IMMUNOTHERAPY IN HEPATOCELLULAR CARCINOMA

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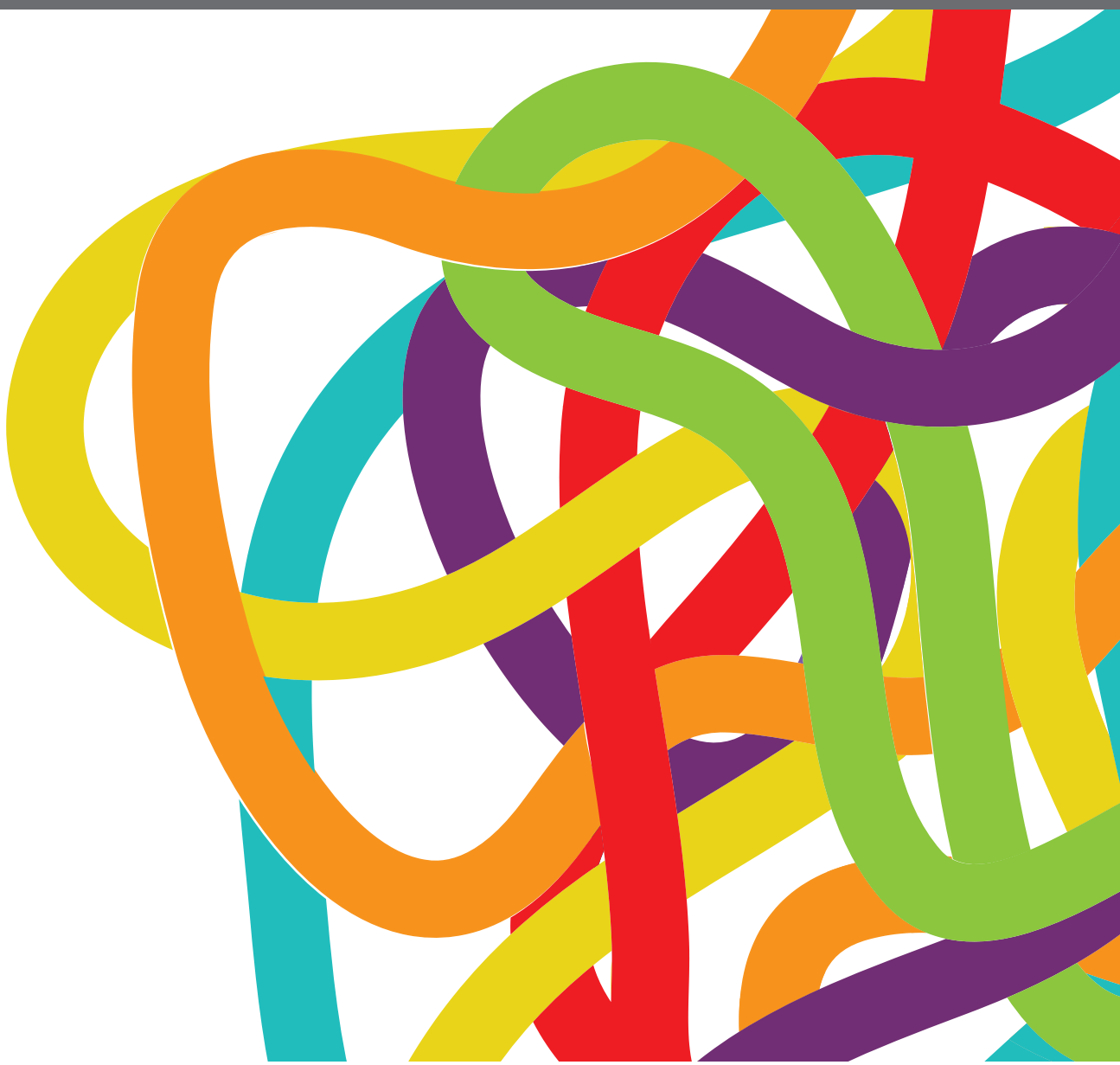

