pathway	Inhibition	Advanced HER2- + GC
Trastuzumab deruxtecan (Enhertu®): Antibody-drug conjugate	HER2 pathway	Inhibition	Subsets of patients with advanced GC
Immunomodulators			
Dostarlimab (Jemperli)	PD-1/PD-L1 pathway	Checkpoint inhibitor	Advanced GC that has dMMR
Nivolumab (Opdivo®)	PD-1/PD-L1 pathway	Checkpoint inhibitor	Subsets of patients with advanced GC
Pembrolizumab (Keytruda®)	PD-1/PD-L1 pathway	Checkpoint inhibitor	Subsets of patients with advanced GC

dMMR: DNA mismatch repair deficiency; GC: Gastric cancer; HER2: Human epidermal growth factor receptor 2; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; VEGF: Vascular endothelial growth factor.

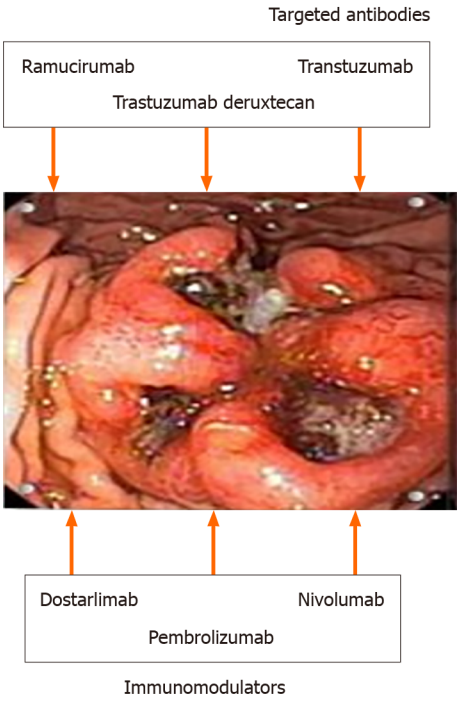

Figure 1 Graphical abstract: Immunotherapy for gastric cancer.

geneity of immunogenicity.

The main individual components of immunotherapy are cytokines, immune checkpoints, monoclonal antibodies, bispecific antibodies, ADCs, and chimeric antigen receptors (CARs). Nivolumab is used as a third-line therapy. Ramucirumab and claudiximab are also effective, particularly as long as they are combined with other therapies[46]. Synergistic activity results primarily from the combination of anti-PD-1 and anti-CTLA-4 antibodies, a combination that has been extensively investigated alongside the inclusion of anti-HER2 antibodies. The limitations and challenges of immunotherapy in GC are real and should be addressed shortly. For example, because GC is heterogeneous, the response to immunotherapeutic agents is affected. Therefore, universal targets should be identified such as identifying reliable biomarkers for personalized therapeutic approaches. Existing biomarkers (PD-L1 expression and MSI) do not accurately predict response to therapy. Immunotherapy has moderate efficacy in the advanced stages of GC. Finally, ICIs may cause autoimmune diseases. There is a lack of animal models of GC with all that this implies for the evaluation of therapeutic strategies.

The mode of action of these therapies is of particular interest since the growth of tumor is restricted by restarting the tumor-immune cycle and restoring the normal immune response of the patient. These strategies are discussed in brief

below[47].

ICI treatment: The use of ICIs is the most widespread treatment for GC since these inhibitors are particularly effective in solid tumors as well as hematological malignancies with long-term responses without significant toxicity. ICIs such as monoclonal antibodies against PD-1 or PD-L1 can prolong the survival of patients with AGC[48]. It is widely accepted that ICIs by inhibiting excessive activation of the immune response, prevent the occurrence of autoimmunity reactions and damage to autologous tissues. These actions of ICIs are used by cancer cells to inhibit T-cell activation to avoid their destruction. ICIs express their antitumor activity by binding monoclonal antibodies to ICIs on the surface of tumor or immune cells, thereby inhibiting pathological regulation and activating the immune system. ICIs can inhibit the interactions between PD-1 and PD-L1, keep T cells activated, activates natural killer (NK) cells, and prevent cancer cells from evading the destructive influence of immune cells. The most important ICIs are considered to be CTLA-4, PD-1, and PD-L1.

Existing data support that PD-L1 exhibits a significant correlation between its expression level and the benefit expected from ICI application in GC. However, its application is not without drawbacks, such as interobserver variability, the immunohistochemical assay used, and the effect of chemotherapy and radiation[49]. Currently, there are six Food and Drug Administration-approved immunotherapy options for GC (Table 1).

(1) PD-1 inhibitors

Nivolumab: Nivolumab is a humanized monoclonal immunoglobulin G4 (IgG4) PD-1 antibody that, upon binding to the PD-1 receptor, enhances the body's immune response by inhibiting the relation of PD-1 with PD-L1 and PD-L2 and inactivating the relevant pathway. Nivolumab in combination with chemotherapy is used as first-line therapy or nivolumab monotherapy as third- or later-line therapy. In the phase III ATTRACTION-2 trial, nivolumab as a third-line treatment improved overall survival (OS) in patients with AGC[50]. Also the combination of 5-fluorouracil and platinum as a first-line treatment improved OS in patients with HER2-negative AGC (global phase III CheckMate-649 study).

Pembrolizumab: Pembrolizumab is a monoclonal antibody directed against PD-1. It is active in tumors with high MSI or high tumor mutational load. Pembrolizumab acts by blocking the interaction between PD-1 and PD-L1. It activates and proliferates immune system T cells, as well as cytokine production. Pembrolizumab is used in previously treated patients with high-grade satellite instability or patients with tumor mutational burden-high AGC or in combination with trastuzumab and chemotherapy for HER2-positive AGC (United States). Pembrolizumab monotherapy, or combined with chemotherapy, has been used as first-line treatment for AGC. Pembrolizumab-based combination chemotherapy did now show better results.

Dostarlimab: Monoclonal antibody used in previously treated patients with MSI-H AGC (United States).

(2) PD-1 combined with chemotherapy

Treatment modalities that should be applied after ICI combination therapy, such as re-administration of ICI or the use of combination therapy with drugs that have a different mechanism of action, are the subject of intensive research. Several clinical studies are ongoing to apply this therapeutic strategy perioperatively and/or postoperatively in patients with early GC[51]. Satisfactory results are obtained from the combination of ICI and chemotherapy with tolerable toxicity. Thus, the combination of camrelizumab and capecitabine and oxaliplatin (CAPOX) has shown encouraging results in patients with metastatic or AGC in phase II clinical trials.

In the ATTRACTION-4 studies, nivolumab in combination with chemotherapy was shown to have satisfactory anticancer activity with an acceptable level of safety. Patients who received nivolumab in combination with s-1 and oxaliplatin and CapeOX experienced longer remission and objective remission rates[52].

Perioperative administration of ICI combined with chemotherapy in resectable locally AGC has shown encouraging results. However, a significant number of patients have shown resistance to ICIs, highlighting the importance of better patient selection or further combination immunotherapy[53,54]. As the number of available drugs increases, it is important to understand the target biomarkers and drug characteristics and select the optimal therapy for each patient;

(3) PD-L1 inhibitors

PD-L1 is a PD-1 Ligand expressed in various tumors that increases the immune-mediated tumor response by inhibiting the combination of PD-1 and PD-L1.

(4) Avelumab

Avelumab is a fully humanized PD-L1 IgG1 monoclonal antibody which, by binding to PD-L1, reduces the level of immunosuppression by blocking the reaction between PD-L1 and the PD-1 receptor[55]. The drug administered alone or as part of a combination therapy demonstrates satisfactory clinical results possibly in subsequent therapies;

(5) Atezolizumab

Atezolizumab is a monoclonal antibody that by binding to PD-L1 on tumor and immune cells infiltrating the tumor, can destroy cancer cells through the activation of T cells. However, in an earlier clinical trial in a small number of patients who were given the drug as a single treatment, the results were disappointing[56].

(6) Durvalumab

Durvalumab is a humanized monoclonal antibody that binds the PD-L1 protein by inhibiting its binding to the PD-1 protein on the T-cell surface. As a result, cancer cells cannot use the PD-L1/PD-1 pathway to avoid being destroyed by the immune cells. The existing data on its efficacy are largely conflicting[57].

Nonspecific enhancer therapy: This treatment involves various immune modifiers that enhance the immune response when given in combination with other therapies thus enhancing the final effect. These agents include cytokines (CKs), lentinan, *Streptococcus* preparation (OK-432), and Bacillus Calmette-Guerin (BCG) vaccine.

(1) CKs

CKs are small molecular proteins produced by immune cells that have been stimulated by incubation with a mitogen. They include IL-2, interferon-γ, tumor necrosis factor α, and granulocyte-macrophage colony-stimulating factor, among others. Their actions consist of activation, proliferation and differentiation of lymphocytes[58]. Of these, low-dose IL-2 has been used for the treatment of patients undergoing radical gastrectomy with postoperative adjuvant chemotherapy. The drug achieved an increase in CD4+ T and NK cells in both the periphery and around the tumor[59]. However, CKs have certain side effects and thus could be used as adjuvant molecules in cancer immunotherapy.

(2) Lentinan

Lentinan is quite often used as an immune adjuvant in the treatment of GC. Its actions are indirectly anticancer as it promotes the maturation, differentiation, and proliferation of immune cells[60]. The drug combined with chemotherapy in patients with AGC can significantly prolong the OS of the patients.

(3) BCG vaccine

The BCG vaccine, which is be prepared from attenuated bovine tuberculosis bacillus, enhances macrophage activity and boosts cellular immunity through activation of CD4+ and CD8+ cells[61]. BCG in combination with 5-fluorouracil, adriamycin, mitomycin C prolongs the survival of patients with AGC[62].

Oncolytic virus therapy: The treatment of malignant tumors using natural or genetically modified oncolytic viruses is a new and interesting type of immunotherapy[63]. Oncolytic viruses are divided into inherent oncolytic wild-type strains, which through their affinity with tumor cells, can proliferate and induce their lysis, and viruses in which by modification of the viral genome the virus becomes tumor-selective. Subsequently, through various processes, the virus loses its ability to replicate in normal cells, while its ability to infect cancer cells is enhanced[64].

In a recent study, Yang *et al*[65] investigated the effect of CF17, a new replication-competent chimeric poxvirus that they administered to intestinal and diffuse GC cell lines. They found that CF17 could infect, and kill cancer cells in dose- and time-dependent manners *in vitro*. In parallel, in the *in vivo* experiments, CF17 treatment resulted in a reduction in tumor burden, prevention of ascites formation, and prolonged survival of the experimental animals. These experimental results suggest that CF17 may be used in future studies to treat patients with GC and malignant ascites[65]. In a very recent study, the same group of investigators demonstrated that CF33 oncolytic viruses are capable of delivering functional proteins and demonstrating effective antitumor activity in GCPM models when administered intraperitoneally, suggesting that similar studies in GC patients with peritoneal metastases can be designed and performed in the future[66].

Hori *et al*[67] investigated the potential activity of adenoviral vectors expressing p53 against peritoneal metastases from diffuse-type GC. They found that the oncolytic adenovirus OBP-702 induced a significantly greater antitumor effect in GC cells compared with Ad-p53 adenovirus, through induction of p53-mediated apoptosis and autophagy and suppression of the tyrosine kinase receptor. The *in vivo* experiment showed that intraperitoneal administration of the oncolytic adenovirus OBP-702 suppressed peritoneal metastasis of NUGC-4 and GCIY cells compared to Ad-p53, and increased the survival of experimental animals[67]. Currently, the treatment of GC with oncolytic viruses is limited to *in vitro* research only. There have been no reports from clinical studies.

Tumor vaccine therapy: Treatment based on vaccine-derived elements of the malignant neoplasm relies on the specific immune response induced by the administration of the cancer antigen, which recognizes the cancer antigen on the surface of the antigen-presenting cells[68]. Tumor vaccines primarily involve dendritic cell vaccine (controversial efficacy), nucleic acid vaccine (satisfactory safety, no application in human GC), peptide/protein vaccine (activate the immune system by combining with MHC molecules or T cells of APC), and tumor-associated antigens vaccine. The latter is of particular interest concerning the future treatment of GC. Tumor-associated antigens are proteins produced by cancer cells. A characteristic of these proteins is that they do not show antigenicity under normal conditions. They may, however, elicit immune responses following the appearance of a mutation[69].

Of particular note is the vaccine against *H. pylori*. This bacterium has been classified as a class I carcinogen by the World Health Organization. Therefore, the development of a vaccine against *H. pylori* is expected to have a significant impact on the incidence of *H. pylori*-related GC. Potential antigens of the microbe for vaccine preparation are individual components of the microbe such as bacterial urease, various virulence factors, outer membrane protein, flagella, *etc.* At present, vaccines based on the microbial elements mentioned above are being tested in experimental models[70].

Neoantigen-based personalized vaccines: The use of personalized vaccines designed to induce *de novo* T-cell responses against neoantigens specific to individual patient malignancies has been an area of systematic research in recent years. Results have shown that these vaccines provide potent immunogenicity in many solid cancers. However, the research field contains many elements that need to be answered. A deeper evaluation of the phenotypes, functionality, and long-term memory potential of vaccine-induced CD4+ and CD8+ T-cells is required to achieve optimization of vaccination strategies. Their use is based on the fact that the mutations that occur in cancer cells generate new autoantigen epitopes called neoepitopes or neoantigens. Their advantages are that they are expressed exclusively by the cancer cells causing the generation of specific reactions and that they are derived from somatic mutations causing the generation of short and long-term reactions directed only towards the cancer cells thus preventing the possibility of recurrence of the neoplasm. Of course, this therapeutic method has some disadvantages related to high cost, time delays in vaccine generation, and inconsistency with the most suitable vaccine delivery platform. Results of clinical applications are expected soon[71].

Adoptive cellular immunotherapy: Adoptive immunotherapy consists of selectively isolating sensitized immune cells (T cells and NK cells) from patients or donors and re-administering them to patients to enhance the proliferation of various immune cells. The cells recognize specific tumor antigens and bind to and destroy them[72]. Adoptive immunotherapy includes the following categories: Cytokine-induced killer (CIK) cells, tumor-infiltrating lymphocytes (TILs), NK cell

therapy, CAR T cells, and T cell receptor-gene engineered T cells[73].

(1) NK cell therapy

NK cells play an important role in the body's defense processes against various viruses and cancer cells, making a decisive contribution to immune regulation processes[74]. NK cells show satisfactory efficacy against solid tumors while effectively preventing tumor metastasis.

(2) TIL cell therapy

TIL cell therapy is a treatment in which lymphocytes derived from the patient's tumor are cultured and amplified *in vitro* and then reintroduced into the patient's body[75].

All relevant studies have been conducted in cell lines and there are no clinical data.

CTLA-4 inhibitors: CTLA-4 is a small molecule expressed on the surface of CD4+ and CD8+ T cells. Its inhibition by specific inhibitors results in the restoration of a normal immune response. CTLA-4 inhibitors currently include two monoclonal antibodies ipilimumab and tremelimumab. Ipilimumab is a fully humanized CTLA-4 antibody inhibitor that will probably find application as part of a combination therapy. Tremelimumab is also a fully humanized IgG2 monoclonal antibody against CTLA-4 which has demonstrated antitumor activity in a variety of solid tumors including gastro-esophageal junction cancer. It appears that this drug has satisfactory safety, which combined with satisfactory efficacy, may increase the survival of patients with AGC.

CIK cells: In recent years, the type of treatment-induced through the so-called “CIK” has been widely discussed. The term “CIK cells” has been increasingly used over the last decade. These cells include features that make them quite a promising therapeutic method for treating cancer due to the antitumor potential of NK cells[76]. Future research in this area will likely focus on the development of more potent antitumor CAR T cells.

CAR T-cell therapy: CAR T-cell therapy is a relatively new approach. CAR T cells, a subset of genetically modified T cells, can recognize specific antigens and their action is independent of major histocompatibility complex (MHC) interactions. Several potential targets for the treatment of GC patients have been identified including claudin 18.2 (CLDN18.2), mesothelin, anthrax toxin receptor 1, and mucin 3A. CAR therapy targets multiple tumor cell targets in GC patients. Among them, intercellular adhesion molecule 1, CAR T cells, and CLDN18.2 CAR T cells have shown good results. However, satisfactory therapeutic responses are not observed in all patients[77].

NK cell therapy: Treatment with NK cells continues to evolve[78]. This treatment is also combined with other forms of immunotherapy to achieve more satisfactory results.

Other biomarker-targeted therapy: Recent studies reporting on new potential molecular targets are investigating the following targeted therapies of AGC: CLDN18.2-targeted therapy, FGFR pathway inhibitors, and EGFR inhibitors. Data on the most important of these, CLDN18.2-targeted therapy, are reported below.

CLDN18.2-targeted therapy: CLDN18.2 is a component of intercellular junctions and is present only in the gastric mucosa. During the processes of carcinogenesis, CLDN18.2 maintains its expression at a rate of 14.1% to 72%, both in gastric adenocarcinoma mucosa and diffuse type cancer[79].

Zolbetuximab is highly promising for the treatment of GC chimeric IgG1 monoclonal antibody that binds to CLDN18.2 thus inducing antibody-dependent cytotoxicity[80]. In several studies, mainly phase II, zolbetuximab showed very satisfactory results. In the MONO study, the drug had an ORR of 9% and a disease control rate of 23% in previously treated patients with GC or EC[81]. In the FAST study, zolbetuximab combined with first-line chemotherapy significantly improved progression-free survival (PFS) and OS in patients with CLDN18.2-positive GC[82]. In the SPOTLIGHT study, zolbetuximab administered with mFOLFOX6 significantly improved median PFS and median OS in patients with CLDN18.2-positive and HER-2-negative AGC[83]. The phase II GLOW study investigated the effect of zolbetuximab with CAPOX as first-line therapy in patients with CLDN18.2-positive, HER2-negative, locally advanced, unresectable, or metastatic GC. The zolbetuximab plus CAPOX combination significantly improved median PFS and median OS[84]. Another promising therapeutic approach targeting CLDN18.2 uses CAR T specific for CLDN18.2. Administration of these cells resulted in partial or complete tumor regression in CLDN18.2-positive patient-derived xenograft models[85]. Therefore, it appears that CLDN18.2 is a novel target for later-line treatment of GC. CLDN18.2 CAR T therapy is expected to become a cornerstone in cellular immunotherapy of solid tumors.

New drugs targeting CLDN18.2 are currently being developed, such as CLDN18.2 bispecific antibodies and ADC analogs. Some of them achieved satisfactory preclinical results representing the field of various clinical studies. The only concern concerning this type of therapy is the kind and severity of adverse effects that may occur since CLDN18.2 is expressed in the normal gastric mucosa.

Tumor microenvironment: The tumor microenvironment differs from that of normal tissues. The main characteristic of this microenvironment is the presence of an inflammatory response ,which favors the accumulation of immune cells and the activation of progenitor cells. The most important cells that promote gastric carcinogenesis through modulation of immune responses and secretion of soluble factors are macrophages. Furthermore, other elements of the stroma such as endothelial cells or blood vessels promote tumor growth through blood flow and secretion of cytokines and chemokines. The fundamental structural and functional changes of cancer-associated fibroblasts and blood vessels are caused by numerous interactions between cancer cells and other stromal components. Nerves and neurotransmitters are also involved in gastric carcinogenesis by acting in the tumor microenvironment[86].

Therefore, it appears that modification of the pro-tumorigenic stroma and creation of an antitumorigenic microenvironment will be promising therapeutic approaches in the future. Many cases of GC exhibit a significant degree of fibrosis due to cancer-associated fibroblasts through the secretion of IL-6, gremlin-1, and other factors. IL-6 activates signal transducer and activator of transcription signaling in cancer cells, inducing tumor growth and metastasis[87]. Therefore, targeting cancer-associated fibroblast-mediated cross-talk would be beneficial in GC. The above leads to the conclusion that a better understanding of all the interactions of the tumor microenvironment is expected to contribute decisively to the discovery of novel therapeutic targets[88].

BIBLIOMETRICS

Bibliometrics is defined as the process of analyzing a large amount of data that aims to identify recent research points and explore cutting-edge trends, revealing the structure of knowledge in a scientific field and listing all data related to scientific productivity and advances achieved in a specific scientific field and a specific period.

In recent years, there has been a boom in bibliometric analysis of articles in various fields. Tools such as CiteSpace and VOSviewer allow visualization of raw data, thus offering comprehensive and intuitive data representation. So-called scientometrics has been applied to analyze literature related to certain fields to identify hotspots and predict future trends [89].

It is an indisputable fact that the scientific medical literature is growing at a rate of 6% per year, a rate that is impossible for even clinicians to keep track of. For example, the scientific articles registered to date in the PubMed database have exceeded 36 million. It is also an undeniable fact that a significant percentage of articles, mainly reviews but also original research articles, repeat the same knowledge published in different journals. The trend toward publication has also resulted in an explosion of biomedical journals that are either listed in PubMed or other databases. This rate of increase in the medical literature is a real challenge for modern doctors, a challenge related both to updating them on the basic data of their specialty and to their ability to review the data of the international bibliography, a necessary element of lifelong updating. Knowledge discovery in databases (KDD) in data analysis consists of a retrospective data analysis[90]. KDD refers to the fluent or automated extraction of knowledge stored in large databases.

CiteSpace II is a system that could be used by clinicians and medical librarians. For the case of clinical research, CiteSpace II proves to be particularly useful for the creation of ontologies, and for the development of evidence-based knowledge bases to support decisions. However, there are several limitations to its use, the most important of which is the learning curve required to define the imaging parameters and the need to provide specialized knowledge. However, despite the existence of some limitations, CiteSpace II is a valuable tool addressed to a variety of users[91].

In this issue of the *World Journal of Gastroenterology,* the article by Li *et al*[1], published through bibliometric analysis using the Web of Science Core Collection database, CiteSpace software (6.1.6) and VOSviewer (1.6.18)m assessed the current status and emerging trends regarding the application of immunotherapy in GC patients. Relevant publications from the period 1999 to January 2023 were analyzed. The countries where the studies were carried out, scientific institutions, scientific journals, authors of the articles, bibliographic references, and keywords used were evaluated. The study included 2013 articles by 11730 authors using 726 keywords, from 617 scientific institutions in 71 countries. In addition, 228 clinical trials on immunotherapy, 137 on cell therapy, 274 on ICIs, and 23 using vaccines against GC were evaluated. Finally, the Impact Index Per Article for the top 10 highly cited papers from Reference Citation Analysis was also presented.

The main finding of the bibliometric analysis of the studies was that the immunotherapy for GC has developed significantly during the last few years. China and the United States account for the highest volume of publications. Especially for China, it was found that it accounts for 53.2% of the publications (1070 research articles) with the most authors and scientific institutions. The 10 institutions with the highest number of publications were located in China. Perhaps the huge population of China, smoking habits, increased incidence of *H. pylori* infection, and increased incidence of GC in the country have led to the large production of related publications, including on immunotherapy. The second place is occupied by the United States with a publication rate of 16.0%. Environmental factors (high-fat diet, obesity, alcohol consumption) are implicated in the etiology of GC in the United States. Finally, Japan, with a publication rate of 11.3%, took third place. In this country, dietary habits (increased consumption of salt and nitrites) combined with genetic factors are probably responsible for the high incidence of the disease. Regarding the annual distribution of scientific articles, an explosive increase in citations was evident, from 22 publications in the year 1999 to 552 in the year 2022. Thirty authors had at least 10 relevant publications while 17 authors collected more than 400 citations. Seven of the ten most frequently cited articles in the international bibliography were published in the last 7 years. Most publications were in journals related to molecular biology, genetics, immunology, nursing, and medicine. As far as clinical trials are concerned, their annual distribution has shown a sharp rise in recent years, especially from the year 2019 onwards. Regarding scientific journals, *Frontiers in Oncology* took first place with 104 scientific publications, followed by *Frontiers in Immunotherapy* with 78 and *Cancers* with 69 publications. *The Journal of Clinical Oncology* collected the highest number of citations (5044 citations), followed by *Cancer Research* (3018 citations). The study also showed that the most important points of progress concerned vaccinations, immune checkpoint therapy, and cell therapy. In particular, ICIs, and CAR T, are the most modern options for the treatment of GC. It seems that MSI, tumor microenvironment, mismatch repair deficiency, dendritic cell functions, and adoptive immunotherapy together with ICIs, are the most important future research directions. It also appears that the combined administration of chemotherapy and immunotherapy constitutes the future of GC drug treatment.

Also in a recent study[92], bibliometric analysis was used to investigate the size and trends of scientific research regarding immunotherapy of all types of cancer from 2000 to 2021. In these 20 years, a total of 18778 articles were published with the number of articles skyrocketing. from 366 in 2000 to 3194 in 2021. The United States had the largest number of publications (35.9%). A total of 976 significant topics were identified and further classified into four different groups (immune mechanism, cancer biology, immunotherapy, and clinical trials). The most important search topics included the terms 'expression,' 'chemotherapy,' 'dendritic cells,' 'pembrolizumab,' and 'open-label.' Liver, breast, lung, and bladder cancer had the greatest number of studies. Of interest is the recent shift from the investigation of pathophysiological mechanisms to the clinical application of immunotherapies, a trend that is predicted to continue in the future.

From these data, it appears that the largest amount of research on immunotherapy of all cancer types is from the United States, although for GC, the data from China are significantly greater in volume compared to the United States counterparts.

FUTURE PERSPECTIVES

Currently, the advances achieved in anti-HER2 and anti-vascular endothelial growth factor receptor therapy are uninterrupted and with a stable outlook. Research efforts include ICIs as monotherapy or in combination with ADCs on which research is increasingly focused. ADCs are potential chemical drugs that selectively target cancer cells by binding specific cell surface receptors with antibodies. Currently, several ADCs are being investigated in GC patient clinical trials targeting receptors such as EGFR, HER-2, HER-3, CLDN18.2, and mucin 1. ADCs combine the specificity of monoclonal antibodies with the potency of cytotoxic agents. They are likely to be the immunotherapy of the future for GC by acting synergistically with chemotherapy.

For future research, the investigation of new prognostic immunotherapy biomarkers to achieve personalized treatment, the further improvement of the combination of immunotherapy with ADC, the application of bispecific immunotherapy antibodies, and the further development of CAR T therapy are suggested. The transmembrane protein CLDN18.2, is, as is well known, the main component of tight junctions, thus contributing to the integrity of the intestinal barrier. The special feature of tumors is the high expression of this protein in the malignant tissue. Its exposure as an extracellular anchor makes it an ideal target for immunotherapy in digestive system cancers[93]. Also, lymphocyte activation gene-3 is a type of immune checkpoint receptor protein. This gene (also known as cluster of differentiation 223), which is mainly expressed in activated immune cells, is highly associated with the appearance and growth of the tumor, thus being a target for the immunotherapy of GC[94].

CONCLUSION

Current treatment modalities for GC include surgery, chemotherapy, radiotherapy, and molecular-targeted therapy. The trends in clinical trials today are the discovery of new biomarkers and the investigation of more specific treatment options. Immunotherapy has evolved into a very important therapeutic modality for GC, with several possibilities for clinical application such as anti-PD-1 monoclonal antibody being mainly part of combined therapy. The application of immunotherapy against PD-1/PD-L1 in GC also opens up great expectations. However, great caution is required as many issues related to GC immunotherapy are still unresolved. The mechanisms regulating the immune responses are extremely complex and obscure. For example, posttranslational modifications, such as glycosylation, acetylation, and phosphorylation, are involved in the direct regulation of PD-1/PD-L1 protein, as well as cross-talk between PD-1/PD-L1-related signal pathways in GC. The application of ICIs is an important research approach in terms of efficacy and safety. It seems that the combination of several therapeutic agents (immunotherapy and surgery, radiotherapy and chemotherapy, combination of immunotherapies, and the discovery of new ICIs) will constitute future therapeutic strategies.

In conclusion, there are reasonable hopes for the development of safer and more effective immunotherapies in GC as well as hopes for the establishment of individualized evaluation of combination therapies.

FOOTNOTES

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EDITORIAL

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Abstract

In this editorial we comment on the article published "Clinical significance of programmed cell death-ligand expression in small bowel adenocarcinoma is determined by the tumor microenvironment". Small bowel adenocarcinoma (SBA) is a rare gastrointestinal neoplasm and despite the small intestine's significant surface area, SBA accounts for less than 3% of such tumors. Early detection is challenging and the reason arises from its asymptomatic nature, often leading to late-stage discovery and poor prognosis. Treatment involves platinum-based chemotherapy with a 5-fluorouracil combination, but the lack of effective chemotherapy contributes to a generally poor prognosis. SBAs are linked to genetic disorders and risk factors, including chronic inflammatory conditions. The unique characteristics of the small bowel, such as rapid cell renewal and an active immune system, contributes to the rarity of these tumors as well as the high intratumoral infiltration of immune cells is associated with a favorable prognosis. Programmed cell death-ligand 1 (PD-L1) expression varies across different cancers, with potential discrepancies in its prognostic value. Microsatellite instability (MSI) in SBA is associated with a high tumor mutational burden, affecting the prognosis and response to immunotherapy. The presence of PD-L1 and programmed cell death 1, along with tumor-infiltrating lymphocytes, plays a crucial role in the complex microenvironment of SBA and contributes to a more favorable prognosis, especially in the context of high MSI tumors. Stromal tumor-infiltrating lymphocytes are identified as independent prognostic indicators and the association between MSI status and a favorable prognosis, emphasizes the importance of evaluating the immune status of tumors for treatment decisions.

Key Words: Programmed cell death 1; Programmed cell death-ligand 1; Programmed death ligand; Small bowel adenocarcinoma; Tumor infiltrating lymphocytes; Tumor microenvironment; Microsatellite instability

Core Tip: Small bowel adenocarcinoma (SBA) is an uncommon gastrointestinal tumor, accounting for fewer than 3% of all cases, even though it constitutes 95% of the gastrointestinal tract. SBA, which is mostly located in the duodenum, typically goes undetected for a long period of time, resulting to a late-stage discovery and a dismal prognosis. The immunological response, consisting of CD4+ and CD8+ T-lymphocytes, is critical in determining the prognosis. Programmed cell death 1/programmed cell death-ligand 1 (PD-L1) pathway, which is known to be involved in immune evasion in cancer, is implicated in SBA, with PD-L1 expression to a variety of prognostic consequences. The complicated interaction of immunological components, including as TILs and regulatory T cells, emphasizes the complexities of SBA.

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INTRODUCTION

Small bowel adenocarcinoma (SBA) is an uncommon condition, accounting for less than 3% of all gastrointestinal neoplasms. Its rarity comes in contrast with the facts that small intestine constitutes 95% of the surface area of the entire gastrointestinal tract. Adenocarcinomas, constituting around 40% of malignant small bowel tumors, predominantly manifest in the duodenum, with a notable prevalence of 50%–55%[1-4]. SBA presents a challenge in terms of early detection, as it is frequently asymptomatic for an extended period (2 to 8 months), leading to late-stage discovery and poor prognosis. Its detection often arises from complications such as intestinal perforation, ileus, and unbridled gastrointestinal hemorrhaging and by the time of the diagnosis, nearly one-third of individuals are presented with distant metastasis and an advances stage[1,2,5-7]. However, the advanced stage of the diagnosis and the lack of effective chemotherapy lead to a poor prognosis. In terms of treatment options, platinum-based combination chemotherapy with 5-fluorouracil is commonly used and mostly palliatively, and five-year overall survival (OS) rate reaches as high as 30% for locally advanced tumors[3,8].

Malignant small bowel tumors, are often associated with genetic disorders such as familial adenomatous polyposis, Lynch syndrome, Peutz–Jeghers syndrome, and juvenile polyposis, or risk factors including chronic inflammatory conditions like Crohn's disease and coeliac disease, along with environmental factors like smoking, alcohol, and certain dietary habits[2,9-11]. The tumorigenesis of SBAs is believed to align with colorectal cancer (CRC), although chronic inflammation may lead to a distinct sequence of inflammation–dysplasia–adenocarcinoma in some cases[2,12]. The small bowel's unique characteristics, including rapid epithelial cell renewal, preventing the accumulation of genetic damage, an active immune surveillance as it is the largest organ of the immune system, contribute to the rarity of these tumors. High intratumoral infiltration of CD3+ and CD8+ cytotoxic T-lymphocytes, along with the presence of tertiary lymphoid structures, is associated with a favorable prognosis[1,2]. Moreover, microsatellite instability (MSI) in SBA, varies between 5% and 35%, exhibiting a high tumor mutational burden, potentially contributing to the unique characteristics of these cancers[2,3,9].

Evading immune surveillance through the Programmed cell death 1 (PD-1)/programmed cell death-ligand 1 (PD-L1) pathway is a hallmark of cancer. PD-L1, a key player in modulating the tumor microenvironment (TME), is upregulated in various solid tumors, including gastrointestinal cancers[1,2]. Studies have shown the efficacy of blocking the PD-1/PD-L1 signaling pathway in gastrointestinal cancers with high MSI (MSI-H), establishing a significant association between MSI-H and PD-L1 expression in SBA[1]. Interestingly, the relationship between PD-L1 expression and prognosis in gastric and CRC remains contentious. While some studies suggest a favorable prognosis associated with PD-L1 expression, others indicate a poorer prognosis in these cancers, with the effectiveness of anti-PD-L1/PD-1 therapy relying on tumor-infiltrating lymphocytes (TILs), mainly composed of CD8+ T cells[1,2]. The abundance of TILs has been linked to improved survival, emphasizing the crucial role of the immune system in combating small bowel cancers[2]. However, the presence of regulatory T cells, characterized by the expression of FoxP3, within TILs introduces an immunosuppressive element, potentially hindering the efficacy of therapy. A high ratio of FoxP3+ to CD8+ T cells is correlated with poor clinical outcomes in digestive system cancers[1]. In this Editorial we elaborate on the TME, the infiltration of immune cells and the immune status of the tumor, stromal and immune cells.

THE ROLE OF PD-1, PD-L1 AND TILS IN SMALL BOWEL ADENOCARCINOMA

PD-1 is an immunoinhibitory receptor expressed on the surface of CD4+ and CD8+ T cells, B cells, natural killer cells, and monocytes. Its binding to PD-L1 Leads to the inhibition of immune suppression in these cells. Tumors expressing PD-L1 are considered immune-active, generating an immunosuppressive microenvironment. Consequently, PD-L1-positive tumors may be associated with a poor prognosis[1,13].

Given that the PD-1/PD-L1 pathway provides a potential immune escape route for tumors, an increase in PD-L1 expression is anticipated in advanced disease. Although PD-1/PD-L1 expression is expected to suppress the immune reaction against tumors, reports on the prognostic value of PD-L1 expression vary across different cancer types[2,3]. PD-

L1 expression has been shown to correlate with a poor prognosis in esophageal cancer, pancreatic carcinoma, hepatocellular carcinoma, renal cell carcinoma, and ovarian cancer. However, in breast cancer and Merkel cell carcinoma, PD-L1 has been found to correlate with a better patient outcome. For lung cancer, melanoma, gastric cancer, and CRC, both positive and negative prediction values have been reported[2,13,14]. One potential reason for this discrepancy in results may be attributed to the wide variation in the definition of PD-L1 positivity, with the cutoff for positive staining ranging from 1% to 50%. Nevertheless, the mere existence of PD-1/PD-L1 factors allows for the possibility of utilizing targeted immunotherapy in the context of precision medicine.

Microsatellite unstable tumors are characterized by an extensive mutational load, resulting in truncating mutations identified by immune surveillance due to misfolded proteins serving as neoantigens[2,15,16]. This leads to an enhanced antitumoral immune reaction and, consequently, improved survival. MSI status is linked to a high number of PD-1 positive immune cells and PD-L1 expression in immune cells[2,3].

PD-L1 can be expressed by tumor cells (PD-L1TC) or peritumoral inflammatory cells, predominantly histiocytes. PD-L1 expression in tumor cells ranges from 25% to 43%, while in tumor-infiltrating immune cells, it can reach as high as 54% [1,3,9,13]. PD-L1 expression is also increased in MSI tumors (86%) compared to those with microsatellite stable (MSS, 21%). Furthermore, PD-L1 expression is associated with the underlying cause of the tumor. Giuffrida *et al*[17] observed that when SBA is associated with coeliac disease or Crohn's disease, the expression is around 35%, while in sporadic cases of SBA, the expression is only 5%. Increased PD-L1TC is associated with a deeper depth of invasion ($P < 0.005$), increased infiltration of T-lymphocytes (CD3+, CD4+, and CD8+), and a 5-year OS of 74%, compared with PD-L1 negative SBAs[1,3,13]. The appearance of PD-L1 in tumor-infiltrating immune cells leads to a better prognosis, reducing the probability of peritoneal metastasis ($P < 0.05$), increasing the 5-year Disease-Specific Survival to 81%, compared to 33%, and the OS to 74% *vs* 27% in PD-L1 negative cases[2]. Thota *et al*[3] observed that 72% of patients with PD-L1 expression in immune cells had necrosis in the invasion border[3]. Moreover, Klose *et al*[13] support that the absence of PD-L1 in SBAs is correlated with female gender, increased tumor recurrence, metastasis, higher staging, and higher rates of postoperative administration of chemotherapy[13].

PD-1 expression is also increased in SBAs, especially in MSI-H tumors. According to Wirta *et al*[2] and Thota *et al*[3], all MSI-H tumors showed increased expression of PD-1, whereas only 75% of MSS tumors did[2,3]. This is directly related to a smaller tumor-node-metastasis (TNM) staging, with only 9% of patients in stage IV and 42% reported to have stage I or II, positively affecting the OS of the patients[2,9]. However, MSI is present in around 32% of SBA patients, and MSI-H varies from 10% to 21.7%, altering the appearance of PD-1, as MSI, in general, is related to PD-1 expression only in 37.5% of cases[13,18,19]. The 5-year OS in patients with MSI is 60%, while in patients with MSS, it is 54%. Pedersen *et al*[19] in the second phase of their multicenter study, using Pembrolizumab in patients with advanced SBA, observed that 2 patients with MSI-H had a confirmed partial response (50%), while only one (3%) from the MSS/MSI-Low group had a confirmed partial response, and 1 had an unconfirmed response. The responders had an average duration of response of 28.5 months in the MSI-H group and 17.5% in the MSS/MSI-Low group[19]. MSI status, along with PD-1 and PD-L1 expression, may be a helpful predictor for the prognosis of patients and for treatment selection.

TILs play a crucial role in the complex microenvironment of SBA. These specialized immune cells are found within the tumor tissue and are integral components of the host's anti-tumor response. In SBA, the presence and activity of TILs are of particular interest, as they are implicated in both the progression and potential control of the disease. In particular, the appearance of CD8+ TILs in the tumor or the stroma is correlated with less lymph node (LN) metastasis, fewer distant metastases, less peritoneal seeding, and an earlier TNM stage ($P < 0.005$)[1]. Increased percentages of TILs are also correlated with the infiltration of B cells, dendritic cells, and natural killer cells, enhancing the immune response. Along those lines, T-reg and T-helper cells might also be present, regulating the immune response and leading to a worse prognosis[5,20,21]. FoxP3 T-reg cells are associated with deeper depth of invasion, but this association is not significant. However, the increased ratio of FoxP3 to CD8+ cells is significantly associated with a worse prognosis, peritoneal, LN, and distant metastasis ($P < 0.005$)[1]. Moreover, low percentages of CD8+ cells lead to peritoneal seeding ($P = 0.003$), worsening the prognosis[9]. Parkes *et al*[14] observed that infiltration of CD3+, CD4+, and CD8+ T-cells leads to a better progression-free survival rate ($P < 0.005$), and CD8+ cells are also associated with increased OS, although the *P*-value was more than 0.05[14]. According to the multicenter cohort of Noh *et al*[9], the best prognosis was observed in patients with high PD-L1 and high CD8+ TILs in SBA[9].

CONCLUSION

The presence of PD-L1 and PD-1 in SBAs, in contrast to other cancer types, contributes to a more favorable prognosis. Patients with SBA and MSI-H tumors exhibit superior OS rates compared to those with MSS tumors. Similarly, individuals with elevated stromal tumor-infiltrating lymphocyte (sTIL) levels in SBA demonstrate extended OS times, establishing sTIL as a robust independent prognostic indicator. Furthermore, MSI status is closely associated with a favorable prognosis, particularly in the context of MSI-H tumors, and is directly correlated with PD-1 expression. The calculation of a Combined Positive Score is pivotal for evaluating the immune status of tumors and establishing a pertinent cut-off value for the treatment of patients with anti-PD-1/PD-L1 factors.

FOOTNOTES

Author contributions: Christodoulidis G, Kouliou MN and Koumarelas KE contributed to this paper; Christodoulidis G designed the overall concept and outline of the manuscript; Christodoulidis G, Kouliou MN and Koumarelas KE contributed to the discussion and design of the manuscript; Christodoulidis G, Koumarelas KE and Kouliou MN contributed to the writing, editing the manuscript, and review of literature.

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EDITORIAL

Management of autoimmune hepatitis induced by hepatitis delta virus

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Abstract

Approximately 12-72 million people worldwide are co-infected with hepatitis B virus (HBV) and hepatitis delta virus (HDV). This concurrent infection can lead to several severe outcomes with hepatic disease, such as cirrhosis, fulminant hepatitis, and hepatocellular carcinoma, being the most common. Over the past few decades, a correlation between viral hepatitis and autoimmune diseases has been reported. Furthermore, autoantibodies have been detected in the serum of patients co-infected with HBV/HDV, and autoimmune features have been reported. However, to date, very few cases of clinically significant autoimmune hepatitis (AIH) have been reported in patients with HDV infection, mainly in those who have received treatment with pegylated interferon. Interestingly, there are some patients with HBV infection and AIH in whom HDV infection is unearthed after receiving treatment with immunosuppressants. Consequently, several questions remain unanswered with the challenge to distinguish whether it is autoimmune or "autoimmune-like" hepatitis being the most crucial. Second, it remains uncertain whether autoimmunity is induced by HBV or delta virus. Finally, we investigated whether the cause of AIH lies in the previous treatment of HDV with pegylated interferon. These pressing issues should be elucidated to clarify whether new antiviral treatments for HDV, such as Bulevirtide or immunosuppressive drugs, are more appropriate for the management of patients with HDV and AIH.

Key Words: Autoimmune hepatitis; Hepatitis delta virus; Bulevirtide; Prednisolone

Core Tip: There are some pressing issues that should be elucidated in order to clarify whether new antiviral treatments for hepatitis delta virus (HDV), such as Bulevirtide, or immunosuppressive drugs, are more appropriate for the management of patients with HDV and autoimmune hepatitis (AIH). Firstly, several questions remain unanswered with the challenge to distinguish whether it is autoimmune or "autoimmune-like" hepatitis being the most crucial. Secondly, it yet remains uncertain whether autoimmunity is induced by the hepatitis B virus or the Delta virus. Finally, if the cause of AIH lies on the previous treatment of HDV with pegylated interferon.

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INTRODUCTION

Hepatitis delta virus (HDV) was first recognized back in late 1970s as new antigen in the serum of hepatitis B surface antigen (HBsAg) positive patients, termed δ, whilst subsequent clinical trials in chimpanzees proved its existence in 1980 [1]. HDV, the smallest pathogenic virus in human virology, consists of a circular single-stranded negative RNA genome and two types of hepatitis delta antigens, small and large (L-HDAg and S-HDAg, respectively), enveloped by HBsAg. Eight HDV genotypes with various geographical distributions were identified. With HBsAg on its surface, HDV enters hepatocytes *via* sodium taurocholate co-transporting polypeptide (NTCP). Genomic HDVRNA then moves to the nucleus, where it replicates *via* a rolling-circle mechanism. During replication, two species of HDAg (L-HDAg and S-HDAg) are formed: S-HDAg triggers further replication of the virus, while L-HDAg inhibits replication and promotes virion packaging through farnesylation[2]. Many clinical studies have shown that HDV infection most often causes progressive liver disease more rapidly than other viral hepatitis, leading to liver cirrhosis in 70% of cases within 5 to 10 years[3]. Furthermore, HDV is independently associated with an increased risk of hepatocellular carcinoma (HCC) than hepatitis B virus (HBV) monoinfection[4] and has been associated with the development of autoimmune hepatitis (AIH), as in the serum of HBV/HDV co-infected patients, positive autoantibodies have been detected, and autoimmune features have been reported[5].

AIH

AIH is an immune-mediated inflammatory disease of the liver, characterized by circulating autoantibodies, increased concentrations of immunoglobulin G (IgG), and specific histological features[6]. The origin of the disease is presumed to be a loss of immunologic tolerance against hepatocytes induced by environmental factors, but also triggered by specific viral infections (Hepatitis B, C, E, A, and Epstein-Barr), genetic factors, exposure to certain drugs (*e.g.*, nitrofurantoin, minocycline), and some individual risk factors (*e.g.*, sex, age, hormonal status, and comorbidities)[7]. The clinical presentation can be extremely heterogeneous, ranging from asymptomatic disease to fulminant hepatitis, leading to acute liver failure, with the acute onset of AIH being the most frequent pattern worldwide[7]. AIH can be classified into two types according to the pattern of autoantibodies detected. AIH type 1 is characterized by the presence of antinuclear antibodies (ANA) and/or smooth muscle autoantibodies (SMA) and sometimes perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA). AIH type 2 is characterized by the presence of antibodies against kidney microsome-1 (anti-LKM1), anti-LKM 3, and/or liver cytosolic type 1[8].

Diagnosis of AIH

AIH is diagnosed based on clinical, serological, and histological data. Clinical data may include nonspecific symptoms such as fatigue, arthralgia, malaise, amenorrhea, or symptoms and signs of hepatic cirrhosis. Laboratory findings included high concentrations of aminotransferases, detection of the specific autoantibodies mentioned above, and hypergammaglobulinemia (IgG). The histological features of AIH are not typical, as they are similar to those observed in active chronic hepatitis, mainly portal lymphoplasmacytic inflammation with interfacial activity and variable degrees of lobular hepatitis, emperipolesis, and hepatocyte rosettes[7,8]. Although imaging studies have a limited role in the diagnosis of AIH, they are useful for the assessment of liver complications, progression to cirrhosis, and screening for HCC. Currently, imaging is used worldwide to evaluate liver disease progression in patients with AIH. Ultrasound elastography is the most useful noninvasive tool for monitoring patients treated for AIH[9].

Treatment of AIH

Once AIH is diagnosed, treatment is initiated. In most cases, untreated AIH may lead to liver failure and death within

five years of onset, whereas properly treated patients have an excellent prognosis. The aim of treatment for AIH is to achieve and maintain disease remission and symptom resolution, thus halting or even reversing liver damage and fibrosis. However, treatment may cause serious side effects and significant impairment of quality of life. Therefore, an individualized approach is required, taking into consideration not only disease-related factors (inflammatory activity and stage of fibrosis), but also patient characteristics and values (such as life expectancy, comorbidities, living conditions, and patients' personal preferences).

Steroids remain the cornerstone of AIH remission, which is defined as a full biochemical response (normalization of both transaminases and IgG concentrations). The recommended initial dosage varies depending mostly on the profile of the patient and/or the stage of AIH. Starting with a dose of 0.5 mg/kg/d seems to have comparable efficacy to the widely accepted initial dose of 1.0 mg/kg/d, with a slightly slower response rate but fewer side effects; thus, doses higher than 0.5 mg/kg/d of prednisolone should be reserved only for severe acute disease. The response to steroid therapy is usually rapid, as transaminase concentrations start falling within a week, while IgG levels subside more slowly due to their prolonged half-life. Steroid tapering should be started as soon as signs of a biochemical response are observed, usually in steps of 5 mg every week, down to 10 mg of prednisolone per day, until a complete biochemical response is achieved. Budesonide can be used as an alternative to prednisolone, showing a slightly slower response and fewer side effects[10, 11].

Although steroids are regarded as first-line treatment, azathioprine is used for maintenance as a corticosteroid-sparing regimen. Azathioprine can induce sustained remission while reducing the side effects of steroid therapy. Therefore, azathioprine should be administered as early as possible following the initial response, usually 7 d to 14 d after steroid treatment. Azathioprine should also be started at a low dose to avoid intolerance; the recommended dose is 50 mg/d, with careful monitoring of side effects, including full blood counts taken every 1 wk to 2 wk. If no side effect is observed, the dose of azathioprine should then be increased to 1-2 mg/kg/d. Once a complete biochemical response is achieved, immunosuppressive therapy should be titrated to the level required to retain the full response, with steroids preferably tapered out completely. In case of relapse during tapering, steroids should be reintroduced at slightly higher dose[12,13]. For patients who cannot tolerate azathioprine, mycophenolate mofetil (MMF) can be used as an alternative agent at the usual dose of 2 g/d[14]. Finally, if the response to MMF is inadequate, multiple salvage therapies have been described, including cyclosporine, tacrolimus, and other immunomodulatory agents (methotrexate, cyclophosphamide, rituximab, and infliximab). Nevertheless, none of the aforementioned agents have been assessed in controlled clinical trials. Therefore, evidence for third-line treatment options remains scarce, and these patients should be preferably managed in large-volume centers with sufficient expertise in AIH[6].

The treatment duration of for AIH is controversial. Considering that AIH is an idiopathic disease that develops in a background of genetic susceptibility, most patients require long-term, most likely lifelong, therapy. Only 10%-20% of patients with AIH can safely discontinue immunosuppressive therapy and maintain remission. Withdrawal of immunosuppressive agents can be attempted when a complete biochemical response has been achieved for more than two years on monotherapy, with alanine transaminase concentrations in the lower range of normal values and IgG concentrations below 12 g/L. As there is a high chance of relapse, patients with complete treatment withdrawal should be closely monitored, especially in the first six months. Late relapses can also occur decades after sustained remission, highlighting the need for lifelong surveillance in virtually all patients[13].

However, there are certain patients for whom immunosuppressive treatment is contraindicated, including cases of AIH with an acute onset and rapid progression to fulminant liver disease or patients who already have end-stage liver disease at the time of diagnosis. In both cases, liver transplantation was the only treatment option[7].

AIH INDUCED BY HDV

The association of HDV with positive autoantibodies and autoimmune features has been suspected for years, although it was initially suggested that hepatitis delta is directly cytopathic and that liver injury is not immunologically mediated [15]. It remains debatable whether the autoimmune features derive from previous treatment with pegylated interferon or whether HDV induces autoimmunity through molecular mimicry and bystander activation. Considerable research has been conducted on the role of HBV in inducing autoimmunity[16]. Most antibodies used for viral hepatitis are non-disease-specific or non-organ-specific (NOSA). These antibodies include ANA, anti-SMA, anti-LKM1, anti-mitochondrial antibodies, and anti-soluble liver antigen/liver-pancreas antibodies. In a recent study, Hermanussen *et al*[16] identified and analyzed three different cohorts: Forty-six patients with AIH, 42 patients with HDV, and 70 patients with HBV. They found that positive NOSA titers were more frequent in AIH than in HDV (96% *vs* 69%) but more frequent in HDV than in HBV (69% *vs* 43%). With respect to individual antibodies, ANA titers were more frequent in patients with AIH than in those with HDV (89% *vs* 76%). In addition, higher titers ≥ 1:320 were noted in 63% of patients with AIH compared with 28% in the HDV cohort. Compared to patients with HBV, ANA titers were more commonly elevated in patients with HDV co-infection (HDV 67% *vs* HBV 43%). No difference was noted in ANA titers between patients with and without detectable HDV-RNA. The same trend was observed for SMA titers, which were more frequently encountered in AIH patients than in HDV and HBV patients (AIH 50% *vs* HDV 16% *vs* HBV 3%). Patients with detectable HDV-RNA RNAtested positive more frequently, and the titers were higher than those in patients with undetectable HDVRNA. Finally, IgG levels increased in 73%, 54% of patients with HDV and 12% of patients with AIH, HDV, and HBV[16]. In addition to this study, previous reports have shown a positive correlation between pegylated interferon treatment in HBV patients and the induction of AIH in approximately 25%-40%[17]. Furthermore, isolated cases of patients with hepatitis delta have shown that AIH is also observed after treatment with pegylated interferon[18]. In contrast, a case of HDV-

RNA flare has been reported in a previously negative patient who received immunosuppressive treatment for AIH[5].

TREATMENT OF CHRONIC HEPATITIS DELTA

Until recently, the management of chronic hepatitis delta (CHD) was encompassed within the HBV guidelines, as CHD was recognized as an HBV-dependent rare disease. It has even been designated an orphan disease because it affects only a small fraction of hepatitis B surface antigen (HBsAg)-positive patients. Advancements in the knowledge of HDV pathogenesis and the HDV life cycle have led to the identification of new therapeutic targets. For the first time since the discovery of HDV in the 70's, HDV-specific antiviral agents have undergone Phase III clinical trials. Bulevirtide (BLV) was the first HDV-specific antiviral agent to receive marketing authorization from the EMA in July 2020. Owing to the complexity of CHD clinical management on the one hand and the newly available knowledge and therapeutic perspectives, the European Association for the Study of the Liver (EASL) commissioned the first international clinical practice guideline (CPG)[19] on the management of HDV-infected patients, which was first announced during the EASL meeting in June 2023.

Pegylated Interferon a

According to the EASL CPG2023, pegylated interferon (PegIFNα) remains a therapeutic option and is recommended for all patients with CHD and compensated liver disease, irrespective of the presence of cirrhosis, for 48 wk (LoE 2, strong recommendation, consensus)[19]. PegIFNα is associated with a decline in both HBV and HDV markers, and recent studies have shown that it significantly reduces HDV-RNA when administered in the early stages of infection[20], suggesting an inhibitory effect on viral entry and suppression of cell division-mediated HDV spread[21]. In contrast, a recent meta-analysis of 13 studies reported a virological response at 24 wk post-treatment in only 29% of patients receiving PegIFNα, and that 50% of these patients developed virological relapse later, up to 10 years after the end of treatment[22,23].

BLV

BLV is a synthetic myristoylated lipopeptide consisting of 47 amino acids in the preS1 domain of the HBV large surface protein that blocks the attachment of HBsAg to the cell entry receptor, NTCP[24]. It has recently been recommended for all patients with CHD and compensated liver disease (LoE 3, strong recommendation and consensus). The optimal dose and duration have not yet been established thus, until further data becomes available, long-term treatment with 2 mg BLV once daily may be considered safe and adequate (LoE 5, weak recommendation, consensus[19]. Real-world evidence from more than 500 patients treated in France, Germany, Austria, and Italy was presented at international meetings. In the French Early Access Cohort study, an increased rate of virological response from month 12 (33%) to month 24 (68%) was observed after BLV[25]. In the German real-world experience study including 114 patients BLV showed high rates of virological response (≥ 2 Log HDV-RNA decline) in 74% of patients, in a mean intervention period of 38.0 wk ± 17.6 wk. Interestingly, BLV was well tolerated without major adverse effects even in five patients with decompensated cirrhosis at baseline who achieved virological and biochemical responses[26].

Combination treatment with BLV plus PegIFNα

The rationale for combination therapy with BLV and PegIFNα relies on the fact that PegIFNα exhibits immunomodulatory and antiviral properties and is able to inhibit cell division-mediated HDV spread, whereas BLV inhibits HDV from entering hepatocytes, thus preventing further infection. This combination is also recommended by EASL CPG 2023 for patients without PegIFNα intolerance or contraindications (LoE 5, weak recommendation, consensus)[19], In the MYR203 study, the patients were treated for 48 wk with combination therapy. Twenty-four weeks after the end of treatment, HDVRNA was undetectable in 53% of the patients who received BLV 2 mg/d. The rates of sustained virological response were 27% and 7% in patients treated with 5 and 10 mg daily respectively[27]. In the ongoing MYR204 study, patients were randomized to receive combination treatment with BLV at a dose of 2 or 10 mg/d for 48 wk and then continued with BLV monotherapy for another 48 wk, or monotherapy with BLV at a dose of 10 mg for 96 wk. In the 24th wk of treatment, patients receiving combination therapy achieved a greater decline in HDV-RNA levels than those treated with bulevirtidine monotherapy (92% when administered PegIFNα + 10 mg BLV *vs* 88% PegIFNα + 2 mg BLV *vs* 72% in BLV 10 mg monotherapy). Although these data are promising, the number of patients was small and some points need to be clarified further[28].

Future treatment options

Better understanding of the HDV life cycle and its interplay with hepatocytes has enabled the development of new antiviral agents as possible candidates for CHD treatment. Besides BLV, an NTCP inhibitor, other promising agents include prenylation inhibitors that inhibit the prenylation of large HDV antigens, which are essential for HDV virion morphogenesis[29], and nucleic acid polymers, which interact with the hydrophobic surface of HBsAg and destabilize its assembly and secretion of subviral particles, leading to the degradation of intracellular HBsAg[30].

DISCUSSION

Over the last few decades, the interaction between viral hepatitis and autoimmune diseases has received increasing

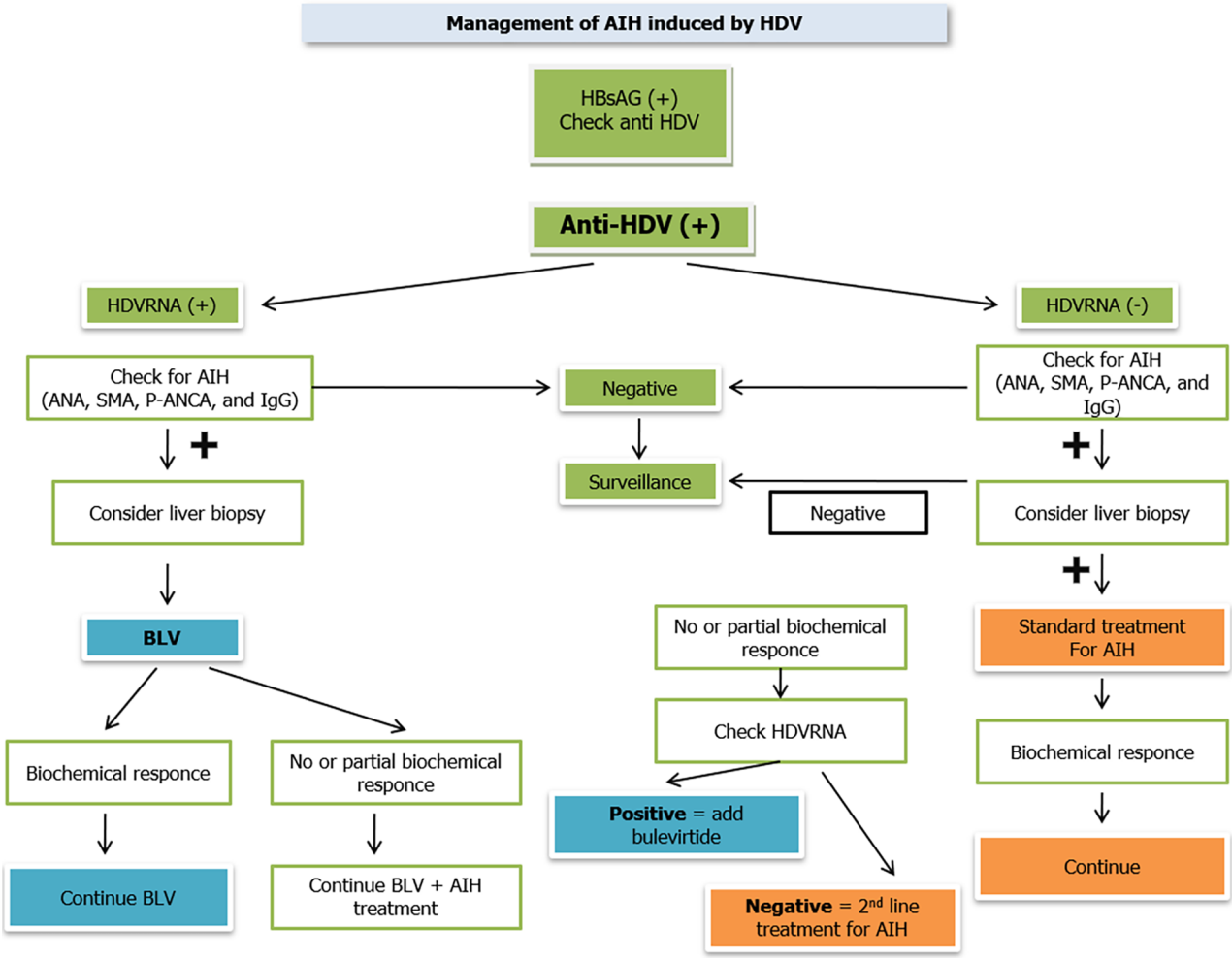

Figure 1 Management of autoimmune hepatitis induced by hepatitis delta virus. Assess all hepatitis B surface antigen (+) patients for anti-hepatitis delta virus (HDV). For anti-HDV positive patients evaluate HDV-RNA status and autoimmune hepatitis (AIH). Consider liver biopsy for patients with serologic evidence of AIH: (1) Initiate BLV for HDV-RNA (+) and AIH (+). If there is biochemical response continue bulevirtide (BLV); If there is partial or no biochemical response use AIH targeted therapies; and (2) If HDV-RNA (-) and AIH (+) initiate treatment for AIH. If there is biochemical response continue AIH treatment; if there is partial or no biochemical response check HDV-RNA and if positive add BLV, otherwise proceed to 2nd line treatment for AIH. HDV: Hepatitis delta virus; HBV: Hepatitis B virus; AIH: Autoimmune hepatitis; ANA: Antinuclear antibodies; SMA: Smooth muscle autoantibodies; BLV: Bulevirtide; ANCA: Anti-neutrophil cytoplasmic antibodies; IgG: Immunoglobulin G; HBsAG: Hepatitis B surface antigen.

attention, with special interest in hepatitis B and D virus co-infection. In some CHD cases, clinical AIH has been reported after treatment with pegylated interferon. Starting from this observation, several questions arise, mainly concerning the exact correlation between the onset of autoimmune disease and hepatitis band D and treatment with pegylated interferon. Since all possible scenarios have been described, and many different cases have been reported in the literature, some steps have been proposed to manage AIH in patients with chronic hepatitis delta. First, all the HBsAg-positive patients were screened for HDV antibodies (anti-HDV). Consequently, HDV-RNA should be tested in all anti-HDV-positive patients, and regardless of the results, all anti-HDV-positive patients should be screened for AIH by testing serological data, suchas ANA, SMA, p-ANCA, and IgG levels, as well as transaminase levels. If serological data indicate AIH, a liver biopsy should be performed. Nevertheless, in anti-HDV-positive patients with detectable HDV-RNA levels and laboratory tests compatible with AIH, treatment with BLV should be initiated as PegIFNα is contraindicated in these cases. If a biochemical response is achieved with transaminase normalization and decreased IgG levels, treatment with BLV should still be continued. If there is a partial or no biochemical response, first-line AIH treatment with prednisolone 0.5 mg/kg of body weight/day should be considered together with BLV. First-line treatment for AIH should be applied in anti-HDV-positive patients with undetectable HDV-RNA levels, positive serological data for AIH, and elevated transaminase levels. If a biochemical response is achieved, prednisolone should be continued in a tapered manner as described previously. If there is a partial or no biochemical response, HDV-RNA should be checked, and if detectable, BLV should be initiated immediately. If it remains undetectable, second-line AIH treatment, that is, MMF, should be considered, but with great caution due to side effects. Despite a satisfactory biochemical response, all anti-HDV patients with undetectable HDVRNA who are being treated for AIH should be closely monitored for HDV reactivation by examining HDVRNA levels at least every 12-24 wk. Moreover, all patients undergoing AIH therapy should receive treatment with nucleos(t)ide analogs to avoid reactivation or exacerbation of chronic hepatitis B (Figure 1).

However, not all patients with CHD and AIH achieve biochemical and/or virological responses when treated with BLV and/or first- or second-line AIH therapy, particularly in patients with decompensated cirrhosis. In these patients, liver transplantation should be considered until new HDV anti-viral agents are available.

CONCLUSION

Therefore, patients with Chronic Hepatitis Delta should be evaluated for AIH. Once AIH is diagnosed, treatment with BLV should be initially administered and continued as long as there is a biochemical response. In patients with partial or no biochemical response, autoimmune therapy with prednisolone should be considered.

FOOTNOTES

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EDITORIAL

Adjuvant therapy for hepatocellular carcinoma: Dilemmas at the start of a new era

Jian-Hong Zhong

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Abstract

Approximately 50%-70% of patients with hepatocellular carcinoma experience recurrence within five years after curative hepatic resection or ablation. As a result, many patients receive adjuvant therapy after curative resection or ablation in order to prolong recurrence-free survival. The therapy recommended by national guidelines can differ, and guidelines do not specify when to initiate adjuvant therapy or how long to continue it. These and other unanswered questions around adjuvant therapies make it difficult to optimize them and determine which may be more appropriate for a given type of patient. These questions need to be addressed by clinicians and researchers.

Key Words: Adjuvant therapy; Hepatocellular carcinoma; Tumor recurrence; Unanswered questions

Core Tip: Several questions need to be addressed by clinical researchers about the use of adjuvant therapy to prolong recurrence-free survival of patients with hepatocellular carcinoma following potentially curative treatment.

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INTRODUCTION

Primary or recurrent hepatocellular carcinoma (HCC) in certain patients can be treated through potentially curative hepatic resection or local ablation[1,2], which is typically defined as complete resection of the tumor, return of alpha fetoprotein levels to normal, and no sign of recurrence 4-8 wk later on contrast-enhanced computed tomography or magnetic resonance imaging[3]. Unfortunately, 50%-70% of patients experience intra- or extrahepatic metastases within five years after such procedures, and these metastases are the most frequent cause of HCC-related death[1,2]. For example, patients with primary HCC in the "very early" or "early" stages according to the Barcelona Clinic Liver Cancer staging system show 5-year recurrence rates of 40.7% after hepatectomy and 29.3% after local ablation[4], and the rate after hepatectomy falls to 18%-25% if the HCC is "intermediate" or "advanced"[5].

Therefore many patients are given adjuvant therapy after curative resection or ablation in order to prolong recurrence-free survival. However, international consensus is lacking about many aspects of adjuvant therapy, including which is the best type for a given type of patient, when it should be performed, and how long it should last. The question has even been raised whether adjuvant therapy is effective at all in certain contexts. These are important questions that need to be addressed through well-designed research and informed discussion.

Who can benefit from adjuvant therapy?

Adjuvant therapy increases treatment costs and risks of adverse events, so it should not be administered routinely to all patients whose tumors have been completely removed by resection or ablation. Instead, national guidelines recommend it for certain types of patients. The Chinese Liver Cancer staging system[3] and the American Association for the Study of Liver Diseases[1] recommend it for patients with factors associated with high risk of recurrence, such as tumor size > 5 cm, presence of > 3 tumors, micro- or macrovascular invasion, or poor tumor differentiation.

Whether these guidelines are optimal is questionable, in light of evidence identifying additional potential risk factors, such as the absence of a tumor capsule, tumor rupture, narrow resection margin (≤ 2 cm) and alpha fetoprotein ≥ 400 ng/mL[1,3]. In addition, the risk factors in guidelines have been associated primarily with recurrence within 6 months after curative treatment, meaning that the guidelines neglect liver cirrhosis and chronic hepatitis, which have been linked primarily to late recurrence[6,7]. The evidence base for all these risk factors should be expanded to the point that they can be taken into account in future versions of guidelines. Another question that should be addressed is whether adjuvant therapy is effective for all etiologies of HCC: For example, immune checkpoint inhibitors may offer limited benefit to patients with HCC linked to non-alcoholic steatohepatitis[8].

Which adjuvant therapies work best?

Based on extensive evidence from randomized controlled trials, Chinese Liver Cancer guidelines mention several adjuvant therapies as effective: Transarterial chemoembolization, hepatic arterial infusion chemotherapy, molecular targeted drugs, and adoptive immunotherapy[3]. In contrast, guidelines from South Korea[9] and the United States[1] do not recommend adjuvant transarterial chemoembolization or hepatic arterial infusion chemotherapy, although the South Korean guidelines do recommend adoptive immunotherapy based on strong evidence, while the United States guidelines mention immune checkpoint inhibition for the first time in the latest revision. Guidelines from the United States and China, but not South Korea, recommend adjuvant antiviral therapy with tenofovir or entecavir for patients with HCC related to chronic infection with hepatitis B virus[1,3].

The evidence base for the efficacy of some adjuvant therapies remains to be solidified. Only one randomized controlled trial has explored adjuvant use of the tyrosine kinase inhibitor sorafenib[10], reporting no significant benefit on recurrence-free or overall survival relative to placebo, and randomized trials of other molecular targeted drugs are ongoing. For example, an evaluation of the adjuvant combination of atezolizumab and bevacizumab has yet to reach the endpoint of median recurrence-free survival[11], although one study suggested that the two therapeutic antibodies may synergize to inhibit tumor angiogenesis, regulatory T proliferation and myeloid cell inflammation[12]. One study has suggested that molecular targeted drugs can potentiate adjuvant immune checkpoint blockade[11]. The current landscape of clinical evidence does not provide multiple, clearly effective treatments based on molecular targeted drugs, which makes it difficult to identify which ones may be optimal for given types of patient. Several network meta-analyses have examined the landscape but failed to converge on clear recommendations for clinical practice because of heterogeneity among patient populations and treatment protocols.

When should adjuvant therapy begin?

This is a key consideration given the inevitable side effects of adjuvant therapy, yet no major guidelines recommend a particular start time. Most randomized controlled trials initiate it 4-8 wk after curative resection. This question should be explored in clinical trials, which should consider that the optimal timing of initiation likely depends on perioperative complications, wound healing, residual liver function, and patient characteristics such as performance status and comorbidities.

How long should adjuvant therapy last?

The evidence base around immune checkpoint blockade and molecular targeted drugs does not clearly indicate minimal or maximal duration of adjuvant treatment. In one trial, sorafenib therapy was scheduled for 48 months, but it lasted closer to 12-13 months because of the lack of efficacy and high frequency of adverse events[10]. In another trial, the combination of atezolizumab and bevacizumab was scheduled for 12 months, and it lasted a median of 11 months[11]. This duration may be too long, at least for certain types of patients: Immune checkpoint inhibitor therapy for 6 months

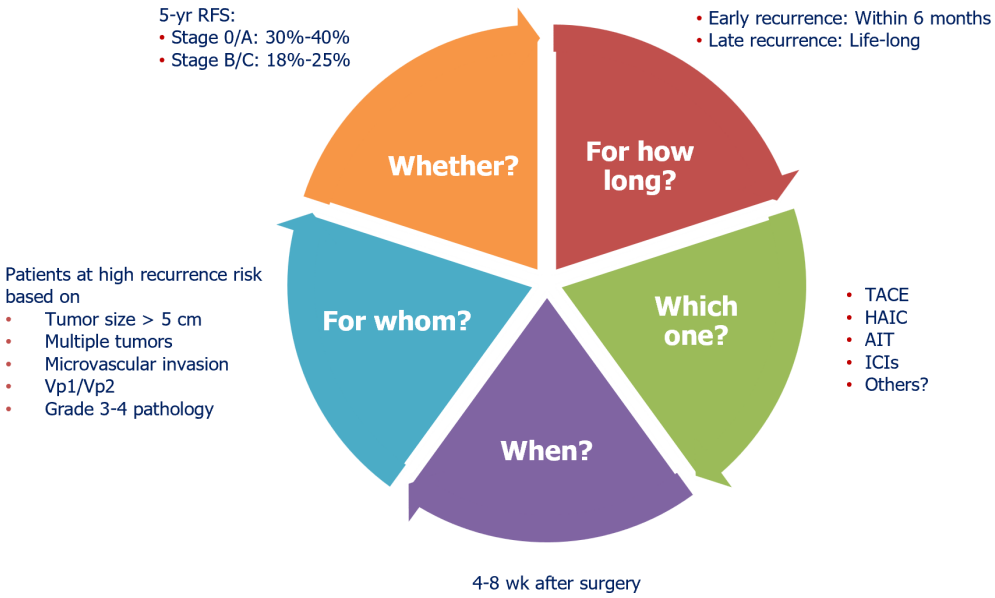

Figure 1 Unanswered questions about adjuvant therapy for patients with hepatocellular carcinoma following potentially curative resection or local ablation. Questions appear within the pie, and evidence-based responses are written around it. AIT: Adoptive immunotherapy; HAIC: Hepatic arterial infusion chemotherapy; ICI: Immune checkpoint inhibitor; RFS: Recurrence-free survival; TACE: Transarterial chemoembolization; Vp1: Segmental portal vein invasion; Vp2: Right anterior or posterior portal vein invasion.

was sufficient to prolong recurrence-free survival in one prospective study[13], and median progression-free survival was shorter than 12 months among patients with unresectable HCC who were treated with immune checkpoint inhibitors alone or together with molecular targeted drugs[14,15].

These observations suggest that 12 months of immune checkpoint inhibition may be excessive and, in any case, that the duration of adjuvant therapy will need to be determined based on its mechanism(s) of action. The indications for transarterial therapy, molecular targeted drugs, adoptive immunotherapy and immune checkpoint inhibition were originally formulated for patients with unresectable HCC, so they may not be optimal for patients whose disease is in an early, resectable stage and who are likely to survive long enough for late recurrence to be a concern. For example, patients with resectable disease who are chronically infected with hepatitis B virus should probably continue antiviral therapy for the long term, perhaps even the rest of their lives[16-18].

CONCLUSION

The costs and adverse effects of adjuvant therapy dictate that clinical researchers better define what therapies should be administered to which patients when and for how long (Figure 1), and that the best evidence be integrated into the next versions of consensus guidelines. This task becomes more urgent as more medical centers administer molecular targeted drugs and immune checkpoint inhibitors to HCC patients[19,20]. Eventually guidelines will also need to take stock of the growing use of neoadjuvant and "conversion" therapies, which promise to make potentially curative treatment accessible to patients with traditionally unresectable HCC.

FOOTNOTES

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OPINION REVIEW

Nonsteroidal anti-inflammatory drugs before endoscopic ultrasound guided tissue acquisition to reduce the incidence of post procedural pancreatitis

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Abstract

Endoscopic ultrasound (EUS) with fine needle aspiration or fine needle biopsy is the gold standard for sampling tissue to diagnose pancreatic cancer and autoimmune pancreatitis or to analyze cyst fluid. The most common reported adverse event of fine needle aspiration and/or fine needle biopsy is acute pancreatitis, which is likely induced by the same pathophysiological mechanisms as after endoscopic retrograde cholangiopancreatography (ERCP). According to the current European Society of Gastrointestinal Endoscopy guideline, nonsteroidal anti-inflammatory drugs are administered prior to ERCP as a scientifically proven treatment to reduce post-ERCP pancreatitis incidence rate. A single suppository of diclofenac or indomethacin prior to EUS guided tissue acquisition (TA) is harmless in healthy adults. Since it is associated with low costs and, most important, may prevent a dreadsome complication, we strongly recommend the administration of 100 mg diclofenac rectally prior to EUS-TA. We will explain this recommendation in more detail in this review as well as the risk and pathophysiology of post-EUS TA pancreatitis.

Key Words: Pancreatitis; Endoscopic ultrasound; Tissue acquisition; Nonsteroidal anti-inflammatory drugs; Pancreatic cancer

Core Tip: Post-endoscopic ultrasound (EUS) pancreatitis has an incidence of 1%-2%. Literature on the effectiveness of diclofenac in preventing a post-EUS-tissue acquisition (TA) pancreatitis is scarce. Based on the pathophysiological mechanism, which is nearly the same in both post-endoscopic retrograde cholangiopancreatography and post-EUS pancreatitis, diclofenac could be effective as prophylaxis of post-EUS-TA pancreatitis. There are several arguments in favor of administration, such as the cost-effective prevention of post-EUS-TA pancreatitis, which could have potentially disastrous consequences. A single suppository of diclofenac has limited side effects. In conclusion, administration of diclofenac prior to EUS-TA procedure should be strongly advised to prevent post-EUS-TA pancreatitis.

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INTRODUCTION

Since its introduction in the early 1990s[1], endoscopic ultrasound guided tissue acquisition (EUS-TA) by fine needle aspiration (FNA) or fine needle biopsy (FNB) is the gold standard for obtaining a tissue specimen for diagnosing pancreatic cancer, with a reported sensitivity and specificity of 77% and 98%, respectively[2,3]. To confirm the diagnosis before deciding on further treatment, EUS-TA of the pancreatic lesion is often indicated[4,5]. Besides being golden standard in obtaining cytology and histology of pancreatic solid masses, EUS-FNA/FNB is also used for cyst fluid analysis[6] and evaluation of autoimmune pancreatitis[7].

A variety of needles with different diameters and needle tip designs are available. Most recent data about the efficacy of EUS-TA recommend the use of a 22-gauge FNB needle in solid masses. In case of an unfavorable position of the endoscope with a sharp angulation of the tip, the more flexible 25-gauge needle can be chosen. For aspiration of cystic fluid, the 22-gauge FNA needles are the preferred ones[8].

Multiple publications have reported the risk of developing post procedural pancreatitis after EUS-TA[9-11]. Diagnosis of pancreatitis is usually based on the revised Atlanta criteria in which two of the following three features are required for the diagnosis of acute pancreatitis: Hyperlipasemia (> 3 times the upper limit of normal); acute abdominal pain; and/or signs of pancreatitis on computed tomography scan[12]. In some publications including EUS-FNA (not EUS-FNB), the reported post-EUS pancreatitis incidence was around 2%[9,10]. To date the most comprehensive approximation of the post-EUS pancreatitis rate is provided by a systematic review and meta-analysis by Tian *et al*[11]. They showed a pooled incidence of pancreatitis of 0.7%[11]. However, this meta-analysis was mainly based on publications from before 2010, and only 30 publications after 2010 were analyzed in this meta-analysis. Table 1 shows seven retrospective and/or prospective trials that were all published after the screening period of the meta-analysis[13-19]. Incidence of post-EUS-TA pancreatitis is comparable to the incidence described in the meta-analysis. Data about post-EUS pancreatitis in relation to the EUS-TA techniques are still scarce though.

The development of acute pancreatitis following a diagnostic EUS-TA may have major consequences for the patient, particularly when there is a suspicion of pancreatic cancer. Delay or even annulment of further diagnostic work-up or treatment drastically reduces the chance of cure, while the survival rate is already low in these patients[20,21].

Acute pancreatitis is also a complication reported after endoscopic retrograde cholangiopancreatography (ERCP). Rectal nonsteroidal anti-inflammatory drugs (NSAIDs) (*i.e.* diclofenac) are administered as prophylaxis to reduce the post procedural ERCP pancreatitis rate by 39%[22]. Assuming a comparable pathophysiological mechanism with the activation of the same inflammatory cascade inside the pancreas as during an ERCP, the preventive effect of diclofenac in EUS-TA could be relevant. Although the reported incidence of post-EUS-TA pancreatitis is lower compared to that after ERCP, the one-time administration of diclofenac post-EUS-TA may make it a worthwhile strategy as it has little to no side effects and is associated with limited costs while potentially avoiding a devastating complication.

Limited literature is available on the preventive value of rectal administration of diclofenac prior to an EUS-TA procedure to protect against post-EUS pancreatitis. In this review we will focus on the clinical consequence of post-EUS pancreatitis and the potential role and impact of diclofenac in its prevention.

PATHOPHYSIOLOGY OF POST-EUS PANCREATITIS

Mechanical injury of the pancreas is multifactorial in origin. It can be caused by manipulation of the ampulla of Vater and pancreatic duct, possibly in combination with increased pressure and overfilling of the ductal system with contrast agents in case of ERCP or direct puncture of the pancreatic parenchyma in case of EUS-TA. In the latter, pancreatitis is most often the result of direct cell damage, while in the former also the development of tissue edema may temporarily hamper the secretion of pancreatic enzymes causing increased ductal and intraparenchymal pressure. These events induce premature activation of pancreatic enzymes causing acute intracellular injury[23]. Both prostaglandins and phospholipase A2 play a key role in the early phase of inflammation[24].

Table 1 Incidence of post endoscopic ultrasound tissue acquisition pancreatitis

Ref.	*n*	Type of study	FNA or FNB	Incidence
Ribeiro *et al*[17], 2018	712	Prospective cohort	FNA and FNB	16/712 (2.2%)
Thomsen *et al*[18], 2022	852	Retrospective cohort	FNB	20/852 (2.3%)
Kandel *et al*[16], 2021	50	Prospective RCT	FNA and FNB	2/50 (4.0%)
van Riet *et al*[19], 2019	608	Prospective RCT	FNA and FNB	2/608 (0.3%)
Gonzalez *et al*[14], 2022	105	Retrospective cohort	FNA and FNB	0/105 (0.0%)
Ishigaki *et al*[15], 2020	154	Retrospective cohort	FNA and FNB	2/154 (1.3%)
Chen *et al*[13], 2022	235	Prospective RCT	FNA and FNB	2/235 (0.9%)

FNA: Fine needle aspiration; FNB: Fine needle biopsy; RCT: Randomized controlled trial.

THE ROLE OF DICLOFENAC IN PREVENTING POST-EUS PANCREATITIS

The use of NSAIDs, either 100 mg diclofenac or 100 mg indomethacin rectally, is recommended by the European Society of Gastrointestinal Endoscopy and the American Society of Gastrointestinal Endoscopy as prophylaxis of a post procedural pancreatitis in patients undergoing ERCP[25,26]. The most optimal timing for the administration of a rectal suppository of diclofenac or indomethacin is just prior to the ERCP[27]. NSAIDs inhibit prostaglandins, phospholipase A2, and neutrophil-endothelial interactions, which will decrease the inflammatory reaction[24]. Both diclofenac and indomethacin reach the maximum concentration between 1-2 h after administration. Both these NSAIDs are mainly bound to albumin (90% *vs* 99%, respectively)[28] and subsequently excreted *via* the hepatobiliary-fecal and kidney pathway. Two hours after administration half of the level of diclofenac has been metabolized[29], while the biological half-life of indomethacin is 5-10 h. In addition, diclofenac is very cheap ($0.19 per supp 100 mg)[30], and a single dose is harmless in healthy adults[31].

However, the use of NSAIDs has limitations. NSAIDs are contraindicated during pregnancy after a gestational age of 30 wk[32], if the glomerular filtration rate is less than 30 mL/min/1.73 m^2, or in case of liver cirrhosis[33,34]. Renal blood flow will be reduced by inhibition of prostaglandins, which can lead to hepatorenal syndrome in patients with liver cirrhosis[34]. If there is a documented allergy to NSAIDs, these should obviously be avoided.

RISK FACTORS FOR POST-EUS PANCREATITIS

Several risk factors are associated with the development of post-EUS pancreatitis. Lee *et al*[35] showed that performing more EUS-guided punctures within one procedure increases the risk of adverse events [odds ratio (OR): 1.24 (1.02-1.50)]. This also applies to performing more than 15 to-and-fro movements per punction [OR: 2.25 (1.07-4.73)]. Performing ERCP on the same day as EUS-guided TA was the greatest risk factor for post procedural pancreatitis [OR: 2.82 (1.31-6.10)]. The excess risk of doing both procedures successively on the same day rather than on separate days however was not discerned. A history of recent acute pancreatitis was also found to be a risk factor for post-EUS pancreatitis (26.6% *vs* 3.3%) [17]. Additionally, the location of the biopsy contributes to the risk of developing post procedural pancreatitis.

Pancreatitis is more common after needle biopsies taken from the uncinate process or the pancreatic head as compared to the body or tail, possibly because in some cases the needle passes a thicker layer of healthy pancreatic parenchyma [36]. Tissue sampling through normal pancreatic parenchyma or through the wall of the main pancreatic duct also increases the risk of post-EUS pancreatitis compared to passage through minimal parenchyma (9.20% *vs* 0.18%). Lastly, patients with pancreatic cancer are less likely to develop post-EUS pancreatitis compared to patients with benign pancreatic diseases, while puncture of solid lesions had a higher overall rate of pancreatitis compared to puncture of cystic lesions (60% of the pancreatitis occurred after puncture of a solid lesion)[17]. In conclusion, both patients with solid lesions and patients with cystic lesions of the pancreas are susceptible to post-EUS pancreatitis. In both cases, there is a similar risk of puncturing through normal parenchyma and/or damaging the pancreatic duct.

FNA *VS* FNB IN RELATION TO PANCREATITIS

Currently, new advances in FNB techniques and increased yield compared to FNA will gradually phase out the use of FNA needles. The advantage of FNB is that fewer needle passes are required to obtain a representative specimen[37,38]. Despite the fact that greater tissue cores are obtained, which could cause hypothetically more damage, a meta-analysis showed that adverse events between FNA and FNB were not significantly different[37]. Rapid on-site evaluation (ROSE) has been advised during an FNA procedure to increase the diagnostic adequacy and thereby reduce the number of repeat procedures[39]. To perform ROSE however, cytopathological evaluation needs to be immediately available, is time con-

suming, and adds to the cost of the procedure. Meta-analysis showed that FNB without ROSE has a similar diagnostic adequacy compared to FNA with ROSE[38].

FUTURE PERSPECTIVE

The only way to answer the question whether diclofenac is useful as a prophylaxis against the development of post-EUS pancreatitis is to conduct a randomized controlled trial (RCT). Ideally, this should be a double-blind, placebo-controlled trial in which one arm receives an NSAID suppository and the other arm receives a rectal placebo prior to the EUS-TA procedure. Patients, researchers, endoscopists, and nurses should be blinded.

Conducting such an RCT has several limitations. Since the incidence of post-EUS pancreatitis is probably between 1% and 2%, many patients need to be included. Aiming to reduce the incidence of post-EUS pancreatitis by 50% from 2% to 1%, with a significance of 5%, a power of 80%, and a 10% drop-out, 2550 patients are required in each arm. Suppose this hypothetically designed trial shows that administration of diclofenac can halve the incidence of post-EUS pancreatitis, then the number needed to treat is 1/100. In other words, 100 patients must receive diclofenac to prevent one post-EUS pancreatitis case. For risk analysis, all known risk factors should be noted and registered.

Therefore, it does not seem to be practically feasible to conduct such a trial. The question is whether such proof is necessary, as the indirect evidence for the protection of post-ERCP pancreatitis is strong and the mechanism of how post-EUS-TA pancreatitis develops seems identical to post-ERCP pancreatitis. Even though post-EUS-TA pancreatitis is a relatively rare event, a cheap and relatively safe diclofenac suppository can lower the incidence and prevent a potentially dreadsome complication that may cause a serious delay in the further work-up and treatment of a patient with a pancreatic mass. Therefore, in our opinion, the associated costs of managing preventable post-EUS-TA pancreatitis are disproportionate compared to standardized prophylactic diclofenac administration prior to EUS-TA.

CONCLUSION

Post-EUS pancreatitis is a rare complication of EUS-FNA/FNB with an incidence of 1%-2%. Despite its low incidence, it may have a significant clinical impact as pancreatitis may run a severe disease course causing delay in further diagnostics or therapy or even worse. Diclofenac suppository is effective as prophylaxis against pancreatitis after ERCP. Literature on the effectiveness of diclofenac in preventing post-EUS-TA pancreatitis is scarce. Based on the pathophysiological mechanism, which is nearly the same in both types of pancreatitis, diclofenac could be effective as prophylaxis for post-EUS-TA pancreatitis.

Unfortunately, an RCT with unfeasible numbers of patients is the only way to answer the question whether there is a significant benefit to the administration of diclofenac. In our opinion, further attempts to investigate the use of NSAIDS in post-EUS-TA pancreatitis prevention have limited added value. There are several arguments in favor of administration, such as the cost-effective prevention of post-EUS-TA pancreatitis, which could have potentially disastrous consequences for the patient. In addition, a single suppository of diclofenac has limited side effects. In conclusion, administration of diclofenac prior to the EUS-TA procedure of a solid or cystic pancreatic lesion should be strongly advised to prevent developing post-EUS-TA pancreatitis.

FOOTNOTES

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REVIEW

Autoimmune pancreatitis: Cornerstones and future perspectives

Camilla Gallo, Giulia Dispinzieri, Nicola Zucchini, Pietro Invernizzi, Sara Massironi

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Abstract

Autoimmune pancreatitis (AIP) is an autoimmune subtype of chronic pancreatitis resulting from the aberrant immune response against the pancreas, leading to inflammation and fibrosis. Although AIP is rare, its incidence is increasing and is often misdiagnosed as other pancreatic diseases. AIP is commonly classified into two types. Type 1 AIP (AIP-1) is typically associated with elevated serum immunoglobulin G4 (IgG4) levels and systemic manifestations, while type 2 AIP is typically a more localized form of the disease, and may coexist with other autoimmune disorders, especially inflammatory bowel diseases. Additionally, there is emerging recognition of a third type (type 3 AIP), which refers to immunotherapy-triggered AIP, although this classification is still gaining acceptance in medical literature. The clinical manifestations of AIP mainly include painless jaundice and weight loss. Elevated serum IgG4 levels are particularly characteristic of AIP-1. Diagnosis relies on a combination of clinical, laboratory, radiological, and histological findings, given the similarity of AIP symptoms to other pancreatic disorders. The mainstay of treatment for AIP is steroid therapy, which is effective in most cases. Severe cases might require additional immunosuppressive agents. This review aims to summarize the current knowledge of AIP, encompassing its epidemiology, etiology, clinical presentation, diagnosis, and treatment options. We also address the challenges and controversies in diagnosing and treating AIP, such as distinguishing it from pancreatic cancer and managing long-term treatment, highlighting the need for increased awareness and knowledge of this complex disease.

Key Words: Autoimmunity; Pancreatitis; Autoimmune pancreatitis; Immunoglobulin G4; Steroids; Relapse

Core Tip: Autoimmune pancreatitis (AIP) is rare and often misdiagnosed. The lymphoplasmacytic sclerosing form, type 1 AIP (AIP-1), represents the pancreatic manifestation of immunoglobulin G4-related disease, while the idiopathic ductal centric form, type 2 AIP (AIP-2), is often associated with inflammatory bowel disease. AIP-1 presents with obstructive jaundice or abnormalities in exocrine and endocrine pancreatic function; AIP-2 usually shows abdominal pain and acute pancreatitis. The atypical mass-forming abnormality of the pancreas implies the need to histologically distinguish AIP form pancreatic ductal adenocarcinoma. Steroids are the first-line therapy for both AIP-1 and AIP-2, rituximab is a good alternative for AIP-1. Given the high relapse rate, long-term maintenance therapy is recommended. Scientific efforts are focusing on target therapies.

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INTRODUCTION

Definition

Autoimmune pancreatitis (AIP) is a relatively rare, specific form of chronic benign pancreatic disease characterized by obstructive jaundice, with or without pancreatic masses, histologic evidence of a specific lymphoplasmacytic infiltrate and fibrosis, and a dramatic response to steroid therapy[1].

Two main forms of AIP have been described: Type 1 AIP (AIP-1), known as lymphoplasmacytic sclerosing pancreatitis, and type 2 AIP (AIP-2), known as idiopathic ductal centric pancreatitis[2], which differ mainly in epidemiology, pathogenesis, clinical presentation, histologic pattern, and natural history.

AIP-1 predominantly affects men in their sixth to seventh decade of life, and is usually painless, although mild epigastric pain may occur in about one third of patients[3]. It represents the pancreatic manifestation of immunoglobulin G4 (IgG4)-related disease (IgG4-RD), a rare, immune-mediated, systemic fibro-inflammatory multi-organ disease that often determines the growth of inflammatory pseudotumors in the affected organs. IgG4-RD usually affects two or more organs, with AIP-1 and IgG4-related cholangitis (IRC) being the most common manifestations (45% of cases overall). However, other possible typical localizations of the disease include retroperitoneal fibrosis, sialadenitis and dacryoadenitis (Mikulicz disease), Riedel's thyroiditis, mediastinal lymphadenopathy, aortic and/or renal involvement, and interstitial lung disease[4]. Based on the distribution of organ involvement, four characteristic IgG4-RD phenotypes can be distinguished: Pancreatic-hepatobiliary disease, which is the most common; retroperitoneal fibrosis and/or aortitis; disease confined to the head and neck; Mikulicz syndrome with systemic involvement[5]. IgG4-RD is characterized by the following histologic features: lymphoplasmacytic infiltrates rich in IgG4+ plasma cells [> 10 per high-power field (HPF)], storiform fibrosis, and obliterative phlebitis. Circulating IgG4 levels may vary, but the ratio of circulating IgG4 to IgG levels is typically > 10%[6].

