The Gut Microbiome and Hepatocellular Carcinoma: Implications for Early Diagnostic Biomarkers and Novel Therapies

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Abstract

Hepatocellular carcinoma (HCC) ranks the third place among all causes inducing cancer-associated mortality, worldwide. HCC nearly exclusively occurs in cases suffering from chronic liver disease (CLD), which results from the vicious cycle of liver damage, inflammation, and regeneration possibly lasting for dozens of years. Recently, more and more investigation on microbiome-gut-liver axis enhances our understanding toward how gut microbiota promotes liver disease and even HCC development. In this review, we summarize the mechanisms underlying the effect of gut microbiota on promoting HCC occurrence, with the focus on key pathways such as bacterial dysbiosis, leaky gut, bacterial metabolites, and microorganism-related molecular patterns, which promote liver inflammation, genotoxicity, and fibrosis that finally lead to cancer occurrence. Furthermore, we discuss gut microbiota's important potential to be the early diagnostic biomarker for HCC. Gut microbiota may be the candidate targets to simultaneously prevent CLD and

HCC occurrence among advanced liver disease cases. We outlook the gut microbiota-targeting treatments in detail to prevent CLD and HCC progression.

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Introduction

Liver cancer (LC) is one of the most common malignant tumors in the world. In 2018, there were 841,080 new cases and 781,631 deaths [1, 2]. With regard to the morbidity and mortality, LC ranks the seventh and third places across diverse cancers, which has caused a great cancer burden, globally [1, 3, 4]. Hepatocellular carcinoma (HCC) represents a frequently occurring primary LC [5]. It is estimated that the HCC morbidity will show an increasing trend by the following 10 years [6]. Generally, HCC is induced by chronic liver disease (CLD) [7], and its major risk factors include hepatitis B or C virus infection, diabetes, nonalcoholic fatty liver disease (NAFLD), alcoholism, as well as additional genetic or metabolic disorders [8, 9]. HCC has a complicated pathogenic mecha-

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Table 1. Changes in microbiota composition associated with HCC in animal models

Models	Disease	Implicated microbiota	Reference
Mice	DEN induced HCC	Altered gut microbiota	[23]
Mice	DEN-CCL4 induced HCC	Altered gut microbiota	[24]
Mice	STZ-HFD induced NASH-HCC	<i>Atopobium</i> spp. ↑, <i>Bacteroides</i> spp. ↑, <i>Bacteroides vulgatus</i> ↑, <i>B. acidifaciens</i> ↑, <i>B. uniformis</i> ↑, <i>Clostridium cocleatum</i> ↑, <i>C. xylanolyticum</i> ↑, <i>Desulfovibrio</i> spp. ↑	[25]
Rat	DEN induced HCC	<i>Escherichia coli</i> ↑, <i>Atopobium</i> cluster ↑, <i>Atopobium</i> ↑, <i>Collinsella</i> ↑, <i>Eggerthella</i> ↑, <i>Coriobacterium</i> ↑, <i>Lactobacillus</i> species ↓, <i>Bifidobacterium</i> species ↓, <i>Enterococcus</i> species ↓	[26]
Mice	HFHC induced NAFLD-HCC	<i>Mucispirillum</i> ↑, <i>Desulfovibrio</i> ↑, <i>Anaerotruncus</i> ↑, <i>Desulfovibrionaceae</i> ↑, <i>Bifidobacterium</i> ↓, <i>Bacteroides</i> ↓	[27]
Mice	DMBA-HFD induced HCC	Altered gut microbiota	[28]
Mice	MYC transgenic spontaneous HCC	Gram-positive bacteria ↑, Bacteria mediating primary-to-secondary bile acid conversion ↑, <i>Clostridium scindens</i> ↑	[32]
Mice	DMBA or DMBA-HFD induced HCC	Gram-positive bacteria	[33]

HFHC, high-fat/high-cholesterol.

nism; the etiology and exact molecular mechanism of HCC has yet to be fully elucidated to date.

As revealed by studies published over the last 10 years, gut microbiota has an important role in human health. Typically, gut microbiota substantially benefits its host, especially in terms of immunity and metabolism [10, 11], but gut microbiota has been increasingly recognized to be related to disease processes [12]. Recently, investigation on the microbiome-gut-liver axis enhances our understanding toward gut microbiota's effect on promoting liver disease occurrence and development, with bidirectional communication between the liver and gut microbiota. Gut microbiota together with their metabolites and products are suggested to have key functions in HCC occurrence. Meanwhile, emerging evidence suggests that the intestinal commensal bacterial species may exert the role of pathogenesis or protection during HCC development. Although still a relatively new area of research, the existing studies indicate that gut microbiome may be the candidate target used to prevent and manage HCC.

