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Research progress of gut microbiota in hepatocellular carcinoma

Keliu Li[1,2] | Jianhua Liu[1,2] | Xiaosong Qin[1,2]

[1]Department of Laboratory Medicine, Shengjing Hospital of China Medical University, Shenyang, China

[2]Liaoning Clinical Research Center for Laboratory Medicine, Shenyang, China

Correspondence
Xiaosong Qin, Department of Laboratory Medicine, Shengjing Hospital of China Medical University, Shenyang, China; Liaoning Clinical Research Center for Laboratory Medicine, No. 36 Sanhao Street, Heping District, Shenyang 110004, China.
Email: qinxs@sj-hospital.org

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Abstract

Background: Hepatocellular carcinoma (HCC) is the sixth most common cancer and the fourth leading cause of cancer-related death in the world. A number of challenges remain for the early detection and effective treatment of HCC. In recent years, microbiota have been proven to be associated with the development of HCC. Many studies have explored the pathogenesis, diagnostic marker, and therapeutic target potential of microbiota in hepatocellular carcinoma. Therefore, we aimed to introduce the research methods and achievements of gut microbiota in hepatocellular carcinoma and discuss the value of gut microbiota in the pathogenesis, diagnosis, and treatment of hepatocellular carcinoma.

Methods: Keywords are used to search relevant articles which were mainly published from 2010 to 2021, and we further selected targeted articles and read the full text.

Results: Gut microbiota involved in promoting the formation and development of hepatocellular carcinoma, and differential gut microbiota and microbial metabolites have the potential to be the biomarkers of hepatocellular carcinoma. Purposefully regulated gut microbiota can improve the prognosis of patients, which is expected to be used in hepatocellular carcinoma.

Conclusion: The study of gut microbiota in hepatocellular carcinoma is definitely worthy of study. In-depth and elaborate research design is crucial for the study of the mechanism of gut microbiota involved in hepatocellular carcinoma, which can provide new directions and targets for the diagnosis, treatment, and prognosis of hepatocellular carcinoma.

KEYWORDS
biomarker, gut–liver axis, hepatocellular carcinoma, metabolomics, microbiome

1 | INTRODUCTION

Primary liver cancer (PLC) includes hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC), and combined hepatocellular cholangiocarcinoma (CHC).[1] Among PLC, HCC accounts for 80%–90% of all cases and is the sixth most common cancer and the fourth leading cause of cancer-related death in the world in 2018.[2] The main causes of HCC include infection by the hepatitis B virus (HBV; 33%), hepatitis C virus (HCV; 21%), alcoholic liver disease (30%), and other pathogenic factors (16%).[3,4] It is estimated that

257 million people worldwide currently suffer from chronic HBV infection and that between 2015 and 2030, 20 million people will have died from HBV-induced acute hepatitis, chronic hepatitis, cirrhosis, and HCC; 5 million will have died from HCC alone.[5] Advanced diagnosis, postoperative vulnerability to recurrence, and limited treatment options are the main causes of poor prognosis for patients with HCC. Enhancing the level of early-stage diagnosis and improving clinical treatment outcomes are critical for ameliorating PLC's currently high mortality.

In recent years, an increasing number of studies have found that gut microbiota and intestinal metabolites are closely associated with a variety of diseases, including inflammatory diseases,[6] metabolic diseases,[7] autoimmune diseases,[8] and tumors.[9] In 1921, B. Hoefert first found that gut microbiota were associated with chronic liver disease.[10] Subsequent studies showed altered gut microbiota composition in patients with chronic liver disease or liver cancer. 16S rDNA sequencing of gut microbiota from patients with chronic hepatitis B and associated cirrhosis and liver cancer revealed significant differences in the microbial community structure from a healthy control group. Briefly, the gut microbiota of the disease group showed a decreased abundance of beneficial bacteria and an increase abundance of harmful bacteria, and there was a decreased abundance of the phylum Firmicutes in patients with chronic hepatitis compared with healthy individuals. In addition, there was an increase abundance of the Bacteroidetes in the disease group[11] a group of gram-negative bacteria that produce lipopolysaccharides (LPS), which can trigger liver inflammation and plays a role in fungal toxin-induced liver inflammation.[12]

Gut microbiota play a variety of roles in the development of liver cancer. Ma et al.[13] found that gut microbiota can use bile acids to control the accumulation of $CXCR6^+$ natural killer T (NKT) cells in the liver, which can inhibit hepatocellular carcinoma growth, by upregulating CXCL16, a chemokine in hepatic sinusoidal cells. In addition, probiotics and prebiotic have shown some potential in the treatment of liver diseases; administration of VSL#3 (a probiotic mixture) significantly improves non-alcoholic fatty liver disease (NAFLD) and reduced body mass index in obese children.[14] Prebiotic can work synergistically with probiotics (the two are known as synbiotic when combined). In one study, synbiotic capsule (seven beneficial bacterial strains and a prebiotic oligofructose) reduced alanine aminotransferase (ALT) levels in NAFLD patients.[15]

Early monitoring of liver cancer uses ultrasonography combined with serological alpha-fetoprotein (AFP) testing, but AFP is also elevated in diseases such as embryogenic tumors and acute and chronic hepatitis; therefore, its use as a diagnostic biomarker can result in a high rate of false negative and false positive.[16] Early-stage hepatocellular carcinoma can be treated with surgical resection or radiofrequency ablation, but the efficacy of this treatment depends on tumor size, number, and location. In addition, most hepatocellular carcinoma develops mainly from liver cirrhosis and thus the patient's physical condition must be evaluated to assess whether the patient is suitable for surgical treatment. In adjuvant treatment of advanced cancer, only sorafenib has been confirmed to improve survival rate.[17]

Therefore, challenges still remain in the early diagnosis and treatment of hepatocellular carcinoma, especially considering that most liver cancer patients are generally diagnosed at an advanced stage. Thus, it is still urgent to find novel diagnostic markers for early diagnosis and more effective therapeutic methods. Therefore, we review the role of gut microbiota in the pathogenesis, diagnosis, and the treatment of hepatocellular carcinoma by introducing the detection methods for gut microbiota-related studies and the main results to underscore the value of gut microbiota research in addressing the above problems.