AIP-2 usually affects younger subjects without sex differences. It manifests as acute symptomatic pancreatitis, with specific involvement of a single organ. AIP-2 is caused by dysimmune fibro-inflammatory infiltration of the middle and small pancreatic ducts (PDs) and pancreatic acini, leading to the formation of pathognomonic granulocytic epithelial lesions (GELs)[7]. In 15%-30% of cases, AIP-2 is associated with inflammatory bowel disease (IBD), typically ulcerative colitis (UC). For this reason, anti-neutrophil cytoplasmic antibodies (p-ANCA and c-ANCA) can often be detected in patients with AIP-2[8], although no specific serological markers are currently available: Serum IgG4 Levels are usually normal or only slightly elevated.

A third type of AIP has recently been described: Type 3 AIP (AIP-3) is a mostly asymptomatic or rarely paucisymptomatic form of pancreatic injury that exclusively affects patients with advanced malignancies. It is an iatrogenic entity caused by a non-specific, inflammatory T-cell mediated immune response against PDs and acini, triggered by immune checkpoint inhibitors (often anti-PD-1 and anti-CTLA4). The disease typically occurs 4-6 months, rarely more than 12 months, after the start of therapy. It is not characterized by pathognomonic histopathologic lesions, and it is usually seronegative, although elevated IgG4 levels have been occasionally described[9].

Epidemiology

Few data are available on the overall prevalence and incidence of AIPs. Among the possible immune-mediated pancreatic disorders, AIP-1 is the most common and accounts for the vast majority of cases[10]; it is more common in Asia than in the United States and European Union[11]. Regarding AIP-1, thanks to the increasing awareness of IgG4-RD and the dissemination of diagnostic guidelines, large-scale epidemiological data have recently been published, mainly from Japan. According to a nationwide epidemiological survey conducted in 2016, AIP-1 showed an incidence of 1-3 cases per 100,000 adults and a prevalence of approximately 10 cases per 100000 adults; compared with previously published data, these results have more than doubled in less than 5 years. The reported male-to-female sex ratio was 2.94:1, and the mean age at diagnosis was 64.8 years[12]. The first raw data published in Italy showed that AIP-1 affects approximately 6% of the general population, and accounts for 61% of AIP cases[3].

On the other hand, AIP-2 is more prevalent in Western countries than in Asia[13], with an estimated prevalence rate of 4.6–6% in acute and chronic pancreatitis and about 1-4 cases per 100000 adults in the general population[2-14]. Only two Asian studies investigated the epidemiology of AIP-2 in IBD patients and reported a prevalence of 0.3%-0.5%[15,16], which is approximately 100-fold higher than in the general population, and may even be underestimated due to the difficulty of diagnosing AIP-2, which often requires histological confirmation. On the other hand, 49%-67% of AIP-2 patients have concomitant IBD, which means that AIP-2 patients have a 12-15-fold higher risk of having a concurrent IBD compared to the general population[17]. According to an Italian multicenter study, AIP-2 accounts for 28% of all AIP cases. Compared to AIP-1, younger people are more likely to be affected, with no significant gender difference between men and women[3].

According to a recent American review on AIP-3, the incidence of AIP-3 among all immune-mediated adverse events with immune checkpoint inhibitors is between 0.6% and 4%[9].

Etiopathogenesis

Despite numerous attempts, the pathogenesis of AIP-1 is still unclear. As it is the pancreatic manifestation of IgG4-RD, it is a multifactorial disease in which both genetic and environmental factors play a pivotal role. Genome-wide association studies in IgG4-RD-affected patients revealed a significant association between mutations in human leukocyte antigen *DRB1* genes encoding macrophage-type toll-like receptors (TLRs) II major histocompatibility complex (MHC)[4]. The overexpression of certain types of TLRs in the pancreas highlights the central role of the innate immune system in the development of AIP-1. Plasmacytoid dendritic cells (pDCs) may also play a key role in the pathogenesis of AIP: They are involved in host defense against microbial infections and are the major source of type 1 interferons (IFN-I)[18]. The unregulated production of IFN-I and, consequently, of interleukine (IL)-33 by pDCs could underlie AIP-1. IL-33, which is also produced by overexpression of certain types of TLRs, may promote activation of mainly Th2 cells and regulatory T cells that produce IL-4 and IL-10, respectively, which in turn are responsible for switching immunoglobulins to the IgG4 subclass[19]. The role of IgG4 in the development of AIP-1 and IgG4- RD is still unclear, but it is hypothesized that IgG4 may play a role in the activation of the complement system after the presence of immune complexes has been demonstrated in IgG4-RD-affected tissues[20].

In addition to the activation of T helper and T reg CD4+ lymphocytes that follows the interaction between TLRs and MHC-II, also the interaction between T follicular helper (Tfh) cells, especially circulating type 1 Tfh cells, and SLAMF7, a member of the Signaling Lymphocyte Activation Molecule family receptors, promotes IgG4 release[21]. SLAMF7 is implicated in homotypic interactions with activated B cells and, thus, it is involved in disease immunopathogenesis. SLAMF7+ CD4+ cytotoxic T cells (CTLs) are unusual CD4+ cells, which have been shown to express cytotoxic mediators that are typically expressed by CD8+ cells, and have been shown to have the potential to both stimulate fibroblast activation and interact with antigen-presenting B cells[22]. Recent studies have shown that SLAMF7+ CD4+ CTLs are increased in the peripheral blood of subjects with active IgG4-RD, and thus represent a key pathological factor in the disease[23].

Furthermore, cellular components that form the fibro-inflammatory pancreatic aggregate include eosinophils, which are attracted to the pancreatic site primarily by the chemotactic action of eotaxin. It is noteworthy that elevated levels of circulating eotaxin-1 and 3 have been detected in AIP-1 patients[24]. The presence of elevated levels of circulating IgE and IgG4 in IgG4-RD and AIP-1 and the presence of eosinophilic infiltrates in the pancreas suggest that, in addition to genetic predisposition, environmental factors play an important role in the development of AIP-1. Prolonged exposure to certain exogenous antigens and molecular mimicry between these antigens and some autoantigens may lead to overactivity of specific types of TLRs that trigger a dysimmune response directed against the endogenous autoantigens[4].

Regarding the pathogenesis of AIP-2, the Th-17 subset of CD4+ effector T cells plays a crucial role in infiltrating the periductal pancreatic tissue, where they release inflammatory cytokines, mainly IL-17, IL-21, IL-22, and IL-23[25]. The reasons leading to this hyperactivation of Th-17 cells and their migration into pancreatic tissue are not yet clear. However, there may be a link with genetic mutations in the genes for multiple endocrine neoplasia 1 and polycystic kidney and liver disease 1, which are frequently found in AIP-2 patients[26]. Moreover, the pathognomonic AIP-2 GELs consist not only of lymphocytes but mainly of neutrophils that migrate and aggregate in the periductal pancreatic tissue, attracted by the chemotactic function of IL-8, which in turn is stimulated by IL-17[27]. IL-8 was overexpressed not only in AIP-2 cases but also in UC patients, suggesting that it is an immunological biomarker for the coincidence of AIP-2 and UC[28].

The increasing awareness of the relationship between specific alterations in the composition of the gut microbiota and the innate immunological response, and thus the development of autoimmune diseases, led to the hypothesis of a possible role of the microbiota in the etiopathogenesis of AIP, particularly *K. Pneumoniae*[29]. This possible gut-pancreas axis could apply not only to AIP-2, for which the correlation data between IBD and changes in the gut microbiota are strong but also to AIP-1[30].

Finally, the etiopathogenesis of AIP-3 is closely related to the administration of checkpoint inhibitors, which trigger a non-specific inflammatory immune response mediated by T cells, mainly CD8+ T cells, resulting in an increased ratio of CD8+/CD4+ T lymphocytes[31,32]. In Figure 1, a concise overview of the etiopathogenetic mechanisms underlying AIP-1, AIP-2, and AIP-3 is provided.

CLINICAL AND SEROLOGICAL FEATURES

The two main forms of AIP described, AIP-1 and AIP-2, have two distinct clinical phenotypes. AIP-1 occurs mainly in older men and is usually painless. According to an international multicenter study, the most common symptom is

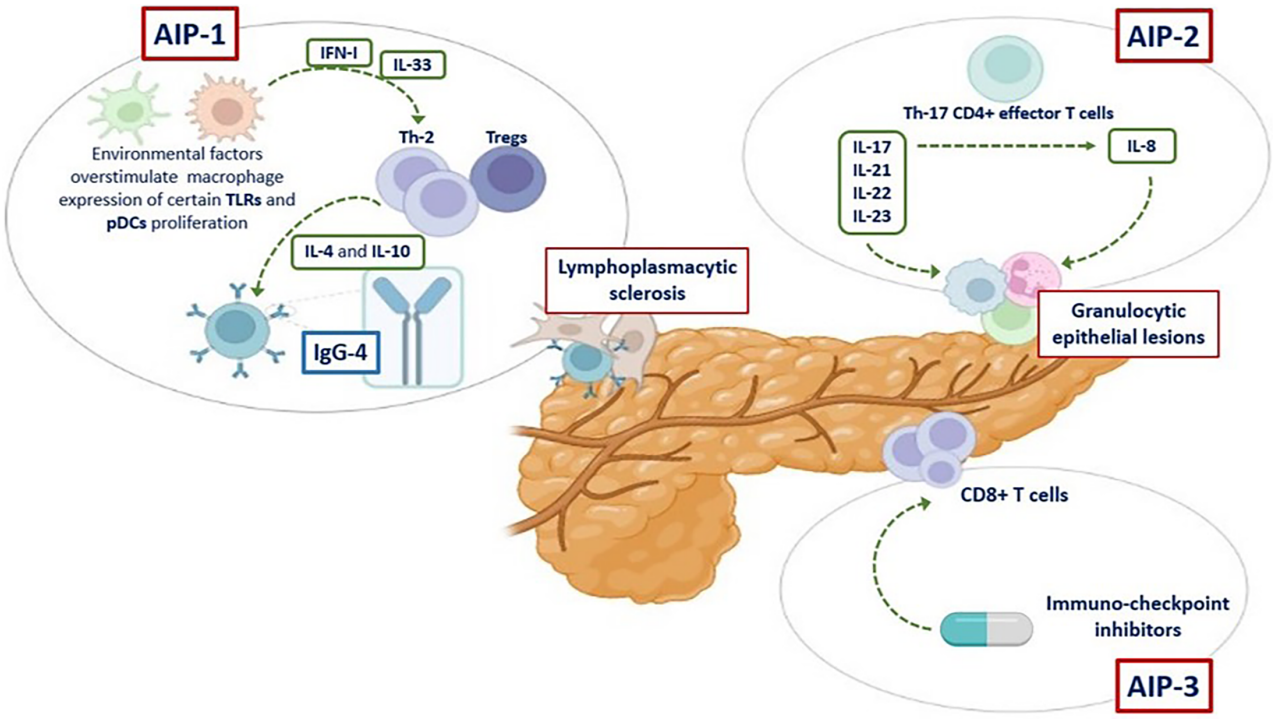

Figure 1 Ethiopathology of different types of autoimmune pancreatitis. Ethiopathological mechanisms of type 1 autoimmune pancreatitis, type 2 autoimmune pancreatitis, and type 3 autoimmune pancreatitis. AIP: Autoimmune pancreatitis; IFN: Interferon; IL: Interleukine; TLRs: Toll-like receptors; pDCs: Plasmacytoid dendritic cells; IgG4: Immunoglobulin G4; AIP-1: Type 1 autoimmune pancreatitis; AIP-2: Type 2 autoimmune pancreatitis; AIP-4: Type 3 autoimmune pancreatitis.

obstructive jaundice, which occurs in 75% of cases[10] and is thought to be due to compression of the common bile duct by the mass/swelling of the pancreatic head or by direct infiltration of biliary wall with lymphocytes and plasma cells [33]. Less commonly, AIP-1 manifests with abdominal symptoms (in nearly 40% of patients), such as abdominal pain or malaise, and more rarely with acute pancreatitis. Other clinical manifestations include weight loss and abnormalities of exocrine and endocrine pancreatic function, with diabetes mellitus that may occur before (33%), concurrently (52%), or after steroid treatment[6]. It may also manifest as diffuse, focal, or segmental enlargement of the pancreas, mimicking PD adenocarcinoma (PDAC), from which it must be differentiated. As it is the pancreatic manifestation of IgG4-RD, AIP-1 usually occurs with the involvement of other organs, such as biliary stricture, renal involvement, orbital pseudotumor, extensive lymphadenopathy, and retroperitoneal fibrosis. The most common clinical presentation of IgG4-RD sees the involvement of the bilio-pancreatic district, such that AIP-1 and IRC occur together in 80% of cases. It should be noted that although the involvement of other organs supports the diagnosis of AIP, the absence of involvement of other organs does not exclude AIP-1, and isolated pancreatic involvement is seen in approximately 50% of patients[34]. IgG4-RD is a multisystemic fibroinflammatory disease characterized by elevated serum concentration of IgG4 and accumulation of IgG4-expressing plasma cells in the affected organs[35]. However, serum IgG4 plays an increasingly minor role in the diagnosis of AIP-1 and IgG4-RD. Recent studies have shown that up to half of patients with biopsy-proven and clinically active IgG4-RD may have normal serum IgG4 concentrations[36]. Furthermore, only 10% of patients with elevated serum IgG4 levels were diagnosed with IgG4-RD, underscoring the lack of specificity of this test[37].

While AIP-1 has a mostly asymptomatic clinical course, AIP-2 manifests more frequently with abdominal pain and acute pancreatitis. Acute pancreatitis occurs in nearly 50% of patients[28]. Other manifestations include painless obstructive jaundice, focal pancreatic masses, and symptomatic PD strictures[38], similar to AIP-1 patients. Compared with AIP-1, AIP-2 typically affects younger patients, with an average age of 40 years, and has no gender predilection. Although AIP-2 can also occur with exclusive pancreatic involvement, a strong association between AIP-2 and concurrent IBD, especially UC, has been reported, as mentioned previously[17]. In most cases, the diagnosis of IBD precedes the diagnosis of AIP-2, but it is unclear whether active IBD plays a role in the development of AIP-2. According to an Italian retrospective study at IBD-AIP, 68% of patients had a prior or concomitant diagnosis of UC, but only 44% had active disease[39]. However, a French study with a similar group of patients shows that 80% of patients had a previous or concomitant diagnosis of IBD, and about 70% had active disease at the onset of AIP[40]. Table 1 resembles the differential characteristics between AIP-1 and AIP-2.

RADIOLOGICAL PRESENTATION

Contrast-enhanced (CE)-computed tomography (CT) and magnetic resonance imaging (MR) (MRI) have proven useful in the imaging diagnosis of AIP. Imaging abnormalities of the pancreas are virtually indistinguishable between AIP-1, AIP-

Table 1 Differential characteristics between type 1 and type 2 autoimmune pancreatitis

	AIP-1	AIP-2
Gender (M:F)	3:1	1:1
Mean age at disease onset	60-70 yr	40-60 yr
Epidemiology	Asia > Western Countries	Western Countries > Asia
Main clinical manifestations	Painless jaundice (75%); Abdominal symptoms (40%)	Abdominal pain and acute pancreatitis (50%)
	Weight loss	
	Diabetes and exocrine pancreatic insufficiency	
Extrapancreatic manifestations	IgG4-related disease extrapancreatic manifestations (50%)	IBD (49%-67%)
	Hepatobiliary disease	
	Retroperitoneal fibrosis and/or aortitis	
	Head and neck involvement	
	Mikulicz syndrome	
Serum IgG4 levels	Elevated (circulating IgG4 to IgG levels typically > 10%) (50%)	Normal (p-ANCA and c-ANCA autoantibodies often positive)
Histologic features	Lymphoplasmacytic infiltrates rich in IgG4+ plasma cells	Granulocytic epithelial lesions
	Storiform fibrosis	
	Obliterative phlebitis	
Steroid therapy	Responsive	Responsive
Relapse	High rate (39%)	Rare

AIP: Autoimmune pancreatitis; IBD: Inflammatory bowel disease; ANCA: Anti-neutrophil cytoplasmatic antibodies; IgG: Immunoglobulin G; AIP-1: Type 1 autoimmune pancreatitis; AIP-2: Type 2 autoimmune pancreatitis.

2, and AIP-3[9,41]. The differential diagnosis between these three different nosographic entities is mainly based on the combination of history, clinical presentation, histopathologic findings, and, in the case of IgG4-RD-involvement of the pancreas, the possible presence of combined characteristic radiologic findings reflecting coexisting pathologies in other affected organs[42]. Furthermore, CE-CT and MRI scans do not always allow a correct differential diagnosis between mass-forming AIP and PDAC, which is challenging because of their common epidemiologic and clinical manifestations [43].

Typical CT features of AIP include focal or diffuse sausage-like swellings of the parenchyma with straight margins, rectangular shape of the tail (cut-tail sign), and consequent loss of the typical lobular structure[44] (Figure 2). An exception is elderly patients, in whom the age-related reduction in pancreatic volume may mask the presence of inflammatory swelling of the organ[45].

Due to the presence of fibrosis, the arterial or pancreatic phase of the CE-CT scan typically shows a homogeneous reduced enhancement of the affected areas compared with the normal pancreatic parenchyma, whereas a gradually increasing enhancement is detectable in the delayed phases of the dynamic scan[46,47]. Small areas of normal pancreatic parenchyma may remain focal in association with the affected lesions: such areas maintain normal arterial blood flow and may therefore be visualized as punctate, speckled, or dotted contrast enhancement in the arterial phase[45]. These findings help to distinguish AIP from PDAC[48].

As a result of the physio-pathological accumulation of the fibrotic component at the periphery of the inflammatory areas (be it the pancreas as a whole or the intrapancreatic pseudotumor lesions), a capsular rim demarcates the swollen pancreas and/or the pseudotumoral affected areas, with a typical reduced enhancement in the arterial phase and a progressively increasing enhancement in the delayed phases[49]. PDAC may sometimes have a peripheral rim, but unlike the rim detectable in AIP, it is usually early enhanced in the arterial phase[50]; therefore, the CE behavior of the perilesional rim of AIP with mass-forming AIP may help distinguish AIP from PDAC.

As a consequence of inflammatory involvement of the main PD (MPD), the arterial phase of the CE-CT scan may show a marked hyperdense demarcation of the MPD walls, which are often also thickened (enhanced duct sign)[51].

In particular, the capsular rim and thickened and hyper-enhanced MPD are usually less common in AIP-2, but these differences are not sufficient to make a differential diagnosis with AIP-1 based on radiologic presentation alone[41].

The typical appearance of AIP at CE-MRI is characterized by a diffuse or focal signal of lower intensity on unenhanced T1-weighted MRI images, with an even more hypointense signal in line with the border delineating the entire pancreas or the affected pseudotumoral areas, which are heavily composed of fibrosis. On T2-weighted images, the areas affected by AIP show moderately higher signal intensity, still demarcated by a low-intensity fibrotic rim (Figure 3). The contrastographic behavior of AIP on MRI is the same as that described for CE-CT (Figure 2)[46,47].

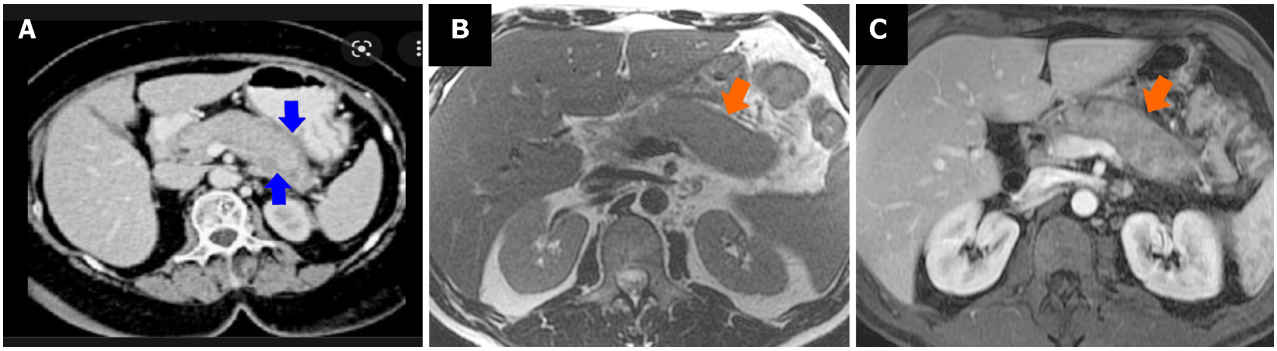

Figure 2 Radiological appearance of autoimmune pancreatitis-part 1. A: Unenhanced computed tomography scan appearance of a diffuse autoimmune pancreatitis (AIP): Sausage-like swelling of the parenchyma, with straight margins, and consequent loss of the typical lobular structure. A hypodense fibrotic capsule-like rim demarcates the swollen pancreas (blue arrow); B: Unenhanced T1 weighted magnetic resonance imaging (MRI) appearance of a diffuse AIP: Sausage-like swelling of the parenchyma, with straight margins, and rectangular shape of the tail (cut-tail sign) (orange arrow); C: Arterial phase of the contrast-enhanced T1 weighted MRI: Homogeneous reduced enhancement of the pancreatic parenchyma, with a more hypointense fibrotic capsule-like rim that demarcates the swollen pancreas (orange arrow).

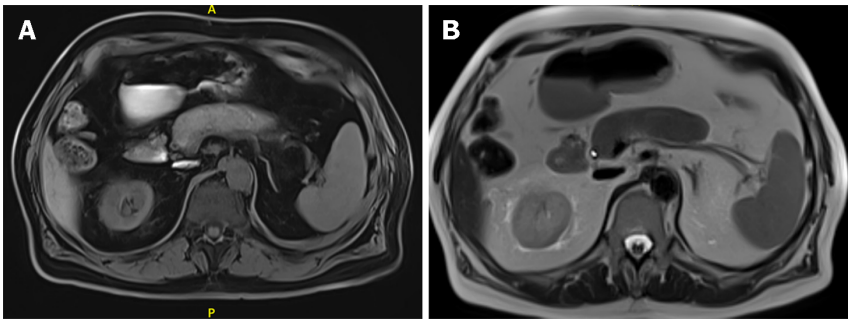

Figure 3 Radiological appearance of autoimmune pancreatitis-part 2. A: Unenhanced T1 weighted magnetic resonance imaging (MRI) images of autoimmune pancreatitis (AIP): Diffuse hypointense pancreas, with an even more hypointense fibrotic capsule-rim; B: Unenhanced T2 weighted MRI images of diffuse AIP: The affected parenchyma shows a moderately higher intensity signal, with a persistently low-intensity fibrotic rim.

On diffusion-weighted images, the presence of highly cellular plasmocyte proliferation is reflected in a homogeneously hyperintense signal of the affected areas, with the mean apparent diffusion coefficient of the lesions being significantly lower in mass-forming AIP than in PDAC[52-54].

Magnetic resonance cholangiopancreatography images show typical multiple and long MPD skip narrowings without upstream dilatation but with prominent side branches of the PD[55], producing a characteristic radiological sign (icicle sign). In the case of mass-forming AIP, the MPD may penetrate the lesion without complete occlusion (the sign of ductal penetration)[56].

MR elastography results vary considerably depending on the pathological phases of AIP: Recent edematous inflammation is associated with lower stiffness values, whereas chronic fibrotic inflammation is associated with higher stiffness values. However, AIP is generally associated with lower median pancreatic stiffness values than PDAC[57,58].

Concerning the MRI differential diagnosis between mass-forming AIP and PDAC, a multicenter nationwide study highlighted the following features of AIP as the most reliable among all those mentioned above: The presence of long and multiple MPD strictures, the absence of upstream dilatation of the stricture, and the detection of PD side branches originating from a strictured segment (sensitivity 44%-71%, specificity 92%-$P < 0.05$)[50]. According to a recent Korean meta-analysis, the absence of MPD dilation has the highest pooled sensitivity (87%, 95%CI = 68%-96%), whereas the presence of a peripancreatic rim has the highest pooled specificity (100%, 95%CI = 88%-100%) in distinguishing the two diseases[43].

According to the results of a recent comparative meta-analysis between CT and MRI in terms of diagnostic accuracy in AIP, MRI had significantly higher summary sensitivity than CT (84% *vs* 59%, $P = 0.02$) but similar specificity (97% *vs* 99%, $P = 0.18$). In the subgroup analysis for mass-forming AIP, sensitivity for discriminating between mass-forming AIP and PDAC was higher for MRI than CT (76% *vs* 50%, $P = 0.28$), but specificity was similar for both methods (97% *vs* 98%, $P = 0.07$)[59].

On 18F-fluorodeoxyglucose (FDG) positron emission tomography (PET)-CT, AIP usually shows markedly increased diffuse uptake, which is different from the typical focal PDAC uptake[60]. Some other 18F-FDG parameters, including the SUV_{max} ratio between the pancreatic lesion and liver and uptake outside the pancreas in other organs, might help to distinguish the two diseases. Indeed, the SUV_{max} ratio between the pancreas and liver in delayed scans is usually higher in PDAC. On the contrary, the presence of increased uptake in the salivary glands, prostate (with a typical "V" shape), and mediastinal, hilar, and para-pancreatic lymph nodes are likely concomitant signs of IgG4- RD with pancreatic

involvement[61,62].

Furthermore, since fibrosis is an important feature of IgG4-RD, 68Ga-fibroblast activation protein inhibitor-PET, which uses a recently introduced agent targeting fibroblast activation protein, proved to have high sensitivity in detecting IgG4-related pancreatic, biliary and lacrimal gland involvement, with significantly higher uptake than 18F-FDG-PET[63].

According to a very recent study focusing on the radiological appearance of AIP-3[32], it is consistently associated with acinar injury and pancreatic volume loss. The parenchymal loss is directly proportional to pancreatic enzyme elevation: higher pancreatic enzymes correspond to major parenchymal loss, while near-normal pancreatic enzymes are associated with near-normal radiological pancreatic aspect. These distinct radiological features suggest AIP-3 to be sustained by a novel mechanism of chronic pancreatic injury.

ENDOSCOPIC ULTRASOUND PRESENTATION AND ENDOSCOPIC ULTRASOUND-GUIDED TISSUE SAMPLING

In the complex scenario of AIP diagnosis, the role of endoscopic ultrasound (EUS) so far can be seen mainly in its ability to biopsy the affected pancreatic parenchyma and thus make a definite AIP diagnosis, which is also different from PDAC. The endoscopic approach of first choice for obtaining pancreatic specimens for histopathological evaluation should be EUS fine-needle biopsy (FNB): According to a recent meta-analysis, FNB needles seem to be more accurate than fine-needle aspiration (FNA) needles in diagnosing AIP, as they guarantee a core biopsy[64,65]. However, the diagnosis of AIP is challenging, even by using EUS and FNA/FNB. The sonographic and cross-sectional findings of AIP closely mimic PDAC, and tissue sampling techniques for diagnosis of AIP still remain suboptimal[66]. Although the diagnostic consistency of histologic diagnosis of type 1 AIP based on the findings obtained by an EUS-guided FNA/FNB is feasible, it remains a challenge and not conclusive[67].

The main EUS findings may be divided into diffuse and focal pictures of AIP. EUS characteristics suggestive of diffuse AIP included diffuse pancreatic enlargement with echo-poor echo texture, hyperechoic foci/stands or lobularity (parenchymal heterogeneity), loss of connection to the splenic vein, hyperechoic MPD walls thickening and peripancreatic hypoechoic margin; stones and cysts similar to those described in chronic alcoholic pancreatitis may occur in the late stages of AIP. In mass-forming AIP, EUS features included focal hypoechoic mass, absence of parenchymal heterogeneity, eventually PD dilation, and vessel involvement. In a recent retrospective study, these pictures were used to construct a prediction diagnostic model, that showed an area under the receiver operating characteristic curve of more than 0.95, with a good capability to distinguish focal AIP from PDAC. By using the optimal cutoff value, the efficacy of the model for diagnosing PDAC showed 83.7%-91.8% sensitivity and 93.3%-95.6% specificity[68]. It is likely that the use of EUS-based convolutional neural networks can help, showing in a recent study, a sensitivity of 99% and a specificity of 98% for distinguishing AIP from normal pancreas, a sensitivity of 94% and a specificity of 71% for distinguishing AIP from chronic pancreatitis, and a sensitivity of 90% and a specificity of 93% for distinguishing AIP from PDAC[66].

EUS elastography may show increased stiffness of the parenchyma. EUS is extremely useful in detecting other typical findings of IgG4- RD AIP, such as changes in the common bile duct and lymphoadenomegaly (Figure 4)[69].

Finally, regarding the natural history of the disease, the typical picture of AIP described above usually improves after steroid treatment: the swelling of the pancreas decreases, the capsular rim disappears, the multiple MPD stenoses improve, and the enhanced duct sign also disappears. Nevertheless, the global CE of the previously affected parenchyma may not completely normalize[54,70]. Table 2 resembles the main radiological features of AIP.

HISTOPATHOLOGICAL CHARACTERISTICS

The main morpho-histological features of AIP-1 are dense lymphoplasmacytic infiltrate of the affected areas, distributed mainly lobule-centered but sometimes involving the periductal areas with a resulting slit-like obstruction of the PD; storiform fibrosis composed of spindle-shaped cells and inflammatory cells on a background of delicate collagen; luminal obliteration of the interlobular vein by the lymphoplasmacytic infiltrate, forming obliterative phlebitis. Interobserver variability in the interpretation of storiform fibrosis and obliterative phlebitis is not negligible; additional elastic staining, such as Elastica van Gieson staining, should be considered because it may help reduce interobserver variability[71]. In contrast to the findings typical of AIP-2, organs affected by AIP-1 do not usually show neutrophilic infiltration or abscess formation.

In addition to these typical morphologic features, which have historically been the primary histologic diagnostic criteria for AIP-1, biopsy or resection specimens of AIP-1 exhibit a highly pathognomonic immunohistochemical pattern: Diffuse and massive IgG4+ plasma cell infiltration with > 10 per HPF in biopsy specimens and > 50 per HPF in surgical specimens. For diagnostic purposes, to date, minimally invasive small biopsies have largely replaced surgical resections, and although this development is an achievement for the field, it represents a major challenge for the surgical pathologist. In fact, according to recent studies, around one-half of all small biopsies do not usually meet the pathological criteria for IgG4-RD, being the lack of both storiform-type fibrosis and obliterative phlebitis the most common reason for diagnostic failure. However, despite the lower pathological quality of biopsy samples, which is mainly due to their smaller size, the IgG4/total IgG ratio on biopsy samples proved the same high diagnostic accuracy when compared to the one on resection specimens. Immunohistochemistry (IHC) for IgG4 and total IgG, and the evaluation of IgG4/total IgG ratio is thus mandatory for IgG4-RD diagnosis, being it the most sensitive tissue-based feature of IgG4-RD[72]. According to a recent

Table 2 Main radiological features of autoimmune pancreatitis

CT scan	**Diffuse or focal sausage-like swelling**
	Cut-tail sign
	Homogeneous reduced enhancement with dotted contrast enhancements of normal parenchyma
	Hypo-enhanced capsule-like rim with delayed enhancement
	Thickened hyperdense MPD walls
MRI	Diffuse or focal lower intensity signal on T1-weighted MRI images, with an even more hypointense capsule-rim
	Moderately higher intensity signal on T2-weighted images, still with a low-intensity fibrotic rim
	DWI homogeneously hyperintensity
MRCP	Multiple and long MPD skip narrowings
	No upstream dilatation
	Side PD branches (icicle sign)
	Duct-penetrating sign, in case of mass-forming AIP
18F-FDG PET-CT	Diffused or focal increased uptake
EUS	Diffuse pancreatic enlargement, with echopoor echotexture, loss of interface with the splenic vein, concomitant intraparenchymal hyperechoic foci and strands
	Hyperechoic MPD walls
	Solitary, irregular, hypoechoic mass, in case of mass-forming AIP, generally in the head of the pancreas, without upstream dilatation of the MPD
Elastography	Magnified parenchymal stiffness

AIP: Autoimmune pancreatitis; CT: Computed tomography; MPD: Main pancreatic duct; MRI: Magnetic resonance imaging; MRCP: Magnetic resonance cholangiopancreatography; FDG: Fluorodeoxyglucose; PET: Positron emission tomography; EUS: Endoscopic ultrasound.

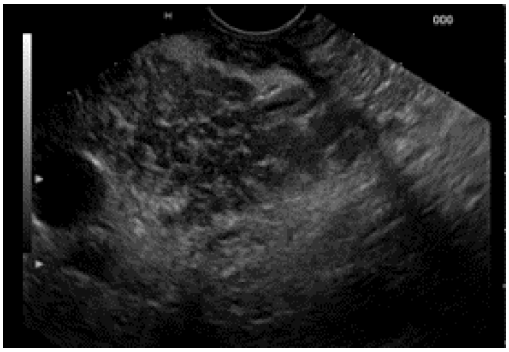

Figure 4 Endoscopic ultrasound appearance of autoimmune pancreatitis. Endoscopic ultrasound aspect of a mass-forming autoimmune pancreatitis: Solitary, irregular hypoechoic mass, located in the head of the pancreas, without upstream dilatation of the main pancreatic duct.

meta-analysis, in fact, the use of IHC for IgG4 in the diagnosis of AIP-1 has a sensitivity and specificity of approximately 70% and 92%, respectively[73].

Although the IgG4/total IgG ratio is emphasized in most diagnostic algorithms, the optimal cutoff has not yet been shared univocally; to date, most studies have utilized a cutoff ranging from 30% to 40% with a higher cutoff corresponding to higher specificity, but proportionally lower sensitivity[74,75].

Of note, treatment can interfere with histological findings and cell counts; on the other hand, also prolonged disease can lead to possible false negative immunohistochemical patterns.

Currently, there are no specific serological markers for AIP-2, so the diagnosis is made based on histology[7]. AIP-2 is characterized by a large inflammatory infiltrate in the pancreas composed mainly of neutrophils but also containing lymphocytes and plasma cells. This inflammation occurs primarily in the PD area, where it forms structures known as GELs[7,76]. AIP-2 can also cause clusters of neutrophils to form inside the ducts. Unlike AIP-1, which is characterized by obliterative phlebitis and storiform fibrosis, these features are less common in AIP-2. In addition, the number of IgG4+ plasma cells is usually not significantly increased in AIP-2, although small pockets of these cells may be present[74].

The absence of established histologic patterns for AIP-3 raises questions about its categorization, even if it is important to consider that the field of AIP is still evolving, and our understanding of the disease continues to expand. Therefore, at this stage, referring to this subtype as AIP-3 allows for the recognition of a distinct subgroup within the spectrum of AIP, even in the absence of well-defined histologic patterns. However, it is crucial to continue research efforts to establish clearer diagnostic criteria and classification systems for AIP-3 and other potential subtypes to improve the accuracy of diagnosis and guide appropriate treatment strategies. Figure 5 Histological samples of AIP-1.

DIAGNOSIS

According to the International Consensus Diagnostic Criteria for AIP, a definitive diagnosis of AIP-1 can be made in diffuse pancreatitis based on clinical, radiological, and serological features. In the presence of atypical mass-forming imaging and concomitant absence of other diagnostic criteria (mainly IgG4-RD extrapancreatic involvement or elevated IgG4+ plasma cells count), histologic evaluation by surgical or EUS-FNB tissue sampling is mandatory to make the definitive diagnosis and differentiate AIP from PDAC. In the latter scenario, the AIP-1 diagnosis can be definitively established if three or more of the following four histologic features are present: lymphoplasmacytic cell infiltration, > 10 per HPF IgG4+ plasma cells (in case of biopsy sampling, otherwise > 50 per HPF in case of surgical specimen), storiform fibrosis, or obliterative phlebitis. In the presence of fewer than three of these histologic features, increased plasma IgG4 cell counts, along with typical imaging features, may help determine the diagnosis: (1) Serum IgG4 level has proven to be a valuable tool in the diagnosis of AIP and it is one of the five cardinal features of Mayo's HISORt criteria for the diagnosis of AIP-1[77], which are based on 5 main diagnostic criteria: Histologic findings, imaging, serology, involvement of other organs, and response to steroid therapy. Indeed, in most cases, AIP patients exhibit significantly elevated levels of serum IgG4, typically exceeding a defined threshold of 135 mg/dL. However, it is important to note that elevated IgG4 levels alone are not sufficient for an AIP diagnosis, as they may also be observed in other conditions, such as IgG4-RD involving multiple organ systems. Therefore, a comprehensive diagnostic approach combining clinical presentation, radiologic imaging, serologic markers (including IgG4 levels), and histopathologic evaluation is critical for accurate diagnosis and differentiation of AIP from mimicking diseases.

With the exact purpose of excluding disease mimics, in 2019 the ACR/EULAR diagnostic criteria were developed. They consist of a three-step classification process: first, at least one of 11 possible organs must be involved in a manner consistent with IgG4-RD; second, 32 clinical, serological, radiological, and pathological exclusion criteria must be verified; third, eight weighted inclusion criteria domains, addressing clinical findings, serological results, radiological assessments, and pathological interpretations, have to be applied. A case meets the classification criteria for IgG4-RD if the entry criteria are met, no exclusion criteria are present, and the total points is ≥ 20[5].

In the case of an uncertain histologic diagnosis, systems for grading the likelihood of AIP (highly suggestive, probable, inconclusive) based on various combinations of features have been proposed, but they remain to be clinically validated [78-81]. A biopsy showing little or no evidence of AIP cannot exclude AIP with certainty unless a positive alternative diagnosis can be made[75].

Ongoing research in the field of AIP is investigating potential future markers for diagnosis. These markers include subclass analysis of IgG4, serum cytokines [such as IL-6 and tumor necrosis factor (TNF)-alpha], serum microRNAs (*e.g.*, miR-21 and miR-375), autoantibodies targeting pancreatic antigens and advanced imaging techniques (*e.g.*, EUS and MRI). However, further research is needed to validate their clinical utility in routine AIP diagnosis. Integration of these markers with existing diagnostic criteria may improve accuracy in diagnosing AIP.

Definitive AIP-2 diagnosis is histologic and requires the presence of GELs; lobular neutrophil infiltration strengthens the diagnosis[41].

AIP-3 can be diagnosed in the presence of a compatible drug history and by excluding other causes of pancreatitis.

INDUCTION THERAPY FOR AIP

According to recent literature data, approximately 25% of cases of AIP show spontaneous resolution of symptoms without medical treatment, with some case series reporting resolution rates up to 55%[82]. Nevertheless, experts from different countries have proposed detailed treatment criteria for acute AIP. A consensus statement published in 2016 stated that therapy is recommended for symptomatic disease (abdominal pain, back pain, fever, obstructive jaundice) or in the case of AIP-1, for asymptomatic patients with persistent pancreatic mass on imaging or persistent liver test abnormalities in case of concomitant IRC[82]. In addition to the above criteria, following the latest United European Gastroenterology (UEG) recommendations, treatment is indicated for subclinical conditions that may lead to severe or irreversible organ failure[75].

Steroids are the first-line therapy for patients with active AIP-1 and 2[75,82]. They inhibit dendritic cell maturation and downstream signal transduction of TLRs; they also inhibit the expression of many proinflammatory cytokines involved in AIP pathogenesis. Based on UEG and Swedish Society of Gastroenterology recommendations, the initial dose of prednisone should be 0.6-0.8 mg/kg per day (typically 30-40 mg/d). The treatment duration at the full dose is one month, with an initial assessment of response to treatment approximately 2 wk after initiation (especially in cases of a diagnostic steroid study). Thereafter, treatment should be gradually tapered by 5 mg/wk to a maintenance dose of 2.5-5 mg/d over 2-3 months[83].

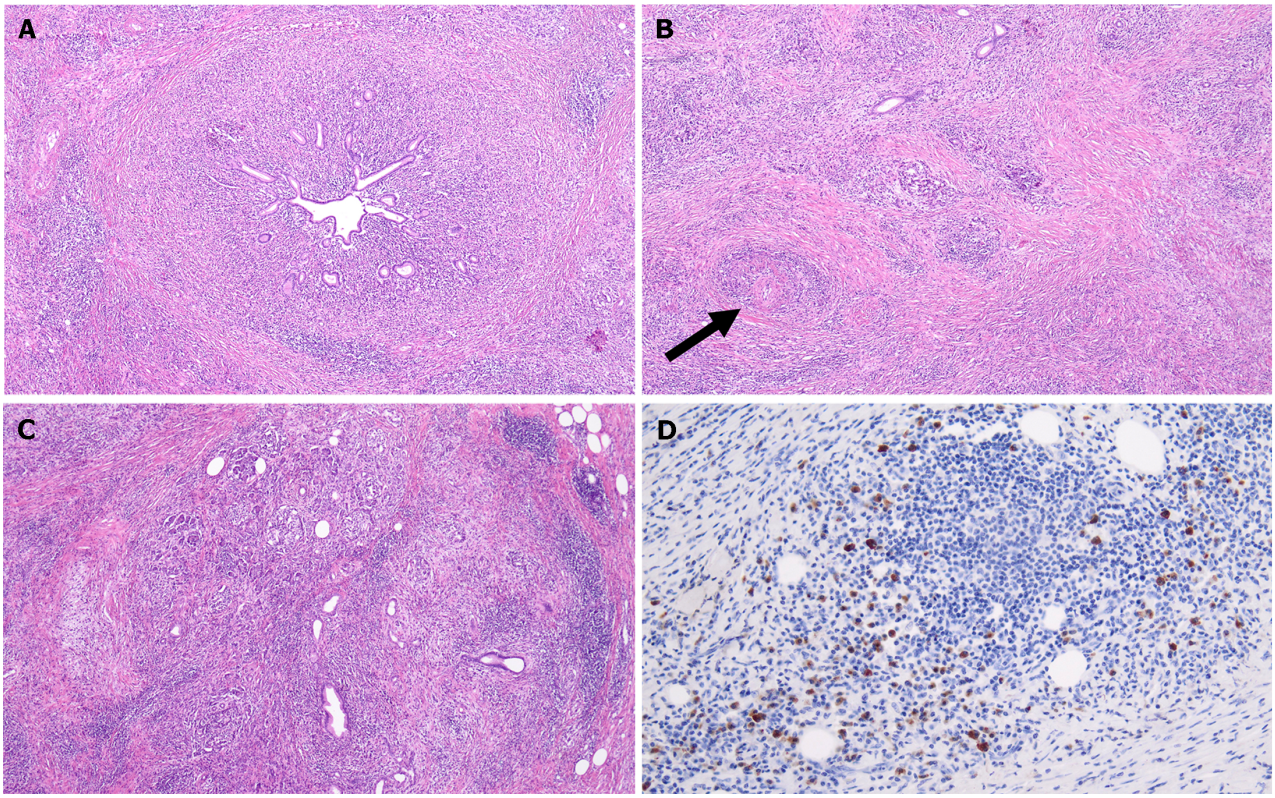

Figure 5 Histology of autoimmune pancreatitis. A: Histological samples of type 1 autoimmune pancreatitis. Hematoxylin eosin (HE) 4, Duct centric lymphoplasmacytic infiltrate; B: HE 10, storiform fibrosis with intense lymphoplasmacytic infiltrate and obliterative phlebitis (arrow); C: HE 4, lobule complete effacement by inflammatory cells and fibrosis; D: Immunoglobulin G4 (IgG4) IIC 20, moderate increase of IgG4+ plasma cells.

Up to 97%-100% of AIP, patients respond to steroid treatment[84,85]. Clinical complete remission is defined as the disappearance of symptoms, normalization of IgG or IgG4 serum levels, and disappearance of typical AIP features on imaging, *i.e.*, mainly shrinkage of the enlarged pancreatic parenchyma and regression of the narrowing of the multistrictured MPD. Incomplete remission is achieved only when one or 2 of these 3 categories are met. Imaging response to steroids is an optional diagnostic criterion, as mentioned earlier. In a small proportion of cases, AIP patients do not show any steroid response[84,85]. Furthermore, some comorbidities may contraindicate long-term steroid treatment[86]. In such cases, rituximab, a monoclonal antibody directed against CD20 B-cell-specific antigen, is the second-line good alternative therapeutic choice for acute AIP-1[75,87], whether monotherapy with immunomodulators (such as azathioprine, 6-mercaptopurine, mycophenolate mofetil, cyclosporine A, tacrolimus, methotrexate, and cyclophosphamide) did not prove sufficient efficacy, but reliable data specifically on AIP-2 are lacking[88].

If rituximab therapy is required for induction remission, the most common regimen includes 1 g of intravenous rituximab on days 0 and 14[75], and it has been shown to guarantee complete remission in up to 83% of patients[89,90]. On the other hand, given their low efficacy as monotherapy, immunomodulators steroid-sparing agents are used mainly in combination with low-dose steroids in steroid-refractory conditions[91].

Minipulse steroid therapy (two administrations of methylprednisolone 500 mg/day for three days with an interval of four days) was described in several Japanese protocols[92].

Specifically concerning AIP-2, colchicine has recently been reported to be a successful treatment option: it inhibits neutrophils and thus reduces the formation of the pathognomonic GELs[93]. There is also emerging evidence suggesting the potential use of biologic medications in the treatment of AIP-2. While corticosteroids remain the first-line therapy for AIP-2, there have been reports of cases where biologic agents, such as anti-TNF-alpha and ustekinumab, have shown promise in managing steroid-refractory or steroid-dependent AIP-2. These patients have often been effectively treated with anti-TNF-alpha agents, which are also indicated for frequent concomitant IBD[7]. In a recent letter by Lauri, two cases of AIP-2 were reported to have been safety and successfully treated with ustekinumab, a monoclonal antibody that targets interleukin-12 and interleukin-23, used for concomitant IBD. This treatment option highlights the potential efficacy of ustekinumab in managing AIP-2, although further research and clinical trials are needed to validate its effectiveness in a larger patient population[94].

AIP-3 therapy is essentially based on the discontinuation of immune checkpoint inhibitors and supportive therapy. Indeed, the role of corticosteroids and immunosuppressants is not well understood. A retrospective study of 82 patients with AIP-3 found no statistically significant differences in the duration of symptoms or hospitalization between patients treated with corticosteroids and patients not treated with them[95]. Furthermore, steroid use in these immunocompromised patients with advanced malignancies carries a significantly increased risk of infectious events[96].

DISEASE RELAPSES

Relapses are significantly more common with AIP-1 (nearly 60% of cases) than with AIP-2 (9%-25%); the relapse rate in AIP-IBD patients appears to be similar to that of isolated AIP-2[97].

The main factors predicting relapse are young age, high serum IgG4 levels at disease onset, persistently high serum levels after treatment, diffuse enlargement of the pancreas, concurrent evidence of IRC, especially if proximal, or extensive multiorgan involvement[98]. Also, elevated levels of circulating IgE and/or eosinophils, and the presence of rich-in-eosinophils pancreatic infiltrate at histology represent other risk factors for disease relapse that need to be considered. Lastly, prolonged exposure to certain exogenous antigens may lead to overactivity of specific types of TLRs that may perpetuate a dysimmune response[4].

Given the high relapse rate in AIP-1, long-term maintenance therapy is recommended, especially for patients with a known high risk of relapse. Current guidelines recommend low-dose (5 mg/d) maintenance treatment with steroids for 2-3 years[99]. To reduce the risk of adverse events and lifetime cumulative steroid dose, the use of steroid-sparing agents is an alternative treatment strategy. According to a recent meta-analysis, nearly 40% of the cases relapse on immunosuppressive agents (azathioprine, mycophenolate mofetil, methotrexate, tacrolimus, and cyclophosphamide). To date, no study that compares the efficacy of different immunosuppressive has been published and the interpretation of the efficacy of conventional immunosuppressive medications is hampered by concomitant glucocorticoid use[100]. Also in the case of maintenance therapy, rituximab with the same induction scheme proved to be superior to immunomodulatory drugs in terms of efficacy[101]: 100% rituximab *vs* 81% azathioprine *vs* 72% other immunosuppressant[100].

In AIP-2, maintenance therapy is unnecessary in most patients unless certified risk factors are present; in the latter case, anti-TNF-alpha agents have been shown to be effective[7].

In cases of proven recurrent AIP, re-administration of a high dose of glucocorticoids with a slow steroid taper is effective[99].

Concerning AIP-3, it is not appropriate to refer to the risk of disease recurrence but rather to emphasize that after the acute event has resolved, the disease evolves in up to 36% of cases to a treatment-emergent stage of glandular atrophy with endocrine and exocrine insufficiency, associated with markedly reduced overall survival[102].

CONCLUSION

Given the multitude of mechanisms that explain the etiopathogenesis of AIP, great scientific efforts are being made to find new effective target therapies. As mentioned earlier, given the chemotactic role of eotaxin on inflammatory cells, attention is being paid to the development of targeted anti-eotaxin therapy[24]. Because increased production of IFN-I and IL-33 by pDCs promotes the chronic inflammation and fibrosis characteristic of AIP and IgG4- RD, neutralization of IFN-I and IL-33 may represent a new therapeutic option for these diseases. The anti-IFN-I therapeutics anifrolumab and sifalimumab and the anti-IL-33 therapeutic etokimab have been successfully used in systemic lupus erythematosus, but reliable data are not yet available for AIP in humans[35].

In addition, targeted therapies against B-cell lineage plasmablasts and CD4+ T cells (such as anti-CD19 inebilizumab, an inhibitor of B-cell activating factor ianalumab, anti-CD80/86 abatacept, anti-LOX2 simtuzumab, anti-SLAMF7 elotuzumab, or anti-CD38 daratumumab) have recently been proposed[103].

Finally, regarding the possible role of the microbiota in the etiopathogenesis of AIP, manipulation of the gut microbiota through prebiotics, probiotics, symbiotics, and fecal microbiota transplantation may represent a future prophylactic perspective, possibly targeting IgG4-RD and/or IBD patients. Indeed, early studies in mice have shown that sterilization of the gut leads to a significant reduction in the accumulation of pDCs in the pancreas that produce IFN-I and IL-33[104].

FOOTNOTES

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MINIREVIEWS

Fecal microbiota transplantation for treatment of non-alcoholic fatty liver disease: Mechanism, clinical evidence, and prospect

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Abstract

The population of non-alcoholic fatty liver disease (NAFLD) patients along with relevant advanced liver disease is projected to continue growing, because currently no medications are approved for treatment. Fecal microbiota transplantation (FMT) is believed a novel and promising therapeutic approach based on the concept of the gut-liver axis in liver disease. There has been an increase in the number of pre-clinical and clinical studies evaluating FMT in NAFLD treatment, however, existing findings diverge on its effects. Herein, we briefly summarized the mechanism of FMT for NAFLD treatment, reviewed randomized controlled trials for evaluating its efficacy in NAFLD, and proposed the prospect of future trials on FMT.

Key Words: Non-alcoholic fatty liver disease; Fecal microbiota transplantation; Randomized controlled trial; Mechanism; Efficacy

Core Tip: There are several reviews contributing to the role of gut microbiome in the pathophysiology, therapeutic potential and clinical trials for non-alcoholic fatty liver disease (NAFLD). However, no consensus is available in the literature about the clinical efficacy of fecal microbiota transplantation (FMT) on NAFLD. This is the first review to summarize recent randomized controlled trials for evaluating the effects of FMT on blood lipid profile, liver function, histological changes and other parameters in patients with NAFLD. We also discuss its therapeutic mechanism and propose the obstacles and prospect of FMT in future trials.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), recently proposed as metabolic dysfunction-associated fatty liver disease (MAFLD), is a chronic liver disease with a global prevalence of about 25% in adult population[1]. NAFLD has a bidirectional association with components of obesity and metabolic syndrome. In view of the ongoing obesity epidemic worldwide, the rise in metabolic syndrome, and other factors, the population of NAFLD along with advanced liver disease is projected to sustain its growth[2]. Although patients with NAFLD at early stages have no self-conscious symptoms, those with advanced NAFLD are at a markedly increased risk of adverse outcomes, such as an increased overall and liver-specific mortality[3]. Due to its large population base, NAFLD is emerging as a rapidly increasing cause of liver-related mortality worldwide[4] and an important cause of end-stage liver disease[5], thus leading to a substantial health economic burden.

The pathophysiology of NAFLD is extremely complicated and involves multiple pathogenic pathways that are not completely elucidated. On the other hand, there is currently no approved therapy for NAFLD, although several drugs are reserved for patients with biopsy-proven non-alcoholic steatohepatitis (NASH). In search of potential novel therapeutic options, the manipulation of the gut microbiome has attracted a lot of attention. Evidence from human observational studies and animal experiments have found the alterations in gut microbiome in NAFLD and indicated numerous pathophysiologic mechanisms relating the gut microbiome to NAFLD[6-9]. In brief, gut dysbiosis damages the intestinal barrier integrity, leading to increased gut permeability to bacterial products and hepatic exposure to harmful substances, thus triggering fat accumulation, hepatic inflammation and fibrosis[10-12].

Considering the close link between the gut microbiome and NAFLD, many therapeutic approaches targeting gut microbiota, such as probiotic, prebiotic or fecal microbiota transplantation (FMT) have been tested for whether they could provide the clinical benefit of alleviation of NAFLD. FMT is the latest mode of gut microbiota manipulation for the treatment of NAFLD[13], which seems to be more effective than other existing approaches of microbiota manipulation. FMT from a healthy donor to the host is aimed to quickly re-establish a healthy gut microbial community, which can help treat gastrointestinal diseases. As evidenced by several animal-based studies, FMT could effectively improve the manifestations of NAFLD[14-16]. On the other hand, there has been an increase in the number of clinical trials evaluating the role of FMT in NAFLD treatment[17-19]. Herein, we briefly summarized the mechanism, randomized controlled trials (RCTs), and prospect of FMT in the treatment of NAFLD.

MECHANISM OF FMT FOR NAFLD

Structure and function of gut-liver axis

The functioning of the gut-liver axis is established on the interaction between the gut, along with its microbiota, and the liver, through the portal vein. The gut microbiota is a complex community consisting of over 100 trillion microbes, prevalently bacteria[20]. The interface between the microbiome and intestinal epithelial cells is the gut mucus barrier, physically separating the microbiota from the epithelial lining, thus providing protection against an exaggerated inflammatory response[21] (Figure 1). Thus, the mucus barrier serves as a first line of defense. Underneath the mucus layer is the intestinal barrier, which is formed by a monolayer of epithelial cells. Adjacent epithelial cells are sealed together by tight junctions[22], and they function as a physical shield preventing bacteria from crossing the gut into the portal circulation. In addition, some mucosal immune cells patrol the epithelium, and cooperate to protect against strikes arising from the microbiota and infectious agents[23]. In case the epithelium is breached, there is another barrier, the gut vascular

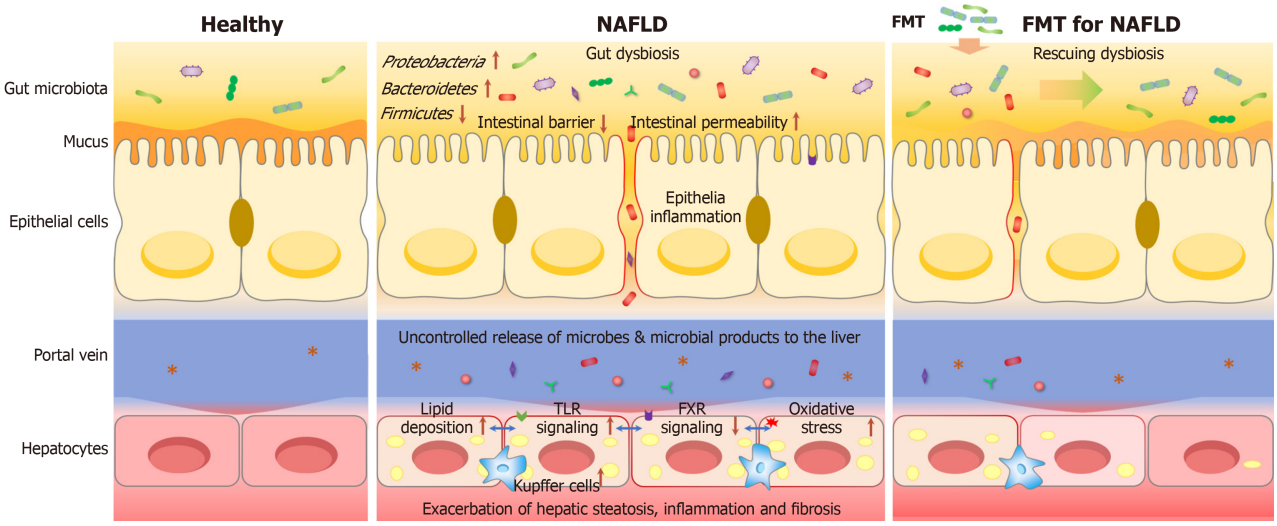

Figure 1 Homeostatic and disrupted gut-liver crosstalk, and mechanism of fecal microbiota transplantation for non-alcoholic fatty liver disease treatment. Left: Healthy/homeostatic condition; Middle: In non-alcoholic fatty liver disease (NAFLD), the intestinal barrier can be disrupted, which facilitates the translocation of microbes and microbial metabolites to the liver, thus promoting hepatic steatosis, inflammation and fibrosis; Right: Fecal microbiota transplantation is used to recover microbial diversity and abundance and restore homeostatic gut-liver crosstalk, and consequently attenuate the symptoms of NAFLD; FMT: Fecal microbiota transplantation.

barrier that prevents bacteria from entering the portal circulation and disseminating systemically[24].

Due to the multiple barriers as mentioned above, most bacteria cannot directly interact with the host, but through the mediation by the bacterial products and metabolites. With the aid of this indirect mediation, the gut microbiota participate in nutrient digestion and absorption, host metabolism, and mucosal and systemic immunity[25]. For instance, gut microbiota breaks down dietary fibers into short-chain fatty acids (SCFAs), which could provide energy support for the host cells. More notably, they also have been shown to regulate lipid metabolism, protect intestinal mucosal barrier, control the differentiation of several immune cells, and participate in the microbicidal activity of macrophages[26-28]. In addition, gut microbiota transform the primary bile acids into secondary bile acids, which act as the natural agonists of intestinal farnesoid X receptor (FXR). FXR engagement can regulate its downstream defense genes to enhance epithelial barrier properties[29], reduce lipogenesis in the liver[30], and improve insulin sensitivity[31]. In summary, the homeostasis constructed by the balance of gut microbiome and the intact physiological barriers is critical for controlling the reciprocal interaction between the gut and the liver to maintain health.

Disruption patterns of gut-liver axis in NAFLD

Current data indicate that altered gut microbiome, along with bacterial components, impair the intestinal barrier and vascular barrier, and facilitate the influx of bacterial products into the portal vein in NAFLD[11,29]. Noticeably, the liver is especially vulnerable to the insults from these bacterial products, which consequently aggravate the hepatic metabolic abnormalities and inflammation (Figure 1).

Several clinical studies have shown that patients with NAFLD have remarkable gut dysbiosis, generally characterized by the over-growth of bacteria and changes in microbiota composition[32]. For instance, recent research has found an increased abundance of *Escherichia coli* and *Bacteriodes vulgatus* in patients with NAFLD[9]. Abnormalities in intestinal microbiota have established a link with the reduced thickness of the mucous layer, as well as the increased intestinal permeability, in patients with NAFLD[33,34]. The damage of intestinal barriers loses control of the passage of bacterial components and metabolites, which can reach the liver *via* the portal vein. Some of these components are agonists of Toll-like receptor (TLR) signal pathway, which results in enhanced hepatic expression of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), thus promoting inflammation and fibrosis[32,35]. The altered gut microbiome has also likely contributed to a decreased production of SCFAs, which promotes lipogenesis and lipid deposition in NAFLD[36]. In addition, gut dysbiosis reduces bioavailability of choline and increases the portal influx of trimethylamine, which also contributes to hepatic steatosis in NAFLD[37]. Disruptive bile acid pool is another consequence of microbiota abnormalities, and it suppresses FXR-mediated signaling in the intestine and the liver, leading to increased lipid accumulation, oxidative stress and inflammation[38,39].

FMT for remoulding gut-liver crosstalk to treat NAFLD

Given these attacks induced by gut dysbiosis which promotes liver steatosis, inflammation and fibrosis, the manipulation of the gut microbiota is being explored as a therapeutic target for NAFLD. Probiotics, prebiotics and symbiotics have been explored for treating patients with NAFLD[40-42]. However, it does not come to a clear and substantial conclusion whether these agents generate significant clinical improvement in NAFLD patients. Moreover, data on adverse effects links to the use of probiotics[43]. These issues have paved the road for FMT application in NAFLD treatment, which is expected to yield better clinical efficacy and less side effects.

The fecal material for FMT contains distal gut microbiota from a healthy donor and is processed into an odorless and tasteless preparation. In clinical practice, the approaches for FMT include oral administration of microflora liquid or capsule, transplanting to the middle digestive tract by endoscopic biopsy hole, and threading through the colonic pathway to the lower digestive tract[44]. FMT is used to replenish a healthy gut microbial environment and restore physiological colonization, leading to the recovery of microbial diversity and abundance[12] (Figure 1). The repair of gut dysbiosis can elevate intestinal permeability through the rebuilding of physiological barriers (*i.e.* mucus barrier, epithelial barrier)[17], and suspending the uncontrolled influx of bacterial products to the liver. The restoration of intestinal structure and function can improve lipid metabolism, decrease insulin resistance, suppress inflammatory response, and consequently attenuate the symptoms of NAFLD (*i.e.* reduction of fat content, liver steatosis, serum transaminase levels and inflammatory infiltrates)[16,45,46]. Finally, FMT has been demonstrated well tolerated and safe over long-term use [47,48].

EVIDENCE FROM RCTS ON FMT IN NAFLD TREATMENT

To review RCTs for NAFLD treatment using FMT, references indexed in databases (PubMed, EMBASE, the Cochrane Library, and Web of Science) were searched with the combination of the following keywords: 'non-alcoholic fatty liver disease/NAFLD/fatty liver/non-alcoholic steatohepatitis/metabolic dysfunction-associated fatty liver disease/MAFLD/steatohepatitis/Liver steatosis/hepatic steatosis', and 'fecal microbiota transplantation/ fecal microbiota transplant/fecal microbiome transplantation/fecal microbiome transplant/FMT'. The eligibility criteria was determined based on PICOS principle (population, interventions, comparisons, outcomes, study designs). The inclusion criteria were as follows: (1) Study design was RCT; (2) participants were diagnosed with NAFLD by either liver histology or noninvasive imaging; and (3) study results included one of the following outcomes: serum cholesterol, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL-C), homeostatic model assessment of insulin resistance (HOMA-IR), body mass index (BMI), or assessment of histological change by any of the following modalities: Liver biopsy, MRI or elastography. Specifically, liver biopsy outcomes included determination of NAFLD activity score (NAS), necro inflammation score, fibrosis score, or steatosis score. MRI outcomes included estimated fibrosis stage, and elastography outcomes included fat attenuation degree or fibrosis stage. The exclusion criteria were as follows: (1) Participants were under 18 years; (2) participants had severe diseases except NAFLD; and (3) study reported incomplete data. As a result, a total of 2778 references were initially identified, and finally, 3 published RCTs that had undergone full-text assessment were included in this review. The detail of study selection was recorded in Figure 2.

Evidence from Included RCTs

The three RCTs were conducted between 2013 and 2021, and were comprised of 117 adult participants with NAFLD. Two trials[17,18] were double-blind while one[19] was open-label. Regarding FMT intervention, two studies performed allogenic FMT through duodenal infusion, while one adopted colonic infusion. Among the outcomes, serum levels of cholesterol, triglycerides, HDL-C and LDL-C were reported in all trials, while only one trial[18] reported the histological change (NAS score, necro inflammation score, fibrosis score, steatosis score) in patients between baseline and endpoint. In all trials, adverse effects of FMT were not reported. Details of the general characteristics of all included RCTs were given in Table 1.

A double-blind RCT conducted by Craven *et al*[17] included 21 patients to compare allogenic with autologous FMT. As a result, no significant changes were found in total triglycerides, cholesterol, HDL-C, LDL-C, HOMA-IR, weight, waist-to-hip ratio, BMI or hepatic proton density fat fraction in patients after FMT. However, patients experienced a significant increase in small intestinal permeability after FMT. The changes in fecal microbiota composition varied by individuals in both groups after FMT. Moreover, a trend toward an increase in the fecal microbiota diversity was found in patients who had improvement in intestinal permeability, although changes in specific taxa were hardly discerned in allogenic transplant. It is noteworthy that the investigators only deployed FMT one time but evaluated its efficacy after 24 wk, and it was difficult to expect one session of FMT to achieve a long-lasting therapeutic effect, which could be one reason for the unsatisfactory results of most outcomes. In addition, this study was also limited by small sample size and lack of histological findings.

In another double-blind RCT conducted by Witjes *et al*[18], 21 participants with hepatic steatosis received either allogenic or autologous FMT. FMT administration was performed using duodenal infusion three times at 8-wk intervals, with a duration of 24 wk. The results indicated a trend toward improvement in the necro-inflammation score, but finally no significant improvement in liver histology following allogenic FMT. Similarly, there was no significant improvement in biochemical parameters. However, significant changes in the expression of some hepatic genes associated with inflammation and lipid metabolism were found in the allogenic FMT group, compared with autologous FMT. The findings from fecal microbiota analysis suggested no significant changes in fecal microbiota diversity and composition between baseline and endpoint, in either group.

Xue *et al*[19] carried out an open-label 4-wk RCT including 75 patients with NAFLD. Participators randomly received allogenic FMT or oral probiotics. The fecal microbiota preparation was administered to patients by colonic infusion per day, for 3 d in total. The results showed no significant difference in blood lipid (*i.e.* triglycerides, cholesterol, LDL-C, HDL-C) and liver function tests before and after treatment in either group. However, a significant decrease in liver fat attenuation degrees was found in the FMT group, while a significant increase was found in the non-FMT group. Microbiota analysis indicated that certain bacterial contents had a trend toward healthy individuals after FMT. Furthermore,

Table 1 Characteristics of included randomized controlled trials

Ref.	Patient population	Diagnosis	Study design	Fecal donor	Intervention	Sample size	Duration in wk	Outcomes
Craven *et al*[17], 2020	Adults, NAFLD	Biopsy, fibroscan, MRI	Double-blind, parallel, RCT	3 donors, healthy, BMI < 25 kg/m^2	Treatment: Allogenic FMT	15	24	HOMA-IR, hepatic PDFF, small intestine permeability, NEFA, cholesterol, HDL-C, LDL-C, triglycerides, glucose, BMI, weight, waist-to-hip ratio
					Control: Autologous FMT	6		
					FMT: Duodenal infusion			
Witjes *et al*[18], 2020	Adults, NAFLD	Ultrasound	Double-blind, parallel, RCT	3 donors, healthy, BMI < 25 kg/m^2, 8-weekly vegan	Treatment: Allogenic FMT	10	24	Histological change (NAS score, necro inflammation score, fibrosis score, steatosis score), intestinal microbiota composition, plasma metabolites, cholesterol, HDL-C, LDL-C, triglycerides, glucose, ALT, AST, monocytes
					Control: Autologous FMT	11		
					FMT: Duodenal infusion			
Xue *et al* [19], 2022	Adults, NAFLD	Fibroscan	Open-label, parallel, RCT	Healthy undergraduate donors	Treatment: Allogenic FMT	47	4	ALT, AST, cholesterol, HDL-C, LDL-C, triglycerides, total bilirubin, Albumin, hepatic fat attenuation, changes in the gut microbiota, HOMA-IR, BMI
					Control: Oral probiotics	28		
					FMT: Colonic infusion			

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; FMT: Fecal microbiota transplantation; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; HOMA-IR: Homeostatic model assessment of insulin resistance; MRI: Magnetic resonance imaging; NAFLD: Non-alcoholic fatty liver disease; NAS score: NAFLD activity score; NEFA: Non-esterified fatty acids; PDFF: Proton density fat fraction; RCT: Randomized controlled trial.

FMT had a greater impact on the microbial community structure in lean NAFLD than in obese NAFLD patients.

Pooled analysis of selected outcomes

The outcomes reported in more than two RCTs were selected for pooled analysis. The mean and/or standard deviation (SD) values of cholesterol, triglycerides, LDL-C, HDL-C, AST, ALT, BMI and HOMA-IR were extracted for assessment. Data were processed to obtain the changes between baseline and endpoint. The pooled effects were reported as weighted mean differences (WMD) with 95% confidence intervals (95%CI). Statistical analysis was performed using STATA 16.0 software (Stata Corp LLC, TX, United States) using a random-effects model. A P value of < 0.05 was considered significant for the test for overall effect.

All three included RCTs[17-19] reported the changes of serum lipid profile (triglycerides, cholesterol, LDL-C and HDL-C) in patients between baseline and endpoint. The pooled results suggested that FMT failed to cause significant improvement of triglycerides (WMD -0.07, 95%CI: -0.47 to 0.33, P = 0.74; Figure 3A), cholesterol (WMD -0.27, 95%CI: -0.75 to 0.21, P = 0.27; Figure 3B), LCL-C (WMD 0.05, 95%CI: -0.31 to 0.42, P = 0.78; Figure 3C), and HDL-C (WMD -0.05, 95%CI: -0.22 to 0.15, P = 0.54; Figure 3D), as compared with control. There was no significant heterogeneity between studies. Two RCTs[18,19] reported the outcomes of ALT and AST, and the pooled analysis suggested that FMT caused no significant reduction in ALT (WMD -0.51, 95%CI: -22.57 to 21.55, P = 0.96; Figure 3E) and AST (WMD -2.78, 95%CI: -10.26 to 4.71, P = 0.47; Figure 3F). Likewise, two RCTs[17,19] reported the outcomes of HOMA-IR and BMI, and pooled analysis found no improvement in them after FMT (HOMA-IR: WMD -1.09, 95%CI: -2.43 to 0.25, P = 0.11, Figure 3G; BMI: WMD -0.64, 95%CI: -2.73 to 1.44, P = 0.54, Figure 3H).

Although these negative results might challenge the potential of FMT in the treatment of NAFLD, their reliability could be attenuated by several limitations. Firstly, only a very small number of RCTs was retrieved, as well as a very small sample size. Moreover, the available data of histological outcome which is the major prognostic index of NAFLD, was insufficient for pooled analysis. Finally, the management of FMT administration to patients varied among studies, which might influence the efficacy of FMT. Therefore, future studies including standardized FMT sessions in more patients with robust outcome measures are still needed.

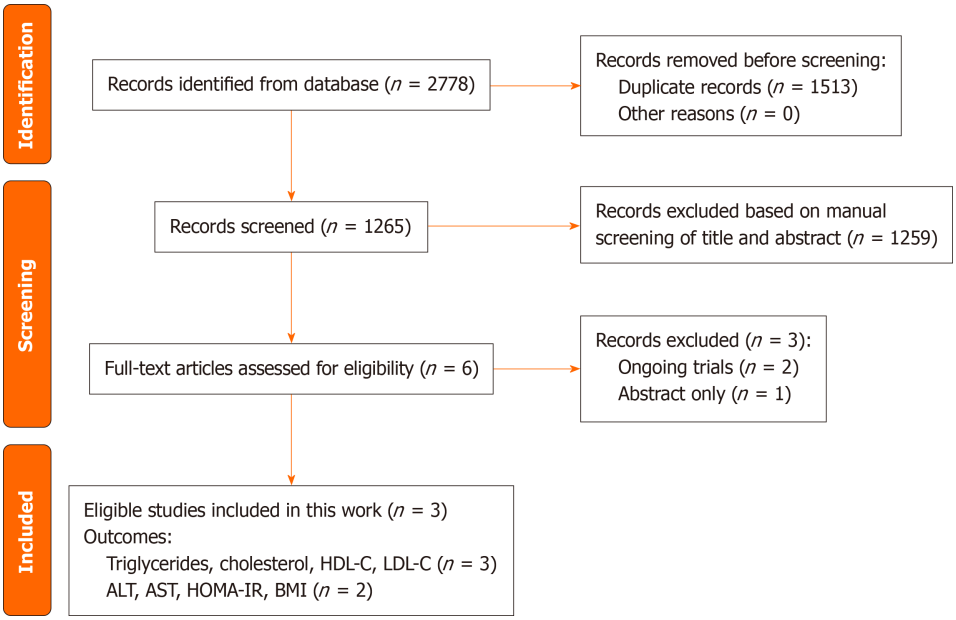

Figure 2 Flow diagram for the process of study selection. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; HDL-C: High-density lipoprotein cholesterol; HOMA-IR: Homeostatic model assessment of insulin resistance; LDL-C: Low-density lipoprotein cholesterol.

PROSPECT

The only recommended treatment for NAFLD is weight loss. However, it is hard to adhere to lifestyle modifications for achieving it, making the need for effective and safe medications more urgent. In past years, numerous pharmacological therapies have been investigated, such as medications targeting the glucagon-like peptide-1 (GLP-1), peroxisome proliferator-activated receptors (PPARs) and intestinal microbiota. There is considerable evidence that the composition of the gut microbials in NAFLD patients changes significantly from control individuals[9,11,49], which contribute to obesity, insulin resistance and liver steatosis[12,16,50]. FMT is an emerging tool for manipulating intestinal microbiota, which is considered as a potential therapeutic approach for NAFLD. Although current outcomes on FMT therapy for NAFLD vary between RCTs and most are gloomy, aforementioned RCTs still demonstrate that patients might benefit from FMT due to improvement of small intestinal permeability, alleviation of hepatic necro-inflammation, and liver fat attenuation. Furthermore, the results from the three RCTs and pooled analysis are hardly considered as robust due to apparent limitations. Therefore, FMT is still expected to be promising in NAFLD treatment, but there are a series of obstacles that still need to be resolved.

Firstly, the alterations of gut microbiota could be variable by each individual with NAFLD, or/and by the stage of NAFLD, and sometimes the results are contradictory[51,52]. Thus, the significant heterogeneity lying in gut microbiota makes the restoring of varied gut microbiomes by a fixed FMT difficult to be achieved, and also hinders the transforming of the success of one FMT directly to another population. Moreover, the characteristics of donor feces, in particular fecal microbiota richness, diversity, and compatibility, have considerable influence on the efficacy of FMT, while the rigorous criteria for donor selection has not yet been determined. Furthermore, repetitive FMT should be required to maintain the improvement of the gut microbiome, however, the management of FMT is still casual across trials. Although the safety of FMT is evidenced, long-term consequences of FMT are unknown as FMT still has rare risks. Therefore, the practice guidelines of FMT still need to be extensively investigated. Finally, FMT combined with other pharmacological therapies are worth considering in future trials since NAFLD involves multiple pathogenic factors.

CONCLUSION

In summary, our work highlighted disruption patterns of the gut-liver structure and function in NAFLD, and how FMT remolds the homeostasis of the gut-liver crosstalk to alleviate NAFLD. Since FMT is suggested as a therapeutic of great potential, increasing the number of clinical trials that are carried out and evaluating its efficacy in NAFLD needs to be a priority. We reviewed the published RCTs to analyze the evidence on the clinical efficacy of FMT in NAFLD patients. FMT failed to yield clinical benefit in blood lipid (*i.e.* triglycerides, cholesterol, LDL-C, HDL-C), and liver function (*i.e.* ALT, AST) parameters. By contrast, the improvement of small intestinal permeability and the alleviation of hepatic necro-inflammation in patients after FMT could be encouraging. Whereas the reliability of the above results is challenged by several limitations, especially small sample size and casual FMT administration protocols, it is believed that some obstacles still need to be resolved before fully inspiring the potential of FMT in the treatment of NAFLD. Future high-quality trials in more patients adopting more scientific management of FMT are essential to further validate the clinical benefit of FMT.

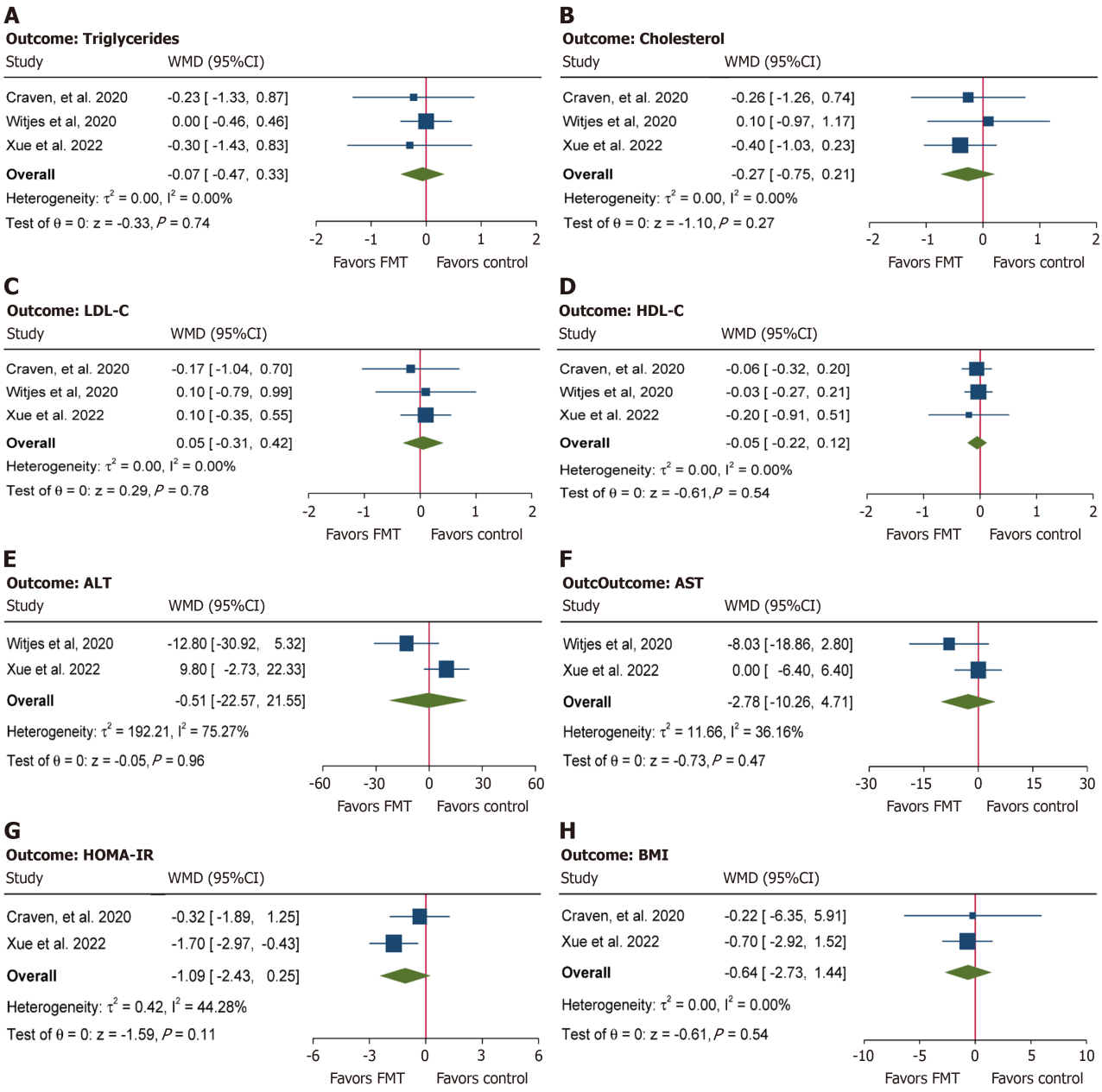

Figure 3 Pooled analysis depicting the effects of fecal microbiota transplantation on serum triglycerides, cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, alanine aminotransferase, aspartate aminotransferase, homeostatic model assessment of insulin resistance and body mass index measured by weighted mean difference in patients with non-alcoholic fatty liver disease. A: Serum triglycerides; B: Cholesterol; C: Low-density lipoprotein cholesterol (LDL-C); D: High-density lipoprotein cholesterol (HDL-C); E: Alanine aminotransferase (ALT); F: Aspartate aminotransferase (AST); G: Homeostatic model assessment of insulin resistance (HOMA-IR); H: Body mass index (BMI). FMT: Fecal microbiota transplantation; NAFLD: Non-alcoholic fatty liver disease; WMD: Weighted mean difference.

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FOOTNOTES

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Co-corresponding authors: Nan-Nan Li and Zheng Li.

Author contributions: Qiu XX and Cheng SL contributed to the acquisition of data; Qiu XX, Cheng SL, Liu YH, Li Y, and Zhang R contributed to the analysis and interpretation of data; Qiu XX, Cheng SL, Liu YH, Li NN, and Li Z drafted the article; Liu YH, Li Y, Zhang R, Li NN, and Li Z revised the article; Li NN and Li Z contributed to the conception and design of the study, and critical revision;

all authors contributed to the final approval of the article. Qiu XX and Cheng SL contributed equally to this work as co-first authors; Li NN and Li Z contributed equally to this work as co-corresponding authors. The reasons for designating co-first or co-corresponding authors are as follows: (1) The research was performed as a collaborative effort, and the designation of co-first/co-corresponding authorship accurately reflects the distribution of responsibilities and contribution to the study; (2) the designation reflects the diversity of expertise and skills of the overall research team; and (3) these authors contributed efforts of equal substance throughout the research process. In summary, we believe that this designation is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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ORIGINAL ARTICLE

Retrospective Study

Transcatheter arterial chemoembolization combined with PD-1 inhibitors and Lenvatinib for hepatocellular carcinoma with portal vein tumor thrombus

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Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) patients complicated with portal vein tumor thrombus (PVTT) exhibit poor prognoses and treatment responses.

AIM

To investigate efficacies and safety of the combination of PD-1 inhibitor, transcatheter arterial chemoembolization (TACE) and Lenvatinib in HCC subjects comorbid with PVTT.

METHODS

From January 2019 to December 2020, HCC patients with PVTT types I-IV were retrospectively enrolled at Beijing Ditan Hospital. They were distributed to either the PTL or TACE/Lenvatinib (TL) group. The median progression-free survival (mPFS) was set as the primary endpoint, while parameters like median overall survival, objective response rate, disease control rate (DCR), and toxicity level served as secondary endpoints.

RESULTS

Forty-one eligible patients were finally recruited for this study and divided into the PTL (n = 18) and TL (n = 23) groups. For a median follow-up of 21.8 months, the DCRs were 88.9% and 60.9% in the PTL and TL groups (P = 0.046), respectively. Moreover, mPFS indicated significant improvement (HR = 0.25; P < 0.001) in PTL-treated patients (5.4 months) compared to TL-treated (2.7 months) patients. There were no treatment-related deaths or differences in adverse events in either group.

CONCLUSION

A triplet regimen of PTL was safe and well-tolerated as well as exhibited

favorable efficacy over the TL regimen for advanced-stage HCC patients with PVTT types I-IV.

Key Words: Hepatocellular carcinoma; Transcatheter arterial chemoembolization; Lenvatinib; PD-1 inhibitor; Portal vein tumor thrombus

Core Tip: Hepatocellular carcinoma (HCC) with portal vein tumor thrombus (PVTT) has a poor prognosis and treatment responses. Assessment of its survival has important clinical implications. Through our research, we discovered that a triplet regimen of PD-1 inhibitor/transcatheter arterial chemoembolization/Lenvatinib was safe and well-tolerated as well as exhibited favorable efficacy over the transcatheter arterial chemoembolization/Lenvatinib regimen for advanced-stage HCC comorbid with PVTT.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed primary malignancies of the liver that leads to high morbidity and mortality rates in adults, especially those with chronic liver diseases and hepatitis infections. In 2020, HCC ranked sixth among all cancers concerning 4.7% of all new cancer cases, and third for 8.3% of all cancer-related deaths[1]. The portal vein tumor thrombus (PVTT) is a commonly occurring complication in at least 16%-30% of HCC patients[2], contributes to poor prognosis, and increases the susceptibility to cancer recurrence, with the median survival of about 2.7-4 months without any interventions[3]. Although both European Association for the Study of the Liver and American Association for the Study of Liver Diseases guidelines suggested that HCC patients complicated with PVTT should be classified as Barcelona Clinic Liver Cancer-C stage and receive systemic treatments[4,5]. However, there is currently no optimal treatment strategy for such patients. Tyrosine kinase and immune checkpoint inhibitors (TKI and ICI, respectively) are generally applied in systemic therapies of HCC, while the combination of transcatheter arterial chemoembolization (TACE) with systemic therapeutics has substantially improved treatment outcomes in advanced-stage patients.

TACE is recommended for treating unresectable HCC[6-8], and its safety and effectiveness have been demonstrated in several clinical trials[9-12]. Despite this, in clinical practices, tumor recurrence and distant metastasis are often encountered in post-TACE HCC patients[13]. Several studies have confirmed better treatment responses when TACE is combined with TKI over TACE alone[14-16]. In the era of rapidly advancing immunotherapy research, studies suggest that ICI and TKI together can promote the recovery of cytotoxic T lymphocytes (CTLs) from exhaustion[17], thereby enhancing antitumor responses[18,19]. Retrospective studies suggest that a triplet regimen of TACE or a combination of hepatic arterial infusion chemotherapy (HAIC), Lenvatinib, and PD-1 inhibitor could be superior to dual therapy in advanced HCC cases[20-22]. However, there is limited data on highly complicated HCC patients, and the success rate of Atezolizumab plus Bevacizumab therapy has not yet been established for this subset of HCC patients[23]. Therefore, we hypothesized that the triplet regimen of TACE plus targeted drugs and ICIs might introduce better prognoses for HCC patients with PVTT. Here, we aimed to compare the safety and efficacy of the triplet regimen of Lenvatinib, PD-1 inhibitor, and TACE *vs* Lenvatinib plus TACE in advanced-stage HCC with type I- IV PVTT.

MATERIALS AND METHODS

Patient selection

Eligible patients were retrospectively enrolled at Beijing Ditan Hospital affiliated with Capital Medical University, between January 2019 and December 2020. Participants were distributed into two treatment groups: The PD-1 inhibitor/TACE/Lenvatinib (PTL) and TACE/Lenvatinib (TL) groups. HCC patients had different stages of PVTT without obstructing the major vein lumen or inferior vena cava. The Chinese Society of Clinical Oncology guidelines for liver carcinoma were followed to confirm the histological and clinical diagnoses of HCC[24]. The diagnosis of PVTT was confirmed either by radiological (*e.g.*, CT and MRI) or pathological methods. As per Cheng's classification, the PVTT could be staged in four types. Type I tumor thrombus involves portal vein branches in segments; type II includes left and right branches of the hepatic portal vein; type III involves the main portal vein, and type IV affects the superior mesenteric vein or inferior vena cava[25].

The inclusion criteria comprised the following: (1) Ages ranged from 18 to 75 years; (2) life expectancy ≥ 3 months; (3) having at least one typical enhanced measurable target lesion of ≥ 1 cm, according to the modified Response Evaluation Criteria in Solid Tumors (mRECIST); (4) Eastern Cooperative Oncology Group-performance status (ECOG-PS) score 0-1; (5) Child-Pugh classification A or B (≤ 7 points); (6) non-surgical indications or refusal of surgical treatment; (7) not receiving radiofrequency ablation, TACE, and any other locoregional treatments 4 wk before the admission; (8) no limit on several liver tumors; and (9) might or might not have extrahepatic metastasis. The exclusion criteria included: (1) A history of other cancers; (2) any contraindications for therapies with TACE, Lenvatinib, and/or PD-1 inhibitor; (3) simultaneous ongoing treatment with other drugs; (4) incomplete patient record data; and (5) other conditions considered unsuitable for inclusion in the study by the investigators. Additionally, patients treated with any locoregional therapies *e.g.*, radiofrequency ablation and radiotherapy) for intrahepatic lesions of HCC 4 wk before the study enrollment were eliminated.

Treatments

TACE: TACE was carried out by 2 professional radiologists (Cai L and Guo J). First, the feeding artery of the tumor was determined by angiography, then a super-selective 5-F catheter was inserted. Then, a mixture of Lipiodol (5-20 mL; Lipiodol Ultra-Fluide; André Guerbet Laboratories) and Lobaplatin (20mg/m^2; Changan Hainan International Pharmaceutical Co., China) was prepared for embolization using absorbable embospheres (300-500mm; Biosphere Medical Inc). The entire intrahepatic tumor burden was treated by TACE. Conventional TACE (cTACE) was repeated on demand if the patient's subsequent follow-ups revealed incomplete necrosis or insufficient Lipiodol uptake.

Systemic therapy: Patients with ≥ 60 kg and < 60 kg (or Child-Pugh class B) of body weights, respectively, received 12 mg and 8 mg of Lenvatinib orally once daily until the day of cTACE. In the absence of any post-TACE symptoms (fever, nausea, vomiting, *etc.*), the medication was resumed after each TACE treatment. Patients in the PTL group received intravenous doses of 200 mg of PD-1 inhibitor (Sintilimab, Camrelizumab, Nivolumab, or Tislelizumab), every 3 wk starting from one to two weeks after TACE. When participants experienced adverse events (AEs) of grade 3 or more, dose modification or temporary interruption of medication was performed, according to the drug labels, until AEs were relieved to grade 1 or none. The drug was discontinued in case of uncontrolled tumor progression or unacceptable AEs.

Follow-up visits

Post-TACE assessments were performed by chest, abdominal, or enhanced CT, MRI, and/or laboratory tests during the follow-up visits every 4-8 wk intervals. Laboratory tests included blood routine, hepatic and kidney function tests, quantitation of urine protein, serum α-fetoprotein, and myocardial enzymes, *etc.* AEs records were retrieved for analysis from the hospital's electronic record system, following the Common Terminology Criteria for Adverse Events (v5.0) guidelines. Transient post-TACE AEs, such as elevated live enzymes, abdominal discomforts, and fever, were not recorded. The end-point of the follow-up study was December 1, 2021.

Endpoints

According to mRECIST criteria, median progression-free survival (mPFS) was the primary endpoint, while secondary endpoints included median overall survival (mOS), objective response rate (ORR), disease control rate (DCR), and safety. The mPFS was defined as the duration from the first TACE intervention to the first tumor progression and all-cause death. The mOS was the time from the first TACE to all-cause death. The ORR was the percentage of participants with complete remission (CR) and partial remission (PR). The DCR was referred to as the sum of CR, PR, and stable disease (SD). Tumor progression (20% increase from baseline examination, mRECIST) and transient hepatic dysfunction to Child-Pugh C or emerging extrahepatic metastases were conceived as markers of disease progression[26].

Statistical analysis

Continuous data are presented as median ± interquartile range or mean ± SD. The baseline characteristics of the two groups were evaluated by independent samples *t*-test or chi-squared (χ^2) test. The Kaplan-Meier and log-rank tests were employed to estimate survival curves and corresponding *P* values. Based on the Cox regression models, multivariate and univariate analyses were performed to identify independent prognostic factors related to mPFS. Any survival-related variables with $P < 0.10$ from univariate analyses were combined into a multivariate Cox proportional hazards model. A two-tailed *P* value of < 0.05 was considered statistically significant. The SPSS v 22.0 (IBM, Inc., New York, NY, United States) was exploited for all analyses.