In addition, it is of critical importance to diagnose HCC cases at the early stage, so as to improve the patient prognosis [13]. However, HCC is usually diagnosed at the late stage that is generally accompanied with hepatic insufficiency and failure [5]. Gut microbiota is advantageous in diagnosing disease, including its noninvasive nature, great efficiency, and accuracy. Furthermore, more and more studies suggest that intestinal microbiota can serve as the biomarker to diagnose numerous disorders,

including type 2 diabetes mellitus, pancreatic cancer, colorectal cancer, heart failure, liver cirrhosis (LC), as well as other central nervous system diseases [14–19]. Thus, gut microbiota may be potentially used to diagnose HCC at the early stage.

The present review first summarizes the existing studies that analyzed intestinal microbiota's possible function in HCC occurrence and progression. Then, we discuss gut microbiota-mediated treatment and early diagnosis for HCC. Some possible highlights for diagnosis and treatment, which could be used for future clinical applications, are included.

Function of Gut Microbiota in HCC

HCC is usually the result of a chronic disease process of the liver and almost never occurs spontaneously in the absence of liver disease. In addition, about 80–90% of HCCs occur in advanced fibrotic or cirrhotic livers, which means that about one-third of patients with compensated LC develop HCC in their lifetime [20, 21]. The hepatitis B or C virus-induced cirrhosis stands for a main risk factor leading to HCC. Nonetheless, with the increased morbidity rates of NAFLD and obesity among the developed countries, HCC has become more and more common in such areas [22]. Chronic liver inflammation is associated with HCC occurrence in patients with viral hepatitis, but the precise mechanism underlying HCC occurrence among NAFLD cases remains unclear.

Table 2. Changes in microbiota composition associated with HCC in human studies

Models	Disease	Implicated microbiota	Reference
Human	HCC	<i>Escherichia coli</i> ↑	[34]
Human	HCC	D _{dys} ↑, <i>Proteobacteria</i> ↑, <i>Desulfococcus</i> ↑, <i>Enterobacter</i> ↑, <i>Prevotella</i> ↑, <i>Veillonella</i> ↑, <i>Cetobacterium</i> ↓	[35]
Human	PLC	<i>Enterobacter ludwigii</i> ↑, <i>Enterococcaceae</i> ↑, <i>Lactobacillales</i> ↑, <i>Bacilli</i> ↑, <i>Gammaproteobacteria</i> ↑, <i>Veillonella</i> ↑, diversity of firmicutes ↓, firmicutes/bacteroidetes ↓, <i>Clostridia</i> ↓, <i>Subdoligranulum</i> ↓	[36]
Human	HCC	<i>Bacteroides</i> ↑, <i>Akkermansia</i> ↓, <i>Bifidobacterium</i> ↓	[37]
Human	PLC	Altered gut microbiota	[38]
Human	HCC	<i>Neisseria</i> ↑, <i>Enterobacteriaceae</i> ↑, <i>Veillonella</i> ↑, <i>Limnobacter</i> ↑, <i>Enterococcus</i> ↓, <i>Phyllobacterium</i> ↓, <i>Clostridium</i> ↓, <i>Ruminococcus</i> ↓, <i>Coprococcus</i> ↓	[39]
Human	HCC	Gut microbial α-diversity ↓, <i>Proteobacteria</i> ↑, <i>Enterobacteriaceae</i> ↑, <i>Bacteroides xylanisolvens</i> ↑, <i>B. caecimuris</i> ↑, <i>Ruminococcus gnavus</i> ↑, <i>Clostridium bolteae</i> ↑, <i>Veillonella parvula</i> ↑, <i>Oscillospiraceae</i> ↓, <i>Erysipelotrichaceae</i> ↓	[40]
Human	HCC	<i>Klebsiella</i> ↑, <i>Haemophilus</i> ↑, <i>Alistipes</i> ↓, <i>Phascolarctobacterium</i> ↓, <i>Ruminococcus</i> ↓	[3]

D_{dys}, degree of dysbiosis; NASH, nonalcoholic steatohepatitis; STZ-HFD, streptozotocin-high fat diet.

So far, some experimental data obtained from human studies and animal models indicate that gut microbiota is related to HCC occurrence (Tables 1, 2). Changes of gut microbiota together with their-derived products and metabolites account for the important factors to promote CLD and HCC occurrence. Here, the mechanisms of microbiome-gut-liver axis in promoting HCC occurrence are discussed as well as outlined in Figure 1.

Experimental Studies

Animal study results indicate a close association between gut microbiota and HCC (Table 1). As early as in 2010, Yu et al. [23] used the HCC toxic murine model and suggested that host microflora consumption following the treatment with antibiotic cocktail (ampicillin, vancomycin, neomycin sulfate, and metronidazole) suppressed carcinogenesis, and the HCC nodule size and number were significantly reduced relative to control mice. Consistent with the above results, Dapito et al. [24] suggested that mice that grew under the germ-free conditions had small and few HCC nodules compared with control mice. Besides, applying the low, nontoxic dose of lipopolysaccharide (LPS) for a long time significantly increased HCC size and number. In addition, they also suggested the direct involvement of toll-like receptor (TLR) 4 in the HCC pathogenic mechanism, with no significant differences in tumor morbidity [23, 24].