2 | METHODS

Here are the related retrieval methods: “gut-liver-axis,” “metabolomics,” “microbiota” OR “microbiome” AND “hepatocellular carcinoma” OR “liver cirrhosis”, “16S rRNA”, “metagenomics”, “MS” AND “metabolomics”, “management” OR “treatment” AND “hepatocellular carcinoma”, “FMT” OR “Fecal microbiota transplantation”. Using the above retrieval methods or the combination of the above keywords to retrieve the literatures and to select the articles by the title and abstract, then select the target articles to read the full text.

3 | RESULTS

3.1 | Gut microbiota

A high taxonomic and functional diversity of microorganisms reside in the human intestine, including bacteria, viruses, fungi, and archaea, with a total number of more than 1×10^{14} microorganisms, which is 10 times the number of human cells. All genomes from all gut microorganisms are called the “microbiome,” which contain 150 times more genes than in the human genome.[18,19] The phyla Bacteroidetes and Firmicutes account for the largest proportion (approximately 90%) of gut bacterial with the remaining abundance consisting of Proteobacteria, Actinomycetes, Fusobacteria, Verrucomicrobia, and Cyanobacterium.[20,21] The human gut is colonized by microorganisms at birth, and changes in composition (which leads to changes in function) as the individual grows. The composition and function of intestinal microorganisms in healthy adults are essentially stable.[19] Most studies demonstrated that gut microbiota composition and structure can be influenced by genes,[22] age,[23] diet,[24] drugs, and exercise.[25]

Numerous studies have shown that the structure of gut microbiota in patients with cancer is significantly different from healthy individuals. Compared with healthy individuals, *Peptostreptococcus*, *Porphyromonas*, *Mogibacterium*, *Anaerococcus*, *Slackia*, *Anaerotruncus*, *Collinsella*, *Desulfovibrio*, *Eubacterium*, and *Paraprevotella*. are more abundant in the feces of colorectal cancer (CRC) patients.[26] The beneficial bacteria *Lactobacillus* and *Bifidobacterium* were significantly reduced in the feces of CRC patients, while harmful bacteria including members of the Enterobacteriaceae and *Fusobacterium nucleatum*, which was previously determined as associated with CRC, were

significantly increased.[27] Altered gut microbiota structure was also found in the feces of lung cancer patients (compared with healthy controls); these microbiota communities contain more *Bacteroides*, *Fusobacteria*, *Cyanobacteria*, *Spirochetes*, and *Lentisphaerae*, but significantly fewer *Firmicutes* and *Verrucomicrobia*.[28]

Some researchers compare the fecal microbiota of patients with HCC cirrhosis to those with non-HCC cirrhosis via bacterial culture, they found a significant increase in *Enterobacter* counts in patients with HCC.[29] In the mouse animal model, after disrupting the gut microbiota with penicillin-induced dysbiosis and destroying the mice intestinal mucosal barrier by dextran sulfate, *Escherichia coli* overgrowth occurred in the mouse intestine and the level of LPS and interleukin-6 increased in circulation. These conditions are known to be capable of promoting diethylnitrosamine (DEN)-induced tumor formation, while administration of high doses of the probiotic VSL#3 (which contains four *Lactobacillus* spp., three *Bifidobacterium* spp., and *Streptococcus thermophilus subsp Salivarius*) significantly inhibited DEN-induced carcinogenesis.[30] Thus, the inflammatory state induced by the imbalance of gut microbiota plays an important role in tumor formation, and inhibition of tumor formation can be achieved by restoring gut microbiota homeostasis.

3.2 | Gut microbiota metabolomics

The study of metabolomics targets all the small molecule metabolites in cells, tissues, organs, and body fluids at the influence of endogenous or exogenous factors.[31] Metabolomics is another important field in the branch of systems biology and has gradually developed after genomics and proteomics. One of the main pathways in which gut microbiota interact with the host is through metabolites (small molecules that are intermediate or end products of microbial metabolism). Currently, metabolomics can elucidate disease mechanisms, identify new diagnostic or prognostic markers, and enhance our understanding of drug-response phenotypes.[32] Serum, plasma, and urine are the most commonly used sample types in metabolomics studies, but there are relatively few studies on the metabolomics of fecal samples. Most studies usually focus on identifying gut microbiota composition via sequencing, while it should be noticed that fecal metabolites are considered the products of co-metabolism of gut microbiota and host, so they can reflect the functional status of the intestinal flora. That means changes in the composition of gut microbiota can lead to the changes in metabolite composition and thereby affect the host phenotype.[33] In a metabolomics study of the gut microbiota of obese mice with high-fat diet, Yoshimoto et al.[34] found that deoxycholate (DCA) increased, which stimulates hepatic stellate cells (HSCs) and activates their senescence-associated secretory phenotype (SASP). Some senescent HSCs with secretory characteristics can secrete inflammatory cytokines, chemokines, and proteases, which will induce an inflammatory state in liver and ultimately promote HCC formation in mice given carcinogenic drugs.

Yang et al.[35] performed 16S rRNA sequencing and gas chromatography–mass spectrometry (GC–MS) analysis on feces samples from CRC patients and healthy individuals. Analysis of sequencing data revealed 76 operational taxonomic unit (OTU) in CRC and healthy subjects; metabolomic analysis showed a higher abundance of sugars and fatty acids in healthy subjects, and a higher abundance of amino acids, polyamines, drugs, and other metabolites in CRC patients. Through correlation analysis of differential microbiota with differential metabolites, polyamines were identified as potential diagnostic biomarker of CRC and obtained an area under the curve of 0.76. Therefore, the analysis of gut microbiota composition and metabolomics can help to characterize more comprehensively the pathogenic role of gut microbiota in disease and provides theoretical direction for seeking diagnostic markers and therapeutic methods in this field.