RESULTS

Patient demographics

A total of 502 HCC cum PVTT cases were diagnosed in Beijing Ditan Hospital, of which 424 patients were not treated with Lenvatinib, 22 subjects were not treated with TACE, and 15 patients had incomplete follow-up data. Finally, 41 of these patients were enrolled in the study, of which 23 were allotted to the TL group and the rest (n = 18) patients to the PTL group (Figure 1). The baseline data of 41 patients are outlined in Table 1. The median age of the patients was 57.6 ± 8.8 years. Thirty-six percent (n = 15) of patients in both groups had extrahepatic metastases, and most patients presented PVTT types III-IV (63.4%, n = 26). All patients had a Child-Pugh classification of A-B (≤ 7 points) and an ECOG score of 0-1. In the majority of cases (92.7%, n = 38), the etiopathology was hepatitis B virus infection. The baseline characteristics

Table 1 Baseline clinical characteristics of patients

Characteristic	PTL (*n* = 18)	TL (*n* = 23)	*P* value
Mean age, yr ± SD	56.9 ± 8.1	58.1 ± 9.4	0.307
Gender, *n* ()			0.690
Male	15 (83.3)	18 (78.3)	
Female	3 (16.7)	5 (21.7)	
Weight, *n* (%)			0.794
< 60 kg	7 (38.9)	8 (34.8)	
≥ 60 kg	11 (61.1)	15 (65.2)	
Etiology, *n* (%)			0.447
HBV	16 (88.9)	22 (95.7)	
Others	2 (11.1)	1 (4.3)	
ECOG-PS, *n* (%)			0.586
0	7 (38.9)	7 (30.4)	
1	11 (61.1)	16 (69.6)	
Child-Pugh class, *n* (%)			0.209
A	18 (100)	21 (91.3)	
B	0 (0)	2 (8.7)	
AFP, *n* (%)			0.273
< 400 ng/mL	7 (38.9)	13 (56.5)	
≥ 400 ng/mL	11 (61.1)	10 (43.5)	
Liver cirrhosis, *n* (%)			0.328
Absent	0 (0)	1 (4.3)	
Present	18 (100)	22 (95.7)	
Extrahepatic metastasis, *n* (%)			0.096
Absent	14 (77.8)	12 (52.2)	
Present	4 (22.2)	11 (47.8)	
Size of largest nodule, *n* (%)			0.855
< 5 cm	2 (11.1)	3 (13.0)	
≥ 5 cm	16 (88.9)	20 (87.0)	
Tumor thrombus			0.373
Branch of portal vein	8 (44.4)	7 (30.4)	
Main portal vein and vena cava	10 (55.6)	16 (69.6)	
Tumor number			0.415
Solitary	1 (5.6)	3 (13.0)	
Multiple	17 (94.4)	20 (87.0)	
ALB			0.740
> 3.5 g/dL	8 (44.4)	9 (39.1)	
≤ 3.5 g/dL	10 (55.6)	14 (60.9)	
Treatment history			
Surgery	0 (0)	0 (0)	-
RFA	2 (11.1)	3 (13.0)	0.856
TACE			0.123

1-2	15 (83.3)	14 (60.9)	
> 2	3 (16.7)	9 (39.1)	
ALT (U/L)	65.8 ± 80.6	45.2 ± 26.6	0.835
AST (U/L)	73.6 ± 74.5	68.5 ± 46.5	0.386
TBIL (mg/dl)	18.8 ± 7.0	17.2 ± 8.9	0.780
PT(s)	12.7 ± 1.2	13.1 ± 1.3	0.988
PTA(s)	83.2 ± 12.6	83.7 ± 11.3	0.640

PTL: PD-1 inhibitor/transcatheter arterial chemoembolization/Lenvatinib; TL: Transcatheter arterial chemoembolization/Lenvatinib; HBV: Hepatitis B Virus; ECOG-PS: Eastern Cooperative Oncology Group-performance status; AFP: Alpha fetoprotein; ALB: Albumin; TACE: Transcatheter arterial chemoembolization; ATL: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; PT: Prothrombin time; PTA: Prothrombin activity.

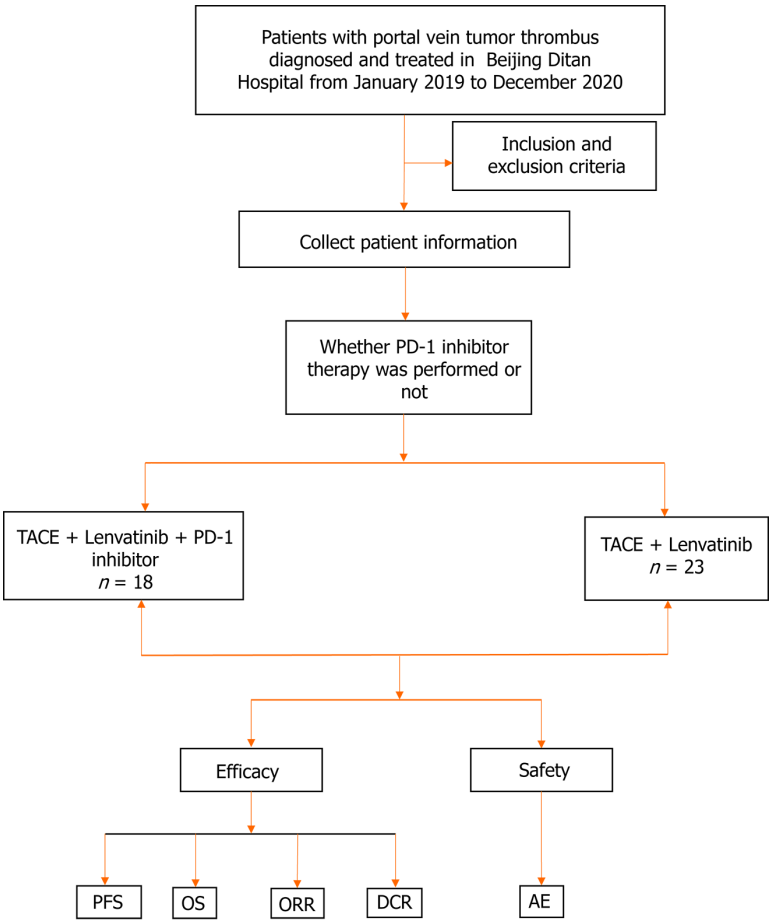

Figure 1 Trial flowchart. Flowchart illustration of patient selection strategy. A total of 41 patients were finally selected, of which 18 patients received treatments with the PD-1 inhibitor/transcatheter arterial chemoembolization (TACE)/Lenvatinib, and 23 patients with the TACE/Lenvatinib regimen. TACE: Transcatheter arterial chemoembolization; AE: Adverse event; PFS: Progression-free survival; OS: Overall survival; ORR: Objective response rate; DCR: Disease control rate.

between the two groups were highly comparable in terms of liver function, demographics, and disease characteristics.

Treatment efficacy

The median follow-up was 21.8 months (95%CI = 14.7-28.8). Patients in the PTL group had significantly longer mPFS (5.4 months; 95%CI = 3.6-7.3) than those in the TL (2.7 months; 95%CI = 1.7-3.6) group (HR 0.25; 95%CI = 0.12-0.52; $P < 0.001$; Figure 2A and B). However, there was no significant difference in mOS between the PTL (9.0 months; 95%CI = 7.07-10.93) and TL (8.27 months; 95%CI = 7.65-8.89) for groups ($P = 0.108$); Figure 2A and B). When stratified by ECOG-PS scores, the patients with ECOG score 0 exhibited a longer mPFS than those with ECOG score 1 (Figure 2C). However, no statistical difference in PFS was demonstrated between patients with ≤ 3 tumors *vs* those with > 3 tumors (5.4 months *vs* 4.0 months, $P = 0.178$).

Table 2 Therapeutic efficacy of response and conversion therapy, *n* (%)

	PTL (*n* = 18)	TL (*n* = 23)	*P* value
CR	1 (5.6)	0 (0)	
PR	2 (11.1)	3 (13.0)	
SD	13 (72.2)	11 (47.8)	
PD	2 (11.1)	9 (39.1)	
ORR	3 (16.7)	3 (13.0)	0.752
DCR	16 (88.9)	14 (60.9)	0.046

PTL: PD-1 inhibitor/transcatheter arterial chemoembolization/Lenvatinib; TL: Transcatheter arterial chemoembolization/Lenvatinib; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; ORR: Objective response rate; DCR: Disease control rate.

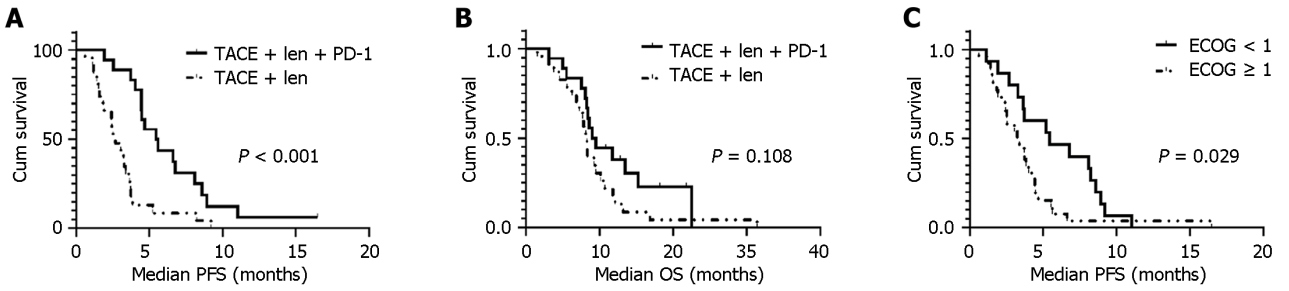

Figure 2 Kaplan-Meier curves estimate prognosis. A: Kaplan-Meier curves estimate median progression free survival by treatment modality; B: Kaplan-Meier curves estimate median overall survival by treatment modality; C: Kaplan-Meier curves estimate median progression free survival for patients with different Eastern Cooperative Oncology Group scores. TACE: Transcatheter arterial chemoembolization; ECOG: Eastern Cooperative Oncology Group; PFS: Progression-free survival; OS: Overall survival.

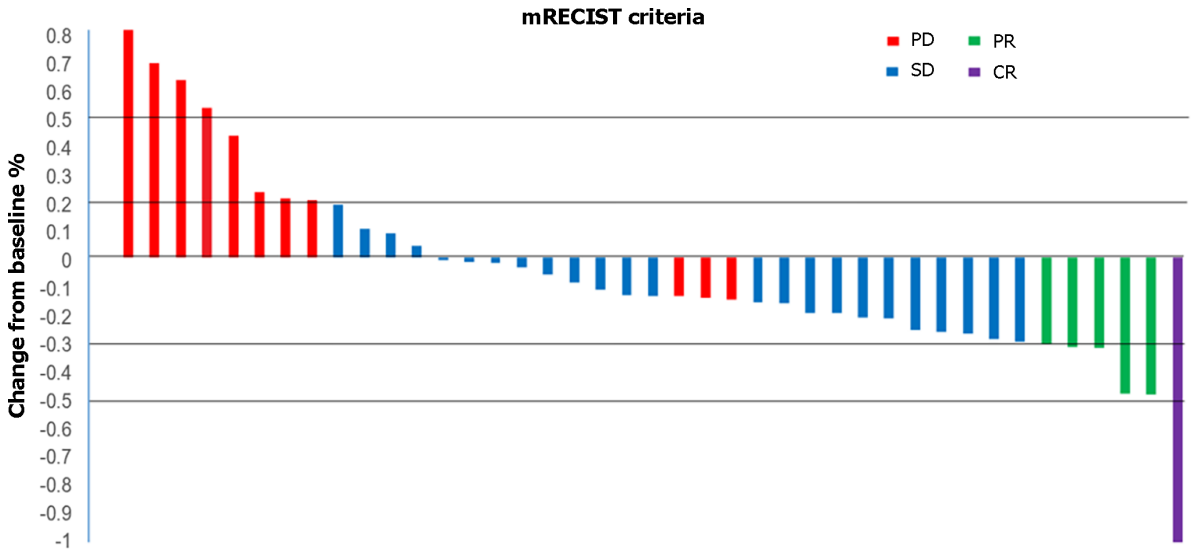

Figure 3 Waterfall plot of maximum tumor response to triple therapy by investigator using the modified Response Evaluation Criteria in Solid Tumors. Waterfall plots showing the maximum level tumor responses to triple therapy by investigators using the mRECIST approach. mRECIST: Modified Response Evaluation Criteria in Solid Tumors; PD: Progressive disease; SD: Stable disease; PR: Partial remission; CR: Complete remission.

The DCR was superior (P = 0.046) in the PTL group (88.9%; 1 CR, 2 PR, and 13 SD) than that in the TL (60.9%; 0 CR, 3 PR, and 11 SD) groups (Table 2). However, the difference in the ORR was not significant (P = 0.752) between the PTL (16.7%; 1 CR, and 2 PR) and TL (13%; 0 CR and 3 PR) groups. Waterfall analysis revealed a reduction in tumor sizes in 70.7% (29/41) of patients as per the investigator's assessment (Figure 3).

Table 3 presents independent prognostic factors related to mPFS. In univariate analysis, age, gender, cirrhosis, Child-Pugh grade, ECOG performance status, tumor thrombus, bilirubin, prothrombin time, and the intervention method were

Table 3 Univariate and multivariate cox proportional hazard model for Median progression-free survival

Characteristic	*N*	Univariate			Multivariate		
		mPFS	95%CI	*P* value	HR	95%CI	*P* value
Treatment				0.000	0.25	0.12-0.52	< 0.001
PTL group	18	5.4	3.61-7.25				
TL group	23	2.7	1.73-3.61				
Gender				0.162	-	-	0.947
Male	33	4.00	2.70-5.31				
Female	8	3.37	2.44-4.30				
Age				0.902	-	-	0.740
≥ 55 yr	26	3.70	3.13-4.28				
< 55 yr	15	3.63	1.96-5.30				
Liver cirrhosis				0.015	-	-	-
Yes	40	3.7	3.39-4.01				
No	1	1.17	-				
Weight				0.537	-	-	-
< 60 kg	15	3.73	3.48-3.98				
≥ 60 kg	26	3.27	1.87-4.67				
Etiology				0.729	-	-	-
HBV	38	3.7	3.16-4.24				
Others	3	4.47	0.36-8.58				
PVTT				0.389	-	-	0.704
I-II types	15	3.7	0.76-6.64				
III-IV types	26	3.7	2.95-4.45				
TACE				0.214	-	-	-
1-2 times	29	3.83	3.18-4.48				
≥ 2 times	12	3.37	2.57-4.17				
ECOG-PS				0.036	2.82	1.29-6.16	0.009
< 1	14	5.43	2.55-8.31				
≥ 1	27	3.27	2.69-3.85				
Child-Pugh grade				0.027	0.10	0.01-1.11	0.061
A	39	3.73	3.28-4.18				
B	2	1.83	-				
AFP				0.177	-	-	-
< 40 0 ng/mL	20	3.63	2.94-4.32				
≥ 400 ng/mL	21	4.43	2.79-6.08				
Extrahepatic spread				0.233	-	-	-
Yes	40	3.7	2.82-4.58				
No	1	3.7	2.54-4.86				
Tumor diameter				0.959	-	-	-
< 5 cm	5	4.43	1.94-6.92				
≥ 5 cm	36	3.70	3.17-4.23				
Anti-viral therapy				0.714	-	-	-

Yes	38	3.7	3.01-4.40				
No	3	3.7	0.82-6.58				
ALB				0.840	-	-	-
> 3.5 g/dL	17	3.37	2.21-4.53				
≤ 3.5 g/dL	24	3.73	3.17-4.30				
Family history of HBV				0.099	-	-	-
Yes	28	4.43	3.60-5.26				
No	13	2.67	1.03-4.28				
PT				0.561	-	-	0.915
< 13 s	25	4.00	2.69-5.31				
≥ 13 s	16	2.53	0.04-5.02				
TBIL				0.964	-	-	0.392
< 1.5 mg/dL	31	3.73	2.86-4.60				
≥ 1.5 mg/dL	10	2.67	1.90-3.45				
ALT				0.907	-	-	-
< 40 μg/mL	22	3.73	2.11-5.35				
≥ 40 μg/mL	19	3.70	3.24-4.16				
AST				0.849	-	-	-
< 40 μg/mL	12	3.23	1.43-5.03				
≥ 40 μg/mL	29	3.70	3.12-4.28				

PTL: PD-1 inhibitor/transcatheter arterial chemoembolization/Lenvatinib; TL: Transcatheter arterial chemoembolization/Lenvatinib; PVTT: Portal vein tumor thrombus; TACE: Transcatheter arterial chemoembolization; ECOG-PS: Eastern Cooperative Oncology Group-performance status; AFP: Alpha fetoprotein; ALB: Albumin; HBV: Hepatitis B virus; PT: Prothrombin time; TBIL: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate transaminase.

significantly associated with mPFS ($P < 0.05$). Finally, we found that the PTL regimen (95%CI = 0.12-0.52; HR = 0.25; $P < 0.001$), and a lower ECOG score (95%CI = 1.29-6.16; HR = 2.82; $P = 0.009$) were favorable factors for prolonged PFS.

Safety

Since post-TACE symptoms of transient AEs disappeared in a short time in most patients, these did not affect the subsequent treatment. We did not summarize these transient AEs related to TACE. Thirty-two patients (78.0) in the two groups experienced treatment-related AEs, including 12 patients (37.5) with high-grade AEs (≥ grade 3; Table 4). The most frequent treatment-related AEs were asthenia, hand-foot skin reaction, proteinuria, hypertension, decreased white blood cell and platelet counts, hypoproteinemia, ascites, nausea, and decreased weight, and most of these AEs were mild to moderate in severity. The incidence of AEs of ≥ grade 3 was similar between the two groups. Immune-related AEs included myocarditis (1 patient, grade 2) and hypothyroidism (2 patients, grade 2). The incidence of dose reduction/discontinuation during treatment was 30.4% (7/23) in the TL group and 27.8% (5/18) in the PTL group.

DISCUSSION

HCC patients complicated with PVTT have a short life expectancy. Due to a high susceptibility toward intravascular and extrahepatic metastases, increased risk of other serious complications, including bleeding from esophageal varices, and impaired liver function caused by primary hepatic occlusion and worsening hypertension[27]. The updated recommendations of the National Comprehensive Cancer Network for advanced-stage HCC refer to systemic therapy[28]. In the era of combination drug therapies, TKIs combined with ICIs, such as Lenvatinib combined with Pembrolizumab or Nivolumab, Cabozantinib combined with Nivolumab or Atezolizumab, have been proven to offer higher efficacy and longer overall survival in HCC patients[29]. The addition of TACE further improves the response of this combination (TKI plus ICI) therapy for advanced-stage HCC subjects[21,22,30]. However, there are no head-to-head studies on the efficacy of TACE or HAIC added with Lenvatinib/PD-1 inhibitor *vs* TACE plus Lenvatinib in a subset of advanced HCC patients complicated with PVTT. Our study identified an advantage of triplet therapy in terms of mPFS (5.4 months *vs* 2.7 months, $P < 0.001$) as well as DCR (88.9% *vs* 60.9%, $P = 0.046$) in HCCs with PVTT types I-IV. And no new additional toxicities were found. These results were complementary to the previous findings[21,22,30], suggesting the triplet

Table 4 Adverse events, *n* (%)

Adverse events	Any grades			High grades (≥ 3)		
	PTL (*n* = 18)	TL (*n* = 23)	*P* value	PTL (*n* = 18)	TL (*n* = 23)	*P* value
Asthenia	7 (38.9)	5 (21.7)	0.186	0 (0.0)	0 (0.0)	-
ALT elevation	14 (77.8)	18 (78.3)	0.665	2 (11.1)	3 (13.0)	0.851
AST elevation	13(72.2)	18 (78.3)	0.971	2 (11.1)	3 (13.0)	0.851
Hand-foot skin reaction	1 (5.6)	3 (13.0)	0.435	0 (0.0)	0 (0.0)	-
Hypertension	2 (11.1)	3 (13.0)	0.856	1 (5.6)	1 (4.3)	0.863
Hypothyroidism	2 (11.1)	0 (0.0)	0.163	0 (0.0)	0 (0.0)	-
Proteinuria	2 (11.1)	3 (13.0)	0.856	0 (0.0)	1 (4.3)	0.383
Dysphonia	0 (0.0)	1 (4.3)	0.383	0 (0.0)	0 (0.0)	-
Decreased WBC	5 (27.8)	4 (17.4)	0.438	1 (5.6)	0 (0.0)	0.264
Decreased PLT	6 (33.3)	4 (17.4)	0.249	0 (0.0)	1 (4.3)	0.383
Hypoproteinemia	4 (22.2)	3 (13.0)	0.451	0 (0.0)	0 (0.0)	-
Infection	1 (5.6)	1 (4.3)	0.863	0 (0.0)	1 (4.3)	0.383
Diarrhea	3 (16.7)	2 (8.7)	0.452	0 (0.0)	0 (0.0)	-
Hepatic encephalopathy	0 (0.0)	1 (4.3)	0.383	0 (0.0)	1 (4.3)	0.383
Myocarditis	1 (5.6)	0 (0.0)	0.264	1 (5.6)	0 (0.0)	0.264
Anorexia	3 (16.7)	4 (17.4)	0.953	0 (0.0)	0 (0.0)	-
Ascites	4 (22.2)	3 (13.0)	0.451	0 (0.0)	0 (0.0)	-
Acute kidney injury	1 (5.6)	1 (4.3)	0.863	1 (5.6)	1 (4.3)	0.863
Nausea	3 (16.7)	5 (21.7)	0.693	0 (0.0)	0 (0.0)	-
Decreased weight	2 (11.1)	4 (17.4)	0.584	0 (0.0)	0 (0.0)	-
Elevated bilirubin	1 (5.6)	1 (4.3)	0.863	1 (5.6)	0 (0.0)	0.264
Alimentary tract hemorrhage	0 (0.0)	1 (4.3)	0.383	0 (0.0)	1 (4.3)	0.383

PTL: PD-1 inhibitor/transcatheter arterial chemoembolization/Lenvatinib; TL: Transcatheter arterial chemoembolization/Lenvatinib; ALT: Alanine aminotransferase; AST: Aspartate transaminase; WBC: White blood cell; PLT: Platelet.

regimen could be utilized in advanced HCC cases, including those with PVTT types I-IV.

The probable mechanism underlying the superiority of the triplet regimen over the dual regimen in treating PVTT in terms of prognosis could be as follows: TACE-induced release of inflammatory factors might have activated the adaptive immunity, or TACE might induce spontaneous T cell responses to regulate the immune environment in the tumor microenvironment. Simultaneously, its combination with ICIs may be more effective in promoting antitumor immune reconstitution[31]. Previous findings have highlighted that tumor immune escape mainly occurs when CTLs are depleted. The immune-promoting activities of anti-VEGF drugs can suppress the expression of PD-1 as well as TIM-3 (mucin domain-containing protein 3) on CTLs, thereby rescuing the CTL population from the depleted state and forming a newly balanced immune environment during treatments[17]. A single-arm investigation has indicated that the PTL regimen could achieve an ORR of 80.6% with manageable toxicity[29].

Although our study demonstrated the superiority of the PTL regimen over the TL, the mPFS was shorter in our study than in the previous study[21]. The following two reasons were considered: (1) This study used advanced-stage HCC patients (63.4% with types III-IV PVTT) with poor prognoses as a sample, while the previous study using a triple regimen did not include all patients with PVTT; and (2) the small sample size used in this study might have caused some variations in the results. We noticed that the mOS of patients treated with PTL was not statistically different compared to that of TL-treated patients. We speculated that the second-line treatment after progression might have exerted a certain impact on the overall survival of patients. For example, the combination with PD-1 inhibitors of patients in the dual group after progression further prolonged the survival time of patients. At the same time, for more accurate and reliable results, randomized controlled trials (RCTs) are needed in the future.

Furthermore, we analyzed the prognostic factors of PFS of the whole cohort and concluded that the treatment mode and the ECOG-PS score were independent factors that could modulate the prognosis of HCC patients. When the ECOG-PS score was lower, the mPFS of patients was better. Moreover, the triplet therapy of the PTL regimen could provide better mPFS to patients than the dual therapy with the TL regimen.

Consistently, the PTL regimen has been shown to offer a similar safety profile in this study[21]. The incidences of treatment-related AEs did not differ considerably between the two treatment modalities. Notably, the triplet therapy regimen was not associated with any recurrences of liver injuries, as well as no grade 3 ir-AEs in our study. A recent study has demonstrated that transient transaminase elevation (*e.g.*, 52% in ALT, or 46% in AST) after TACE could be associated with objective responses[32], which can guide clinical practice. It means that patients with severe liver injury may have limited efficacy from TACE, maybe the severe liver injuries induce liver function deterioration which can hinder the administration of systemic drugs. However, due to limited data sources, no such association was investigated in this study.

Some critical limitations of this study are as follows. First, it was a single-center, small-sample, retrospective, and investigator-biased study. Second, the variability in TACE frequency might have affected the results of this study. Third, the cohort size was too small to obtain reliable statistical power for this study. And lastly, the use of different PD-1 blockers could have influenced the consistency of treatment procedures.

CONCLUSION

In conclusion, we found that the triple therapy with PTL could not only significantly improve mPFS and DCR in advanced HCC patients with PVTT but also prove to be better safety and tolerability. Therefore, based on the previous as well as current findings, it may be concluded that the PTL regimen has powerful potential in obtaining satisfactory treatment outcomes in advanced-stage HCC patients complicated with PVTT types I-IV. Prospective multi-center RCTs are warranted to further confirm the clinical efficacy of the triplet regimen over conventional procedures for HCC with PVTT.

ARTICLE HIGHLIGHTS

Research background

The incidence and mortality of hepatocellular carcinoma (HCC) are among the highest in the world. There are a large number of advanced liver cancer patients with portal vein cancer embolus, and the prognosis is worse. Therefore, it is necessary to explore the treatment plan that can prolong the survival of liver cancer patients with portal vein cancer embolus.

Research motivation

To compare the efficacies and safety levels of the PD-1 inhibitor/TACE/Lenvatinib (PTL) regimen and TACE/Lenvatinib (TL) regimen for HCC subjects comorbid with portal vein tumor thrombus (PVTT), providing a choice for exploring the combination drug regimen that can prolong the survival of HCC with PVTT.

Research objectives

Our research aims to compare the efficacies and safety levels of the PTL regimen and TL regimen for HCC subjects comorbid with PVTT. We found a triplet regimen of PTL was safe and well-tolerated as well as exhibited favorable efficacy over the TL regimen for advanced-stage HCC patients with PVTT types I-IV. The triple therapy regimen may better improve the prognosis of advanced liver cancer patients with portal vein cancer suppository and extend their survival time, which is of great significance.

Research methods

We selected HCC patients with PVTT type I-IV, TACE was carried out by 2 professional radiologists.

Research results

The triple therapy regimen may better improve the prognosis of advanced liver cancer patients with portal vein cancer suppository and extend their survival time. Large-scale prospective studies are needed to further validate the efficacy and safety of the triple therapy regimen in the future.

Research conclusions

A triplet regimen of PTL was safe and well-tolerated as well as exhibited favorable efficacy over the TL regimen for advanced-stage HCC patients with PVTT types I-IV. The combination therapy of TACE, TKI and PD-1 inhibitor was used for advanced liver cancer patients with portal vein cancer embolus.

Research perspectives

Large-scale prospective studies are needed to further validate the efficacy and safety of the triple therapy regimen in the future.

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FOOTNOTES

Co-first authors: Hong-Xiao Wu and Xiao-Yan Ding.

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ORIGINAL ARTICLE

Retrospective Study

Immunoglobulin G-mediated food intolerance and metabolic syndrome influence the occurrence of reflux esophagitis in *Helicobacter pylori*-infected patients

Li-Hui Wang, Bin-Bin Su, Sheng-Shu Wang, Guan-Chao Sun, Kun-Ming Lv, Yi Li, Hui Shi, Qian-Qian Chen

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Abstract

BACKGROUND

Reflux esophagitis has an increasing prevalence and complex and diverse symptoms. Identifying its risk factors is crucial to understanding the etiology, prevention, and management of the disease. The occurrence of reflux esophagitis may be associated with food reactions, *Helicobacter pylori* (*H. pylori*) infection, and metabolic syndromes.

AIM

To investigate the risk factors for reflux esophagitis and analyze the effects of immunoglobulin (Ig) G-mediated food intolerance, *H. pylori* infection, and metabolic syndrome on reflux esophagitis.

METHODS

Outpatients attending the Second Medical Center of the PLA General Hospital between 2017 and 2021 were retrospectively enrolled. The patients' basic information, test results, gastroscopy results, *H. pylori* test results, and IgG-mediated food intolerance results were collected. Multivariate logistic regression analysis was used to analyze risk factors for reflux esophagitis. Statistical

mediation analysis was used to evaluate the effects of IgG-mediated food intolerance and metabolic syndrome on *H. pylori* infection affecting reflux esophagitis.

RESULTS

A total of 7954 outpatients were included; the prevalence of reflux esophagitis, IgG-mediated food intolerance, *H. pylori* infection, and metabolic syndrome were 20.84%, 61.77%, 35.91%, and 60.15%, respectively. Multivariate analysis showed that the independent risk factors for reflux esophagitis included IgG-mediated food intolerance (OR = 1.688, 95%CI: 1.497-1.903, $P < 0.00001$) and metabolic syndrome (OR = 1.165, 95%CI: 1.030-1.317, $P = 0.01484$), and the independent protective factor for reflux esophagitis was *H. pylori* infection (OR = 0.400, 95%CI: 0.351-0.456, $P < 0.00001$). IgG-mediated food intolerance had a partially positive mediating effect on *H. pylori* infection as it was associated with reduced occurrence of reflux esophagitis ($P = 0.0200$). Metabolic syndrome had a partially negative mediating effect on *H. pylori* infection and reduced the occurrence of reflux esophagitis ($P = 0.0220$).

CONCLUSION

Patients with IgG-mediated food intolerance and metabolic syndrome were at higher risk of developing reflux esophagitis, while patients with *H. pylori* infection were at lower risk. IgG-mediated food intolerance reduced the risk of reflux esophagitis pathogenesis in patients with *H. pylori* infection; however, metabolic syndrome increased the risk of patients with *H. pylori* infection developing reflux esophagitis.

Key Words: Gastroesophageal reflux; Esophagitis; Food intolerance; Metabolic syndrome; *Helicobacter pylori*; Chemokines

Core Tip: This retrospective study investigated the effects of IgG-mediated food intolerance, *Helicobacter pylori* (*H. pylori*) infection, and metabolic syndrome on reflux esophagitis. In 7954 outpatients, the prevalence of reflux esophagitis was 20.84%. Patients with IgG-mediated food intolerance and metabolic syndrome are at higher risk of developing reflux esophagitis, while patients with *H. pylori* infection are at lower risk. IgG-mediated food intolerance reduces the risk of reflux esophagitis pathogenesis in patients with *H. pylori* infection; however, metabolic syndrome increases the risk of patients with *H. pylori* infection developing reflux esophagitis.

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a range of diseases arising from the reflux of gastroduodenal content, and its pathogenesis is related to the immuno-inflammatory cascade[1]. Reflux esophagitis (RE), a phenotype of GERD, is defined as a visible mucosal rupture in the distal esophagus[2]. RE accounts for 30% of GERD cases, with an increasing prevalence and complex and diverse symptoms which greatly affect the quality of life; its long-term treatment requirements consume significant healthcare resources and create a socioeconomic burden[3]. Identifying the risk factors for RE is crucial to understanding the etiology, prevention, and management of the disease.

Food intolerance (FI) refers to the discomfort caused by a food or food component at a dose that is normally tolerated; this is responsible for most adverse food reactions. FI can be asymptomatic and manifest only as a high immunoglobulin (Ig) G response to stimulation by specific food antigens[4]. Certain foods can cause an imbalance between pro-inflammatory and anti-inflammatory cytokines, which is conducive to the production of an inflammatory environment and activation of the immune system[5]; this phenomenon may affect the development of RE. Metabolic syndrome is a risk factor for RE[6,7]. The effects of *Helicobacter pylori* (*H. pylori*) infection on reflux remain controversial[8-10]. This study aimed to explore the risk factors for RE and analyze the effects of IgG-mediated FI, *H. pylori* infection, and metabolic syndrome on RE.

MATERIALS AND METHODS

Research methods

This was a retrospective cross-sectional study. Outpatients attending the Second Medical Center of PLA General Hospital between 2017 and 2021 were enrolled.

The inclusion criteria were as follows: (1) Successful gastroscopy at our hospital; (2) successful completion of the IgG-mediated FI test; and (3) successful completion of the *H. pylori* test.

The exclusion criteria were as follows: (1) Missing test results; and (2) severe cardiovascular, cerebrovascular, or consciousness impairment preventing completion of the examination.

Basic information

The data collected from outpatient records included the following: (1) Basic information: age, sex, systolic blood pressure, diastolic blood pressure, smoking history, and drinking history; (2) test results: white blood cell count, hemoglobin, C-reactive protein, folic acid, homocysteine, brain natriuretic peptide precursor, total cholesterol, fasting blood glucose, creatinine, total protein, and 25-hydroxyvitamin D3; (3) diseases: metabolic syndrome and *H. pylori* infection; (4) gastroscopy results: presence of RE; and (5) IgG-mediated FI results.

Diagnostic criteria

Diagnostic criteria for reflux esophagitis: RE, diagnosed by the presence of mucosal rupture, was detected by gastroscopy and classified according to the current international common Los Angeles grading (LA) standard. LA-A type is categorized by the presence of one or more mucosal breakages ≤ 5 mm in length, LA-B type is categorized by the presence of one or more mucosal breakages > 5 mm in length with no fusion lesion, LA-C type is categorized by the presence of mucosal breakages with fusion lesions < 75% of the esophageal circumference, and LA-D type is categorized by the presence of mucosal breakages with fusion lesions ≥ 75% of the esophageal circumference[3].

Diagnostic criteria for IgG-mediated FI: ELISA was performed to detect delayed allergens, namely, specific IgG antibodies for 21 foods (beef, chicken, cod, corn, crab, egg, mushroom, milk, pork, rice, shrimp, soybean, tomatoes, wheat, brewer's yeast, garlic, ginger, onion, cottage cheese, red pepper, and sesame). All reagents were equilibrated to room temperature (20 °C-28 °C) and a standard curve was drawn. The absorbance value of each microwell was read with a microplate reader and the concentration of IgG antibodies was evaluated based on the absorbance of each well and the standard curve. IgG-positive food was defined as IgG antibody concentrations ≥ 50 U/mL. IgG-mediated FI was diagnosed when there was ≥ 1 IgG-positive food[11].

Diagnostic criteria for metabolic syndrome: Metabolic syndrome was diagnosed by the presence of at least three of the following: (1) Abdominal obesity (namely, central obesity): waist circumference ≥ 90 cm for men and ≥ 85 cm for women; (2) hyperglycemia: fasting blood glucose ≥ 6.1 mmol/L or blood glucose ≥ 7.8 mmol/L obtained 2 h after sugar loading or patients diagnosed with diabetes who were receiving treatment; (3) hypertension: blood pressure ≥ 130/85 mmHg (1 mmHg = 0.133 kPa) or patients diagnosed with hypertension who were receiving treatment; (4) fasting triacylglycerol ≥ 1.70 mmol/L; and (5) fasting high-density lipoprotein cholesterol < 1.04 mmol/L[12].

Diagnostic criteria for *H. pylori* infection: The diagnostic criteria for *H. pylori* infection included the use of a ^{13}C-urea breath test or the histologic examination of gastric biopsy specimens as diagnostic tools[13].

Statistical analysis

Normally distributed continuous variables are presented as means ± SD, and independent *t*-tests were used for between-group comparisons. Categorical variables are presented as numbers or percentages, and χ^2 tests were used for between-group comparisons. Multivariate logistic regression analysis was used to analyze risk factors for RE. Mediation effect analysis was used to evaluate the effects of IgG-mediated FI and metabolic syndrome on the occurrence of RE caused by *H. pylori* infection. In this study, SPSS (version 26.0; IBM Corp., Armonk, NY, United States) was applied to organize and statistically analyze the data; a *P*-value < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics

A total of 7954 outpatients were included; 1658 (20.84%) had RE. Notably, RE had a high prevalence among older patients, men, and patients with smoking and alcohol consumption histories. Furthermore, comparing the diseases and test results between the non-RE and RE groups, the RE group demonstrated higher positive IgG-mediated FI, metabolic syndrome, white blood cell count, hemoglobin, homocysteine, fasting blood glucose, and creatinine levels and a lower rate of *H. pylori* infection; all *P*-values were < 0.05 (Table 1).

Multivariate analysis of influencing factors in reflux esophagitis

Multivariate analysis showed that the risk factors for RE included IgG-mediated FI and metabolic syndrome and that *H. pylori* infection was a protective factor for RE (Figure 1).

Mediation effect analysis of IgG-mediated FI on H. pylori infection affecting reflux esophagitis

The x^2 test was used to analyze IgG-mediated FI and *H. pylori* infection. The incidence of IgG-mediated FI was significantly lower in the *H. pylori*-infected group (1700/2856, 59.52%) than in the *H. pylori*-non-infected group (3213/5098, 63.02%; *P* = 0.002). The exposure variable was *H. pylori* infection, the outcome variable was RE, and IgG-mediated FI was used as the mediating variable in the mediation effect analysis. The results showed that the total effect of *H. pylori*

Table 1 Baseline characteristics

Characteristics	Non-reflux esophagitis (*n* = 6296)	Reflux esophagitis (*n* = 1658)	*P* value
Basic information			
Age (yr), mean ± SD	49.76 ± 8.00	50.81 ± 7.83	< 0.001
Sex (male) [*n* (%)]	4177 (66.34)	1504 (90.71)	< 0.001
Systolic blood pressure (mmHg), mean ± SD	123.40 ± 137.49	126.12 ± 16.78	0.421
Diastolic blood pressure (mmHg), mean ± SD	80.58 ± 11.57	82.63 ± 11.06	< 0.001
Drinking history [*n* (%)]			< 0.001
No	2567 (40.77)	387 (23.34)	
Yes	3592 (57.05)	1231 (74.25)	
Abstain	137 (2.17)	40 (2.41)	
Smoking history [*n* (%)]			< 0.001
No	4073 (64.69)	788 (47.53)	
Yes	1777 (28.22)	720 (43.43)	
Abstain	446 (7.08)	150 (9.05)	
Test results			
White blood cell count (10^9/L), mean ± SD	5.87 ± 1.44	6.21 ± 1.55	< 0.001
Hemoglobin (g/L), mean ± SD	143.31 ± 15.33	150.17 ± 12.61	< 0.001
C-reactive protein (mg/L), mean ± SD	0.16 ± 0.36	0.16 ± 0.31	0.999
Folic acid (ng/mL), mean ± SD	9.50 ± 4.39	8.58 ± 4.05	< 0.001
Homocysteine (μmol/L), mean ± SD	12.35 ± 9.38	13.46 ± 6.96	< 0.001
Brain natriuretic peptide precursor (pg/mL), mean ± SD	34.59 ± 41.53	30.29 ± 49.06	< 0.001
Total cholesterol (mmol/L), mean ± SD	4.76 ± 0.90	4.72 ± 0.95	0.103
Fasting blood glucose (mmol/L), mean ± SD	5.50 ± 1.17	5.68 ± 1.20	< 0.001
Creatinine (μmol/L), mean ± SD	67.92 ± 13.97	72.21 ± 12.79	< 0.001
Total protein (g/L), mean ± SD	70.14 ± 5.39	69.82 ± 5.25	0.031
25-hydroxyvitamin D3 (ng/mL), mean ± SD	19.79 ± 6.01	20.13 ± 6.14	0.040
Diseases [*n* (%)]			
IgG-mediated food intolerance	3781 (60.05)	1132 (68.28)	< 0.001
Helicobacter pylori infection	2478 (39.36)	378 (22.80)	< 0.001
Metabolic syndrome	3671 (58.31)	1113 (67.13)	< 0.001

Ig: Immunoglobulin.

infection on RE was -0.122064, the direct effect of *H. pylori* infection on RE was -0.119349, the mediation effect of *H. pylori* infection on RE through IgG-mediated FI was -0.002715, and the mediation effect accounted for 0.022242 of the total effect; the effects showed statistically significant differences (all *P* values < 0.05) (Table 2). IgG-mediated FI had a partially positive mediating effect on *H. pylori* infection in reducing the occurrence of RE.

Mediation effect analysis of metabolic syndrome on H. pylori infection affecting reflux esophagitis

The x^2 test was used to analyze metabolic syndrome and *H. pylori* infection; the incidence of metabolic syndrome was significantly higher in the *H. pylori*-infected group (1767/2856, 61.87%) than in the *H. pylori*-non-infected group (3017/5098, 59.18%; *P* = 0.019). The exposure variable was *H. pylori* infection, the outcome variable was RE, and metabolic syndrome was used as the mediating variable for the mediation effect analysis. The results showed that the total effect of *H. pylori* infection on RE was -0.121571, the direct effect of *H. pylori* infection on RE was -0.122715, the mediation effect of *H. pylori* infection on RE through metabolic syndrome was 0.001144, and the mediation effect accounted for -0.009413 in the total effect; the effects showed statistically significant differences (all *P*-values < 0.05) (Table 3). Metabolic syndrome had a partially negative mediating effect on *H. pylori* infection in reducing the occurrence of RE.

Table 2 Mediation effect analysis: Immunoglobulin G-mediated food intolerance

Indicators	Effect value	95%CI	*P* value
Total effect	-0.122064	-0.138193, -0.104456	< 0.0001
Average direct effect	-0.119349	-0.135905, -0.102256	< 0.0001
Average mediation effect	-0.002715	-0.004145, -0.000316	0.0200
Average mediation effect percentage	0.022242	0.002679, 0.034859	0.0200

Table 3 Mediation effect analysis: Metabolic syndrome

Indicators	Effect value	95%CI	*P* value
Total effect	-0.121571	-0.138064, -0.104781	< 0.0001
Average direct effect	-0.122715	-0.139268, -0.105907	< 0.0001
Average mediation effect	0.001144	0.000182, 0.002307	0.0220
Average mediation effect percentage	-0.009413	-0.018807, -0.001479	0.0220

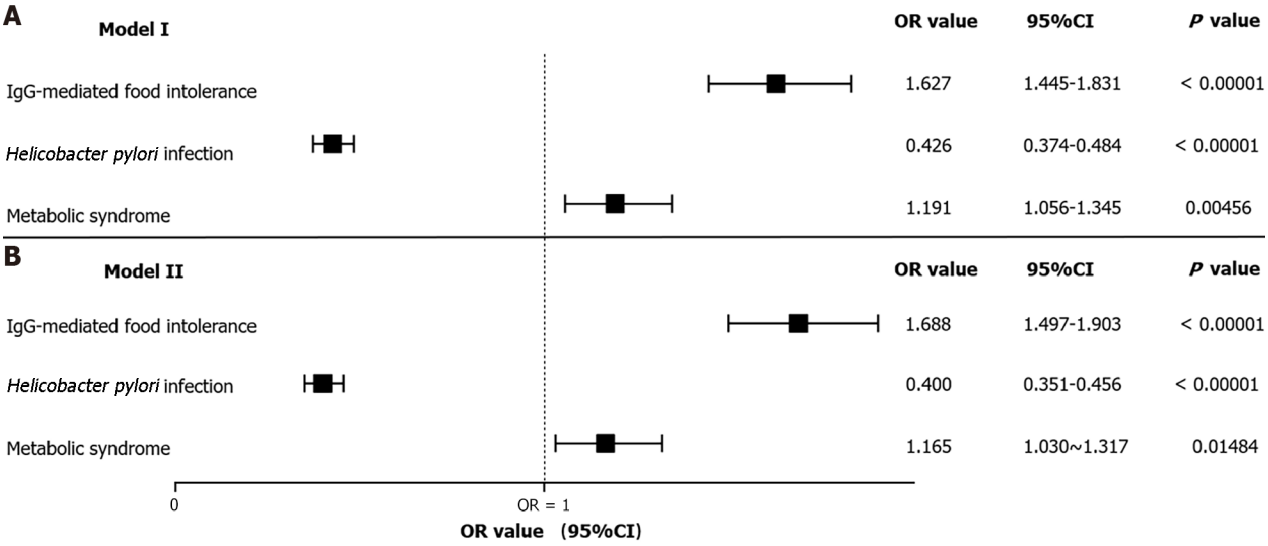

Figure 1 Multivariate analysis of influencing factors in reflux esophagitis. A: Model I was adjusted for age and sex; B: Model II was adjusted for age, sex, white blood cell count, hemoglobin, C-reactive protein, homocysteine, brain natriuretic peptide precursor, total protein, creatinine, 25-hydroxyvitamin D3, smoking history, and drinking history. Ig: Immunoglobulin.

DISCUSSION

RE, also known as erosive esophagitis, is a type of GERD with a complex etiology and an increasing prevalence. Research on its pathogenesis has progressed in recent years, ranging from the invasion of reflux and the destruction of the anti-reflux barrier to immunity, inflammation and biomarkers on a systemic scale[14]. RE demonstrates a high rate of relapse after treatment and is associated with esophageal strictures, bleeding, carcinogenesis, and other complications, which affect the quality of life and long-term prognosis of patients. Therefore, it is critical to explore the risk factors for RE and implement early clinical interventions. The population prevalence of FI is 20%[4], resulting from reactions to food components, non-celiac gluten sensitivity, or defects in enzymes and transport[15]. Furthermore, food may induce inflammatory responses, which are crucial in the development of RE. Metabolic syndrome and its associated components are also risk factors for RE[6], and *H. pylori* infection plays an essential role in RE[8,10]. Therefore, the occurrence of RE may be associated with food reactions, *H. pylori* infection, and metabolic syndromes. This study focused on three relatively common clinical diseases, evaluated risk factors associated with the occurrence of RE, and explored the pathogenic effects of IgG-mediated FI, *H. pylori* infection, and metabolic syndrome on RE to provide a clinical basis for the etiology of the disease; this is crucial knowledge to prevent the occurrence of reflux and improve patients' quality of life.

Our study is the first to investigate the effects of IgG-mediated FI on RE in a large population and confirm that IgG-mediated FI contributes to the development of RE. Studies have suggested that diet may activate the immune system and alter the levels of inflammatory markers in the blood; increased pro-inflammatory cytokines and decreased anti-inflam-

matory cytokines have been linked to diet-associated immune system activation[16]. Furthermore, the promotion and release of inflammatory cytokines and cells during inflammatory reactions involve continuous interdependent cellular communication, which is crucial in the pathogenesis of GERD[1,17]. When FI occurs, a decrease in anti-inflammatory cytokines and the release of inflammatory cells and factors cause reflux; however, further studies are necessary to better understand this phenomenon. Most patients with FI are asymptomatic, which results in such patients receiving less attention[18]. In this study, the prevalence of IgG-mediated FI, which is crucial in the development of RE, was 61.77%; therefore, it is imperative to critically evaluate IgG-mediated FI. Screening for IgG-mediated FI in patients diagnosed with RE or in high-risk groups and avoiding related foods that induce FI in daily life may be crucial in reducing the occurrence of reflux.

This study suggests that metabolic syndrome is a risk factor for RE, consistent with other studies[6,7]. The pathological commonality between the diseases associated with metabolic syndrome is a chronic low-grade inflammation state, which activates various inflammatory signal cascades and leads to the activation of NF-kB, Jun amino-terminal kinase, and inflammatory processes; this results in the recruitment of immune cells, which further aggravates inflammation[19,20]. The mechanism by which metabolic syndrome promotes the development of RE may be associated with the mechanical effect of increased abdominal pressure caused by a large amount of adipose tissue. Furthermore, substances secreted by adipose tissue, such as TNF-α, IL-6, leptin, and insulin-like growth factor-1, significantly contribute to the occurrence, development, and carcinogenesis of GERD[6]. Therefore, controlling metabolic factors such as blood glucose, blood pressure, lipid levels, and body weight can significantly reduce the occurrence of RE.

In this study, *H. pylori* infection was considered a protective factor against RE. The possible mechanism has been analyzed: *H. pylori* infection causes gastric mucosal atrophy and acid production damage, and bacterial ammonia production neutralizes acidic substances and reduces acid reflux. Additionally, it leads to the activation of vagal receptors on the fundus and cardia, consequently increasing serum gastrin secretion, improving lower esophageal sphincter pressure, reducing reflux of gastric contents, and protecting the esophageal mucosa. The effect of *H. pylori* infection on reflux is controversial; some studies have suggested that *H. pylori* infection is a risk factor for RE, while others suggested that there is no relationship between them[8-10]. The possible reasons for the controversy between this study and other studies could be that other studies mostly assessed the impact of *H. pylori* eradication on RE through quality of life and reflux symptoms, and the drugs used to eradicate *H. pylori* significantly reduced the symptoms of reflux, which affected accuracy and confidence in the results. Therefore, screening for *H. pylori* infection is not routinely required for the clinical treatment of RE, and the treatment options for *H. pylori* infection are not indicated as anti-reflux therapy; this is consistent with the recommendations of the 2013 guidelines of the American College of Gastroenterology[21].

This study confirmed the effect of IgG-mediated FI and metabolic syndrome in reducing the occurrence of RE caused by *H. pylori* infection. A study conducted in southwest China revealed that *H. pylori* infection was negatively associated with food-specific IgG in eggs, milk, and wheat; this is consistent with the results of this study[22]. *H. pylori* infection stimulates the production of Foxp3+ regulatory T cells, which have strong immunosuppressive properties, control the degree of self and non-autoantigen reactions, and produce immune tolerance, consequently protecting against *H. pylori* infection. Immune tolerance suppresses the immune response caused by FI, which reduces the occurrence of RE. In people with FI, increased intestinal permeability allows food substances to enter the circulation; concurrently, the immune system recognizes certain food molecules as harmful substances and initiates an immune response to these substances, resulting in food-specific IgG[23], which further activates the immune effect and enhances the protective effect of *H. pylori* infection against the occurrence of RE. Therefore, IgG-mediated FI reduces the incidence of RE in patients with *H. pylori* infection. In addition, the results showed that *H. pylori* infection increased the incidence of metabolic syndrome. *H. pylori* infection can upregulate the expression of various inflammatory factors (including C-reactive protein, tumor necrosis factor, and various interleukins), promote insulin resistance, and initiate metabolic syndromes[24]. Metabolic syndrome results in chronic over-nutrition and excess energy, exceeding the metabolic capacity of tissues, which leads to metabolic stress and weakens the protective effect of *H. pylori* infection against reflux. Therefore, metabolic syndrome increases the incidence of RE in patients with *H. pylori* infection.

Lifestyle modification, dietary control, and pharmacotherapy are the primary treatment options for RE; however, the complex pathogenesis of RE and the critical role of the immuno-inflammatory cascade in its development may complicate the management of symptoms and increase susceptibility to relapse after treatment. This study evaluated the risk factors for RE and explored the effects of IgG-mediated FI, *H. pylori* infection, and metabolic syndrome on RE. Patients with IgG-mediated FI and metabolic syndrome were at a higher risk of RE; however, patients with *H. pylori* infection had a lower risk of RE. IgG-mediated FI reduced the risk of RE in patients with *H. pylori* infection; however, metabolic syndrome increased the risk of RE in patients with *H. pylori* infection. Therefore, in the management of RE, preventing the induction of IgG-mediated FI and optimal diagnosis and treatment of metabolic syndrome may reduce the occurrence of RE; this represents a strategy to achieve prevention and early treatment of RE in the future.

This was a single-center retrospective clinical study, and the test results showed certain deviations. Therefore, prospective and multicenter studies should be conducted to evaluate the factors influencing RE. The mechanisms underlying the effects of IgG-mediated FI, *H. pylori* infection, and metabolic syndrome in RE should be evaluated regarding the molecular perspective and the associated pathways.

CONCLUSION

Patients with IgG-mediated FI and metabolic syndrome are at a higher risk of RE; however, patients with *H. pylori* infection have a lower risk of RE. IgG-mediated FI reduces the risk of RE in patients with *H. pylori* infection; however,

metabolic syndrome increases the risk of RE in patients with *H. pylori* infection.

ARTICLE HIGHLIGHTS

Research background

Reflux esophagitis has an increasing prevalence and complex and diverse symptoms. Identifying its risk factors is crucial to understanding the etiology, prevention, and management of the disease.

Research motivation

The occurrence of reflux esophagitis may be associated with food reactions, *Helicobacter pylori* (*H. pylori*) infection, and metabolic syndromes.

Research objectives

To investigate the risk factors for reflux esophagitis and analyze the effects of IgG-mediated food intolerance, *H. pylori* infection, and metabolic syndrome on reflux esophagitis.

Research methods

This retrospective study analyzed endoscopic images of outpatients attending the Second Medical Center of PLA General Hospital between 2017 and 2021, classified them into non-RE and RE groups, and further explored the differences in IgG-mediated food intolerance, *H. pylori* infection, and metabolic syndrome of the different groups.

Research results

In 7954 outpatients, the prevalence of reflux esophagitis was 20.84%.

Research conclusions

Patients with IgG-mediated food intolerance and metabolic syndrome are at higher risk of developing reflux esophagitis, while those with *H. pylori* infection are at lower risk. IgG-mediated FI reduces the risk of RE in patients with *H. pylori* infection, while metabolic syndrome increases the risk of RE in these patients.

Research perspectives

In the management of RE, preventing the induction of IgG-mediated FI and optimal diagnosis and treatment of metabolic syndrome may reduce the occurrence of RE.

FOOTNOTES

Co-first authors: Li-Hui Wang and Bin-Bin Su.

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Author contributions: Su BB, Lv KM, Li Y, Shi H, and Chen QQ are gastroenterologists; Wang SS is a statistician; Wang LH and Sun GC are master degree candidates; Shi H and Chen QQ performed the disease diagnosis; Wang LH, Su BB, Shi H, and Chen QQ designed the research study; Su BB and Wang SS performed the primary literature and data extraction; Wang LH, Su BB, and Wang SS analyzed the data and wrote the manuscript; Sun GC, Lv KM, Li Y, Shi H, and Chen QQ revised the manuscript for important intellectual content; and all authors read and approved the final version.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the PLA General Hospital (Ethics audits No. S2022-414-01).

Informed consent statement: Patients were not required to provide informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to test by written consent.

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

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ORIGINAL ARTICLE

Retrospective Study

Evaluating the influence of sarcopenia and myosteatosis on clinical outcomes in gastric cancer patients undergoing immune checkpoint inhibitor

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Abstract

BACKGROUND

The development and progression of gastric cancer (GC) are closely linked to the nutritional status of patients. Although immunotherapy has been demonstrated to be clinically effective, the relationships of sarcopenia and myosteatosis with the use of immune checkpoint inhibitors (ICIs) in patients with gastric cancer remain to be characterized.

AIM

To assess the effects of sarcopenia and myosteatosis on the clinical outcomes of patients with GC undergoing treatment with an ICI.

METHODS

We performed a retrospective study of patients who were undergoing immunotherapy for GC. For the evaluation of sarcopenia, the optimal cut-off value for the skeletal muscle index was established using receiver operating characteristic analysis of data obtained from pre-treatment computed tomography images at the L3 vertebral level. Myosteatosis was defined using the mean skeletal muscle density (SMD), with a threshold value of < 41 Hounsfield units (HU) for patients with a body mass index (BMI) < 25 kg/m² and < 33 HU for those with a BMI ≥ 25 kg/m². The log-rank test was used to compare progression-free survival (PFS) and overall survival (OS), and a Cox proportional hazard model was used to identify prognostic factors. Nomograms were developed to predict the PFS and OS of patients on the basis of the results of multivariate analyses.

RESULTS
We studied 115 patients who were undergoing ICI therapy for GC, of whom 27.4% had sarcopenia and 29.8% had myosteatosis. Patients with sarcopenia or myosteatosis had significantly shorter PFS and OS than those without these conditions. Furthermore, both sarcopenia and myosteatosis were found to be independent predictors of PFS and OS in patients with GC administering an ICI. The prediction models created for PFS and OS were associated with C-indexes of 0.758 and 0.781, respectively.

CONCLUSION
The presence of sarcopenia or myosteatosis is a reliable predictor of the clinical outcomes of patients with GC who are undergoing treatment with an ICI.

Key Words: Gastric cancer; Sarcopenia; Myosteatosis, Immune checkpoint inhibitor; Prognostic factor; Overall survival; Progression-free survival

Core Tip: We performed a retrospective study to evaluate the use of sarcopenia and myosteatosis for the prediction of the prognosis of patients with gastric cancer who are being treated with an immune checkpoint inhibitor (ICI). We studied 115 patients with complete sets of clinical data and imaging information and analyzed their muscle cross-sectional area at the L3 Level. We determined the optimal cut-off area value to identify sarcopenia, and myosteatosis was defined using mean skeletal muscle densities of < 41 Hounsfield units (HU) for patients with a body mass index (BMI) < 25 kg/m² and < 33 HU for those with a BMI ≥ 25 kg/m². We found that muscle loss and muscle steatosis are independent predictors of the outcomes of patients with gastric cancer being treated with an ICI.

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INTRODUCTION

Gastric cancer (GC) is the fifth most prevalent cancer globally and is a major global health concern[1]. Although the global incidence and mortality rate associated with GC are decreasing, particularly because of advancements in preventive measures, such as a reduction in the prevalence of *Helicobacter pylori* and improvements in food preservation and storage, East Asia retains high incidence and mortality rates[2-7]. The therapeutic options for GC are expanding, with the inclusion of immune checkpoint inhibitors (ICIs) alongside conventional chemotherapy and targeted agents[8]. For instance, navulizumab in combination with chemotherapy is now a first-line treatment for GC, and pembrolizumab in combination with trastuzumab and chemotherapy is the first-line treatment for patients with HER2-positive GC[9,10]. The advent of ICIs has prompted extensive research aimed at identifying prognostic factors for the success of ICI therapy, and parameters including programmed cell death protein 1 (PD-1)/programmed death ligand 1 (PD-L1) expression, microsatellite instability (MSI), tumor mutational load (TMB), and Epstein-Barr virus (EBV) infection status, have been considered. However, the assessment of these parameters is often expensive and complex[11-13]. Consequently, there is a compelling need for straightforward, cost-effective predictors of the prognosis of patients with advanced GC who are undergoing ICI therapy.

Sarcopenia, which is commonly recognized in patients with cancer, is characterized by the gradual depletion of skeletal muscle and its degeneration. This condition has detrimental effects on metabolism and immunity, resulting in compromised tolerance of, and a poor prognosis associated with, various cancer treatments, including chemotherapy, targeted therapy, and immunotherapy[14,15]. Skeletal muscle mass represents a quantitative and objective measure of the nutritional status of a patient and has been shown to be of prognostic value in patients with a range of cancers, such as GC, hepatocellular carcinoma, and esophageal carcinoma[16-21].

In addition to muscle loss, the presence of myosteatosis, which is characterized by increases in inter- and intramuscular fat content, is also of relevance[22]. This pathological change often accompanies excessive muscle loss and is exacerbated by factors such as aging and obesity, which lead to metabolic abnormalities that can affect the outcomes of treatments[23,24]. Myosteatosis has been shown to be associated with inferior overall survival (OS) in patients with several types of cancer, including hepatocellular carcinoma, GC, and colorectal cancer[25].

In the present study, we used cross-sectional computed tomography (CT) images obtained at the level of the third lumbar vertebra (L3) to evaluate the sarcopenia and myosteatosis of patients with GC who were undergoing immunotherapy, with the aim of investigating the prognostic value of the presence of these conditions with respect to the clinical outcomes of the patients.

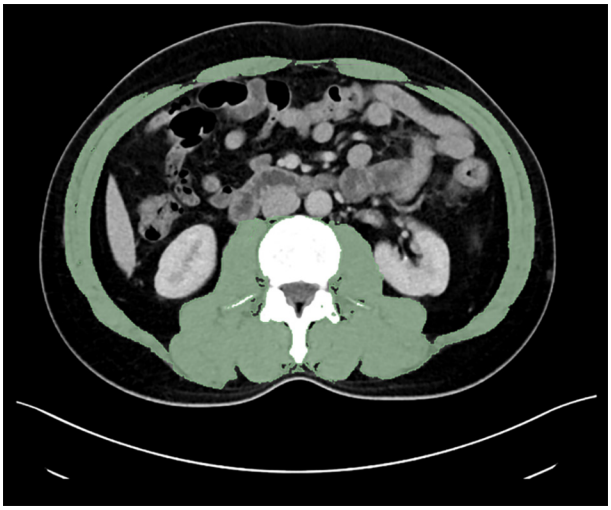

Figure 1 Example of a computed tomography image used for skeletal muscle measurements.

MATERIALS AND METHODS

Participants

Patients diagnosed with GC who underwent immunotherapy with an ICI between February 2016 and October 2022 at our institution were eligible for inclusion in the study. A comprehensive set of data, including demographics, clinical attributes, tumor characteristics (tumor size and stage), laboratory parameters, L3 skeletal muscle area, and mean CT radiodensities, were extracted from the medical records of each participant. The institutional review board provided approval for this analysis, and the requirement for informed consent was waived owing to its retrospective nature. Patients undergoing ICI immunotherapy with a PD-1 blocking antibody, including anti-PD-1 and anti-PD-L1 antibodies, with combination therapy including one of these, or with an ICI in conjunction with chemotherapy and/or other agents in phase III clinical trials, were included in the study. The exclusion criteria comprised prior immunotherapy and the inability to undergo pre-treatment CT examination.

Data collection

The primary endpoints of the study were progression-free survival (PFS) and OS. The timing of these endpoints was determined through telephone follow-up, and the final follow-up consultation was held in December 2022. PFS was defined as the difference between the timing of random assignment to a clinical trial and that of disease progression, which was primarily assessed using enhanced CT. If no evidence of disease progression was identified, the final date of follow-up was used to calculate PFS. OS was calculated as the difference between the timing of the commencement of immunotherapy and the death of the patient.

Evaluation of sarcopenia and myosteatosis

Sarcopenia and myosteatosis were evaluated by a radiologist with over a decade of experience and no knowledge of the clinical outcomes of the participants. The CT data for the participants were imported into 3D Slicer (version 4.10.2, www.slicer.org) to measure the cross-sectional area of the skeletal muscle at the L3 level and the mean skeletal muscle density [SMD, in Hounsfield units (HU)] across the entire muscle region. Skeletal muscle was identified and quantified using HU thresholds ranging from −29 to 150[26]. The L3 muscle region included the psoas major, erector spinae, quadratus lumborum, transversus abdominis, internal and external abdominal oblique muscles, and rectus abdominis. The cross-sectional area was automatically calculated by adding the data for each tissue pixel together and multiplying this by the pixel surface area (Figure 1). Skeletal muscle index (SMI) was calculated as the total L3 skeletal muscle area (cm^2) divided by the square of the participant's height (m^2). Given the lack of established diagnostic criteria for sarcopenia, the optimal cut-off value was determined using receiver operating characteristic (ROC) analysis, and participants with an SMI below this threshold were classified as having sarcopenia. The optimal cut-off value for SMI was calculated to be 27.36 for men and 31.10 for women. Myosteatosis was defined using a mean SMD < 41 HU for participants with a BMI < 25 kg/m^2 and < 33 HU for those with a BMI ≥ 25 kg/m^2[27].

Statistical analysis

Data are presented as mean ± SD for normally distributed continuous data or median for non-normally distributed continuous data. Categorical data were analyzed using Pearson's chi-square or Fisher's exact tests, and continuous datasets for participants with or without sarcopenia and myosteatosis were compared using Student's *t*-test or the Mann–Whitney *U*-test, as appropriate. Kaplan–Meier survival curves were used to evaluate survival outcomes, and Cox's regression analysis was used to identify potential prognostic factors for PFS and OS in univariate analyses. Cox's regression analysis and the parameters that were significant on univariate analysis were then used to identify

independent prognostic factors associated with OS and PFS. Multivariate logistic regression analyses were then performed to construct models for the prediction of 1-, 3-, and 5-year OS and PFS. We used the R statistical package (version 4.1.3, R Foundation for Statistical Computing, Vienna, Austria) and SPSS (version 25.0, IBM, Inc., Armonk, NY, United States) to analyze the data. A two-sided *P*-value < 0.05 was considered to represent statistical significance.

RESULTS

Characteristics and laboratory parameters of participants with or without sarcopenia and myosteatosis

A total of 115 patients with GC who were undergoing ICI treatment were included in the study [89 (77.4%) men and 26 (32.6%) women]. Of these, 29 (25.2%) had stage III GC and 86 (74.8%) had stage IV GC. When we compared the participants with or without sarcopenia, we found that the former were significantly older ($P < 0.001$), had a lower BMI ($P < 0.001$), and were predominantly male ($P < 0.001$). The participants with myosteatosis tended to be older than those without ($P = 0.001$) (Table 1).

Analysis of the laboratory indices showed that participants with sarcopenia had lower creatinine (Crea) concentrations ($P = 0.004$) than those without. In addition, the participants with myosteatosis had higher globulin (GLOB) concentrations ($P = 0.047$), lactate dehydrogenase (LDH) activities ($P = 0.012$), and D-dimer (DDi) concentrations ($P = 0.002$), and lower pre-albumin (PALB) concentrations ($P = 0.010$) than those without (Table 2).

Results of the univariate and multivariate Cox's regression analyses

Univariate analysis identified BMI, total protein (TP), PALB, eosinophil count (Eosi), carbohydrate antigen 724 (CA724) concentration, carbohydrate antigen 125II (CA125II) concentration, sarcopenia, and myosteatosis as potential prognostic factors for OS. Similarly, BMI, alkaline phosphatase (ALP) activity, total bilirubin (TBIL) concentration, indirect bilirubin (IDBIL) concentration, PALB, lymphocyte count (Lym), Eosi, CA724, CA125, TNM stage, sarcopenia, and myosteatosis were identified as potential prognostic factors for PFS. All these potential prognostic factors were then included in multivariate analyses. In these, TP, Eosi, CA724, CA125, sarcopenia, and myosteatosis were found to be independent prognostic factors for OS; ALP, Eosi, CA724, CA125, TNM stage, sarcopenia, and myosteatosis were found to be independent prognostic factors for PFS (Table 3).

Effects of sarcopenia and myosteatosis on survival

The participants with sarcopenia had significantly shorter PFS (median, 15.40 months *vs* 26.20 months, $P < 0.001$) and OS (median, 25.97 months *vs* 38.78 months, $P < 0.001$) than those without (Figure 2). Similarly, the participants with myosteatosis had shorter PFS (median, 16.43 months *vs* 24.30 months, $P = 0.011$) and OS (median, 21.43 months *vs* 33.57 months, $P = 0.001$) than those without (Figure 3).

Given that 74.8% of the participants had stage IV GC, a subgroup analysis was conducted, and this yielded results that confirmed the adverse effects of sarcopenia and myosteatosis on both PFS ($P = 0.002$ *vs* $P = 0.011$, respectively) and OS ($P < 0.001$ *vs* $P = 0.005$, respectively) in this subset of participants (Figures 4 and 5).

Nomograms

We used multivariate Cox regression analysis to construct an optimized prediction model for PFS. In addition to the presence of sarcopenia and myosteatosis, ALP, Eosi, CA724, CA125, and TNM stage were identified to be predictors of PFS, and we used these variables to construct a nomogram for PFS (Figure 6A). Similarly, for OS, the predictive model constructed included sarcopenia and myosteatosis, as well as TP, Eosi, CA724, and CA125 (Figure 7A). Both the predictive models for PFS and OS exhibited good C-indexes of 0.758 and 0.781, respectively. First-year calibration curves showed a close alignment of the observed outcomes and those predicted using the presence of sarcopenia or myosteatosis (Figures 6B and 7B). To validate the predictive utility of these parameters, the nomograms were subjected to area under the curve (AUC) analysis for 1- and 3-year intervals, yielding AUC values of 0.769 and 0.850 for PFS, and 0.843 and 0.904 for OS, respectively (Figures 6C and 7C). In addition, decision curve analysis (DCA) including diverse threshold probabilities demonstrated that the net benefit for the prediction of PFS was maximal within the range 0.040–0.978, peaking at 0.282. Similarly, for OS, the optimal DCA threshold was within the range 0.022–0.900, peaking at 0.260 (Figures 6D and 7D). This meticulous analysis affirmed the robustness and clinical utility of the predictive models for the outcomes of patients undergoing ICI therapy.

DISCUSSION

GC is highly prevalent but often presents with non-specific clinical features, resulting in a delay to diagnosis and the administration of ineffective treatments, especially in older patients[28,29]. Systemic chemotherapy has been the primary approach to the treatment of advanced GC, but it yields limited survival benefits, with a median survival of approximately 1 year[30,31]. In recent years, ICIs have emerged as promising therapeutic options for patients with advanced cancer, showing efficacy and safety in clinical trials. Some ICIs, such as pembrolizumab, avelumab, sindilizumab, tirilizumab, and ipilimumab, have been approved for administration in combination with targeted therapies for advanced GCs[32-34]. Notably, nabulizumab in combination with chemotherapy yielded excellent outcomes in the Chinese subgroup of the CheckMate 649 clinical trial[12]. To date, parameters such as PD-1/PD-L1 expression, MSI, TMB, and

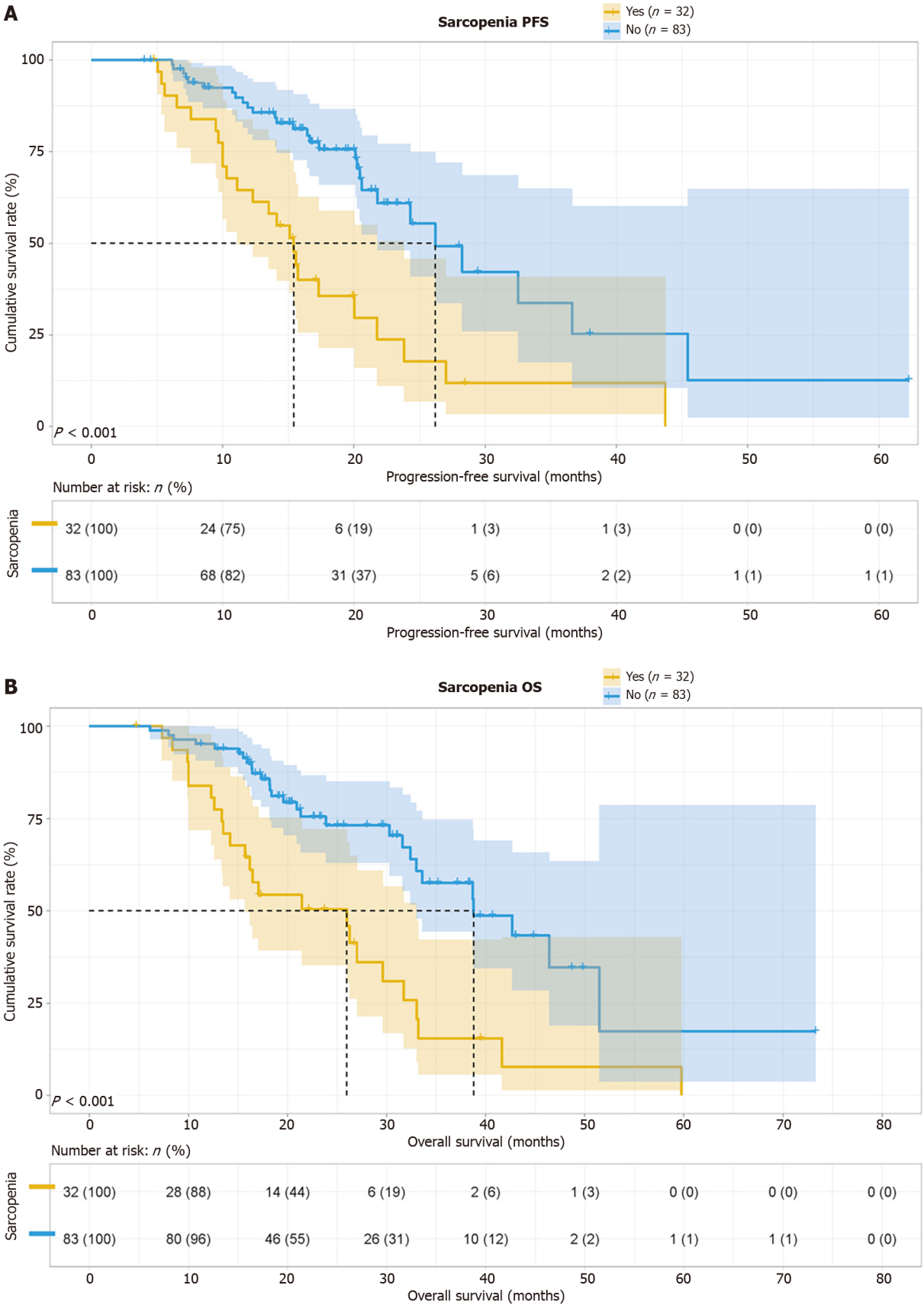

Figure 2 Survival curves for (A) progression-free survival and (B) overall survival in the presence or absence of sarcopenia. PFS: Progression-free survival; OS: Overall survival.

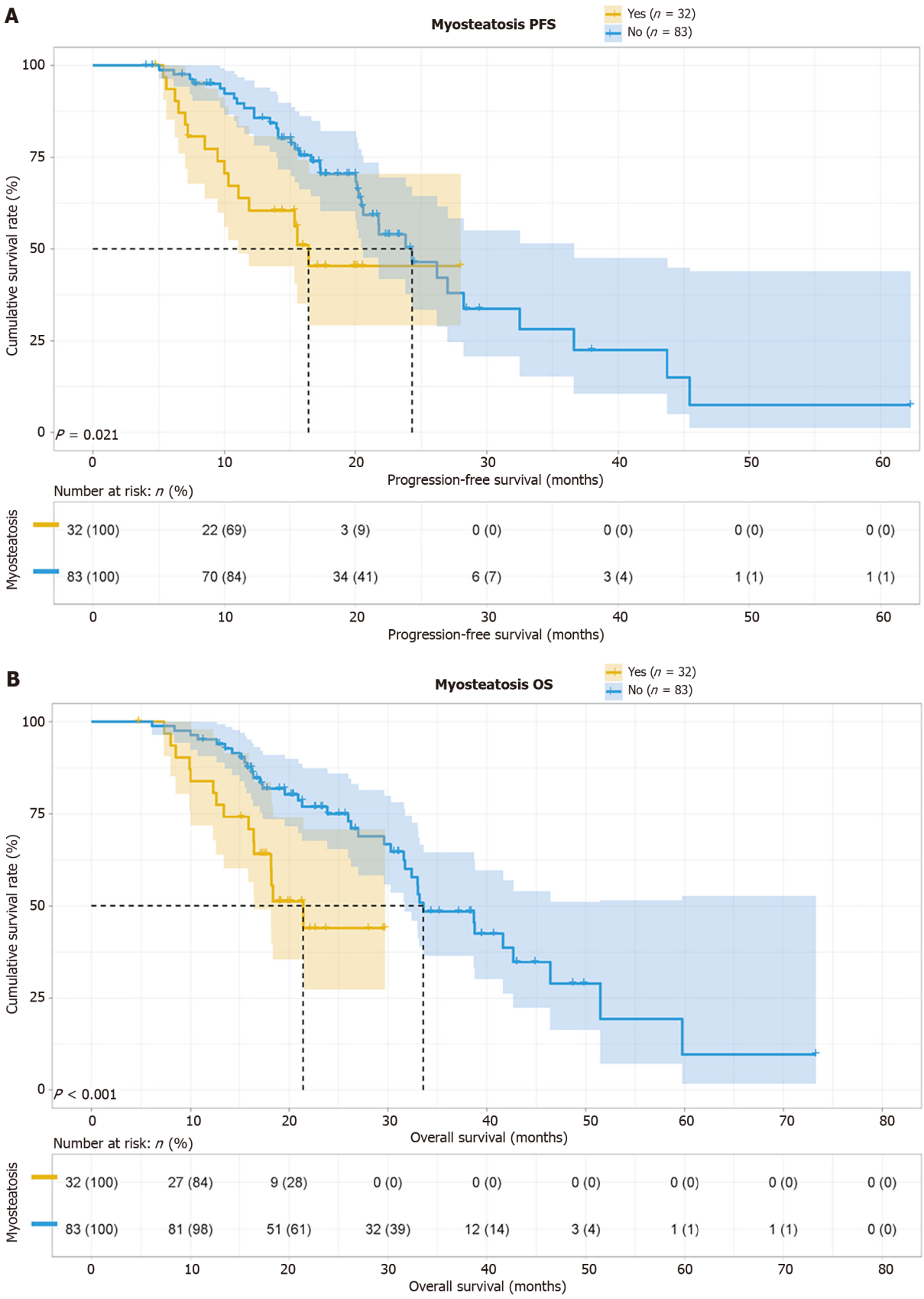

Figure 3 Survival curves for (A) progression-free survival and (B) overall survival in the presence or absence of myosteatosis. PFS: Progression-free survival; OS: Overall survival.

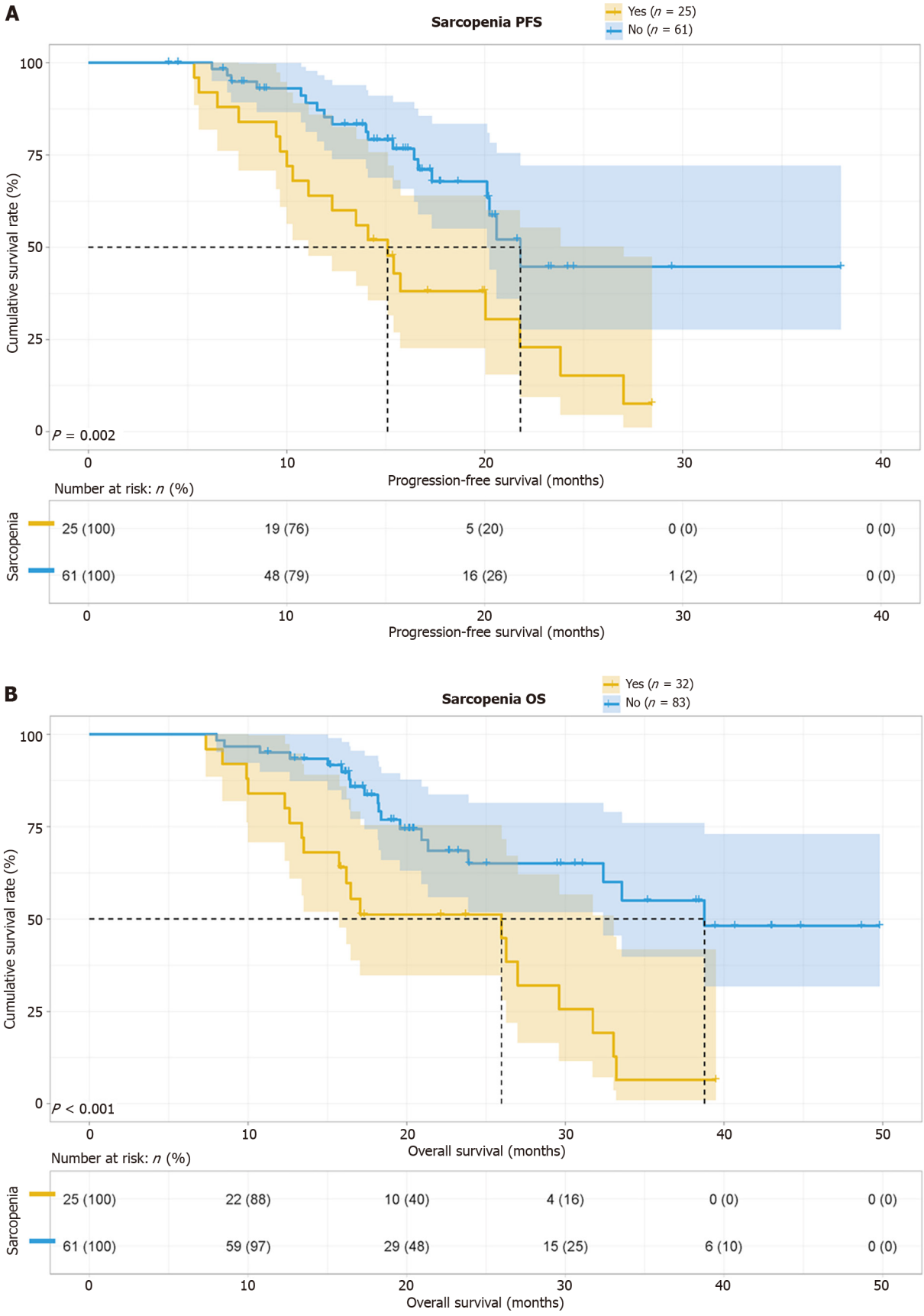

Figure 4 Survival curves for (A) progression-free survival and (B) overall survival in participants with gastric cancer in the presence or absence of sarcopenia. PFS: Progression-free survival; OS: Overall survival.

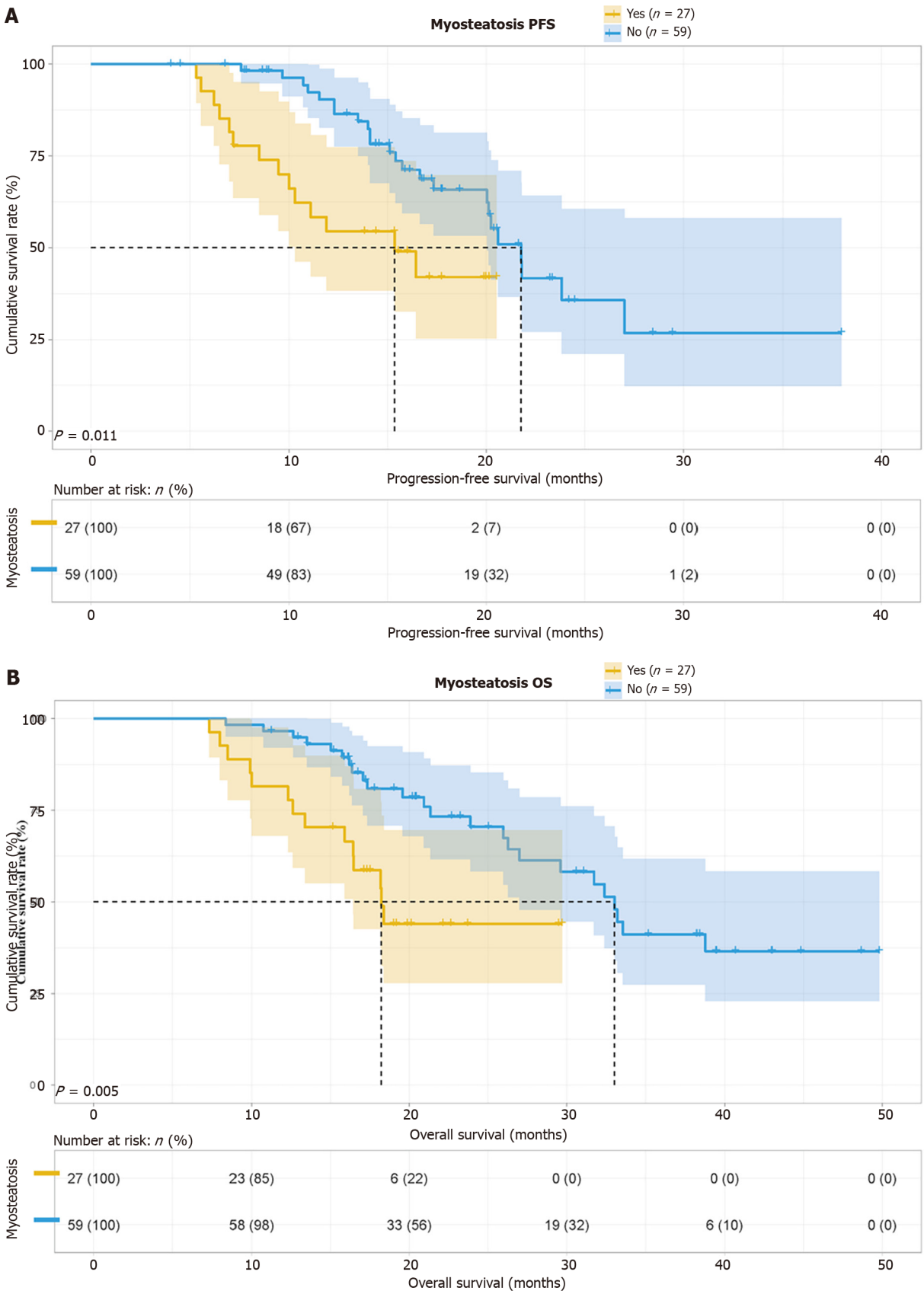

Figure 5 Survival curves for (A) progression-free survival and (B) overall survival in participants with advanced gastric cancer in the presence or absence of myosteatosis. PFS: Progression-free survival; OS: Overall survival.

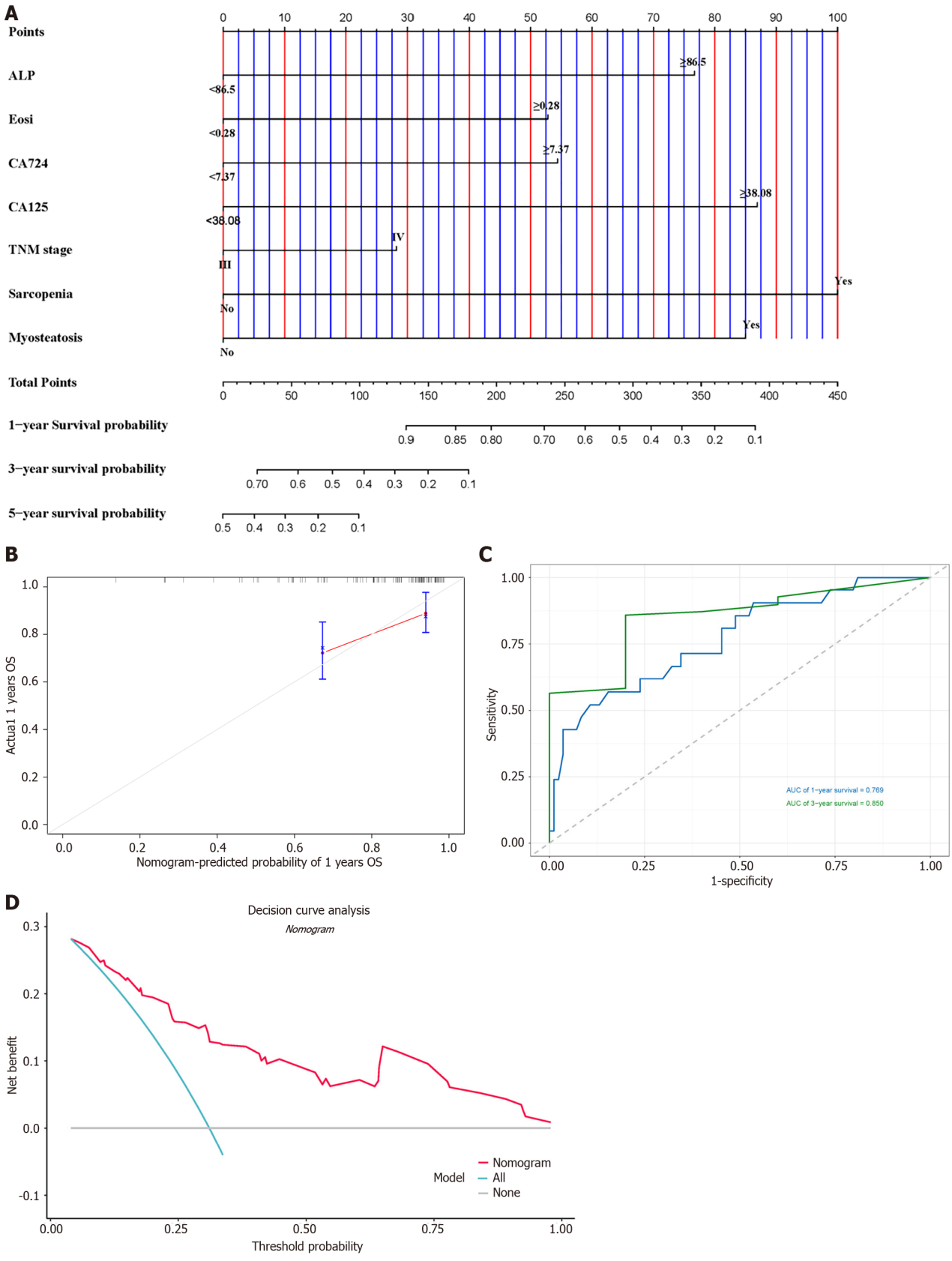

Figure 6 Nomogram for progression-free survival. A: Nomogram for progression-free survival (PFS); B: One-year and 3-year area under the curves for PFS; C: Calibration curves for PFS; D: Decision curve analysis for PFS. AUC: Area under the curve; PFS: Progression-free survival; OS: Overall survival; ALP: Alkaline phosphatase; Eosi: Eosinophil count; CA724: Carbohydrate antigen 724; CA125: Carbohydrate antigen 125.

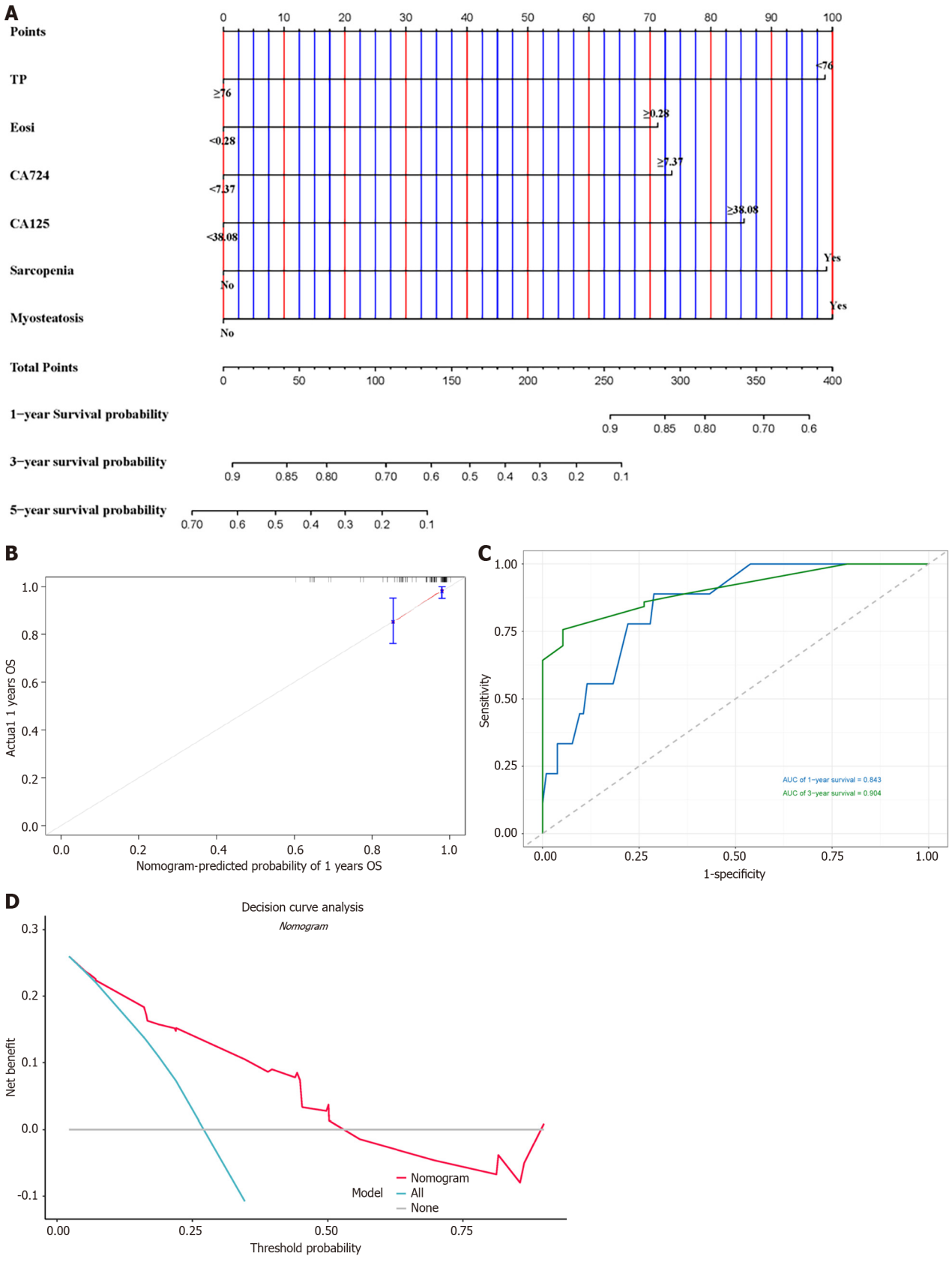

Figure 7 Nomogram for overall survival. A: Nomogram for overall survival (OS); B: One-year and 3-year area under the curves for OS; C: Calibration curves for OS; D: Decision curve analysis for OS. AUC: Area under the curve; OS: Overall survival; TP: Total protein; Eosi: Eosinophil count; CA724: Carbohydrate antigen 724; CA125: Carbohydrate antigen 125.