In keeping with these results, Xie et al. [25] used the mouse model of nonalcoholic steatohepatitis-HCC induced by streptozotocin-high fat diet and found the sig-

nificantly altered gut microbial structure in the liver disease occurrence process. Meanwhile, diverse bacterial species, including *Atopobium* spp., *Bacteroides acidifaciens*, *Bacteroides* spp., *Bacteroides uniformis*, *Bacteroides vulgatus*, *Clostridium xylanolyticum*, *Clostridium cocleatum*, and *Desulfovibrio* spp., showed significant elevated levels within the model mice, which were in direct proportion to pathophysiological characteristics and LPS contents. As demonstrated by Zhang et al. [26], intestinal mucosal injury, intestinal inflammation, and gut dysbacteriosis were observed in the rat model of diethylnitrosamine (DEN)-induced HCC. The gut microbial imbalance is mainly manifested as the significant increases of Gram-negative bacteria *Escherichia coli* and *Atopobium* cluster including the genera *Atopobium*, *Coriobacterium*, *Collinsella*, *Eggerthella*, as well as the significant decrease of *Bifidobacterium*, *Enterococcus*, and *Lactobacillus* species. Recently, by investigating how dietary cholesterol drives NAFLD-HCC by regulating gut microbiota as well as the corresponding metabolites, Zhang et al. [27] found that the high content of dietary cholesterol resulted in steatosis, steatohepatitis, fibrosis, and, finally, HCC in succession in the mice, and insulin resistance was also observed. The occurrence of NAFLD-HCC induced by cholesterol was related to intestinal flora dysbiosis. Typically, the microbial composition was clearly clustered depending on different stages of steatosis, steatohepatitis, or HCC. The sequential increases in *Mucispirillum*, *Desulfovibrio*, *Anaerotruncus*, and *Desulfovibrionaceae* were seen, but *Bacteroides* and *Bifidobacterium* were not seen in mice fed

Fig. 1. The mechanism of gut microbiota on the pathogenesis of HCC. Gut microbiota dysbiosis can lead to the increase of gut permeability, which eventually can lead to microbial translocation and increasing hepatic exposure to microbiota-derived products and metabolites. Subsequently, the progression of liver disease and the development of HCC were promoted via multiple mechanisms. LPS binding with TLR4 that may be directly involved in the pathogenesis of HCC. In addition, the increase of TCA and decrease of IPA may induce hepatic lipid accumulation, inflammation and cell proliferation, which can eventually lead to HCC. Meanwhile, the increase of DCA may induce HCC development by causing hepatic DNA damage. At the same time, LTA may enhance SASP of HSCs collaboratively with DCA to upregulate the expression of SASP factors and COX-2 through TLR 2, thus creat-

ing a tumor-promoting microenvironment. Furthermore, COX-2-mediated PGE₂ production suppresses the antitumor immunity through EP4 receptor, thereby contributing to HCC progression. Moreover, the increase of TMAO may induce HCC development. Besides, the decrease of SCFAs may lead to HCC progression. TLR, toll-like receptor; TCA, taurocholic acid; IPA, 3-indolepropionic acid; LTA, lipoteichoic acid; SASP, senescence-associated secretory phenotype; COX-2, cyclooxygenase-2; LCA, lithocholic acid; ω-MCA, ω-muricholic acid; GLCA, glycolithocholate; TMAO, trimethylamine-N-oxide; SCFAs, short-chain fatty acids; IFN, interferon.

with the high-fat/high-cholesterol (HFHC), as confirmed in patients with hypercholesteremia. The changes in gut bacteria metabolites induced by dietary cholesterol include the reduced level of 3-indolepropionic acid and the elevated level of taurocholic acid. Germ-free mice given gavage of stools derived from high-fat/high-cholesterol-fed mice had lipid accumulation, cell proliferation, and inflammation in the liver. Additionally, atorvastatin abolished the gut microbial dysbiosis induced by cholesterol while totally preventing the formation of NAFLD-HCC.

Yoshimoto et al. [28] investigated the occurrence of HCC in obese mice and discovered that genetic or dietary obesity caused changes in gut microbiota, thus elevating the content of secondary bile acid deoxycholic acid (DCA), the metabolite identified to induce DNA injury [29]. Meanwhile, DCA circulation in the intestine and liver enhances senescence-associated secretory phenotype (SASP) within hepatic stellate cells (HSCs) [30], while this can then produce diverse tumor-promoting and inflammatory factors in the liver, thereby enhancing HCC formation in chemical carcinogen-exposed mice. It should be noted that hindering DCA synthesis and decreasing gut bacteria can effectively prevent HCC formation in obese mice. Consistent findings are obtained in SASP inducer-deficient or senescent HSCs-depleted mice [31], which indicate the important function of DCA-SASP axis in HSCs during the formation of HCC induced by obesity. Similarly, Ma et al. [32] used a primary liver model as well as 3 liver metastasis models and discovered that the changes in commensal gut bacteria caused the anticancer effect in a liver-selective manner. The selectively increased hepatic CXCR6⁺ natural killer T (NKT) cells were seen, which was not affected by the mouse strain, sex, and liver tumor present. Besides, the hepatic NKT cells accumulated exhibited the activation phenotype, which generated greater amounts of interferon- γ when they were stimulated by antigen. According to studies in vivo that used antibody-mediated NKT-deficient and cell depletion mouse models, NKT cells inhibited the liver tumor development. Furthermore, the accumulation of NKT cells was modulated via the expression of CXCL16, the solo ligand for CXCR6, on the hepatic sinusoidal endothelial cell, which forms the lining of liver capillaries and the first barrier for the blood coming from the gut entering the liver. Primary bile acids can upregulate CXCL16, while secondary bile acids can downregulate it. The vancomycin-based antibiotic therapy can remove G⁺ bacteria, including those that mediate the conversion of primary bile acids to secondary bile acids, can sufficiently cause the accumulation of hepatic NKT cells, and can