Gut microbiota are involved in the metabolism of many substances in the intestine, such as fermenting undigested polysaccharides to produce short-chain fatty acids (SCFAs) that can induce intestinal epithelial cells to produce connexins and mucins to maintain and strengthen the physical barrier function of the intestine.[36] SCFAs can also induce innate lymphocyte cells and $CD4^+$ T cells to produce IL-22 that help to mitigate intestinal inflammatory damage.[37] The gut microbiota can convert primary bile acids synthesized by the liver into secondary bile acids, and an imbalance in bile acid homeostasis due to altered gut microbiota structure will contribute to the development of disease.[38] An imbalance of gut microbiota can result in the release of endotoxins (especially LPS), leading to a low level of inflammation and insulin resistance in the body.[25]

Behary et al.[39] used metagenomics and metabolomics studies to characterize the gut microbiota of patients with NAFLD-associated cirrhosis, with or without HCC. They found that the increased abundance of *Bacteroides caecimuris* and *Veillonella parvula* in NAFLD-HCC patients distinguished them from NAFLD-cirrhosis patients. Five bacteria were enriched in NAFLD-HCC (*B. caecimuris*, *Veillonella parvula*, *Clostridium bolteae*, *Bacteroides xylanisolvens*, and *Ruminococcus gnavus*) and all were found to produce SCFAs. Accordingly, metabolomics data showed that feces from NAFLD-HCC patients were enriched with SCFAs: acetate, butyrate, and formate. In addition, in vitro cell culture with bacterial extracts from both groups revealed that gut microbiota and SCFAs can produce a microenvironment that supports immunosuppression.

Notably, a combination of gut microbiota analysis and metabolomics can also be used in disease diagnosis. Yang et al.[35] performed microbiome and metabolomic analysis of fecal samples from CRC patients and healthy individuals and found that certain gut-associated metabolites, for example, polyamines (cadaverine and putrescine) may possess the potential to diagnose CRC.

3.3 | Study on the mechanism of gut microbiota in liver cancer

3.3.1 | Gut–liver axis

There is an inseparable relationship between the intestine and the liver, through the connection of biliary tract and portal vein, they

interact to form the gut–liver axis (Figure 1). The liver acts on the intestine by secreting substances such as primary bile acids and immunoglobulin A (IgA), while the intestine returns secondary bile acids, food metabolites, and bacterial metabolites to the liver through the portal vein.[40,41] The local microbiome, intestinal epithelial cells, and resident immune cells in the gut interact in complex ways and all participants actively contribute to gastrointestinal homeostasis. In this system, bacteria-derived metabolites act as important signals that continuously promote the normal function of the epithelial barrier and immune cells.[42]

3.3.2 | Microorganism bile acid–liver axis

Metabolites in the intestine include those produced by bacteria processing dietary substances, those produced by the host and biochemically modified by intestine bacteria, and those synthesized de novo by gut microorganisms.[42] Bile acids in the intestine are synthesized and secreted by the liver and then become secondary bile acids after they are metabolized by anaerobic bacteria. Farnesoid X receptor (FXR) is a nuclear hormone receptor and the main receptor for bile acids. Other bile acid receptors include the G-protein-coupled bile acid receptor (TGR5), the pregnane X receptor (PXR), the vitamin D3 receptor (VDR), and the constitutive androstane receptor (CAR)[43,44] FXR is mainly reside in the ileum and liver, and bile acid synthesis and secretion are controlled by negative feedback by the FXR in the ileum and liver. Sayin et al.[45] analyzed the bile acid composition of the entire enterohepatic system of germ-free, conventionally fed mice and found that these mice had smaller intestinal bile acid pools and reduced expression levels for most of the enzymes involved in bile acid synthesis. The authors also demonstrated that intestinal microbiota contributed to bile acid synthesis by reducing the levels of taurine-conjugated muricholic acids (T-MCAs) and promoting the expression of FXR-dependent fibroblast growth factor 15 (FGF15) in the ileum. FGF15 then inhibits the expression of cholesterol 7α-hydroxylase (CYP7A1) in the liver and ultimately reduces bile acid synthesis. Thus, intestinal microbiota not only regulate secondary bile acid production in the gut but also regulate bile acid synthesis in the liver, which then affects the structure of gut microbiota. Wang et al.[46] found significant differences between the gut microbiota in feces from a cholic acid (CA)-fed mouse model for CRC and diet-untreated, control $Apc^{min/+}$ mice. The authors also found that CA feeding accelerated CRC formation.

Approximately 95% of bile acids are reabsorbed in the intestine, primarily through the apical sodium-dependent bile acid transporter (ASBT), in the form of conjugated-bile acids in the distal ileum and recirculated through the portal vein to the liver, where they are then secreted. This process is known as the "enterohepatic cycle" of bile acids and occurs approximately six times a day in humans. Bile acids regulate the composition of the microbiota, which in turn regulate the size and composition of the bile acid pool. Xie et al.[47] observed a variety of bile acids increased in a streptozotocin-high fat-induced non-alcoholic steatohepatitis (NASD)-HCC mouse model at week 12 and 20, including taurocholate (TCA), deoxycholate (DCA), glycocholate (GCA), tauroursodeoxycholate (TDCA), taurolithocholate (TLCA), taurochenodeoxycholate (TUDCA), and taurochenodeoxycholate (TCDCA), the last of which had the most pronounced increase of all the bile acids tested. In addition, the incidence of hepatic malignant lesions and liver size were reduced after the administration of cholestyramine (which increases the excretion of hydrophobic bile acids), and histopathological and blood biochemical parameters showed disease reversal and decreased levels of DCA, TCA, and TCDCA in liver and plasma. In the gut microbiota analysis, Firmicutes and Actinobacteria abundance were elevated and the abundance of Bacteroidetes and *Aspergillus* were significantly lower in the feces of the NASD-HCC model rats compared with those in the control group. The abundance of the genera *Clostridium*, *Bacteroides*, and *Desulfovibrio* were significantly increased. All bacterial taxa listed are involved in the deconjugation of bile acids, dihydroxylation, and the breakdown of bile acids to CO_2 and H_2O. Studies have also shown that DCA-, LCA-, or TCDCA-treated HepG2 cell lines can accelerate the growth rate of normal hepatocytes in a high-sugar, high-fat microenvironment, which may lead to malignant transformation of hepatocytes. Moreover, DCA, LCA, and TCDCA increased the expression of the oncoprotein c-Myc in WRL-68 cells