Table 1 Participant characteristics

	Sarcopenia			Myosteatosis		
	Yes (*n* = 32)	No (*n* = 83)	*P* value	Yes (*n* = 32)	No (*n* = 83)	*P* value
Item, mean (SD)						
Age	57.44 (11.18)	57.40 (8.57)	< 0.001	62.31 (7.69)	55.52 (9.24)	0.001
BMI	19.88 (4.39)	22.75 (3.07)	< 0.001	22.04 (3.85)	21.91 (3.85)	0.986
Sex, *n* (%)			< 0.001			0.703
Male	15 (46.9)	74 (89.2)		24 (75.0)	65 (78.3)	
Female	17 (53.1)	9 (10.8)		8 (25.0)	18 (21.7)	
Primary tumor site, *n* (%)			0.900			0.640
Upper 1/3	6 (18.8)	13 (15.7)		5 (15.6)	14 (16.9)	
Middle 1/3	7 (21.9)	23 (27.7)		6 (18.8)	24 (28.9)	
Low 1/3	18 (56.2)	45 (54.2)		20 (62.5)	43 (51.8)	
Whole	1 (3.1)	2 (2.4)		1 (3.1)	2 (2.4)	
Pathology, *n* (%)			0.166			0.344
Adenocarcinoma	6 (18.8)	22 (26.5)		5 (15.6)	23 (27.7)	
Others[1]	5 (15.6)	4 (4.8)		2 (6.3)	7 (8.4)	
Unknown	21 (65.6)	57 (68.7)		25 (78.1)	53 (63.9)	
TNM stage, *n* (%)			0.608			0.141
III	7 (21.9)	22 (26.5)		5 (15.6)	24 (28.9)	
IV	25 (78.1)	61 (73.5)		27 (84.4)	59 (71.1)	
PD-1, *n* (%)			0.281			0.698
Positive	5 (15.6)	5 (6.1)		4 (12.5)	6 (7.2)	
Negative	2 (6.3)	7 (8.4)		2 (6.3)	7 (8.4)	
Unknown	25 (78.1)	71 (85.5)		26 (81.2)	70 (84.4)	
PD-L1, *n* (%)			0.281			0.710
Positive	5 (15.6)	5 (6.1)		4 (12.5)	6 (7.2)	
Negative	2 (6.3)	8 (8.4)		2 (6.3)	8 (9.6)	
Unknown	25 (78.1)	70 (85.5)		26 (81.2)	69 (83.2)	
AFP, *n* (%)			0.702			0.221
< 2.92 ng/mL	19 (59.4)	46 (55.4)		21 (65.6)	44 (53.0)	
≥ 2.92 ng/mL	13 (40.6)	37 (44.6)		11 (34.4)	39 (47.0)	
CEA, *n* (%)			0.933			0.315
< 4.24 ng/mL	9 (28.1)	24 (28.9)		7 (21.9)	26 (31.3)	
≥ 4.24 ng/mL	23 (71.9)	59 (71.1)		25 (78.1)	57 (68.7)	
CA199, *n* (%)			0.859			0.295
< 17.63 U/L	21 (65.6)	53 (63.9)		23 (71.9)	51 (61.4)	
≥ 17.63 U/L	11 (34.4)	30 (36.1)		9 (28.1)	32 (38.6)	
CA724, *n* (%)			0.704			0.955
< 4.40 U/L	20 (62.5)	55 (66.3)		21 (65.6)	54 (65.1)	
≥ 4.40 U/L	12 (37.5)	28 (33.7)		11 (34.4)	29 (34.9)	
CA125II, *n* (%)			0.811			0.756
< 21.94 U/L	26 (81.3)	69 (83.1)		27 (81.9)	68 (84.4)	

≥ 21.94 U/L	6 (18.8)	14 (16.9)		5 (18.1)	15 (15.6)	
Sarcopenia, *n* (%)						0.057
Yes				13 (59.4)	19 (22.9)	
No				19 (40.6)	64 (77.1)	
Myosteatosis, *n* (%)			0.057			
Yes	13 (40.6)	19 (22.9)				
NO	19 (59.4)	64 (77.1)				

[1]Others comprised mucinous carcinoma, signet ring cell carcinoma, and mixed carcinoma. BMI: Body mass index; CEA: Carcinoembryonic antigen; CA199: Carbohydrate antigen 199; CA724: Carbohydrate antigen 724; CA125II: Carbohydrate antigen 125II; PD-1: Programmed cell death protein 1; PD-L1: Programmed death ligand 1.

EBV infection status have been used to identify suitable candidates for ICI therapy, but the nutritional status of the patients has a significant effect on their treatment outcomes[11-13]. Malnutrition and the related symptoms negatively affect the prognosis and the quality of life of patients with cancer. Therefore, we conducted a study using CT-derived data to assess the muscle status of patients with GC undergoing immunotherapy, and especially of those who were negative for prognostic markers such as PD-1 and PD-L1.

Sarcopenia, which reflects malnutrition and involves chronic inflammation, is common in patients with cancer, and features muscle loss and a decrease in fat mass[35]. It significantly impacts quality of life, induces anxiety and depression, and results in poorer clinical outcomes[36,37]. Furthermore, patients with sarcopenia may experience more severe toxic side effects during chemotherapy, owing to alterations in body composition and muscle loss caused by tumor-specific therapy[38-40]. In the present study, sarcopenia in patients with GC who were undergoing ICI therapy was shown to be associated with both PFS and OS. A previous study similarly showed that sarcopenia is associated with shorter PFS and OS in patients with microsatellite-stable GC being treated with a PD-1 inhibitor[41]. In 2021, Kim *et al*[42] also studied patients with advanced GC who were being treated with navulizumab and pabolizumab. They divided the patients into those with or without sarcopenia and discussed the prognostic value of the neutrophil-to-lymphocyte ratio between the groups[42], but did not evaluate the relationship between sarcopenia and prognosis.

Myosteatosis is characterized by excessive fat accumulation in skeletal muscle and is often used to describe low muscle mass in patients[43]. It has predictive value in patients with various diseases and is consistently associated with poor prognoses, including for colon, liver, and pancreatic cancer[44-47]. Several previous studies of the relationship between immunotherapy and myosteatosis have generated important findings. In patients with metastatic melanoma who were being treated with nivolumab, low SMD was found to be associated with shorter OS[48], and another study of patients being treated with ipilimumab revealed that patients with high SMD experienced more immunity-related adverse events but superior objective responses to treatment, whereas low SMD was found to be associated with a worse prognosis[49]. The skeletal muscle microenvironment plays a critical role in skeletal muscle repair, and this involves monocytes, neutrophils, and lymphocytes. Myosteatosis, which reflects low muscle mass, is associated with impaired muscle repair, leading to compromised immunity[50]. This, in turn, may result in poorer responsiveness to immunotherapy, a higher risk of toxic side effects, and poorer clinical outcomes. However, the specific mechanisms involved and the variations in the effects of myosteatosis in patients with various types of cancer warrant further investigation.

Accumulating evidence indicates the involvement of immune cell mitochondria in the effects of ICIs, as well as in the development of sarcopenia and myosteatosis. Previous studies have shown that cancer cells can appropriate the mitochondria of immune cells, thereby facilitating their survival within the immune microenvironment, the evasion of immune surveillance, and resistance to therapeutic interventions[51]. The commandeering of mitochondria from immune cells, and particularly T cells, has been shown to increase the expression of PD-1, which contributes to its anti-tumor effects[52]. Moreover, compromised mitochondrial function can lead to the overexpression of PD-1 in T cells[53], and the inhibition of the hijacking of mitochondria by immune cells has been demonstrated to improve anti-tumor responses in mice with mammary cancer that were treated with an anti-PD-1 antibody[54]. Notably, a previous study has also shown that muscle loss and muscle fat degeneration are indicative of poor mitochondrial function in muscle cells, which can be extrapolated to other normal human cells, including immune cells[55]. Consequently, the presence of sarcopenia and muscle steatosis may present challenges for successful therapy with ICIs.

There are several important considerations regarding studies of the prognostic implications of sarcopenia and myosteatosis. For instance, there is no well-established value of SMI that can be used in the diagnosis of sarcopenia, and therefore in most studies, ROC-derived cut-off values have been used[22]. This approach, in combination with the use of differing cut-off values in patients with different types of cancer, may affect the identified relationships between muscle-related conditions and clinical outcomes. In the case of myosteatosis, mean SMD is an objective index, but some studies have shown variations in mean SMD according to whether measurements were made using unenhanced contrast-enhanced CT images or those obtained during the arterial or portal venous phases of enhancement. Thus, the CT protocol used can introduce bias into SMD measurements. To minimize such bias, we consistently obtained images during the portal-venous phase. In future investigations of the relationships between muscle conditions and cancer, two key challenges should be addressed: The standardization of CT protocols and the optimal diagnostic criteria for sarcopenia and myosteatosis.

Table 2 Laboratory data for the participants, *n* (%)

Item, mean (SD)	Sarcopenia		*P* value	Myosteatosis		*P* value
	Yes (*n* = 32)	No (*n* = 83)		Yes (*n* = 32)	No (*n* = 83)	
ALT (U/L)	20.31 (16.87)	22.89 (21.41)	0.404	20.07 (15.82)	22.98 (21.70)	0.660
AST (U/L)	27.41 (23.12)	25.99 (18.25)	0.954	29.84 (28.16)	25.05 (15.15)	0.831
γ-GGT (U/L)	66.22 (159.76)	60.37 (88.60)	0.378	97.19 (174.69)	48.43 (72.81)	0.067
ALP (U/L)	115.02 (109.27)	114.35 (67.34)	0.216	116.33 (66.18)	113.84 (85.98)	0.651
TBIL (μmol/L)	14.47 (9.57)	14.44 (8.10)	0.584	14.56 (9.70)	14.40 (8.04)	0.350
DBIL (μmol/L)	3.69 (4.51)	3.18 (2.43)	0.476	3.49 (3.51)	3.26 (3.00)	0.627
IDBIL (μmol/L)	10.79 (5.90)	11.26 (6.19)	0.554	11.08 (6.65)	11.15 (5.90)	0.438
TP (g/L)	70.80 (7.83)	68.31 (7.55)	0.077	69.42 (7.63)	68.84 (7.74)	0.798
ALB (g/L)	39.45 (4.70)	38.61 (6.41)	0.189	37.44 (4.50)	39.38 (6.40)	0.053
GLOB (g/L)	31.30 (4.86)	30.15 (5.00)	0.184	31.93 (4.76)	29.91 (4.96)	0.047
PALB (g/L)	200.44 (66.25)	210.51 (65.47)	0.640	182.16 (59.81)	217.55 (65.33)	0.010
Urea (mmol/L)	5.75 (1.89)	5.94 (1.63)	0.501	6.31 (1.98)	5.73 (1.56)	0.182
CREA (μmol/L)	72.00 (15.17)	79.40 (16.50)	0.013	78.78 (15.45)	76.78 (16.83)	0.375
UA (μmol/L)	297.28 (88.63)	316.28 (92.69)	0.169	318.66 (92.09)	308.04 (91.78)	0.636
LDH (U/L)	229.03 (204.48)	233.55 (213.03)	0.836	301.75 (274.39)	205.52 (173.55)	0.012
WBC (109/L)	7.28 (3.82)	7.59 (5.32)	0.769	7.58 (3.60)	7.48 (5.39)	0.280
NEU (109/L)	4.91 (3.57)	4.72 (2.17)	0.658	5.26 (3.36)	4.59 (2.26)	0.208
Lym (109/L)	1.69 (0.58)	1.60 (0.56)	0.476	1.57 (0.53)	1.65 (0.58)	0.493
Mono (109/L)	0.47 (0.22)	0.53 (0.21)	0.150	0.55 (0.18)	0.49 (0.22)	0.113
Eosi (109/L)	0.12 (0.13)	0.13 (0.11)	0.173	0.13 (0.10)	0.12 (0.12)	0.364
Baso (109/L)	0.03 (0.02)	0.02 (0.01)	0.145	0.03 (0.02)	0.02 (0.01)	0.276
Hb (g/L)	124.03 (21.17)	126.69 (27.63)	0.575	121.03 (30.31)	127.85 (23.97)	0.245
RBC (1012/L)	4.19 (0.66)	4.45 (0.73)	0.103	4.26 (0.80)	4.42 (0.68)	0.363
Plt (109/L)	255.41 (98.83)	256.23 (91.47)	0.967	279.75 (110.41)	246.84 (84.53)	0.213
Fbg (g/L)	3.67 (0.98)	4.04 (2.78)	0.902	4.78 (4.13)	3.61 (1.14)	0.051
DDi (mg/L)	1.54 (2.90)	1.28 (2.14)	0.135	1.83 (3.18)	1.17 (1.96)	0.002

ALT: Alanine transaminase; AST: Aspartate aminotransferase; γ-GGT: γ-glutamyl transferase; ALP: Alkaline phosphatase; TBIL: Total bilirubin; DBIL: Direct bilirubin; IDBIL: Indirect bilirubin; TP: Total protein; ALB: Albumin; GLOB: Globulin; PALB: Pre-albumin; Urea: Urea nitrogen; CREA: Creatinine; UA: Uric acid; LDH: Lactate dehydrogenase; WBC: White blood cell count; NEU: Neutrophil count; Lym: Lymphocyte count; Mono: Monocyte count; Eosi: Eosinophil count; Baso: Basophil count; Hb: Hemoglobin; RBC: Red blood cell count; Plt: Platelet count; Fbg: Fibrinogen; DDi: D-dimer.

In the present study, we found that 27.4% of patients with GC who were undergoing ICI treatment had sarcopenia, 29.8% had myosteatosis, and 10.5% of those with sarcopenia also had concurrent myosteatosis. Furthermore, Kaplan–Meier analysis demonstrated that patients with sarcopenia and/or myosteatosis had shorter PFS and OS. Multivariate analysis identified TP, Eosi, CA724, CA125, sarcopenia, and myosteatosis as independent prognostic factors for OS; and TBIL, DBIL, Eosi, CA125, sarcopenia, and myosteatosis were found to independently affect PFS. We were able to develop models that can accurately predict the prognosis of patients undergoing ICI treatment, with C-indexes of 0.758 for PFS and 0.781 for OS. In the future, the accuracy of the prediction may be improved by using multidimensional integrated analyses based on CT radiomics, body composition, markers of inflammation, and gene expression.

Although valuable insights have been provided by the present study, certain limitations should also be acknowledged. First, it was a retrospective, single-center study, and therefore the results require corroboration by multicenter prospective studies. Second, the inclusion of patients treated with a range of ICI regimens introduced variability regarding treatment efficacy, and therefore the results may need validation using a cohort undergoing a uniform treatment regimen. However, to date, there have been few studies of the prognostic implications of sarcopenia and myosteatosis in patients with GC who are undergoing ICI therapy, and the present study has provided novel insight into the prediction of the prognosis of such patients and has improved understanding of the relevance of these muscle

Table 3 Results of the univariate and multivariate analyses to identify parameters predictive of progression-free survival and overall survival

Parameters	OS				PFS			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	Hazard ratio (95%CI)	*P* value	Hazard ratio (95%CI)	*P* value	Hazard ratio (95%CI)	*P* value	Hazard ratio (95%CI)	*P* value
ALT (U/L)	1.191 (0.680-2.088)	0.541			1.312 (0.748-2.301)	0.343		
AST (U/L)	3.316 (0.457-24.065)	0.236			4.063 (0.560-29.469)	0.165		
γ-GGT (U/L)	0.914 (0.522-1.601)	0.753			0.907 (0.511-1.610)	0.739		
ALP (U/L)	1.673 (0.933-2.998)	0.084			1.962 (1.096-3.513)	0.023	1.540 (0.722-3.282)	0.264
TBIL (μmol/L)	1.578 (0.861-2.893)	0.140			2.108 (1.155-3.848)	0.015	3.752 (1.104-12.750)	0.034
DBIL (μmol/L)	1.245 (0.705-2.197)	0.450			1.337 (0.763-2.343)	0.310		
IDBIL (μmol/L)	1.383 (0.785-2.434)	0.261			1.870 (1.066-3.281)	0.029	0.847 (0.276-2.603)	0.772
TP (g/L)	0.389 (0.165-0.917)	0.031	0.310 (0.126-0.765)	0.011	0.502 (0.214-1.179)	0.114		
ALB (g/L)	0.985 (0.542-1.791)	0.961			1.212 (0.667-2.201)	0.528		
GLOB (g/L)	0.380 (0.118-1.223)	0.105			0.345 (0.107-1.114)	0.075		
PALB (g/L)	0.518 (0.293-0.915)	0.024	0.757 (0.414-1.384)	0.366	0.524 (0.299-0.919)	0.024	0.560 (0.288-1.088)	0.087
Urea (mmol/L)	0.693 (0.362-1.328)	0.269			0.711 (0.369-1.368)	0.307		
CREA (μmol/L)	0.882 (0.486-1.600)	0.679			1.007 (0.557-1.822)	0.981		
UA (μmol/L)	0.571 (0.256-1.275)	0.172			0.641 (0.284-1.446)	0.284		
LDH (U/L)	1.094 (0.556-2.152)	0.794			1.056 (0.543-2.051)	0.873		
WBC (10^9/L)	1.500 (0.835-2.696)	0.175			1.450 (0.809-2.598)	0.212		
NEU (10^9/L)	1.352 (0.728-2.512)	0.339			1.334 (0.719-2.475)	0.360		
Lym (10^9/L)	0.581 (0.323-1.043)	0.069			0.555 (0.308-0.998)	0.049	0.692 (0.345-1.391)	0.301
Mono (10^9/L)	0.727 (0.418-1.262)	0.257			0.730 (0.421-1.267)	0.264		
Eosi (10^9/L)	3.205 (1.520-6.758)	0.002	2.398 (1.116-5.153)	0.025	2.856 (1.374-5.936)	0.005	3.022 (1.341-6.809)	0.008
Baso (10^9/L)	1.151 (0.656-2.019)	0.625			1.408 (0.797-2.489)	0.239		
Hb (g/L)	1.062 (0.604-1.866)	0.835			1.010 (0.576-1.772)	0.973		
RBC (10^{12}/L)	0.951 (0.542-1.667)	0.860			0.946 (0.538-1.666)	0.849		
Plt (10^9/L)	0.578 (0.308-1.085)	0.088			0.590 (0.314-1.110)	0.102		
Fbg (g/L)	0.044 (0.000-	0.282			0.44 (0.000-	0.238		

	12.949)				7.917)			
DDi (mg/L)	1.906 (0.978-3.715)	0.058			1.883 (0.978-3.627)	0.058		
AFP ng/mL	0.830 (0.473-1.454)	0.514			0.871 (0.496-1.529)	0.631		
CEA ng/mL	1.507 (0.796-2.855)	0.208			1.175 (0.622-2.219)	0.620		
CA199 U/L	1.605 (0.927-2.780)	0.091			1.756 (1.015-3.037)	0.044	1.395 (0.719-2.706)	0.325
CA724 U/L	2.051 (1.176-3.578)	0.011	2.459 (1.334-4.534)	0.004	1.846 (1.055-3.228)	0.032	1.951 (1.056-3.606)	0.033
CA125 II U/L	2.408 (1.294-4.482)	0.006	2.763 (1.302-5.862)	0.008	2.735 (1.445-5.179)	0.002	2.419 (1.094-5.348)	0.029
BMI (kg/m^2)	0.533 (0.309-0.921)	0.024	1.165 (0.584-2.322)	0.665	0.467 (0.268-0.815)	0.007	1.232 (0.581-2.610)	0.586
Age (< 53.50 *vs* ≥ 53.50)	0.978 (0.550-1.737)	0.939			1.276 (0.721-2.256)	0.403		
Sex (Male *vs* Female)	1.288 (0.689-2.410)	0.428			1.275 (0.682-2.385)	0.447		
TNM stage (III *vs* IV)	1.586 (0.820-3.067)	0.170			2.044 (1.031-4.052)	0.041	2.313 (1.007-5.317)	0.048
Sarcopenia (Yes *vs* No)	2.896 (1.670-5.021)	< 0.001	3.569 (1.808-7.045)	< 0.001	3.021 (1.737-5.253)	< 0.001	4.036 (1.959-8.316)	< 0.001
Myosteatosis (Yes *vs* No)	2.662 (1.357-5.225)	0.004	3.172 (1.471-6.839)	0.003	2.104 (1.118-3.962)	0.021	2.624 (1.256-5.483)	0.010

ALT: Alanine transaminase; AST: Aspartate aminotransferase; γ-GGT: γ-glutamyl transferase; ALP: Alkaline phosphatase; TBIL: Total bilirubin; DBIL: Direct bilirubin; IDBIL: Indirect bilirubin; TP: Total protein; ALB: Albumin; GLOB: Globulin; PALB: Pre-albumin; Urea: Urea nitrogen; CREA: Creatinine; UA: Uric acid; LDH: Lactate dehydrogenase; WBC: White blood cell count; NEU: Neutrophil count; Lym: Lymphocyte count; Mono: Monocyte count; Eosi: Eosinophil count; Baso: Basophil count; Hb: Hemoglobin; RBC: Red blood cell count; Plt: Platelet count; Fbg: Fibrinogen; DDi: D-dimer; PFS: Progression-free survival; OS: Overall survival.

conditions to the outcomes of immunotherapy.

CONCLUSION

Sarcopenia and myosteatosis, which reflect the body's response to trophic inflammation, are useful predictors of the prognosis of patients with GC who are undergoing treatment with ICI. The clinical course of patients with sarcopenia and myosteatosis has the potential to involve a number of unfavorable outcomes, including shorter PFS and OS. In summary, the evaluation of muscle mass by CT imaging has the potential to yield robust predictors of the prognosis of patients with GC being treated with ICIs.

ARTICLE HIGHLIGHTS

Research background

The evolution and progression of gastric cancer (GC) is closely associated with the nutritional status of patients. The laboratory indices currently used to assess the nutritional status of patients have limitations.

Research motivation

The presence or absence of sarcopenia and myosteatosis are objective indicators of the nutritional status of patients, and muscle mass status influences the effectiveness of immune checkpoint inhibitors (ICIs) therapy.

Research objectives

This study aims to investigate the effects of sarcopenia and sarcopenia on the clinical prognosis of patients with GC being treated with ICIs.

Research methods

We studied 115 patients with GC who underwent ICI therapy between 2016 and 2022. The third lumbar vertebrae skeletal muscle cross-sectional area and the mean skeletal muscle density were assessed using 3D Slicer. We then analyzed the relationships of sarcopenia and myosteatosis with the prognosis of the patients.

Research results

Patients exhibiting sarcopenia and/or myosteatosis demonstrated poorer clinical outcomes, and nomograms formulated on the basis of these conditions had substantial prognostic value.

Research conclusions

The presence of sarcopenia and/or myosteatosis was validated for the prediction of the clinical outcomes of patients with GC undergoing ICI therapy.

Research perspectives

Screening for sarcopenia and myosteatosis should help identify patients with advanced GC who would benefit from treatment with ICIs.

FOOTNOTES

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Author contributions: Deng GM and Song HB contributed equally to this work; Deng GM, Song HB, Du ZZ, Xue YW, Song HJ and Li YZ designed the research study; Deng GM, Song HB, Du ZZ and Li YZ performed the research; Xue YW, Song HJ and Li YZ provided data and funding for the experiment; Deng GM and Song HB analyzed the data and wrote the manuscript; all authors have read and approve the final manuscript.

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ORIGINAL ARTICLE

Observational Study

Mitochondrial dysfunction affects hepatic immune and metabolic remodeling in patients with hepatitis B virus-related acute-on-chronic liver failure

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Abstract

BACKGROUND

Immune dysregulation and metabolic derangement have been recognized as key factors that contribute to the progression of hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF). However, the mechanisms underlying immune and metabolic derangement in patients with advanced HBV-ACLF are unclear.

AIM

To identify the bioenergetic alterations in the liver of patients with HBV-ACLF causing hepatic immune dysregulation and metabolic disorders.

METHODS

Liver samples were collected from 16 healthy donors (HDs) and 17 advanced HBV-ACLF patients who were eligible for liver transplantation. The mitochondrial ultrastructure, metabolic characteristics, and immune microenvironment of the liver were assessed. More focus was given to organic acid metabolism as well as the function and subpopulations of macrophages in patients with HBV-ACLF.

RESULTS

Compared with HDs, there was extensive hepatocyte necrosis, immune cell infilt-

ration, and ductular reaction in patients with ACLF. In patients, the liver suffered severe hypoxia, as evidenced by increased expression of hypoxia-inducible factor-1α. Swollen mitochondria and cristae were observed in the liver of patients. The number, length, width, and area of mitochondria were adaptively increased in hepatocytes. Targeted metabolomics analysis revealed that mitochondrial oxidative phosphorylation decreased, while anaerobic glycolysis was enhanced in patients with HBV-ACLF. These findings suggested that, to a greater extent, hepatocytes used the extra-mitochondrial glycolytic pathway as an energy source. Patients with HBV-ACLF had elevated levels of chemokine C-C motif ligand 2 in the liver homogenate, which stimulates peripheral monocyte infiltration into the liver. Characterization and functional analysis of macrophage subsets revealed that patients with ACLF had a high abundance of $CD68^+$ $HLA\text{-}DR^+$ macrophages and elevated levels of both interleukin-1β and transforming growth factor-β1 in their livers. The abundance of $CD206^+$ $CD163^+$ macrophages and expression of interleukin-10 decreased. The correlation analysis revealed that hepatic organic acid metabolites were closely associated with macrophage-derived cytokines/chemokines.

CONCLUSION

The results indicated that bioenergetic alteration driven by hypoxia and mitochondrial dysfunction affects hepatic immune and metabolic remodeling, leading to advanced HBV-ACLF. These findings highlight a new therapeutic target for improving the treatment of HBV-ACLF.

Key Words: Acute-on-chronic liver failure; Hypoxia-inducible factor-1α; Mitochondria; Metabolic phenotype; Immune cells

Core Tip: Our data were obtained from liver of patients with hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF), whose mitochondrial function, metabolites, and immune microenvironment were less susceptible to any confounding factors caused by other failing organs. Widely infiltrating macrophages were originated from peripheral circulating monocytes in the liver of patients with HBV-ACLF. Mitochondrial oxidative phosphorylation was decreased, and anaerobic glycolysis was enhanced in patients with HBV-ACLF. Liver of patients made greater use of the extra-mitochondrial glycolytic pathway for providing energy. Bioenergetic alteration driven by hypoxia and mitochondrial dysfunction contribute to hepatic immune and metabolic remodeling, may leading to organ failure and poor clinical prognosis in patients with advanced HBV-ACLF.

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INTRODUCTION

Acute-on-chronic liver failure (ACLF) is a syndrome characterized by acute decompensation of chronic liver disease associated with organ failure and high short-term mortality. Hepatitis B virus (HBV)-related ACLF is the most common type of liver failure in the Asia-Pacific region[1]. It is characterized by excessive immune response due to HBV reactivation. HBV-ACLF leads to acute hepatic decompensation in patients with chronic liver disease or cirrhosis[2]. The excessive immune response is caused by molecular patterns that are associated with bacterial pathogens and damage, which activate pattern recognition receptors of the innate immune system. The core immune mechanisms in HBV-ACLF involve the activation of the innate immune system and impaired adaptive immune response[2].

The interactions between HBV reactivation, immune dysregulation, and inflammatory response form an intricate network that iad the pathogenesis of ACLF. Each of these processes has a tremendous demand for energy and nutrients. Therefore, the liver needs to adjust its metabolic patterns to generate sufficient energy for supporting viral replication, inflammatory response, and immune cell activation. Dysregulation of this process causes metabolic disturbance and energy crisis, which might result in organ failure. Recent transcriptome and metabolomics studies have shown that there are significant changes in metabolic pathways, including lipid metabolism, fatty acid metabolism, and oxygen homeostasis, in all stages of HBV-ACLF[2]. Decreased oxidative phosphorylation and increased fatty acid β-oxidation were also observed in HBV-ACLF[3]. Mitochondria are the central hubs of energy production and metabolic processes that mainly regulate oxidative phosphorylation and produce ATP. Remodeling mitochondrial metabolism is essential for regulating innate immunity and inflammatory response. Mitochondrial dysfunction is a hallmark of ACLF, which controls the metabolism of leukocytes in patients with acute decompensated cirrhosis and ACLF[4]. Profound alterations in metabolic pathways have been observed in patients with HBV-ACLF. It has also been noted that mitochondrial dysfunction might reprogram energy metabolism in ACLF.

We hypothesized that mitochondrial structure and function are altered in patients with HBV-ACLF, causing a shift in cellular energy metabolism, metabolic patterns, and immune response in patients with ACLF. In this study, the tissue levels of organic acid metabolites and innate immune cells in patients with advanced HBV-ACLF were measured. The mitochondrial ultrastructure was assessed using transmission electron microscopy (TEM). Biomarkers of mitochondrial dysfunction were measured to monitor mitochondrial impairment.

MATERIALS AND METHODS

Patients

The study used liver tissue samples from 17 patients with HBV-ACLF (a group hereafter called "ACLF") and 16 healthy donors (HDs) at The Second Xiangya Hospital of Central South University. Patients with HBV-ACLF met the diagnostic criteria for ACLF suggested by the China Medical Association[5]. Based on this criterion, reactivation of hepatitis B virus causes progressive acute jaundice and coagulation dysfunction, which can be accompanied by hepatic encephalopathy, ascites, electrolyte imbalance, infection, hepatorenal syndrome, and the hepatopulmonary syndrome. Other symptoms and complications, such as extrahepatic organ failure, may also exist. The jaundice rapidly progresses, with a serum total bilirubin (TBIL) ≥ 10 × upper limit of normal or a daily increase of ≥ 17.1 μmol/L. Hemorrhagic manifestations present with prothrombin time (PT) activity (PTA) ≤ 40% [or international normalized ratio (INR) ≥ 1.5]. The inclusion criteria used in this study were as follows: (1) Patients with HBV-ACLF eligible for liver transplantation; (2) model for end-stage liver disease (MELD) score more than or equal to 15[5]; and (3) 18–70-year-old male or female. The exclusion criteria were as follows: (1) Super-infection or co-infection with other hepatotropic and non-hepatotropic viruses; (2) previous application of any immunomodulatory agents or cytotoxic/immunosuppressive drugs within the last three months; (3) hepatocellular carcinoma or extrahepatic malignancies; and (4) co-existence of other liver diseases, such as alcoholic liver disease, Wilson disease, drug-induced liver injury, or autoimmune hepatitis.

All liver tissues were obtained after liver transplantation. Donor livers were lavaged and trimmed before transplantation. The native liver tissues were collected after trimming, before they were washed with an ice-cold PBS solution. The study protocol was approved by the Ethics Committee of the First Hospital of Hunan University of Chinese Medicine (No. HN-LL-SWST-15), and written informed consent was obtained from all participants.

Clinical data collection

Demographic and laboratory data were collected, including age, gender, white blood cell (WBC), neutrophil count, platelet count (PLT), TBIL, aspartate transaminase (AST), alanine transaminase (ALT), albumin (ALB), creatinine, blood urea nitrogen, PT, INR, PTA, hypersensitive C-reactive protein (hs-CRP), procalcitonin (PCT), lactate (LAC), and erythrocyte sedimentation rate. ACLF risk scores were calculated. All laboratory data were collected from the most recent examinations before surgery.

Collection of liver tissue homogenate and enzyme-linked immunosorbent assay

To collect tissue homogenate, surgically excised liver tissues were freshly harvested and cut into small pieces after PBS (0.01 M, pH 7.4) flushing. The 0.1 g liver tissues were transferred into a glass homogenizer with 0.9 mL of pre-cooled PBS. After the liver tissues were thoroughly ground to homogenization, the supernatant was collected and filtered through a 0.22-μm filter to remove any impurities before detection.

Following the manufacturers' instructions (Adsbio, Jiangsu, China), ELISA kits were used to detect the concentrations of growth differentiation factor 15 (GDF15; 2305H16), fibroblast growth factor 21 (FGF21; 2305H22), interleukin-1β (IL-1β; 2305H19), tumor necrosis factor-α (TNF-α; 2305H12), interleukin-10 (IL-10; 2305H25), transforming growth factor β (TGF-β; 2305H33), and chemokine C-C motif ligand 2 (CCL2; 2305H36) in liver tissue homogenate.

Histopathology

Surgically excised liver tissues were kept in 4% paraformaldehyde. Fixed liver tissues were embedded in paraffin, cut into 3 μm sections, and stained with H&E. The samples were finally imaged and scored under light microscopy. The histopathological scores were assessed based on the "Guideline for diagnosis and treatment of liver failure (2018)." The ACLF pathological score was given according to the extent of liver tissue necrosis. Normal liver tissue structure received a score of 0; spotty necrosis, fusion necrosis, and bridging necrosis received a score of 2; submassive hepatic necrosis affecting approximately 1/2-2/3 of hepatic parenchyma received a score of 3; and massive hepatic necrosis affecting more than 2/3 of the hepatic parenchyma received a score of 4.

Immunohistochemical staining

Immunohistochemical staining of liver sections was performed. Several 3-μm sections were prepared from paraffin-embedded blocks of tissue. Sections were dewaxed, rehydrated by fractionated alcohol series, and heated in a microwave oven for 5 min in 10 mm sodium citrate (pH 6.0) to recover antigens. After antigen retrieval, paraffin sections were placed in 3% H_2O_2 and blocked for 10 min to eliminate endogenous peroxidase activity. Then, the samples were blocked and incubated with 10% goat serum for 30 min. The rabbit anti-human HIF-1α (1:200, Bioss, Beijing, China) was added as the primary antibody and incubation was done overnight at 4 °C, in a humidified box. Then, the secondary antibody was added dropwise before incubation at 37°C for 30 min. DAB was added as the substrate to enhance color development. When the color change was observed, the staining solution was immediately washed off with tap water. Hematoxylin

was counterstained for 3 min, differentiated with 1% hydrochloric acid and alcohol, and rinsed with tap water for 10 min. Gradient alcohol dehydration was performed. Xylene was transparent, and the film was mounted with a neutral gum. The number of HIF-1α positive cells was quantified, and images were captured under a microscope. The Image J software (NIH Image, Bethesda, MD, United States) was used for analysis.

Immunofluorescence staining

For double immunofluorescence staining, paraffin-embedded tissues were cut into 3.5 mm sections. Each section was deparaffinized and blocked with EDTA (pH 8.0) antigen repair solution (1:49, Aifang, Changsha, China) at 96°C for 20 min. Then, the sections were incubated overnight at 4°C with the following primary antibodies: Mouse anti-human CD68 (1:200, Aifang, Changsha, China), mouse anti-human Ki67 (1:200, Boster, Wuhan, China), rabbit anti-human HLA-DR (1:300, Abcam, Cambridge, United Kingdom), rabbit anti-human CD206 (1:200, Aifang, Changsha, China), and mouse anti-human CD163 (1:150, Aifang, Changsha, China). The samples were then incubated with FITC tag goat-anti-rabbit IgG (1:500), FITC tag goat-anti-mouse IgG (1:500), cy3 tag goat-anti-rabbit IgG (1:500), and cy3 tag goat-anti-mouse IgG (1:500) for 1 h. The sections were slightly dried and incubated with the DAPI staining solution (Solarbio, Beijing, China) at room temperature and in the dark, for 10 min. After DAPI staining for 5 min, the sections were washed with PBS three times. Each of the three washing procedureswas done for 5 min. The washed sections were then sealed with an anti-fluorescence attenuated sealing solution that contained DAPI. Fluorescence images were scanned using a digital pathology scanner (KF-FL-020, Zhejiang, China) and images were captured. The results were analyzed using the Image J software (NIH Image, Bethesda, MD, United States).

TEM

The liver tissue was dissected into 1-mm pieces and fixed in 4% paraformaldehyde and 0.1 M sodium cacodylate buffer (pH 7.2) containing 2% glutaraldehyde, at 4°C overnight. After three times of washing in buffer, the samples were post-fixed in 2% osmium tetroxide and 1% uranyl acetate for 2 h, rinsed in water, dehydrated in ascending ethanol series and 100% acetone, before it was infiltrated and embedded in eponate. Ultrathin sections were cut using a Leica EM UC7. The sections were exposed to the primary stain (5% aqueous uranyl acetate). They were then exposed to the secondary stain (lead citrate) and visualized using a 120 kv TEM HT7800 (Hitachi, Japan). Five random fields of view were imaged per group to quantify the size and number of mitochondria. Mitochondria were identified based on their morphology. Mitochondrial length, width, and cross-sectional area were measured using Image Pro-Plus 6.0 software (Media Cybernetics, Silver Spring, MD, United States). Mitochondrial count analysis was performed at the original magnification of 7000 .

Targeted metabolomics

Ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system (Waters XEVO TQ-S Micro, Waters Corporation) was used for targeted metabolomics analysis of 16 organic acids in liver samples. Briefly, 17 liver samples from patients with HBV-ACLF and 12 liver samples from HDs were collected and stored in an Eppendorf Safelock microcentrifuge tube. The centrifuge tube was placed in a low-temperature centrifuge and centrifuged at 12000 rpm at 4 °C for 5 min. Then, 50 μL of supernatant were taken and 50 μL of propionic acid isotope internal standard (IS; 5 μg/mL) and 50 μL of 3-nitrophenylhydrazine (250 mmol/L, prepared with 50% methanol/aqueous solution) were added to it. Thereafter, 50 μL of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (150 mmol/L, prepared with 75% methanol/aqueous solution (containing 7.5% pyridine)) was added and placed in a shock mixer at 30°C for 30 min. The supernatant was extracted by adding 50 μL of 2, 6-di-tert-butyl-p-cresol methanol solution (2 mg/mL) and 250 μL of 75% methanol solution at 12000 rpm for 5 min, and then used for LC-MS analysis.

Chromatographic separation was performed using Waters ACQUITY UPLC I-CLASS ultra-high performance liquid chromatography with a Waters UPLC BEH C18 column (2.1 mm × 100 mm I.D., 1.7 μm; Waters Corp., Milford, MA, United States) at a column temperature of 40°C. The mobile phase was composed of 0.1% formic acid aqueous solution (A). The methanol: Isopropyl alcohol ratio was 8:2 (B). The flow rate was 0.30 mL/min, and the injection volume was 5 μL.

Multireaction monitoring of 16 organic acids (IS) was performed using the Waters XEVO TQ-S Micro series four-bar mass spectrometry system. The optimal Mass Spectrometric parameters were as follows: Ion source voltage was 3.0 kV, temperature was 150°C; desolvation temperature was 450°C, desolvation gas flow rate was 1000 L/h; cone-hole gas flow rate was 10 L/h.

Data acquisition and processing were conducted using the MassLynx 4.1 software (Waters Corp., Milford, MA, United States), and the concentration of each metabolite was calculated based on the standard curve. The calculated concentrations of organic acids were imported into the SIMCA software (v. 14.1, Umetrics, Sweden) for multivariate analyses, including orthogonal partial least squares-discriminant analysis (OPLS-DA) and replacement test. Finally, differential metabolites were used for pathway enrichment analysis.

Statistical analysis

Statistical analyses were performed using the SPSS software (ver. 21.0; IBM Corp., Armonk, NY, United States), and the figures were produced using Prism 8.0 (GraphPad Software, San Diego, CA, United States). All values are presented as mean ± SE of mean, median (interquartile range), or numbers (%). For single comparisons, the unpaired Student's *t*-test was used for data with normal distribution, and the Mann-Whitney test was used for data with non-normal distribution. Categorical variables were compared using chi-squared tests. The Pearson test was used for determining the correlations for normally distributed variables. The Spearman test was applied to measure the correlations of non-normal variables. A

P value of less than 0.05 indicated statistical significance.

RESULTS

Baseline characteristics

Table 1 summarizes the baseline characteristics, demographic, and laboratory features of patients. The two groups were comparable with regard age and sex, and all patients in the ACLF group were male. All patients with ACLF had liver cirrhosis, and five cases in the HD group had metabolic associated fatty liver disease. Compared with the HD group, the ACLF group had lower levels of WBC, PLT, ALB, sodium, and PT, but higher levels of TBIL, AST, ALT, and INR. Moreover, the PTA of the ACLF group was lower than the lower limit of normal, and the hs-CRP, PCT, LAC, and NH3 were more than the upper limit of normal. The mean MELD score of patients in the ACLF group was 28.65, with each individual having a MELD score of greater or equal to 19, suggesting a high risk of end-stage liver disease. In the ACLF group, the mean MELD-Na score was 32.83, and the mean COSSH-ACLF II score was 7.69. More than 65% of patients with ACLF were exposed to an intermediate to high risk of end-stage liver disease (Table 1).

Liver histopathology

Under the light microscope, the following observations were made: the structure of the liver tissue of some HDs was complete and clear; the structure of liver lobules was clear and normal; the volume of hepatocytes was uniform; the nuclei were in the middle; and the cytoplasm was red-stained. The hepatocytes were arranged radially around the central vein, and there was no sign of necrosis, steatosis, fibroplasia, and inflammatory infiltration. The liver of the others showed diffuse fatty lesions, disordered lobular structure, deranged hepatic cords, ballooned or swollen hepatocytes, loose cytoplasm, diffuse lipid droplets of different sizes in the cytoplasm, and a small number of punctate necrosis. Pathologic assessment of the liver in patients with ACLF showed destruction of the normal structure as well as collapse or incomplete collapse of reticular scaffolds. The liver tissue showed massive or submassive necrosis, along with abundant inflammatory cell infiltration. The percentage of normal hepatocytes significantly decreased. Hepatic sinusoids were significantly dilated, congested, and even hemorrhagic. Circular or triangular portal areas were observed around necrosis. Massive bile ducts, in the form of tubes or branches, were observed around the edge of the necrotic zone and the confluence area. Cholestasis and bile plugs were seen in some of the lumens. The hepatic lobules were structurally disorganized, with massive extracellular matrix hyperplasia. Residual hepatocytes were wrapped and segmented into nodules of varying sizes by fibrous septae. Hepatocytes in the residual nodules showed various degrees of hepatocyte ballooning. Some hepatocytes were eosinophilic, and apoptotic bodies and binucleated hepatocytes were visible (Figure 1A). Histopathological scoring showed that hepatocyte necrosis markedly increased in the liver of patients with ACLF compared with the HDs, and the difference was statistically significant (Figure 1B).

Upregulated hypoxia markers in patients with ACLF

The transcription factor HIF-1α mediates the adaptive response to hypoxia. In normoxic conditions, HIF-1α is hydroxylated and rapidly degraded. Hypoxia upregulates HIF-1α and promotes its translocation to the nucleus to form a complex with HIF-1β. This promotes the transcription of genes that are essential for hypoxic adaptation[6]. The expression of key mediators of hypoxia in the liver samples was examined through immunohistochemistry to assess whether patients with ACLF suffered from anoxia whammy. The results from immunohistochemical staining demonstrated that the expression of HIF-1α was elevated in the cytoplasm and nucleus of hepatocytes in patients with ACLF compared with the HDs. This showed significant hypoxic injury in patients with ACLF (Figure 2).

Altered mitochondrial ultrastructure in the liver of patients with ACLF

TEM images with different magnifications are shown in Figure 3A. In HDs, mitochondrial morphology, cristae, and matrix were intact and orderly arranged. Compared to normal mitochondrial conformation in HDs, mitochondrial ultrastructure was severely disorganized in the liver of patients with ACLF. Some mitochondria were severely swollen and transformed into vacuolated structures with abnormal mitochondrial morphology. The mitochondrial outer membrane was mostly incomplete, while the cristae were sparse, disorganized, or absent. The density of the mitochondrial matrix was lower and filled with an electron-dense material in patients with ACLF, suggesting extensive mitochondrial degeneration. A small number of autophagosomes and autophagic lysosomes were seen in the liver of patients with ACLF (Figure 3A). Compared with HDs, the liver of patients had an increased number of mitochondria in each microscopic field. The length, width, and area of the mitochondria also increased (Figure 3B and C), as part of the repair response after liver injury.

Mitochondrial dysfunction in patients with ACLF

TEM showed severe ultrastructural damage of mitochondria in the livers of patients with ACLF. GDF15 and FGF21 Levels in tissue homogenates were examined to reveal mitochondrial dysfunction. GDF15 and FGF21 are circulating markers for mitochondrial disorders and are widely used in diagnosis of mitochondrial diseases[7-9] and other serious diseases, such as liver failure[4,10,11], heart failure[12,13], and sepsis[14,15]. Their expression levels positively correlated with disease severity. The expression of GDF15 in liver homogenates of patients with ACLF increased compared to the HD group (Figure 3D). This revealed mitochondrial dysfunction in patients with ACLF. Nevertheless, FGF21 Levels were significantly lower in patients with ACLF compared to the HDs (Figure 3E). In contrast, plasma FGF21 levels were higher

Table 1 Baseline characteristics of the included patients

Variable	HD (*n* = 16)	ACLF (*n* = 17)	*P* value
Age (yr)	47.24 ± 2.01	52.18 ± 4.16	0.2964
Gender [male, *n* (%)]	14 (87.5)	17 (100)	0.227
Other liver disorders, *n* (%)			
Cirrhosis	0 (0)	17 (100)	< 0.0001
MAFLD	5 (31.25)	0 (0)	0.044
None	11 (68.75)	0 (0)	< 0.0001
Laboratory measurements			
WBC ($\times 10^9$)	14.01 (7.69)	7.86 ± 1.07	< 0.0001
NEUT (%)	78.20 (19.50)	71.90 (14.95)	0.1705
PLT ($\times 10^9$)	199.44 ± 26.26	70.00 ± 8.80	0.0002
TBIL (μmol/L)	10.16 ± 1.01	332.05 ± 51.60	< 0.0001
AST (IU/L)	36.00 (35.55)	94.50 (83.15)	0.0106
ALT (IU/L)	24.00 (41.05)	67.50 (67.8)	0.0281
ALB (g/L)	42.98 ± 0.86	37.25 ± 1.26	0.0007
Sodium (mmol/L)	142.32 ± 1.43	135.40 ± 1.48	0.0023
CREA (μmol/L)	71.00 (28.40)	64.00 (39.50)	0.8717
BUN (mmol/L)	5.69 (3.24)	5.40 (6.30)	0.8094
PT (S)	12.29 ± 0.36	27.36 ± 2.28	< 0.0001
INR	1.02 ± 0.04	2.56 ± 0.26	< 0.0001
PTA (%)	ND	28.00 (31.00)	ND
hs-CRP (mg/L)	ND	8.41 (8.73)	ND
PCT (ng/mL)	ND	0.51 (0.37)	ND
LAC (mmol/L)	ND	2.78 ± 0.21	ND
NH_3 (μmol/L)	ND	84.9 ± 10.19	ND
ESR (mm/h)	ND	10.00 (9.5)	ND
ACLF risk scores			
MELD score	ND	28.65 ± 1.42	ND
MELD category, *n* (%)			
1: ≥ 40	ND	1 (5.9)	ND
2: 30-39	ND	8 (47.1)	ND
3: 20-29	ND	7 (41.1)	ND
4: 15-19	ND	1 (5.9)	ND
MELD-Na (score)	ND	32.83 ± 2.42	ND
COSSH-ACLF II score	ND	7.69 ± 0.30	ND

All values are expressed as mean ± SEM, median (IQR), or number (percentages). Data with normal distribution were compared using unpaired student's *t* -test, and the Mann-Whitney test was used for data without non-normal. Categorical variables were compared using chi-squared tests.
MAFLD: Metabolic associated fatty liver disease; NEUT: Neutrophilic granulocyte; PLT: Platelet; TBIL: Total bilirubin; AST: Aspartate transaminase; ALT: Alanine transaminase; ALB: Albumin; CREA: Creatinine; BUN: Blood urea nitrogen; PT: Prothrombin time; INR: International normalized ratio; PTA: Prothrombin time activity; hs-CRP: Hypersensitive C-reactive protein; PCT: Procalcitonin; LAC: Lactate; ESR: Erythrocyte sedimentation rate; MELD: Model for end-stage liver disease; MELD-Na: Model for end-stage liver disease includes serum sodium; COSSH-ACLF II: Chinese group on the study of severe hepatitis B- acute-on-chronic liver failure II; HD: Healthy donor; ACLF: Acute-on-chronic liver failure; ND: Not determined.

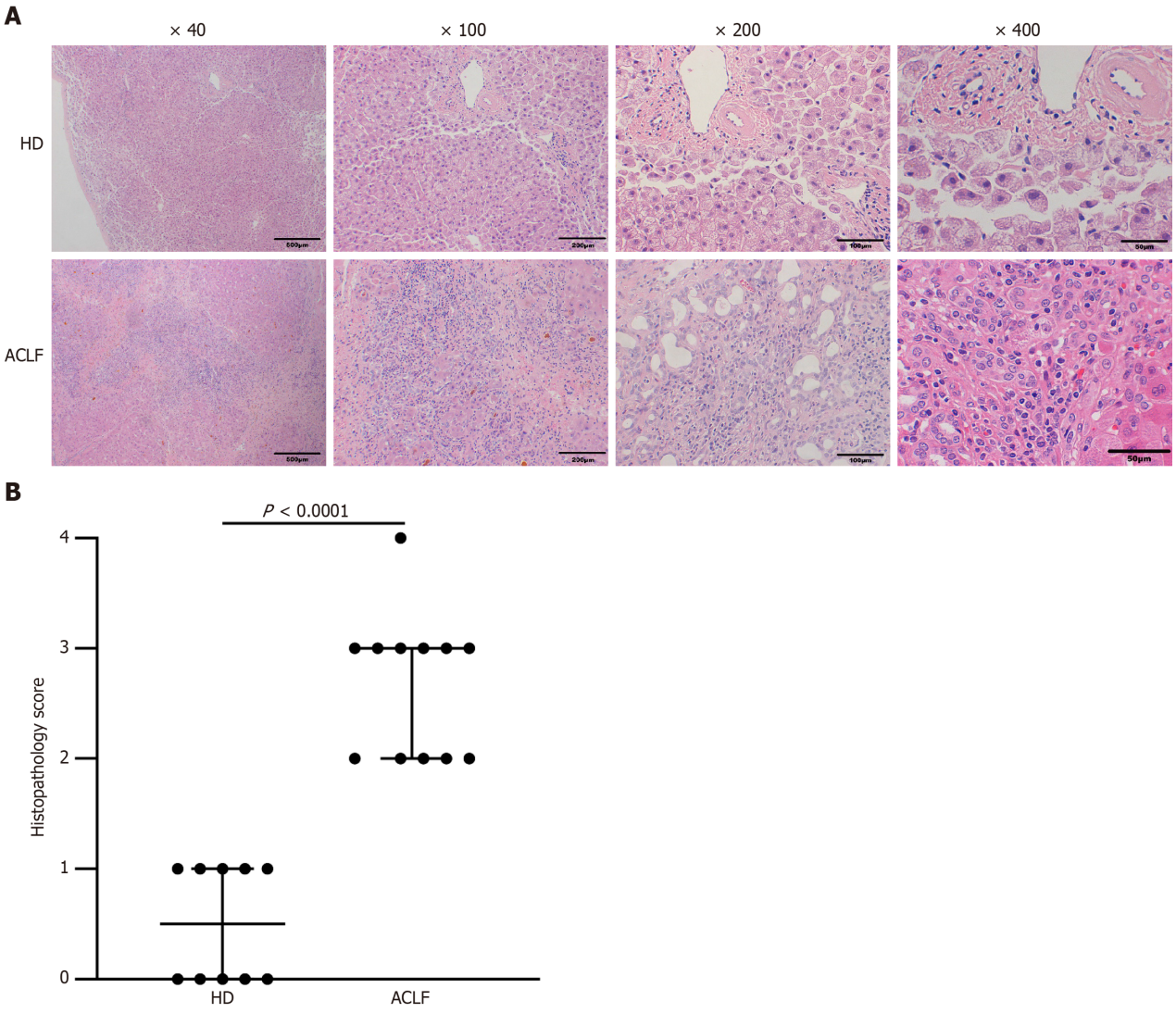


Figure 1 Liver histopathology in healthy donors and patients with acute-on-chronic liver failure. A: HE staining of the liver tissue from healthy donors (n = 10) and patients with acute-on-chronic liver failure (n = 12); B: Histopathological Scoring. Data were compared using Mann-Whitney test. ACLF: Acute-on-chronic liver failure; HD: Healthy donor.

in patients with ACLF in some studies[6,11]. Some studies reported that plasma levels of FGF21 were not associated with the severity of ACLF[11].

Metabolic profiles of liver in patients with ACLF

Mitochondria drive metabolic adaption to environments. This study investigated whether mitochondrial dysfunction in patients with ACLF was accompanied by metabolic change. Therefore, the levels of organic acid metabolites in tissues of HDs and ACLF patients were determined through targeted metabolomics. A total of 16 different types of organic acids were quantified. The concentrations of the 16 organic acids were imported into the SIMCA software for OPLS-DA. The results are shown in the OPLS-DA scatter diagram (Figure 4A). The OPLS-DA model effectively differentiated between HD and ACLF samples, suggesting that there were differences in total organic acid metabolites between HDs and patients with ACLF, with lower inter-sample variability among patients with ACLF. The 200 permutation tests of our data demonstrated no overfitting in the OPLS-DA model [Q2 = (0.0, −0.474); Figure 4B]. The hierarchical cluster analysis showed that the 16 organic acids could differentiate between ACLF patients and HDs (Figure 4C). Volcano plots were used to identify fumarate, methylmalonic acid, succinate, α-ketoglutaric acid, LAC, phenylacetic acid, and ethylmalonic acid as differential metabolites (Figure 4D).

Statistical analysis of data from targeted metabolomics revealed seven differential organic acids. Compared with HDs, fumarate and methylmalonic acid levels decreased in patients with ACLF, whereas succinate, α-ketoglutaric acid, LAC, phenylacetic acid, and ethylmalonic acid levels increased in these patients (Figure 4E). The LAC /pyruvate ratio, a surrogate marker for the cytosolic NADH/NAD^+ ratio and a hallmark of the redox state[16] was also calculated. The LAC /pyruvate ratio was significantly elevated in patients with ACLF compared to HDs (Figure 4E), suggesting enhanced anaerobic glycolysis. The liver in patients with ACLF patients used the extra-mitochondrial pathways to generate ATP. The seven differential organic acids were imported into the MetaboAnalyst website (https://www.metaboanalyst.ca/) for metabolic pathway enrichment analysis. The pathway enrichment analysis identified six pathways (P < 0.05) enriched

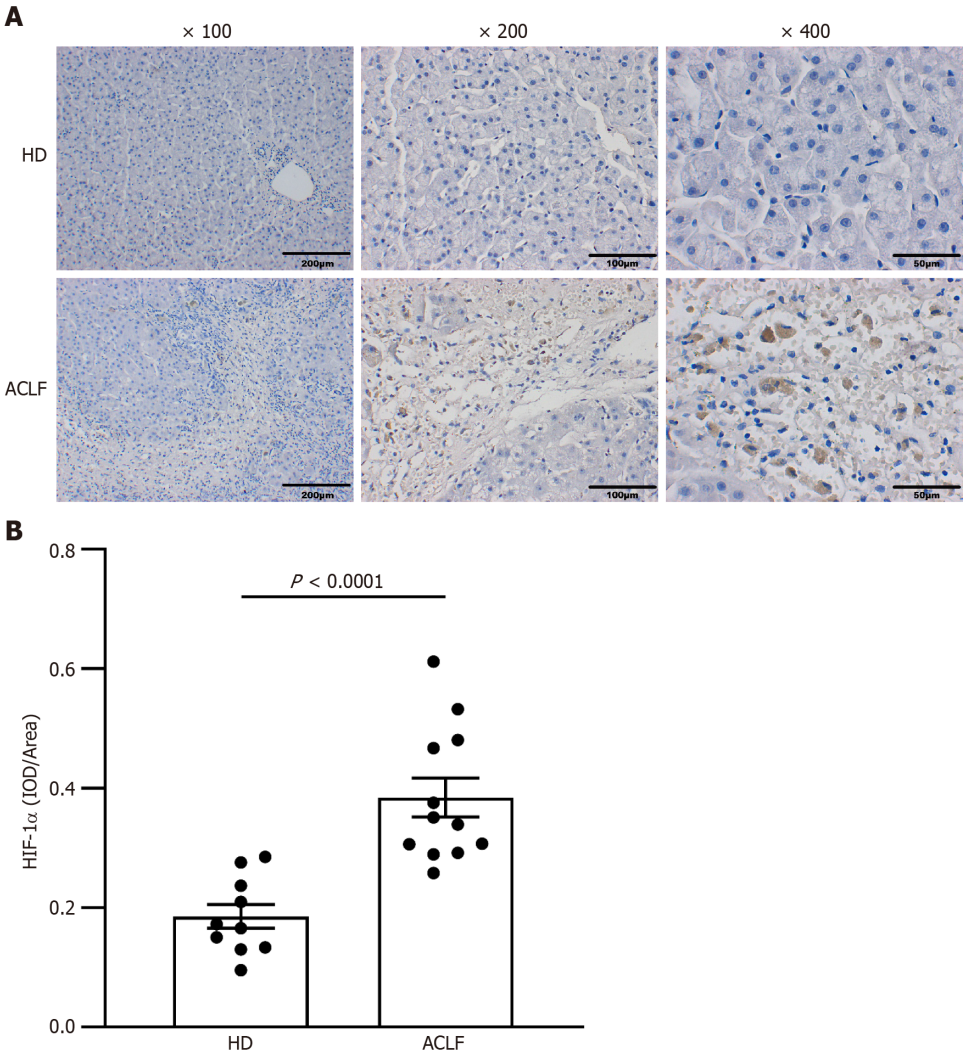


Figure 2 Expression of hypoxia-inducible factor-1α increased in the liver tissues of patients with acute-on-chronic liver failure. A: Immunohistochemical staining of hypoxia-inducible factor-1α (HIF-1α); B: Semi-quantitative analysis of HIF-1α expression. Data were compared using unpaired student's *t*-test. ACLF: Acute-on-chronic liver failure; HD: Healthy donor; HIF-1α: Hypoxia-inducible factor-1α.

in ACLF (Figure 4F). These pathways play key roles in glucose metabolism, fatty acid metabolism, and amino acid metabolism.

Widespread macrophages activation in the liver of patients with ACLF

Mitochondrial dysfunction affected liver metabolism in patients with ACLF. Double immunofluorescence staining with anti-ki67 and anti-CD68 antibodies was used to label proliferating macrophages and determine whether metabolic transformation affected hepatic macrophages (Figure 5A). There was no statistically significant difference in cell proliferation ($Ki67^+$) between HDs and patients with ACLF (Figure 5B). However, there was significant macrophage ($CD68^+$) infiltration in the liver of ACLF patients (Figure 5C). Double immunofluorescence staining with Ki67 and CD68 showed no significant differences in the number of proliferating macrophages between the groups (Figure 5D), suggesting that the infiltrating macrophages originated from peripheral circulating monocytes rather than liver-resident macrophages (*i.e.*, Kupffer cells). CCL2, a key chemokine that regulates monocyte/macrophage migration and infiltration, was overexpressed in the liver of patients with ACLF (Figure 5E), suggesting an increased capacity of circulating monocytes for hepatic infiltration in patients with ACLF.

Impaired macrophage polarization in the liver of patients with ACLF

Macrophages can acquire different phenotypes depending on environmental and immune signals. They are mainly classified into two major groups: (1) Classically activated macrophages endowed with pro-inflammatory and microbicidal functions and (2) alternatively activated macrophages with anti-inflammatory and tissue remodeling properties. The classically activated macrophages ($CD68^+$ $HLA\text{-}DR^+$) and alternatively activated macrophages ($CD163^+$ $CD206^+$) were labeled *via* double immunofluorescence staining (Figure 6A and B) to determine whether there was a phenotypic change in hepatic macrophages. The results showed that the abundance of $CD68^+$ $HLA\text{-}DR^+$ macrophages increased (Figure 6C), while the abundance of $CD163^+$ $CD206^+$ macrophages decreased in patients with ACLF compared to HDs (Figure 6D). Macrophages were polarized toward the classically activated phenotype (Figure 6E). The results from

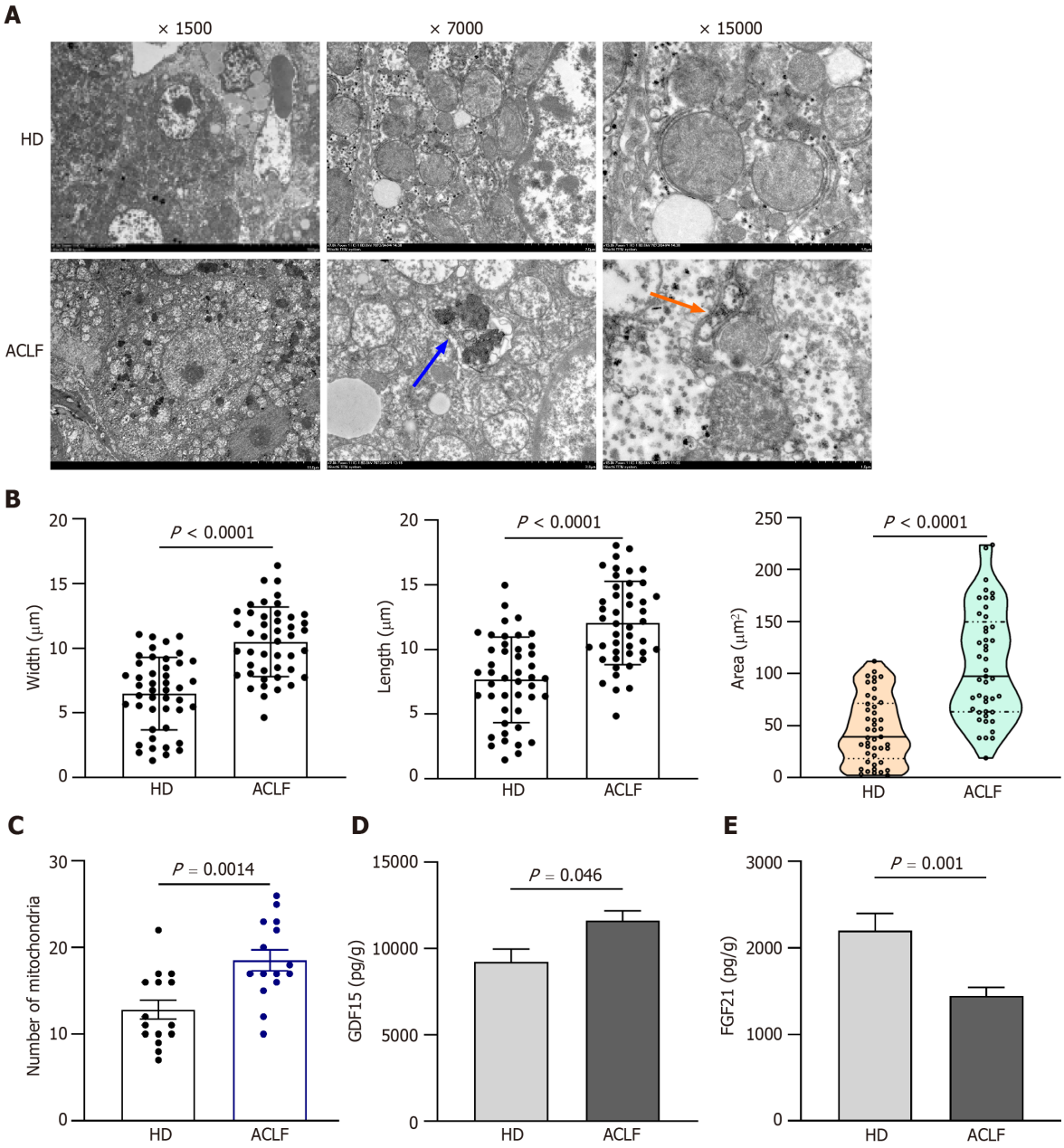
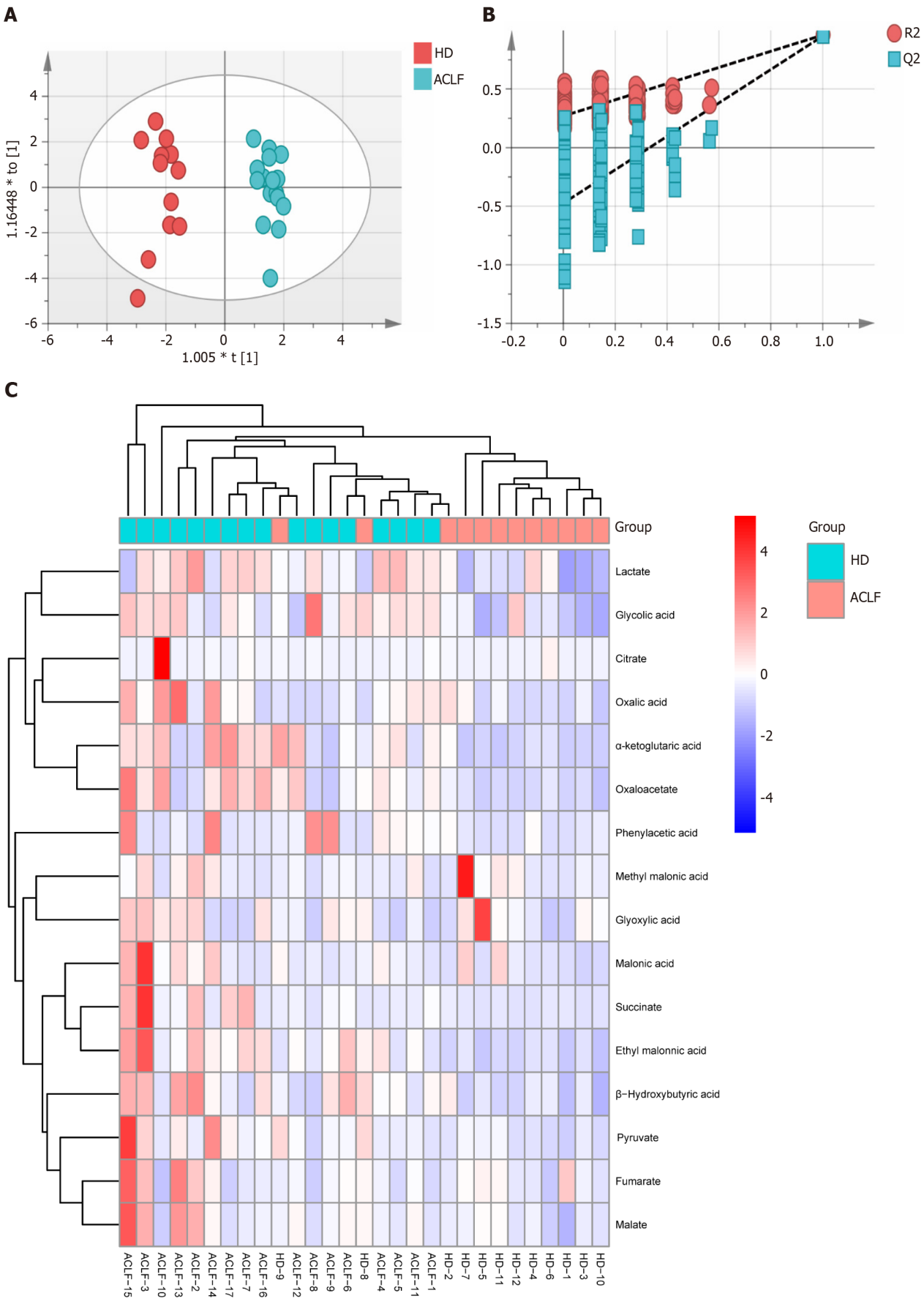


Figure 3 Mitochondrial morphology and function in the liver of patients with acute-on-chronic liver failure. A: Representative electron microscopy images of hepatic mitochondria from 3 patients with acute-on-chronic liver failure (ACLF) and 3 healthy donors (HDs). Orange arrows indicate autophagosome; the blue arrow indicates autophagic lysosome; B: Length, width, and area of each mitochondrion; C: Number of mitochondria at original magnification of 7000 ; D: Growth differentiation factor 15 levels in liver homogenates of 12 HDs and 17 patients with ACLF; E: Fibroblast growth factor 21 levels in liver homogenates. Data with normal distribution were compared using unpaired student's *t*-test, and the Mann-Whitney test was used for non-normal data. ACLF: Acute-on-chronic liver failure; HD: Healthy donor; GDF15: Growth differentiation factor 15; FGF21: Fibroblast growth factor 21.

the cytokine assay were consistent with those from immunofluorescence. Compared with HDs, patients with ACLF exhibited elevated expression of IL-1β and TGF-β1 (Figure 6F). IL-1β is a $CD68^+$ $HLA\text{-}DR^+$ macrophage-derived pro-inflammatory cytokine and TGF-β1 is a potent pro-fibrotic factor. As a $CD163^+$ $CD206^+$ macrophage-derived anti-inflammatory cytokine, IL-10 was downregulated in patients with ACLF (Figure 6F). There was no significant difference in TNF-α levels between the groups (Figure 6F).

The relationship between metabolic remodeling and macrophage activation

Macrophages undergo extensive metabolic rewiring upon activation, increasing the abundance of specific metabolites [17]. They act as co-factors for enzymes and posttranslationally modify histones. They also affect the function of transcription factors, enzymes, and other key proteins, thereby regulating cellular phenotypes[18]. The correlation between 16 organic acid metabolites and macrophage-derived cytokines/chemokines was analyzed to investigate whether the metabolic changes affected the immune inflammatory response in patients with ACLF (Figure 7A). The results showed that IL-1β levels positively correlated with those of pyruvate, fumarate, malate, ethyl malonic acid, glyoxylic acid, and β-Hydroxybutyric acid, while negatively correlating with those of citrate ($P < 0.05$). The correlation


A
HD
ACLF
1.16448 * to [1]
1.005 * t [1]
B
R2
Q2
C
Group
Lactate
Glycolic acid
Citrate
Oxalic acid
α-ketoglutaric acid
Oxaloacetate
Phenylacetic acid
Methyl malonic acid
Glyoxylic acid
Malonic acid
Succinate
Ethyl malonnic acid
β-Hydroxybutyric acid
Pyruvate
Fumarate
Malate
ACLF-15
ACLF-3
ACLF-10
ACLF-13
ACLF-2
ACLF-14
ACLF-17
ACLF-7
ACLF-16
HD-9
ACLF-12
ACLF-8
ACLF-9
ACLF-6
HD-8
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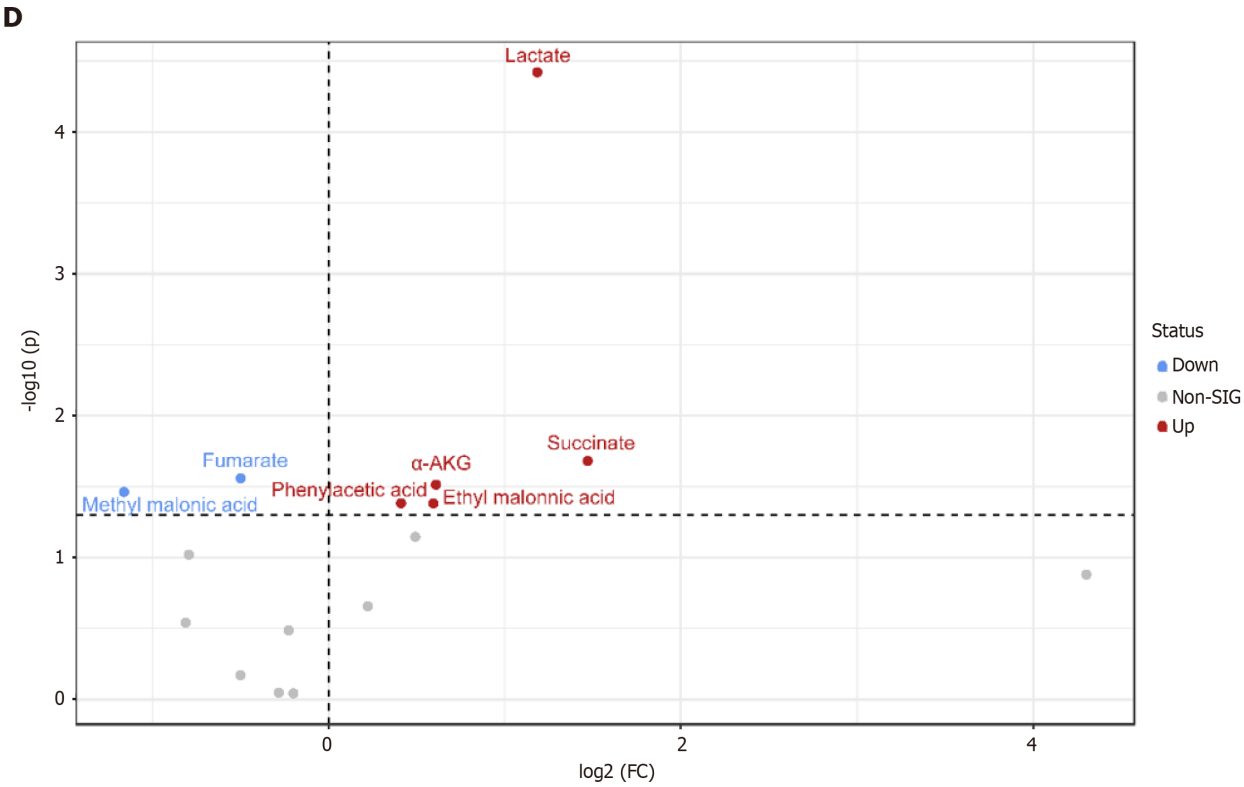
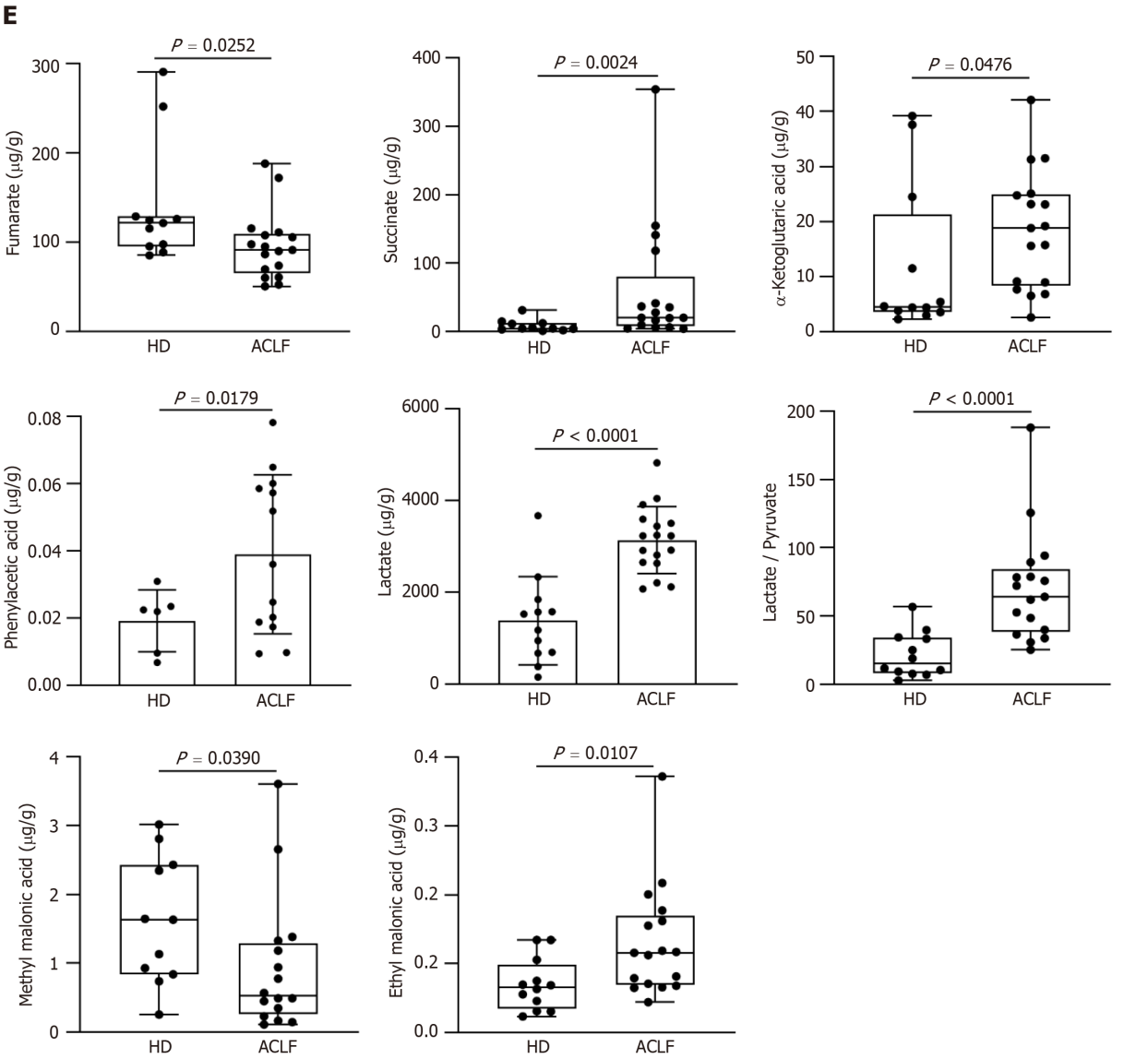

D
Lactate
Succinate
α-AKG
Fumarate
Phenylacetic acid
Ethyl malonnic acid
Methyl malonic acid
-log10 (p)
log2 (FC)
Status
Down
Non-SIG
Up


E
P = 0.0252
Fumarate (μg/g)
P = 0.0024
Succinate (μg/g)
P = 0.0476
α-Ketoglutaric acid (μg/g)
P = 0.0179
Phenylacetic acid (μg/g)
P < 0.0001
Lactate (μg/g)
P < 0.0001
Lactate / Pyruvate
P = 0.0390
Methyl malonic acid (μg/g)
P = 0.0107
Ethyl malonic acid (μg/g)
HD
ACLF

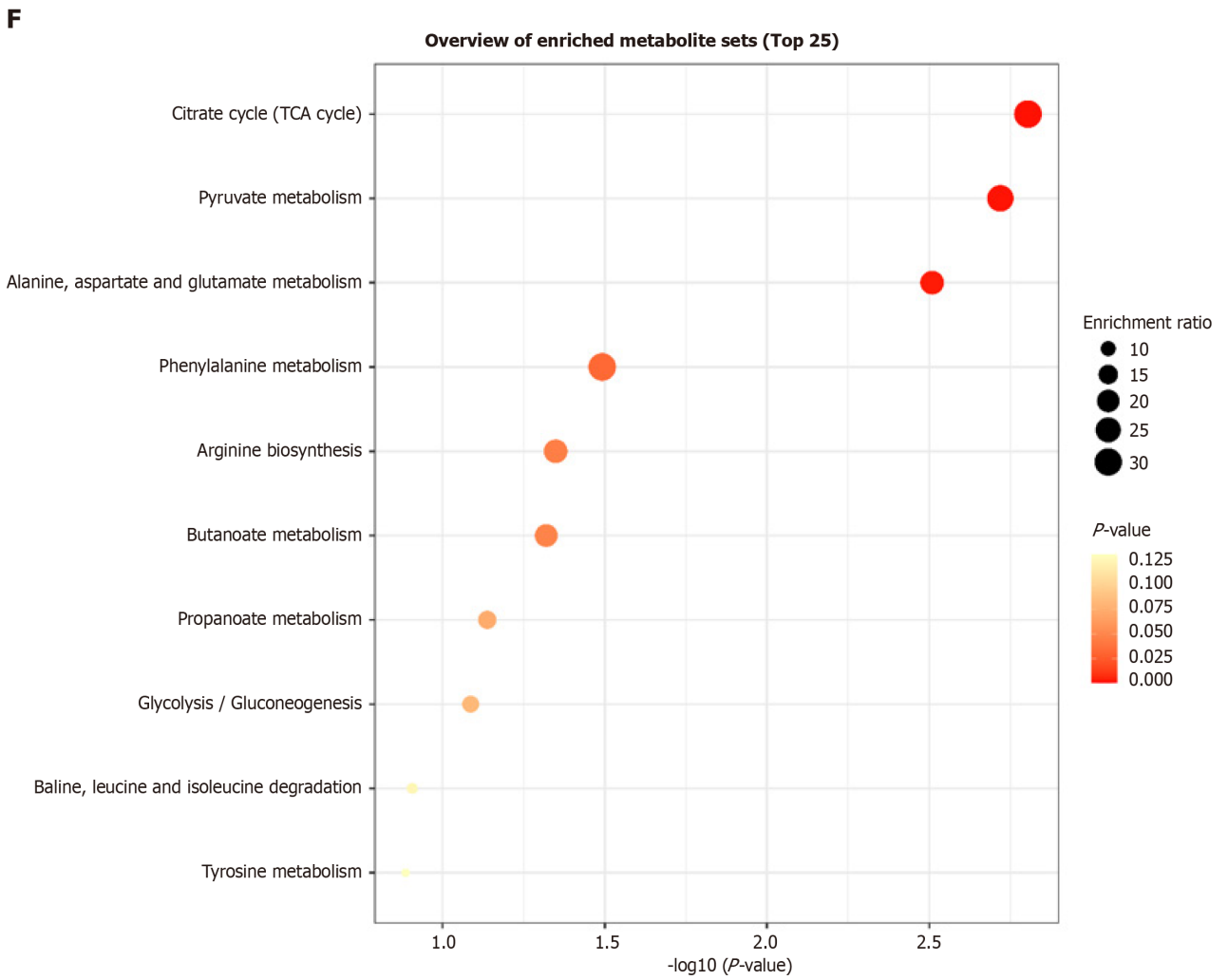


Figure 4 Metabolic profiles of the liver of patients with acute-on-chronic liver failure. A: Orthogonal partial least squares-discriminant analysis scatter plot; B: Permutation tests; C: Hierarchical clustergram of organic acid metabolites; D: Volcano plot comparing organic acid metabolites between patients with acute-on-chronic liver failure (n = 17) and healthy donors (n = 12); E: Quantitative analysis of differentially expressed organic acid metabolites; F: Kyoto Encylopedia of Genes and Genomes pathway enrichment analysis. Data with normal distribution were compared using unpaired student's t-test, and the Mann-Whitney test was used for non-normal data. ACLF: Acute-on-chronic liver failure; HD: Healthy donor.

coefficients were 0.671, 0.671, 0.738, 0.603, 0.736, 0.559, and -0.644, respectively (Figure 7B). TNF-α levels positively correlated with those of LAC (P < 0.05), with a correlation coefficient of 0.665 (Figure 7B). IL-10 levels negatively correlated with those of pyruvate, fumarate, malate, and methyl malonic acid (P < 0.05), and the correlation coefficients were -0.635, -0.612, -0.535, and -0.621, respectively (Figure 7B). TGF-β1 Levels positively correlated with those of fumarate, malate, methyl malonic acid, ethyl malonic acid, and glyoxylic acid, while negatively correlating with citrate (P < 0.05). The correlation coefficients were 0.568, 0.618, 0.589, 0.544, 0.557, and -0.518, respectively (Figure 7B). CCL2 levels positively correlated with those of glycolic acid (P < 0.05), with a correlation coefficient of 0.665 (Figure 7B). Organic acid metabolites strongly and positively correlated with pro-inflammatory cytokines and negatively correlated with anti-inflammatory cytokines. IL-1β was strongly associated with tricarboxylic acid (TCA) cycle intermediates. Succinate is a novel driver of inflammation, which can be sensed by succinate receptor 1[19,20]. The intracellular succinate in macrophages stabilizes HIF-1α, thereby enhancing IL-1β production in normoxic conditions[21]. Lactate not only provides energy for cell growth but also acts as an important signaling molecule for regulating the function of immune cells[22]. In hypoxic conditions, macrophages exhibit enhanced migratory capacity partly through HIF-1α signaling to redirect pyruvate from the TCA cycle to LAC production, thereby enhancing localized energy generation[23].

DISCUSSION

Viral, immune, and metabolic processes are involved in the development and progression of HBV-ACLF. The metabolic characteristics of patients with HBV-ACLF profoundly change, mainly due to hyperammonemia and hypoxia[3]. Severe systemic inflammation in ACLF is associated with the accumulation of metabolites in the body, along with profound metabolic alterations, such as mitochondrial dysfunction[24]. Mitochondrial dysfunction governs immunometabolism in

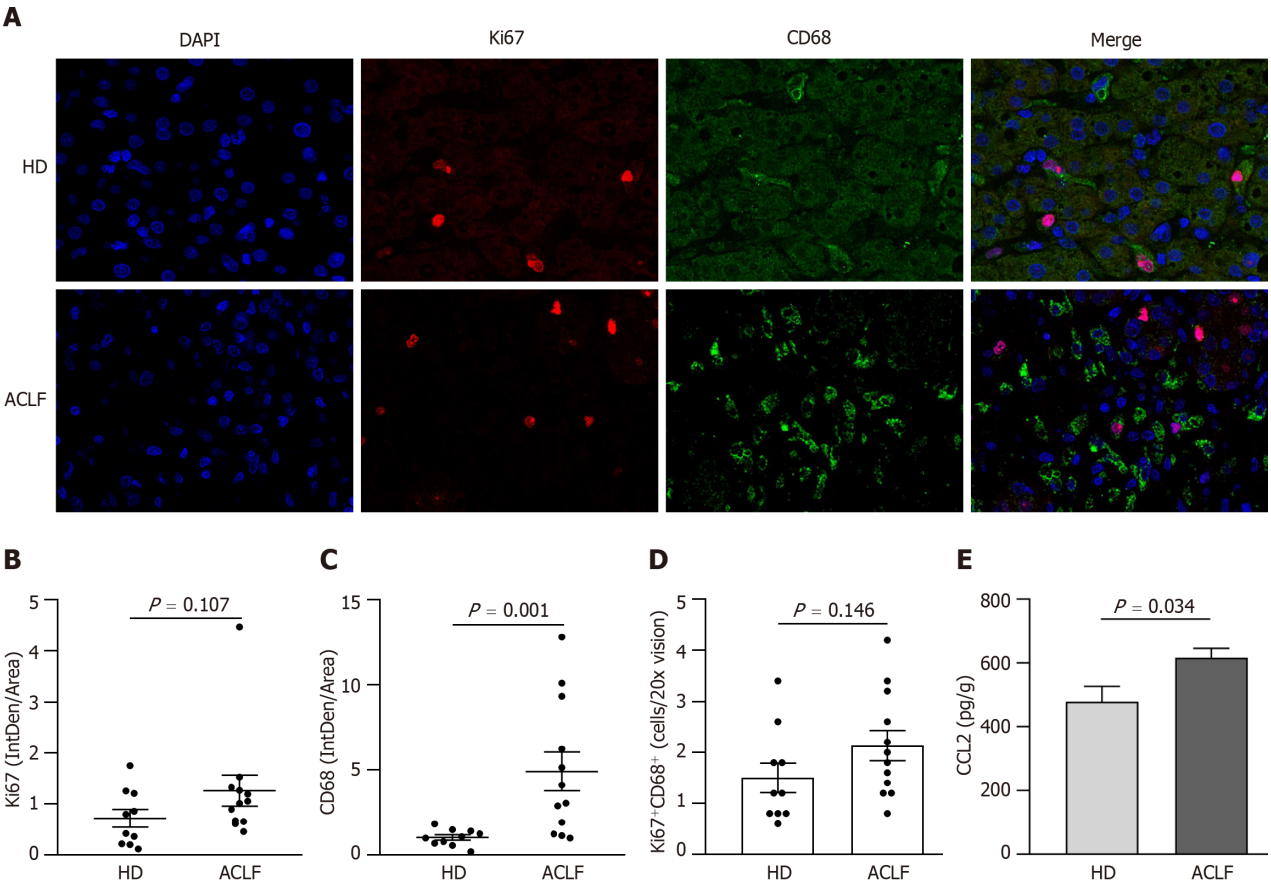


Figure 5 Macrophages were widely activated in the liver of patients with acute-on-chronic liver failure. A: Representative microscopic images of the double immunofluorescence staining of Ki67 and CD68 in the liver (400 fold). The red fluorescence signal represented Ki67; the green fluorescence signal represented CD68; and the blue fluorescence signal represented DAPI; B: Ki67 immunofluorescence intensity in the liver of healthy donors (n = 10) and patients with acute-on-chronic liver failure (n = 12); C: CD68 immunofluorescence intensity in the liver; D: The mean number of Ki67$^+$ CD68$^+$-positive cells in five × 200 fields; E: Chemokine C-C motif ligand 2 levels in liver homogenates. Data with normal distribution were compared using unpaired student's t-test, and the Mann-Whitney test was used for non-normal data. ACLF: Acute-on-chronic liver failure; HD: Healthy donor.

the leukocytes of patients with ACLF[4]. This study is the first to describe changes in the bioenergetics of the liver in patients with advanced HBV-ACLF. Bioenergetic alteration driven by hypoxia and mitochondrial dysfunction contribute to hepatic immune and metabolic remodeling, which may cause organ failure and poor clinical prognosis in patients with advanced HBV-ACLF. In this study, the data were obtained from the liver of patients with HBV-ACLF, whose mitochondrial function, metabolites, and immune microenvironment were less susceptible to any confounding factors caused by other failing organs.

Massive inflammatory cell infiltration was observed in the liver tissue of HBV-ACLF patients in histopathological assessment. CCL2 levels were elevated in the liver tissue of HBV-ACLF, indicating increased chemotaxis of peripheral monocytes. Consistent with our findings, a multicenter, prospective cohort study showed an increased activation of the innate immune system in HBV-ACLF[2]. Immunofluorescence further confirmed that circulating monocyte-derived macrophages widely infiltrated the liver of patients with HBV-ACLF. Moreover, extensive ductular reactions (DR) were noted around the necrotic zone of the liver and around portal areas. DR is pathologically recognized as bile duct hyperplasia and is associated with trans-differentiation of hepatocytes. DR may play an important role in hepatic regeneration[25], though it does not exist in all hepatobiliary diseases. Extracellular matrix, inflammatory cell infiltration, and activated myofibroblasts are also involved in the pathogenesis of DR[26-28]. This implies that DR is closely associated with liver regeneration after severe or prolonged injury.

Mitochondria, which are the main oxygen consumers in cells, are the primary organelles that are affected by hypoxia. In this study, hypoxia significantly changed the ultrastructure of mitochondria as evidenced by mitochondrial swelling and ridge destruction. The number of mitochondria increases as an adaptive response to chronic non-specific cellular damage. FGF21 and GDF15 levels were also determined as biomarkers for the severity of mitochondrial dysfunction. For the first time, a reduction in FGF21 levels was noted in patients with HBV-ACLF. FGF21 and GDF15 levels increase in patients with ACLF as independent predictors of adverse clinical events[6,14]. It's important to note that these data were obtained from peripheral blood.