suppress the growth of liver tumor. Bile acid-metabolizing bacteria (*Clostridium scindens*) colonization and secondary bile acids (lithocholic acid or ω -muricholic acid) supplementation can reverse both the accumulation of NKT cells and inhibition of liver tumor growth in mice containing the changed gut commensal bacteria. In the healthy liver tissues of primary LC cases, the level of chenodeoxycholic acid (a primary bile acid) is positively related to CXCL16 level, while glycolithocholate (a secondary bile acid) is negatively correlated with CXCL16 level, which suggests that such results may be applicable to human beings.

Recently, Loo et al. [33] reported that hepatic translocation of obesity-induced lipoteichoic acid (the G⁺ gut microbial component) enhanced HCC formation through generating the oncogenic microenvironment. In addition, lipoteichoic acid promoted the SASP of HSCs collaboratively with DCA (the gut microbial metabolite induced by obesity) to upregulate cyclooxygenase-2 (COX-2) and SASP factors via TLR-2. It was interesting that prostaglandin E₂ (PGE₂) generation induced by COX-2 inhibits the anticancer immune response via EP4 receptor, thus facilitating HCC occurrence. Additionally, the overexpressed COX-2 and excessive PGE₂ generation were observed in HSCs from HCCs patients with noncirrhotic, nonalcoholic steatohepatitis, which indicated the potentially consistent mechanism in human beings. Altogether, the COX-2 pathway induced by gut microbiota generates lipid mediators. PGE₂ within the senescent HSCs in tumor microenvironment has an important function in suppressing anticancer immunity.

Taken together, these experimental studies reveal the significant alterations of gut microbiota during liver disease occurrence process. Meanwhile, these findings provided a link between the gut microbiota and their-derived products, metabolites, and immune responses in the liver. Thus, the associations of gut microbial ecology with liver pathology possibly stand for the potential diagnostic and therapeutic targets for CLD and HCC.

Clinical Studies

More and more clinical trials also show that gut microbial composition is associated with HCC occurrence. Additionally, gut microbiota has an important potential as an early biomarker to diagnose HCC. In 2016, Grąt et al. [34] discovered that gut microbial profiles related to HCC among patients with cirrhosis were featured by the elevated *E. coli* abundances in the feces. Meanwhile, predicting HCC presence based on fecal *E. coli* counts may be very potential. Consequently, *E. coli* overgrowth in the intes-

tine possibly facilitates HCC formation. The dysbiosis degree related to primary HCC elevates as cancer development stage increases. By introducing the more comprehensive integrated index called the degree of dysbiosis (D_{dys}), Ni et al. [35] found that primary HCC cases had higher fecal microbial levels of pro-inflammatory bacteria compared with healthy individuals. Notably, D_{dys} elevated remarkably among primary HCC cases relative to normal subjects. Additionally, D_{dys} tended to elevate as the primary HCC stage increased, even though the difference was not significant between diverse primary HCC stages. Similarly, in order to investigate gut microbial alterations at different primary LC (PLC) stages, the association of intestinal microbiota with PLC is illustrated. By comparing intestinal microbiota of PLC patients, LC patients, and healthy control subjects, Zhang et al. [36] found that the Firmicutes diversity at phylum level decreased from HC to LC and PLC patients. Across the diverse species, *Enterobacter ludwigii* was enriched in PLC patients, which increased by 100 folds relative to those in LC and HC groups. The Firmicutes/Bacteroidetes ratio remarkably declined as disease progressed. Meanwhile, *Clostridia* was the dominant gut microbial species in HC group, while *Lactobacillales*, *Enterococcaceae*, *Gamma-proteobacteria*, and *Bacilli* can serve as the markers to diagnose PLC. In addition, correlation analysis was conducted to investigate the relations of gut microbial diversity with clinical factors, which showed that *Veillonella* was significantly positively correlated with alpha fetoprotein (AFP) of PLC patients, while *Subdoligranulum* was negatively correlated with AFP.