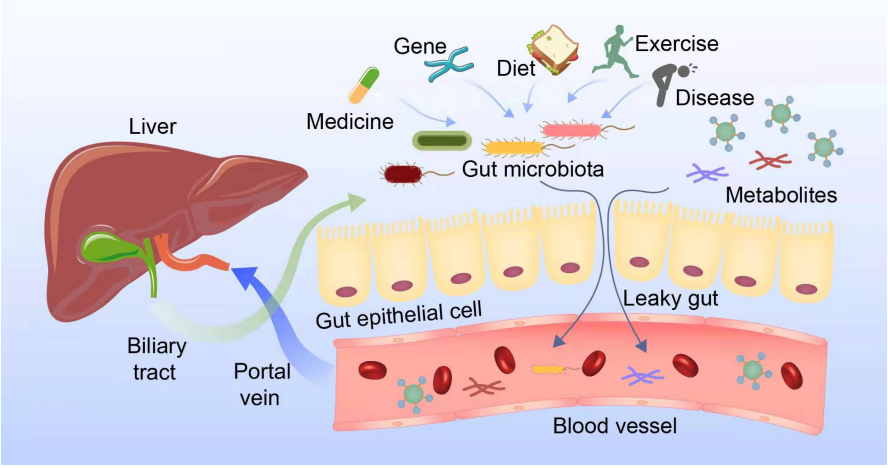

FIGURE 1 Gut-liver axis

and decreased the expression of the tumor suppressor gene CEBPα in HepG2 cells, suggesting that several hydrophobic bile acids may synergistically promote hepatocarcinogenesis. Ma et al.[13] found hepatic NKT cells accumulation with an associated inhibition of tumor growth after the addition of an antibiotic mixture to the drinking water of mice. The authors concluded that the antibiotics removed the bacteria responsible for converting primary bile acids to secondary bile acids; this result was later validated in human cases. To summarize the carcinogenic mechanism between gut microbiota, bile acids, and liver cancer, the gut microbiota uses bile acids as messengers to control the chemokine-dependent accumulation of hepatic NKT cells and antitumor immunity in the liver.

3.3.3 | Disruption of the intestinal barrier

The intestinal mucosa is the physical and chemical barrier that separates the gut microbiota from the liver and systemic circulation. An intact and healthy mucosal barrier prevents translocation of gut microbiota and intestinal pathogens from invading the epithelium and triggering a series of inflammatory reactions. Destruction of this barrier allows translocation of microbiota and invasion of intestinal pathogens into the epithelium triggering an inflammatory response in the body.

The efficacy of this physical barrier is dependent on the intestinal epithelium and its mucosal structure. The intestinal epithelial cells are tightly attached to neighboring cells by apical junctional proteins, including claudins, ocludins, and junctional adhesion molecules (JAM).[48,49] Although the adhesion is tight, the junctional structure does allow for small gaps between epithelial cells; apical junctional proteins are characterized by electric selectivity and allow the passage of small solute molecules which can also regulate intestinal mucosal permeability through a non-selective pathway.[50]

The thickness of the mucus layer varies in different parts of the intestine. Goblet cells, which are responsible for producing mucus, are distributed intermittently across the intestinal monolayer of epithelial cells. Mucus in the small intestine is rarefied and thus porous and allows bacteria to enter, but the immune barrier of the intestinal epithelium disallows commensal bacteria and pathogens from invading the epithelium.[51] In contrast, the colon has two mucus layers, an outer "loose" layer, which is the site of bacterial colonization, and an inner dense layer, where no bacteria are present.[52] Paneth cells at the base of the intestinal crypts are capable of producing antimicrobial peptides (AMPs), including defensins, cathelicidins, and the regenerating gene (Reg) IIIα/β/γ (RegIIIγ). AMP has antimicrobial activity and can act broadly on bacteria to inhibit cell division, interfere with microbial metabolism, and disrupt ATP synthesis.[53] A mucus layer containing AMP covers the tightly connected intestinal epithelial cells, and the two features combined constitute the first barrier to microbial invasion or translocation. Antibody secreting cells in the lamina propria of the gut secrete secretory IgA (sIgA), which becomes concentrated in the outer mucus layer and non-covalently cross-links microorganisms, facilitating their removal. Besides, sIgA helps to prevent microbial adhesins from interacting with the epithelium. sIgA also specifically inhibits pathogens by directly recognizing the receptor binding domain.[54]