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IMMUNOTHERAPY IN HEPATOCELLULAR CARCINOMA

Topic Editors:
Weijia Fang, Zhejiang University, China
Ka On Lam, The University of Hong Kong, SAR China
Victor Ho Fun Lee, The University of Hong Kong, SAR China

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EDITORIAL
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Editorial: Immunotherapy in Hepatocellular Carcinoma

Bin Li[1†], *Ka-On Lam*[2†], *Victor Ho-Fun Lee*[2*] *and Weijia Fang*[1*]

[1] Department of Medical Oncology, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China,
[2] Department of Clinical Oncology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China

Keywords: hepatocellular carcinoma, immunotherapy, PD-1, liquid biopsy, tumor immune microenvironment, gut microbiota

Editorial on the Research Topic

Immunotherapy in Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer with high incidence and mortality worldwide (1). Curative treatment options for patients with early-stage HCC remain reasonable, including surgical resection, ablation or transplantation (2). However, a large proportion of HCC patients are often diagnosed at advanced stage and with limited effective treatments (2). And the objective response rates and prognosis of advanced HCC treated with several recommended targeted therapies are still dismal only until recently (3–5). Newly-developed immunotherapies especially immune checkpoint inhibitors (ICIs) have definitely evolved the treatment strategy of advanced HCC, due to the encouraging efficacy and acceptable toxicity. Although this is a significant progress, further improvement is still an unmet need.

In this Research Topic, two comprehensive reviews introduce the clinical application of ICIs in the treatment of HCC and underlying clinical challenges, which draw a picture named "the past, present and future of HCC immunotherapy" (Donisi et al.; Zhang et al.).

Hepatitis B virus (HBV) infection is a primary risk factor for the development of HCC (6). And the clinical performance of ICIs in such particular population is still not very clear. A real-world study is carried out in an endemic area of HBV infection, which demonstrates acceptable toxicity and favorable efficacy of nivolumab monotherapy in unresectable HCC (Sung et al.). Of interest, there exist significant intratumoral heterogeneity and disturbed immune microenvironment in HCC. And the striking heterogeneous responses of multiple lesions from a single patient to nivolumab immunotherapy are also thoroughly studied. A retrospective analysis was carried out to evaluate the clinical outcomes of recurrent hepatitis B virus-related HCC who received nivolumab plus chemotherapy or targeted treatment (Chen et al.). After multiple lines of therapy, nivolumab-based therapy still displayed antitumor activity and there were less frequent treatment-related adverse events of any grade in recurrent HCC patients, even for with HBV infection.

Indeed, HCC is a notorious tumor. Although current immunotherapy has brought about better clinical outcomes, the improvements are often modest and the corresponding scope of clinical application is not fully optimized. Nowadays, various immunotherapy-based combination strategies have shown early promising anti-tumor activity (Donisi et al.). And more researches of combined immunotherapy are designed to deal with the complexity of HCC.

Locoregional therapies not only achieve local control in HCC but also could initiate an immune response by exposing neo-tumor-associated antigens *via* necrosis of the tumor cells (7). During programmed cell death protein 1 (PD-1) blockade immunotherapy, HCC patients achieve disease control or atypical progressive diseases (different responses in multi-lesions of the same individual).

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Liang Qiao,
Westmead Institute for Medical Research, Australia

***Correspondence:**
Weijia Fang
weijiafang@zju.edu.cn
Victor Ho-Fun Lee
vhflee@hku.hk

[†]These authors have contributed equally to this work

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Thus, a proof-of-concept clinical trial was carried out to explore whether subtotal thermal ablation could increase the response rate of anti-PD-1 monotherapy and improve survival in this special population (Lyu et al.).

HCC is inclined to invade adjacent vasculature in particular the portal vein causing portal vein tumor thrombosis (PVTT) (8). Radiotherapy (RT), a standard option for HCC with PVTT, gradually change its role from a palliative treatment to a curative one. Thus, a randomized controlled study is designed by Hu et al. to confirm the efficacy and safety of combining stereotactic body RT (SBRT) with camrelizumab and apatinib in first line treatment for HCC patients with PVTT.