FGF21 levels may be affected by mitochondrial dysfunction in other damaged organs. A series of different models demonstrated that FGF21 has differential tissue-specific effects and might be a modulator of stress signaling in mild-to-moderate mitochondrial dysfunction. However, the effects of FGF21 are dispensable in severe mitochondrial dysfunction [8]. FGF21 is mainly synthesized and secreted by hepatocytes. Decreased FGF21 levels may be attributed to hepatic

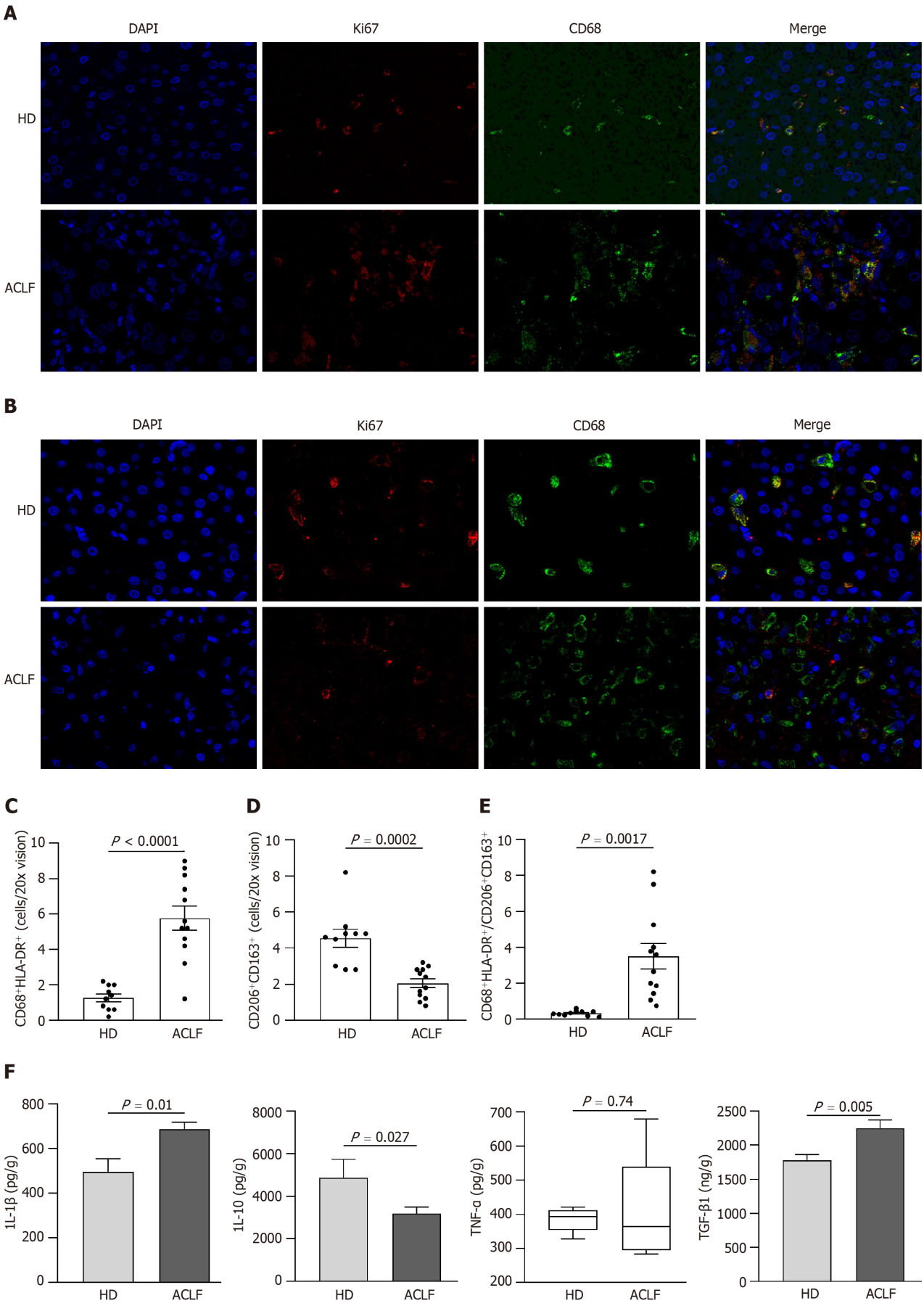


Figure 6 Generalized macrophages activation showed a classically activated phenotype in the liver of patients with acute-on-chronic liver failure. A: Representative microscopic images of double immunofluorescence staining of CD68 and HLA-DR in the liver (400 fold). The red fluorescence signal

represented CD68; the green fluorescence signal represented HLA-DR; and the blue fluorescence signal represented DAPI; B: Representative microscopic images of the double immunofluorescence staining of CD206 and CD163 in the liver (400 fold). The red fluorescence signal represented CD206; the green fluorescence signal represented CD163; and the blue fluorescence signal represented DAPI; C: The mean number of $CD68^+$ $HLA\text{-}DR^+$-positive cells in five × 200 fields; D: The mean number of $CD206^+$ $CD163^+$-positive cells in five × 200 fields; E: The ratio of $CD68^+$ $HLA\text{-}DR^+/CD206^+$ $CD163^+$-positive cells in healthy donors and patients with acute-on-chronic liver failure; F: The levels of macrophage-derived cytokines (interleukin-1β, tumor necrosis factor-α, interleukin-10, and transforming growth factor-β1) in liver homogenates. Data with normal distribution were compared using unpaired student's *t*-test, and the Mann-Whitney test was used for non-normal data. ACLF: Acute-on-chronic liver failure; HD: Healthy donor; IL-1β: Interleukin-1β; IL-10: Interleukin-10; TNF-α: Tumor necrosis factor-α; TGF-β1: Transforming growth factor.

insufficiency and massive hepatocyte necrosis in patients with ACLF. GDF15 is a protein that is mainly secreted by activated macrophages[29]. The extensive hepatic infiltration of inflammatory cells was consistent with increased levels of GDF15. A meta-analysis showed that GDF15, which is a biomarker of mitochondrial dysfunction, has higher diagnostic accuracy than FGF21[30]. In conclusion, the FGF21 Levels in the serum do not have diagnostic and prognostic significance in patients with advanced HBV-ACLF. More studies are needed to carefully assess the role of serum biomarkers in predicting serious adverse events in patients with ACLF.

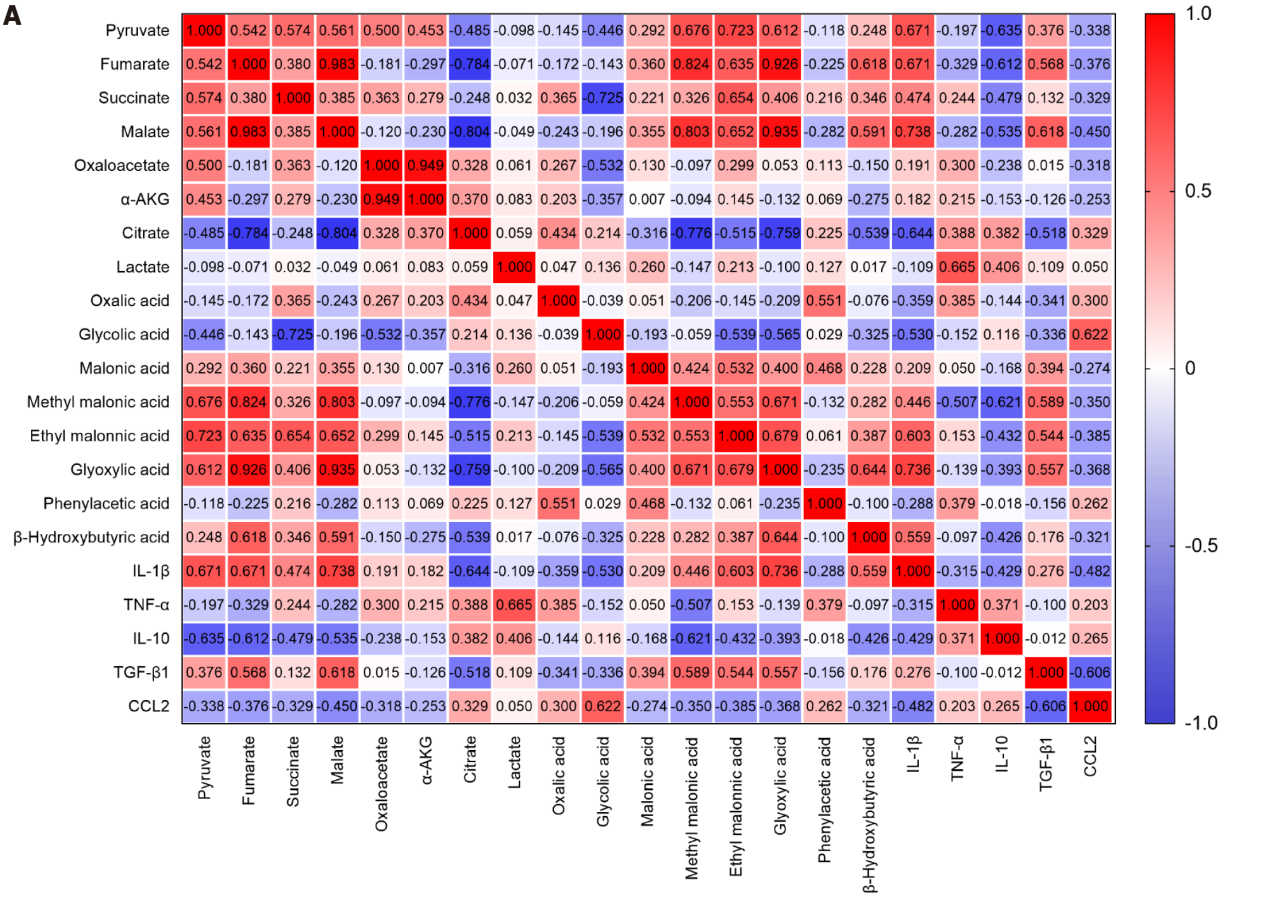
Mitochondrial dysfunction in ACLF severely impairs ATP production so the liver of patients with ACLF provides more energy *via* glycolysis[4]. Decreased fumarate levels and increased succinate levels may be attributed to the inhibition of the TCA cycle. Selective impairments were observed in the conversion of succinate into fumarate in the TCA cycle of leukocytes obtained from patients with ACLF[4]. Similar impairments were observed between isocitric acid and α -ketoglutaric acid[4], and α-ketoglutaric acid accumulation may be due to glutamine supplementation[3]. These results illuminate the metabolic characteristics of the liver of patients with ACLF. These characteristics include enhanced glycolysis and glutamine anaplerosis, inhibited oxidative phosphorylation, and TCA cycle disruption. In contrast, another clinical study found that glycolysis was repressed in the liver of patients with HBV-ACLF[3]. In the same study, patients with ACLF were only admitted to combined medicine. The severity and clinical characteristics of patients were different from those of patients with advanced HBV-ACLF. We hypothesized that glycolysis undergoes a dynamic transition during HBV-ACLF progression. In addition, enhanced glycolysis may not be only a passive transition after mitochondrial dysfunction but also an active choice in hypoxia. In hypoxic conditions, stably expressed HIF-1α mediates glucose metabolism reprogramming *via* multiple pathways, thereby transforming energy production from oxidative phosphorylation to glycolysis. For example, HIF-1α increases glucose conversion into LAC by increasing the expression of glucose transporters and glycolytic enzymes[31,32]. HIF-1α decreases mitochondrial oxidation and promotes glycolysis in macrophages[33].

Macrophage activation is characterized by pronounced metabolic adaptation. Classically activated macrophages secrete proinflammatory mediators. A shift from impaired TCA cycle and oxidative phosphorylation to glycolysis also takes place. In contrast, alternatively activated macrophages secrete anti-inflammatory cytokines and are characterized by enhanced oxidative phosphorylation and fatty acid oxidation[34]. Notably, organic acid metabolites strongly correlated with macrophage-derived cytokines/chemokines, linking metabolism to immunity in ACLF. This study was not designed to elucidate whether metabolic remodeling is the underlying cause or the consequence of immune activation, but there is enough evidence supporting that impaired TCA cycle and enhanced glycolysis reinforce inflammatory responses. High levels of succinate in cells have been shown to stabilize and activate the HIF-1α and its downstream targets by inhibiting prolyl hydroxylases. This pathway induces IL-1β secretion in macrophages[35,36]. Accelerated glycolysis may guarantee a competitive bioenergetic state and provide energy for classically activated macrophages to release proinflammatory cytokines[37]. We demonstrated that imbalanced macrophage polarization existed in the pathogenesis of HBV-ACLF. Classically activated macrophages release high amounts of cytokines, leading to tissue damage and organ failure. It can be a new immunometabolic therapeutic strategy to regulate macrophage polarization by inhibiting the glycolysis pathway.

In summary, our findings provided direct mechanistic evidence of tissue hypoxia, mitochondrial dysfunction, metabolic remodeling, and imbalanced immune homeostasis in patients with advanced HBV-ACLF. The interaction between these pathophysiological mechanisms forms an interconnected complex network. HIF-1α may be the core target in this network. Regulating liver metabolism reprogramming and promoting the polarization of alternatively activated macrophages may attenuate liver inflammation and promote tissue repair. This provides insights to treatment strategies that are worth exploring.

CONCLUSION

The results indicated that bioenergetic alteration driven by hypoxia and mitochondrial dysfunction affects hepatic immune and metabolic remodeling, leading to advanced HBV-ACLF. These findings highlight a new therapeutic target for improving the treatment of HBV-ACLF.


A
Pyruvate 1.000 0.542 0.574 0.561 0.500 0.453 -0.485 -0.098 -0.145 -0.446 0.292 0.676 0.723 0.612 -0.118 0.248 0.671 -0.197 -0.635 0.376 -0.338
Fumarate 0.542 1.000 0.380 0.983 -0.181 -0.297 -0.784 -0.071 -0.172 -0.143 0.360 0.824 0.635 0.926 -0.225 0.618 0.671 -0.329 -0.612 0.568 -0.376
Succinate 0.574 0.380 1.000 0.385 0.363 0.279 -0.248 0.032 0.365 -0.725 0.221 0.326 0.654 0.406 0.216 0.346 0.474 0.244 -0.479 0.132 -0.329
Malate 0.561 0.983 0.385 1.000 -0.120 -0.230 -0.804 -0.049 -0.243 -0.196 0.355 0.803 0.652 0.935 -0.282 0.591 0.738 -0.282 -0.535 0.618 -0.450
Oxaloacetate 0.500 -0.181 0.363 -0.120 1.000 0.949 0.328 0.061 0.267 -0.532 0.130 -0.097 0.299 0.053 0.113 -0.150 0.191 0.300 -0.238 0.015 -0.318
α-AKG 0.453 -0.297 0.279 -0.230 0.949 1.000 0.370 0.083 0.203 -0.357 0.007 -0.094 0.145 -0.132 0.069 -0.275 0.182 0.215 -0.153 -0.126 -0.253
Citrate -0.485 -0.784 -0.248 -0.804 0.328 0.370 1.000 0.059 0.434 0.214 -0.316 -0.776 -0.515 -0.759 0.225 -0.539 -0.644 0.388 0.382 -0.518 0.329
Lactate -0.098 -0.071 0.032 -0.049 0.061 0.083 0.059 1.000 0.047 0.136 0.260 -0.147 0.213 -0.100 0.127 0.017 -0.109 0.665 0.406 0.109 0.050
Oxalic acid -0.145 -0.172 0.365 -0.243 0.267 0.203 0.434 0.047 1.000 -0.039 0.051 -0.206 -0.145 -0.209 0.551 -0.076 -0.359 0.385 -0.144 -0.341 0.300
Glycolic acid -0.446 -0.143 -0.725 -0.196 -0.532 -0.357 0.214 0.136 -0.039 1.000 -0.193 -0.059 -0.539 -0.565 0.029 -0.325 -0.530 -0.152 0.116 -0.336 0.622
Malonic acid 0.292 0.360 0.221 0.355 0.130 0.007 -0.316 0.260 0.051 -0.193 1.000 0.424 0.532 0.400 0.468 0.228 0.209 0.050 -0.168 0.394 -0.274
Methyl malonic acid 0.676 0.824 0.326 0.803 -0.097 -0.094 -0.776 -0.147 -0.206 -0.059 0.424 1.000 0.553 0.671 -0.132 0.282 0.446 -0.507 -0.621 0.589 -0.350
Ethyl malonnic acid 0.723 0.635 0.654 0.652 0.299 0.145 -0.515 0.213 -0.145 -0.539 0.532 0.553 1.000 0.679 0.061 0.387 0.603 0.153 -0.432 0.544 -0.385
Glyoxylic acid 0.612 0.926 0.406 0.935 0.053 -0.132 -0.759 -0.100 -0.209 -0.565 0.400 0.671 0.679 1.000 -0.235 0.644 0.736 -0.139 -0.393 0.557 -0.368
Phenylacetic acid -0.118 -0.225 0.216 -0.282 0.113 0.069 0.225 0.127 0.551 0.029 0.468 -0.132 0.061 -0.235 1.000 -0.100 -0.288 0.379 -0.018 -0.156 0.262
β-Hydroxybutyric acid 0.248 0.618 0.346 0.591 -0.150 -0.275 -0.539 0.017 -0.076 -0.325 0.228 0.282 0.387 0.644 -0.100 1.000 0.559 -0.097 -0.426 0.176 -0.321
IL-1β 0.671 0.671 0.474 0.738 0.191 0.182 -0.644 -0.109 -0.359 -0.530 0.209 0.446 0.603 0.736 -0.288 0.559 1.000 -0.315 -0.429 0.276 -0.482
TNF-α -0.197 -0.329 0.244 -0.282 0.300 0.215 0.388 0.665 0.385 -0.152 0.050 -0.507 0.153 -0.139 0.379 -0.097 -0.315 1.000 0.371 -0.100 0.203
IL-10 -0.635 -0.612 -0.479 -0.535 -0.238 -0.153 0.382 0.406 -0.144 0.116 -0.168 -0.621 -0.432 -0.393 -0.018 -0.426 -0.429 0.371 1.000 -0.012 0.265
TGF-β1 0.376 0.568 0.132 0.618 0.015 -0.126 -0.518 0.109 -0.341 -0.336 0.394 0.589 0.544 0.557 -0.156 0.176 0.276 -0.100 -0.012 1.000 -0.606
CCL2 -0.338 -0.376 -0.329 -0.450 -0.318 -0.253 0.329 0.050 0.300 0.622 -0.274 -0.350 -0.385 -0.368 0.262 -0.321 -0.482 0.203 0.265 -0.606 1.000
Pyruvate Fumarate Succinate Malate Oxaloacetate α-AKG Citrate Lactate Oxalic acid Glycolic acid Malonic acid Methyl malonic acid Ethyl malonnic acid Glyoxylic acid Phenylacetic acid β-Hydroxybutyric acid IL-1β TNF-α IL-10 TGF-β1 CCL2
1.0
0.5
0
-0.5
-1.0
B
r = 0.671 P = 0.006
Pyruvate (μg/g)
1L-1β (pg/g)
r = 0.671 P = 0.006
Fumarate (μg/g)
1L-1β (pg/g)
r = 0.738 P = 0.002
Malate (μg/g)
1L-1β (pg/g)
r = -0.603 P = 0.015
Ethyl malonic acid (μg/g)
1L-1β (pg/g)
r = -0.736 P = 0.002
Glyoxylic acid (μg/g)
1L-1β (pg/g)
r = -0.559 P = 0.027
β-Hydroxybutyric acid (μg/g)
1L-1β (pg/g)
r = 0.568 P = 0.024
Fumarate (μg/g)
TGF-β1 (ng/g)
r = 0.618 P = 0.013
Malate (μg/g)
TGF-β1 (ng/g)
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Methyl malonic acid (μg/g)
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Ethyl malonic acid (μg/g)
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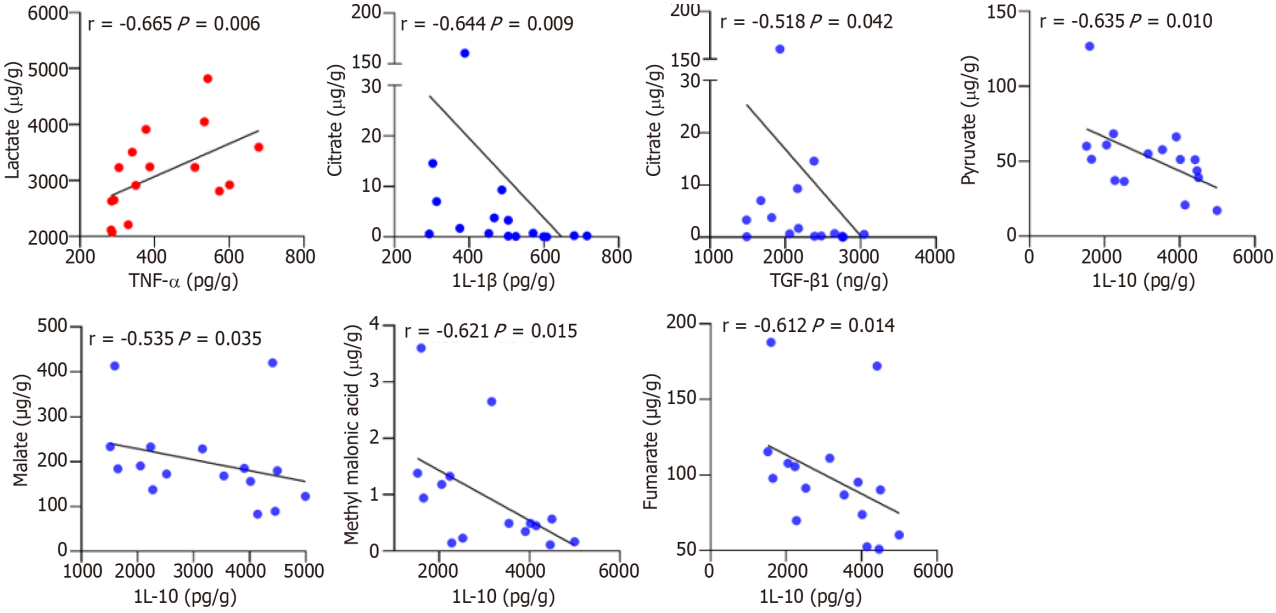

Figure 7 Correlation plot between the 16 organic acid metabolites and macrophage-derived cytokines/chemokines in patients with acute-on-chronic liver failure (*n* = 17). A: Correlation plot between the 16 organic acid metabolites and cytokines/chemokines; B: Linear correlation scatter plot between the organic acid metabolites and cytokines/chemokines (*P* 0.05). The Spearman test was used to measure the correlations of non-normally distributed variables. IL-1β: Interleukin-1β; IL-10: Interleukin-10; TNF-α: Tumor necrosis factor-α; TGF-β1: Transforming growth factor. CCL-2: Chemokine C-C motif ligand 2.

ARTICLE HIGHLIGHTS

Research background

Immune dysregulation and metabolic derangement have been recognized as key factors that contribute to the progression of hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF).

Research motivation

Currently, the mechanisms underlying immune and metabolic derangement in patients with advanced HBV-ACLF are unclear.

Research objectives

To identify the bioenergetic alterations in the liver of patients with HBV-ACLF causing hepatic immune dysregulation and metabolic disorders.

Research methods

This study evaluated the mitochondrial ultrastructure, metabolic characteristics, and immune microenvironment of the liver of the subjects.

Research results

There was extensive hepatocyte necrosis, immune cell infiltration, and ductular reaction in the liver of patients with ACLF. Hypoxia significantly changed the ultrastructure of mitochondria as evidenced by mitochondrial swelling and ridge destruction. Mitochondrial oxidative phosphorylation decreased, while anaerobic glycolysis was enhanced in patients with HBV-ACLF. Circulating monocyte-derived macrophages widely infiltrated the liver of patients with HBV-ACLF. Patients with ACLF had a high abundance of $CD68^+$ $HLA\text{-}DR^+$ macrophages and elevated levels of both interleukin-1β and transforming growth factor-β1 in their livers. The abundance of $CD206^+$ $CD163^+$ macrophages and expression of interleukin-10 decreased.

Research conclusions

Bioenergetic alteration driven by hypoxia and mitochondrial dysfunction affects hepatic immune and metabolic remodeling, leading to advanced HBV-ACLF.

Research perspectives

Regulating liver metabolism reprogramming and promoting the polarization of alternatively activated macrophages may attenuate liver inflammation and promote tissue repair. This provides insights to treatment strategies that are worth exploring.

FOOTNOTES

Author contributions: Zhang Y and Tian XL performed experiments, analysed data and wrote the paper; Wu DS and Li Q performed experiments and analysed data; Li JQ and Chen B designed experiments, performed experiments and edited the paper.

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Institutional review board statement: The study protocol was approved by the Ethics Committee of the First Hospital of Hunan University of Chinese Medicine (No. HN-LL-SWST-15), and written informed consent was obtained from all participants.

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

Conflict-of-interest statement: The authors declare no conflicts of interest that pertain to this work.

Data sharing statement: All data generated or analyzed during this study are included in this published article.

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

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Metadherin promotes stem cell phenotypes and correlated with immune infiltration in hepatocellular carcinoma

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Abstract

BACKGROUND

Metadherin (*MTDH*) is a key oncogene in most cancer types, including hepatocellular carcinoma (HCC). Notably, *MTDH* does not affect the stemness phenotype or immune infiltration of HCC.

AIM

To explore the role of *MTDH* on stemness and immune infiltration in HCC.

METHODS

MTDH expression in HCC tissues was detected using TCGA and GEO databases. Immunohistochemistry was used to analyze the tissue samples. *MTDH* was stably knocked down or overexpressed by lentiviral transfection in the two HCC cell lines. The invasion and migration abilities of HCC cells were evaluated using Matrigel invasion and wound healing assays. Next, we obtained liver cancer stem cells from the spheroids by culturing them in a serum-free medium. Gene expression was determined by western blotting and quantitative reverse transcription PCR. Flow cytometry, immunofluorescence, and tumor sphere formation assays were used to characterize stem-like cells. The effects of *MTDH* inhibition on tumor growth were evaluated *in vivo*. The correlation of *MTDH* with immune cells, immunomodulators, and chemokines was analyzed using ssGSEA and TISIDB databases.

RESULTS

HCC tissues expressed higher levels of *MTDH* than normal liver tissues. High *MTDH* expression was associated with a poor prognosis. HCC cells overexpressing *MTDH* exhibited stronger invasion and migration abilities, exhibited a stem cell-like phenotype, and formed spheres; however, *MTDH* inhibition

attenuated these effects. *MTDH* inhibition suppressed HCC progression and CD133 expression *in vivo*. *MTDH* was positively correlated with immature dendritic, T helper 2 cells, central memory $CD8^+$ T, memory B, activated dendritic, natural killer (NK) T, NK, activated $CD4^+$ T, and central memory $CD4^+$ T cells. *MTDH* was negatively correlated with activated $CD8^+$ T cells, eosinophils, activated B cells, monocytes, macrophages, and mast cells. A positive correlation was observed between the *MTDH* level and *CXCL2* expression, whereas a negative correlation was observed between the *MTDH* level and *CX3CL1* and *CXCL12* expression.

CONCLUSION

High levels of MTDH expression in patients with HCC are associated with poor prognosis, promoting tumor stemness, immune infiltration, and HCC progression.

Key Words: Metadherin; Hepatocellular carcinoma; Cancer stem cells; Immune infiltration

Core Tip: This study demonstrated that high metadherin (*MTDH*) expression is associated with poor prognosis in hepatocellular carcinoma (HCC). High *MTDH* expression increased the invasion and migration abilities of HCC cells and promoted stemness and self-renewal. Moreover, *MTDH* influenced immune cell infiltration and chemokine levels. These results provide additional evidence for the potential role of *MTDH* as a molecular marker for HCC.

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INTRODUCTION

Primary liver cancer is one of the six most common cancers and the third leading cause of cancer-related deaths worldwide. About 75%-85% of primary liver cancer cases are caused by hepatocellular carcinoma (HCC)[1]. The treatment of liver tumors, is limited by its complex pathogenesis, postoperative recurrence, and drug resistance[2]. The poor prognosis of patients with this disease is attributable to metastasis and high recurrence rates. Therefore, it is crucial to understand the relevant mechanisms underlying HCC and identify new therapeutic approaches and targets.

According to several studies, a small proportion of tumor cells, including those in HCC and colorectal cancer, are capable of self-renewal, proliferation, and differentiation, and they are referred to as cancer stem cells (CSCs)[3,4]. The surface markers of CSCs in HCC include CD133, CD90, and EpCAM. High expression levels of CSC markers of CSCs increase stem cell characteristics and tumor sphere-forming capacity[5-7]. An infiltration of multiple immune cells occurs in HCC, including T lymphocytes[8], B cells[9], dendritic cells (DCs)[10] , and natural killer (NK) cells[11]. The HCC consists of these tumor-infiltrating immune cells. The type and number of immune cells have prognostic value and can influence the response to immunotherapy.

Metadherin (*MTDH*), also known as astrocyte elevated gene-1 or lysine-rich *CEACAM1*, is a key oncogenic gene in most cancer types[12]. In malignant tumors, *MTDH* promotes proliferation capacity[13], migration[14], cell survival, and angiogenesis[15], as well as poor prognosis, in lung, prostate, and breast cancers. The importance of *MTDH* in HCC has been demonstrated in numerous studies[16,17]; however, the effects of the expression of *MTDH* on stem cell characteristics and immune cell infiltration in HCC remain unclear.

Compared with normal liver tissues, liver cancer tissues showed higher levels of *MTDH* expression. Notably, *MTDH* expression was associated with poor prognosis. Our findings showed that *MTDH* was expressed at higher levels in tumor spheres than in adherent cells. *MTDH* expression in HCC cells positively correlated with *CD133*, *Oct4*, and *Nanog* expression in stem cells. Furthermore, the inhibition of *MTDH* expression inhibited tumor growth. Our study confirmed that *MTDH* is associated with immune cell infiltration, as confirmed by the analysis of the ssGSEA and TISIDB databases.

MATERIALS AND METHODS

HCC Samples

All the gene expression profile files and the clinical information were obtained from two public databases. TCGA (The Cancer Genome Atlas database, https://portal.gdc.cancer.gov/) contains information on transcriptional gene expression in human cancer and healthy tissues. After removing samples with incomplete clinical information, 369 liver tumor tissues and 50 healthy liver tissues were analyzed. In the GEO (Gene Expression Omnibus, www.ncbi.nlm.nih.gov/geo/), incompletely annotated samples were excluded from the dataset of GSE14520 (sequencing platform: GPL3921). The

analysis included 210 samples, comprising both tumor samples and their corresponding non-cancer pairs. The clinical information of all the samples is presented in Table 1. Liver tumor tissue microarrays of nine paired samples (HLiv-H030PG03) were purchased from Shanghai Xinchao (Shanghai, China).

Differential expression and survival analyses

TCGA data were converted to TPM (transcripts per million) values for subsequent analyses. For GSE14520, data normalization and log2 transformation were performed. Probe IDs were converted to gene symbols using a platform annotation file. Genes with multiple probes are represented by their maximum expression values. Data pre-processing was performed, followed by differential expression and statistical analyses using R software. A survival curve analysis was performed using the R packages "Survival" and "Survminer" with the best cut-off value for *MTDH* calculated for both datasets. R packages "ggstatsplot" and "corrplot" were used for data visualization.

Cell culture

Huh7 cells were purchased from the Chinese Academy of Sciences Cell Bank and, MHCC-97H cells were obtained from the Liver Cancer Institute at Fudan University Zhongshan Hospital. All the cells were cultured in DMEM medium [10% fetal bovine serum (FBS)], 100 U/mL penicillin, and 100 U/mL streptomycin in an incubator with 5% CO_2.

Sphere formation assay

Tumor spheres were cultured in serum-free medium (SFM) consisting of 20 ng/mL EGF (PeproTech), DMEM/F12 (HyClone), 20 ng/mL bFGF (PeproTech), and 20 μL/mL B27 (Gibco). Single cells (1×10^4) were seeded in a 6-well ultra-low adsorption plate (Corning) containing 1.5 mL of serum-free culture medium. All the cells were incubated for 14 d in incubators with 5% CO_2 at 37°C. Inverted microscopes were used to count tumor spheres larger than 50 mm.

Extraction of proteins and western blotting

To prepare proteins from the cells, the cells were lysed in RIPA solution (CWBIO) containing a protease inhibitor cocktail solution. The protein concentration in the solution was determined using a BCA kit (Beyotime). Proteins were separated by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Beyotime Millipore) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). PVDF membranes were closed with 5% skim milk at room temperature (RT) for 1 h, followed by overnight incubation with primary antibody at 4°C. The membranes were washed thrice with TBST containing 0.1% Tween-20, followed by incubation with secondary antibodies (EarthOx) (1:5000) for 1 h. After washing thrice with TBST, for 5 min, the blots were visualized with chemiluminescence reagents (Beyotime). The antibodies used were anti-MTDH (CST, 14065T), anti-CD133 (YT5192, ImmunoWay), anti-NANOG (CST, 4903T), and anti-GAPDH (YM3215, ImmunoWay).

Total RNA extraction and real-time quantitative reverse transcription PCR

TRIzol (Takara) was used to extract RNA from the cells, and the cDNA (complementary DNA) composition was determined using the PrimeScript™ RT Reagent Kit (TaKaRa). Real-time quantitative reverse transcription PCR (qRT-PCR) was performed using SYBR Premix Ex Taq (TaKaRa Bio). A CFX96 Real-Time PCR Detection System (Bio-Rad) was used for qRT-PCR analysis. PCR was performed using the primers purchased from Sangon Biotechnology (Shanghai, China). The $^{2-\Delta\Delta}$Ct method was used to calculate the data. Table 2 shows primer sequences.

Cell transfection

To establish a cell line with stable overexpression and suppression of *MTDH*, the cell were infected with a lentivirus. MTDH-overexpressing lentiviral vectors, MTDH-RNAi lentiviral vectors, and blank control lentiviral vectors were acquired from GeneChem (Shanghai, China). On the previous day, 3×10^4 cells were grown in each well of a six-well plate. When the cells reached 30% confluence, they were transfected according to the manufacturer's protocols at Multiplicity of Infection of 5. After 96 h, fluorescence microscopy and western blotting were used to verify the transfection efficiency. Puromycin screening was then performed for 2 wk (GeneChem).

Immunofluorescence

The cells were seeded in a 24-well plate. Paraformaldehyde solution (4%) was used to fix the cells for 20 min after 24 h of incubation. For cell membrane permeabilization, the cells were treated with 0.3% TritonX-100 (Sigmautes) for 15 min. The cells were incubated overnight with anti-NANOG antibody at 4°C after 120 min incubation in 5% BSA in TBST. Next, the cells were washed thrice with PBS and incubated with DyLight 649 AffiniPure Goat Anti-Rat IgG (1:200, Abbkine) for 1 h in a dark humidified box. The final counterstaining was done using DAPI (4',6-diamino-2-phenylindole) for 10 min. Images were captured using a fluoresce microscope (Nikon).

Wound healing assay

MHCC-97H and Huh7 cells were cultured in six-well plates. Subsequent experiments were performed when the cell density in the wells reached 90%. After scratching the cells with a 200-mL pipette, 2% FBS cell culture medium was used for continued culture. All the cells were incubated with 5% carbon dioxide in an incubator at 37°C. A microscope was used to photograph the wounded areas.

Table 1 Description of the datasets used in this study

Clinical characteristics	GSE14520		TCGA	
	Total (*n* = 210)	%	Total (*n* = 369)	%
Age				
< 60	169	80.48	168	45.53
≥ 60	41	19.52	200	54.20
Unknown	–	–	1	0.27
Gender				
Female	26	12.38	120	32.52
Male	184	87.62	249	67.48
T stage				
T1	–	–	179	48.51
T2	–	–	94	25.47
T3	–	–	80	21.68
T4	–	–	13	3.52
Unknown	–		3	0.81
N stage				
N0	–	–	250	67.75
N1	–	–	4	1.08
Nx	–		114	30.89
Unknown	–	–	1	0.27
M stage				
M0	–	–	264	71.55
M1	–	–	4	1.08
Mx	–	–	101	27.37
Stage				
I	90	42.86	169	45.80
II	75	35.71	86	23.31
III	43	20.48	85	23.04
IV	0	0.00	5	1.36
Unknown	2	0.95	24	6.50
Status				
Alive	130	61.90	241	65.31
Dead	80	38.10	128	34.69
Unknown	–	–	0	0.00
HBV status				
AVR-CC	52	24.76	–	–
CC	152	72.38	–	–
Unknown	6	2.86	–	–

HBV: Hepatitis B virus; AVR: Active viral replication chronic carrier; CC: Chronic carrier.

Invasion and migration assays

The migration and invasion abilities of the cells were assessed using 24-well transwell chambers with or without Matrigel (BD Biosciences). The supplements were kept in 5% CO_2 at 37°C for 12 h. Next, 2 × 10^5 cells were re-suspended in 200 μL of SFM and placed in transwell cups with an 8-micron pore membrane (BD). Simultaneously, cell culture medium containing 10% FBS was added to the lower layer to make a total volume of 600 μL. The chambers were maintained in 5% CO_2 for 24 h at 37°C. The cells that failed to pass through the pores and remained in the upper chamber were carefully wiped off with a cotton swab. Migrating cells were fixed in 4% paraformaldehyde solution for 20 min. Next, the cells remaining in the chambers were stained in 200 μL 0.1% crystal violet for 30 min, followed by counting with a microscope in five random areas.

Flow cytometry

The cells were washed with PBS after trypsin digestion. Next, they were suspended in 80 μL of PBS, followed by the addition of 2 μL PE-CD133 antibody (Miltenyi Biotec) and 20 μL of FcR Blocking Reagent (Miltenyi Biotec). Under light-proof conditions, the mixture was incubated at 4°C for 10 min. Next, all the cells were washed thrice in PBS, followed by resuspension with 500 μL of PBS. The mixture was analyzed using a FACS Calibur Flow cytometer (BD Biosciences).

Animal experiments

BALB/c-nu mice were obtained from the Animal Experiment Center of Chongqing Medical University. The experiments were conducted in the animal facilities of the Animal Experiment Center of Chongqing Medical University (12-h light/dark cycle, 27 ± 2°C, 50% ± 10% humidity). Nude mice were randomly divided into two groups of three mice each. MHCC-MTDH-LV cells (2 × 10^6 cells) were re-suspended in a complete culture medium. Next, 100 μL of cell suspension were subcutaneously injected into nude mouse (female, 4–6 wk old). The tumors were detached after 4 wk. Next, the tumor volume was calculated using the following formula: Length × width^2/2. Tumor tissues were subjected to immunohistochemical staining (IHC) analysis. This study was approved by the Ethics Committee of the Second Hospital of Chongqing Medical University.

IHC

All the slides were dewaxed and gradually hydrated. Antigen extraction was performed using citrate buffer under high pressure and temperature conditions. All the slides were rinsed with PBS and incubated with goat serum for 10 min at RT. Next, the slides were exposed to anti-MTDH and anti-CD133 at 4°C overnight. Subsequently, horseradish peroxidase-labeled streptavidin was added, followed by the addition of DAB reagent. Tap water was dehydrated using an ethanol gradient. The slides were then washed twice with xylene and wrapped in neutral balsam. Protein expression levels were assessed using the ImageJ software. The average optical density was calculated by measuring the integrated optical density and the area of each image, which reflected the concentration per unit area of the target protein.

ssGSEA

The infiltration abundances of 28 immune cell species in TCGA samples were quantified using ssGSEA. The gene set data for the immune cells were downloaded from the website (https://www.cell.com/cms/10.1016/j.celrep.2016.12.019/). Box plots were constructed to compare immune cell differences between the groups. The R package used was "GSVA" [18], "ggplot2."

TISIDB database

TISIDB is an online analysis site for the analysis of tumor-immune interactions (http://cis.hku.hk/TISIDB/)[19]. To further investigate the immunological impact of MTDH in cancer, we analyzed and evaluated the TISIDB database through the "Immunomodulators" and "chemokine modules".

Statistical analysis

All the experiments were conducted at least thrice. Data were analyzed using R 4.1.2 and GraphPad Prism software. Student's t-test was used to compare two groups of continuous variables. The Kruskal-Wallis and Wilcox tests were used for non-parametric tests. A P value < 0.05 indicated statistical significance.

RESULTS

MTDH is upregulated in HCC and positively associated with poor prognosis

To identify aberrant *MTDH* expression in HCC, we downloaded microarray gene profiling data from GEO (GSE14520/GPL3921) and TCGA. In 50 normal liver tissues and 369 Liver cancer tissues from TCGA, *MTDH* mRNA expression was upregulated in liver tumor tissues. Similarly, *MTDH* expression was increased in the tumor in GSE14520 (n = 420; Figure 1A and B). *MTDH* expression was examined in human HCC (n = 9) and paracancerous tissues (n = 9) using IHC. According to these results, HCC tissues overexpressed *MTDH*, compared with the para-cancerous tissues (Figure 1C). Furthermore, we constructed the Kaplan-Meier curve of HCC using "Survival" and "Survminer" R packages. Shorter survival times were associated with higher MTDH (Figure 1D and E). A higher expression of *MTDH* was observed in liver cancer tissues than in normal liver tissues or para-cancerous tissues, which had impact on the prognosis of patients with liver cancer.

Table 2 Primers used in this study

Gene	Forward	Reverse
Metadherin	5′-CCAGGCTCCTTCATCAACTT-3′	5′-AthAAGCAGCCACCAGAGATTG-3′
CD133	5′-TGGATGCAGACCTTGACAACGT-3′	5′-ATACCTGCTACGACAGTCGTGGT-3′
Nanog	5′-AATACCTCAGCCTCCAGCAGATG-3′	5′- TGCGTCACACCATTGCTATTCTTC -3′
Oct4	5′-CAGGAGGCATTGCTGATGAT-3′	5′-GAAGGCTGGGGCTCATTT-3′
GAPDH	5′-CAGGAGGCATTGCTGATGAT-3′	5′- GAAGGCTGGGGCTCATTT -3′

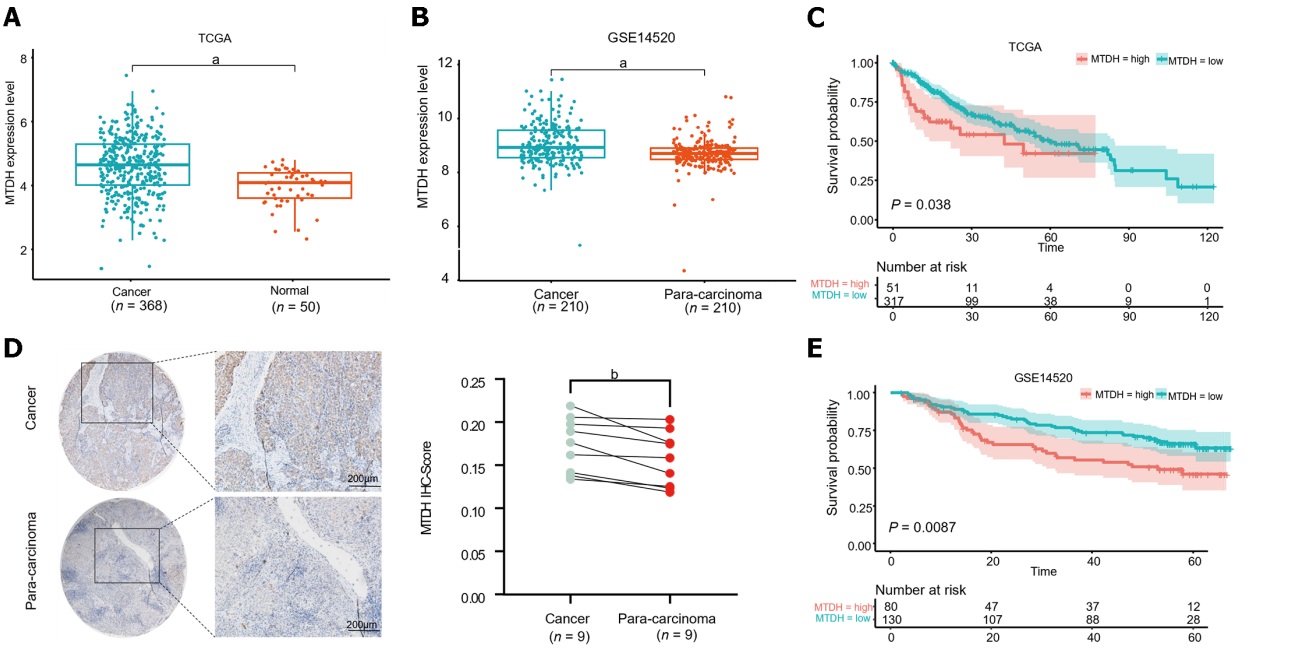

Figure 1 Metadherin overexpression has been linked to a worse prognosis in hepatocellular carcinoma. A and B: Metadherin (*MTDH*) mRNA expression in normal liver tissues compared with liver cancer tissues; C: Images of para-carcinoma (n = 9) and cancer tissues (n = 9) stained with *MTDH* by Immunohistochemical staining (scale bar = 200 μm); D and E: Patient's overall survival curves according to *MTDH* expression. $^{a}P < 0.0001$, $^{b}P < 0.01$. Normal: Normal liver tissues; Cancer: Liver cancer tissues; Para-carcinoma: Para-cancerous tissue; MTDH: Metadherin; IHC: Immunohistochemical staining.

MTDH promotes HCC cell migration and invasion

Two cell lines, Huh7 and MHCC-97H, were transfected with the related lentiviruses. Specifically, we used a blank control (LV-NC) *vs* overexpression (LV-OE) and a blank control (LV-NC) *vs* knockdown (LV-RNAi), to obtain stable overexpression and suppression of *MTDH*. Western blotting was performed to verify MTDH protein expression after transfection (Figure 2A). The migration and invasion assays showed that *MTDH* overexpression significantly accelerated the migration and invasion of MHCC-97H and Huh7 cells (Figure 2B). Conversely, *MTDH* knockdown cells exhibited lower potential for migration and invasion (Figure 2C). The wound healing assay confirmed that *MTDH* overexpression significantly promoted wound healing, whereas *MTDH* knockdown suppressed scratch wound healing in HCC cells (Figure 2D). These results indicate that high *MTDH* expression correlates positively with the migration and invasive abilities of HCC cells.

MTDH is associated with the CSCs phenotypes in HCC

To determine whether *MTDH* is related to the stem cell phenotype, we examined *MTDH* and HCC stemness using TCGA database. TCGA data showed a significant positive correlation between *MTDH* expression in HCC tissues and *CD133*, *NANOG*, and *Oct4* expression (n = 369; Figure 3A). Our previous research confirmed that liver CSCs (LCSCs) can be enriched using a serum-free stem cell medium to promote tumor sphere formation. Next, we performed SFM on MHCC-97H and Huh7 cells, followed by western blotting and qRT-PCR to determine MTDH expression in stem cell spheres and attached cells. Compared with liver cancer-adherent cells, LCSCs expressed increased mRNA levels of MTDH and the stem cell markers (*CD133*, *Oct4*, and *Nanog*; Figure 3B). The results showed that the protein expression levels of MTDH, CD133, and NANOG were higher than those in liver cancer adherent cells (Figure 3C). These results showed that MTDH was highly expressed in LCSCs.

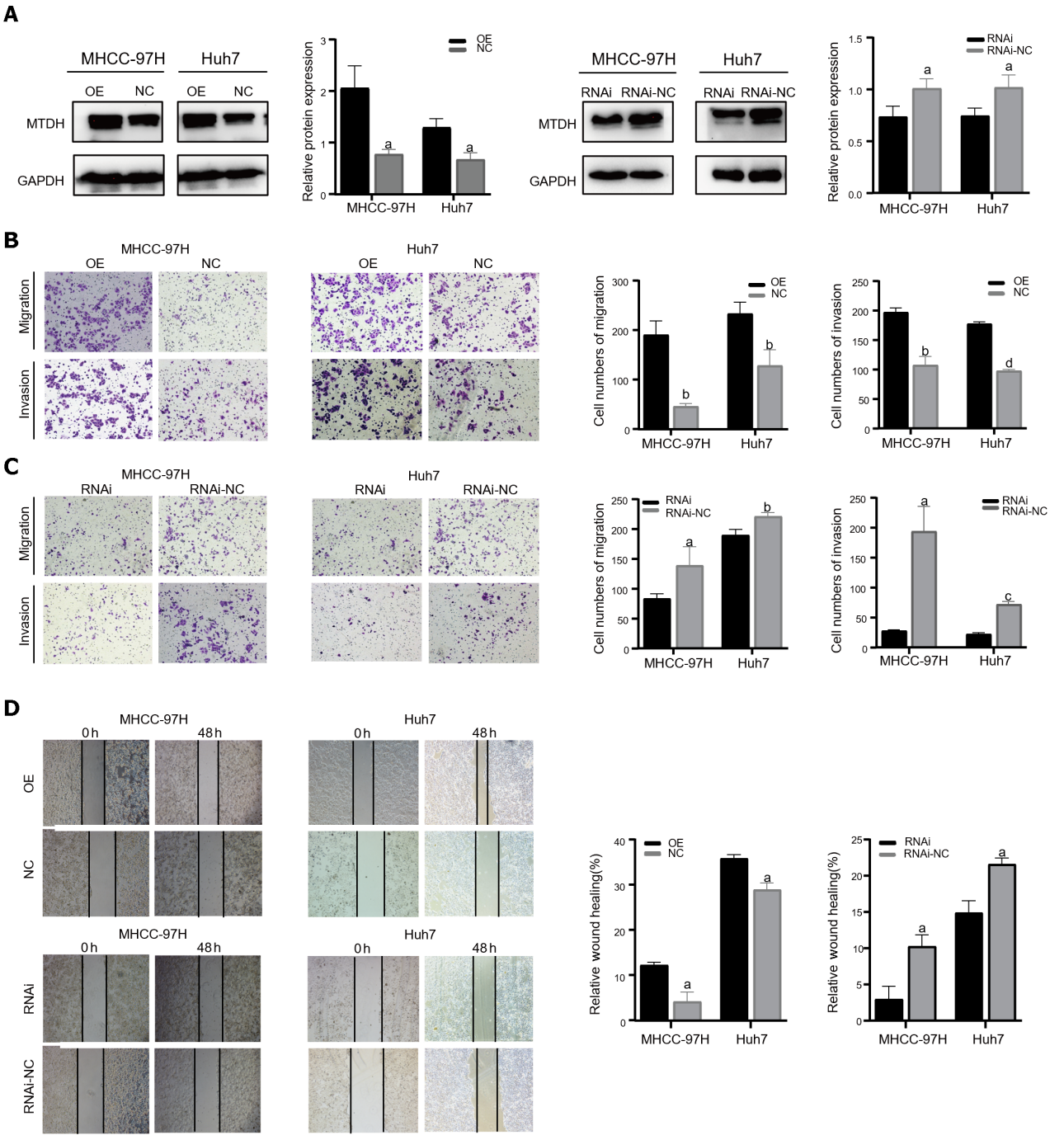

Figure 2 Metadherin promotes proliferation, migration, and invasion of hepatocellular carcinoma cells. A: In Huh7 and MHCC-97H cells, Western blotting revealed the efficacy of Metadherin overexpression and knockdown; B and C: Characteristic images of trans-well invasion assays 24 h after culture; D: Images depicting scratch width at 0 h and 48 h post-scratch in cells captured using inverted microscopy. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. OE: Metadherin overexpression group; NC: Overexpression control group; RNAi: Metadherin knockdown group; RNAi-NC: MTDH knockdown control group.

Overexpression of MTDH enhances the LCSCs phenotypes

TTo confirm that MTDH maintained stem-like phenotypes in HCC cells, stem cell markers were detected in MHCC-97H and Huh7 cell lines overexpressing MTDH (*CD133*, *Oct4*, *Nanog*). Through PCR experiments, we demonstrated that the markers of CSCs markers were higher in MTDH-overexpressing cells than in the NC groups (Figure 4A). MTDH-overexpressing Huh7 and MHCC-97H cells also expressed high levels of CD133 and Nanog protein (Figure 4B). A The sphere culture assay showed the formation of more spheres in MTDH-overexpressing Huh7 and MHCC-97H cells than in control cells (Figure 4C). Flow cytometry revealed that MTDH overexpression effectively increased the number of $CD133^{+}$ HCC cells (Figure 4D). In addition, MTDH overexpression effectively enhanced CSCs phenotypes in HCC cells.

MTDH knockdown inhibits LCSCs phenotypes

The PCR results demonstrated that the mRNA expression of stem cell markers in MTDH-RNAi cells was lower than that in the NC group (Figure 5A). In contrast to the NC group, MTDH-RNAi cells showed lower expression of *CD133* and

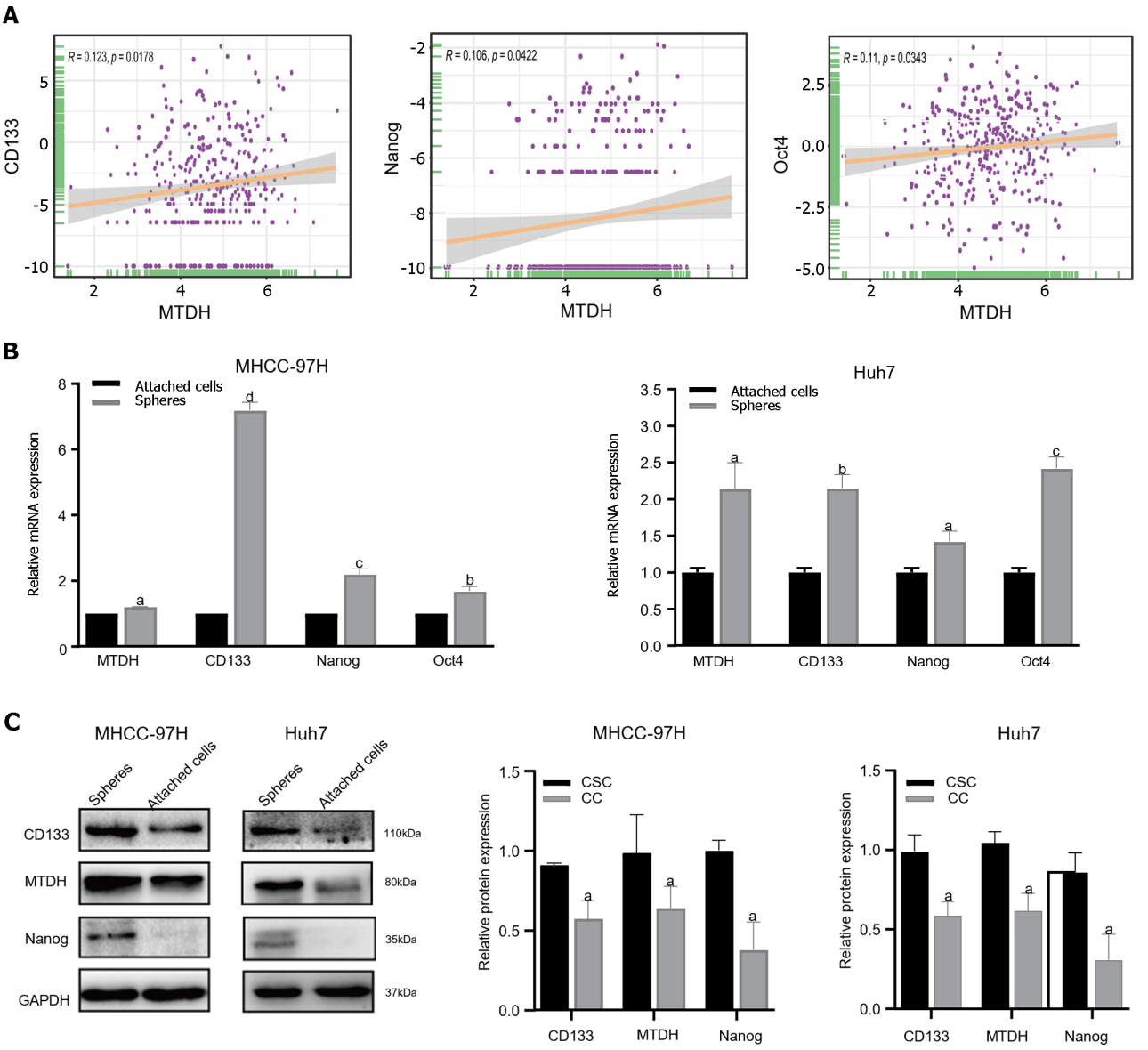

Figure 3 Metadherin correlates with the stemness properties in hepatocellular carcinoma. A: Correlation between Metadherin (*MTDH*) and *CD133*, *Nanog*, *Oct4* in TCGA; B: Through quantitative reverse transcription PCR, the expression levels of *MTDH*, *CD133*, *Nanog*, *Oct4* were measured in attached cells and tumor spheres in 97H and Huh7 cell lines. In tumor spheres, all the four genes were expressed at increased levels; C: MTDH, CD133, and Nanog were higher in attached cells than in tumor spheres. Gene GAPDH served as the internal reference. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$.CSC: Cancer stem cells; CC: Cancer cells.

Nanog (Figure 5B). The downregulation of MTDH reduced the numbers of both MHCC-97H and Huh7 cell spheres, compared with those in the control LV-NC group, as indicated by the results of the sphere culture assay (Figure 5C). The results of the immunofluorescence showed that Nanog fluorescence intensity was greater in the NC group than in the MTDH-RNAi group (Figure 5D). Taken together, these results confirm that the inhibition of *MTDH* expression attenuates the acquisition of the HCC stem cell phenotype.

MTDH knockdown reduces tumor growth and CD133 expression in vivo

Male BALB/c nude mice were injected with LV-NC or LV-MTDH-RNAi MHCC-97H cells to evaluate the effect of *MTDH* on tumor growth. The MTDH-RNAi group exhibited a smaller tumor volume than the 97H-NC group, confirming that *MTDH* promotes tumor growth (Figure 6A and B). Images of hematoxylin and eosin-stained tumor tissues of nude mice are shown in Figure 6C. To further clarify the role of *MTDH* in the tumor stem cell phenotype, CD133 and MTDH proteins were detected by IHC in the tumor tissues of nude mice. We found that CD133 expression decreased with reduced MTDH expression (Figure 6D). Therefore, these results further suggest that *MTDH* overexpression promotes liver tumor growth and the CSCs phenotype.

MTDH expression and immune cell infiltration

A significant association was observed between immune cell infiltration and survival in patients with HCC. Using ssGSEA, we quantified immune cell infiltration scores in HCC samples from TCGA to understand the relationship

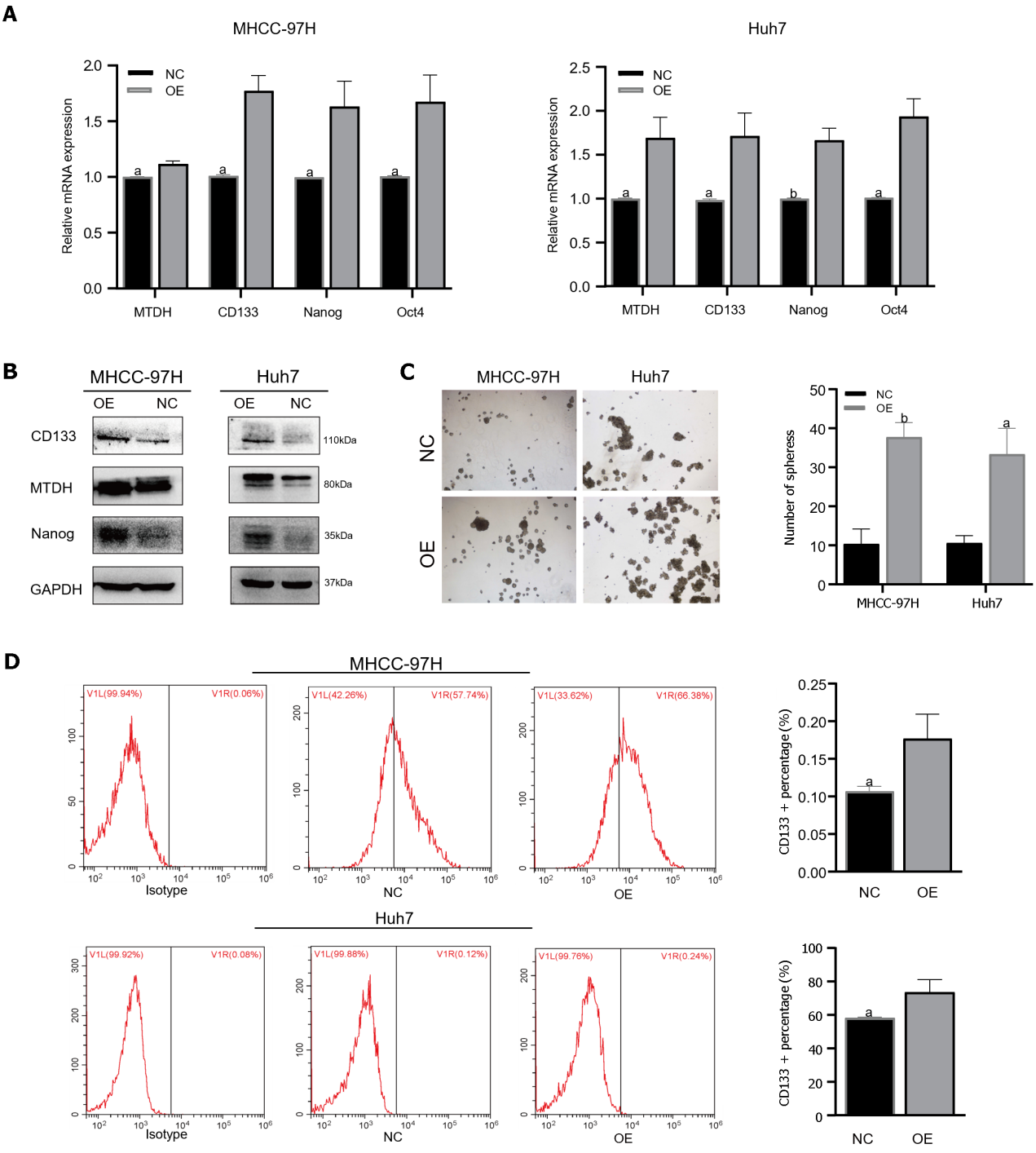

Figure 4 Metadherin overexpression promotes stem cell phenotypes and self- renewal in hepatocellular carcinoma cell lines. A and B: Through quantitative reverse transcription PCR polymerase chain reaction and Western blot, we determined Metadherin expression and stemness markers; C: The typical pictures of sphere formation assays from 97H-overexpression and Huh7-overexpression cells; D: Flow cytometric analysis of CD133$^+$ cells in 97H-overexpressing and Huh7-overexpressing cells. $^aP < 0.05$, $^bP < 0.01$. OE: Metadherin overexpression group; NC: Overexpression control group; MTDH: Metadherin.

between *MTDH* and infiltration. Firstly, the results demonstrated a significantly higher infiltration of activated B cell, immature B, memory B, activated CD8 T, gamma delta T, effector memory CD8 T, central memory CD8 T, and effector memory CD4 T cells in healthy tissue than in HCC. Similarly, higher infiltration of eosinophils, immature DCs, myeloid-derived suppressor cells, monocytes, macrophages, mast cells, monocytes, neutrophils, NK cells, NK T cells, and T helper (Th) cells were observed in healthy tissue than in HCC (Figure 7A). These findings suggest that immune cells are essential in the progression of HCC. Based on the median *MTDH* expression, we divided the liver cancer samples in TCGA into high and low *MTDH* expression groups and assessed immune cell infiltration in both groups. Notably, the levels of memory B ($P < 0.0001$), immature dendritic ($P < 0.001$), Th2 ($P < 0.001$), and central memory CD4$^+$ T ($P < 0.01$) cells were higher in the high *MTDH* expression group than that in the low group. In the low *MTDH* expression group, activated CD8 T cells ($P < 0.0001$), macrophages ($P < 0.01$), activated B cells ($P < 0.01$), effector memory CD8 T cells ($P < 0.01$), mast cells ($P < 0.01$), eosinophils ($P < 0.05$), monocytes ($P < 0.05$), and Th1 cells ($P < 0.05$) were significantly increased

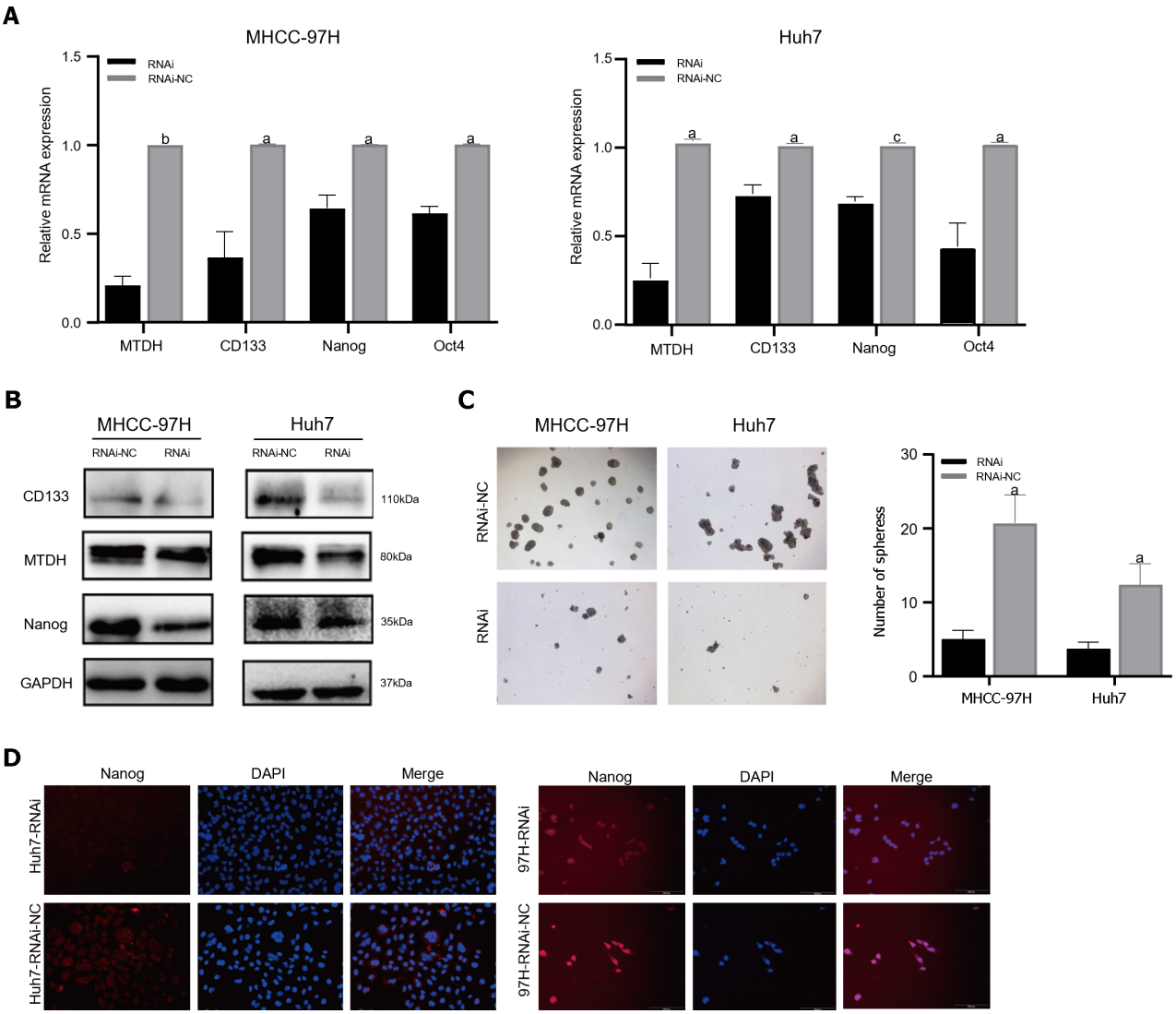

Figure 5 Metadherin downregulation inhibits hepatocellular carcinoma stem cell phenotypes. A: mRNA expression of Metadherin (*MTDH*), *CD133*, *Nanog*, and *Oct4* in 97H and Huh7-RNAi; B: In comparison with that of MTDH-LV-RNAi, the protein expression of CD133 and Nanog was elevated in MHCC-97H-NC and Huh7-NC cells; C: Images depicting sphere formation by 97H-RNAi and Huh7-RNAi cells; D: Immunofluorescence images of *Nanog* (red) in 97H-LV and Huh7-LV samples. DAPI (blue) was used to stain the nuclei. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. RNAi: Metadherin knockdown group; RNAi-NC: Metadherin knockdown control group.

(Figure 7B).

We examined the relationship between immune cells and *MTDH* expression (Figure 8A). Immature dendritic ($r = 0.28$, $P < 0.0001$; Figure 8B), Th2 ($r = 0.26$, $P < 0.0001$; Figure 8C), memory B ($r = 0.25$, $P < 0.0001$; Figure 8D), central memory CD4 T ($r = 0.22$, $P < 0.0001$; Figure 8E), central memory CD8 T, NK T, activated dendritic, activated CD4 T, and NK cells also showed a positive relationship with *MTDH*. Activated CD8 T cells ($r = -0.23$, $P < 0.0001$; Figure 8F), eosinophils ($r = -0.13$, $P < 0.05$; Figure 8G), activated B cells ($r = -0.12$, $P < 0.05$; Figure 8H), monocytes ($r = -0.12$, $P < 0.05$; Figure 8I), macrophages, and mast cells showed a negative correlation with *MTDH*.

Correlation of MTDH expression with immunomodulators and chemokines

ICIs (immune checkpoint inhibitors) are gaining increasing attention as tumor immunotherapy strategies for different types of cancers, facilitating improved prognosis in some patients. *MTDH* and the various immunosuppressive agents in the TISIDB database did not show significant correlation, as indicated by our online analysis (Figure 9A). However, in the analysis of correlation with immunostimulants (Figure 9B), a positive correlation was found between *MTDH* and *MICB* ($r = 0.207$, $P = 5.76 \times 10^{-5}$; Figure 9C), *NT5E* ($r = 0.201$, $P = 9.8 \times 10^{-5}$; Figure 9D), and *TNFSF14* ($r = 0.143$, $P = 0.00576$; Figure 9E). Based on these results, *MTDH* may be involved in the regulation of tumor immunity.

Chemokines and their receptors induce cell migration. The expression of *MTDH* correlated with that of chemokines and receptors in immune cells, based on the data from the TISIDB database (Figure 10A and B). In HCC, *MTDH* expression positively correlated with *CXCL2* ($r = 0.224$, $P = 1.34 \times 10^{-5}$; Figure 10C) and negatively correlated with *CX3CL1* ($r = 0.245$, $P = 1.73 \times 10^{-6}$; Figure 10D) and *CXCL12* ($r = 0.208$, $P = 5.4 \times 10^{-5}$; Figure 10E). However, *MTDH* expression was not significantly correlated with chemokine receptor expression.

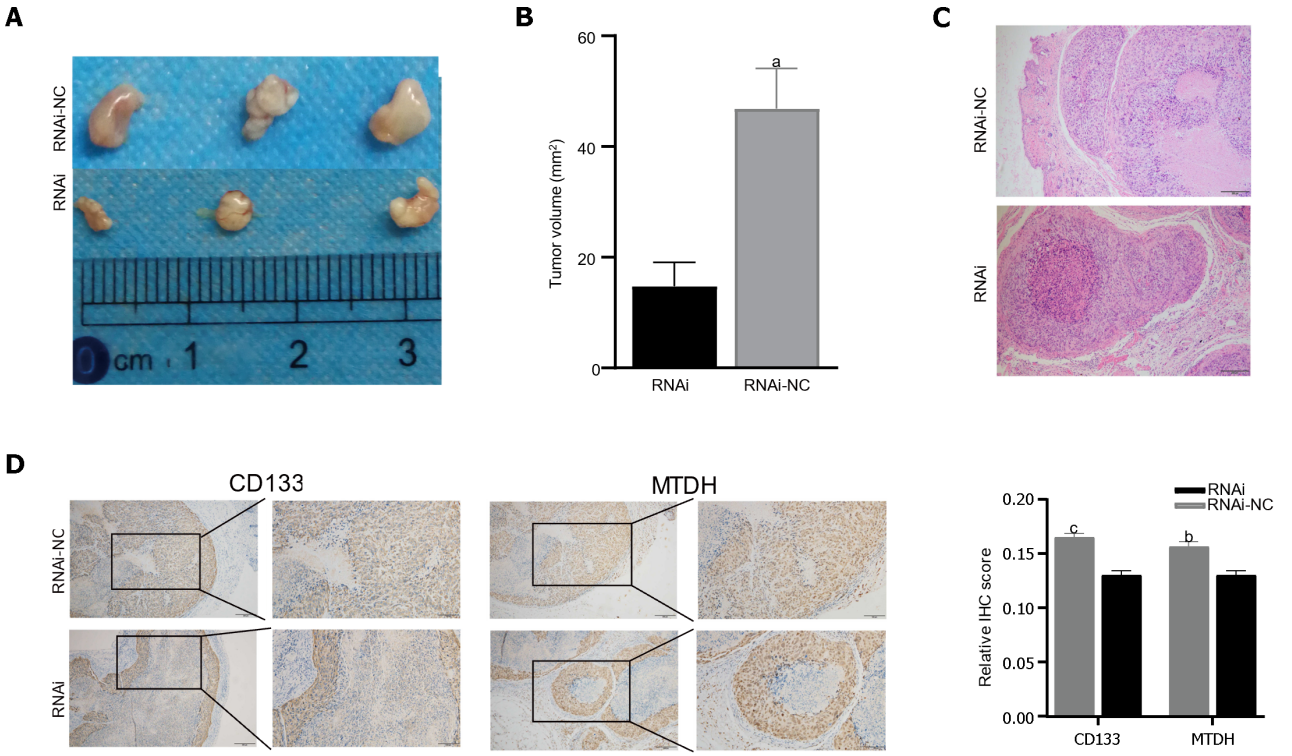

Figure 6 Metadherin stimulates tumorigenesis *in vivo*. A: Tumors derived from nude mice injected with 97H-RNAi (n = 3), 97H-NC cell (n = 3); B: Tumor volume showed that the inhibition of Metadherin (*MTDH*) significantly inhibited tumor growth; C and D: Representative immunohistochemical staining (IHC) images of tumors from nude mice stained with CD133 and MTDH. Histograms show the IHC score. Histograms show the IHC score (scale bars = 200 μm). $^{a}P < 0.01$, $^{b}P < 0.001$, $^{c}P < 0.0001$. RNAi: Metadherin knockdown group; RNAi-NC: Metadherin knockdown control group.

DISCUSSION

This study demonstrated that *MTDH* expression was higher in HCC tissues than in normal liver tissues and was associated with shorter survival time, stronger migration, and invasive ability. This study also focused on the effects of *MTDH* on stemness acquisition and immune infiltration of HCC cells. *MTDH* promotes stemness in HCC cells and high *MTDH* expression may impede the effectiveness of cancer immunotherapy. The findings of this study will provide additional information that can enhance the understanding of the prognosis and treatment of HCC patients.

It has been reported that the expression level of *MTDH* correlates with serum alpha-fetoprotein level[20], microvascular infiltration, tumor differentiation, and TNM stage[21]. In addition, the 1-year, 3-year and 5-year overall survival (OS) rates of the high-expression group were significantly lower than those of the low-expression group, and the cumulative recurrence rate was significantly higher than that of the low-expression group[21]. Univariate and multivariate analyses identified AEG-1 as an independent prognostic factor for OS and recurrence[22]. The findings of Yoo *et al*[22] showed that *MTDH* expression gradually increased in phases I-IV and from high differentiation to low differentiation. Differential expression, immunohistochemistry, and survival analysis showed similar results. The findings indicate that *MTDH* is closely associated with poor clinical prognosis of HCC. Additional studies have revealed that *MTDH* participates in proliferation, tumor progression, invasiveness[23], and metastasis[13]. We performed invasion, migration, and scratch assays on HCC cell lines with overexpressed and under-expressed *MTDH*. The results suggest that *MTDH* plays a role in HCC progression, which is consistent with the report of Yoo *et al*[22].

CSCs are capable of promoting metastasis and enhancing resistance to tumor therapies, including liver cancer therapy [24]. *CD133*, *NANOG*, *Oct4*, and several other molecular markers related to stemness maintenance in human CSCs have been reported[25,26]. Notably, *CD133* is a surface marker for LCSCs. The co-expression of *Nanog* and *OCT4* is associated with aggressive tumor behavior and worse clinical outcomes in HCC cells. A study by Hu *et al*[27] found strong correlations among *MTDH*, *CD133*, and *SOX2* Levels in gliomas. However, the contribution of *MTDH* to the tumor stem cell phenotype in HCC remains unclear. We explored the correlation between *MTDH* and stem cell markers in HCC using correlation analysis and experimental studies. The three stemness markers in the TCGA LIHC dataset exhibited positive correlation with *MTDH* expression. Additionally, MTDH expression increased in HCC spheres. The overexpression of *MTDH* in HCC cells enhanced their self-renewal ability, increased the proportion of $CD133^{+}$ cells, and promoted the expression of tumor stemness markers. However, after *MTDH* knockdown, stem cell markers were less expressed, and self-renewal was suppressed. The tumorigenic experiments were performed using nude mice. The primary tumor size was reduced in the *MTDH*-suppressed group, with decreased CD133 protein expression. These results confirm that *MTDH* plays a regulatory role in HCC stem cells Therefore, in this study, we demonstrated that *MTDH* promoted an increase in tumor stem cells in HCC cells, which could lead to a worse prognosis.

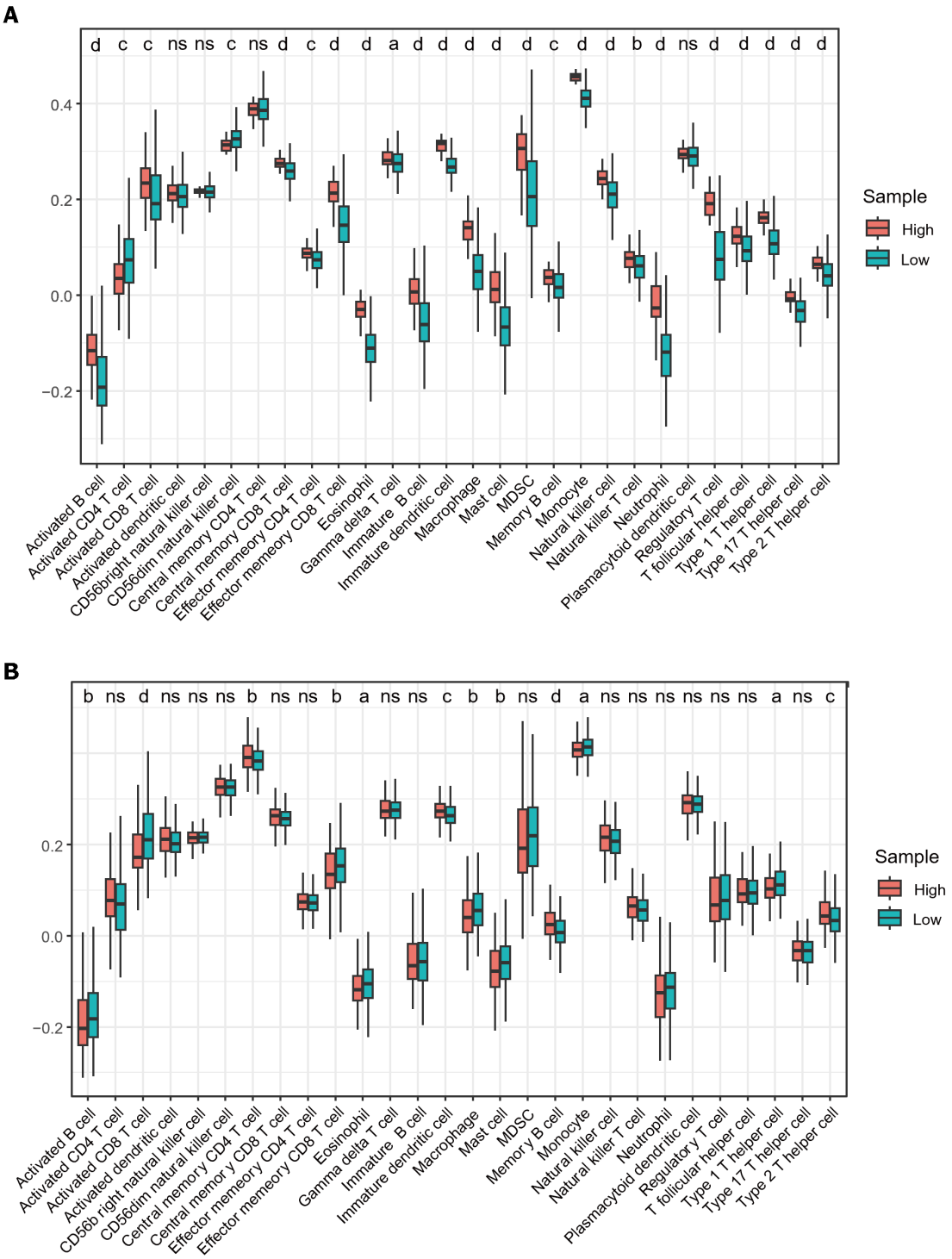

Figure 7 Infiltration of immune cells in TCGA samples using ssGSEA. A: Immune cell infiltration between LIHC samples and normal samples; B: Different immune cell infiltration patterns in high and low expression samples of Metadherin. ns: Not significant. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$, [d]$P < 0.0001$.

MTDH can influence the Wnt/β-catenin, Ha-ras, and PI3K/Akt pathways. The Wnt/β-catenin pathway maintains the CSCs phenotype[28]. In gastric cancer, *MTDH* forms a complex with *β-Catenin* and *LEF1*, facilitating the promotion of β-catenin protein translocation and activation of genes downstream of Wnt signaling[29]. CD133 glioma cells overexpressing *MTDH* maintain stemness and drug resistance through Wnt/β-catenin protein signaling[27]. The precise mechanism through which *MTDH* regulates CSCs in HCC should be further elucidated in the future.

MTDH increases the expression of PD-L1 and up-regulates the transcriptional activity of PD-L1 through the β-catenin/lev-1 signaling pathway. Patients with HCC and high *MTDH* and PD-L1 expression may benefit more from PD-1 monoclonal therapy. This suggests that *MTDH* is associated with immunity against HCC[30]. Immunotherapy resistance in tumor cells is related to components outside the tumor cells in the tumor microenvironment. Mature DCs can induce specific immune responses in the body, acting as anti-infection and anti-tumor agents. Conversely, immature DCs can inhibit the function of antigen-specific effector T cells in the body to further induce immune tolerance. There are different

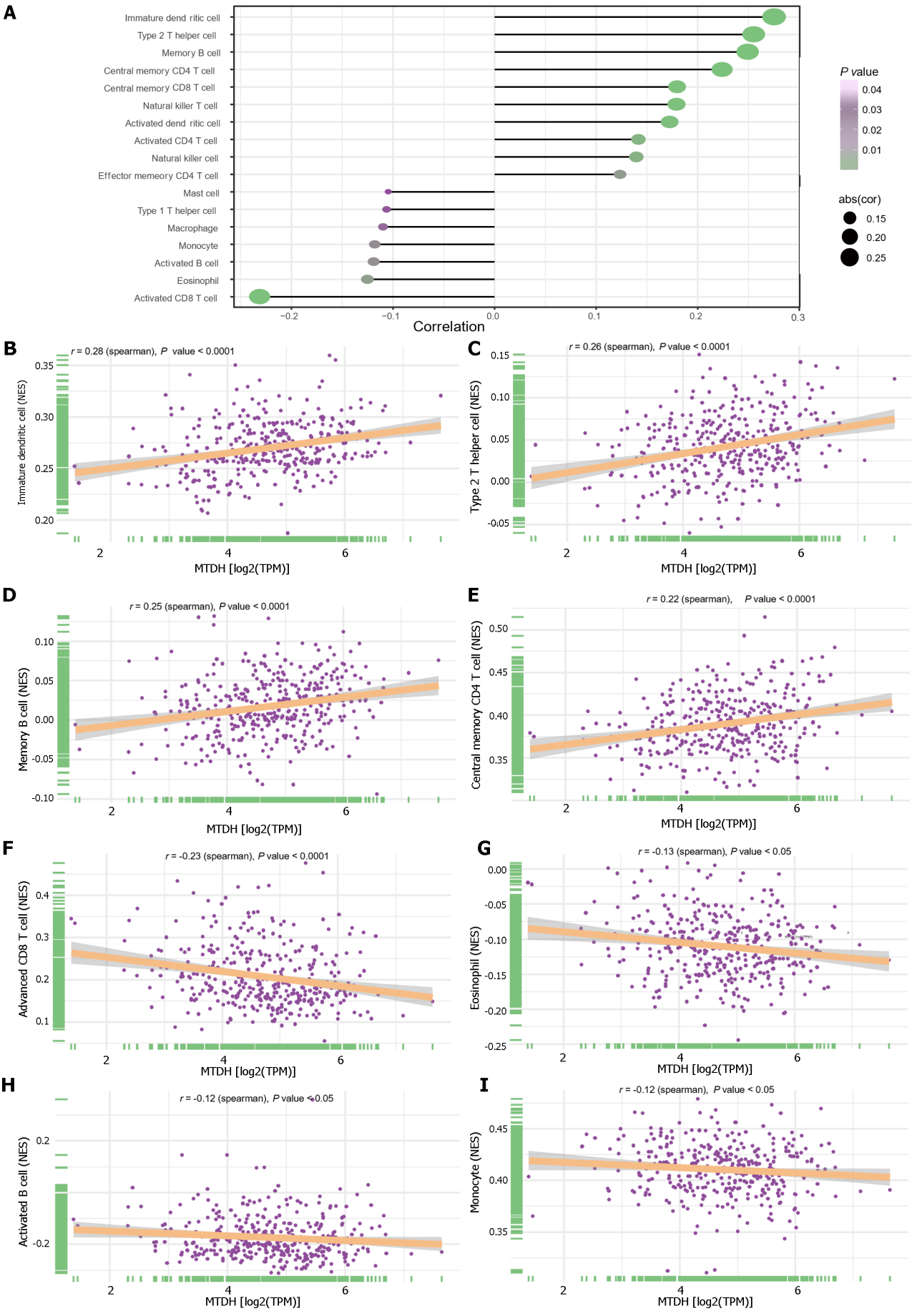

Figure 8 Immune infiltration and Metadherin expression levels. A: Metadherin (*MTDH*) expression levels correlated with infiltration of immune cells; B:

Correlations between *MTDH* and immature dendritic cells (r = 0.28, P < 0.0001); C: Correlations between *MTDH* and T helper 2 cells (r = 0.26, P < 0.0001); D: Correlations between *MTDH* and memory B cells (r = 0.25, P < 0.0001); E: Correlations between *MTDH* and central memory CD4 T cells (r = 0.22, P < 0.0001); F: Correlations between *MTDH* and activated CD8 T cell (r = -0.23, P < 0.0001); G: Correlations between *MTDH* and eosinophils (r = -0.13, P < 0.05); H: Correlations between *MTDH* and activated B cells (r =-0.12, P < 0.05); I: Correlations between *MTDH* and monocytes (r = -0.12, P < 0.05). MTDH: Metadherin.

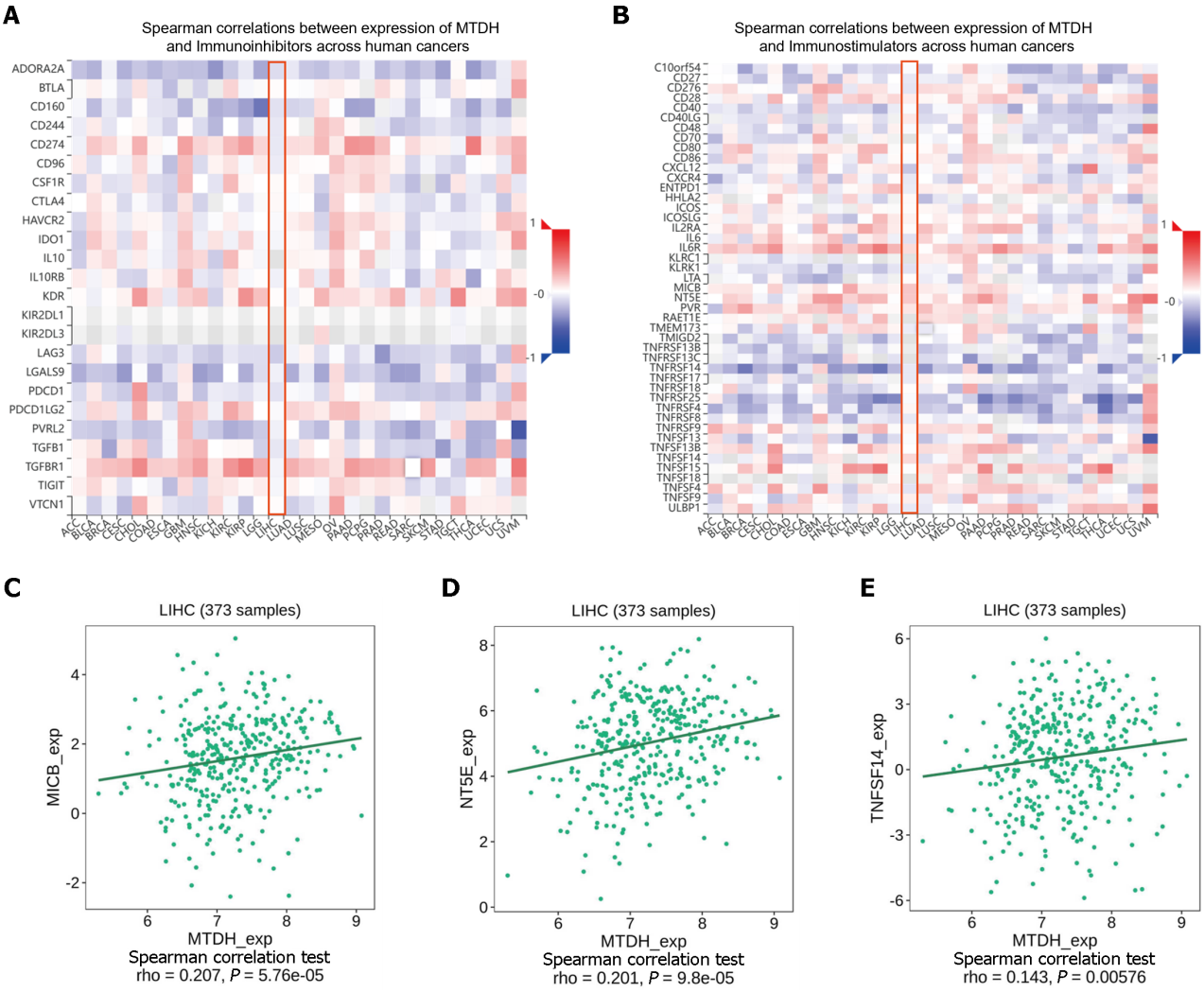

Figure 9 Correlation between Metadherin expression and immunomodulators. A and B: Heat map showing the correlation between Metadherin (*MTDH*) and immunosuppressive agents and immunostimulants in hepatocellular carcinoma; C: Correlations between *MTDH* and *MICB*; D: Correlations between *MTDH* and *NT5E*; E: Correlations between *MTDH* and *TNFSF14*. MTDH: Metadherin.

subpopulations of $CD4^+$ Th cells, which include Th1, Th2, and Th17 cells. It has been shown that Th2 cytokines (IL-4 and IL-10) promote tumor growth and metastasis, while Th1 cytokines (IL-2 and TNF-α) are associated with a good prognosis in HCC[31,32]. We found that a high level of *MTDH* expression positively correlated with an increase in Th2 cells and immature DCs. We speculated that *MTDH* may increase immune tolerance and metastasis of tumor cells by regulating the infiltration level of immature DC or Th2 cells, which in turn leads to poor prognosis in patients. In contrast, *MTDH* exhibited negative correlation with the infiltration levels of activated $CD8^+$ T cells, eosinophils, activated B cells, and monocytes. These findings suggest that *MTDH* plays a critical role in regulating tumor immune infiltration in HCC.