Disruption of the intestinal barrier, known as "leaky gut," allows bacteria and bacterial products to cross the intestinal mucus and epithelium, which can then trigger an intestinal immune response. When the liver is exposed to various microbial-associated molecular patterns (MAMPs; i.e., bacteria and bacterial products), pattern recognition receptors (PRRs) will bind to them and induce a sustained inflammatory response and promote liver injury, fibrosis, cirrhosis, and oncogenic transformation[55] The most widely studied MAMP is LPS, which is a cell wall component of gram-negative bacteria that binds to the PRR and ultimately leads to the activation of pro-inflammatory transcription factors.[47] Many studies have suggested that the pro-inflammatory effects of LPS promote liver injury and drive the progression of liver disease. Ponziani et al.[56] found that patients with NAFLD-related cirrhosis and HCC had higher gut-derived inflammation than patients with NAFLD-related cirrhosis without HCC, and that the former had significantly higher calprotectin levels as well as certain inflammatory factors and lacked protective bacteria. Macrophages in the liver, as well as HSCs, can express TLR4 (a type of PRRs), which specifically recognizes LPS and releases the inflammatory mediators IL-1, IL-6, and tumor necrosis factor, which can subsequently lead to liver injury.[45] In a prospective cohort study, Fedirko et al.[57] analyzed serum anti-LPS levels in patients with HCC and confirmed a significant positive correlation between antibody response rate and the risk of hepatocarcinogenesis, thus suggesting that the ectopic distribution of gut microbiota metabolites due to intestinal leakage plays an important role in the development of liver cancer. In a study by Dapito et al.,[58] genetic TLR4 inactivation, intestinal sterilization, or aseptic state reduced the development of HCC by approximately 80%. However, long-term treatment with low-dose LPS significantly promoted the development of HCC, confirming the hypothesis that special intestinal microbiota and TLR promote HCC.

4 | METHODS FOR THE STUDY OF GUT MICROBIOTA AND ITS METABOLOMICS

4.1 | Analytical methods for studying gut microbiota

Basic research on gut microbiota mainly uses sequencing to classify microbiota and to predict or annotate their gene function. Next-generation sequencing (NGS), which has the advantages of large-scale parallel sequencing and high-throughput sequencing to achieve DNA or RNA sequence detection. The second- and third-generation sequencing technologies are both considered NGS. Advantages of NGS over traditional sequencing methods include higher throughput with sample multiplexing and faster turnaround time for large sample volumes, and its cost is lower.[59] With the increasing demand for gene sequencing research, many companies have launched a variety

of sequencers. With technological progress, many sequencing technologies have been eliminated due to high cost, low throughput, and low accuracy. The Illumina system is currently one of the most widely used NGS platforms, and the main sequencing instruments include Miseq, HighSeq, and NovaSeq. MiSeq is one of the smallest benchtop sequencers, which can be used for targeted gene sequencing (amplicon sequencing and targeted enrichment), shotgun metagenomics, and various gene expression analyses and is a cost-effective tool that has become the most widely used NGS platform.[60] Third-generation sequencing technologies include Pacific Biosciences' single-molecule real-time sequencer and Oxford Nanopore Technologies' nanopore sequencer. Although third-generation sequencers produce longer reads compared with second-generation sequencers, they have a higher error rate and they are more expensive, and produce sequencing results that are difficult to compare with sequences in existing databases.[59] However, third-generation sequencing has some unique advantages, such as the ability to detect modified nucleotides and identify modified bases for epigenetic studies. Moreover, the sequencing accuracy can be improved by performing additional sequencing with second-generation sequencers.[61] Thus, which sequencing technology is appropriate for a given study requires considering the study's goals and limitations.

16S rDNA gene sequencing and shotgun metagenomics are two of the most commonly used second-generation sequencing technologies and are both used to analyze the composition and relative abundance of intestinal microorganisms. Unlike traditional methods of gut microbiota identification, which require isolation and culturing, these two methods classify microorganisms as Operational Taxonomic Units (OTUs). 16S rDNA sequencing involves selecting one or several variation regions of 16S ribosomal RNA of prokaryotes and use universal primers to design the conserved regions for PCR amplification, and then conduct sequencing analysis and species identification of the highly variable regions. Metagenomic sequencing aims to sequence all microbial genes in a given sample and compared with 16S rDNA gene sequencing, it has higher resolution and accurate results, which facilitates the discovery of new genetic species and can describe the complexity of gut microbiota in more detail. Besides 16S sequencing will lead to slight bias of binding sites due to different primer binding choices, which will eventually lead to bias of prediction.[62] Metagenomic sequencing yielded far more species than 16S rDNA sequencing. Metagenomic sequencing is more restrictive and conserved in the classification of OTU, and it can be better classified even at lower sequencing depth. Metagenomic sequencing had high resolution at the species level, while 16S rDNA could only identify the genus level.[63]

4.2 | Metabolomics analysis technology

The main techniques currently applicable for metabolomic analysis are liquid chromatography-mass spectrometry (LC–MS), gas chromatography–mass spectrometry (GC–MS), and capillary electrophoresis–mass spectrometry (CE-MS). The biological samples available for study are serum or plasma, cerebrospinal fluid, urine, feces, tissues, and saliva.[64] CE-MS is not commonly used in metabolomics studies due to the low reproducibility of experimental results. GC–MS is suitable for identifying thermally stable and volatile metabolites as well as derivable metabolites. LC–MS is more suitable for unstable and nonvolatile components, and its application to non-targeted metabolite analysis is that it has much higher coverage than GC–MS and is more commonly used in metabolomics studies. Therefore, different detection techniques are applicable to different metabolites.[64,65] Due to the high complexity of metabolites in most samples, a single analytical technique cannot be used to detect all metabolites. Mass spectrometry coupled with gas chromatography or liquid chromatography or combined with capillary electrophoresis can provide higher peak volumes and enable qualitative and quantitative analysis. A study which used a combination of the above-mentioned methods achieved a more comprehensive analysis of sample metabolites.[66]

Metabolomic studies involve both targeted and untargeted methods; targeted metabolomics aims to identify and quantify a limited number (tens to hundreds) of known metabolites, such as those commonly found in clinical analyses or in samples obtained based on the basis of prior studies; untargeted metabolomics focuses on obtaining data on as much metabolites data as possible, annotating metabolites, and exploring the metabolic changes involved in the study.[67] MS analytical platforms include triple quadrupole mass spectrometer, time of flight mass spectrometer (TOF), and ion trap mass spectrometer. Triple quadrupole mass spectrometer is typically used for targeted analysis in which the identities of hundreds of known metabolites are measured; this method is able to identify target metabolites with high sensitivity but possesses poor quantitative accuracy compared with the latter two techniques. In contrast, TOF and ion trap mass spectrometry are typically used for untargeted metabolic analysis and can detect approximately thousands of metabolite peaks, but quantification of all detected metabolites increases the complexity of data analysis and calculation.