In line with these results, to explore the gut microbial characteristics related to HCC patients with NAFLD cirrhosis, by studying the gut microbial profiles, circulating mononuclear cells, inflammatory status, and intestinal permeability in HCC cases with cirrhosis induced by NAFLD, cases suffering from cirrhosis induced by NAFLD, and normal subjects were studied. Ponziani et al. [37] showed that HCC cases had elevated fecal calprotectin contents, with similar intestinal permeability to that in cases with cirrhosis without HCC. The C-C motif chemokine ligand (CCL) 3, CCL4, CCL5, interleukin 8, and interleukin 13 levels in plasma increased among HCC cases, which were related to the activation of circulating monocytes. For all patients with cirrhosis, the *Streptococcus* and *Enterobacteriaceae* were significantly enriched in fecal microbiota, while the *Akkermansia* abundance decreased relative to those in normal subjects. *Ruminococcaceae* and *Bacteroides* were enriched in HCC patients compared with cirrhotic patients, whereas the abundance

of *Bifidobacterium* decreased. The abundance of *Bifidobacterium* and *Akkermansia* was negatively correlated with the calprotectin content, which in turn was related to cellular and humoral inflammatory factors. *Bacteroides* also came to similar results.

Liu et al. [38] demonstrated that serum trimethylamine-N-oxide (TMAO) (a choline derived metabolite produced by gut microbiota) content increased in PLC patients compared with controls. The increased trimethylamine-N-oxide content in serum predicted a higher risk of PLC. Such relation was more potent among patients showing decreased serum choline contents. More large-sample-size prospective research is warranted for confirming the above results. Likewise, to characterize gut dysbiosis in non-LC-induced HCC (NLC-HCC) and LC-induced HCC (LC-HCC) elucidates the role of gut microbiota in HCC pathogenic mechanism. By comparing fecal microbiota in hepatitis patients, LC patients, LC-HCC patients, NLC-HCC patients, and healthy controls, Zheng et al. [39] discovered the remarkable reduction of fecal microbial α -diversity among LC cases; besides, there were significant differences in 27 genera and 3 phyla between LC and other groups (hepatitis, HCC, and normal groups). Besides, beta-diversity was largely different in LC compared with other groups. HCC group had markedly higher gut microbial diversity compared with LC group. Characterizing the fecal microbiota of LC-HCC and NLC-HCC, the fecal microbial diversity of LC-HCC group, but not NLC-HCC group, significantly increased compared with LC group. There were 13 genera related to HCC tumor size. The abundances of butyrate-producing genera (*Clostridium*, *Ruminococcus*, and *Coprococcus*) declined, whereas those of LPS-producing genera (*Neisseria*, *Enterobacteriaceae*, and *Veillonella*) increased among LC-HCC patients. Besides, 3 biomarkers (*Enterococcus*, *Phyllobacterium*, *Limnobacter*) might be utilized to diagnose disease, accurately.

In recent, by characterizing gut microbiota among cases suffering from NAFLD-induced cirrhosis, in the presence or absence of HCC, and evaluating the role in peripheral immune reaction based on the in vitro model, Behary et al. [40] found that dysbiosis characterized microbial communities in cases suffering from NAFLD-induced cirrhosis, and the compositions and functions were changed in the process of HCC development. For NAFLD-HCC cases, the microbial gene functions promote the generation of short-chain fatty acids (SCFAs), as verified through metabolomic study. Moreover, studies in vitro indicate that the NAFLD-HCC microbiota-derived, rather than control group-derived bacterial extracts, trigger

Table 3. New potential gut microbial biomarkers for the early diagnosis of HCC

Models	Disease	Gut microbial biomarkers	Reference
Human	HCC	<i>Escherichia coli</i>	[34]
Human	HCC	<i>Enterococcaceae</i> , <i>Lactobacillales</i> , <i>Bacilli</i> and <i>Gammaproteobacteria</i>	[36]
Human	HCC	<i>Enterococcus</i> , <i>Limnobacter</i> , and <i>Phyllobacterium</i>	[39]
Human	HCC	30 microbial markers	[3]

30 microbial markers: OTU10, OTU12, OTU28, OTU57, OTU58, OTU63, OTU78, OTU86, OTU87, OTU96, OTU97, OTU128, OTU136, OTU209, OTU285, OTU291, OTU310, OTU372, OTU373, OTU427, OTU451, OTU624, OTU664, OTU748, OTU927, OTU968, OTU976, OTU1032, OTU1091, and OTU1294.

the T-cell immunosuppressive phenotype, which is characterized by the attenuated CD8⁺ T cells and expanded regulatory T cells.

All in all, as suggested by these clinical studies, gut microbiota has a key function in HCC occurrence and development. Furthermore, gut microbiota may be early diagnostic biomarker of HCC, and its manipulation is an efficient strategy to prevent and treat HCC.