Not limited to selective combination, some new directions of immunotherapy are being discovered. Zhang et al. review gut microbiota and related potential treatment options for liver cancer. Certain bacterial species could improve anti-tumor immunity and enhance the efficacy of immunotherapy by modulating the components of bile acids. And modulating gut microbial components is considered to be a potential strategy to improve the efficacy of immunotherapy for HCC treatment.

Keeping the balance of cellular senescence is closely related to the occurrence and progression of HCC. With more in-depth research on cellular senescence, dual effects of cellular senescence and underlying mechanism of induced immune surveillance are gradually unmasked. A novel review by Liu et al. summarize the latest advances about hepatocellular senescence, and bring up some emerging intervention strategies in senescence-related therapy which HCCs that may benefit from tumor immune microenvironment remodeling. For instance, activating immune surveillance, recruiting functional immune cell types and eliminating atypical proliferative hepatocyte may act as the key elements of these senescence based "new immunotherapy".

HCC is a highly aggressive disease with a poor prognosis and anti-PD-1 blockades prolong the median overall survival of advanced HCC to about 13-15 months (9, 10). On the basic of tumor immune microenvironment (TIME) phenotypes and differentially expressed gene clusters, Chen et al. construct a TIME score model. And further analysis reveals TIME score is positively associated with clinicopathologic features and somatic gene mutations. In another study of Chen et al., a nomogram constructed by potential prognostic factors including age, ECOG status, hepatectomy status, and transcatheter arterial chemoembolization (TACE) use, is performed to distinguish high-risk group and low-risk group of HCC patients. These prediction model certainly exhibit robust prognostic value for HCC. Moreover, Zhang et al. also reveal that specific group of bacteria or change of gut microbiome could be promising biomarkers used for diagnosis and prognosis of HCC.

Recently, more and more molecules and proteins have been unveiled, which are closely associated with the carcinogenesis and development of HCC as well as the tumor immune microenvironment. In this topic, Tang et al. summarize the major biological functions of circRNAs in liver cancer and emphasize the circRNAs-induced immune escape, by predominantly affecting natural killer cells.

These multi-angle articles collected in this Research Topic of Frontiers in Oncology, present an attractive scope of what is novel, promising, and controversial in the HCC immunotherapy field. We hope these valuable work would aid clinicians to understand and select immunotherapy options more wisely for the better management of HCC patients.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Real-World Outcomes of Nivolumab in Patients With Unresectable Hepatocellular Carcinoma in an Endemic Area of Hepatitis B Virus Infection

Pil Soo Sung [1,2], Jeong Won Jang [1,2]*, Jaejun Lee [1,3], Soon Kyu Lee [1,2], Hae Lim Lee [1,4], Hyun Yang [1,3], Hee Chul Nam [1,2], Sung Won Lee [1,4], Si Hyun Bae [1,3], Jong Young Choi [1,2], Nam Ik Han [1,4] and Seung Kew Yoon [1,2]**

[1] The Catholic University Liver Research Center, College of Medicine, The Catholic University of Korea, Seoul, South Korea, [2] Division of Gastroenterology and Hepatology, Department of Internal Medicine, College of Medicine, Seoul St. Mary's Hospital, The Catholic University of Korea, Seoul, South Korea, [3] Division of Gastroenterology and Hepatology, Department of Internal Medicine, College of Medicine, Eunpyeong St. Mary's Hospital, The Catholic University of Korea, Seoul, South Korea, [4] Division of Gastroenterology and Hepatology, Department of Internal Medicine, College of Medicine, Bucheon St. Mary's Hospital, The Catholic University of Korea, Seoul, South Korea

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Edited by:
Ka on Lam,
The University of Hong Kong,
Hong Kong

Reviewed by:
Daniel Lin,
Thomas Jefferson University,
United States
Johan Nicolay Wiig,
Oslo University Hospital, Norway