MTDH significantly correlated with the immunostimulants (*MICB*, *NT5E*, and *TNFSF14*). Chemokines initiate lymphocyte infiltration early in the development of malignancies to enhance the activities of antitumor agents. Chemokines reduce apoptosis, promote proliferation, enrich CSCs in tumors, and increase the resistance of tumor cells to therapy[33]. Analyzing the TISIB database revealed a positive correlation between *MTDH* and *CXCL2* expression levels and a negative correlation between *MTDH*, *CXCL12*, and *CX3CL1* expression levels. *CXCL2* promotes the invasion and migration of HCC cells[34]. The recurrence rate of intrahepatic or extrahepatic metastases is lower in patients with HCC expressing high levels of *CX3CL1* and its receptor, *CX3CR1*[35]. HPMEC (human lung microvascular endothelial cells) exhibit an MTDH-mediated attraction towards suspension-cultured cells through the CXCR4/CXCL12 axis, suggesting that MTDH promotes HCC cell metastasis through the CXCR4/CXCL12 pathway[36]. Our analysis showed that MTDH expression negatively correlated with *CCXCL12* and *CXCR4*, but not significantly with *CXCR4*. This finding was

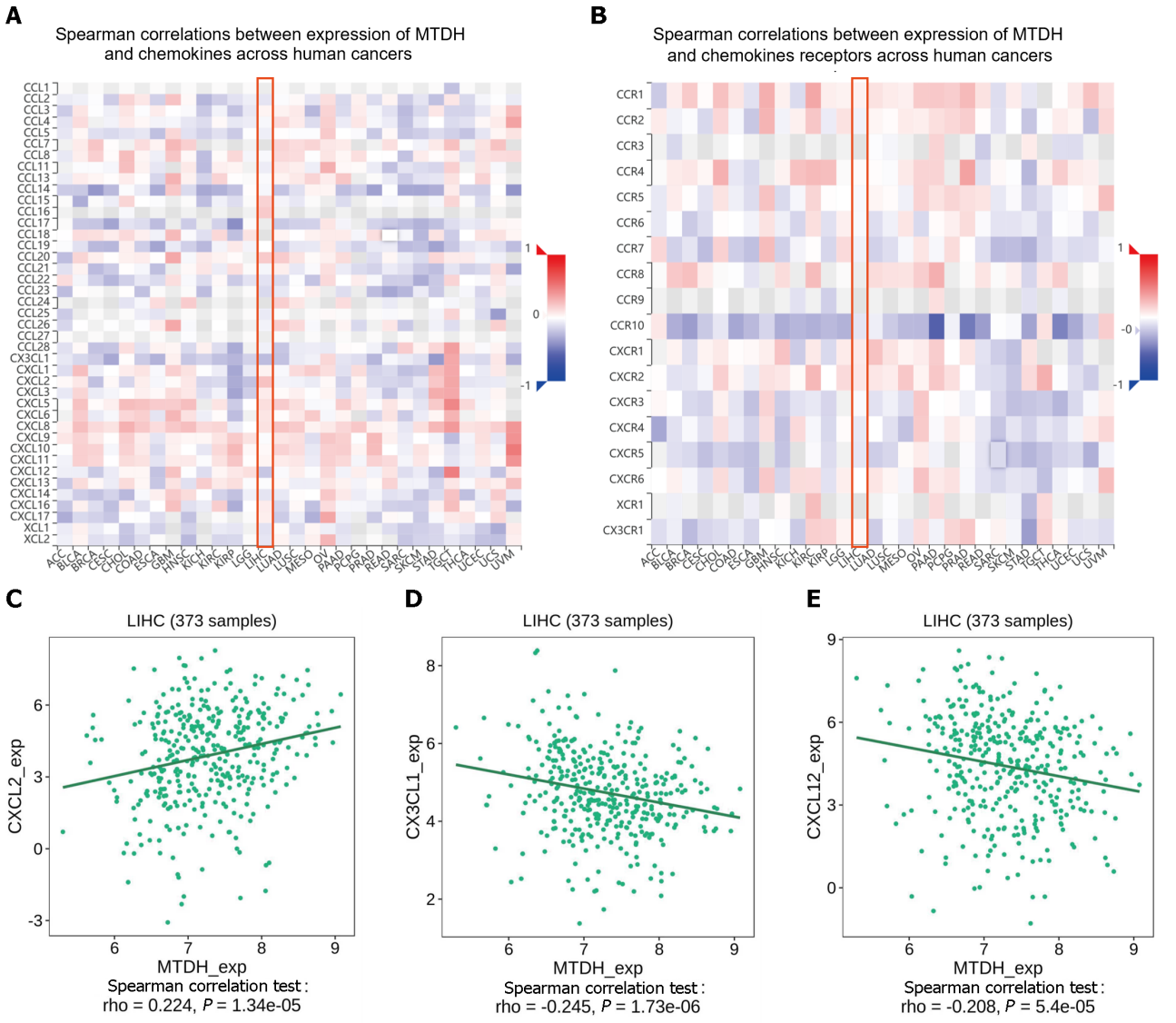

Figure 10 Chemokines and chemokine receptor correlates with Metadherin. A and B: A correlation analysis of Metadherin (*MTDH*) and chemokines and the receptors in LIHC is presented as a heat map; C: Correlations between *MTDH* and *CXCL2*; D: Correlations between *MTDH* and *CX3CL1*; E: Correlations between *MTDH* and *CXCL12*. MTDH: Metadherin.

inconsistent with our anticipated results. Exploring the interactions and the associated mechanisms between *CCXCL12* and *MTDH* will further elucidate the role of *MTDH* in HCC.

This study had some limitations. The exact mechanism through which *MTDH* influences HCC stem cells needs further investigation both *in vivo* and *in vitro*. Additionally, although we found that *MTDH* expression was closely associated with immune infiltration and prognosis, a more in-depth investigation is essential to elucidate the exact mechanism of MTDH-mediated immune infiltration.

CONCLUSION

High *MTDH* expression is associated with poor prognosis in HCC. *MTDH* may influence HCC progression through the regulation of tumor stemness and immune infiltration, providing additional evidence for the possible role of *MTDH* as a potential molecular marker of HCC.

ARTICLE HIGHLIGHTS

Research background

Metadherin (*MTDH*) is a key oncogene in most cancer types, including hepatocellular carcinoma (HCC). Tumor stem cells are associated with tumorigenesis, metastasis, cell proliferation, and postoperative recurrence. The type and number

of immune cells in the HCC microenvironment have prognostic value and can influence the response to immunotherapy. The impacts of *MTDH* expression on stem cell characteristics and immune cell infiltration in HCC remain unclear.

Research motivation

MTDH is a key oncogene in most cancers. It is important to explore the impact of *MTDH* on the prognosis of HCC patients and determine whether it affects tumor progression by influencing stem cell phenotype and immune infiltration.

Research objectives

This study aimed to investigate the effects of *MTDH* on tumor stemness and immunity in HCC.

Research methods

Differential expression of *MTDH* in tissues was detected using TCGA and GEO databases, and immunohistochemistry was performed on HCC and para-cancerous tissue samples. *MTDH* was stably downregulated or overexpressed by lentiviral transfection in both HCC cell types. Invasiveness and migration were assessed using stromal infiltration and wound healing assays. HCC stem cells were obtained by culturing spheroids in a serum-free medium. Flow cytometry, immunofluorescence, and sphere formation assays were used to identify stem-like cells. Relevant gene expression was detected through western blotting and real-time quantitative reverse transcription-PCR. The effect of *MTDH* inhibition on tumor growth was investigated using *in vivo* tumor formation assays. Correlations between *MTDH* and immune cells, immunomodulators, and chemokines were analyzed using ssGSEA and the TISIDB database.

Research results

This study confirmed that the expression of *MTDH* in HCC tissues was higher than that in normal liver tissues and that the high expression of *MTDH* resulted in poor prognosis of patients with HCC. HCC cells overexpressing *MTDH* exhibited stronger invasion and migration abilities, exhibited a stem cell-like phenotype, and formed spheres, whereas *MTDH* inhibition attenuated these effects. *MTDH* inhibition suppressed tumor growth and CD133 expression *in vivo*. Correlation analysis showed that *MTDH* exhibited positive correlation with immature dendritic cells (DCs), T helper (Th)2 cells, central memory $CD8^+$ T cells, memory B cells, activated DCs, natural killer (NK) T cells, NK cells, activated $CD4^+$ T cells, and central memory $CD4^+$ T cells. The results also showed that *MTDH* exhibited negative correlation with activated $CD8^+$ T cells, eosinophils, activated B cells, monocytes, macrophages, and mast cells. Correlation analysis of *MTDH* expression with immunomodulators and chemokines showed that *MTDH* levels positively correlated with *CXCL2* expression and negatively correlated with *CX3CL1* and *CXCL12* expression.

Research conclusions

In HCC, *MTDH* expression is increased, leading to poor prognosis. *MTDH* promotes the acquisition of tumor cell stemness and tumor growth in HCC, influencing immune infiltration and immunotherapy.

Research perspectives

Through database analysis, as well as *in vivo* and *in vitro* experiments, we confirmed that *MTDH* leads to poor prognosis in patients with HCC, promotes the acquisition of the tumor stem cell phenotype, and influences immune infiltration. The exact mechanism through which *MTDH* influences HCC stem cell and immune cell infiltration requires further exploration. This may provide a scientific basis for further understanding of the prognosis and treatment of patients with HCC.

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FOOTNOTES

Author contributions: Wang YY and Shen MM performed the experiments and wrote the manuscript; Gao J revised the manuscript; all the authors have approved the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Lipid metabolism-related long noncoding RNA RP11-817I4.1 promotes fatty acid synthesis and tumor progression in hepatocellular carcinoma

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Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) is one of the most common types of tumors. The influence of lipid metabolism disruption on the development of HCC has been demonstrated in published studies.

AIM

To establish an HCC prognostic model for lipid metabolism-related long non-coding RNAs (LMR-lncRNAs) and conduct in-depth research on the specific role of novel LMR-lncRNAs in HCC.

METHODS

Correlation and differential expression analyses of The Cancer Genome Atlas data were used to identify differentially expressed LMR-lncRNAs. Quantitative real-time polymerase chain reaction analysis was used to evaluate the expression of LMR-lncRNAs. Nile red staining was employed to observe intracellular lipid levels. The interaction between RP11-817I4.1, miR-3120-3p, and ATP citrate lyase (ACLY) was validated through the performance of dual-luciferase reporter gene and RIP assays.

RESULTS

Three LMR-lncRNAs (negative regulator of antiviral response, RNA transmembrane and coiled-coil domain family 1 antisense RNA 1, and RP11-817I4.1) were identified as predictive markers for HCC patients and were utilized in the construction of risk models. Additionally, proliferation, migration, and invasion were reduced by RP11-817I4.1 knockdown. An increase in lipid levels in HCC cells was significantly induced by RP11-817I4.1 through the miR-3120-3p/ACLY axis.

CONCLUSION

LMR-lncRNAs have the capacity to predict the clinical characteristics and prognoses of HCC patients, and the discovery of a novel LMR-lncRNAs, RP11-817I4.1, revealed its role in promoting lipid accumulation, thereby accelerating the onset and progression of HCC.

Key Words: Hepatocellular carcinoma; Lipid metabolism; Immune microenvironment; Prognostic markers; Metabolic reprogramming

Core Tip: In the current study, we investigated the functions of lipid metabolism-related long noncoding RNAs (LMR-lncRNAs) in assessing and forecasting the prognosis of patients with hepatocellular carcinoma (HCC), and established an accurate and reliable lipid metabolism-related risk score model for prediction. In particular, we focused on a novel LMR-lncRNA, RP11-817I4.1, which proved its important regulatory role in HCC lipid metabolism and tumor progression and showed therapeutic potential. Our findings will be extremely beneficial in understanding the probable molecular biological processes of HCC and discovering novel prognostic indicators and molecular targets.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is among the most common malignant tumors and ranks as the fourth leading cause of cancer-related fatalities globally[1]. Although surgical resection is an effective therapy for HCC, even patients in the early stages experience higher recurrence rates[2]. HCC is characterized by its rarity, poor prognosis, and frequent recurrence. Despite significant advancements in improving the prognosis of HCC patients, the 5-year survival rate remains low[3]. Therefore, further exploration of the pathogenesis and development of HCC, along with the discovery of novel diagnostic and prognostic indicators, is essential to identify potential therapeutic targets for enhancing patient survival and achieving precision treatment.

Mounting evidence underscores the significance of metabolic reprogramming in both the initiation and progression of carcinomas[4,5]. Due to their rapid growth and adaptability to changes in the tumor microenvironment (TME), cancer cells necessitate metabolic reprogramming to meet their high-energy demands[6]. One of the most prominent metabolic dysregulations observed in tumor cells is lipid metabolic reprogramming, a phenomenon that has garnered increasing attention[7]. HCC serves as a significant site for lipid metabolism and is associated with various lipid metabolic abnormalities[8]. Previous studies have established a connection between HCC and abnormal metabolic alterations, including increased de novo fatty acid synthesis, reduced oxidation, and modified phosphatidylcholine metabolic activity [9]. These metabolic pathways generate intermediate energy sources that facilitate HCC cell growth, proliferation, and metastasis[10]. An in-depth examination of the alterations in the TME is imperative for assessing the prognostic and therapeutic implications for HCC patients, as the proliferation of HCC cells in the body is intricately linked to the support provided by the tumor milieu[11]. Emerging evidence suggests that the TME of HCC may include numerous metabolic irregularities, with altered lipid metabolism being the most prominent among them, which has been gaining significant attention in recent years.

Noncoding RNAs longer than 200 nucleotides are long noncoding RNAs (lncRNAs)[12]. Recent studies suggest that lncRNAs may modulate fatty acid metabolism and affect tumor development[13]. Moreover, the sterol regulatory element binding protein (SREBP) transcription factor, apolipoprotein, triglyceride (TG) metabolism, and macrophage cholesterol absorption and efflux are other pathways through which lncRNAs impact lipid metabolism[14,15]. For example, it has been revealed that metastasis associated lung adenocarcinoma transcript 1 binds to SREBP-1c in hepatoma carcinoma and can maintain the stability of nuclear SREBP-1c protein through the ubiquitin-protease pathway, leading to lipid metabolism abnormalities in HepG2 hepatocytes[16]. In general, lncRNAs participate in the regulation of numerous genes associated with lipid metabolism in tumor cells[17,18].

The objective of this research was to assess the potential predictive value of lipid metabolism-related lncRNAs (LMR-lncRNAs) in HCC patients by integrating clinical data with the expression levels of relevant lncRNAs. Subsequently, three lncRNAs [negative regulator of antiviral response (NRAV), RNA transmembrane and coiled-coil domain family 1 antisense RNA 1 (TMCC1-AS1), and RP11-817I4.1] that were differentially expressed in the The Cancer Genome Atlas (TCGA) cohort and among lipid metabolism-related genes were used to establish the lipid metabolism-related risk score model (LMRRSM) for predicting the prognosis of HCC using univariate and multivariate Cox regression and Least absolute shrinkage and selection operator (LASSO) regression analyses. Receiver Operating Characteristic Curve (ROC) were generated to assess the specificity and sensitivity of the models. We developed a personalized prognostic characteristic model for patients with HCC by comprehensively analyzing the expression status and prognostic landscape of LMR lncRNAs. Quantitative real-time polymerase chain reaction (qRT–PCR) confirmed the differential expression of RP11-817I4.1 between HCC cells and adjacent normal tissues. Loss-of-function assays revealed the biological characteristics of the lncRNA RP11-817I4.1 in hepatoma cell lines. According to these results, a prognostic model and crucial LMR-lncRNAs might be developed as potential biomarkers and serve as indices for HCC prognosis.

MATERIALS AND METHODS

Data source and preprocessing

Normalized RNA-seq data from 50 normal and 374 HCC samples were obtained from the TCGA[19]. Furthermore, the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA) databases yielded a list of eight lipid metabolism-related pathways and 323 lipid metabolism-related genes. One study of clinical relevance omitted 135 patients from the TCGA cohort because of inadequate clinical information.

Identification of differentially expressed LMR-lncRNAs

With the conditions of a |coefficient of correlation| > 0.6 and $P < 0.001$, Pearson's correlation analyses were conducted to investigate the coexpression of lipid metabolism-related genes and lncRNAs. The "limma" package of R was used to search for lncRNAs that were differentially expressed between tumor and normal tissues. We defined the lncRNA difference analysis data as follows: "false discovery rate (FDR)< 0.05, log2| fold change (FC)| > 1, and $P < 0.05$."

Construction of the LMRRSM

To identify LMR-lncRNAs associated with survival, we employed the R package "survival" to conduct univariate Cox regression analysis of the differentially expressed lncRNAs and overall survival (OS) in HCC patients. Subsequently, to mitigate overfitting and select the optimal LMR-lncRNAs, we applied a LASSO regression model. Following collinearity assessment, stepwise multivariate Cox regression analysis was employed to establish the LMR-lncRNA-derived risk signature in HCC patients. The following algorithm, which was based on the combination of Cox coefficients and lncRNA expression data, was used to calculate risk scores for the TCGA cohort. The risk score was calculated as follows: risk score = expression of lncRNA 1 α1 + expression of lncRNA 2 α2 + expression of the lncRNA *n*αn. The regression coefficient of the LMR-lncRNAs is represented by the symbol α in the risk signature. Using the median values of the risk scores, HCC patients in the TCGA cohort were categorized into either high-risk or low-risk groups. Survival analysis was subsequently performed to evaluate the survival rates of the high- and low-risk groups. Further investigation was conducted to analyze the differences in prognosis between the two risk groups. A reasonable cutoff threshold was defined as a P value < 0.05. The prediction accuracy and reliability were also assessed using ROC curves.

To validate the association between LMR-lncRNAs and clinical characteristics, we evaluated the associations between the risk scores and clinical features. In the univariate Cox regression analysis, factors, including age, sex, grade, stage, T stage, N stage, M stage, and the risk scores derived from the lipid metabolism-related signature were all taken into account to assess the prognostic relationship of LMRRSM within the TCGA cohort. Subsequently, through multivariate analysis, we assessed the independent prognostic potential of the risk scores. Next, we investigated the association between the risk score and clinicopathological features to determine the significance of the predictive models for the progression of HCC.

Analysis of the GeneExpression Profiling Interactive Analysis database

The GeneExpression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn/) can be used for cancer or normal gene expression profiling and interaction analyses. We used the GEPIA database to evaluate LMR-lncRNA expression in HCC and normal tissue samples. Furthermore, GEPIA was used to evaluate the survival of patients with HCC stratified by the lipid metabolism-related. A P value less than 0.05 was used.

GO and KEGG enrichment analysis

Functional enrichment analyses were performed using the clusterProfiler package. A P-value < 0.05 indicated significant enrichment. We employed the R packages "GOplot" and "ggplot2" to visualize the most prominently enriched GO terms and KEGG pathways, respectively. GSEA was used to investigate the connection between the expression of RP11-817I4.1 and signaling pathway activity.

Evaluation of immune environment characteristics

To further illustrate the potential impact of the predictive risk score on the tumor immune microenvironment, immune-

related scores for different risk groups were calculated using the R software tool ESTIMATE. Single sample gene set enrichment analysis (ssGSEA), which measures the enrichment levels of 29 immune characteristics for every HCC patient through the form of ssGSEA scores, was carried out using the R package "GSVA." Previous studies have furnished evidence regarding immune cell signatures[20,21]. Subsequently, in the high- and low-risk groups, we explored the relationship between immune cell infiltration and immune-related pathways.

The TIMER online database (https://cistrome.shinyapps.io/timer/) was used to reanalyze gene expression data from the TCGA to assess the abundance of six subtypes of tumor-infiltrating immune cells[22]. As a result, we determined the extent of immune infiltration in HCC patients and examined the relationship between the risk score and immune cell infiltration.

Patients and tissue samples

Between June 2021 and July 2022, we obtained 80 pairs of HCC samples and their corresponding nontumor tissues with explicit consent from HCC patients at the Second Affiliated Hospital in Kunming, China. None of the participants in this study received preoperative chemotherapy or radiation. The diagnosis of HCC was independently confirmed by two pathologists. The ethical committee of the Second Affiliated Hospital of Kunming Medical University approved this study, which was conducted in accordance with the Helsinki Declaration.

Cell lines and cell culture

HCC cell lines (Hep-G2, Hep-3B, Li-7, Huh-7, and HCC-LM3) and the normal human hepatic cell line L02 were sourced from the Chinese Academy of Sciences Cell Bank in Shanghai, China. Li-7, Huh-7, HCC-LM3, Hep-G2, Hep-3B, and L02 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). All cell culture media were supplemented with penicillin (100 U/mL) and streptomycin (100 U/mL) and maintained at 37 °C in a 5% CO_2 incubator.

qRT–PCR

TRIzol reagent was used to collect and lyse the cells (Invitrogen, CA, United States). A PrimeScript RT Kit (TaKaRa, Osaka, Japan) and RNA (1 μg) were used to generate reverse-transcribed complementary DNA (cDNA). For real-time-quantitative polymerase chain reaction, Fast SYBR Green Master Mix was used (Bio-Rad, CA, United States). The reaction cycle conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. GAPDH served as the internal loading control, and the relative quantification $2^{-\Delta\Delta CT}$ technique was employed to calculate the relative levels. The primer sequences used in this study were as follows: RP11-817I4.1: Forward: *5'-GTAGTGGCTGCTGCTGTTAGG-3'* Reverse: *5'-TTCAACGGTGGCAAACTCAAAG-3'* Actin sequence: Forward: *5'-ATCATGTTTGAGACCTTCAACA-3'* Reverse: *5'-CATCTCTTGCTCGAAGTCCA-3'*-Actin was utilized as the internal control.

Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis is commonly used to separate extracted proteins. The samples were loaded into the wells of the gel, and an electric current was applied to separate the proteins based on their size and charge, resulting in the formation of distinct bands. Subsequently, the separated proteins were transferred onto a PVDF membrane by applying an electric current to the membrane. The transferred membrane was treated with a protein-blocking agent (bovine serum albumin) to prevent nonspecific binding. ATP citrate lyase (ACLY) recombinant rabbit monoclonal antibody (702731; Thermo Scientific) was added to a mixture containing the blocking agent, after which the proteins were allowed to bind to the transferred membrane. The antibody specifically binds to the target protein, leading to the formation of an immune complex. To remove unbound antibodies and other non-specific binding substances, the transferred membrane underwent multiple TBST washes. Images were captured using a chemiluminescence imaging system, and quantitative analysis was conducted. The expression levels of the target proteins in the samples were quantified based on optical density or fluorescence intensity.

Subcellular fractionation assay

Nuclear and cytoplasmic RNA was separated from each fraction using a PARIS Kit (Life Technologies, United States). Next, the nuclear and cytoplasmic RNA molecules isolated from each sample were identified *via* qRT-PCR. The nuclear and cytoplasmic markers GAPDH and Neat1 were identified.

Cell transfection

To downregulate RP11-817I4.1, three human small interfering RNAs (RP11-817I4.1-sh1, *UAAAUCAUUACCACUUUGGUU*; RP11-817I41-sh2, *AUUAUCACUACCAAUUUCCUU*; RP11-817I41-sh3, *UUGUUUGCCACUUCUGCCCUU*; and control shRNA (si-NC, *TTCTCCGAACGTGTCACGT*) were manufactured by Qingke (SL100568, SignaGen, United States). Lipofectamine® 3000 (Sigma, United States) was employed for all cell transfections, which were carried out for 48 h at 37 °C. The cells were then utilized for subsequent experiments 48 h after transfection.

Cell colony formation assays

After 24 h of transfection, 500 tumor cells/well were plated into 6-well plates and cultivated for colony formation at 37 °C with 5% CO_2. The cells were fixed for 5 min with 10% formaldehyde and stained for 1 h with 0.1% crystal violet. The count of colonies was conducted after a 2-wk period. Each experiment was performed in triplicate, and the means of the results were computed.

Wound healing assay

Cell migration was assessed *via* wound healing assays. Transfected cells were placed in 6-well plates [5 × 10^5 cells/well containing 5% (v/v) FBS]. Subsequently, a 200 μL pipette tip was employed to gently scrape the cell monolayer that had reached 95%-100% confluence. Using a light inverted microscope, the wound was inspected at 0 and 24 h, and the results were recorded as W0 and 24, respectively (magnification, 40 ×). Cell migration (%) was estimated using the following formula: (W024)/W0 × 100.

Transwell migration and invasion experiments

The transfected cells were centrifuged and suspended before being introduced into the upper layer of the Transwell system for use in the Transwell migration test. Following that, 600 μL of complete medium containing 20% FBS was added to the lower compartment. After 24-48 h of incubation, the transplanted membranes were stained with 0.1% crystal violet and preserved in methanol. Subsequently, the stained cells were counted and photographed under a microscope, and the average value was determined. Similarly, in the Transwell invasion test, the Matrigel was removed, and the top chamber was precoated for the migration experiment. The migration assay was carried out in the following stages.

Nile red staining

After the tissues were homogenized, the cells were placed in 6-well plates with 4% paraformaldehyde solution, washed with phosphate-buffered saline (PBS), and stained with Nile red solution (7385-67-3; Solarbio, Beijing, China) in the dark. Images were acquired using IF microscopy after the samples had been subjected to two PBS washes and stained with 4′,6-diamidino-2-phenylindole.

Dual-luciferase reporter assay

We designed and synthesized a dual-luciferase reporter vector containing these gene sequences. This vector typically includes genes for two luciferases (firefly luciferase and green fluorescent protein), along with the promoter and terminator sequences of RP11-817I4.1 and ACLY. The constructed dual-luciferase reporter vector was transfected into liver cancer cells. After the addition of miRNA mimics or miRNA inhibitors, luciferase activity was assessed using a luciferase assay kit (16186, Thermo Scientific) following the manufacturer's instructions. This involved separately measuring the activities of firefly luciferase and green fluorescent protein. The competitive relationship between miRNAs and mRNAs was evaluated by comparing the differences in luciferase activity among the various groups.

RNA immunoprecipitation

Cell lysates were harvested and prepared from liver cancer cells. Then, the specific anti-AGO2 antibody (MA5–23515; Thermo Scientific) was added to the protein A/G agarose beads to form antigen-antibody complexes. The cell lysate was coprecipitated with antigen-antibody complexes to enrich the complex containing AGO2. The coprecipitated complexes were washed and dissolved, followed by colloidal sol/colloidal removal. The proteins were removed, and the remaining RNA was extracted by proteinase treatment. The isolated RNA was reverse-transcribed into cDNA using reverse transcriptase (RT), and quantitative polymerase chain reaction was employed to detect the presence and quantification of the target mRNA and miRNA within the AGO2 complex. Specific primers and probes were used to quantitatively measure the expression levels of the target mRNAs, lncRNAs, and miRNAs.

Transcriptomics

The samples (cells or tissues) were prepared, and RNA was extracted. The isolated RNA was transcribed to synthesize cDNA using RT and random primers. This conversion process allowed the mRNA to be transformed into cDNA. An RNA library suitable for sequencing was subsequently constructed by adding adapter sequences and performing PCR amplification. The prepared RNA library was subjected to high-throughput sequencing (*via* the Illumina HiSeq platform). Afterward, we carried out preprocessing of the sequencing data, which involved the elimination of low-quality reads and adapter sequences. Bioinformatic tools were employed to align and quantify the preprocessed sequencing data against a reference genome or transcriptome. Significantly differentially expressed genes were determined through differential expression analysis (log-fold change > 1, FDR value < 0.05).

Subcutaneous tumor model

We chose 6-wk-old male BALB/c mice from the Animal Experimental Center of Kunming Medical University, ensuring that the animals were purebred, healthy, and met the experimental criteria. Tumor cell lines were transfected with the RP11-817I4.1-sh1 plasmid for *in vitro* cultivation, ensuring the health and purity of the cells. The cultured tumor cells were harvested, and an appropriate cell suspension was obtained through washing and centrifugation. The sterile operating room was filled, and the operating area and tools were prepared in advance. Isoflurane was used to anesthetize the mice, ensuring that they were painless. The tumor cell suspension was subcutaneously injected into the left axilla of each animal. After a period of 3 wk, the mice were humanely euthanized, and specimens were collected. All procedures and steps related to animal experiments were subject to rigorous review and approval by the Ethics Review Committee for Animal Experiments at Kunming Medical University (Approval Number: kmmu20211188).

Statistical analysis

For all the statistical studies, GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, United States) and SPSS 24.0 (SPSS, Inc., Chicago, IL, United States) were used. At least three separate experiments were conducted for each group.

Continuous data are reported as the mean ± SD. For statistical analysis, the unpaired Student's *t*-test was employed to compare continuous variables. Variations across experimental groups were assessed using either Student's *t*-test or one-way ANOVA. Survival times across groups were evaluated using Kaplan-Meier (K-M) survival analysis or univariate Cox regression analysis. *P*-values were calculated for both sides, and a confidence threshold of 0.05 was considered statistically significant. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

RESULTS

The study's workflow is depicted in Figure 1A, with each step elaborated upon in the subsequent sections.

Differential expression of LMR-lncRNAs

Using RNA sequencing data from HCC patients and 323 lipid metabolism-related genes, we identified 31 differentially expressed LMR-lncRNAs *via* the R "limma" package; 29 of these lncRNAs were upregulated, and 2 were downregulated. The results are shown as heatmaps and volcano plots (Figure 1B and C).

Construction of the LMRRSM and analysis

To further understand the connection between LMR-lncRNAs and patient prognosis, we constructed a three-lncRNA model based on LMR-lncRNAs to predict disease prognosis and survival in patients with HCC. The expression of ten LMR-lncRNAs was found to be strongly correlated with OS *via* univariate Cox regression analysis. A forest plot of the hazard ratio (HR) revealed that, whereas one lncRNA was protective, nine lncRNAs were risk factors (Figure 1D). Subsequently, LASSO regression analysis of the LMR lncRNAs associated with OS was performed to evaluate the predictive ability of the model (Figure 1E and F). Thus, after LASSO regression, only four lncRNAs were found among the 10 significant LMR-lncRNAs in the univariate Cox regression model. Ultimately, a predictive signature consisting of three LMR-lncRNAs, NRAV, TMCC1-AS1, and RP11-817I4.1 was chosen to construct a prognostic model *via* multivariate Cox regression analysis (Figure 1G). Similarly, the coefficients of the three lncRNAs were 0.134336124, 0.663916403, and 0.703331257. The three lncRNAs had HRs of 1.143777206, 1.942384618, and 2.020472221. The total risk score was calculated as follows: (0.134336124 × expression level of NRAV) + (0.663916403 × expression level of TMCC1-AS1) + (0.703331257 × expression level of RP11-817I4.1). Figure 1H-J shows that the expression of the three lncRNAs was upregulated in HCC tissues according to the GEPIA database. Additionally, GEPIA revealed that HCC patients with higher NRAV, TMCC1-AS1, or RP11-817I4.1 expression had worse prognoses (Figure 1K-M).

Relationships between clinical characteristics and risk scores

Through univariate and multivariate Cox regression analyses, we established that risk scores could serve as independent prognostic factors. As illustrated in Figure 2A, the univariate Cox regression analysis demonstrated significant associations between OS and stage, T stage, M stage, and risk score. However, according to our multivariate analysis, only the risk score was significantly associated with OS (Figure 2B). We observed that the advanced-stage (Figure 2C) and T-stage (Figure 2D) tumors had higher expression levels of NRAV; the expression levels of TMCC1-AS1 increased in the advanced-stage tumors (Figure 2E); and the advanced-stage (Figure 2F) and T-stage (Figure 2G) tumors had higher expression levels of RP11-817I4.1. In addition, we determined that the risk score could be used as an independent prognostic factor. Additionally, correlation analysis revealed strong associations between risk score and disease stage (Figure 2H), grade (Figure 2I), and T stage (Figure 2J) ($P < 0.05$).

Clinical outcomes of the different risk groups

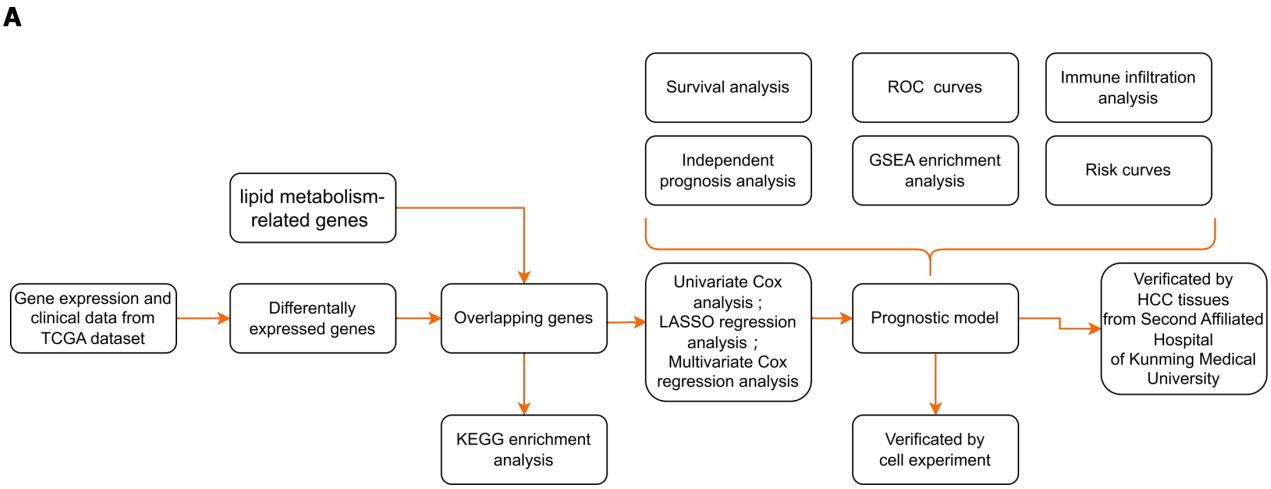
Based on the median risk scores, HCC patients were divided into low- and high-risk groups. The prognostic risk scores for each HCC sample were computed using the formula mentioned above. Figure 3A-C show the dispersion of the survival status, risk score, and matching heatmap of the expression of the three lncRNAs in the TCGA cohort. As the risk score increased, the expression levels of NRAV, TMCC1-AS1, and RP11-817I4.1 increased (Figure 3A). Figure 3C demonstrates that patients with a higher risk score had a considerably greater mortality rate than those with a lower risk score. In addition, K-M survival curve analysis revealed that patients with HCC in the high-risk subgroup had poorer outcomes than those in the low-risk subgroup (Figure 3D). Subsequently, we evaluated the time-dependent ROC curves to confirm the accuracy of the LMRRSM. At 1, 2, and 3 years, the AUC values of the prognostic model in the TCGA cohort were 0.757, 0.713, and 0.666, respectively (Figure 3E). Overall, our findings indicate that the LMRRSM can accurately predict the occurrence and progression of HCC.

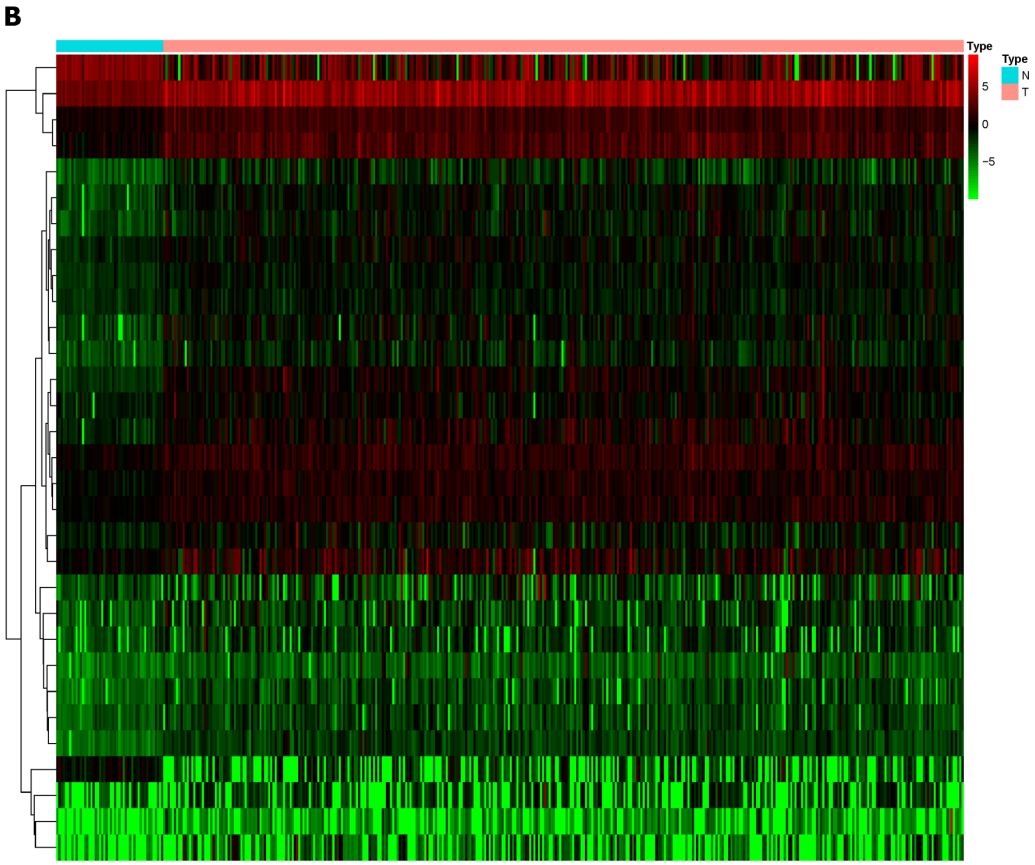
Identification of potential pathways and biological functions of DEGs between risk score groups

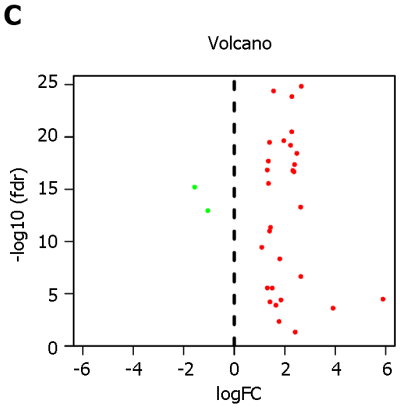
To further explore the biological processes and pathways associated with the risk score, GO and KEGG analyses were performed on the differentially expressed genes (DEGs) between the high- and low-risk groups. According to the GO-BP analysis, the genes with differential expression were mostly enriched in extracellular matrix organization, extracellular structure organization, and nuclear division (Figure 3F). The DEGs identified *via* KEGG analysis were mostly related to the PI3K/Akt signaling pathway, the cell cycle, and focal adhesion (Figure 3G).

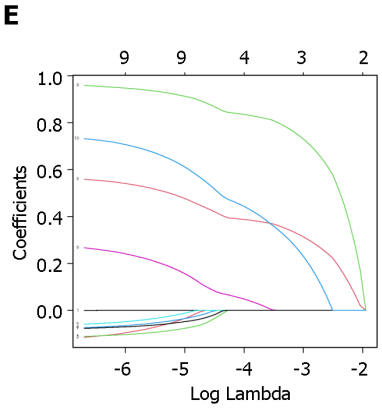
The immune landscape of the high- and low-risk groups

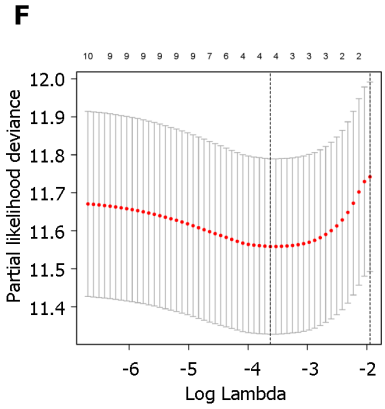
We assessed the activity or enrichment levels of immune cells, functions, or pathways in the different risk score groups


A
Survival analysis
ROC curves
Immune infiltration analysis
Independent prognosis analysis
GSEA enrichment analysis
Risk curves
lipid metabolism-related genes
Gene expression and clinical data from TCGA dataset
Differentially expressed genes
Overlapping genes
Univariate Cox analysis ; LASSO regression analysis ; Multivariate Cox regression analysis
Prognostic model
Verificated by HCC tissues from Second Affiliated Hospital of Kunming Medical University
KEGG enrichment analysis
Verificated by cell experiment

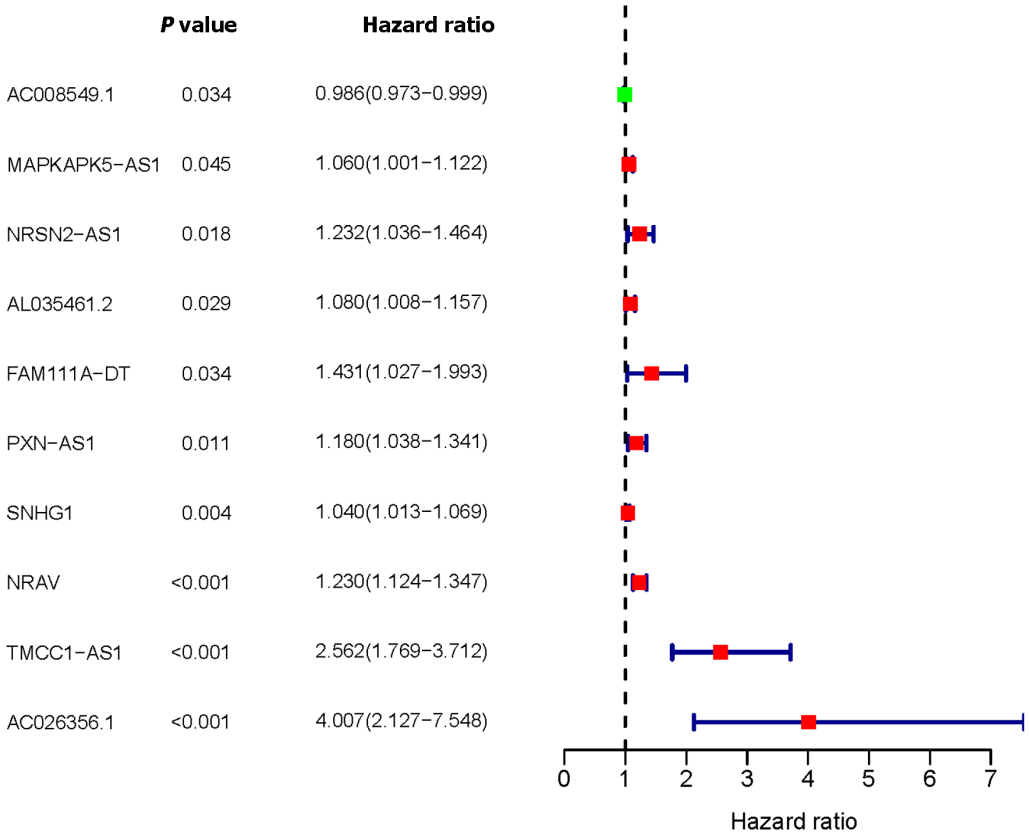

B
Type
N
T
5
0
−5


C
Volcano
-log10 (fdr)
logFC

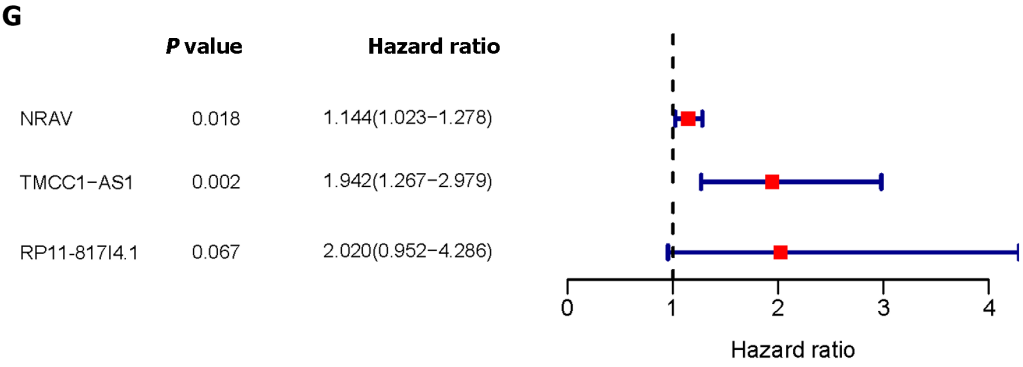

E
Coefficients
Log Lambda


F
Partial likelihood deviance
Log Lambda

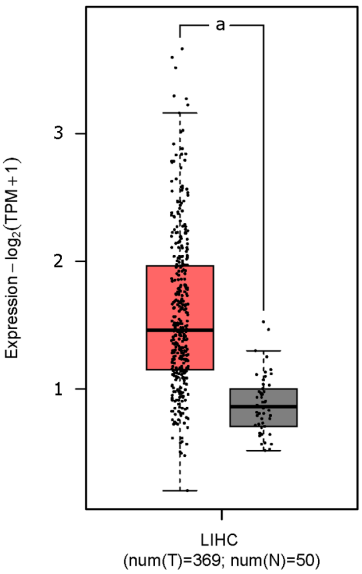
D


P value
Hazard ratio
AC008549.1 0.034 0.986(0.973−0.999)
MAPKAPK5−AS1 0.045 1.060(1.001−1.122)
NRSN2−AS1 0.018 1.232(1.036−1.464)
AL035461.2 0.029 1.080(1.008−1.157)
FAM111A−DT 0.034 1.431(1.027−1.993)
PXN−AS1 0.011 1.180(1.038−1.341)
SNHG1 0.004 1.040(1.013−1.069)
NRAV <0.001 1.230(1.124−1.347)
TMCC1−AS1 <0.001 2.562(1.769−3.712)
AC026356.1 <0.001 4.007(2.127−7.548)
0 1 2 3 4 5 6 7
Hazard ratio

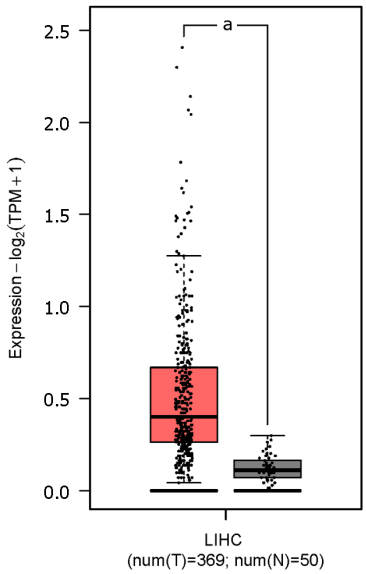
G


P value
Hazard ratio
NRAV 0.018 1.144(1.023−1.278)
TMCC1−AS1 0.002 1.942(1.267−2.979)
RP11-817I4.1 0.067 2.020(0.952−4.286)
0 1 2 3 4
Hazard ratio

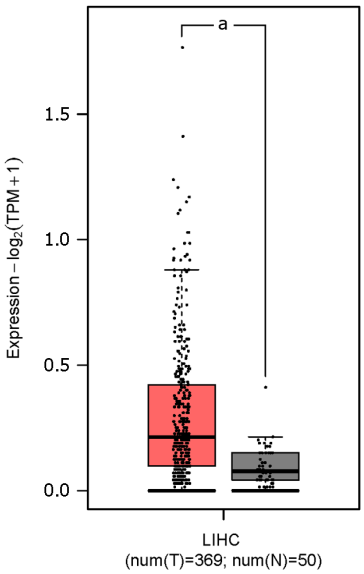
H


a
Expression − log2(TPM+1)
LIHC
(num(T)=369; num(N)=50)

I


a
Expression − log2(TPM+1)
LIHC
(num(T)=369; num(N)=50)

J


a
Expression − log2(TPM+1)
LIHC
(num(T)=369; num(N)=50)

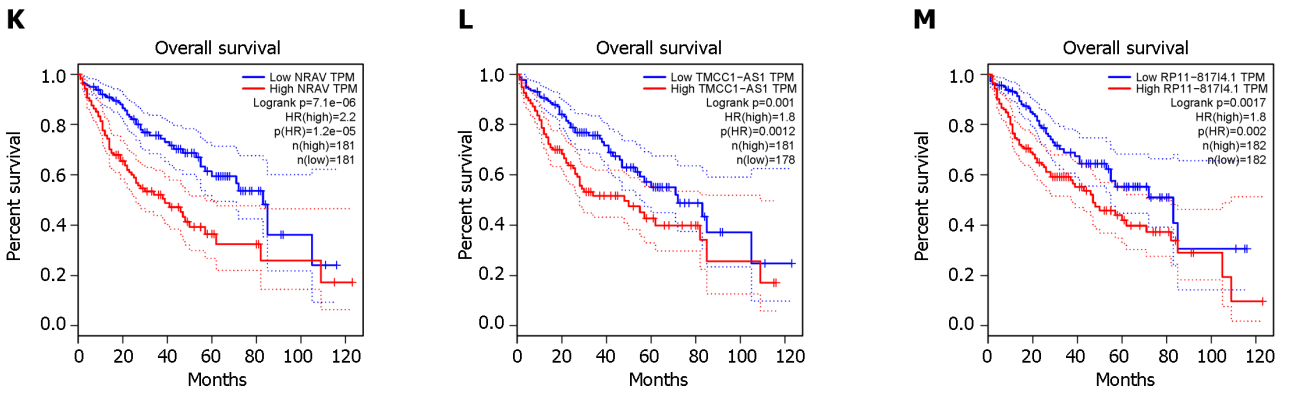


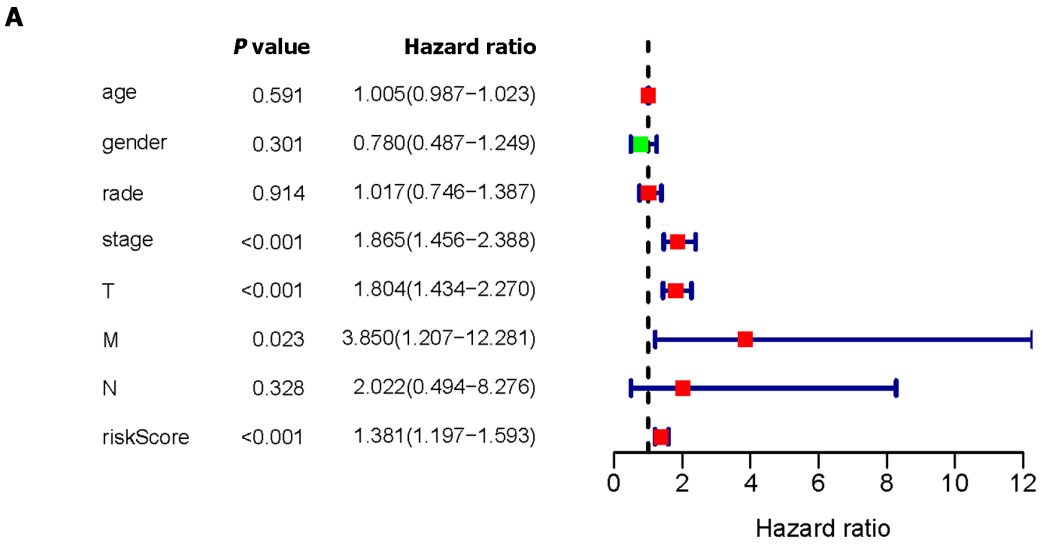
Figure 1 Differentially expressed lipid metabolism-related and prognosis-related long non-coding RNAs. A: Flow chart of the analysis process in our study; B and C: Heatmap and volcano plot demonstrating differentially expressed lipid metabolism-related long non-coding RNAs (lncRNAs) between Hepatocellular carcinoma (HCC) tissues and nontumor tissues. Red, positive regulation; green, negative regulation; D: Forest plot of hazard ratios (HR) constrating the prognostic value of lncRNAs. The dashed line indicates that the location of the HR was 1; E: Construction and analysis of the prognostic risk model. Plots for Least absolute shrinkage and selection operator expression coefficients of 10 Lipid metabolism-related lncRNAs; F: Cross-validation plot for the penalty term; G: The HRs and *P* values from the multivariate Cox regression analysis are shown in the forest plot; H-J: Three differentially expressed lncRNAs between HCC and nontumor tissues in the GeneExpression Profiling Interactive Analysis database; K-M: Survival curves of patients with differential expression of three lncRNAs in GEPIA. HCC: Hepatocellular carcinoma; Lasso: Least absolute shrinkage and selection operator; KEGG: Kyoto Encyclopedia of Genes and Genomes; NRAV: Negative regulator of antiviral response; TMCC1-AS1: RNA transmembrane and coiled-coil domain family 1 antisense RNA 1; FC: Fold change; FDR: False discovery rate; TPM: Transcripts Per Kilobase of exon model per Million mapped reads; TCGA: The Cancer Genome Atlas; ROC: Receiver operating characteristic.

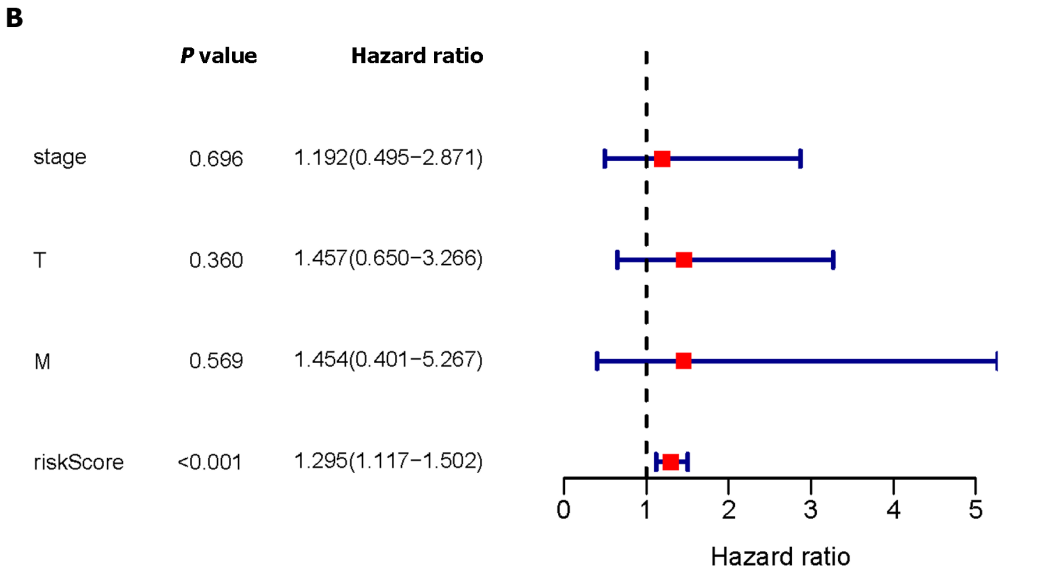
using ssGSEA scores, which were derived from the analysis of 29 immune-associated gene sets (Figure 4A). We effectively calculated scores for immune cell infiltration, stromal content, and tumor purity using the ESTIMATE methodology (Figure 4B). By comparing stromal content, tumor purity, and immune cell infiltration scores, we elucidated the relationship between distinct risk groups and immune cell infiltration. The results showed that there were no significant differences in stromal scores, estimated scores, ESTIMATE scores, tumor purity, or immune scores between the high-risk and low-risk patients (Figure 4C-F). According to the ssGSEA results, the percentages of aDCs, iDCs, macrophages, Tfhs, Th2 cells, and Tregs were significantly greater in the high-risk group than in the control group, whereas the percentages of infiltrating B cells, mast cells, and NK cells were significantly lower. Additionally, we found that when risk scores improved, some immunological traits (such as antigen presenting cell costimulatory, C-C chemokine receptor, checkpoint, and major histocompatibility complex class I classes) were significantly more active, whereas others [such as cytolytic activity and interferon (IFN) classes I and II] were repressed (Figure 4G and H). We conducted a further analysis of the relationship between the risk score and immune cell infiltration. Correlation analysis revealed that the risk score exhibited strong associations with CD8+ T cells (r = 0.236; P = 4.3e-6), macrophages (r = 0.359; P = 1.038e-12), neutrophils (r = 0.339; P = 2.034e-11), dendritic cells (r = 0.315; P = 5.43e-10), CD4+ T cells (r = 0.139; P = 0.008), and B cells (r = 0.213; P = 3.675e-05) (Figure 4I-N). These findings suggest that risk signatures based on the LMRRSM may provide new insights into the TME, immune responses, and immune infiltration in HCC patients.

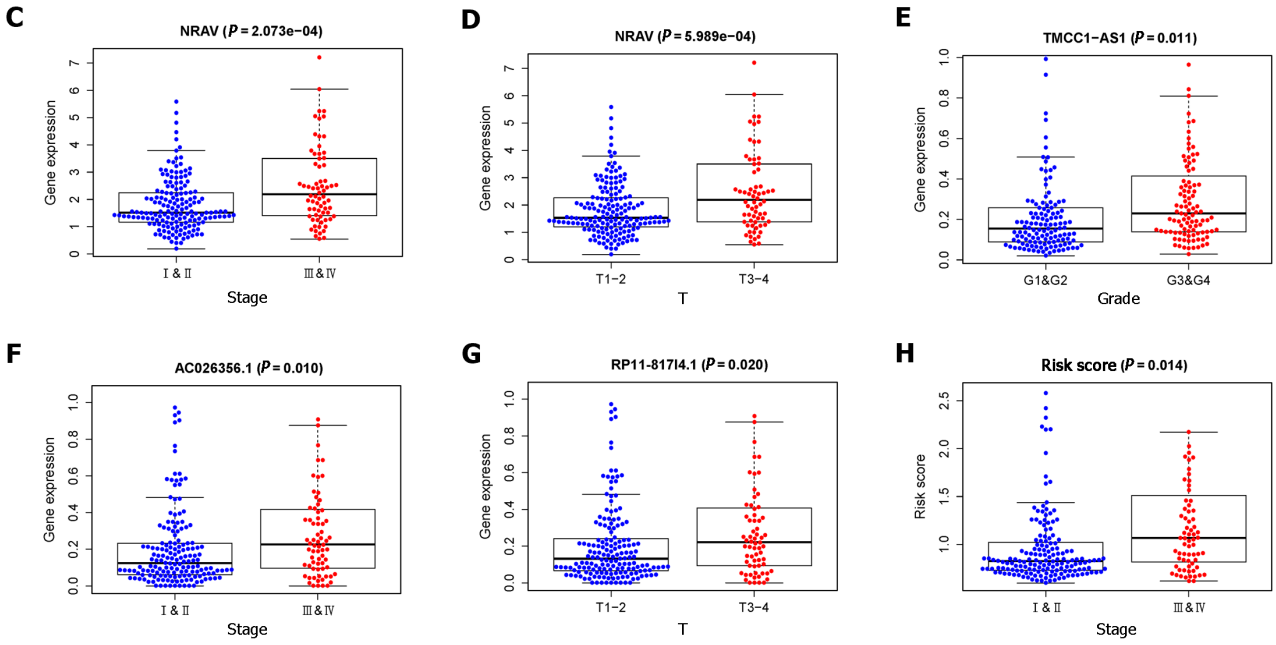
RP11-817I4.1 affects the biological behaviors of HCC cells

qRT-PCR was employed to assess the expression of RP11-817I4.1 in human HCC tissues and HCC cell lines. The qRT-PCR analysis revealed that RP11-817I4.1 exhibited upregulation in HCC tissues compared to adjacent normal tissues (Figure 5A). Additionally, we separated the HCC patients into two groups based on the median RP11-817I4.1 expression level: High- and low-expression groups. K-M survival analysis revealed that the high-expression group had a considerably worse OS than the low-expression group (Figure 5B). And RP11-817I4.1 was overexpressed in HCC cell lines (Hep-3B, Li-7, HCC-LM3, and Huh-7) but not in the normal hepatic cell line L02 (Figure 5C). Among the six cell lines, RP11-817I4.1 displayed significantly higher expression in HCC-LM3 and Huh-7 cells; consequently, these cell lines were selected for subsequent experiments. To further investigate the potential function of RP11-817I4.1 in HCC development, we evaluated the subcellular localization of RP11-817I4.1 in HCC cells. Figure 5D demonstrates that RP11-817I4.1 was primarily localized in the cytoplasm of Huh-7 and HCC-LM3 cells.

To further explore the role of RP11-817I4.1 in the occurrence and development of HCC, we used three shRNAs to specifically inhibit the expression of RP11-817I4.1 in Huh-7 and HCC-LM3 cells. The efficiency of RP11-817I4.1 transfection in Huh-7 and HCC-LM3 cells was confirmed by qRT-PCR, and RP11-817I4.1-sh1 and RP11-817I4.1-sh2 were confirmed to be the most efficient (Figure 5E). Colony formation assays demonstrated that the silencing of RP11-817I4.1 inhibited HCC cell growth and colony formation (Figure 5F). Subsequently, RP11-817I4.1 silencing significantly reduced the migratory and invasive capabilities of Huh-7 and HCC-LM3 cells (Figure 5G and H). The subcutaneous tumor experiment further provided evidence that RP11-817I4.1 knockdown led to a suppression of tumor size and weight *in vivo* (Figure 5I and J). Immunostaining with Ki67 revealed that RP11-817I4.1 knockdown significantly restrained the *in vivo* proliferative capacity of HCC cells (Figure 5K). Taken together, these findings confirmed the involvement of RP11-817I4.1 in the progression of HCC both *in vitro* and in *vivo*.


A
P value
Hazard ratio
age 0.591 1.005(0.987−1.023)
gender 0.301 0.780(0.487−1.249)
rade 0.914 1.017(0.746−1.387)
stage <0.001 1.865(1.456−2.388)
T <0.001 1.804(1.434−2.270)
M 0.023 3.850(1.207−12.281)
N 0.328 2.022(0.494−8.276)
riskScore <0.001 1.381(1.197−1.593)
0 2 4 6 8 10 12
Hazard ratio


B
P value
Hazard ratio
stage 0.696 1.192(0.495−2.871)
T 0.360 1.457(0.650−3.266)
M 0.569 1.454(0.401−5.267)
riskScore <0.001 1.295(1.117−1.502)
0 1 2 3 4 5
Hazard ratio


C
NRAV (P = 2.073e−04)
Gene expression
I & II
III & IV
Stage
D
NRAV (P = 5.989e−04)
Gene expression
T1−2
T3−4
T
E
TMCC1−AS1 (P = 0.011)
Gene expression
G1&G2
G3&G4
Grade
F
AC026356.1 (P = 0.010)
Gene expression
I & II
III & IV
Stage
G
RP11-817I4.1 (P = 0.020)
Gene expression
T1−2
T3−4
T
H
Risk score (P = 0.014)
Risk score
I & II
III & IV
Stage

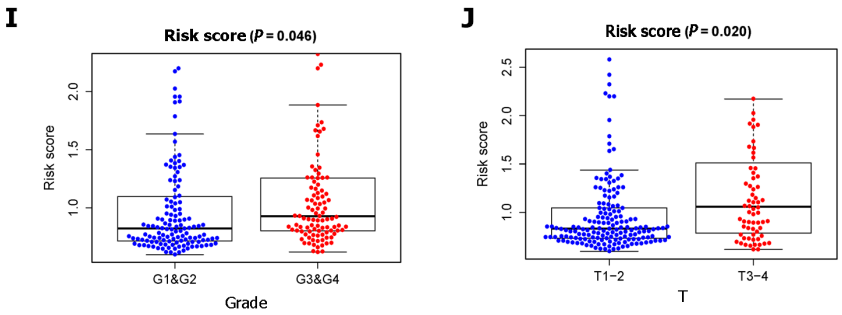


Figure 2 Analysis of the prognostic risk model. A: Univariate regression analysis of hepatocellular carcinoma (HCC) incidence and the relationships between age, sex, grade, stage, T stage, M stage, N stage and the riskscore; B: Multiple regression analysis of HCC and the relationships between stage, T stage, M stage, and the riskscore; C-J: Correlation of the prognostic lipid metabolism-related signature with clinicopathological characteristics. NRAV: Negative regulator of antiviral response; TMCC1-AS1: RNA transmembrane and coiled-coil domain family 1 antisense RNA 1; NRAV: Negative regulator of antiviral response; TMCC1-AS1: RNA transmembrane and coiled-coil domain family 1 antisense RNA 1.

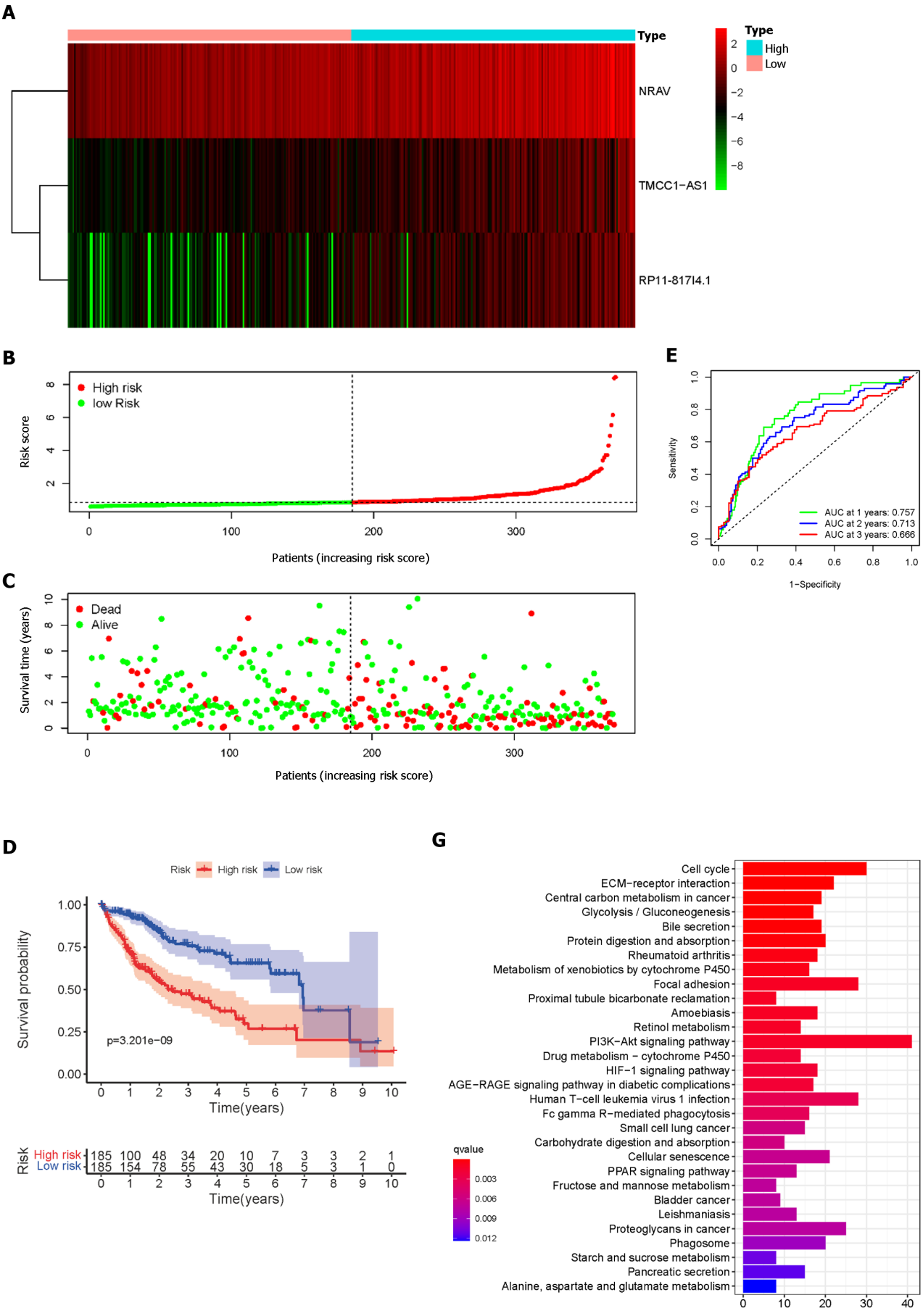
RP11-817I4.1 silencing suppressed lipid anabolism in HCC cells

Patients with HCC were divided into high- and low-expression groups according to the expression of RP11-817I4.1. Using GSEA, we detected enrichment pathways for genes that were coexpressed with high- or low-expression groups in HCC. The results indicated a negative correlation between the activity of fatty acid metabolism signaling pathways and the expression of RP11-817I4.1. A heatmap showed that the genes related to the signaling pathway of fatty acid metabolism were considerably active in the low-expression groups (Figure 6A). To gain further insight into the function of RP11-817I4.1, we conducted transcriptomic analysis on HCC-LM3 cells transfected with shNC or RP11-817I4.1-sh1 plasmids and subsequently conducted KEGG enrichment analysis of the DEG. As shown in Figure 6B, these genes were enriched mainly for fatty acid degradation and fatty acid metabolism. These pathways indicate a crucial role for RP11-817I4.1 in the regulation of lipid metabolism. Consequently, we assessed the intracellular TG content in HCC cell lines transfected with siRP11-817I4.1. The results demonstrated that the levels of intracellular TGs and free fatty acids (FFA) were significantly lower in the RP11-817I4.1-silenced cells compared to the control cells (Figure 6C and D). Nile red staining also revealed that the suppression of RP11-817I4.1 Led to reduced cellular lipid accumulation in Huh7 and HCC-LM3 cells (Figure 6E), the same changes were found in intracellular cholesterol (Figure 6F). According to the correlation analysis of mRNA expression profiles based on TCGA data, RP11-817I4.1 and ACLY, the key genes involved in lipid synthesis, exhibited the highest correlation (r = 0.46) (Figure 6G). These findings were further substantiated by the analysis of 80 patient samples from the Second Affiliated Hospital of Kunming Medical University, where the correlation between RP11-817I4.1 and ACLY was even more pronounced (r = 0.55) (Figure 6H). We then observed that both the mRNA and protein expression of ACLY were inhibited by RP11-817I4.1 knockdown (Figure 6I and J). In summary, our findings indicate that RP11-817I4.1 fosters lipid accumulation in HCC cells.

RP11-817I4.1 regulates ACLY expression by sponging miR-3120-3p

Through nucleocytoplasmic fractionation, we previously established that RP11-817I4.1 is primarily located in the cytoplasm (Figure 5D), suggesting its potential role in regulating ACLY expression through a ceRNA mechanism[23]. Subsequently, utilizing the miRDB and TargetScan databases, we identified miR-3120-3p as a potential interacting miRNA that binds to both RP11-817I4.1 and ACLY mRNAs (Figure 7A). qRT-PCR demonstrated that knockdown of RP11-817I4.1 Led to an upregulation in the expression of miR-3120-3p, while overexpression of RP11-817I4.1 had the opposite effect (Figure 7B and C). Moreover, analysis of clinical specimens unveiled a strong negative correlation between the expression levels of RP11-817I4.1 and miR-3120-3p (Figure 7D). Subsequently, we designed luciferase reporter gene plasmids based on the binding sites of RP11-817I4.1 and miR-3120-3p to validate their interactions (Figure 7E). The results indicated that the miR-3120-3p mimic significantly reduced the luciferase activity of RP11-817I4.1-WT cells, whereas the miR-3120-3p inhibitor had the opposite effect (Figure 7F). Neither the miR-3120-3p mimic nor the miR-3120-3p inhibitor had an impact on the luciferase activity of RP11-817I4.1-MUT cells (Figure 7F). These findings collectively demonstrated that RP11-817I4.1 binds to miR-3120-3p and inhibits its expression.

Subsequently, we assessed the regulatory role of miR-3120-3p in ACLY. We observed that the miR-3120-3p mimic significantly reduced the protein level of ACLY mRNA, while the miR-3120-3p inhibitor had the opposite effect (Figure 7G-I). Following this, we designed luciferase reporter gene plasmids based on the binding sites of ACLY and miR-3120-3p (Figure 7J). Luciferase reporter gene assays revealed that the miR-3120-3p mimic significantly diminished the luciferase activity of ACLY-WT cells, whereas the miR-3120-3p inhibitor had the opposite effect (Figure 7K). Neither the miR-3120-3p mimic nor the miR-3120-3p inhibitor impacted the luciferase activity of the ACLY-MUT cells (Figure 7K). Additionally, we conducted RNA immunoprecipitation (RIP) experiments with the Argonaute 2 protein, the binding partner of miRNAs, which confirmed the direct interaction between RP11-817I4.1, miR-3120-3p, and ACLY (Figure 7L). These findings collectively demonstrated that RP11-817I4.1 regulates ACLY expression by acting as a sponge for miR-3120-3p.


A
Type
NRAV
TMCC1-AS1
RP11-817I4.1
Type
High
Low
B
High risk
low Risk
Risk score
Patients (increasing risk score)
C
Dead
Alive
Survival time (years)
Patients (increasing risk score)
D
Risk
High risk
Low risk
Survival probability
p=3.201e-09
Time(years)
High risk 185 100 48 34 20 10 7 3 3 2 1
Low risk 185 154 78 55 43 30 18 5 3 1 0
Time(years)
E
Sensitivity
1-Specificity
AUC at 1 years: 0.757
AUC at 2 years: 0.713
AUC at 3 years: 0.666
G
Cell cycle
ECM-receptor interaction
Central carbon metabolism in cancer
Glycolysis / Gluconeogenesis
Bile secretion
Protein digestion and absorption
Rheumatoid arthritis
Metabolism of xenobiotics by cytochrome P450
Focal adhesion
Proximal tubule bicarbonate reclamation
Amoebiasis
Retinol metabolism
PI3K-Akt signaling pathway
Drug metabolism - cytochrome P450
HIF-1 signaling pathway
AGE-RAGE signaling pathway in diabetic complications
Human T-cell leukemia virus 1 infection
Fc gamma R-mediated phagocytosis
Small cell lung cancer
Carbohydrate digestion and absorption
Cellular senescence
PPAR signaling pathway
Fructose and mannose metabolism
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Leishmaniasis
Proteoglycans in cancer
Phagosome
Starch and sucrose metabolism
Pancreatic secretion
Alanine, aspartate and glutamate metabolism
qvalue

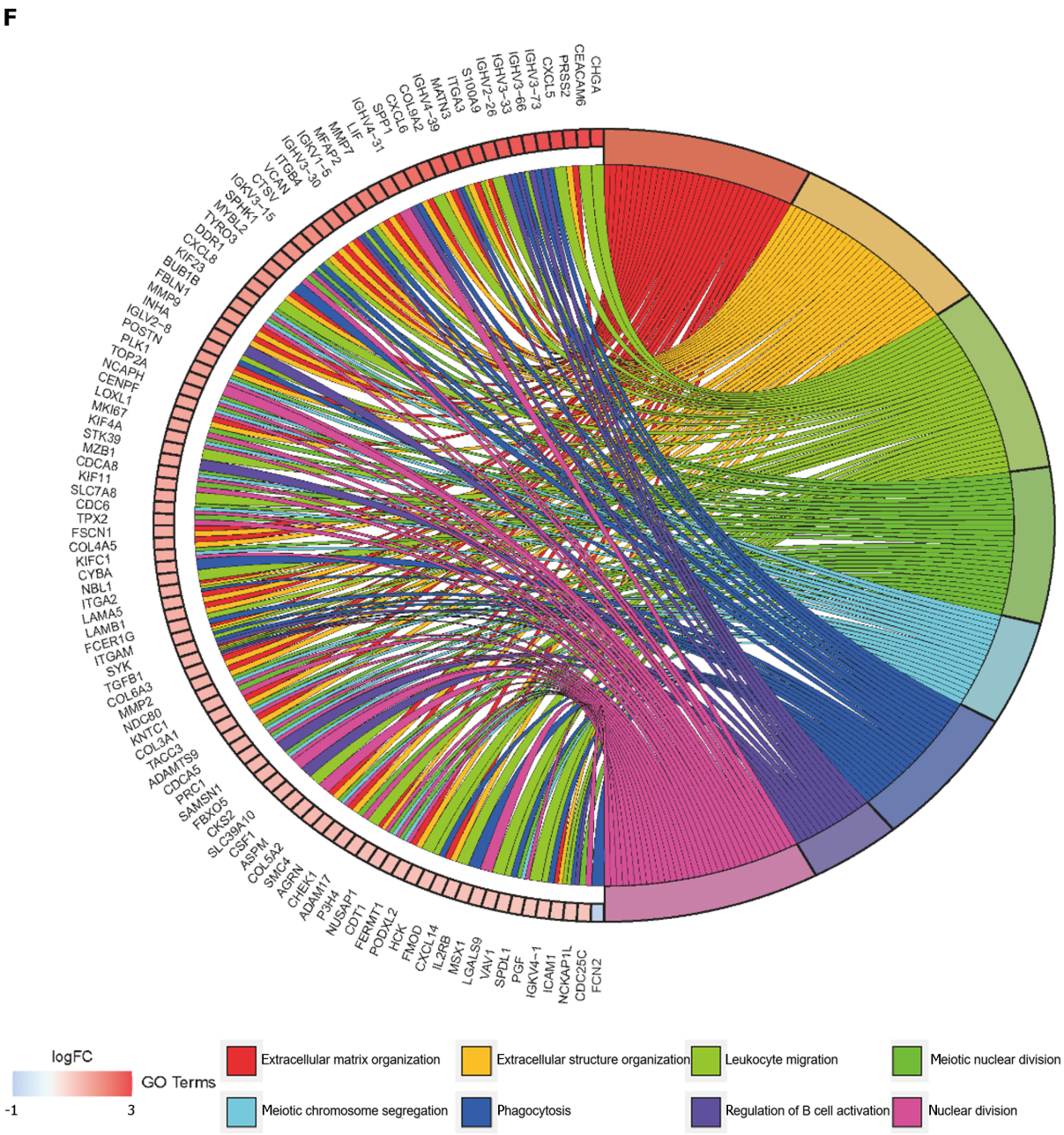
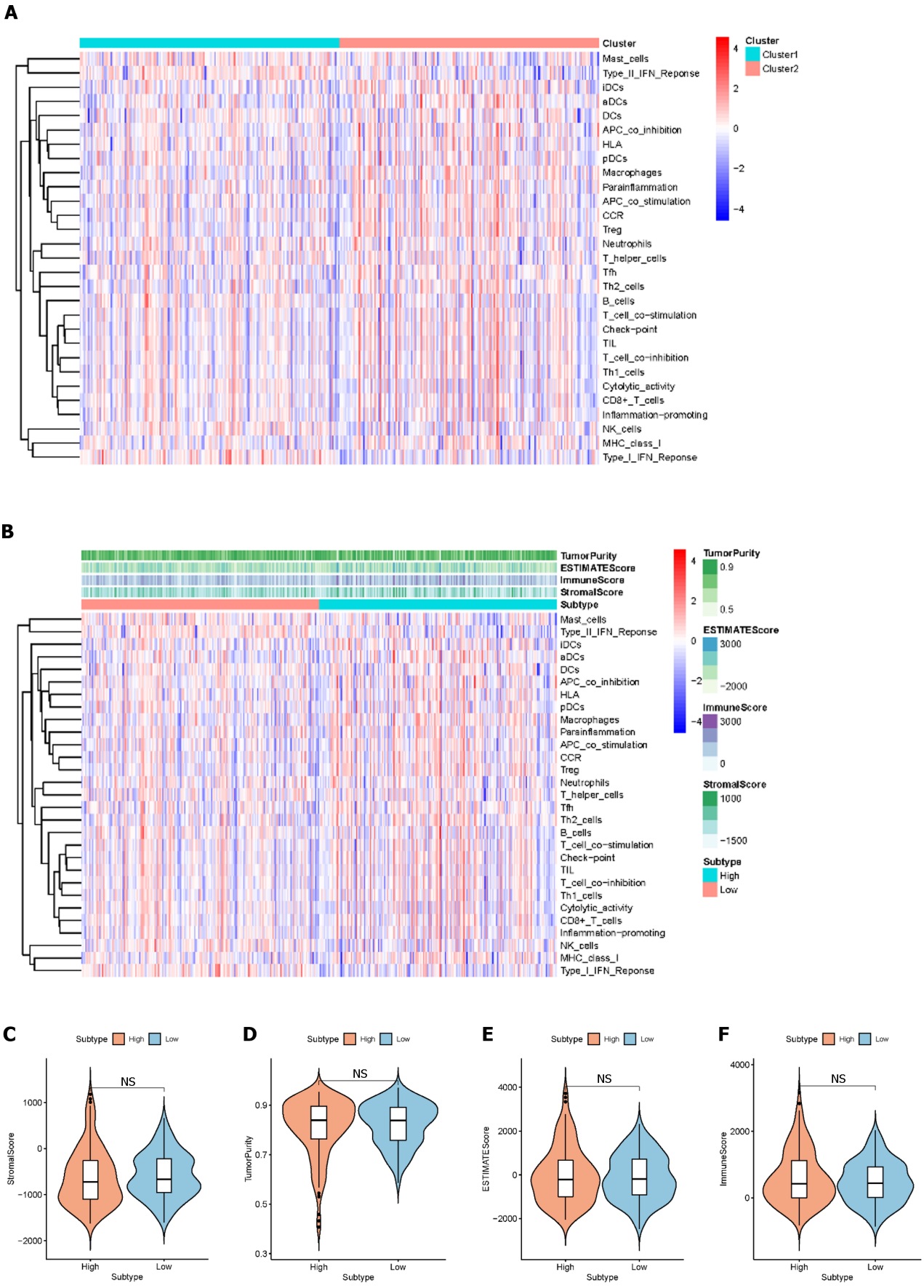


Figure 3 Lipid metabolism-related long noncoding RNAs in hepatocellular carcinoma patients from the The Cancer Genome Atlas cohort. A: The distribution of patients in the high-risk group and low-risk group; B: Heatmap demonstrating the distribution of the expression of the three long non-coding RNAs in the The Cancer Genome Atlas (TCGA) cohort; C: Survival status of the high-risk and low-risk groups; D: Kaplan-Meier survival curve of overall survival in HCC patients in the TCGA cohort according to the median cutoff value; E: Receiver Operating Characteristic Curves at 1, 2, and 3 years in the TCGA cohort; F and G: Functional enrichment analysis of the lipid metabolism-related risk signatures: Gene Ontology-biological process analysis, Kyoto Encyclopedia of Genes and Genomes analysis. NRAV: Negative regulator of antiviral response; TMCC1-AS1: RNA transmembrane and coiled-coil domain family 1 antisense RNA 1; AUC: Area under curve.

The RP11-817I4.1/miR-3120-3p/ACLY axis regulates HCC lipid metabolism and tumor progression

To confirm the functional role of the RP11-817I4.1/miR-3120-3p/ACLY axis in HCC, functional rescue experiments were conducted. Clonogenic assays demonstrated that the decreased proliferation capacity of HCC cells due to RP11-817I4.1 knockdown could be rescued by a miR-3120-3p inhibitor or overexpression of ACLY (Figure 8A). Furthermore, the reduced invasion and migration abilities of HCC cells resulting from RP11-817I4.1 knockdown could be rescued by the miR-3120-3p inhibitor or ACLY overexpression (Figure 8B and C). In terms of lipid metabolism, the decreases in the intracellular TG, FFA, and total cholesterol levels caused by RP11-817I4.1 knockdown were reversed by the miR-3120-3p inhibitor or ACLY overexpression (Figure 8D-F). Moreover, Nile red staining illustrated that the decrease in neutral lipids resulting from RP11-817I4.1 knockdown could be reversed by the miR-3120-3p inhibitor or ACLY overexpression (Figure 8G). Subcutaneous tumor assays provided evidence that the reduced *in vivo* proliferation capability of HCC cells upon RP11-817I4.1 knockdown could be rescued by a miR-3120-3p inhibitor or ACLY overexpression (Figure 8H and I).


A
Cluster
Mast_cells
Type_II_IFN_Reponse
iDCs
aDCs
DCs
APC_co_inhibition
HLA
pDCs
Macrophages
Parainflammation
APC_co_stimulation
CCR
Treg
Neutrophils
T_helper_cells
Tfh
Th2_cells
B_cells
T_cell_co-stimulation
Check-point
TIL
T_cell_co-inhibition
Th1_cells
Cytolytic_activity
CD8+_T_cells
Inflammation-promoting
NK_cells
MHC_class_I
Type_I_IFN_Reponse
Cluster
Cluster1
Cluster2
4
2
0
-2
-4
B
TumorPurity
ESTIMATEScore
ImmuneScore
StromalScore
Subtype
Mast_cells
Type_II_IFN_Reponse
iDCs
aDCs
DCs
APC_co_inhibition
HLA
pDCs
Macrophages
Parainflammation
APC_co_stimulation
CCR
Treg
Neutrophils
T_helper_cells
Tfh
Th2_cells
B_cells
T_cell_co-stimulation
Check-point
TIL
T_cell_co-inhibition
Th1_cells
Cytolytic_activity
CD8+_T_cells
Inflammation-promoting
NK_cells
MHC_class_I
Type_I_IFN_Reponse
4
2
0
-2
-4
TumorPurity
0.9
0.5
ESTIMATEScore
3000
-2000
ImmuneScore
3000
0
StromalScore
1000
-1500
Subtype
High
Low
C
Subtype High Low
NS
StromalScore
1000
0
-1000
-2000
High
Low
Subtype
D
Subtype High Low
NS
TumorPurity
0.9
0.7
0.5
0.3
High
Low
Subtype
E
Subtype High Low
NS
ESTIMATEScore
4000
2000
0
-2000
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F
Subtype High Low
NS
ImmuneScore
4000
2000
0
High
Low
Subtype

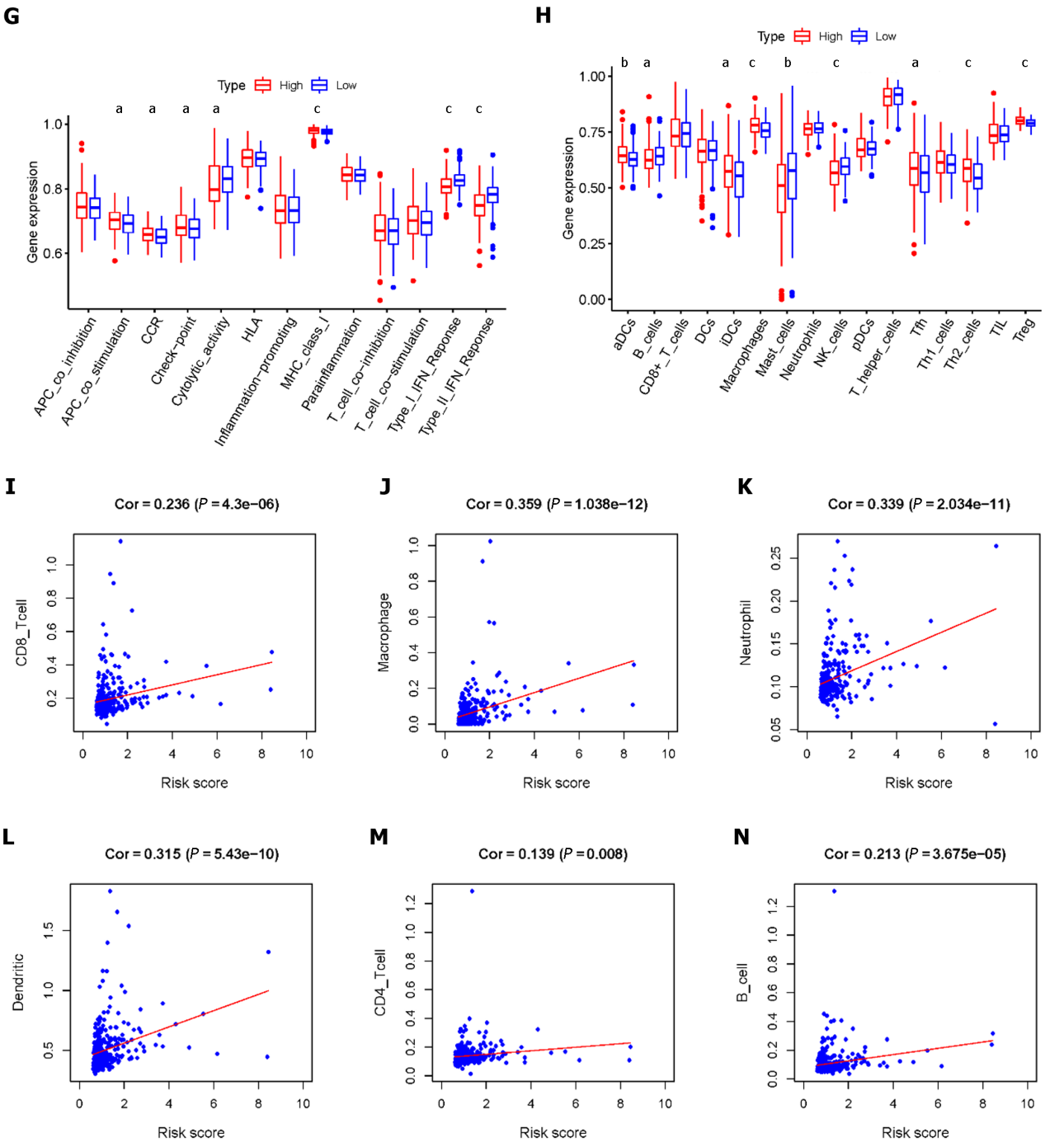
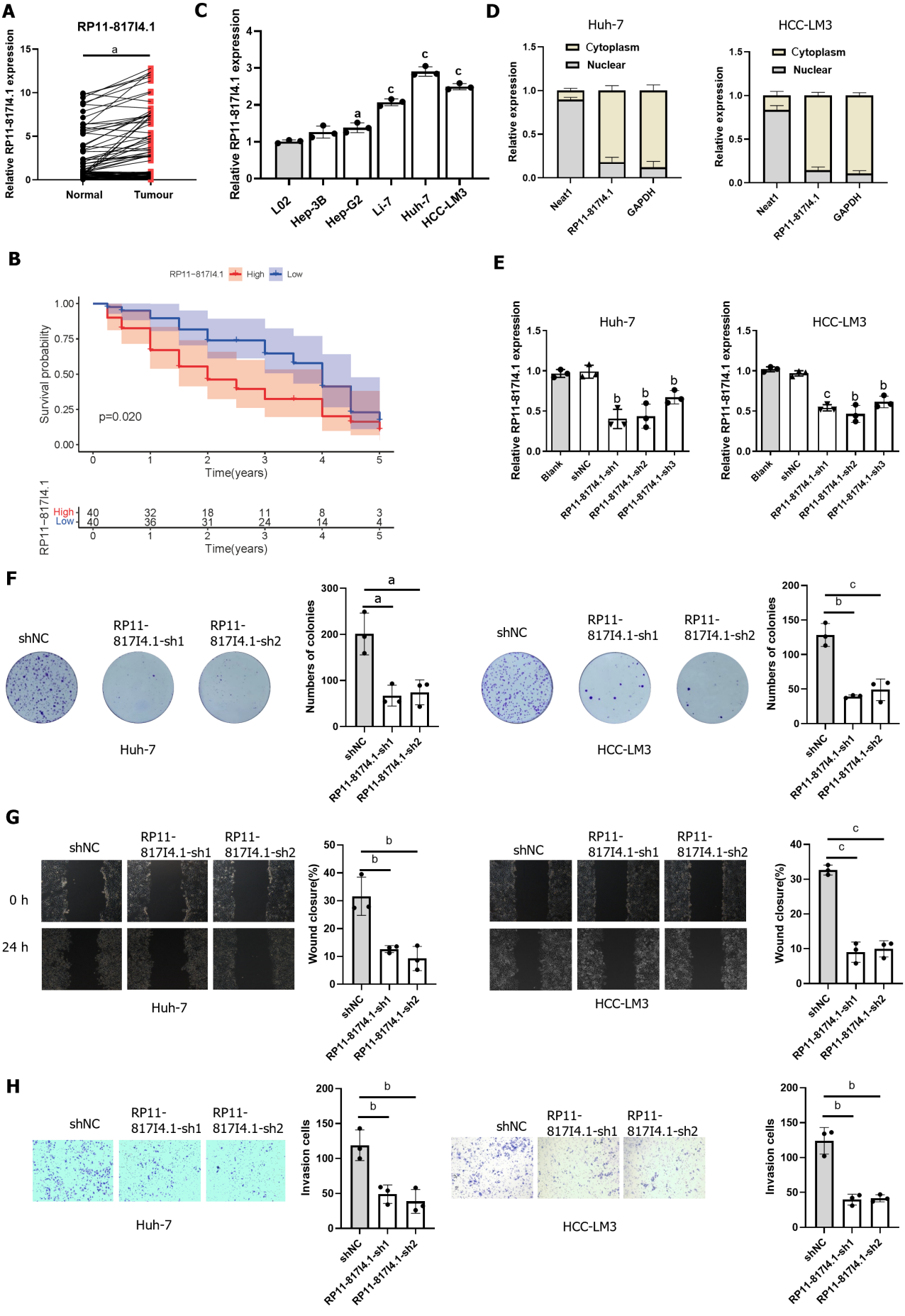


Figure 4 Lipid metabolism-related risk signatures are closely related to tumor environment characteristics and immune infiltration in hepatocellular carcinoma. A: Immune-related risk signature component expression; B: The correlations between risk groups and the level of tumor immune infiltration are shown based on the immune score, stromal score, and tumor purity score; C-F: Comparison of the ESTIMATE score, stromal score, immune score, and tumor purity between the high-risk group and low-risk group; G: Comparison of immune cell abundances between the high-risk and low-risk groups of hepatocellular carcinoma (HCC) patients; H: Comparison of immune-related functions between the high-risk and low-risk groups of HCC patients; I-N: Relationships between the risk score and infiltration abundances of six types of immune cells. Cor: Correlation.