5 | PROGRESS IN THE STUDY OF GUT MICROBIOTA AND ITS METABOLISM IN DIAGNOSTIC MARKERS OF HEPATOCELLULAR CARCINOMA

Currently, early detection of liver cancer relies on ultrasound and serum AFP indicators; however, the false-negative and false-positive rate of serological monitoring indicators are high. Due to the nonspecific clinical symptoms of liver cancer and the lack of conventional and effective early monitoring tools, the best treatment period has often passed by the time the cancer is detected, resulting in limited treatment options for patients, poor treatment prognosis, and a high mortality rate. Intestinal gut microbiota diversity and metabolism are involved in the development of hepatocellular carcinoma, and

many studies have explored their potential as diagnostic markers for hepatocellular carcinoma (Table 1).

In a study of gut microbiota in a Chinese population with early hepatocellular carcinoma patients and chronic liver disease at different stages, Ren et al.[68] found increased intestinal microbial diversity, a decreased abundance of butyrate-producing bacteria, and an increased abundance of LPS-producing bacteria in the feces of early HCC patients compared with cirrhotic patients. Based on 30 potential microbial markers of HCC, HCC classifier models were constructed and the diagnostic potential of specific gut microbiota markers for early and even late-stage HCC was validated at verification phase. The potential of microbial markers for the diagnosis of HCC was also confirmed in independent cohorts across different regions. In a follow-up study by the same authors,[69] paired data from the liver transcriptome and intestinal microbiome were obtained and analyzed. The abundance of *Bacteroides*, *Lachnospiracea incertae sedis*, and *Clostridium XIVa* were significantly elevated in the non-small cell liver cancer group and were sufficient to distinguish the small liver cancer group from the non-small liver cancer group. In addition, most of the microbiota involved were able to metabolize cholic acid, chenodesoxycholic acid, 3-dehydrocholic acid, 7-dehydroanthropodeoxycholic acid, and taurocholic acid. Cao et al.[70] found 6 statistically different potential metabolic biomarkers in the fecal metabolomics study of patients with liver cirrhosis and liver cirrhosis-HCC and healthy controls, including chenodeoxycholic acid, urobilin, urobilinogen, keto-lithocholic acid (KLCA), lysophosphatidylcholines (LPC), including lyso-palmitoylphosphatidylcholine (LPC C18:0) and lyso-stearicphosphatidylcholine (LPC C16:0), which can be used to distinguish the disease group from the healthy group. Compared with healthy people, the fecal chenodeoxycholic acid of patients with liver cirrhosis and HCC decreased, and the concentration of lysophosphatidyl choline increased significantly. Multivariate statistical analysis showed that although liver cirrhosis was present in all cases, hepatocellular carcinoma was still the main cause of metabolic changes. Ponziani et al.[56] studied NAFLD-related cirrhosis, NAFLD-related cirrhosis patients with HCC and healthy individuals, they discovered that in patients with liver cirrhosis, *Enterobacteriaceae* and *Streptococcus* were enriched, while *Akkermania* was reduced. In the HCC group, *Bacteroides* and *Rumenococci* increased, and *Bifidobacteria* decreased. Zheng et al.[71] found that *Enterococcus*, *Limnobacter*, and *Phyllobacterium* can be used for accurate diagnosis of HCC in the study of the gut microbiota of patients with hepatitis, cirrhosis, and HCC.

Visconit et al.[72] conducted metagenomic shotgun sequencing of feces of 1004 twins, and conducted metabolomics analysis of feces and blood of corresponding subjects. The result showed that unrelated subjects shared on average almost twice the number of metabolic pathways (82%) as the number of OTUs (43%). Then, after analyzing metabolites in blood (673) and feces (713), it was found that the metabolic pathways utilized by the microbiota were related to 43% of metabolites in the blood and 95% of the metabolites in the feces. This result suggests that the study of feces metabolism cannot be ignored. Acting as biochemical transformers, intestinal microbiota are capable of converting the complex chemical space presented by dietary and host nutrients into an environment of metabolites.[73] Zhang et al.[74] fed C57BL/6 male littermate mice with high fat/high cholesterol (HFHC), high fat/low cholesterol, and normal forage for 14 months. In the process of HFHC feeding, which in mice leads to the development of fatty degeneration, steatohepatitis, fibrosis and finally HCC, *Myxospirillum*, *Desulfovibrio*, *Anaerotruncus*, and *Vibrio desulphuricans* significantly increased in abundance while *Bifidobacterium* and *Bacteroides* decreased in abundance. Moreover, serum taurocholic acid and 3-indolepropionic acid were reduced, and the administration of lipid-lowering drugs and gut microbiota regulation in model mice could effectively inhibit tumor progression. This suggests that the cholesterol diet drives NAFLD-HCC formation by inducing alterations in intestinal microbiota and metabolites in mice. If feces, a non-invasive and easily obtained routine sample can be used as a reference indicator for early-stage diagnosis of HCC, it will bring great well-being and economic value to patients and society. Of course, more explicit results are required to support the clinical application, and it is also need that corresponding database can be improved and microbiological analysis techniques can be routinely applied.