Gut Microbiota for the Early Diagnosis of HCC

Early diagnosis of HCC is accompanied by a variety of treatment options and typically leads to good outcomes. Biomarkers such as AFP, des-gamma-carboxy prothrombin, and *Lens culinaris* agglutinin A-reactive fraction of AFP (AFP-L3) are identified to be the markers specific to HCC [41, 42]. The novel potential biomarkers, including aldo-keto reductase family 1 member 10 [43], have been studied to diagnose HCC and predict its prognosis. Notably, gut microbiota as a novel biomarker is reported. Gut microbiota is advantageous in diagnosing disease, like the noninvasiveness, high efficiency, and accuracy. Gut microbial alterations may serve as biomarkers of HCC disease since they are related to liver disease development, ranging from cirrhosis/fibrosis to cancer [44, 45]. In recent, some studies regarding the association of gut microbiota with HCC have preliminarily indicated that it is important to identify microbiome biomarkers based on gut microbial alterations in CLD to diagnose HCC at an early stage (Table 3).

Recently, in order to characterize gut microbiome among HCC cases and assess the possibility to use it as the noninvasive biomarker to diagnose HCC, by characterizing gut microbiota, identifying the biomarkers, and constructing the HCC classifiers among early HCC patients, cirrhosis patients, and normal subjects, validating

the findings in normal subjects, early HCC cases, and advanced HCC cases, as well as further verifying diagnosis potential in HCC from Xinjiang and Zhengzhou, Ren et al. [3] found that fecal microbial diversity in early HCC with cirrhosis showed an increase compared with that in cirrhosis. In addition, compared with cirrhosis, the abundance of phylum Actinobacteria elevated in early HCC. Accordingly, the abundances of 13 genera such as *Parabacteroides* and *Gemmiger* increased in early HCC compared with cirrhosis. By contrast, the abundances of Butyrate-producing genera showed a decrease, whereas those of LPS-producing genera increased in early HCC patients compared with normal subjects. Moreover, the authors identified the optimal 30 microbial markers between non-HCC and early HCC cases. Notably, the potential potential of gut microbial markers in diagnosing early or even advanced HCC was verified. Importantly, microbial markers successfully achieved a cross-region verification of HCC in Northwest China and Central China.

Altogether, the study has first characterized gut microbiome among HCC cases established the diagnosis model, and validated the use of microbial markers to successfully diagnose HCC in a cross-region manner. Gut microbiota-targeted biomarkers are the candidate noninvasive approaches to diagnose HCC in the early stage.

Targeting Microbiota for HCC Prevention

At present, no available treatment can be applied to prevent HCC in addition to the treatment of underlying disease. As the gut-microbiota-liver axis contributes greatly to CLD and HCC development, it is a promising target in prevention measures (Fig. 2), supported by many preclinical articles showing that the HCC incidence dramatically decreases by about 80% in rat and

Fig. 2. The mechanism of gut microbiota target therapy to HCC. Modulating gut microbiota may suppress Th17 cell differentiation and their secreted IL-17 weakened the angiogenesis, and subsequently suppressed HCC progression. In addition, regulating gut microbiota may promote SCFAs production and ultimately can inhibit HCC development. Meanwhile, modulating gut microbiota may increase the production of propionate that may improve HCC not only through a cAMP level-dependent pathway but also

by binding with GPR43. Furthermore, regulating gut microbiota may induce an anti-HCC effect, with an increase of hepatic CXCR6⁺ NKT cells and heightened IFN- γ production. At the same time, CXCR6⁺ NKT cell accumulation was regulated by CXCL16 expression of liver sinusoidal endothelial cells, which was controlled by gut microbiome-mediated primary-to-secondary bile acid conversion. cAMP, cyclic Adenosine monophosphate; GPR, G protein coupled receptor; IFN, interferon; IL, interleukin.

mouse models [23, 24, 28]. Further, several small-scale clinical trials also reveal that antibiotic treatment like rifaximin and norfloxacin can extend the survival time of cirrhotic cases [46–49]. Targeting the gut microbiota for HCC prevention is particularly attractive, since it can use some approaches with a high safety profile and low risk of severe adverse effects, like probiotics, prebiotics, synbiotics, fecal microbiota transplantation (FMT), and antibiotics.

Probiotics

Probiotics are live microorganisms, which benefit the host if they are given in sufficient doses [50]; for instance, they improve gut microbial balance and stimulate bacte-

rial products and metabolites [51]. The popularity of probiotics has expanded exponentially recently, due to their health promoting effects and their abilities to prevent or treat different diseases [52–61]. Likewise, many studies suggest that probiotics are effective on the treatment of liver diseases in animal models and human beings. At present, probiotics are just studied based on the murine HCC models, and relevant data in human beings are unavailable (Table 4).