DISCUSSION

HCC is a type of primary liver tumor that is common worldwide and has a poor prognosis. Research on the molecular etiology and biological features of this disease is critical[8]. A growing body of research suggests that metabolic reprogramming linked to lipid metabolism is a characteristic feature of malignant tumors[24]. Cancer cells employ lipid metabolism pathways to proliferate, survive, invade, and metastasize, as well as to obtain nutrients, biofilm constituents, and signaling molecules essential for the TME[25]. lncRNAs regulate various biological processes through intricate interactions among multiple mechanisms. Previous studies have identified several lncRNAs as crucial regulatory factors in lipid metabolism and signaling processes[16]. Therefore, lipid metabolism-related markers hold significant potential as


A
RP11-817I4.1
Relative RP11-817I4.1 expression
Normal
Tumour
C
Relative RP11-817I4.1 expression
L02
Hep-3B
Hep-G2
Li-7
Huh-7
HCC-LM3
D
Huh-7
HCC-LM3
Cytoplasm
Nuclear
Relative expression
Neat1
RP11-817I4.1
GAPDH
B
RP11-817I4.1 High Low
Survival probability
p=0.020
Time(years)
E
Relative RP11-817I4.1 expression
Blank
shNC
RP11-817I4.1-sh1
RP11-817I4.1-sh2
RP11-817I4.1-sh3
F
shNC
RP11-817I4.1-sh1
RP11-817I4.1-sh2
Huh-7
HCC-LM3
Numbers of colonies
G
0 h
24 h
Wound closure(%)
H
Invasion cells

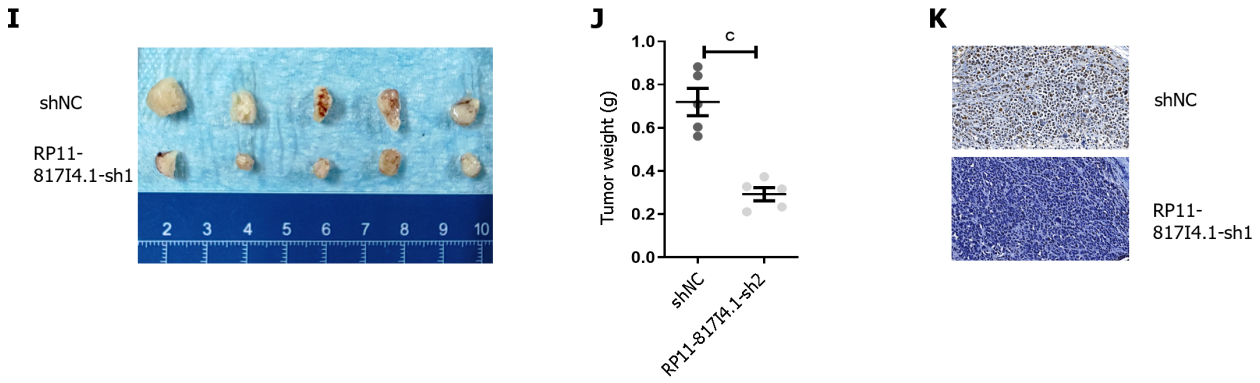


Figure 5 RP11-817I4.1 is overexpressed in hepatocellular carcinoma tissues and cell lines and is correlated with poor survival. A: Quantitative real-time polymerase chain reaction (qRT-PCR) evaluation of RP11-817I4.1 expression in hepatocellular carcinoma (HCC) tissue samples compared with that in matched adjacent normal tissue samples; B: Kaplan-Meier analysis showed that high RP11-817I4.1 expression was associated with poor overall survival in HCC patients; C: Expression levels of RP11-817I4.1 in different HCC cell lines Hep-G2, Hep-3B, Li-7, Huh-7, and HCC-LM3: compared with those in the normal human hepatic cell line L02; D: The localization of RP11-817I4.1 was identified in Huh-7 and HCC-LM3 cells *via* a subcellular fractionation assay; E: RP11-817I4.1 expression levels were determined *via* qRT-PCR assays after Huh-7 and HCC-LM3 cells were transfected with empty vector, shNC, RP11-817I4.1-sh1, RP11-817I4.1-sh2, or RP11-817I4.1-sh3; F: The effects of RP11-817I4.1 knockdown on Huh-7 and HCC-LM3 cell proliferation were assessed *via* colony formation assays; G: A wound healing assay was performed to evaluate cell mobility; H: Cell invasion was assessed and quantified by performing Transwell invasion assays; I: HCC-LM3 cells were transfected with either shRNA or the RP11-817I4.1-sh1 plasmid to establish an HCC subcutaneous tumor model; J: We measured the weight of the HCC subcutaneous tumor model; K: Ki67 staining was performed in the HCC subcutaneous tumor model. The data are presented as the mean ± SD and are representative of three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. HCC: Hepatocellular carcinoma.

prognostic indicators. Research has shown that lipid metabolism-related lncRNA signatures can serve as independent prognostic markers in various solid tumors[26-29]. However, the prognostic significance and relationship between lipid metabolism status and risk signatures in HCC patients have not been determined. Hence, it is imperative to conduct additional fundamental and clinical investigations to establish the clinical applications of LMR-lncRNAs in diagnosis and treatment. This research aims to elucidate the link between lipid metabolism and HCC development and lay the foundation for utilizing LMR-lncRNAs as novel targets for HCC treatment and prognosis in the future.

In this study, LMR-lncRNAs were identified *via* bioinformatic analysis of HCC data from TCGA, and a lipid metabolism-related prognostic model was established. Following the analysis, 31 LMR-lncRNAs were identified. Among them, three LMR-lncRNAs (NRAV, TMCC1-AS1, and RP11-817I4.1) were validated as components of the risk signature through Cox regression and LASSO regression analysis. This risk signature effectively stratified HCC patients into low- and high-risk groups. Furthermore, the risk score displayed significant predictive value for OS in both univariate and multivariate Cox regression analyses. Furthermore, the risk signature based on three lncRNAs performed well in classifying the risk groups of HCC patients in the TCGA cohort, according to KM, ROC curve, and risk diagram analyses.

Recent research has shown that risk signatures based on lncRNAs linked to lipid metabolism can serve as a dependable indicator for categorizing risk groups in various types of tumors. Similar to our study, Wu *et al*[30] revealed that lncRNA signatures connected to lipid metabolism may stratify patients with colon cancer into various risk categories. Similarly, another study demonstrated that LMR-lncRNA signatures could effectively categorize patients with human lung adenocarcinoma into different response groups, where the risk scores from these signatures emerged as the most substantial predictors of pathological remission[31]. In our investigation, a prognostic model built with LMR-lncRNAs, referred to as LMRRSM, displayed the capability to accurately forecast the prognosis of HCC patients. According to these findings, individuals with HCC who fell into the high-risk score category had a worse prognosis than those with HCC in the low-risk score category ($P < 0.001$). Additionally, according to univariate and multivariate Cox regression analyses, the risk score maintained its independent predictive significance in comparison to well-established clinicopathological prognostic markers. In the TCGA cohort, the prognostic model had a high level of accuracy for 1-, 2-, and 3-year OS. According to our biological function analysis, the genes associated with the DEGs among the risk score groups were enriched mainly in the PI3K-Akt signaling pathway. This indirectly indicated that the risk score was closely related to the PI3K-Akt signaling pathway. It is worth noting that the PI3K/Akt signaling pathway plays a crucial role in the proliferation and survival of cancer cells. According to previous reports, the PI3K/Akt signaling pathway is abnormally activated in 30%-50% of patients with HCC[32].

Our focus was directed towards the TME, a vital component in the context of cancer. The TME has been recognized as a significant factor in tumor biology[33]. Variations in the lipid metabolism of tumors, which comprises an abundance of metabolites and lipid metabolic products, may be responsible for both local immunosuppression in the TME and tumor progression[34]. Zhang *et al*[35] conducted an *in vitro* assay to mimic the interaction between tumor-associated macrophages and HCC within the TME. Their research revealed that M2 monocyte-derived macrophages promoted HCC cell migration in a manner dependent on fatty acid oxidation, which was linked to increased interleukin-1β secretion. Notably, immune checkpoint protein PD-L1, along with other immunosuppressive signals such as Toll-like receptor 4 and CD48/2B4, are expressed by macrophages and impact local immunity by deactivating CD8+ T cells, facilitating the recruitment of Tregs, and suppressing NK cell activity[36,37]. These findings align with our own results. The patients

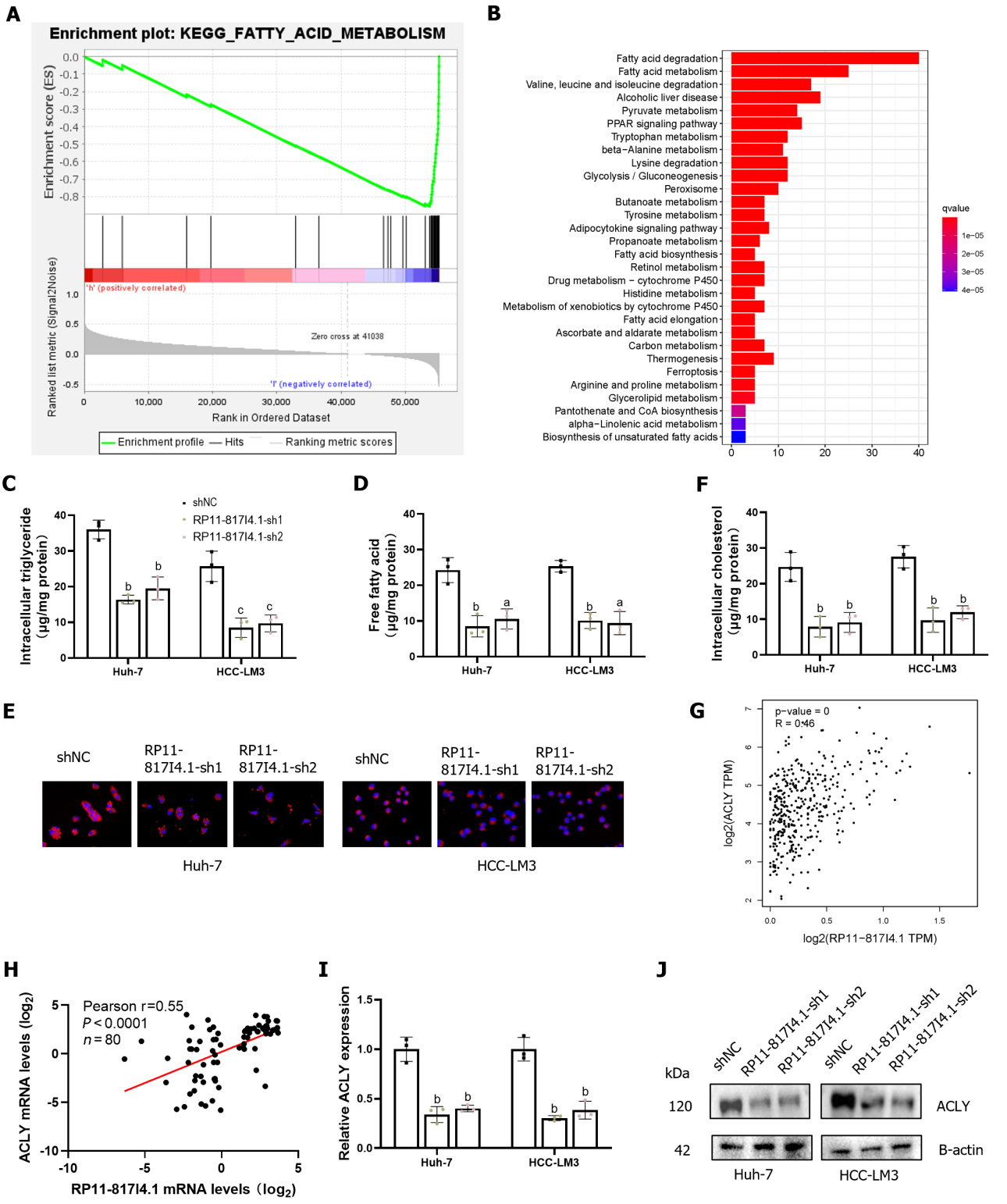


Figure 6 RP11-817I4.1 is associated with fatty acid metabolism in hepatocellular carcinoma. A: Gene set enrichment analysis showed that the activity of the fatty acid metabolism signaling pathway was negatively correlated with the expression of RP11-817I4.1; B: Kyoto Encyclopedia of Genes and Genomes analysis of the transcriptomic analysis of hepatocellular carcinoma (HCC)-LM3 cells transfected with shNC or the RP11-817I4.1-sh1 plasmid revealed that the differentially expressed genes were enriched mainly in fatty acid degradation and fatty acid metabolism; C: Free fatty acid; D: Levels were measured in Huh7 and HCC-LM3 cells expressing shNC, RP11-817I4.1-sh1, or RP11-817I4.1-sh2; E: Cellular neutral lipids were measured in Huh7 and HCC-LM3 cells expressing shNC, RP11-817I4.1-sh1, or RP11-817I4.1-sh2 by double staining with Nile red and DAPI. Magnification, 320 ×; F: Cellular cholesterol levels were measured in Huh7 and HCC-LM3 cells expressing shNC, RP11-817I4.1-sh1, or RP11-817I4.1-sh2; G: Correlation analysis between RP11-817I4.1 and ATP citrate lyase (ACLY) in the The Cancer Genome Atla database; H: Correlation analysis between RP11-817I4.1 and ACLY at the Second Affiliated Hospital of Kunming Medical University; I: Changes in ACLY mRNA levels after RP11-817I4.1 knockdown; J: Changes in the ACLY protein level after RP11-817I4.1 knockdown. The data are presented as the mean ± SD and are representative of three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. ACLY: ATP citrate lyase; TPM: Transcripts Per Kilobase of exon model per Million mapped reads.

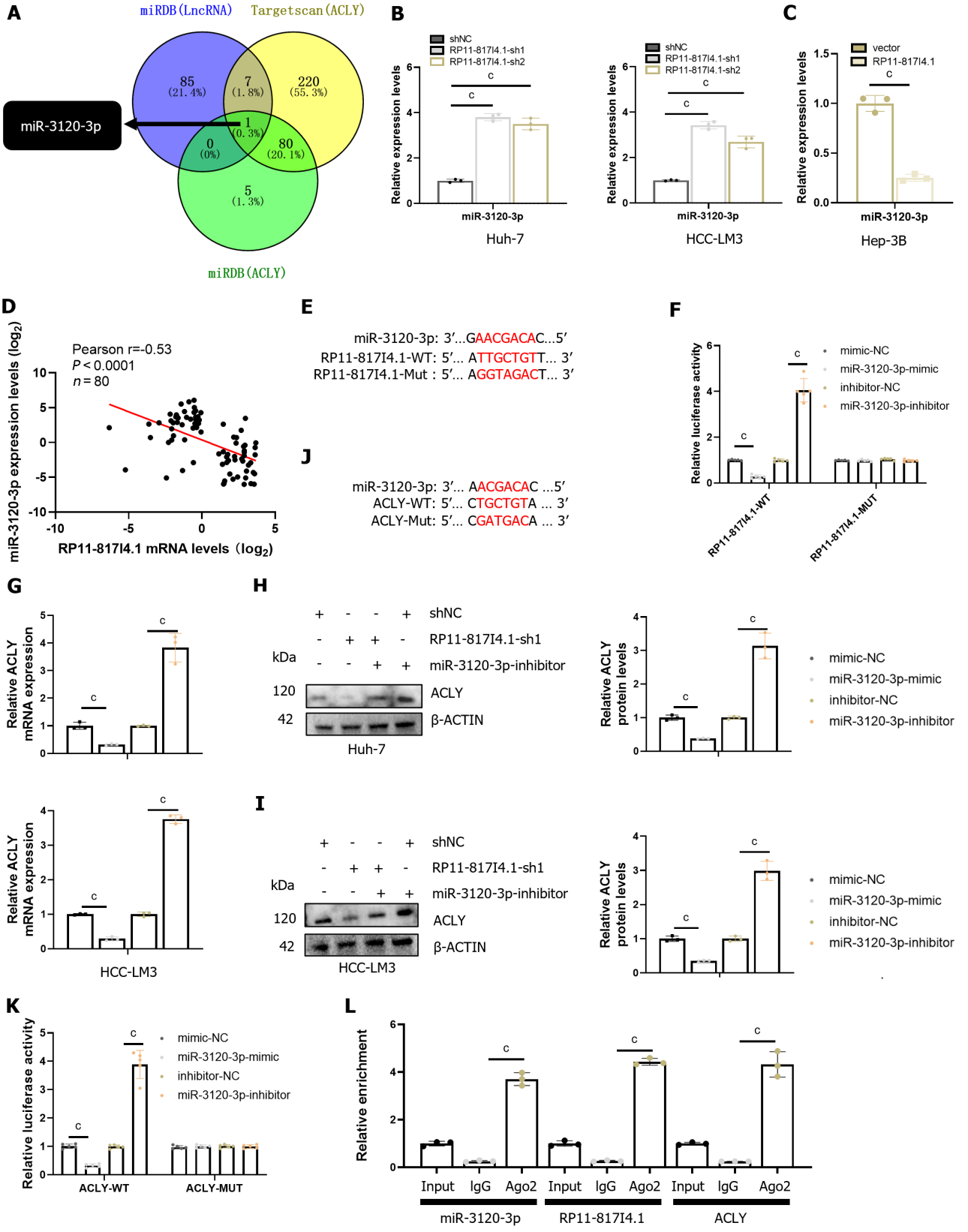
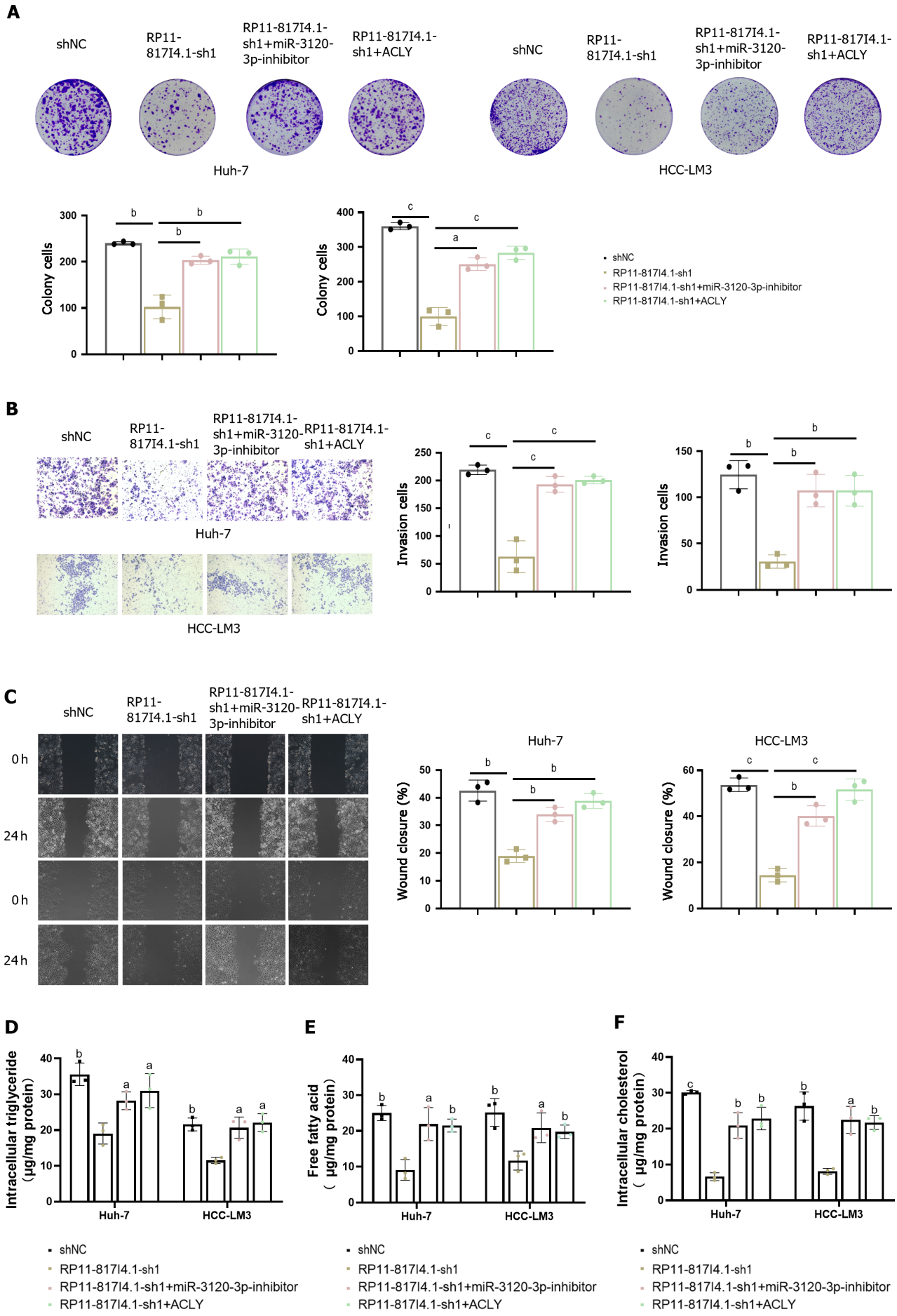


Figure 7 RP11-817I4.1 promotes ATP citrate lyase expression by sponging miR-3120-3p. A: MiRNA analysis based on the miRDB and TargetScan databases; B: Changes in the expression of miR-3120-3p after knockdown of RP11-817I4.1; C: Changes in the expression of miR-3120-3p after the overexpression of RP11-817I4.1; D: Correlation analysis between RP11-817I4.1 and ATP citrate lyase (ACLY) at the Second Affiliated Hospital of Kunming Medical University; E: Design of the luciferase reporter gene plasmid for the binding site of RP11-817I4.1 and miR-3120-3p; F: Luciferase reporter gene experiment using the plasmid designed in E; G: The impact of miR-3120-3p expression intervention on ACLY mRNA levels; H and I: The impact of miR-3120-3p silencing on ACLY protein levels; J: Design of the luciferase reporter gene plasmid for the binding site of ACLY and miR-3120-3p; K: Luciferase reporter gene experiment using the plasmid designed in J; L: Immunoprecipitation of RP11-817I4.1, miR-3120-3p, and ACLY mRNA using an AGO-2 antibody. The data are presented as the mean ± SD and are representative of three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. WT: Wild-type; MUT: Mutant; ACLY: ATP citrate lyase.


A
shNC
RP11-817I4.1-sh1
RP11-817I4.1-sh1+miR-3120-3p-inhibitor
RP11-817I4.1-sh1+ACLY
Huh-7
HCC-LM3
Colony cells
B
Invasion cells
C
0 h
24 h
Wound closure (%)
D
Intracellular triglyceride (μg/mg protein)
E
Free fatty acid (μg/mg protein)
F
Intracellular cholesterol (μg/mg protein)

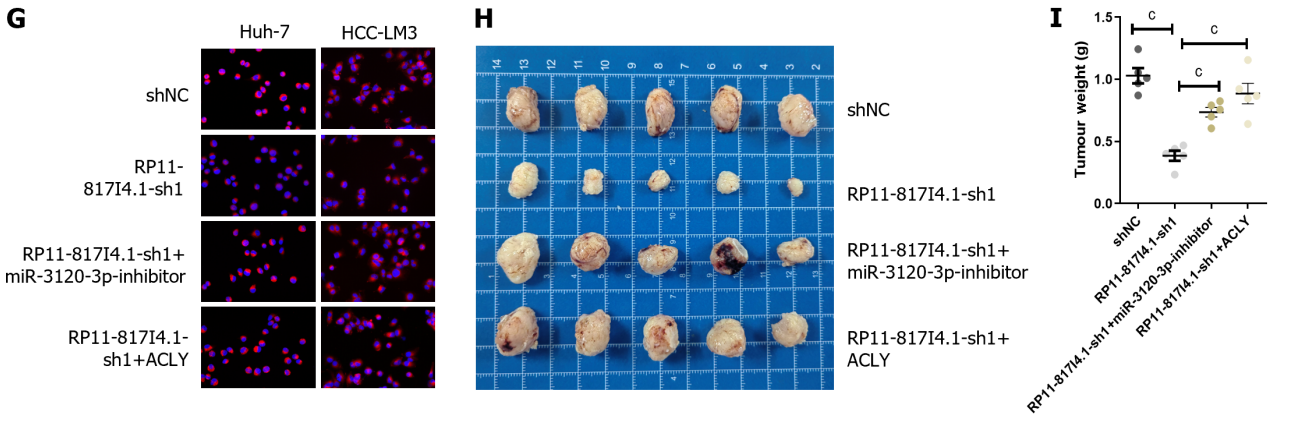


Figure 8 The RP11-817I4.1/miR-3120-3p/ATP citrate lyase axis promotes lipid synthesis and tumor progression in HCC. A: Colony formation experiment showing the effect of miR-3120-3p and ATP citrate lyase (ACLY) on the change in hepatocellular carcinoma (HCC) proliferation caused by RP11-817I4.1; B: Transwell experiment showing the effect of miR-3120-3p and ACLY on HCC invasion caused by changes in RP11-817I4.1 cell ability; C: Scratch experiments showing the effect of miR-3120-3p and ACLY on the change in HCC cell migration caused by RP11-817I4.1; D-F: The effects of miR-3120-3p and ACLY on the changes in triglyceride; free fatty acid and cholesterol; levels in HCC cells caused by RP11-817I4.1; G: Nile red staining showing the effects of miR-3120-3p and ACLY on the changes in neutral lipid levels in HCC caused by RP11-817I4.1; H: The subcutaneous tumor model shows the effects of miR-3120-3p and ACLY on the changes in HCC growth caused by RP11-817I4.1 *in vivo*; I: Weight detection of HCC cells in the subcutaneous tumorigenesis model. The data are presented as the mean ± SD and are representative of three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. ACLY: ATP citrate lyase; HCC: Hepatocellular carcinoma.

were typically divided into two groups according to LMRRSM. We then examined the connection between immune infiltration and LMRRSM. There were more aDCs, iDCs, macrophages, Tfh cells, Th2 cells, and Tregs in the high-risk group. In the low-risk group, there were significantly higher numbers of B cells, mast cells, and NK cells. Consequently, LMRRSM is closely connected to the immunological condition of patients with HCC.

In this study, three lncRNAs were identified as risk signatures: NRAV, TMCC1-AS1, and RP11-817I4.1. Previous reports have linked certain molecular components of this signature to the development and regulation of tumor growth. NRAV, for instance, is primarily found in the cytoplasm and plays a role in regulating vesicle-transporting protein activity[38]. NRAV reduction is part of the human antiviral innate immune response to viral infections and is achieved through the control of IFN-stimulated gene transcription[39]. Additionally, the lncRNA NRAV is elevated in HCC and may promote cell growth and migration by suppressing miR-199a-3p, thereby enhancing the expression of CISD2 and activating the Wnt/β-catenin signaling pathway[40]. In patients with HCC, the lncRNA TMCC1-AS1 has been repeatedly associated with prognosis. Elevated expression of TMCC1-AS1 is strongly correlated with poor OS and disease-free survival in HCC patients. At the molecular level, epithelial-mesenchymal transition is activated by upregulating the expression of TMCC1AS1 in HCC cell lines, which decreases E-cadherin expression and elevates Ki67, proliferating cell nuclear antigen, Ncadherin, and vimentin expression[41].

Currently, there are no studies on the lncRNA RP11-817I4.1, and its biological function and potential mechanism in HCC remain unclear. However, this study has provided insights into RP11-817I4.1. It revealed that the expression of RP11-817I4.1 was significantly increased in HCC tissues and cell lines, suggesting that RP11-817I4.1 may play a role in promoting the progression of HCC. Notably, Kaplan-Meier survival curves were generated for RP11-817I4. The expression of RP11-817I4.1 was found to be strongly correlated with OS and has the potential to serve as a standalone predictive biomarker for patients with HCC. Additionally, this study showed that RP11-817I4.1 enhanced the proliferation, migration, and invasion of HCC-LM3 and Huh-7 cells, indicating that RP11-817I4.1 plays a critical role in the regulation of hepatocellular tumorigenesis. The present study focused on the signaling pathway involved in fatty acid metabolism to investigate the potential mechanism by which RP11-817I4.1 promotes abnormalities in lipid metabolism in HCC cells. The present study showed that inhibiting RP11-817I4.1 expression resulted in a considerable increase in intracellular TG levels in HCC lines. Specifically, we demonstrated that RP11-817I4.1 positively regulates the expression of ACLY, a key gene involved in lipid synthesis, by sponging miR-3120-3p, thereby promoting lipid synthesis and HCC progression. These findings suggest that RP11-817I4.1 promotes HCC progression by promoting lipid synthesis.

CONCLUSION

In the present study, we investigated the functions of LMR-lncRNAs in assessing and predicting the prognosis of patients with HCC and established an accurate and reliable LMRRSM for prediction. In particular, we focused on a novel LMR-lncRNA, RP11-817I4.1, which has been proven to play an important regulatory role in HCC lipid metabolism and tumor progression and has therapeutic potential. Our findings will be extremely beneficial for understanding the probable molecular biological processes involved in HCC and discovering novel prognostic indicators and molecular targets.

ARTICLE HIGHLIGHTS

Research background

Published studies have demonstrated the impact of disturbances in lipid metabolism on the progression of hepatocellular carcinoma (HCC), which is a prevalent form of cancer.

Research motivation

Long noncoding RNAs (lncRNAs) modulate fatty acid metabolism and influence HCC development through pathways involving transcription factors and lipid-related processes. Understanding the role of metabolic reprogramming and lncRNAs in HCC may lead to novel diagnostic markers and therapeutic targets.

Research objectives

This study aims to investigate a novel lncRNA, RP11-817I4.1, revealed its role in promoting lipid accumulation, thereby accelerating the onset and progression of HCC.

Research methods

The study identified three lipid metabolism-related lncRNAs (LMR-lncRNAs), negative regulator of antiviral response (NRAV), RNA transmembrane and coiled-coil domain family 1 antisense RNA 1 (TMCC1-AS1), and RP11-817I4.1, as predictive markers for HCC and used them to construct risk models. Knockdown of RP11-817I4.1 decreased proliferation, migration, and invasion in HCC cells. RP11-817I4.1 was found to increase lipid levels in HCC cells through the miR-3120-3p/ATP citrate lyase (ACLY) axis, highlighting its role in lipid metabolism and HCC progression.

Research results

The study identified three LMR-lncRNAs, NRAV, TMCC1-AS1, and RP11-817I4.1, as predictive markers for HCC patients and utilized them in constructing risk models. Knockdown of RP11-817I4.1 resulted in reduced proliferation, migration, and invasion of HCC cells. Moreover, RP11-817I4.1 was found to significantly increase lipid levels in HCC cells through the miR-3120-3p/ACLY axis. These findings provide valuable insights into the molecular processes of HCC, uncover novel prognostic indicators and molecular targets, and highlight the therapeutic potential of RP11-817I4.1 in the context of HCC treatment.

Research conclusions

In this study, the functions of LMR-lncRNAs were investigated for their role in assessing and predicting the prognosis of HCC patients, leading to the establishment of an accurate and reliable lipid metabolism-related risk score model for prediction. Of particular interest was the novel LMR-lncRNA, RP11-817I4.1, which was found to regulate HCC lipid metabolism and tumor progression, showing potential as a therapeutic target. The findings hold significant promise for understanding the molecular processes in HCC, identifying novel prognostic indicators, and uncovering molecular targets for potential therapeutic interventions.

Research perspectives

Lipid metabolism-related lncRNA RP11-817I4.1 may be a potential therapeutic target, and the development of small molecule drugs targeting RP11-817I4.1 may help improve the prognosis of HCC patients.

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FOOTNOTES

Co-first authors: Ren-Yong Wang and Jia-Ling Yang.

Co-corresponding authors: Jin-Ze Li and Hong Zhu.

Author contributions: Wang RY, Yang JL, Xu N, Liang DM, Li JZ and Zhu H designed research; Wang RY, Yang JL, Xu J and Yang SH performed research; Wang RY and Yang JL contributed new reagents or analytic tools; Wang RY, Yang JL, Li JZ and Zhu H analyzed data; Wang RY, Yang JL, Li JZ and Zhu H wrote the paper. All authors contributed to the study design, interpretation of the investigations, data analysis, and manuscript review. Wang RY and Yang JL are listed as co-first authors because they made equal and significant contributions throughout the research process, being jointly responsible for key aspects such as experimental design and data analysis. On the other hand, Li JZ and Zhu H are designated as co-corresponding authors due to their crucial roles in the research design and experimental processes, overseeing the entire study's planning and supervision, as well as being responsible for interpreting the data and publishing the results. In summary, the authorship order reflects their actual contributions and roles in the research.

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Institutional review board statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Second Affiliated Hospital of Kunming Medical University (2021128).

Institutional animal care and use committee statement: All animal experimental procedures and steps were strictly reviewed by the Ethics Review Committee for Animal Experiments at Kunming Medical University (Approval Number: kmmu20211188).

Conflict-of-interest statement: There are no conflicts of interest to disclose in this article.

Data sharing statement: The data supporting the conclusions of this study can be obtained from the corresponding author under reasonable requirements. Normalized RNA-seq data of 50 normal and 374 HCC samples were obtained from TCGA (https://www.cancer.gov/ccg/research/genome-sequencing/tcga).

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SYSTEMATIC REVIEWS

Quality of life after pancreatic surgery

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Abstract

BACKGROUND

Pancreatic surgery is challenging owing to the anatomical characteristics of the pancreas. Increasing attention has been paid to changes in quality of life (QOL) after pancreatic surgery.

AIM

To summarize and analyze current research results on QOL after pancreatic surgery.

METHODS

A systematic search of the literature available on PubMed and EMBASE was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. Relevant studies were identified by screening the references of retrieved articles. Studies on patients' QOL after pancreatic surgery published after January 1, 2012, were included. These included prospective and retrospective studies on patients' QOL after several types of pancreatic surgeries. The results of these primary studies were summarized inductively.

RESULTS

A total of 45 articles were included in the study, of which 13 were related to pancreaticoduodenectomy (PD), seven to duodenum-preserving pancreatic head resection (DPPHR), nine to distal pancreatectomy (DP), two to central pancreatectomy (CP), and 14 to total pancreatectomy (TP). Some studies showed that 3-6 months were needed for QOL recovery after PD, whereas others showed that 6-12 months was more accurate. Although TP and PD had similar influences on QOL, patients needed longer to recover to preoperative or baseline levels after TP. The QOL was better after DPPHR than PD. However, the superiority of the QOL between patients who underwent CP and PD remains controversial. The decrease in exocrine and endocrine functions postoperatively was the main factor affecting the QOL. Minimally invasive surgery could improve patients' QOL in the early

stages after PD and DP; however, the long-term effect remains unclear.

CONCLUSION
The procedure among PD, DP, CP, and TP with a superior postoperative QOL is controversial. The long-term benefits of minimally invasive versus open surgeries remain unclear. Further prospective trials are warranted.

Key Words: Quality of life; Pancreaticoduodenectomy; Duodenum-preserving pancreatic head resection; Distal pancreatectomy; Central pancreatectomy; Total pancreatectomy

Core Tip: This review summarizes and analyzes current research results on quality of life (QOL) after pancreatic surgery. The article covers the discussion and analysis of the QOL of various pancreatic surgeries. Which kind of surgical procedure has better QOL is controversial. The long-term benefits on QOL of minimally invasive surgery over open surgery are controversial.

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INTRODUCTION

The pancreas, located in the retroperitoneum, is a glandular organ with endocrine and exocrine functions. It can be divided into four main parts: Head, neck, body, and tail. Pancreatic surgery can be divided into pancreaticoduodenectomy (PD), duodenum-preserving pancreatic head resection (DPPHR), distal pancreatectomy (DP), central pancreatectomy (CP), and total pancreatectomy (TP). Pancreatic surgery is challenging due to the organ's complex anatomical structure, peripheral vascularity, and intractable postoperative complications. Following the standardization of surgical steps and improvements in relevant medical techniques and surgical instruments, the safety of pancreatic surgery has significantly improved. Perioperative morbidity, mortality, and other related indicators have become more acceptable. However, owing to the organ's essential role in digestion, absorption, and blood glucose regulation, changes in the quality of life (QOL) of patients after pancreatic surgery have attracted the attention of surgeons.

More patients with non-malignant pancreatic diseases are willing to undergo surgical treatment because of the acceptable safety. In this case, from the perspective of the patient postoperatively, the significance of rehabilitation reflects the traditional perioperative outcome and QOL[1]. The QOL is a new concept that extends beyond health. Although there is no consensus on its conception[2], we can consider it a multi-dimensional architecture that incorporates objective and individual subjective views of aspects of one's physical, psychological, and social well-being[3-5]. It includes evaluating physical health, and many subscales, such as emotion, job, culture, family, sociability, economy, cognition, happiness, sex, and some symptoms[6]. Since people have realized the importance of QOL, many QOL scales have emerged, including the European Organization for Research and Treatment of Cancer QLQ-C30, European Quality of Life 5-dimension, 36-item Short, *etc*. However, it is challenging to follow up on patients' QOL once they are discharged from the hospital. Consequently, most relevant studies had small sample sizes or lacked long-term follow-up results. Moreover, a summary of studies on QOL after pancreatic surgery is lacking.

This study assessed the QOL in patients who underwent PD, DPPHR, DP, CP and TP. We conducted this study to describe the existing findings on the QOL after pancreatic surgery to make it easier for surgeons and patients to decide on a surgical approach. In addition, we attempted to identify controversial results to encourage further targeted research.

MATERIALS AND METHODS

Search strategy

This systematic review was conducted using PubMed and EMBASE databases, according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guideline[7]. Two authors (Li and Zhen) independently screened the articles after removing duplicates. Our search algorithm combined the terms: (1) "Pancreatic surgery" OR "pancreatoduodenectomy" OR "duodenum-preserving pancreatic head resection" OR "distal pancreatectomy" OR "central pancreatectomy" OR "total pancreatectomy"; and (2) "Quality of life". Only articles written in English were included. The references of retrieved articles were screened for any relevant articles.

Inclusion and exclusion criteria

Inclusion criteria: Articles involving the QOL of patients who underwent PD, DPPHR, DP, CP, and TP were included. The exclusion criteria were as follows: (1) Articles not within the scope of interest of this review; (2) Overlapping patient data; (3) Articles not published in English; and (4) Articles published before January 1, 2012.

RESULTS

Literature search

The search results are shown in Figure 1. A total of 1515 potential studies were identified: 1313 from PubMed, 190 from EMABASE, and 12 additional references through a manual search. After excluding duplicates, 1453 articles were left. However, after screening titles and abstracts, 872 articles were excluded because they were outside the scope of this review. We also excluded article that were inaccessible (n = 127). A total of 454 full-text articles were collected, of which 312 were excluded for language (n = 11), not addressing the QOL after PD, DPPHR, DP, CP, or TP (n = 301), or being published before January 1, 2012 (n = 97). After the selection process, 45 clinical studies were included. The 45 articles included 13 on PD, seven on DPPHR, nine on DP, two on CP, and 14 on TP.

Study characteristics for PD

Thirteen studies, including three randomized controlled trials (RCTs), four prospective observational studies, and six retrospective studies on PD were assessed. Six studies focused on perioperative QOL in patients with PD. Two RCTs and one retrospective study reported postoperative QOL changes after two years (Table 1). Some studies demonstrated that patients' QOL significantly diminished within one month post-operatively and nearly recovered to preoperative or baseline levels at three months after PD regardless of the pathology type[8-11], others reported that six months even one year was a more accurate period[12-14]. For long-term survivors, gastrointestinal symptoms such as bloating and indigestion are factors that affect their long-term QOL, and some of these symptoms are caused by pancreatic exocrine insufficiency after PD instead of post-operative complications[15,16]. Studies have reported that nearly half of the survivors required pancrelipase after PD[9,17,18]. Pancrelipase can improve nutritional status; however, its capacity to improve QOL is controversial[9,13,19].

Most studies have demonstrated that no differences between pylorus-preserving PD (PPPD) and conventional PD overall mortality, morbidity, survival, and QOL[20-24]. Studies have also shown that preoperative body weight loss, impaired preoperative pancreatic exocrine function, longer operative time, intraoperative radiotherapy, pancreatic carcinoma, and postoperative diarrhea may result in delayed QOL recovery[25].

Laparoscopic PD (LPD) could provide better QOL for patients with better functional status within six months postoperatively[26]. However, this advantage disappears after six months[27].

Study characteristics for DPPHR

Table 2 summarizes the results of the included articles on DPPHR. The sample sizes of the seven studies were 74, 80, 25, 40, 85, 17, and 16. Only one study examined the change in QOL within one year. One group of researchers reported that DPPHR and PD were comparatively effective in improving long-term QOL postoperatively[28-30]. Another group held that DPPHR could bring about better outcomes in the form of less frequent nausea, pain, and diarrhea, better physical status, working ability, and global QOL[31].

Studies have found that the Frey and the Berne approach had the advantages of shorter operation time and hospital stay duration compared to the Beger's. However, none showed any obvious difference in improving the patients' postoperative QOL[32-35].

Study characteristics for DP

Nine studies included patients who underwent DP (Table 3). Two studies reported the perioperative QOL of patients who underwent DP, and seven mainly compared the differences between open and minimally invasive methods. Studies have shown that minimally invasive DP (MIDP) results in shorter hospital stays and functional recovery time compared to open DP (ODP)[36,37]. The MIDP group had better short-term QOL than the ODP group for up to 30 d postoperatively [38,39]. However, which is better for long-term QOL of > 1 year is controversial. During this period, some studies demonstrated no difference between MIDP and ODP[38,40], while others reported that MIDP could bring about better QOL for patients regarding physical, cognitive, social, and role functions, and symptoms, such as nausea, vomiting, and insomnia[41,42].

Laparoscopic spleen-preserving DP (LSPDP) and laparoscopic DP with splenectomy (LDPS) had similar perioperative outcomes[43]. Patients who underwent LSPDP had significantly better vitality than those who underwent LDPS, and were less likely to contract the common cold and flu[44,45].

The modified Appleby improved the ratio of R0 resection, relieved pain and improved patients' overall QOL[46].

Study characteristics for CP

Details of the two studies on CP with sample sizes of 36 and 42 are included in Table 3. Laparoscopic CP can help patients maintain better working and living conditions than open CP[47]. While comparing DP and PD, some researchers thought that CP showed a significant benefit in specific symptoms, such as loss of appetite, insomnia, nausea, and vomiting[48]. Others held different opinions that CP was associated with better pancreatic function but the same or even worse long-

Table 1 Articles retrieved from literature reporting quality of life after pancreaticoduodenectomy

Ref.	Year	Country	Study design	Relevant patients	Total patients	Moments of assessment	Operation type	Questionnaires
Chan *et al*[12]	2012	Mexico	Prospective single-center study	37	37	PRE, 1, 3, 6 and 12 months	PD	SF-36
Gerstenhaber *et al*[11]	2013	Israel	Retrospective, single-center study	70	168	At discharge, 3, 6 and 12 months	PD	EORTC QLQ-C30
Rees *et al*[14]	2013	United Kingdom	Prospective single-center study	41	53	PRE, 6 wk, 3, 6, 12, 18 and 24 months	PD	EORTC QLQ-C30; EORTC QLQ-PAN26
Park *et al*[77]	2016	Korea	Retrospective, single-center study	10	15	10.5 (3, 18) yr	PPPD	EORTC QLQ-C30; EORTC QLQ-PAN26
Fong *et al*[9]	2017	United States	Retrospective, single-center study	245	305	9.1 (5.1, 21.2) yr postoperatively	PD	EQ-5D-5L; EORTC QLQ-C30
Laitinen *et al*[8]	2017	Finland	Prospective single-center study	47	47	PRE, 3, 6, 12, 18 and 24 months	PD	EORTC QLQ-C30; EORTC QLQ-PAN26
Heerkens *et al*[15]	2018	Netherlands	Prospective single-center study	118	137	1, 3, 6 and 12 months	PD	RAND-36; EORTC QLQ-C30; EORTC QLQ-PAN26
Diener *et al*[80]	2017	Germany	Multicenter, randomized controlled trial	226	250	24 months	PD and DPPHR	EORTC QLQ-C30; EORTC QLQ-PAN26
Allen *et al*[13]	2018	United States	Retrospective, global study	927	7605	2.0 (0.7, 4.3) yr	PD	SF-36; GSRS
Klaiber *et al*[24]	2020	Germany	Prospective, randomized controlled trial	96	188	PRE, 1 months, 34.3 (16, 57) months	PD and PPPD	EORTC QLQ-C30; EORTC QLQ-PAN26
Balduzzi *et al*[16]	2020	Italy	Retrospective, single-center study	47	75	60 (12, 240) months	PD	Pancreatitis Quality of Life Instrument; DSMQ
Jung *et al*[18]	2022	South Korea	Retrospective, single-center study	122	122	12 months	PD	EORTC QLQ-C30; EORTC QLQ-PAN26
Qin *et al*[27]	2023	China	Prospective, randomized controlled trial	656	200	3 yr	PD	EQ-5D-3L

EORTC: European Organization for Research and Treatment of Cancer; PRE: Preoperative quality of life; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy; DPPHR: Duodenum-preserving pancreatic head resection; SF-36: 36-item Short; EQ-5D: European Quality of Life 5-dimension.

term QOL and significantly increased post-operative morbidity and risk than DP or PD[49,50].

Study characteristics for TP

The two studies on TP were prospective observational studies (Table 4). Two articles showed the results of QOL within one year. It has been extensively verified that the perioperative and long-term outcomes of TP are comparable to those of PD regarding morbidity, mortality, survival rates, and QOL, regardless of patient age or tumor pathology[51-55]. One study demonstrated that the long-term post-operative QOL of patients who underwent TP was lower than that of the general population[56], however, more studies reported no significant differences[57,58]. Regarding pain relief, especially for most patients with narcotic-dependent, TP could alleviate pain largely such that half of the patients with chronic pancreatitis patients could be relieve from narcotics and return to normal life a year after surgery. However, it is a continuous improvement process. Over time, an increasing number of patients no longer required narcotics to control their abdominal pain[59-63]. More than half of the patients reported that their bowel habits had changed; therefore, they needed to take pancreatin[64-66]. A quarter to more than half of the patients, especially children, achieved insulin independence after islet cell autotransplantation (IAT)[67]. Although the insulin independence rate could decline over time, most patients could almost control their glycemic stability with an acceptable dose of insulin[60,61,68-70]. The stable control of glucose provides a more enjoyable life with better QOL for patients to have a normal social, work and study life [71].

Table 2 Articles retrieved from literature reporting quality of life after duodenum-preserving pancreatic head resection

Ref.	Year	Country	Study design	Relevant patients	Total patients	Moments of assessment	Operation type	Questionnaires
Bachmann *et al*[33]	2014	Germany	Randomized controlled trail	74	74	16 (14, 18) yr	DPPHR	EORTC QLQ-C30
Tan *et al*[35]	2016	China	Retrospective, single-center study	80	156	50 months	The Frey and modified Frey	EORTC QLQ-C30
Pothula *et al* [81]	2014	India	Prospective single-center study	25	25	PRE, 12 months	The Frey	SF-36
Klaiber *et al* [32]	2016	Germany	Prospective single-center study	40	65	129 (111, 137) months	The Beger and modified Beger	EORTC QLQ-C30; EORTC QLQ-PAN26
Keck *et al*[29]	2012	Germany	Prospective, randomized controlled trial	85	85	> 5 yr	The Frey and Beger	EORTC QLQ-C30
Fischer *et al* [30]	2015	United States	Retrospective, single-center study	17	45	40.7 (23.7, 53.7) months	DPPHR	EORTC QLQ-C30
Aimoto *et al* [82]	2013	Japan	Retrospective, single-center study	16	16	70.8 months for the Frey, 119.8 months for PPPD	The Frey and PPPD	EORTC QLQ-C30

EORTC: European Organization for Research and Treatment of Cancer; DPPHR: Duodenum-preserving pancreatic head resection; PPPD: Pylorus-preserving pancreaticoduodenectomy; PRE: Preoperative quality of life; SF-36: 36-item Short.

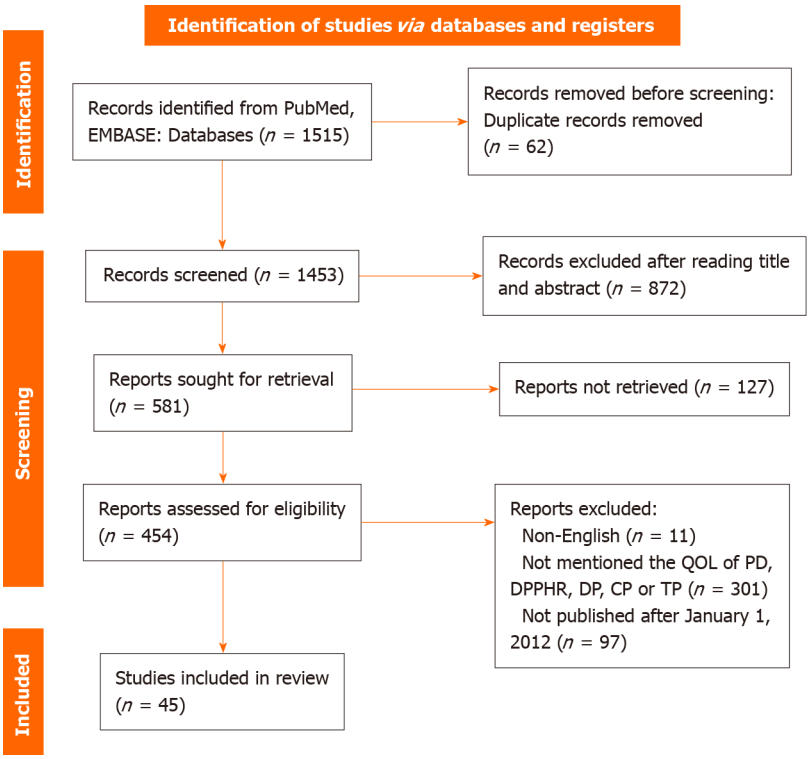

Figure 1 PRISMA flow diagram. QOL: Quality of life; PD: Pancreaticoduodenectomy; DPPHR: Duodenum-preserving pancreatic head resection; DP: Distal pancreatectomy; CP: Central pancreatectomy; TP: Total pancreatectomy.

DISCUSSION

PD

PD, developed by Kausch[72] and Whipple *et al*[73], is a major surgical procedure used to treat middle and lower-segment cancers of the common bile duct and the periampullary region. The safety of PD has improved significantly in recent years. The mortality rate of PD has decreased from > 50% to < 5%, and the incidence of surgical complications has also decreased significantly[74]. Under these circumstances, attention gradually shifted from safe hospital discharge to

Table 3 Articles retrieved from literature reporting quality of life after distal pancreatectomy and central pancreatectomy

Ref.	Year	Country	Study design	Relevant patients	Total patients	Moments of assessment	Operation type	Questionnaires
van Hilst *et al*[38]	2019	Netherlands	Prospective, randomized controlled trial	63	108	1 yr	ODP and LDP	EQ-5D; EORTC QLQ-C30; EORTC QLQ-PAN26
De Rooij *et al*[36]	2019	Netherlands	Multicenter, randomized controlled trial	108	111	From postoperative day 3 to 30	ODP and MIDP	EQ-5D-3L; EORTC QLQ-C30
Korrel *et al* [40]	2021	Netherlands	Multicenter, randomized controlled trial	62	84	44 (39, 50) months	ODP and MIDP	EQ-5D; EORTC QLQ-C30; EORTC QLQ-PAN26
Zhang *et al* [44]	2021	China	Retrospective, single-center study	102	110	106 (62, 189) months	LSPDP and LDPS	SF-36
Ricci *et al* [41]	2015	Italy	Retrospective, single-center study	54	81	12 months	ODP and LDP	EORTC QLQ-C30
De Pastena *et al*[42]	2021	Italy	Multicenter, randomized controlled trial	79	152	52 months	LDP and RDP	EQ-5D; EORTC QLQ-C30
Choi *et al* [45]	2012	Korea	Retrospective, single-center study	61	72	23 (3, 76) months	LSPDP and LDPS	-
Braga *et al* [39]	2015	Italy	Retrospective, single-center study	100	170	1 and 3 months	LDP	SF-8
Kwon *et al* [43]	2016	Korea	Retrospective analysis of prospective gathered data, single-center	104	111	PRE, at discharge, 3, 6 and 12 months	LSPDP and LDPS	EORTC QLQ-C30
Zhang *et al* [47]	2017	China	Retrospective, single-center study	36	36	45 (4, 216) months	LCP and OCP	SF-36
Lv *et al*[50]	2018	China	Retrospective, single-center study	42	42	53 (21, 117) months	CP and DP	EORTC QLQ-C30

EORTC: European Organization for Research and Treatment of Cancer; ODP: Open distal pancreatectomy; LDP: Laparoscopic distal pancreatectomy; MIDP: Minimally invasive distal pancreatectomy; LSPDP: Laparoscopic spleen-preserving distal pancreatectomy; LDPS: Laparoscopic distal pancreatectomy with splenectomy; RDP: Robotic distal pancreatectomy; LCP: Laparoscopic central pancreatectomy; OCP: Open central pancreatectomy; PRE: Preoperative quality of life; SF-36: 36-item Short; EQ-5D: European Quality of Life 5-dimension.

the recovery of QOL. Therefore, an increasing number of studies have assessed the changes in patients' QOL after PD. However, these studies came from different countries with different demographic characteristics and almost always had small sample sizes, especially prospective studies. As shown in Table 1, seven of the studies had a sample size of < 100 participants, and only one had a sample size of more than 300.

PPPD was first performed in 1943 by Watson[75] and was popularized by Traverso and Longmire[76]. Although the merits of PPPD versus classic PD are still debated, especially regarding perioperative risk, PPPD provides surgeons with another option[77]. Most studies have demonstrated that PPPD and PD have similar effects on patients' QOL. Factors leading to the delayed recovery of QOL, such as preoperative body weight loss and impaired preoperative pancreatic exocrine function, are currently being explored.

Traditionally, PD was performed openly. Since the first case described by Gagner and Pomp[78] in 1994, many surgeons have explored the advantages of LPD and open PD (OPD). Our previous multi-center, open-label, RCT proved that LPD was associated with a shorter length of stay, similar short-term morbidity, and mortality rates as OPD. Due to the better safety of LPD and the maturity of surgical techniques, an increasing number of surgeons are focusing on comparing the differences in QOL between LPD and OPD. LPD have a better QOL advantage than OPD in the first six months, however, our new study showed that this advantage disappears three years postoperatively[27]. However, owing to the difficulty in collecting data, most related research data are unrepresentative. Therefore, high-quality RCTs should be performed in the future.

DPPHR

PD was surgeons' first choice for benign or low-grade malignant lesions of the pancreatic head until the emergence of DPPHR. For these patients, since Beger *et al*[79] developed DPPHR in the early 1970s, another choice has emerged; with DPPHR, more organs are preserved, which could result in better endocrine and exocrine function postoperatively. Therefore, many studies have focused on prioritizing PD and DPPHR. Except for the perioperative parameters, whether DPPHR is superior to PD regarding QOL is still controversial[80]. Most researchers believe that DPPHR and PD relieve

Table 4 Articles retrieved from literature reporting quality of life after total pancreatectomy

Ref.	Year	Country	Study design	Relevant patients	Total patients	Moments of assessment	Operation type	Questionnaires
Wilson *et al* [61]	2014	United States	Retrospective, single-center study	112	166	At least 5 yr (60 to 132 months)	TPIAT	SF-36
Pulvirenti *et al*[54]	2019	Italy and United States	Retrospective, multicenter study	94	329	63 (20, 109) months	TP	SF-36; EORTC QLQ-PAN26
Hartwig *et al* [57]	2015	Germany	Retrospective, single-center study	81	434	24, 48, 72, 96 and 120 months	TP	EORTC QLQ-C30; EORTC QLQ-PAN26
Chinnakotla *et al*[70]	2014	United States	Retrospective, single-center study	30	75	PRE, 3, 6, 12 months and annually post-operative	TPIAT for children	Rand-36
Stoop *et al* [64]	2020	Netherlands	Retrospective, single-center study	53	145	21 (13, 54) months	TP	EORTC QLQ-C30; EORTC QLQ-PAN26; Problem Areas in Diabetes; Diabetes Treatment Satisfaction Questionnaire
Chinnakotla *et al*[60]	2014	United States	Retrospective analysis of prospective gathered data, single-center	80	484	3, 6, 12 and 24 months	TPIAT	RAND-36
Bellin *et al* [67]	2015	United States	Retrospective, single-center study	> 100	> 100	12, 24 and 36 months	TPIAT for children	SF-36
Wu *et al*[65]	2016	China	Retrospective, single-center study	36	186	5.9 yr	TP	SF-36; Audit of Diabetes Dependent QoL; EORTC QLQ-PAN26
Watanabe *et al*[58]	2015	Japan	Retrospective, single-center study	25	44	21 (2, 222) months	TP	SF-36
Walsh *et al* [62]	2012	United States	Prospective single-center study	20	20	12 (6.75, 24) months	TPIAT	Visual Analogue Pain Scale; 20 Point Depression Anxiety Stress Scale; 10-point Pain Disability Index
Scholten *et al* [53]	2019	Netherlands	Retrospective, multicenter study	60	148	3 and 5 yr	TP	EQ-5D; EORTC QLQ-C30
Casadei *et al* [56]	2016	Italy	Prospective single-center study	119	257	TP 28 (18, 36) months, PD 27 (14, 27) months	TP and PD	EQ-5D-5L
Barbier *et al* [66]	2013	United States	Retrospective, single-center study	25	56	35 (4, 168) months	TP	EORTC QLQ-C30; EORTC QLQ-PAN26
Solomina *et al*[71]	2017	United States	Retrospective analysis of prospective gathered data, single-center	20	20	28 (2, 38) months	TPIAT	SF-36

TPIAT: Total pancreatectomy and islet cell auto-transplantation; PRE: Preoperative quality of life; EORTC: European Organization for Research and Treatment of Cancer; SF-36: 36-item Short; EQ-5D: European Quality of Life 5-dimension; TP: Total pancreatectomy.

obstruction of the pancreatic head, which was the cause of the symptoms. Therefore, they have no significant influence on long-term QOL postoperatively[28,29]. Another study also suggested that increased digestive tract reconstruction during PD surgery lead to lower exocrine function and worse QOL postoperatively[31]. However, this study had poor representativeness because of its smaller sample size and earlier publication time.

Modifications of the original Beger procedure appeared, such as those by Frey and Berne, as people realized its superiority[81,82]. Compared to the Beger, Frey and Berne were technically more straightforward. All patients maintained the same pancreatic volume and exocrine and endocrine functions. Therefore, they had advantages regarding operation time and duration of hospital stay but showed no noticeable difference in improving postoperative QOL[32-35]. In conclusion, surgeons can choose any of them based on their expertise and intraoperative findings. Owing to the shorter operation time and length of hospital stay, modifications to the original Beger procedure should be preferred.

DP

DP is the standard surgical method for treating tumors of the pancreatic body or tail. Traditionally, it has been performed using an open approach. However, due to technological developments in laparoscopic and robotic instruments, MIDP is routinely performed by surgeons worldwide. Nearly all studies have demonstrated that MIDP can result in better QOL

than ODP perioperatively. However, which is better in the long-term remains controversial. Larger sample sizes and more convincing studies have reported no long-term differences between MIDP and ODP[38,40].

While performing DP, the traditional approach is to remove the spleen because it is closely attached to the distal pancreas anatomically. As people realize the function of the spleen, an increasing number of surgeons are choosing to perform LSPDP for benign and low-malignancy tumors of the distal pancreas. Due to the preservation of the spleen in LSPDP, it is clear that LSPDP is superior to LDPS regarding QOL[44,45].

Appleby surgery was first performed in 1976 for the treatment of progressive carcinoma[83] of pancreatic body and tail. Owing to the difficulty of Appleby technology and the advent of neoadjuvant therapy, the number of Appleby surgeries is decreasing; therefore, there is a lack of relevant studies concerning QOL after Appleby.

CP

Guillemin successfully performed CP by anastomosis to both pancreatic remnants with an omega-shaped jejunal loop in 1957[84]. Letton and Wilson[85] completed the procedure in two patients with pancreatic injury with a Roux-en-Y jejunal loop anastomosis to the tail and closure to the head remnant[85]. An increasing number of surgeons prefer to perform this procedure in cases where the lesion is limited to the pancreatic neck or body. A normal pancreas has significantly less parenchymal loss, which means that more pancreatic function can be retained. According to previous studies, functional recovery and mean QOL are comparable to those of a standard control population[48]. It is generally believed that patients who underwent CP have a better QOL, but a higher perioperative risk[47-50]. However, studies on the QOL after CP are lacking.

TP

Since Rockey[86] performed the first TP in a patient with pancreatic cancer in 1942, some surgeons have attempted to perform the same procedure. However, owing to poor perioperative outcomes and QOL in the beginning, the feasibility of TP has been questioned. Many studies have been conducted to answer this question. The safety of TP has improved dramatically owing to mature surgical techniques and other factors. Impaired exocrine function is also one of the reasons why the feasibility of TP has been questioned. However, the optimization of pancreatin improved the patients' exocrine function. Another reason is the high risk of brittle diabetes. Many factors are associated with insulin independence, such as non-hereditary chronic pancreatitis, younger age, lower body surface area, and higher total islet equivalents. The pancreas is the only organ that produces insulin. Due to the removal of the entire pancreas, TP causes great damage to patients' ability to maintain stable blood sugar levels. To solve this problem, a new technology, the IAT, was first described in 1977. In IAT, islet cells are isolated from patients and transplanted into the portal vein. With the advent of pancreatin and IAT, the endocrine and exocrine functions of patients after TP have significantly improved[64-66]. It seems unlikely that TP can maintain or improve patients' QOL. However, this was only possible if the patient had preoperative endocrine and exocrine pancreatic dysfunction or chronic pain. TP improved the QOL of these patients to some extent. In conclusion, TP can be considered in selected patients with neoplasms involving the entire pancreas or refractory chronic pancreatitis, regardless of the age of patients and pathology of the neoplasms.

CONCLUSION

Due to the importance of the endocrine and exocrine functions of the pancreas, surgeons have attempted to preserve normal pancreatic tissue and surrounding organs. Therefore, different surgical procedures have been developed depending on the location of the neoplasms. However, regardless of the procedure type, perioperative outcomes were generally acceptable. PD and TP had similar effects on patients' QOL. The time that patients needed to recover to the preoperative or baseline level was 3-6 months after PD, but longer after TP. At this stage, more than half of the patients still required pancreatin to relieve gastrointestinal symptoms. Most studies have demonstrated that PPPD has a similar influence on perioperative and long-term outcomes as PD. DPPHR could provide better QOL with less pain, nausea, and diarrhea symptoms, and better physical and working status. In addition, owing to the higher incidence of perioperative complications in CP than in PD, whether CP could provide a better QOL remains debatable. As far as minimally invasive surgery is concerned, it seems that they could indeed produce better QOL in the early stages after PD and DP, but the long-term outcomes still need to be confirmed by more studies. In DP, preservation of the spleen can preserve the immunological function of the patients to defeat the usual virus.

This study has some shortcomings. We did not complete a systematic analysis of the data from previous studies, but only analyzed their conclusions. The scope of our study was not comprehensive enough, and some surgical procedures were not included. However, our goal was to provide directions for future research.

It is so big a project to collect data about patients' postoperative QOL levels that the majority of studies do not have enough cases. It is not easy to contact patients *via* e-mail or phone once they are discharged from hospital. This means that incomplete data are common, especially when collecting long-term outcomes. As shown in the table, the rate of loss to follow-up was high, and there was a lack of prospective studies, especially randomized controlled studies. We propose conducting well-designed prospective analyses to verify our results.

ARTICLE HIGHLIGHTS

Research background

Pancreatic surgery is challenging because of the anatomical characteristics of pancreas. With the progress of medical standards, the perioperative outcomes have been greatly improved these years. More and more attention has been paid to the changes of quality of life (QOL) after pancreatic surgery. There is a lack of summary of QOL after various kinds of pancreatic surgery. With the purpose of describing the results of existing researches concerning QOL of pancreatic surgery we conducted this study.

Research motivation

Understanding which kind of pancreatic surgery has better QOL can provide some basis for clinical surgical decision.

Research objectives

This review aimed to summarize and analyze current research results on QOL after pancreatic surgery including pancreaticoduodenectomy, duodenum-preserving pancreatic head resection, distal pancreatectomy, central pancreatectomy and total pancreatectomy after January 1, 2012. It provides some directions for future researches based on the results of the controversy over patients' QOL after surgery. And it also provides some basis for clinical surgical decision-making.

Research methods

A systematic review was conducted in PubMed and EMBASE Database, according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses guideline. And references of the retrieved articles were screened for any relevant articles. We extracted the results of these articles and summarized them.

Research results

This review summarizes and analyzes current research results on QOL after pancreatic surgery. The article covers the discussion and analysis of the QOL of various pancreatic surgery. Which kind of surgical procedure has better QOL is controversial. The long-term benefits on QOL of minimally invasive surgery over open surgery are controversial.

Research conclusions

Comparison and summary of QOL in patients with different types of pancreatic surgery. We included not only the results of the same surgical procedure, but also the results between different procedures.

Research perspectives

More well-designed prospective analyses of patients' QOL after pancreatic surgery are needed.

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FOOTNOTES

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META-ANALYSIS

Prevalence and clinical impact of sarcopenia in liver transplant recipients: A meta-analysis

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Abstract

BACKGROUND

The prevalence of sarcopenia in patients undergoing liver transplantation (LT) remains to be determined partly because of different diagnostic criteria. Sarcopenia has recently been recognized as a new prognostic factor for predicting outcomes in LT candidates.

AIM

To estimate the prevalence of sarcopenia and evaluate its clinical effect on LT candidates.

METHODS

This systematic search was conducted in PubMed, Web of Science, Embase, and Cochrane Library for original English-language articles that investigated the prevalence and influence of sarcopenia in patients undergoing LT from database inception to November 30, 2022. Cohort studies of the definition of sarcopenia that estimate sarcopenia prevalence and evaluate its effect on clinical outcomes and the risk of mortality were included.

RESULTS

Twenty-five studies involving 7760 patients undergoing LT were included. The pooled prevalence of sarcopenia in patients undergoing LT was 40.7% [95% confidence intervals (95%CI): 32.1–49.6]. The 1-, 3-, and 5-year cumulative probabilities of post-LT survival in patients with preoperative sarcopenia were all lower than those without sarcopenia ($P < 0.05$). Sarcopenia was associated with an increased risk of post-LT mortality in patients undergoing LT (adjusted hazard ratio: 1.58; 95%CI: 1.21–2.07). Patients with preoperative sarcopenia had a longer intensive care unit stay, a high risk ratio of sepsis, and serious post-LT complications than those without sarcopenia.

CONCLUSION
Sarcopenia is prevalent in a substantial proportion of patients undergoing LT and is strongly and independently associated with higher a risk of mortality risk.

Key Words: Sarcopenia; Liver transplantation; Mortality; Clinical outcomes

Core Tip: The prevalence and effect of sarcopenia on patients undergoing liver transplantation (LT) remains to be determined partly because of different diagnostic criteria. Twenty-five studies involving 7760 patients undergoing LT were included in this meta-analysis. The pooled prevalence of sarcopenia in patients undergoing LT was 40.7%. Sarcopenia was associated with an increased risk of post-LT mortality in patients undergoing LT.

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INTRODUCTION

According to the Global Burden of Disease project, liver disease accounts for approximately 2 million deaths annually worldwide, including one million patients who died from complications of cirrhosis and one million patients who died from liver cancer and viral hepatitis[1]. Liver transplantation (LT) has become the standard treatment for patients with decompensated end-stage liver disease (ESLD)[2]. However, less than 10% of global organ transplantation needs are met at current rates of transplantation[1]. With the widespread shortage of human organs, rigorous selection of LT candidates is essential[3]. Therefore, choosing which patients are clinically suitable for LT is one of the hardest challenges for clinicians. Waiting-list mortality and post-LT survival are key determinant factors in waiting-list placement[3]. The model for ESLD (MELD) score is the most common tool used to predict outcomes in patients for LT[4]. Although the MELD score has a strong predictive value for pre-LT outcomes, it underestimates disease severity in approximately 15%–20% of patients with cirrhosis, leading to an inaccurate prediction of post-LT outcomes[4]. A common but often overlooked complication in patients with ESLD is malnutrition[5]. Undeniably, patients who are malnourished are more likely to suffer from adverse outcomes and have a high mortality risk[6,7]. Given the importance of nutritional status, an appropriate nutritional assessment must be established to determine the effect of nutritional status on patients undergoing LT.

Sarcopenia is a progressive and generalized loss of skeletal muscle mass, strength, and function and is a major component of malnutrition[8]. A previous study discovered that patients with cirrhosis have a high protein oxidation rate and a low carbohydrate oxidation rate, leading to an imbalance in skeletal muscle protein synthesis and breakdown[9]. Hepatocellular dysfunction and portosystemic shunting also result in biochemical and hormonal perturbations in patients with ESLD that contribute to sarcopenia[7]. The prevalence of sarcopenia in patients with ESLD ranges from 30% to 70%, depending on the liver disease etiology, disease stage, and diagnostic criteria[10]. Regardless of how sarcopenia is defined, it is a robust predictor of clinically relevant adverse outcomes, including poor quality of life, mortality in patients on the LT waitlist, longer stays in the hospital or intensive care unit, high incidence of infection following LT, and higher overall healthcare costs[11].

Over the past few years, sarcopenia has become a topic of prolific exploration in patients with ESLD[11]. A meta-analysis published in 2016 indicated that sarcopenia was associated with post-LT mortality; however, overlapping patients were included in this article[3]. Since then, several large and rigorously designed studies with long-term follow-up have been published. Considering the large variability in the prevalence of sarcopenia, the effect of sarcopenia on a broader range of clinically important LT-related outcomes remains unclear. Thus, this meta-analysis aimed to systematically evaluate the literature about patients who underwent LT to summarize the diagnostic criteria for sarcopenia, estimate its prevalence, and assess its effect on clinical outcomes.

MATERIALS AND METHODS

This meta-analysis was conducted based on the PRISMA checklist and was registered in PROSPERO (CRD42022379765).

Search strategy and Selection criteria

A systematic search was conducted in PubMed, Web of Science, Embase, and Cochrane Library for original English-language articles that investigated the prevalence and effect of sarcopenia on patients undergoing LT from database

inception to November 30, 2022. The search keywords and search strategies for all the included databases are shown in Supplementary Table 1. To find additional potential studies, the reference lists of the included articles were also manually searched. In addition, the study only included human studies.

The inclusion criteria were as follows: (1) Patients who underwent LT; (2) a definite diagnosis of sarcopenia based on either muscle mass or muscle function parameters; (3) studies that included the prevalence of sarcopenia in patients who underwent LT and clinical outcomes for post-LT patients; and (4) prospective or retrospective cohort studies. The exclusion criteria were as follows: (1) Case reports or review articles; (2) unclear definition of sarcopenia; (3) studies that only examined the effect of sarcopenia on pre-LT patients or patients awaiting LT; (4) duplicate studies; and (5) studies with insufficient data or unclear study information. Two reviewers independently read the full text and screened the articles that met the inclusion and exclusion criteria. The discrepancies between reviewers regarding inclusion were settled through consensus or by consulting with third-party experts.

Data extraction and quality assessment

Data were independently extracted and coded from each included study by two researchers (Jiang MJ and Wu MC) using an Excel spreadsheet. The basic information gathered from the studies included in this analysis encompasses the first author, year of publication, study design, study location, age or sex distribution, total number of participants, definition of sarcopenia, crude prevalence of sarcopenia, clinical characteristics, including the etiology of liver disease, liver function, presence of hepatocellular carcinoma, follow-up duration, and relevant outcomes. The quality of the included studies was also scored by at least two authors (Jiang MJ, Wu MC, Duan ZH, and Xiao TX) independently using the Newcastle–Ottawa Scale (NOS; 9 items in total, out of 9 points)[12]. Disagreements were resolved by consensus or discussion with a third author (Wu J, Li J, and Meng QH).

Statistical analysis

The prevalence of sarcopenia was determined through a meta-analysis. Subgroup data were collected according to the method used to define sarcopenia, sex, region, etiology of liver disease, and severity of liver disease. The primary outcome of this meta-analysis was mortality risk in patients undergoing LT with sarcopenia. The effect of sarcopenia on the incidence of post-LT survival was evaluated using the pooled unadjusted hazard ratio (HR) or adjusted HR and 95% confidence intervals (95%CI). The 90-day, 1-year, 3-year, and 5-year cumulative mortality were also pooled for patients with and without sarcopenia using the Freeman–Tukey double arcsine transformation method[13]. Continuous outcome data evaluated using homogenous metrics (*e.g.*, same test instrument) were summarized as weighted mean difference (WMD) and 95%CI. Dichotomous variables were tested using both risk ratios (RR) and 95%CI. Random-effects meta-regression was used to test the difference between two groups. Sensitivity analysis was used to evaluate the stability of the model. Egger's and Begg's tests combined with the observation funnel plot were used to evaluate publication bias. Heterogeneity was statistically assessed using the I^2 measurement and Cochran's Q statistic. A random effects model was used if heterogeneity was high (P value of Q statistic ≤ 0.1 or I^2 ≥ 50%). Stata 16 software was used for the meta-analysis. Two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of the included studies

A detailed flowchart of the literature search is shown in Figure 1. Of the 7573 records identified from four databases (PubMed, n = 1,042; EMBASE, n = 4312; Cochrane Library, n = 289; Web of Science, n = 1930), 1863 duplicates and 5408 ineligible titles/abstracts were excluded. Of the other 302 articles that underwent full-text review, 25 retrospective cohort studies with data on 7760 patients were included. The detailed characteristics of all the studies in this meta-analysis are summarized in Table 1. Overall, 10 of the 25 studies were from Asia, 6 from Europe, 5 from North America, and 2 from Africa. Only one study was from Australia, and one was a multicenter international study. The number of patients of the included studies ranged from 47 to 2816. The mean age of the patients ranged from 41.6 to 57.0 years among the included researches. All studies were rated high quality with an NOS score of ≥ 7 (Supplementary Table 2).

Diagnosis of sarcopenia

All the studies included in this meta-analysis used a low muscle mass as the basis of diagnosis. No studies have used low muscle strength or low physical performance as diagnostic criteria. Twenty-four studies used skeletal muscle area-based CT to diagnose sarcopenia, and only one study used dual-energy X-ray absorptiometry (DEXA) to assess sarcopenia. Fifteen studies reported the cross-sectional muscle area with the third lumbar-skeletal muscle index (L3-SMI), whereas the psoas muscle area or psoas muscle index (PMI) was reported in nine studies. Twenty-three studies used different diagnostic criteria based on sex. As the most used diagnostic method for sarcopenia, the cutoff values of L3-SMI ranged from 39.0-52.4 cm^2/m^2 in men and from 28.9-42 cm^2/m^2 in women. A summary of the diagnostic criteria used to assess sarcopenia in the included studies is presented in Table 1.

Prevalence of sarcopenia

The prevalence of sarcopenia was reported in 25 studies (n = 7760) and ranged from 11.0% to 78.3%, yielding a pooled prevalence of 40.7% (95%CI: 32.1-49.6; Figure 2A). Given the significant heterogeneity, subgroup analyses of sarcopenia rates were conducted for different definitions, sexes, regions, basic diseases, and Child–Pugh class (Table 2). The

Table 1 Characteristics of the included studies

Ref.	Country	Methods for measuring muscle mass	Definition of sarcopenia	Sample size (*n*)	Mean age (yr)	Male (%)	HCC (%)	BMI (Kg/m²)	Follow-up time	NOS
Kumar *et al*[2], 2020	India	CT: L3-SMI	L3-SMI < 52.4 cm^2/m^2 for male and < 38.5 cm^2/m^2 for female	115	46.30 ± 10.20	90.4	-	24.5 ± 4.3	90 d	7
Bhanji *et al*[8], 2019	United States	CT: L3-SMI	L3-SMI < 50 cm^2/m^2 in men and < 39 cm^2/m^2 in women	293	51.95 ± 11.00	70.3	30.4	27.51 ± 5.73	13	8
Shafaat *et al*[26], 2023	United States	CT: L3-SMI	L3-SMI < 50 cm^2/m^2 in men, < 39 cm^2/m^2 in women	454	57.00 ± 8.89	65.0	-	29.0 ± 5.7	11yr	9
Sim *et al*[27], 2022	Korea	CT: L3-SMI	L3-SMI < 39.9cm^2/m^2 in men, < 28.9 cm^2/m^2 in women	2816	53.00 ± 7.41	75.0	52.8	24.2 ± 3.1	7.8 yr	8
Prakash *et al*[28], 2022	India	CT: L4-PMTH	L4-PMTH < 1 mm/m for men and < 10.4 mm/m for women	51	46.40 ± 9.10	96.1	37.3	23.34 ± 5.1	-	7
Wu *et al*[29], 2021	Taiwan China	CT: L3-PMI	L3-PMI < 2.63 cm^2/m^2 for female	122	52.32 ± 7.98	55.0	24.6	24.83 ± 4.45	10 yr	8
Kuo *et al*[30], 2019	United States	CT: L3-SMI	L3-SMI < 48 cm^2/m^2 for men	126	53.00 ± 8.89	63.0	14.0	28 ± 6.67	10.6 yr	8
Golse *et al*[31] 2017	France	CT: L3-4 PMA	L3-4 PMA < 1464 mm^2 in women and < 1561 mm^2 in men	256	5.003 ± 10.50	76.6	40.0	25.3 ± 10.5	8 yr	8
Beumer *et al*[32], 2022	Multicenter international study	CT: L3-SMI	L3-SMI < 37 cm^2/m^2 for women with a BMI < 25 kg/m^2 or 42 cm^2/m^2 for women with a BMI ≥ 25 kg/m^2, and < 45 cm^2/m^2 for men with a BMI < 25 kg/m^2, or < 51 cm^2/m^2 for men with a BMI ≥ 25 kg/m^2	528	57.00 ± 9.00	86.0	100.0	26.67 ± 5.02	5 yr	8
Pinto Dos Santos *et al*[33], 2020	Germany	CT: L3-PSMI	L3-PSMI < 18.6 cm^2/m^2	368	56.80 ± 9.70	69.3	44.6	25.2 ± 4.37	10 yr	7
Izumi *et al*[34], 2017	Japan	CT: L3-PMI	L3-PMI < 612.5 mm^2/m^2 in men and < 442.9 mm^2/m^2 in women	47	54.00 ± 10.00	51.1	23.4	-	120 d	7
Hamaguchi *et al* [35], 2017	Japan	CT: L3-SMI	L3-SMI < 40.31 cm^2/m^2 in men and < 30.88 cm^2/m^2 in women	250	54.00 ± 14.07	44.8	33.0	22.7 ± 3.63	5 yr	8
Irwin *et al*[36], 2021	South Africa	CT: L3-SMI	L3-SMI < 39 cm^2/m^2 for women and < 50 cm^2/m^2 for men	106	-	60.4	-	-	1 yr	9
Tan *et al*[14], 2022	China	CT: L3-PMI	L3-PMI < 6.25 cm^2/m^2 for man	70	41.60 ± 9.70	100.0	-	22.9 ± 2.9	10 yr	8
Masuda *et al*[37], 2014	Japan	CT: L3-PMA	< 800 cm^2 for men and < 380 cm^2 for women	204	54.32 ± 9.60	50.49	-	23.6 ± 3.4	8 yr	7
Miarka *et al*[38], 2021	Poland	CT: L3-SMI	L3-SMI < 50 cm^2/m^2 for men and 39 cm^2/m^2 for women	98	55.00 ± 8.00	76.5	26.5	27 ± 4	1224 d	7

Montano-Loza *et al* [39], 2014	Canada	CT: L3-SMI	L3-SMI ≤ 41 cm^2/m^2 for women and ≤ 53 cm^2/m^2 for men with a BMI ≥25 kg/m^2 and L3 SMI ≤ 43 cm^2/m^2 for patients with a BMI < 25 kg/m^2	248	55.00 ± 1.00	68.0	39.0	27.19 ± 2.23	5 yr	9
Kalafateli *et al*[23], 2017	United Kingdom	CT: L3-PMI	L3-PMI < 340 mm^2/m^2 for men and < 264 mm^2/m^2 for women	232	54.00 ± 12.00	69.8	25.0	25 ± 6.43	1 yr	8
Czigany *et al*[40], 2020	Germany	CT: L3-SMI	L3-SMI < 50 cm^2/m^2 in men and < 39 cm^2/m^2 in women	225	54.00 ± 12.00	66.7	28.0	27 ± 5	90 d	8
Hey *et al*[41], 2022	Australia	DEXA: APLM	APLM < 7.26 kg/m^2 for male and < 5.5 kg/m^2 for female	469	55.00 ± 10.59	72.1	26.0	-	1 yr	8
Lindqvist *et al*[42], 2019	Sweden	CT: L3-SMI	L3-SMI < 43 cm^2/m^2 for men with BMI < 25 kg/m^2 and < 53 cm^2/m^2 for BMI > 25 kg/m^2 and < 41 cm^2/m^2 for women in all BMI ranges	53	57.00 ± 11.11	69.8	52.8	25.1 ± 5.3	1 yr	8
Riccardo *et al*[43], 2019	Japan	CT: L3-SMI	L3-SMI < 42 cm^2/m^2 for men and L3-SMI < 38 cm^2/m^2 for women	138	57.00 ± 13	56.5	-	24.8 ± 5	10 yr	8
Andrea *et al*[21], 2013	United States	CT: L3-4 SMI	L3-4 SMI ≤ 38.5 cm^2/m^2 for women and ≤ 52.4 cm^2/m^2 for men	338	55.00 ± 10.00	65.9	-	28 ± 6	1021.2 d	9
Young *et al*[44], 2018	Korea	CT: L3-PMTH	L3-PMTH < 15.5 mm/m	92	53.33 ± 5.75	67.4	100.0	24.17 ± 2.81	36 months	8
Hassan *et al*[45], 2022	Egypt	CT: L3-SMI	L3-SMI < 52.4 cm^2/m^2 for male and < 38.5 cm^2/m^2 for female	61	48.70 ± 12.50	75,4	-	23.9 ± 3.9	6 months	7

HCC: Hepatocellular carcinoma; NOS: Newcastle–Ottawa Scale; L3: The caudal end of the third lumbar vertebra; L3-SMI: Third lumbar-skeletal muscle index; PMA: The Area of the Psoas Muscle; PMTH: The psoas muscle thickness to height ratio; PSMI: Paraspinal muscle index; APLM: Appendicular lean mass; PMI: Psoas muscle index.

subgroup analysis based on the definition of sarcopenia showed that the prevalence of sarcopenia was 41.5% (95%CI: 29.6–53.9) when defined by L3-SMI, 36.4% (95%CI: 16.2–59.5) by L3-PMI, and 41.5% (95%CI: 26.7–57.1) by other definitions. A subgroup analysis by sex was also performed, which revealed that male patients had a higher pooled prevalence of sarcopenia (43.3%, 95%CI: 31.1–55.9) than female patients (33.1%, 95%CI: 21.6–45.6). Moreover, 23 studies were analyzed by regional subgroup, and Africa had the highest pooled prevalence of sarcopenia among patients undergoing LT (57.6%, 95%CI: 50.0–65.1). Among the different primary liver diseases, patients with alcoholic liver disease had the highest prevalence of sarcopenia (52.2%, 95%CI: 36.2–68.2). Finally, the prevalence for patients with Child–Pugh class C (54.3%, 95%CI: 43.9–64.8) was higher than those with Child–Pugh class B (38.9%, 95%CI: 30.8–47.0) or A (30.4%, 95%CI: 26.0–35.0).

Cumulative post-LT survival in patients with and without sarcopenia

The 90-day, 1-year, 3-year, and 5-year cumulative probabilities of post-LT survival in patients with sarcopenia were 92.9% (95%CI: 88.9–96.9), 79.8% (95%CI: 72.8–86.8), 74.3% (95%CI: 68.0–80.5), and 63.6% (95%CI: 56.5–70.6), respectively (Supplementary Figures 1-4). By comparison, they were 96.5% (95%CI: 94.7–98.3), 92.7% (95%CI: 90.2–96.2), 93.4% (95%CI: 90.6–96.2), and 79.5% (95%CI: 73.2–85.8), respectively, in patients without sarcopenia (Supplementary Figures 1-4). The 1-, 3-, and 5-year cumulative probabilities of post-LT survival in patients with preoperative sarcopenia were all lower than those without preoperative sarcopenia ($P < 0.05$). However, regardless of whether patients had sarcopenia, no difference was found in the 90-day cumulative probabilities of survival post-LT ($P = 0.289$; Table 3).

Table 2 The pooled overall prevalence of sarcopenia in study subgroups

Subgroup	Studies (*n*)	Sarcopenia (*n*)	Prevalence [% (95%CI)]	I^2 (%)	*P* value
L3-SMI	15	1562	41.5 (29.6-53.9)	98.6	< 0.001[a]
L3-PMI	4	139	36.4 (16.2-59.5)	95.6	< 0.001[a]
Others	6	538	41.5 (26.7-57.1)	97.0	< 0.001[a]
Sex					
Male	17	1280	43.3 (31.1-55.9)	98.9	< 0.001[a]
Female	15	366	33.1 (21.6-45.6)	95.7	< 0.001[a]
World region					
Europe	6	458	37.7 (26.6-49.5)	94.0	< 0.001[a]
Asia	10	734	38.8 (23.3-55.5)	98.3	< 0.001[a]
North America	5	661	47.8 (33.4-62.4)	96.7	< 0.001[a]
Africa	2	96	57.6 (50.0-65.1)	0.0	< 0.001[a]
Disease types					
Viral	8	407	32.3 (18.9-47.2)	97.0	< 0.001[a]
ALD	11	477	52.2 (36.2-68.2)	96.8	< 0.001[a]
NAFLD	5	54	47.2 (25.7-68.6)	85.6	< 0.001[a]
AIH/PSC/PBC	4	141	33.6 (19.0-48.2)	63.5	0.042[a]
HCC	10	399	35.9 (18.6-53.2)	98.4	< 0.001[a]
Other	7	123	41.2 (22.6-59.8)	92.2	< 0.001[a]
Child-Pugh class					
A	4	121	30.4 (26.0-35.0)	44.6	0.144
B	5	157	38.9 (30.8-47.0)	56.8	0.055
C	7	446	54.3 (43.9-64.8)	88.2	< 0.001[a]

[a]*P* value < 0.05.

L3-SMI: Third lumbar-skeletal muscle index; L3-PMI: Third lumbar- psoas muscle index; ALD: Alcoholic liver disease; NAFLD: Nonalcoholic fatty liver disease; AIH: Autoimmune hepatitis; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cholangitis; HCC: Hepatocellular carcinoma.

Table 3 Pooled 90 d and 1, 3, and 5-year cumulative survival probabilities in patients with and without sarcopenia

Survival [% (95%CI)]	With sarcopenia	Without sarcopenia	*P* value
90-day	92.9 (88.9-96.9), 5 studies 422 patients	96.5 (94.7-98.3), 5 studies 891 patients	0.289
1-year	79.8 (72.8-86.8), 10 studies 894 patients	92.7 (90.2-95.2), 10 studies 3475 patients	0.015[a]
3-year	74.3 (68.0-80.5) 4 studies 185 patients	93.4 (90.6-96.2) 4 studies 291 patients	0.003[a]
5-year	63.6 (56.5-70.6) 8 studies 730 patients	79.5 (73.2-85.8) 8 studies 1316 patients	0.006[a]

[a]*P* value < 0.05.

The *P* value was produced using the random-effects meta-regression method.

Association between sarcopenia and post-LT mortality

Based on univariate analysis of data from 9 studies (n = 4845), sarcopenia was associated with an increased risk of post-LT mortality, with a pooled unadjusted HR of 1.72 (95%CI: 1.33–2.24, I^2 = 60.7%, P = 0.009; Supplementary Figure 5). In data from multivariate analysis (nine studies, n = 4430), sarcopenia was still significantly associated with increased post-LT mortality with a pooled adjusted HR of 1.58 (95%CI: 1.21–2.07, I^2 = 54.4%, P = 0.025; Figure 2B).

Impact of sarcopenia on clinical outcomes

Seven studies involving 1,369 patients reported data on the length of ICU stay that were available for meta-analysis.

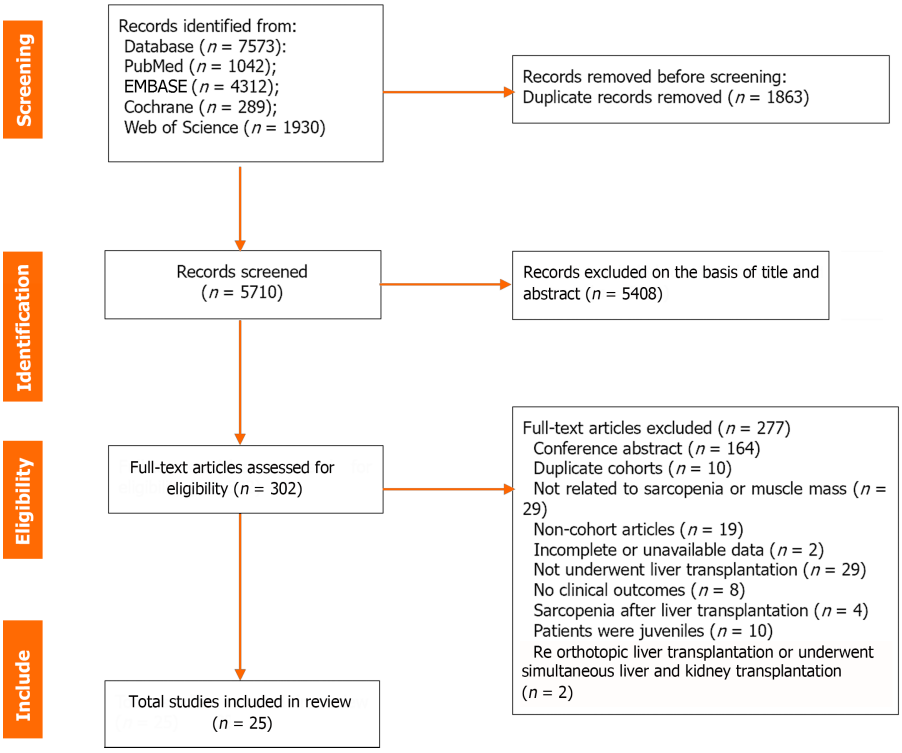

Figure 1 Literature identification process.

Patients with preoperative sarcopenia had longer ICU stays than those without sarcopenia post-LT (WMD: 4.503, 95%CI: 2.218–6.788, $P < 0.001$) (Supplementary Figure 6). Six studies involving 1001 patients reported data on the length of stay (LOS) and were included in the meta-analysis. LOS was not different in patients with or without preoperative sarcopenia (WMD: 9.352, 95%CI: 2.557–15.261, $P = 0.162$; Supplementary Figure 7). Data from four studies involving 606 patients were available for meta-analysis of developing sepsis post-LT, showing that patients with preoperative sarcopenia had a higher risk of sepsis post-LT than those without sarcopenia (RR: 2.00, 95%CI: 1.143–3.503, $P = 0.015$; Supplementary Figure 8). Four studies involving 643 patients reported data of the Clavien–Dindo classification and were included in the meta-analysis. Patients with preoperative sarcopenia had a higher RR of serious postoperative complications (Clavien–Dindo classification ≥ 3) than those without preoperative sarcopenia (RR: 1.287, 95%CI: 1.05–1.583, $P = 0.017$; Supplementary Figure 9).

Sensitivity analysis, meta-regression, and publication bias

Sensitivity analyses that excluded one study at a time and then pooled the remaining studies showed adjusted HRs ranging from 1.49 to 1.70, suggesting that our results were robust (Supplementary Figure 10). Meta-regression analyses showed no association of pooled adjusted HR with sample size ($P = 0.819$), percentage of male patients ($P = 0.660$), average follow-up time ($P = 0.746$), different regions ($P = 0.786$), diagnostic method ($P = 0.553$), and NOS ($P = 0.865$; Supplementary Table 3). The funnel plot was symmetrical (Figure 3). Egger's ($P = 0.526$) and Begg's ($P = 0.348$) tests suggested no significant statistical evidence of publication bias.

DISCUSSION

LT is the only potential treatment for ESLD[14]. To increase the survival rates of LT candidates, preoperative risk assessment for risk is essential. Most patients with ESLD who undergo LT are physically deconditioned, with low functional capacity, malnutrition, sarcopenia, and frailty[10,15]. Sarcopenia is a common symptom of ESLD that strongly affects adverse outcomes and mortality in this population[11]. Although a meta-analysis also evaluated the association between skeletal muscle mass and mortality in patients with cirrhosis, studies of post-LT patients were excluded[5]. Another systematic review reported sarcopenia-impaired outcomes in patients awaiting or undergoing LT; many patients evaluated for LT but did not undergo LT were also included in this meta-analysis[3]. However, since then, a number of large, rigorously designed, and long-term follow-up studies have provided updated data. Therefore, in this study, a more comprehensive search was performed, a much larger pool of potential studies was screened, studies with overlapping cohorts and only waiting-LT patients were excluded, and a comprehensive range of subgroup and sensitivity analyses was performed to summarize the prevalence, post-LT survival, and outcomes of sarcopenia in patients who underwent LT.