6 | GUT MICROBIOTA AND THE TREATMENT OF HCC

Common treatments for liver cancer include surgical resection, chemotherapy, radiation therapy, radiofrequency ablation, hepatic artery chemoembolization, molecularly targeted therapy, and liver transplantation, which is the most extreme treatment option. However, the difficulty of early detection of liver cancer limits which of these treatments can be used.[17] The multi-kinase inhibitor sorafenib is recommended worldwide as a front-line treatment for advanced HCC, but the average survival time of patients has increased by only 3 months since it was approved for use. Extended administration of sorafenib produced drug resistance in cancer cells and possesses many side effects,[75] and practitioners often combine sorafenib treatment with other anticancer treatments and drugs.[76] Immune checkpoint inhibitors were approved by the US Federal Drug Administration in 2017 as a second-line treatment drug for advanced HCC with sorafenib resistance. The drug has a safety profile and improves overall survival of patients.[77] The emergence of the molecularly targeted drug Lenvatinib, as well as second-line drugs and the combination of molecularly targeted drugs with immune checkpoint inhibitor drugs, has brought new hope for the treatment of patients with advanced HCC. However, limited treatment options and efficacy still mean that research into new therapeutic approaches that more rapidly, effectively, and economically inhibit hepatocellular carcinoma progression and improve the disease is urgently needed. Dysbiosis gut microbiota plays an important role in the occurrence and development of hepatocellular carcinoma, which makes it a therapeutic research field worthy of attention.

TABLE 1 Differential gut microbiota and microbial metabolites in hepatocellular carcinoma

Biomarker type	Technique	Subjects	Biomarker for HCC	Reference	Function
Gut microbiota	16S rRNA Miseq sequencing	75 early HCC; 40 cirrhosis; 75 healthy controls	*Bifidobacterium* ↓† *Butyrate*-producing bacterial genera ↓	Ren[68]	A diagnostic biomarker for HCC
	16S rRNA Miseq sequencing	113 HBV -related HCC; 100 healthy volunteers	*Bacteroides* ↑ ‡ *Lachnospiracea incertae sedis* ↑ *Clostridium XIVa* ↑	Huang[69]	The microbiome can differentiate small HCC patients from patients with non-small HCC.
	16S rRNA gene sequencing	21 patients with NAFLD -related cirrhosis and HCC; 20NAFLD-related cirrhosis without HCC; 20healthy controls	*Bacteroides* ↑ *Ruminococcaceae* ↑ *Enterococcus* ↑ *Phascolarctobacterium* ↑ *Oscillospira* ↑ *Bifidobacterium* ↓	Ponziani[56]	The gut microbiome can be a diagnostic biomarker for HCC.
	16S rRNA gene sequencing	24 hepatitis; 24 liver cirrhosis; 75 HCC; 20 healthy controls	*Enterococcus* ↑ *Limnobacter* ↑ *Phyllobacterium* ↑	Zheng[71]	The gut microbiota could be used for precision diagnosis of HCC
	Shotgun metagenomic sequencing	32 NAFLD-HCC; 28 with NAFLD cirrhosis; 30 non-NAFLD control	*B. caecimuris* ↑ *V. parvula* ↑	Bebary[39]	The specially accumulated gut microbiota can distinguish NAFLD -HCC from NAFLD cirrhosis.
	16S rRNA gene sequencing	30 HCC-cirrhosis patients; 38 cirrhotic patients without HCC; 27 healthy volunteers	*Veillonella dispar* ↑ *Faecalibacterium prausnitzii* ↑ *Ruminococcus gnavus*↓ *Clostridium* ↑ *CF231 genus* of *Paraprevotella* ↑ *Alphaproteobacteri-a* ↓ *Verrucomicrobia* ↓ *Akkermansia muciniphila* ↓	Lapidot[85]	Gut Microbiome composition can distinguish between patients with HCC-cirrhosis and healthy controls. The gut microbiome was significantly altered in cirrhotic patients.
Microbial metabolites	UPLC/Q-T-OF MS; LC–MS/MS	22 liver cirrhosis; 23 HCC; 23 healthy control	CDCA ↓ Urobilin ↓ Urobilinogen ↓ ketolithocholic acid ↓ LPC C18:0 ↑ LPC C16:0 ↑	Cao[70]	Fecal metabolomics has potential for the diagnosis of liver cirrhosis and HCC.
	LC–MS/MS; ^{1}H-NMR	32NAFLD-HCC; 28with NAFLD cirrhosis; 30 non-NAFLD controls	Acetate ↑ Butyrate ↑ Formate ↑	Bebary[39]	Metabolites increased in NAFLD-HCC

Note: †: downregulated; ‡: up-regulated.

Abbreviations: ^{1}H-NM, hydrogen nuclear magnetic resonance; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LC–MS, liquid chromatography–mass spectrometry; LPC, lyso-palmitoylphosphatidylcholine; NAFLD, non-alcoholic fatty liver disease; rRNA, ribosomal ribonucleic acid; UPLC/Q-TOF MS, ultra performance liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry.

6.1 | Antibiotics and probiotics

Yamada et al.[78] fed mice with steatohepatitis-inducing high-fat diet (STHD-01) which induced the formation of NASH in mice and these mice further progressed into HCC. In the process of STHD-01 feeding, the gut microbiota structure showed significant changes, including an increase in the abundance of *Bacteroides* and a decrease in the abundance of *Bifidobacterium*, *Prevotella*, and *Streptococcus*. The incidence of HCC was reduced in mice with steatohepatitis after treatment with antibiotics. Therefore, the development of HCC is accompanied by certain gut microbiota changes, and the use of antibiotics can inhibit the occurrence of HCC. Antibiotics can be used to remove harmful bacteria from the gut or floral transplantation methods can increase the ratio of beneficial bacteria to correct gut microbiota disorders in HCC patients. Similarly, in a study conducted by Zhang et al.,[74] germ-free mice fed with feces excreted by high fat/high cholesterol (HFHC)-induced HCC mice for 14 months showed hepatic lipid accumulation, inflammation, and cell proliferation, suggesting that gut microbiota are involved in triggering liver inflammation. Furthermore, Atorvastatin restored cholesterol-induced dysbiosis of the gut microbiota and completely prevented the development of NAFLD-HCC. Thus, the correction of microbial dysbiosis has a deterrent effect on the development of HCC. Moreover, Ma et al.[13] used vancomycin to remove the gram-positive bacteria that mediated the conversion of primary bile acids to secondary bile acids, which was sufficient to induce aggregation of hepatic NKT cells and inhibit HCC growth. In contrast, supplementing secondary bile acids or increasing the colonization of bile-acid-metabolizing bacteria can reverse the accumulation of NKT cells and its inhibitory effect on tumor growth in mice. Artificial supplementation with intestinal probiotics can reduce the size of liver tumors and reduce the risk of hepatocarcinogenesis and development. Many important angiogenic factors and receptors were significantly downregulated in mice fed with a probiotic mixture, and the expression hypoxia-inducible factor-1 increased. Analysis of gut microbiota in probiotic-fed mice treated in advance with preventive probiotic mixtures revealed an increase in SCFA-producing bacteria, as well as a functional shift to a more anti-inflammatory metabolic environment.[79]