As early as 2012, Zhang et al. [26] found that VSL#3 (containing *Bifidobacterium breve*, *Streptococcus thermophilus*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus pa-*

Table 4. Targeting microbiota therapeutic strategies for HCC

Models	Disease	New strategies	Implicated microbiota	Reference
Mice	DEN induced HCC	VSL#3	G [−] bacteria groups ↓, <i>Escherichia coli</i> ↓, <i>Atopobium</i> cluster ↓, <i>Bacteroides fragilis</i> group ↓, <i>Prevotella</i> ↓	[26]
Mice	Hepa1-6 transplanted tumor	Prohep	<i>Alistipes</i> ↑, <i>Butyrivimonas</i> ↑, <i>Mucispirillum</i> ↑, <i>Oscillibacter</i> ↑, <i>Parabacteroides</i> ↑, <i>Paraprevotella</i> ↑, <i>Prevotella</i> ↑, <i>Bacteroides fragilis</i> ↑, <i>Alistipes shahii</i> ↑, <i>Parabacteroides distasonis</i> ↑, <i>Akkermansia muciniphila</i> ↑	[62]
Mice	BaF3 transplanted tumor	Inulin-type fructans	No	[64]
Mice	DEN induced HCC	An antibiotic cocktail (ampicillin, vancomycin, neomycin sulfate, and metronidazole)	No	[23]
Mice	DEN-CCL4 induced HCC	An antibiotic cocktail (ampicillin, neomycin, metronidazole and vancomycin)	No	[24]
Mice	DMBA-HFD induced HCC	An antibiotic cocktail (ampicillin, neomycin, metronidazole and vancomycin)	No	[28]
Mice	MYC transgenic spontaneous HCC	An antibiotic cocktail (vancomycin, neomycin, and primaxin) or vancomycin	<i>Verrucomicrobiales</i> ↑, <i>Clostridiales</i> ↓, <i>Bacteroidales</i> ↓	[32]
Mice	Inulin-induced HCC	Vancomycin	<i>Lachnospiraceae</i> ↓, <i>Ruminococcaceae</i> ↓, <i>Bifidobacteria</i> ↓, <i>Clostridium</i> cluster XIVa ↓	[68]

VSL#3: *Streptococcus thermophiles*, *Bifidobacterium breve*, *B. longum*, *B. infantis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei*, and *L. delbrueckii* subsp. *Bulgaricus*. Prohep: *Lactobacillus rhamnosus*GG, *Escherichia coli* Nissle 1917 and heat-inactivated VSL#3. G[−], gram-negative.

racasei) administration alleviated HCC occurrence induced by DEN through the restoration of gut homeostasis and the mitigation of hepatic and intestinal inflammation, thereby preventing cirrhosis from progressing into HCC.

Recently, Li et al. [62] discovered the effect of probiotics on inhibiting HCC development in mouse model. Administration of probiotics mixture Prohep (comprising heat-inactivated VSL#3, *E. coli* Nissle 1917, and *Lactobacillus rhamnosus* GG) into mice bearing tumor xenografts reduced liver tumor size, and shifted gut microbial composition to beneficial bacteria including *Oscillibacter* and *Prevotella*, as well as anti-inflammatory factors produced by the above bacteria. Apart from the reduced tumor size, administration of probiotics can decrease the production of angiogenic factors. With regard to the underlying pathways involved in the mechanism, mice administered with probiotics had decreased gut Th17 cell level and Th17 recruitment into the tumor site. Moreover, probiotics' antitumor activity was related to the generation of SCFAs, as evidenced by the enriched SCFA-related pathway discovered in mice treated with probiotics. Altogether,

probiotics treatment can regulate the microbiota and affect the regulation of the T-cell differentiation in the gut, while these then change the pro-inflammatory cytokine contents within the extraintestinal tumor microenvironment.

Prebiotics

Prebiotics represent the nonabsorbent oligosaccharide substances, including lactulose. They function to accelerate beneficial bacterial growth and suppress harmful bacterial growth, thus adjusting the balance of gut microbiota [63]; in addition, they can lead to the production of SCFAs and regulate the immune response. Thus, prebiotics may prevent or treat HCC. But so far, there is only one study that has investigated the effects of prebiotics on HCC (Table 4). Bindels et al. [64] demonstrated that treatment with inulin-type fructans decreased the infiltration of hepatic BaF3 cells, mitigated inflammation while increasing portal propionate content in mice given transplantation of Bcr-Abl-transfected BaF3 cells. Besides, propionate can suppress the growth of BaF3 cells in vitro by the cAMP-dependent pathway. Further, when

free fatty acid receptor 2, the Gi/Gq-protein-coupled receptor also referred to as GPR43 that binds to propionate, is activated, it can decrease the proliferation of BaF3 as well as other human cancer cells. All in all, these results support prebiotics as the novel antitumor treatment strategy.

Synbiotics

Synbiotics are the complex constituted by prebiotics and probiotics, which have been suggested to show higher efficient than prebiotics or probiotics alone [65]. Similarly, synbiotics may improve HCC. At present, no study has investigated the ameliorating effect of synbiotics on HCC.