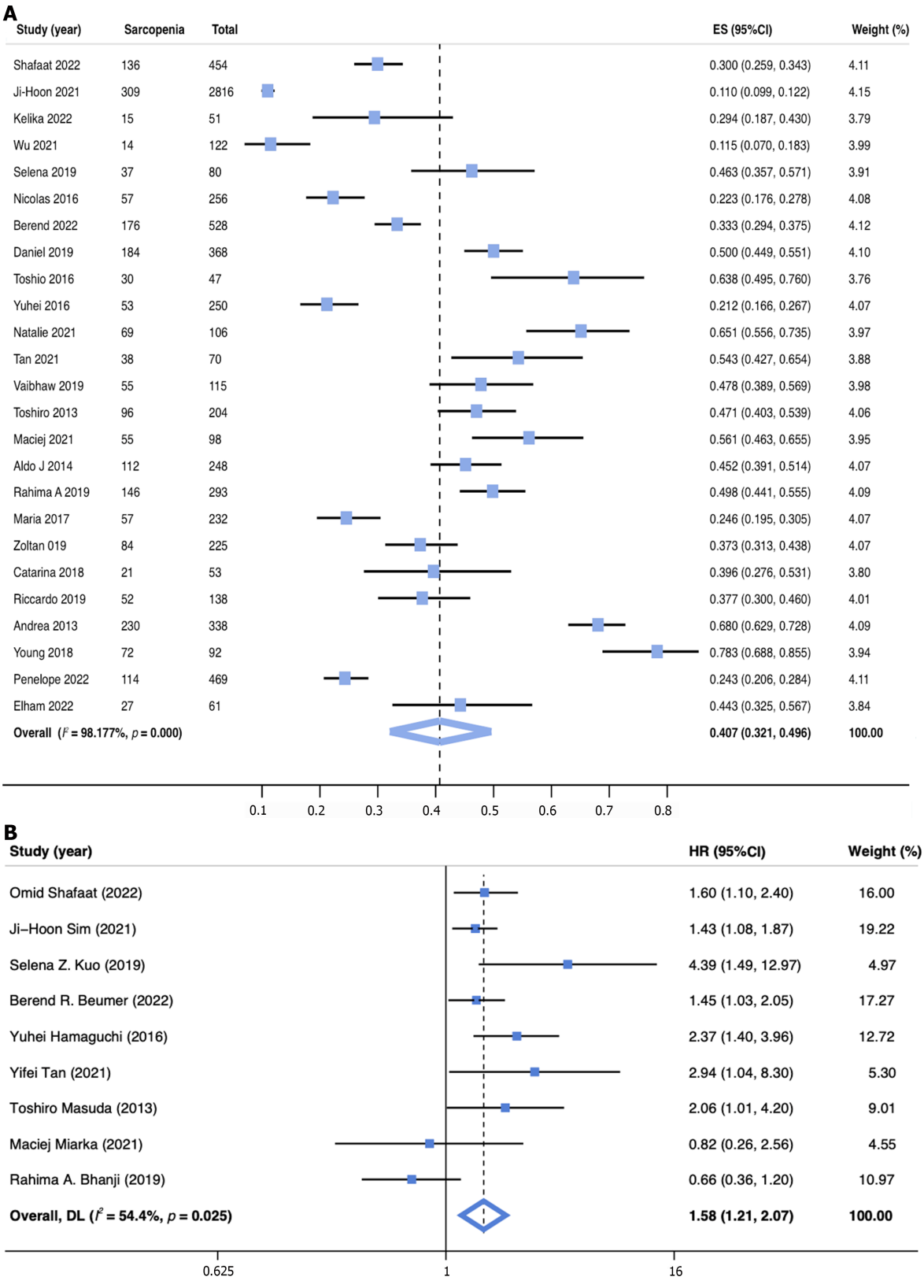

Figure 2 The prevalence and clinical impact of sarcopenia in patients underwent liver transplantation. A: The pooled overall prevalence of sarcopenia in patients underwent liver transplantation in the included studies; B: Forest plot for multivariate analysis assessing the association between sarcopenia and mortality risk.

As a major component of malnutrition, sarcopenia is a strong predictor of morbidity and mortality in patients with ESLD[16]. Despite the importance of sarcopenia, no consensus has been established regarding how to accurately measure and define sarcopenia in clinical settings[17]. The European Working Group on Sarcopenia defined sarcopenia as "a progressive and generalized skeletal muscle disorder associated with an increased likelihood of adverse outcomes including falls, fractures, disability, and mortality", combining both muscle mass and muscle strength or muscle

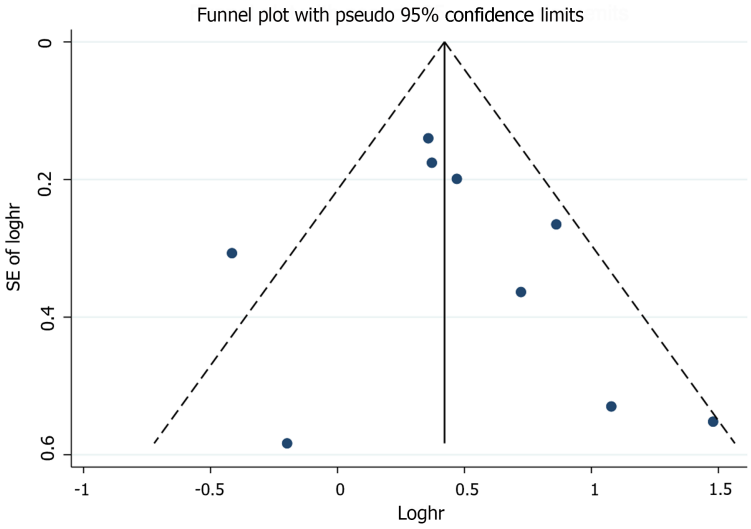

Figure 3 Funnel plot with 95% pseudo-confidence limits for all included studies.

performance[18]. In this study, 24 studies used skeletal muscle area measured by CT scans as the method to diagnose sarcopenia, and only one study used DEXA to assess sarcopenia. None of the included studies used low muscle strength or low physical performance as diagnostic criteria in this meta-analysis. While most working groups recommend considering both muscle mass and muscle function for the diagnosis of sarcopenia, most studies in patients with liver disease have investigated sarcopenia using measures of muscle mass alone[10,18,19]. Based on the available data on liver disease, some guidance developed a consensus definition for the operationalization of sarcopenia in liver disease as the phenotypic manifestation of loss of muscle mass alone[10,11]. In the future, muscle strength or physical performance should also be included in the diagnosis of sarcopenia, and consistent tests should be conducted to diagnose sarcopenia.

Owing to the varied definitions of sarcopenia, a wide range of the prevalence of sarcopenia in patients undergoing LT was reported[3]. In this meta-analysis, the overall pooled prevalence of sarcopenia for patients who underwent LT was 40.6%. In another meta-analysis, the prevalence of sarcopenia ranged from 22.2% to nearly 70% in patients undergoing LT or evaluated for LT[3]. A previous meta-analysis excluded post-LT patients, and sarcopenia affected 37.5% of patients with cirrhosis[5]. The etiology of liver disease has been associated with differences in the prevalence of sarcopenia[10,20]. Our finding is consistent with previous studies that have shown that patients with alcohol-associated liver disease (ALD) had a lower baseline muscle mass[20,21]. A previous study reported that sarcopenia is related to the severity of liver disease as estimated by the Child–Pugh class[22]. In another study, the muscle mass index was negatively correlated with the Child–Pugh score[16]. In this meta-analysis, patients with ALD and those in the Child–Pugh C class had the highest prevalence of sarcopenia of > 50%. Our study is in line with the results from a recent study that showed that sarcopenia is common in patients with ESLD and worsens with the progression of liver disease[16]. All studies that reported the prevalence of sarcopenia separately for different sexes included in this meta-analysis indicated a higher prevalence among men. Sex is believed as the most important factor influencing muscle mass in the general population[16]. Therefore, future studies to define sarcopenia are needed for clinical application with consideration of sex, age, ethnicity, and basic diseases.

The North American Expert Opinion Statement on Sarcopenia in LT recommends using sarcopenia to predict the prognosis of LT[11]. However, various clinical outcomes, such as overall mortality in evaluated patients, waitlist mortality in listed patients, post-LT mortality in patients undergoing LT, and short-term *vs* long-term outcomes, confound the comparison between published studies and the development of generalized definitions[11]. Our studies mainly focused on post-LT mortality and complications. From this meta-analysis, no difference in the 90-day cumulative probabilities of survival post-LT was found between patients with or without sarcopenia. However, the 1-, 3-, and 5-year cumulative probabilities of post-LT survival in patients with sarcopenia were all lower than those in patients without sarcopenia. In our meta-analysis, sarcopenia was associated with a pooled HR of 1.58 (95%CI: 1.21–2.07) for post-LT mortality similar to a prior meta-analysis with a pooled HR of 1.84 (95%CI: 1.11–3.05) for post-LT mortality[3]. Although multiple studies have shown sarcopenia to be associated with post-LT mortality[3,23,24], data reporting preoperative sarcopenia associated with adverse post-LT outcomes are limited. In this meta-analysis, patients with preoperative sarcopenia had longer ICU stays than those without sarcopenia post-LT. In addition, patients with preoperative sarcopenia had a higher RR of sepsis and serious post-LT complications than those without sarcopenia. This indicates that preoperative sarcopenia may play an important role in the clinical outcomes of patients undergoing LT. Given the lack of an objective metric of sarcopenia, some guidelines do not recommend using sarcopenia as a contraindication against LT [10,11]. However, sarcopenia may guide the decision about LT in an attempt to minimize liver-related complications and optimize overall patient recovery. Therefore, it is important to incorporate sarcopenia into the management and treatment of LT candidates to optimize nutrition and physical activity[25].

This study has several limitations. First, the wide inclusion criteria in this study generated significant heterogeneity that could not be explained. A random-effects model with subgroup analyses was used whenever possible to minimize the effect of heterogeneity. Subgroup analysis, meta-regression, and sensitivity analysis were used to identify the possible

sources of heterogeneity. Second, significant heterogeneity in the means of defining sarcopenia and the diagnostic criteria employed was noted among the included studies. Thus, future studies should build uniform cutoff thresholds based on ethnicity to assess sarcopenia. Third, the included articles and all diagnostic protocols lacked an assessment of muscle strength and physical performance. Future prospective studies using the criteria including muscle mass and muscle strength/physical performance must determine whether the predictive power is improved after employing a more comprehensive algorithm to diagnose sarcopenia. Fourth, the number of studies on some variables for clinical outcomes was limited, so the application and promotion of the combined results were also restricted to a certain extent. Fifth, the etiology of liver diseases is an important factor associated with mortality. However, we could not analyze the effect of liver disease etiology on our results because the HR in each study was not reported separately according to etiology. Finally, although the quality of the included studies was evaluated using the NOS statement entries during the search and screening processes, some subjectivity remained in the evaluation of the literature because of the lack of accepted quality evaluation criteria, which may lead to some selection bias in the included studies.

CONCLUSION

This meta-analysis demonstrated that sarcopenia affects 40% of LT recipients. This study also showed that sarcopenia was associated with a 1.58-fold higher risk of post-LT mortality. Sarcopenia was also associated with long-term survival rates and adverse post-LT outcomes. Because of the high prevalence and adverse post-LT outcomes, sarcopenia should be considered a part of the initial evaluation of LT candidates. More future studies are needed are needed to incorporate sarcopenia or muscle mass index/function into a formal prognostic scale for LT patients.

ARTICLE HIGHLIGHTS

Research background

Liver transplantation (LT) has become the standard treatment for patients with end-stage liver disease (ESLD). With the widespread shortage of human organs, rigorous selection of LT candidates is essential. Over the past few years, sarcopenia has become a topic of prolific exploration in patients with ESLD. Sarcopenia has recently been recognized as a new prognostic factor for predicting outcomes in LT candidates. Therefore, this study aimed to estimate the prevalence of sarcopenia and evaluate its clinical effect on LT candidates.

Research motivation

As a major component of malnutrition, sarcopenia is a strong predictor of morbidity and mortality in patients with ESLD. However, the link between sarcopenia and LT candidates is not well studied.

Research objectives

This meta-analysis aimed to systematically evaluate the literature about patients who underwent LT to summarize the diagnostic criteria for sarcopenia, estimate its prevalence, and assess its effect on clinical outcomes.

Research methods

This systematic search was conducted in PubMed, Web of Science, EMBASE, and Cochrane Library for original English-language articles that investigated the prevalence and influence of sarcopenia on patients undergoing LT from database inception to November 30, 2022. The prevalence of sarcopenia was determined through a meta-analysis. The effect of sarcopenia on the incidence of post-LT survival was evaluated using the pooled unadjusted hazard ratio (HR) or adjusted HR and 95% confidence intervals.

Research results

Twenty-five studies involving 7760 patients undergoing LT were included. The pooled prevalence of sarcopenia in patients undergoing LT was 40.7%. The 1-, 3-, and 5-year cumulative probabilities of post-LT survival in patients with preoperative sarcopenia were all lower than those without sarcopenia ($P < 0.05$). Sarcopenia was associated with an increased risk of post-LT mortality in patients undergoing LT. Patients with preoperative sarcopenia had a longer intensive care unit stay, a high risk ratio of sepsis, and serious post-LT complications than those without sarcopenia.

Research conclusions

Sarcopenia is prevalent in a substantial proportion of patients undergoing LT. This study also showed that sarcopenia was associated with a 1.58-fold higher risk of post-LT mortality. Sarcopenia was also associated with long-term survival rates and adverse post-LT outcomes.

Research perspectives

Because of the high prevalence and adverse post-LT outcomes, sarcopenia should be considered a part of the initial evaluation of LT candidates. More studies are needed to incorporate sarcopenia into a formal prognostic scale for LT recipients.

FOOTNOTES

Co-first authors: Min-Jie Jiang and Mu-Chen Wu.

Author contributions: Jiang MJ, Wu MC, and Meng QH conceived and designed the experiments; Jiang MJ, and Wu MC analyzed the data; Duan ZH, Wu J, Xu XT, and Li J collected the information; Jiang MJ and Wu MC wrote the paper; Meng QH reviewed and edited the paper; all authors contributed to preparing the manuscript and approved the contents. Jiang MJ and Wu MC contributed equally to this work as co-first authors. The reasons for designating Jiang MJ and Wu MC as co-first authors are twofold. First, the research was performed as a collaborative effort, and the designation of co-corresponding authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper. This also ensures effective communication and management of post-submission matters, ultimately enhancing the paper's quality and reliability. Second, Jiang MJ and Wu MC contributed efforts of equal substance throughout the research process. The choice of these researchers as co-corresponding authors acknowledges and respects this equal contribution, while recognizing the spirit of teamwork and collaboration of this study. In summary, we believe that designating Jiang MJ and Wu MC as co-first authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

Conflict-of-interest statement: All authors have no conflict of interest.

PRISMA 2009 Checklist statement: This meta-analysis was conducted based on the PRISMA checklist and was registered in PROSPERO (CRD42022379765).

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SCIENTOMETRICS

Bibliometrics analysis based on the Web of Science: Current trends and perspective of gastric organoid during 2010-2023

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Abstract

BACKGROUND

Three-dimensional organoid culture systems have been established as a robust tool for elucidating mechanisms and performing drug efficacy testing. The use of gastric organoid models holds significant promise for advancing personalized medicine research. However, a comprehensive bibliometric review of this burgeoning field has not yet been published.

AIM

To analyze and understand the development, impact, and direction of gastric organoid research using bibliometric methods using data from the Web of Science Core Collection (WoSCC) database.

METHODS

This analysis encompassed literature pertaining to gastric organoids published between 2010 and 2023, as indexed in the WoSCC. CiteSpace and VOSviewer were used to depict network maps illustrating collaborations among authors, institutions and keywords related to gastric organoid. Citation, co-citation, and burst analysis methodologies were applied to assess the impact and progress of research.

RESULTS
A total of 656 relevant studies were evaluated. The majority of research was published in gastroenterology-focused journals. Globally, Yana Zavros, Hans Clevers, James M Wells, Sina Bartfeld, and Chen Zheng were the 5 most productive authors, while Hans Clevers, Huch Meritxell, Johan H van Es, Marc Van de Wetering, and Sato Toshiro were the foremost influential scientists in this area. Institutions from the University Medical Center Utrecht, Netherlands Institute for Developmental Biology (Utrecht), and University of Cincinnati (Cincinnati, OH, United States) made the most significant contributions. Currently, gastric organoids are used mainly in studies investigating gastric cancer (GC), *Helicobacter pylori*-infective gastritis, with a focus on the mechanisms of GC, and drug screening tests.

CONCLUSION
Key focus areas of research using gastric organoids include unraveling disease mechanisms and enhancing drug screening techniques. Major contributions from renowned academic institutions highlight this field's dynamic growth.

Key Words: Gastric organoid; Gastric disease; Bibliometrics analysis; Gastric cancer; Gastritis

Core Tip: This study highlights the pivotal role of organoid technology in gastric disease research, emphasizing its growth in publications and citations. Key contributions from leading researchers and institutions, particularly in understanding gastric cancer and *Helicobacter pylori*-infective gastritis, mark advances in the field. Focused on deciphering cancer mechanisms and improving drug screening, this area of exploration provides crucial insights for future gastroenterology research.

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INTRODUCTION

The term "organoid" was first introduced as an anatomical diagnosis of tumors in a study of tumor mechanisms in 1946 [1]. Organoid generally refers to tissues or structures that are similar to organs used *in vitro* biology. A gastric organoid is a specific cluster of cells derived from gastric or pluripotent stem cells[2,3]. Gastric organoids are highly similar to gastric epithelial tissue in terms of cellular components, tissue architecture, and specific functions, and have corresponding functional characteristics, which enable the replication of gastric epithelial tissues *in vitro*[4]. Gastrointestinal organoid technology represents a technical revolution compared with conventional cell biology methods. In conventional cell culture, a single-cell clone is cultured in a two-dimensional environment. However, in gastric organoid culture, many cell clones from the stomach can be cultured in a three-dimensional (3D) environment exhibiting a higher degree of similarity to the *in vivo* microenvironment, thus providing a more accurate and physiologically pertinent model for study[5,6]. Gastric organoids cultured *in vitro*, especially those derived from human tissues, provide a new platform for the study of human gastric physiology and diseases[7]. Since Barkerfirst described a protocol using mouse leucine-rich repeat-containing G-protein coupled receptor 5+ (Lgr5+) gastric stem cells to construct gastric organoids in 2010, gastric organoids have attracted increasing attention in the field of digestive tract research[8].

In recent years, numerous living biobanks of tumor organoids have been established. For instance, a human gastric cancer (GC) organoid biobank encompasses 34 patients and 63 organoid lines, capturing the heterogeneity of tumor subtypes and enabling therapeutic screening[9]. These established organoid biobanks serve as biomaterial for human cancer research and personalized medicine. They have been widely used in fundamental research investigating gastric physiology and pathology, clinical areas, such as diagnostic processes, pharmacological screening and development, as well as gene therapy applications[10,11]. Hans Clevers from Utrecht University in the Netherlands is a pioneer in the field, having made significant contributions to the technology[12].

The Web of Science Core Collection (WoSCC) database houses many published academic studies. Because these publications are fragmented, strong conclusions from studies investigating gastric organoids should coherently bring research findings together. In the present study, we used bibliometric analysis to generate a network map of author(s), institution(s), keywords, and co-cited references derived from publications addressing gastric organoids. We then systematically analyzed the main topics based on the results of this analysis to build an in-depth and comprehensive understanding of research investigating gastric organoids.

MATERIALS AND METHODS

Data collection

Literature sources included in this study were retrieved from the WoSCC database. The database was searched by combining the terms "organoid", "gastroid", "spheroid", "gastric", "gastritis", and "stomach". Studies published from 2010 up to September 2023 were searched to form the basis of data processing and analysis.

Data analysis

Data extracted from the studies retrieved in the literature search of the WoSCC database included author information, title, keywords, abstract, and references, and underwent preliminary screening. Quantitative variables were analyzed using spreadsheet software (Excel 2019, Microsoft Corporation, Redmond, WA, United States). Citespace version 6.1.R3, developed by Chen Chaomei, and VOSviewer version 1.68, developed by Nees Jan van Eck and Ludo Waltman, were used for scientific knowledge mapping and analysis[13,14]. VOSviewer 1.68 was used to calculate citations and publications of authors and institutions, and to depict the keyword network graph[15,16]. Citespace 6.1.R3 was used to calculate co-cite and citation frequencies, centrality, keyword bursts, references, as well as to depict a network graph of authors, institutions, and a timeline graph of keywords[17].

Citation analysis is a statistical method of illustrating the quantity of cited studies to identify patterns and measurement characteristics. Generally, the more often a study is cited, the greater its impact. Co-citation is when 2 studies are cited by ≥ 1 study at the same time, which indicates how closely the 2 studies are related[18,19]. Moreover, the greater the number of studies cited simultaneously, the closer the relationship between the two. Centrality is an indicator of the importance of a node in a network, and documents with high centrality are often the key hubs connecting two different domains. Evolution of a field of knowledge can be indicated by references with citation bursts. A burst is a keyword/citation reference that has received scholarly attention in a particular field. The timeline is presented as a blue bar, while the interval, when a topic is found to have a burst, is presented as a red section indicating the year it started, the year it ended, and the time of the burst. Higher intensity indicates a higher citation frequency.

Each circle on the network map represents an "individual", and the size of the circle represents the active degree and the number of publications of the keyword. The closer the distance between an "individual" and an "individual", the closer the relationship between them. The increased number of connected lines indicates the increased number of co-citations between the "individual"[14].

RESULTS

General information

A total of 656 articles were retrieved in the literature search. The successful culture of gastric-like organs was first reported in 2010, and relevant research developed slowly and steadily in the ensuing years[8]. The number of publications and citations exhibited stable growth from 2010 to 2016 and then a sharp increase after 2017 (Figure 1A). Researchers discovered the great advantage of the organoid model in the past 5 years; therefore, relevant publications and citations have increased rapidly. Most articles have been high quality and were published in *Gastroenterology*, *International Journal of Molecular Science*, *Cancers*, *Cellular and Molecular Gastroenterology and Hepatology*, *Nature Communications*, *Cancer Research*, *Cancer Science*, and *Gut* (Figure 1B). These journals focus mainly on cancer and gastroenterological diseases.

Authors

A total of 474 investigators published research in this area, with 32 publishing > 5 studies. Yana Zavros, Hans Clevers, James M Wells, Sato Toshiro, and Koo Bon-kyoung were the 5 most productive authors. Hans Clevers, Huch Meritxell, Johan H van Es, Marc Van de Wetering, and Sato Toshiro were the 5 most popular scientists. Hans Clevers, Yana Zavros, and Richard Peek demonstrated high centrality (0.04). Most of the productive and influential investigators were from The Netherlands and the United States, mainly from the University Medical Center Utrecht (Utrecht, Netherlands) and the University of Cincinnati (Cincinnati, OH, United States), respectively. Table 1 summarizes the top 15 most cited researchers and their respective countries.

Hans Clevers, Sato Toshiro, Sina Bartfeld, N Barker, Huch Meritxel, and Marc van de Wetering established a close and vital cooperative network. HHN Yang worked closely with T Seldlitz, G Vlachogiannis worked closely with L Broutier, and another network was formed between S Takaishi and A Jemal. The co-authorship network diagram is presented in Figure 2A.

Institutions

A total of 314 institutions published research on this topic, and 54 published > 5 studies. The top 5 most-productive institutions in this context were the University of Cincinnati, Hiroshima University, University of Michigan (Ann Arbor, MI, United States), Vanderbilt University (Nashville, TN, United States), and the University Medical Center Utrecht. The top 5 most cited institutions were the University Medical Center Utrecht, the Netherlands Institute for Developmental Biology Utrecht, University of Cincinnati, University of Hong Kong, and the University of Michigan. The top 15 most-cited institutions, which were mostly from the Netherlands, United States, China, and Japan, are summarized in Table 2. The University of Cincinnati had the highest centrality (0.25), followed by the University Medical Center Utrecht (0.18).

Table 1 The top 15 most cited scholars in gastric organoid field

Rank	Author	Publications	Citations	Country	Centrality
1	Clevers, Hans	30	5144	Netherland	0.08
2	Huch, Meritxell	10	3987	German	0.04
3	Van es, Johan	7	3244	Netherland	0.00
4	Van de Wetering, Marc	6	3175	Netherland	0.00
5	Sato, Toshiro	14	3141	Japan	0.08
6	Stange, Daniel	12	1764	German	0.01
7	Barker, Nick	13	1707	Netherland	0.03
8	Koo, Bon-kyoung	15	1618	Netherland	0.25
9	Bartfeld, Sina	13	1405	German	0.03
10	Zavros, Yana	46	1312	United States	0.04
11	Wells, James	22	1040	United States	0.01
12	Spence, Jason	7	973	United States	0.00
13	Peek, Richard	5	715	United States	0.05
14	Schumacher, Michael	6	657	Japan	0.00
15	Chen, Zheng	12	615	United States	0.03

Table 2 The top 15 most cited institutions in gastric organoid field

Rank	Institution	Country	Publications	Citations	Centrality
1	University Medical Center Utrecht	Netherland	19	5083	0.18
2	Netherlands Institute for Developmental Biology Utrecht	Netherland	8	3064	0.00
3	University of Cincinnati	United States	33	1451	0.25
4	University of Hong Kong	Hongkong, China	6	1148	0.08
5	University of Michigan	United States	22	1104	0.04
6	Cincinnati Children's Hospital Medical Center	United States	16	999	0.07
7	Columbia University	United States	11	986	0.05
8	Vanderbilt University	United States	22	941	0.25
9	Stanford University	United States	11	881	0.02
10	Cambridge University	United Kingdom	7	758	0.08
11	Harvard University	United States	12	731	0.08
12	Shinshu University	Japan	17	625	0.07
13	Keio University	Japan	13	602	0.13
14	Nanjing med University	China	14	600	0.20
15	Miami University	United States	7	556	0.00

The United States had the most extensive cooperative network. The University of Cincinnati, Vanderbilt University, University of Michigan, and Cincinnati Children's Hospital Medicine Center formed the centers of each United States-collaborating institution. Chinese and Japanese scientists contributed the most to research from Asian institutions. Nanjing University (Nanjing, Jiangsu Province, China), Sun Yat-sen University (Guangzhou, Guangdong Province, China), and Fudan University (Shanghai, China) played central roles in the Chinese institutional network of collaborations, radiating out to other institutions. Japanese institutions were dominated, in this context, by Hiroshima University (Hiroshima, Japan), with more collaborations with the University of Cincinnati and other Japanese and Korean institutions.

The University Medical Center Utrecht in the Netherlands has a network of collaborations with European institutions. A network diagram illustrating institutional collaboration is presented in Figure 2B.

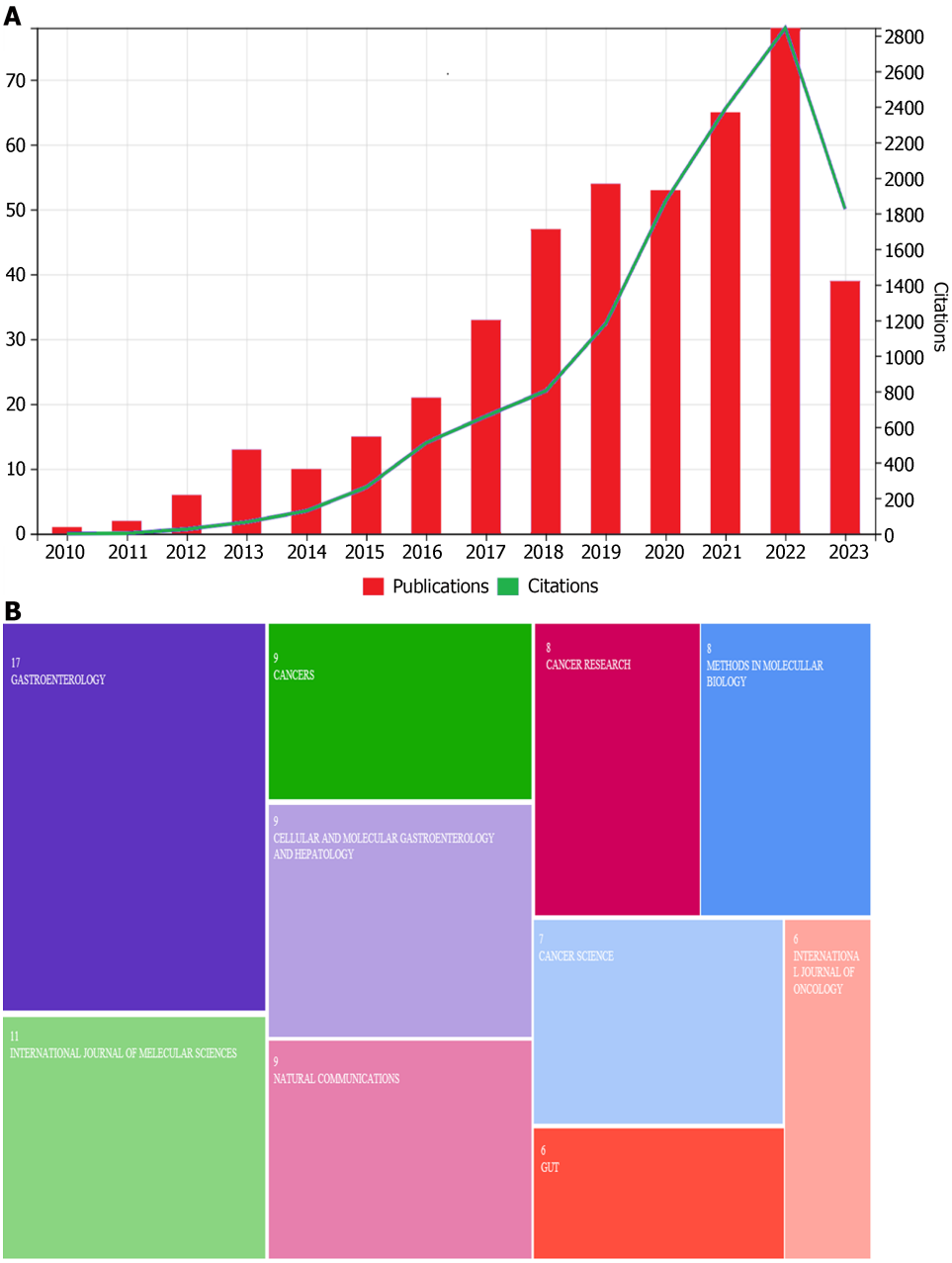

Figure 1 Publication and citation trends in gastric organoid. A: The number of publications and citations per year from 2010 to 2023. The red bars represent the number of publications, and the green line indicates the number of citations, showing an overall upward trend in both metrics over the given period; B: Citations in different medical journals in a recent year, with each color-coded section corresponding to a different journal. The size of each section reflects the number of citations, and the number is provided within each, with *Gastroenterology* and *Cancers* being among the journals with the highest citation counts.

Keywords and hotspots for gastric organoids

VOSviewer was used to construct a keyword network graph. Based on the network analysis the high-frequency keywords of published articles were clustered into 4 categories. The orange area (cluster #1, "Gastric organoid formation") included the main terms related to gastric organoid construction such as stem cells, gastric epithelium, pyloric gland, and fundic gland. The green area (cluster #2, "Gastric cancer organoid formation") explored gastric organoid construction such as tumor stem cells, tumor proliferation and differentiation, and immunohistochemistry. The blue area (cluster #3, "Applications of gastric cancer organoids") occupied a large panel and described the application of organoids in GC therapeutic research, which indicated the study of tumor drug resistance, gene mutations, signaling pathways, and immunohistochemistry. It contains proteins such as WNT, kif11, and pi3k. Another interesting finding in these network diagrams is the red cluster, which is related to the application of organoids in gastritis (cluster #4, "Application of organoid in gastritis research"), including *Helicobacter pylori* (*H. pylori*) infection, inflammatory pathway activation, and cagA proteins (Figure 3). Many early studies investigating organoids focused on the formation and differentiation of GC organoids, whereas more recent studies focused on the treatment and mechanism of gastritis and GC.

After filtering out non-relevant and repetitive keywords, such as "expression" and "identification", the 20 most popular keywords in the field of gastric organoids were shortlisted and are summarized in Table 3. GC and *H. pylori*-

Table 3 The 20 most popular keywords in the field of gastric organoids

Keyword	Freq	Centrality
Gastric cancer	177	0.06
Stem cell	110	0.04
Cancer stem cell	45	0.10
Differentiation	41	0.06
Helicobacter pylori	32	0.01
Metastasis	30	0.19
Proliferation	30	0.04
Resistance	29	0.06
Epithelial cell	27	0.16
Self-renewal	26	0.11
Apoptosis	22	0.07
Progression	22	0.04
Protein	20	0.03
Progenitor cell	18	0.04
Gene expression	18	0.05
Chief cell	17	0.04
Migration	16	0.01
Chemotherapy	16	0.07
Epithelial mesenchymal transition	14	0.02
Mutation	15	0.03

related gastritis were identified as the most prevalent diseases. Stem cells, cancer stem cells, epithelial cells, progenitor cells, and chief cells were identified as the main cellular "hotpot". Cellular events related to cancer development and progression, such as metastasis, proliferation, apoptosis, migration, epithelial-mesenchymal transition, and mutation have received more attention.

In addition, burst keywords were considered to be the indicators of emerging trends. Overall, it can be classified into 3 phases, beginning from 2010 to 2014 with high intensity in drug resistance, cancer stem cell, and self-renewal, followed by that from 2015 to 2020 with epithelium, carcinoma, epithelial-mesenchymal transition, *H. pylori*, inflammation, and regeneration year by year, and the third phase from 2020 until present with mutation, response, tumor microenvironment, and paclitaxel. Figure 4 depicts the keywords with the strongest citation bursts in this field.

The timeline view of the keywords in gastric organoid is presented in Figure 5. There were a total of 17 main research clusters incorporating gastric organoid applications. In addition, the typical labels in each cluster are reported in Table 4.

Co-cited references and references burst

A total of 492 references were co-cited, 11 of which were co-cited > 30 times. Table 5 summarizes the top 10 co-cited studies, and Figure 6 illustrates the main elements of these important studies investigating gastric organoids and the significant findings. McCracken *et al*[20] used human pluripotent stem cells to form gastric organoids by adding essential cellular factors [such as EGF, BMP4, WNT5a, and fibroblast growth factors (*e.g.*, FGF10)]. Bartfeld *et al*[6] used flow cytometry to sort 4 specific cell lines, pit mucous cells, gland mucous cells, chief cells, and enteroendocrine cells, and successfully cultured the corresponding organoid lines. Schlaermann *et al*[7] pioneered a culture protocol for the spheroid formation of the gastric corpus and body, which can be used as an *in vitro* model of *H. pylori* infection. Vlachogiannis *et al*[21] compared the responses of 21 patients with gastrointestinal tumors and patient-derived organoids (PDOs) to targeted drugs or chemotherapy and found that sensitivity and specificity to predict drug treatment for patients were 100% and 93%. Yan *et al*[9] established an organoid biobank and recorded differentially expressed genes between tumor organoids and paired tumor tissues. Seidlitz *et al*[22] and Nanki *et al*[23] demonstrated that human and mouse GC organoid models can mimic the typical human GC characteristics and altered signal pathways, demonstrating their potential role as biomarkers of treatment response.

Burst analysis displayed the minimum duration of the bursts as 1 year, although the longest was 5 years. Of these citations, 28% (7/25) of the bursts occurred before 2015, with 36% (9/25) of literature bursts in 2015 and 36% (9/25) after 2017[24]. Twelve percent (3/25) of the citation outbreaks occurred in the past 2 years. Of the top 25 references, the strongest citation burst (14.3) was for studies by Barker *et al*[25], with the longest citation burst. Barker *et al*[25] reported

Table 4 The typical labels in each cluster

No.	Cluster label	Year	Label
0	Stem cell	2015	Stem cell; cancer; differentiation; model; acid gastric cancer; signet ring carcinoma; matrix metalloproteinase; spdef; transcription factor; stem cell; regeneration; gastric cancer; progenitor; *in vitro* expansion
1	Mouse	2018	Gastric cancer; transcription factor; regeneration; tumor suppressor genes; identification *in vitro*; expansion; liver; leptin deficiency; en-y gastric bypass; mouse; liver; infection; bariatric surgery; gastrointestinal cancer
2	Noncanonical nuclear factor-kappa B	2019	Gastric cancer; her2; capecitabine; open label; oxaliplatin drug screening; cancer organoids; precision medicine; *Helicobacter pylori*; living biobanks; cancer organoids; organoid; drug screenin; tptep1; secretion
3	Gastric cancer	2013	Gene; differentiation; receptor; lgr5; r spondin, gastric cancer; morphogenetic protein; epidermal growth factor receptor; *Helicobacter pylori*; tissue engineering; tumor microenvironment; receptor; gastric intestinal metaplasia; gene; csc markers (6.04, 0.05)
4	Targeted therapy	2016	Gastric cancer; cancer stem cell; endothelial growth; mammary stem cell; gastric cancers, cancer stem cells; translational research; tumor suppressor genes; gastric organoids; cancer microenvironment; abcg2; cancer stem cell; lgr4; acbp-3; cycle arrest
5	Polypeptide expression	2017	Cancer; progression; microenvironment; mmp 9; angiogenesis, gene expression; endoderm; gastrointestinal tract; mouse small intestine; gata4; gene expression; angiogenesis; epstein barr virus; toripalimab; mmp 9
6	Cell	2015	Goblet cell carcinoid; crypt cell adenocarcinoma; appendix; mucinous; neuroendocrine; amphophilic; crypt cell adenocarcinoma; appendix; amphophilic; mucinous; neuroendocrine
7	Expression	2012	Survival; tumor suppressor; her2; poor prognosis; amplification, organoids; stomach; chemotherapy; gastroids; proliferation; amplification; gastroids; myc; inactivation; conditional mouse model
8	Synthetic lethality	2019	Gastric cancer; gastric organoids; gland fission; gastric stem cells; mtor; mtor; gland fission; gastric stem cells; gastric organoids; gastric cancer
9	Autophagy	2014	Gastric cancer; *Helicobacter pylori*; base excision repair; stomach neoplasms; gastric stem cells, nf kappa; trefoil protein; *Helicobacter pylori*; robust model; atrophic gastritis; NF kappa b; *Helicobacter pylori*; inflammation; antral epithelium; dynamic histology
10	Cancer organoids	2020	Gastric cancer; resistance; cancer; cetuximab; heterogeneity, cancer stem cell; lauren classification; mucin phenotype expression; cluster; transcription factor; gastric cancer; cd44; stomach; cancer stem cell; chemoresistance
11	Tumor microenvironment	2017	Gastric cancer; t-cell infiltration; recombinant protein; precision oncology; cancer model, stem cell; stomach; promote; cancer; gene expression; targeted therapy; self-renewa; promote; anti-egfr single domain antibody; cancer evolution
12	abcg 2	2012	Gastric cancer; cancer stem cells; *Helicobacter pylori*; beta catenin; wound repair identification; stem cell; expression; inhibition; stomach; polypeptide expressing metaplasia; contribute; network; immune response; inhibition
13	Gene expression	2013	Gastric cancer; drug sensitivity; shaker; albumin-bound paclitaxel; stomach, breast cancer; model; growth; drug; identification; cell; breast cancer; growth; recombinant protein; irgd
14	Crypt cell adenocarcinoma	2018	Expression; stomach; stem cell; identification; progenitor cell, gastric cancer; stem cells; cancer biology; beta-galactoside alpha; sialic acid; expression; identification; pathogenesis; metaplasia; progenitor cell
15	Amplification	2015	Gastric cancer; cell adhesion; cell matrix interaction; magnetic resonance imaging; hereditary diffuse gastric cancer, synthetic lethality; discoidin domain receptor; diffuse gastric cancer; magnetic resonance imaging; hereditary diffuse gastric cancer; synthetic lethality; hdgc; e cadherin; e-cadherin; chemoprevention
16	mTOR	2015	Gastric cancer; stem-like subtype; adult mouse; cluster; cathepsin c ovarian; shaker; dynamic; fluid; peritoneal; autophagy; hypoxia inducible factor 1 alpha; cancer; metastasis; fibroblasts

that single Lgr5+ stem cells generated gastric-like organs *in vitro* and that the WNT pathway promoted Lgr5+ stem cell transformation, thereby promoting gastric adenoma formation. The second-highest citation burst appeared in a study by Stange *et al*[19], with a burst strength of 11.39 from 2015 to 2017. Stange *et al*[19] reported that Troy+ cells at the gland base can generate all gland cells, which represents remarkable plasticity ability in the field of epithelial stem cell biology. Molecular characterization of gastric adenocarcinoma also demonstrated a citation burst from 2018 to 2019, with a strength of 10.83[26]. In the past 2 years, Steel *et al*[27] reported that patient tissues exhibited a response to combination drug therapy similar to PDOs and PDOs, which resembled the original patient's tissue gene mutation, which made a citation burst. Another review from Drost *et al*[28], which commented on current cancer organoid protocols and the method as a reliable model for cancer research also presented a burst. Figure 7 depicts the citation bursts for the 25 most-cited references.

DISCUSSION

Professor Hans Clevers, a prolific scientist in the field of adult stem cells and organoid technology, identified *Lgr5* as a marker of intestinal stem cells and established the first *in vitro* 3D organoid culture system for intestinal stem cells with his team. This pioneered the area of organoid research as a disease model for diagnostics, basic science, and preclinical

Table 5 The Top10 co-cited literature

Ref.	Title	Journal	Freq	Centrality
Bartfeld *et al*[6], 2015	*In vitro* expansion of human gastric epithelial stem cells and their responses to bacterial infection	*Gastroenterology*	50	0.10
Bray *et al*[24], 2018	Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries	*CA Cancer J Clin*	49	0.00
Yan *et al*[9], 2018	A Comprehensive Human Gastric Cancer Organoid Biobank Captures Tumor Subtype Heterogeneity and Enables Therapeutic Screening	*Cell Stem Cell*	45	0.05
Vlachogiannis *et al*[21], 2018	Patient-derived organoids model treatment response of metastatic gastrointestinal cancers	*Science*	43	0.06
Seidlitz *et al*[22], 2019	Human gastric cancer modelling using organoids	*GUT*	43	0.02
Schlaermann *et al*[7], 2016	A novel human gastric primary cell culture system for modelling *Helicobacter pylori* infection *in vitro*	*GUT*	37	0.09
Mccracken *et al*[20], 2014	Modelling human development and disease in pluripotent stem-cell-derived gastric organoids	*Nature*	35	0.05
Nanki *et al*[23], 2018	Divergent Routes toward Wnt and R-spondin Niche Independency during Human Gastric Carcinogenesis	*Cell*	32	0.07
Cancer Genome Atlas Research Network[26], 2014	Comprehensive molecular characterization of gastric adenocarcinoma	*Nature*	31	0.05
Stange *et al*[19], 2013	Differentiated Troy+ chief cells act as reserve stem cells to generate all lineages of the stomach epithelium	*Cell*	28	0.12

testing. Sato Toshiro exhibited the highest centrality among Asian researchers. He was a postdoctoral researcher in Hans Clevers' laboratory and was involved in the initial establishment of "mini-guts" in culture, after which he returned to Keio University (Tokyo, Japan) to focus on digestive tract organoids. The most predominant researchers were from the Clevers' laboratories, including Sina Bartfeld, Johan H Van es, Marc Van de Wetering, and Huch Meritxell[29-31].

The main institutions were those of the more influential authors, such as the University Medical Center Utrecht, University of Cincinnati, University of Michigan, and Keio University. The first research investigating gastrointestinal organoids establishment from the University of Cincinnati was in collaboration with Keio University, where Toshiro Sato worked[32]. Institutions in Europe, the United States, and Japan were more willing to form collaborative networks. Notably, Chinese institutions formed closer collaborations within the country than with external agencies. Chinese researchers are characterized by a lack of influence, which may be attributed to the lack of external collaboration(s), thus necessitating encouragement to form partnerships with institutions around the world[33,34].

The network diagram of keywords revealed that the study of gastric organoids is currently being applied to 2 diseases: *H. pylori*-infective gastritis and GC. The timeline and burst analysis of keywords reflect past research hotspots and possible future research trends. Based on changes in keywords in this field in this decade, research investigating gastric organoids has gradually shifted from the exploration of model building in the early years to basic research and clinical applications, with the main focus on the mechanism of GC and drug sensitivity testing. In addition, research investigating *H. pylori* has received attention. The gastric organoid is expected to become a stable and powerful preclinical model in the future.

The results of co-cited literature in this decade suggest a shift from the earliest attempts to successfully construct gastric-like organs to a greater focus on the establishment of gastric organ biobanks. Work by Barker *et al*[8,25] was the initiating study in this field, laying the foundation for culturing gastric glands in culture dishes and the culture protocol by Bartfeld *et al*[6], which has become the reference standard for most studies[22,23,35,36]. In clinical applications, isolating GC tissues from patients to establish an organoid sample bank requires high technical expertise[9]. In addition, current technology cannot acquire pure cancer organoids because PDOs are always mixed with healthy tissues[23]. These limitations are offset by many advantages, such as their 3D physiology and the ability to test patient tumor tissues over time to facilitate clinical decision making. Several studies have compared organoid and patient responses to chemotherapeutics and achieved encouraging results[9,22,23,27]. Studies investigating larger-population cohorts are needed to confirm the accuracy of the predictive value of organoids to antitumor drugs. Drug screening for tumor organoids is more likely to be used as a predictive model similar to antibiotic susceptibility testing. Until then, larger samples require diagnostic tests to determine their sensitivity and specificity. To achieve effective clinical translation, clinical research investigating tumor organoids should focus on improving the efficiency and accuracy of drug screening and reappearing the tumor characteristics of patients to the greatest extent. Although these challenges need to be addressed, the overall outlook for predicting clinical outcomes is promising[38,39].

There were some limitations to the present study. Because gastric organoid research is an emerging field, the literature base is still in its infancy; as such, results of bibliometric analyses are inevitably limited. Second, there are currently some frontier technologies in gastric organoids; however, bibliometrics cannot highlight them because they are too innovative and have not been cited enough. These include organoid-on-chips[40] and clustered regularly interspaced palindromic repeats (*i.e.*, "CRISPR")-associated protein 9-mediated base editing organoids. Jeong developed an innovative human

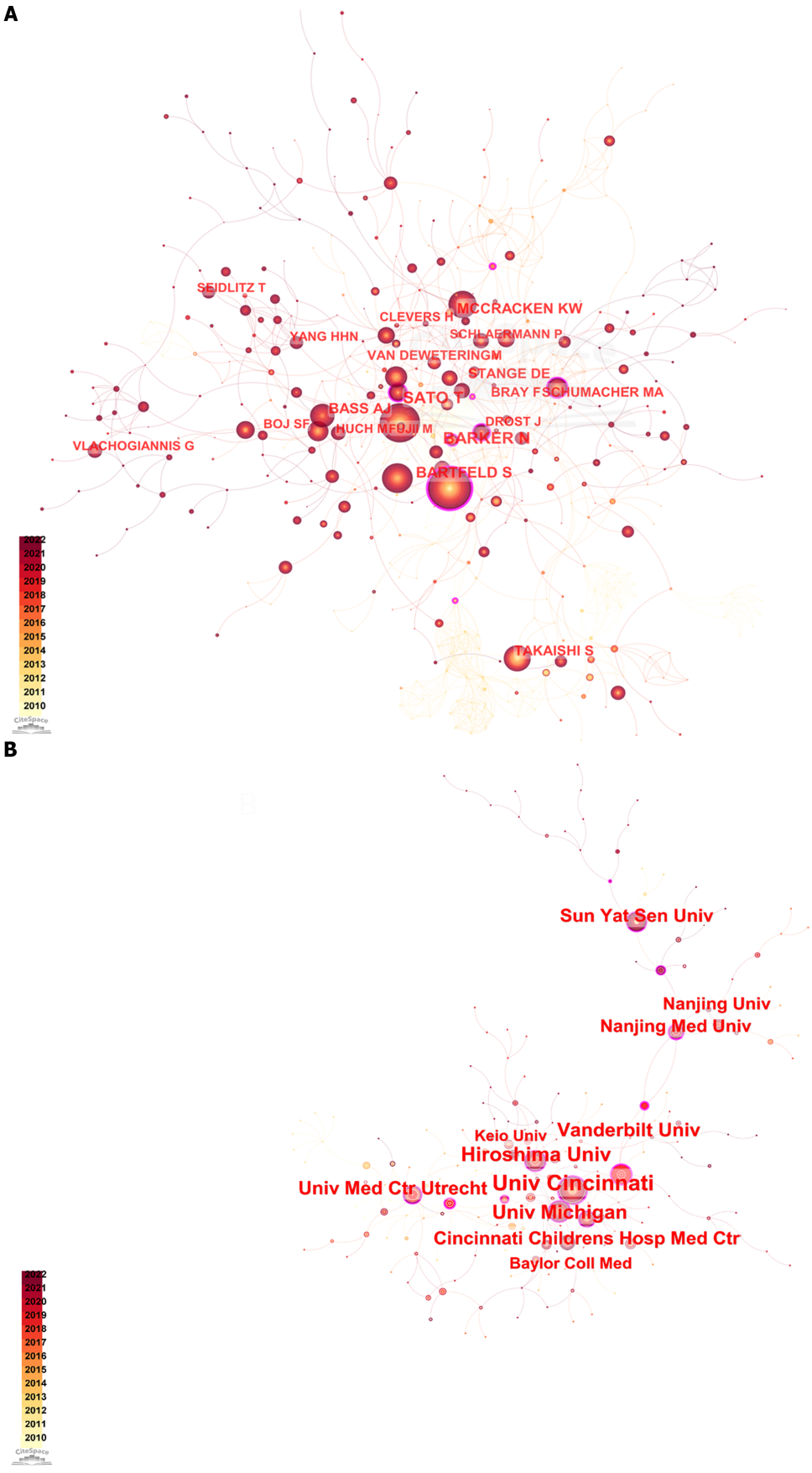

Figure 2 Network analysis of key scholars and institutions in gastric organoid research (2010-2023). A: Depicts the collaboration and citation network among key scholars in the field, where the size of the nodes represents the citation count, and the color gradient indicates the year of publication (from light for 2010 to dark for 2023); B: The network positioning and relationships of major research institutions, with node size and color coding similarly representing the volume of citations and the temporal progression of research.

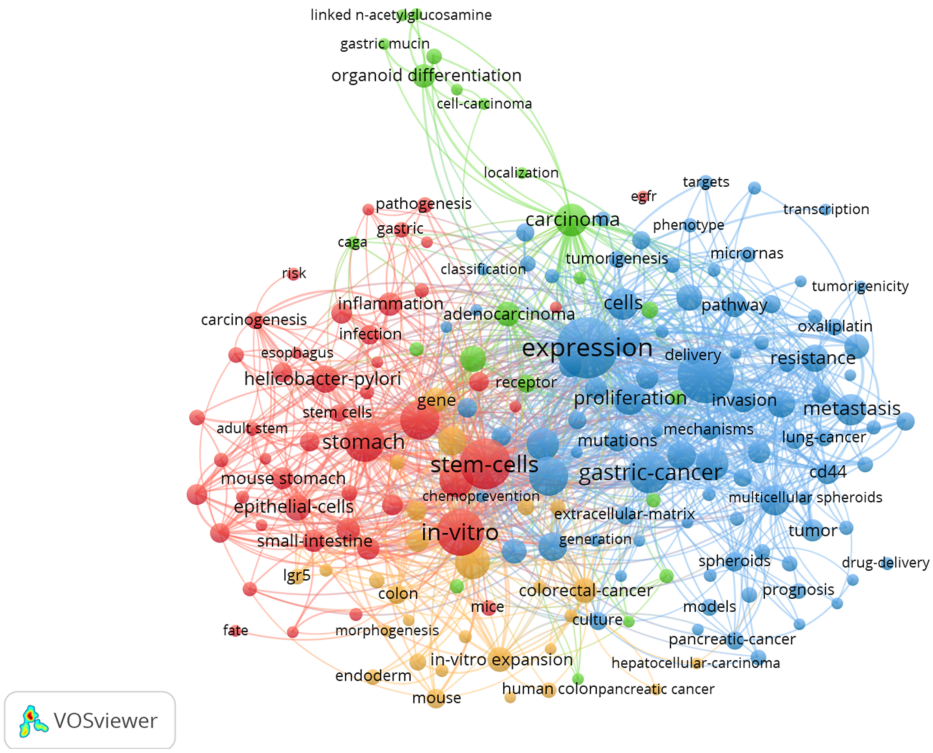

Figure 3 Keyword co-occurrence network in gastric organoid research. This visualization maps the interconnectivity of keywords within the gastric organoid literature, with node size indicating the frequency of keyword occurrence and edge thickness representing the strength of the keyword co-occurrence.

Top 20 keywords with the strongest citation bursts

Keywords	Year	Strength	Begin	End	2010 - 2022
drug resistance	2010	2.29	**2010**	2014	
cancer stem cell	2010	2.63	**2013**	2014	
self renewal	2010	2.47	**2013**	2014	
epithelium	2010	2.69	**2014**	2017	
induction	2010	2.79	**2015**	2018	
carcinoma	2010	2.2	**2016**	2016	
epithelial mesenchymal transition	2010	2.94	**2017**	2019	
invasion	2010	2.81	**2018**	2018	
helicobacter pylori	2010	2.42	**2018**	2020	
survival	2010	2.38	**2018**	2020	
inflammation	2010	2.22	**2018**	2019	
regeneration	2010	2.94	**2019**	2020	
mechanism	2010	2.81	**2019**	2020	
tissue	2010	2.52	**2019**	2020	
protein	2010	3	**2020**	2022	
pathway	2010	2.95	**2020**	2022	
mutation	2010	2.71	**2021**	2022	
response	2010	2.24	**2021**	2022	
tumor microenvironment	2010	2.24	**2021**	2022	
paclitaxel	2010	2.2	**2021**	2022	

Figure 4 Top 20 keywords with the strongest citation bursts. The bar chart highlights keywords that have experienced significant surges in citations over specific time periods, with the length and color of the bars indicating the duration and intensity of the citation bursts, respectively.

stomach microphysiological system-on-a-chip (hsMPS), which exhibits a significantly improved gastric mucosal barrier. This advancement results from integrating MPS technology with organoid structures. In this hsMPS model, epithelial cells obtained from human antral organoids are co-cultivated with primary gastric mesenchymal stromal cells, isolated from gastric tissues, in an environment with regulated fluid dynamics[41]. Chen *et al*[42] utilized CRISPR technology in GCOs to reveal that SOX9-marked gastric stem cells play a pivotal role in the malignant transformation process, with biased symmetric cell division being crucial for their malignancy, particularly in regulating symmetric cell division and influencing the expansion of GC cells. Finally, the latest culture method described by Eicher *et al*[43], who differentiated human pluripotent stem cells into three germ layers, neuroglial, mesenchymal, and epithelial precursors, and subsequently cultured organoids to overcome the limitation of the lack of non-epithelial cells in organoids.

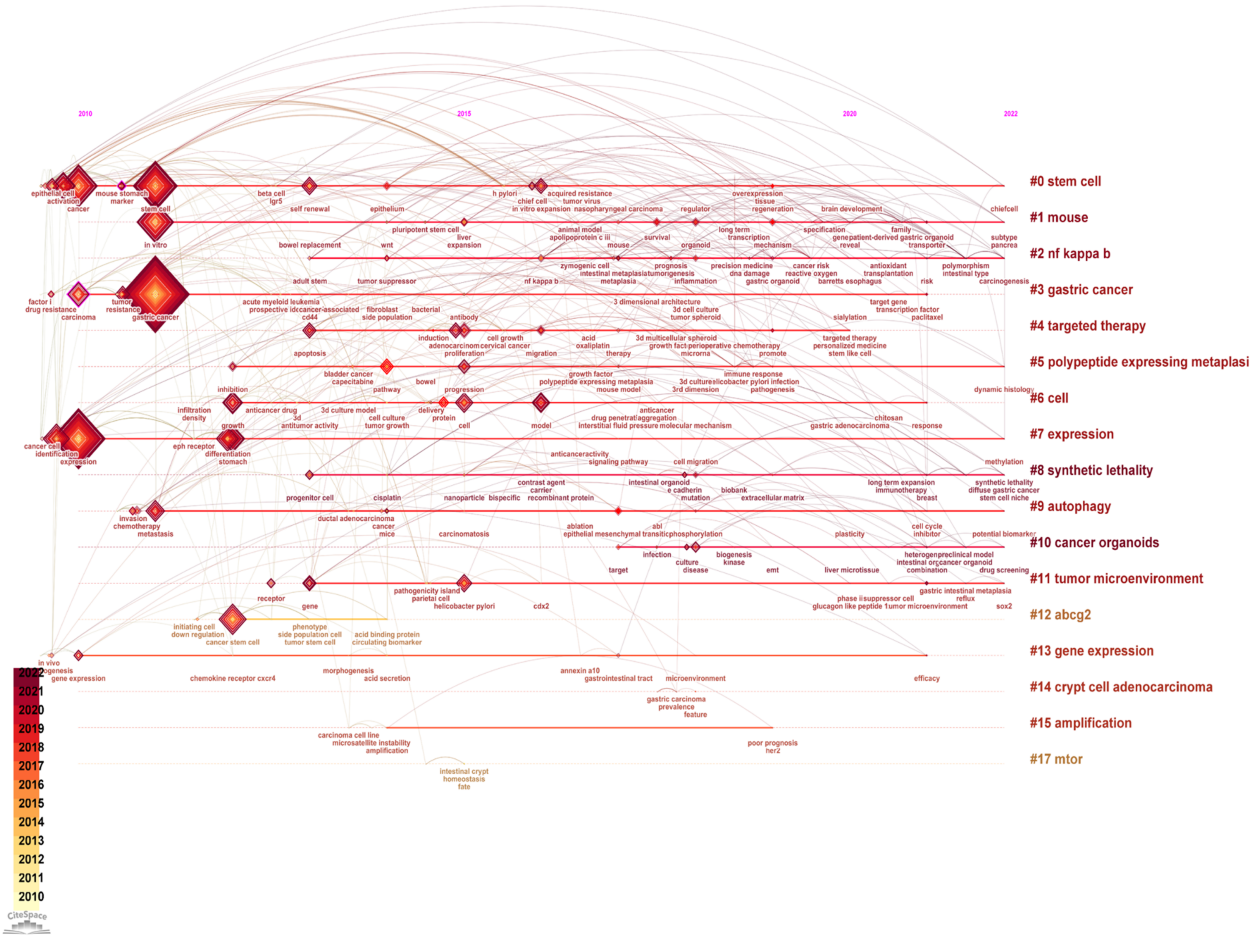

Figure 5 Timeline visualization of thematic evolution in gastric organoid research. This timeline graphically represents the evolution of research themes in the field of gastric organoids, with cluster size indicating the number of publications and connecting lines representing thematic shifts over time.

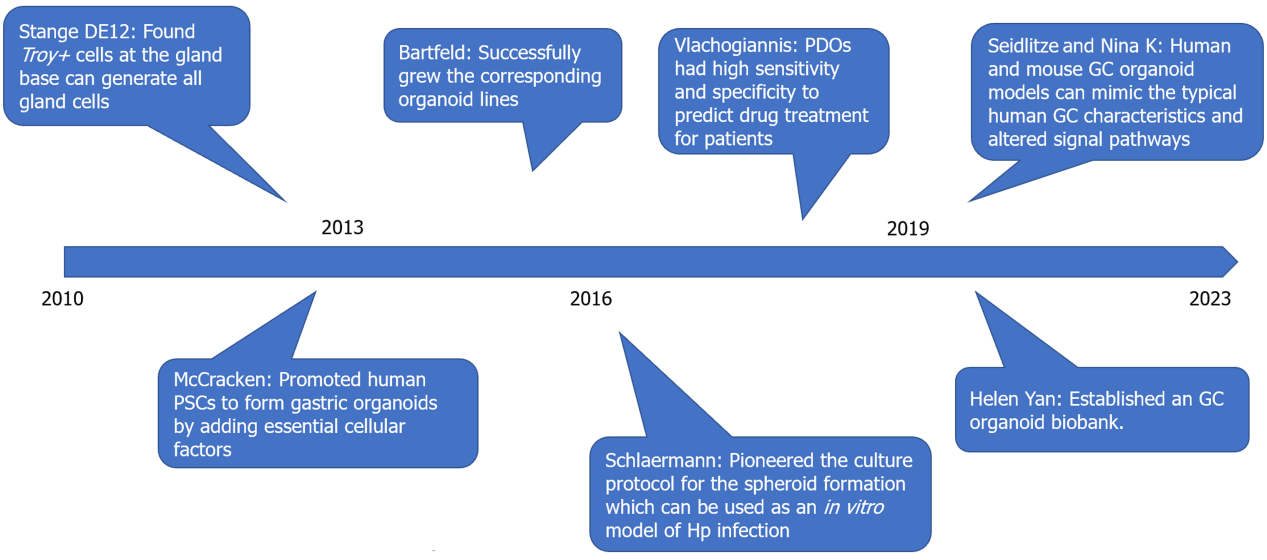

Figure 6 Milestone timeline in gastric organoid research. A chronological display of significant milestones in gastric organoid research, with each entry detailing a breakthrough and its impact on the field. PDO: Patient-derived organoids; GC: Gastric cancer; Hp: *Helicobacter pylori*; PSC: Pluripotent stem cell.

Top 25 references with the strongest citation bursts

References	Year	Strength	Begin	End	2010 - 2022
Barker N, 2010, CELL STEM CELL, V6, P25, DOI 10.1016/j.stem.2009.11.013, DOI	2010	14.3	2010	2015	
Sato T, 2009, NATURE, V459, P262, DOI 10.1038/nature07935, DOI	2009	5.87	2010	2014	
Takaishi S, 2009, STEM CELLS, V27, P1006, DOI 10.1002/stem.30, DOI	2009	7.39	2012	2014	
Sato T, 2011, NATURE, V469, P415, DOI 10.1038/nature09637, DOI	2011	4.88	2012	2016	
Huch M, 2013, NATURE, V494, P247, DOI 10.1038/nature11826, DOI	2013	4.15	2013	2016	
Arnold K, 2011, CELL STEM CELL, V9, P317, DOI 10.1016/j.stem.2011.09.001, DOI	2011	4.02	2013	2016	
Sato T, 2011, GASTROENTEROLOGY, V141, P1762, DOI 10.1053/j.gastro.2011.07.050, DOI	2011	6.47	2014	2016	
Stange DE, 2013, CELL, V155, P357, DOI 10.1016/j.cell.2013.09.008, DOI	2013	11.39	2015	2017	
Mccracken KW, 2014, NATURE, V516, P400, DOI 10.1038/nature13863, DOI	2014	9.25	2015	2019	
Schwank G, 2013, CELL STEM CELL, V13, P653, DOI 10.1016/j.stem.2013.11.002, DOI	2013	6.22	2015	2017	
Khurana SS, 2013, J BIOL CHEM, V288, P16085, DOI 10.1074/jbc.M112.445551, DOI	2013	4.78	2015	2017	
Wang K, 2014, NAT GENET, V46, P573, DOI 10.1038/ng.2983, DOI	2014	4.72	2015	2019	
Li XN, 2014, NAT MED, V20, P769, DOI 10.1038/nm.3585, DOI	2014	4.3	2015	2017	
Yui SR, 2012, NAT MED, V18, P618, DOI 10.1038/nm.2695, DOI	2012	4.3	2015	2017	
Mahe MAXIMEM, 2013, CURR PROTOC MOUSE BIOL, V3, P217, DOI 10.1002/9780470942390.mo130179, DOI	2013	4.01	2015	2018	
Koo BK, 2012, NAT METHODS, V9, P81	2012	3.98	2015	2016	
Bartfeld S, 2015, GASTROENTEROLOGY, V148, P126, DOI 10.1053/j.gastro.2014.09.042, DOI	2015	6.71	2017	2020	
Van DEWETERINGM, 2015, CELL, V161, P933, DOI 10.1016/j.cell.2015.03.053, DOI	2015	5.86	2017	2020	
Bertaux-skeirik N, 2015, PLOS PATHOG, V11, P0, DOI 10.1371/journal.ppat.1004663, DOI	2015	4.03	2017	2020	
Bass AJ, 2014, NATURE, V513, P202, DOI 10.1038/nature13480, DOI	2014	10.83	2018	2019	
Torre LA, 2015, CA-CANCER J CLIN, V65, P87, DOI 10.3322/caac.21262, DOI	2015	5.29	2018	2019	
Ferlay J, 2015, INT J CANCER, V136, P0, DOI 10.1002/ijc.29210, DOI	2015	4.2	2018	2020	
Bray F, 2018, CA-CANCER J CLIN, V68, P394, DOI 10.3322/caac.21492, DOI	2018	9.44	2020	2022	
Steele NG, 2019, CELL MOL GASTROENTER, V7, P161, DOI 10.1016/j.jcmgh.2018.09.008, DOI	2019	6.23	2020	2022	
Drost J, 2018, NAT REV CANCER, V18, P407, DOI 10.1038/s41568-018-0007-6, DOI	2018	4.78	2020	2022	

Figure 7 Top 25 references with the strongest citation bursts. This list showcases the most influential publications in gastric organoid research, with the strength of the citation bursts providing a quantitative measure of their impact over the years indicated.

CONCLUSION

Our study revealed a research area in continuous growth, highlighting a clear trend of increasing focus on GC and *H. pylori*-infective gastritis. The analysis demonstrated that gastric organoids, as a research platform, has made significant strides in decoding disease mechanisms, optimizing drug screening processes, and developing new therapeutic methods. By evaluating the increase in related publications, the rise in citation rates, and the shifts in keywords, we confirmed that gastric organoid research is an active and evolving branch within the field of gastroenterology. Significant academic contributions have come from top research institutions worldwide, particularly those in the Netherlands and the United States, which have led the way in advancing basic research and clinical applications. Additionally, the influence of individual researchers, such as Hans Clevers and Sato Toshiro, has highlighted the importance of personal contributions in driving scientific progress. Gastric organoid research has demonstrated its importance on multiple levels within gastroenterology and the broader field of biomedical science. It not only offers a platform that more accurately simulates the human physiological state but also opens new avenues for personalized medicine in the future. With technological advancements and methodological improvements, gastric organoid research is expected to continue as a focal point of biomedical innovation, particularly in understanding complex disease mechanisms and developing new treatment strategies.

ARTICLE HIGHLIGHTS

Research background

This study conducts a comprehensive bibliometric analysis of gastric organoid research from 2010 to 2023, shedding light on its evolution and emerging trends.

Research motivation

To systematically map the progress and key developments in gastric organoid research, the study highlights the field's significance.

Research objectives

To analyze and understand the development, impact, and direction of gastric organoid research using bibliometric methods.

Research methods

Employed a combination of bibliometric tools and analytical techniques to assess publications and trends in gastric organoid research.

Research results

Identified key contributors and institutions in the field, highlighted the application of gastric organoids in studying

gastric cancer and *Helicobacter pylori*-related gastritis.

Research conclusions

The study underscores the critical role of gastric organoids in advancing our understanding of disease mechanisms and drug screening processes.

Research perspectives

Future research should further explore the potential of gastric organoids in personalized medicine and enhance our comprehension of gastric diseases.

FOOTNOTES

Co-first authors: Kai-Lin Jiang and Yue-Bo Jia.

Co-corresponding authors: Bei-Bei Liang and Jiang-Hong Ling.

Author contributions: Jiang KL and Jia YB contributed to study conception and design; Liu XJ and Yang KM contributed to data collection; Jiang KL contributed to draft manuscript and data analysis; Jia QL and Guo LK contributed to figure revision; Wang XX contributed to make significant revisions to the thesis; Wu CH and Ling JH contributed to fund; Liang BB and Ling JH contributed to approval of the final version and quality of the paper for publication.

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CASE REPORT

Cronkhite-Canada syndrome with esophagus involvement and six-year follow-up: A case report

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Abstract

BACKGROUND

Cronkhite-Canada syndrome (CCS) is a rare, noninherited disease characterized by gastrointestinal polyposis with diarrhea and ectodermal abnormalities. CCS polyps are distributed through the whole digestive tract, and they are common in the stomach and colon but very uncommon in the esophagus.

CASE SUMMARY

Here, we present a case of a 63-year-old man with skin hyperpigmentation accompanied by diarrhea, alopecia, and loss of his fingernails. Laboratory data indicated anemia, hypoalbuminemia, hypocalcemia, hypokalemia, and positive fecal occult blood. Endoscopy showed numerous polyps scattered throughout the digestive tract, including the esophagus. He was treated with nutritional support and glucocorticoids with remission of his symptoms.

CONCLUSION

Comprehensive treatment led by hormonal therapy can result in partial or full remission of clinical symptoms. Treatment should be individualized for each patient according to their therapy response. Surveillance endoscopy is necessary for assessing mucosal disease activity and detecting malignant transformation.

Key Words: Cronkhite-Canada syndrome; Gastrointestinal polyposis; Hormonal therapy; Prognosis; Case report

Core Tip: Cronkhite-Canada syndrome is a rare, noninherited disease characterized by gastrointestinal polyposis with diarrhea and ectodermal abnormalities. We report a case of a 63-year-old man with numerous polyps scattered throughout the digestive tract, including the esophagus. Comprehensive corticosteroid treatment can result in partial or full remission of clinical symptoms.

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INTRODUCTION

First reported in 1955, Cronkhite-Canada syndrome (CCS), also known as polyposis pigmentation-alopecia-onycholrophia syndrome, is characterized by gastrointestinal polyposis with diarrhea and ectodermal abnormalities[1]. It is a rare, noninherited disease with an incidence of 1 in a million[2]. More than 500 cases have been reported worldwide, with most of the cases being reported in Asian countries[3]. Although the incidence of CCS is low, it is associated with high mortality, mainly because of the difficulty in diagnosis and delay in treatment[4]. The diagnosis of CCS is based on history, physical examination, endoscopic findings of gastrointestinal polyposis, and histopathology. Studies have suggested that corticosteroids and immunomodulators are the most effective treatments thus far[5,6]. Long-term surveillance is needed because of the relatively poor prognosis and the increased risk of intestinal malignancy[7].

CASE PRESENTATION

Chief complaints

A 63-year-old male was admitted to our hospital with a two-month history of skin hyperpigmentation and one month of diarrhea.

History of present illness

The patient complained of skin hyperpigmentation for two months and loose stool two to four times per day for one month. There was no associated blood in the stool, abdominal distension, nausea, vomiting, appetite loss, or weight loss. The abnormal skin pigmentation was unevenly distributed throughout the body, with the face and both sides of the hands mainly affected.

History of past illness

Segmental resection of the small intestine was performed twenty-three years prior because of severe abdominal trauma. He was diagnosed with syphilis two years previously and treated with benzathine penicillin. Other medical records revealed bradycardia and varicose veins in the lower limbs.

Personal and family history

The patient has an 80-pack-year history and a 40-year history of drinking approximately 100 g of wine per day. There was no family history of gastrointestinal polyposis.

Physical examination

On physical examination, he had alopecia, nail dystrophy (Figure 1), and hyperpigmentation of the skin, especially on his face and bilateral upper extremities (Figure 2).

Laboratory examinations

Laboratory tests revealed normal routine blood results (Table 1). The fecal occult blood test was positive (+), and C-reactive protein was elevated. Serum albumin and serum total protein were decreased. The levels of serum potassium and calcium were also reduced. Bacterial culture of the stool found no salmonella, shigella, or fungus. Cortisol secretion rhythm, erythrocyte sedimentation rate, immunoglobulin, liver function tests, and other blood chemistry panels were normal. Electromyography suggested peripheral nerve damage (sensory fibers) in the upper and lower extremities.

Imaging examinations

A computer tomography scan of the abdomen showed a partially thickened gastric wall, enterostasis, and slightly enlarged inguinal lymph nodes.

The results of gastroscopy showed multiple polyp hyperplasia of the gastric body, gastric angle, gastric antrum, and descending part of the duodenum (Figure 3B and C). Granular apophyses below the dentate line were observed by the

Table 1 Laboratory findings

Parameters	Findings	Normal range	Remarks
White blood cell count (/L)	8.47×10^9	$(3.5\text{-}9.5) \times 10^9$	Normal
Hemoglobin (g/L)	132	130-175	Normal
Platelet count (/L)	271×10^9	$(125\text{-}350) \times 10^9$	Normal
C-reactive protein (mg/L)	51.5	0-8	High
Fecal occult blood test	Positive	Negative	Positive
Serum albumin (g/L)	36.2	40-55	Low
Serum total protein (g/L)	63.3	65-85	Low
Serum potassium (mmol/L)	3.1	3.5-5.3	Low
Serum calcium (mmol/L)	1.86	2.11-2.52	Low

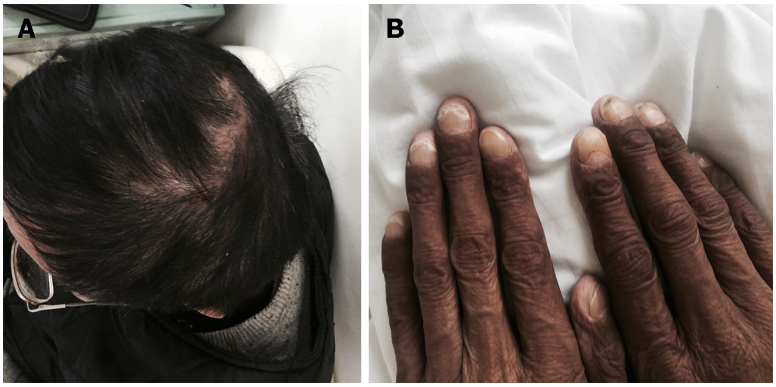

Figure 1 Clinical presence of ectodermal abnormalities. A: Alopecia; B: Nail dystrophy.

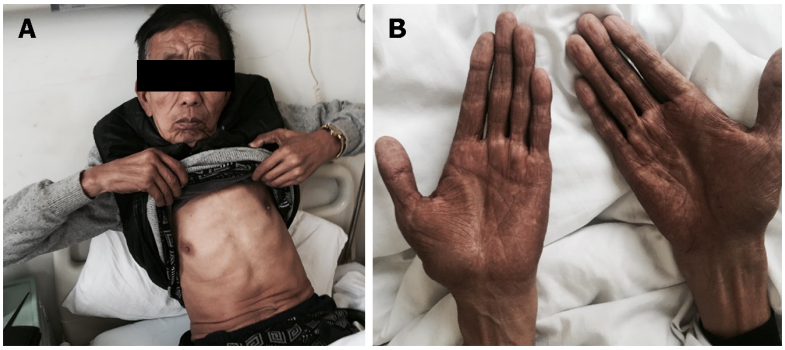

Figure 2 Hyperpigmentation of the skin. A and B: It was most evident on his face (A) and bilateral upper extremities (B).

endoscopist, although they are not as evident in Figure 3A. The results of the colonoscopy showed that mucosa erosion and multiple apophyses with the appearance of polypuses were observed in the ileocecal valve, and numerous polyps were found in the whole colon, among which the largest polyp (4.0 cm) was found 20 cm from the anus (Figure 3D-F).

Histological examination of biopsy specimens obtained from the duodenal bulb was consistent with hyperplastic polyps, and biopsy specimens from the gastric antrum mucosa displayed moderate chronic mucosal inflammation, expansion of the glands, and mild intestinal metaplasia of the glandular epithelium (Figure 4A). Biopsy specimens obtained from the colon suggested tubular adenomas and low-grade intraepithelial neoplasia (Figure 4B).

FINAL DIAGNOSIS

Given the diffuse polyps in the gastrointestinal tract and ectodermal abnormalities, along with the age of onset and family history, the final diagnosis of CCS was made.

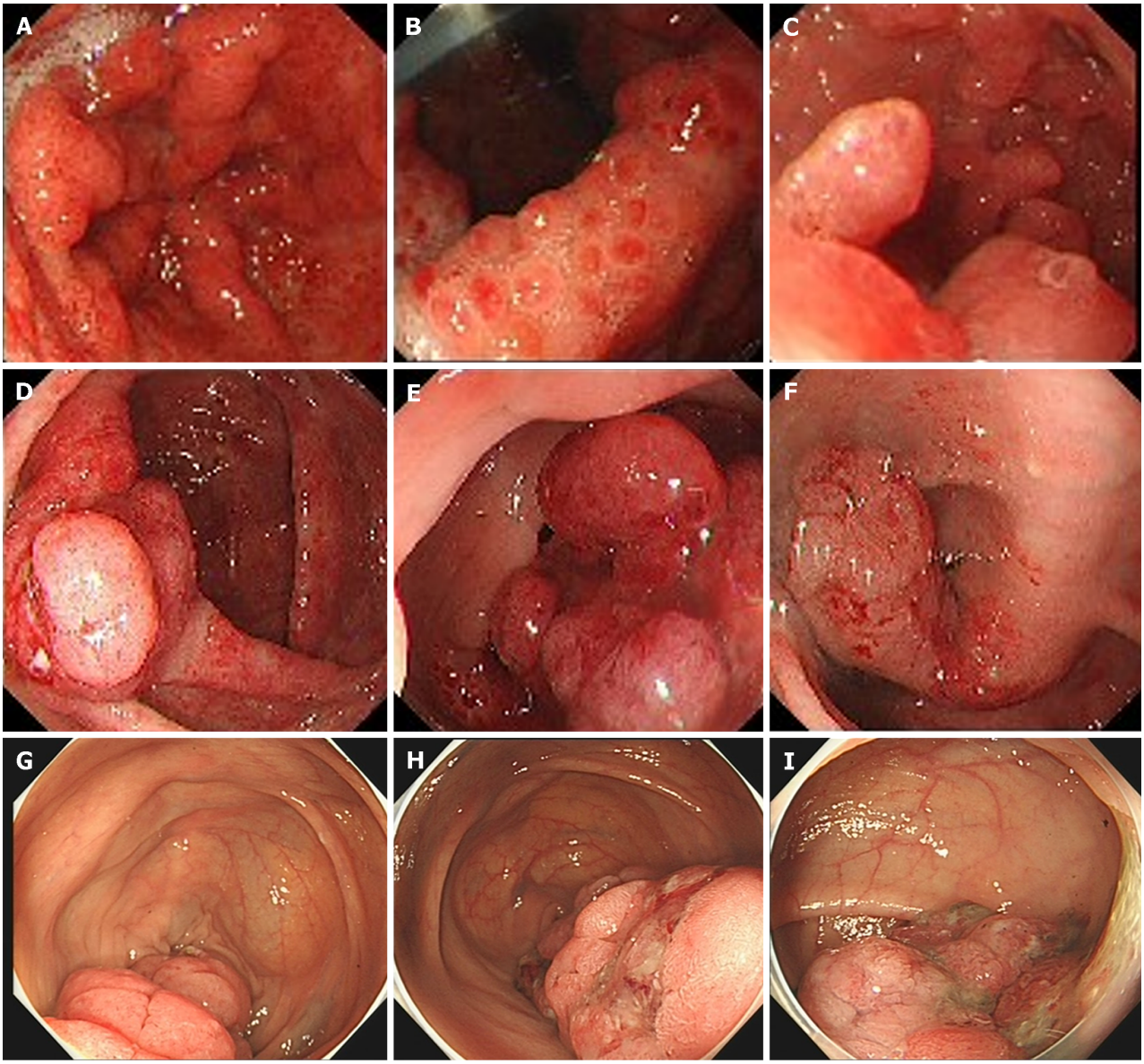

Figure 3 Endoscopy results. A: Granular apophyses below the dentate line were observed by the endoscopist, although they were not so obvious; B and C: Gastroscopy showed multiple polyps hyperplasia of the stomach and the duodenum; D-F: Colonoscopy showed that mucosa erosion and multiple apophyses with the appearance of polypuses were observed in the ileocecal valve, and numerous polyps were found in the whole colon; G-I: The endoscopic findings showed no improvement at 6-year follow-up.

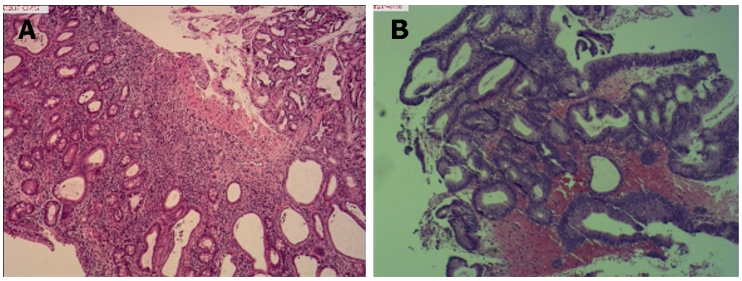

Figure 4 Histological examination of biopsy specimens obtained from duodenal bulb and the colon. A: Duodenal bulb; B: The colon. Haematoxylin and eosin staining (× 10).

TREATMENT

The patient was treated with corticosteroids (oral administration of prednisone initiated at 40 mg/d), nutritional support including vitamins and compound amino acid injection 18AA-II, proton pump inhibitors, gastric mucosal protective agents, antidiarrheal drugs, and *Bacillus licheniformis* capsule to modulate intestinal flora.

OUTCOME AND FOLLOW-UP

The patient was followed up irregularly for approximately six years and had adhered to the advice provided by the chief physician. The symptoms of diarrhea, alopecia, nail dystrophy, and skin hyperpigmentation were relieved within one month (Figure 5). However, the endoscopic findings showed no improvement (Figure 3G-I). The fecal occult blood test remained positive, and his hemoglobin gradually dropped to 91 g/L (normal range, 130-175 g/L) in the following years. Due to the side effects of corticosteroids, he unfortunately developed osteonecrosis of the femoral head, and it was suggested that he undergo total hip arthroplasty. However, the patient refused the surgery, considering the operation risk and his health condition. In total, the patient developed an ileus twice: One was a colonic obstruction with a right inguinal hernia, and the other was a small bowel obstruction with a right inguinal hernia. Conservative therapy was applied, and further examinations, including colonoscopy, were advised to investigate any malignant transformation of the polyps. Nevertheless, the patient refused because he could not tolerate the endoscopy. The most severe complication was an infection of the cervical, oral, and maxillofacial spaces, and he was admitted to the intensive care unit. The patient recovered after catheter drainage of the abscess and other anti-inflammatory treatment. Since diagnosis, the dose of prednisone has been decreased gradually. It has remained at the minimum effective level (10 mg per day) to control the symptoms of diarrhea, alopecia, nail dystrophy, and hyperpigmentation. Although his anemia and malnutrition had not improved, he gained weight and survived a COVID-19 infection.

DISCUSSION

CCS is a rare nonfamilial polyposis syndrome characterized by epithelial disorders in both the gastrointestinal tract and epidermis[8]. The etiology and pathogenesis are still unclear at present[9]. There is no consensus on the treatment of CCS [10]. In this study, we report a case with esophageal involvement and long-term outcomes to better understand CCS in Chinese patients.

There are no definitive criteria for diagnosing CCS, which usually depends on medical history, physical examination, endoscopic examination, and pathological results[11]. For our patient, who first went to the department of endocrinology, the differential diagnosis for the hyperpigmentation included primary adrenal insufficiency, POEMS syndrome, Peutz-Jegher syndrome (PJS), hemochromatosis and some connective tissue diseases. The hyperpigmentation of primary adrenal insufficiency is usually distributed throughout the body but can also be localized. In general, it is more obvious in exposed parts and portions of the skin that are easy to rub (such as the face, hand, palmprint, areola, and so on). The pigmentation of the tongue surface, buccal mucosa, lips, oral mucosa, gums, and cicatrix was also deepened but not distinct from normal skin. However, only a few studies described the hyperpigmentation in CCS, mainly as brownish changes with a clear boundary. Colored spots sometimes occur on the face, body, limbs, palms, soles of the foot, and oral mucosa[12].

When there are multiple polyps in the gastrointestinal tract, CCS usually needs to be differentiated from PJS, juvenile polyposis syndrome, familial adenomatous polyposis, Turcot syndrome, Cowden syndrome, and other diseases. PJS is characterized by the association of gastrointestinal (GI) polyposis, mucocutaneous pigmentation, and cancer predisposition[13]. PJS-type hamartomatous polyps are most common in the small intestine but can also occur in the stomach, large bowel, and extraintestinal sites[14]. Mucocutaneous hyperpigmentation presents in childhood as dark blue to dark brown macules around the mouth, eyes, and nostrils, in the perianal area, and on the buccal mucosa. Hyperpigmented macules on the fingers are usually common[15].

CCS polyps are distributed throughout the whole digestive tract, and they are common in the stomach and colon, less common in the small intestine and rectum, and uncommon in the esophagus[16]. In a Japanese nationwide survey, the esophagus was involved in 12.3% of cases, in contrast to prior reports[17]. However, in a retrospective study of 103 Chinese cases, the ratio was 4/103, much lower than the Japanese data[18]. Multiple changes, including but not limited to polyps, may occur in the esophagus, which might not be focused on except when there are polyps. As in our case, the endoscopist observed granular apophyses below the dentate line.

There is no consensus on the treatment of CCS, and it is still in the exploratory stage. Current medical therapies include corticosteroids, nonsteroidal anti-inflammatory drugs, immunomodulators, proton pump inhibitors, H2-receptor antagonists, antibiotics, surgery, 5-aminosalicylate acid, antitumor necrosis factor α agents, eradication of *Helicobacter pylori*, nutritional support or a combination of these therapies[19]. Steroid therapy is the mainstay of medical treatment. A retrospective analysis confirmed that oral corticosteroid therapy (30-49 mg/d) appeared to be effective for active CCS, and in most circumstances, a slow reduction in dosage was suggested[17]. However, the long-term efficacy and side effects of low-dose corticosteroids still require observation. In both the Japanese nationwide survey and the largest single-center cohort of Chinese patients, nearly 40% of patients failed to achieve long-term clinical remission after corticosteroid administration, and relapse occurred during or after the cessation of corticosteroid use[17,18]. A proportion of patients were prescribed low-dose (5-10 mg/d) corticosteroids or immunosuppressants to counteract the tendency to relapse[19].

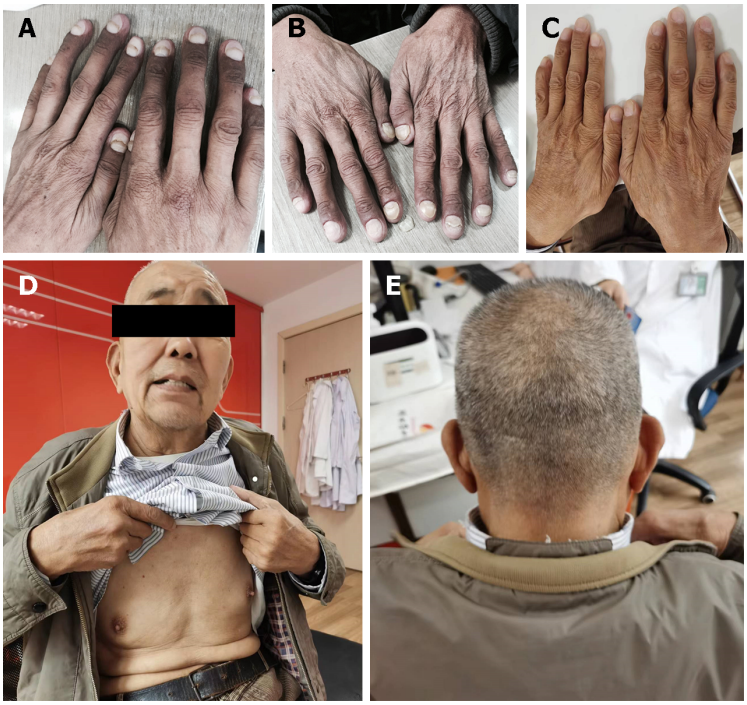

Figure 5 Improvement of the clinical symptoms. A-E: Hyperpigmentation of the skin, nail dystrophy, and alopecia were relieved at one-month (A), five months (B), and four years (C-E) follow-up.

In addition, the symptoms and endoscopic findings of CCS may have different hormonal responses[11]. For our patient, whose symptoms were relieved before the improvement in polyps after treatment, long-term endoscopy surveillance is needed to check the sensitivity to corticosteroids and to detect mucosal disease activity and any malignant transformation of polyps. Adverse events secondary to corticosteroid treatment were frequently reported in the medical records. Even though we tried to reduce the dosage from 10 mg/d to 5 mg/d and withdraw corticosteroids, the GI symptoms, hyperpigmentation, alopecia, and nail dystrophy relapsed. Despite the adverse effects of corticosteroids, they are still the most important therapy with the most clinical evidence and efficacy. The widespread use of corticosteroids may have contributed to the decrease in 5-year mortality from 55% to 16% and the general improvement of outcomes[4,18].

CONCLUSION

CCS is a rare disease with major clinical features of gastrointestinal polyps, diarrhea, skin hyperpigmentation, alopecia, and nail atrophy. Comprehensive treatment led by corticosteroid therapy can result in partial or full remission of clinical symptoms. Treatment should be individualized for each patient according to their responses to the therapy. Surveillance endoscopy is necessary for assessing mucosal disease activity and detecting malignant transformation.

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FOOTNOTES

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LETTER TO THE EDITOR

Monitoring of hepatocellular carcinoma

Imen Akkari, Hanen Jaziri

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Abstract

Screening for hepatocellular carcinoma in patients at risk is an evidence-based approach; however, adherence to the monitoring protocol recommended by international guidelines is difficult. Hence, there is a need to use the best screening options and refine the selection of patients at risk in the future.

Key Words: Hepatocellular carcinoma; Cirrhosis; Risk factors; Surveillance; Imaging; Diagnosis

Core Tip: Hepatocellular carcinoma is a public health problem, and the majority of cases occur in patients with cirrhosis. The screening method for this disease has been the subject of several studies. This Editorial discusses the study titled "Hepatocellular carcinoma surveillance: An evidence-based approach" that was published in *World Journal of Gastroenterology* in 2019.

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TO THE EDITOR

We read with great interest the article by Harris *et al*[1] on the surveillance of hepatocellular carcinoma (HCC). We agree with authors' insight that surveillance should be

performed in cirrhotic and high-risk hepatitis B patients to allow for earlier diagnosis of HCC; however, some issues need to be mentioned.

First, according to that study, the use of alpha-fetoprotein (AFP) in addition to ultrasound is thought to improve the sensitivity of HCC detection. Del Poggio *et al*[2] also recommend the use of AFP screening, as it guides the choice between the continuation of standard ultrasound or performing second-level imaging. However, this strategy is not recommended by European guidelines. The increased cost of screening is among the arguments against the addition of AFP[3]. On the other hand, it is well known that AFP has variable sensitivity and specificity according to its level, as altered levels can be noted without relationship to HCC and a high value has been reportedly observed in only 10%-20% of HCC cases in the initial stages[3,4]. Harris *et al*[1] cited another limitation of using this biomarker, namely the need to determine many thresholds depending on the sub-population.

Second, the use of other radiologic modalities for primary screening in obese persons and those with non-alcoholic fatty liver disease[1] seems to be very difficult to apply in real life. In addition to the radiation exposure that occurs with repeated computed tomography examinations, adherence to this screening program is also difficult as these imaging techniques are not easily accessible, particularly in developing countries. Contrast-enhanced ultrasound is a good surveillance option for patients at high risk for HCC[5]. Therefore, Harris *et al*[1] recommend the use of alternative imaging if the standard ultrasound is limited.

Third, Harris *et al*[1] reported that screening for HCC is underutilized in some demographics (non-Caucasian race and low socioeconomic status). In another study, < 2% of patients received guideline-concordant biannual HCC surveillance [6]. This deficiency in the screening program was detailed in the study by Del Poggio *et al*[2]. According to the authors, real-life implementation of screening programs is far from optimal; therefore, a new strategy was proposed to improve the detection of HCC by primary care physicians and involve performing surveillance in a subspecialist setting. The aim of this approach is to improve the results of real-life screening and reduce HCC mortality.

Finally, prediction models of patients at high risk for HCC[7] and artificial intelligence are emerging approaches in medicine that will be an important element in the management of liver diseases. These advances can be useful in screening patients at high risk for HCC development[8]. Refining the selection of patients at risk into sub-groups of very high, moderate, or low risk could improve HCC screening. The use of abdominal computed tomography and magnetic resonance imaging or better contrast-enhanced ultrasound, could serve as alternative methods in the high-risk population.

In summary, rigorous biannual monitoring by standard ultrasound of at-risk patients remains the first-line screening method. Artificial intelligence protocols to predict the development of HCC in at-risk patients could contribute to the selection of patients at high risk requiring a particular monitoring protocol.

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