6.2 | Fecal microbial transplantation

Fecal microbial transplant (FMT), also known as fecal transplantation, is a procedure where feces from a healthy donor are transferred into the intestine of a diseased patient. Clinicians place small amounts of liquefied and filtered feces into the colon via colonoscopy or use other methods such as feeding tubes, coloclysis, or capsules.[80] Khoruts et al.[81] used colonoscopy in a patient with a *Clostridium difficile* infection to place feces from the patient's healthy husband into the patient's colon. Fourteen days after transplantation, the bacterial composition in the recipient's feces was highly similar to that of the donor, with Bacteroidetes strains and uncharacterized butyrate-producing bacteria increasing at the same time the regression of the patient's symptoms was observed. The similarity of the intestinal microbiota of recipients and donors who receive flora transplantation suggests that donor bacteria rapidly occupy their usual ecological niches, thereby restoring the structure and function of the recipient's microbial community. Orally administered FMT capsules were found to be safe and well tolerated in patients with cirrhosis and recurrent hepatic encephalopathy (HE) and could restore microbial diversity and function. However, its effectiveness needs to be further investigated.[82] Disturbances in the intestinal microbiota of HFD-fed mice were corrected after FMT and increases in the abundance of beneficial bacteria from the genus Christensenellaceae and lactobacillus were observed. FMT also increased the expression of intestinal junction protein ZO-1 and reduced endotoxemia in HFD-fed mice.[83] Although the research of FMT transplantation in patients with HCC were not retrieved, it is evident that FMT has the potential to restore the structure and function of beneficial intestinal flora and has been shown to be effective in liver-associated diseases and mouse models. Therefore, an accurate representation of the gut microbiota of liver cancer patients is still needed. As far as is known, the gut microbiota of HCC patients who have progressed from chronic liver disease involves changes in the abundances of multiple bacteria and, when considering that geography, diet, lifestyle habitats, and exercise can affect the composition of the gut microbiota and to develop individualized programs that can improve the structure and function of individual gut microbiota.

6.3 | Immune checkpoint inhibitors

Immune checkpoint inhibitors target immunomodulatory molecules on the surface of T cells (or their ligands) to enhance antitumor immune responses and have been approved for the treatment of patients with HCC. Zheng et al.[84] used metagenomic sequencing to study the dynamic characteristics and specificity of the intestinal microbiota during the immunotherapy of anti-programmed cell death protein 1 (anti-PD-1) in HCC. Before treatment, Bacteroidetes were the most common intestinal bacteria in both groups, followed by Firmicutes and Proteobacteria. The composition of gut microbiota in patients who responded to anti-PD-1 treatment was relatively stable compared with the composition before treatment. Meanwhile, Proteobacteria in the feces of patients who did not respond began to increase in abundance as early as the third week and became the most abundant taxon by the twelfth week. The feces of patients who responded to treatment were enriched with four *Lactobacillus* species (*L. oris*, *L. mucosae*, *L. gasseri*, and *L. vaginalis*), *Bifidobacterium dentium*, and *Streptococcus thermophilus*, all of which are probiotic lactic acid bacteria that positively impact host metabolism and immunity. At the same time, they found the enrichment of one *Lachnospiraceae* and two *Ruminococcaceae* species (*Lachnospiraceae* bacterium 7_1_58FAA, *Ruminococcus obeum*, *Ruminococcus bromii*) and *Akkermansia*

muciniphila. Commensal *A. muciniphila* and *Ruminococcaceae* can reduce intestinal permeability and systemic immunosuppression, so they are the beneficial bacteria.

This suggests that specific intestinal microorganisms have the potential to intervene in the therapeutic effects of anti-PD-1, and the specific mechanisms of this phenomenon might be explored in animal models to identify additional beneficial microbiota that can improve non-responsiveness to anti-PD-1 therapy in HCC patients.

7 | DISCUSSION

In recent years, the relationship between gut microbiota and human disease has become a hot spot for gut microbiome research. Based on the research of gut microbiota dysbiosis and HCC, some researchers are now using probiotics or FMT to restore imbalanced gut microbiota as a potential adjuvant for the treatment of HCC. Maintaining gut homeostasis, as well as a balanced gut microbiota, is critical, and fully characterizing the metabolic pathways of microbial metabolites is important for further understanding of the gut microbiota as a virtual metabolic organ. However, to date most studies on gut microbiota are based on fecal samples from patients or animal models, whereas many of the key changes associated with the gut–liver axis may occur in the small intestine; therefore, studies of microbiota at different anatomical sites can provide a more thorough analysis of alterations in gut microbiota. Moreover, diet has an important influence on gut microbiota and bacterial metabolism. However, the diet of human subjects is difficult to control, and many studies have investigated the diets of subjects, but have not fully controlled this confounding factor. Therefore, it may be necessary to collect samples after a period of unified dieting in subjects, or to use animal models to exclude diet as a confounding factor so as to gain a more accurate understanding of HCC associated changes in gut microbiota, which will provide valuable new ideas for the diagnosis, treatment, and prognosis of liver cancer.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

There is no new data were created or analyzed in this study, so data sharing is not applicable.

ORCID

Keliu Li https://orcid.org/0000-0002-8510-9552

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