Fecal Microbiota Transplantation

FMT is a process that administers fecal matter solution obtained from a normal donor to the recipient intestinal tract, so as to directly change the microbial composition of the latter and induce the health benefits or treat a certain disorder [53, 54]. Since FMT has successfully been utilized among cases suffering from *C. difficile* infection, which can restore eubiosis and achieve better clinical improvements than those of standard antibiotic therapy, FMT is currently being assessed in some clinical studies to treat numerous other disorders, such as irritable bowel syndrome, inflammatory bowel disease, cardiovascular disease, and metabolic diseases [53, 54, 58, 66]. Recently, FMT has been shown to have a role in some types of cancer, such as non-small cell lung cancer, colorectal cancer, and melanoma [59, 67]. In particular, FMT can enhance efficacy of immune checkpoint inhibitors therapy against these types of cancer. Likewise, FMT may treat or prevent HCC. Furthermore, FMT to enhance the antitumor effect of immune checkpoint inhibitors is a potential strategy for HCC treatment. However, there is currently no data to support this premise, and a number of hurdles must be overcome.

Antibiotics

Since antibiotics can target several pathways through which the gut microbiota promotes HCC occurrence and development (Table 4), they may be one of the most effective strategies to interrupt the tumor-promoting gut-liver axis in CLD. Reducing the total gut bacterial quantity and removing the bacteria with a highly translocated ability can decrease bacterial translocation, thus inhibiting pro-inflammatory signals arising from leaky gut. Meanwhile, selective antibiotics can suppress generation of bacterial metabolites that promote HCC, like DCA,

through decreasing the gut bacterial quantity related to the production of these metabolites [28].

Continuous intestinal sterilization through the oral antibiotic cocktail including neomycin, ampicillin, vancomycin, and metronidazole efficiently decreased the number and size of HCC tumors induced by DMBA-HFD or DEN-CCL4 in mice [24, 28]. Consistent with these observations, Ma et al. [32] found that the antibiotic cocktail (ABX, containing vancomycin, primaxin, neomycin) or vancomycin treatment elicited the liver-selective anticancer effect, with an increase of hepatic CXCR6⁺ NKT cells and enhanced interferon- γ synthesis upon antigen stimulation in MYC transgenic spontaneous HCC mouse model. Meanwhile, NKT cells mediated liver-selective tumor inhibition. The accumulation of NKT cells was regulated by CXCL16 expression of liver sinusoidal endothelial cells, which was controlled through intestinal microbiome-mediated the conversion of primary bile acids to secondary bile acids.

Recently, Singh et al. [68] demonstrated that vancomycin treatment could suppress the development of LC in inulin-fed mice with TLR5 (T5KO) deficiency. Meanwhile, vancomycin treatment led to the selective depletion of gut microbiota, including *Bifidobacteria* of phylum Actinobacteria, as well as G⁺*Lachnospiraceae* and *Ruminococcaceae* of phylum Firmicutes, which ferment fibers, as well as *Clostridium* cluster XIVa, which generate the secondary bile acids. The lack of LC in vancomycin-treated mice is closely related to the large loss circulating secondary bile acids.

Conclusions and Future Perspective

The microbiota-gut-liver axis has a key function in liver disease pathogenic mechanism, like HCC. Increasing evidence has supported the role of the gut microbiota in HCC occurrence and development. Meanwhile, gut microbiota is the potential biomarker used to diagnose HCC early. Therefore, manipulating gut microbiota may be a new approach for HCC treatment or prevention. Probiotics, prebiotics, synbiotics, FMT, and antibiotics may be the innovative, cost-effective, safe, and noninvasive manners for HCC prevention and treatment. Nonetheless, there are numerous problems to be solved with regard to microbiota's effect on HCC genesis. Further research is warranted to investigate on how the microbiota are assembled as well as on which factors contribute to their long-run stability in both health and illness. Meanwhile, more efforts are needed to develop treat-

ment targeting specific gut microbial species or gut microbiota-derived metabolites. Although high-throughput sequencing can be conducted to detect gut microbiota existing within the sample with no need of culture, its results just represent the association between gut microbiota and disease. It is still a significant challenge to move from association to causation. Some specific gut microbial species should be cultured for causation test. As a result, culturomic technique is greatly needed. Furthermore, due to the complicated gut microbial communities, multi-omics analysis that includes transcriptomics, metabolomics, and proteomics allows to overview the whole disease landscape. Additionally, many clinical trials must concentrate on identifying the best bacterial biomarkers to diagnose HCC in an early stage. Parallely, more well-designed clinical trials evaluating gut microbiota-mediated therapies are necessary for guaranteeing the safe outcome and improve outcomes of HCC treatment. At last, in order to gain the recognition of the wider medical community and investigate the possibility of using probiotics as an alternative therapeutic method for cancer, more laboratory-based mechanistic studies and extensive human clinical trials are needed to evaluate the intestinal microbiota and appropriately selected useful bacterial strains. Therefore, more efforts are needed to translate the current knowledge regarding the function

of microbiota-gut-liver axis in promoting HCC into diagnostic, prognostic, and therapeutic strategies in patients.

Conflict of Interest Statement

The authors report no conflicts of interest.

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Author Contributions

Yongbo Kang conceived, collected data, drafted, and approved the final manuscript. Yue Cai and Ying Yang reviewed, provided critical comments, and approved the final manuscript.

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