***Correspondence:**
Pil Soo Sung
pssung@catholic.ac.kr
Jeong Won Jang
garden@catholic.ac.kr
Seung Kew Yoon
yoonsk@catholic.ac.kr

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Real-world results of nivolumab monotherapy against HCC are lacking in the hepatitis B virus (HBV)-endemic, Asia-Pacific regions. Moreover, heterogeneous responses to immune checkpoint inhibitors have rarely been described in advanced HCC. The aim of this study is to evaluate the efficacy and safety of nivolumab monotherapy in a real-world setting in 33 Korean patients with unresectable HCC. In our cohort, twenty-nine patients (88%) showed HBsAg positivity. At the time of nivolumab initiation, 4 among 33 patients (12%) were classified as Barcelona Clinic Liver Cancer (BCLC)-B stage and 29 (88%) as BCLC-C stage, respectively. Prior sorafenib treatment was given to 31 (94%) patients, and 13 (39%) received prior regorafenib treatment. For the liver reserve, patients were classified as Child–Pugh class A (79%) and B (21%), respectively. Grade 3 toxicities occurred in one patient, who developed pneumonitis after 5 cycles of nivolumab treatment. Best overall responses were complete response in 2 patients out of the 33 enrolled patients (6%), partial response in 4 patients (12%) and stable disease in 4 patients (12%). With 29 patients having images for the response evaluation, the objective response rate was 21.4%. The median overall survival (OS) of the cohort was 26.4 weeks (range 2.3–175.1). Achieving objective responses, pre-treatment small tumors (maximal diameter <5 cm) and favorable liver function as assessed by Albumin–Bilirubin grade were significant factors for the favorable OS. Interestingly, differential responses to nivolumab among multiple tumors in a single patient were noted in 6 patients (18%). In these patients, small metastatic tumors were regressed, although their larger tumors did not respond to nivolumab monotherapy. In summary, nivolumab treatment seems clinically efficacious in treating unresectable HCC in an endemic area of HBV infection. Further prospective evaluation is required to overcome the heterogeneous efficacy of nivolumab monotherapy according to the baseline tumor burden.

Keywords: hepatocellular carcinoma, nivolumab, objective response, tumor size, tumor heterogeneity

INTRODUCTION

Worldwide, hepatocellular carcinoma (HCC) is the fourth most common cause of cancer-related mortality. There are more than 850,000 new cases of liver cancer annually, 90% of which are HCC (1). Risk factors for HCC include chronic hepatitis B (CHB) and chronic hepatitis C (CHC), non-alcoholic fatty liver disease (NAFLD) and excessive alcohol ingestion (1). In Asian countries, where hepatitis B virus (HBV) infection is prevalent and accounts for 80% of victims, a considerable number of patients receive only supportive care or, at best, palliative treatments (2).

Sorafenib, a multi-tyrosine kinase inhibitor, has been the only drug available in the last decade to combat HCC (3). Recently, three tyrosine kinase inhibitors have demonstrated improved outcomes: lenvatinib in the first-line, and regorafenib and cabozantinib after first-line failure (4). Although they showed some promising results in terms of efficacy, their use may be limited due to the adverse effects and the potential decrease in the liver reserve. Immune checkpoint inhibitors are intended to target the programmed cell death protein-1 (PD-1), programmed death-ligand 1 (PD-L1), or cytotoxic T lymphocyte-associated protein-4 (CTLA-4) (5). Clinical trials with nivolumab and pembrolizumab in unresectable HCC, representative anti-PD-1 antibodies, had been anticipated to show prolonged survivals in patients treated with these drugs. However, only 14 to 18% of patients treated with pembrolizumab or nivolumab monotherapy had objective tumor responses (6–8). More recently, a phase 3 randomized, multi-centre study (CheckMate 459) evaluating nivolumab monotherapy versus sorafenib as a first-line treatment of unresectable HCC, did not achieve its primary endpoint of overall survival (OS) (9, 10). Moreover, unlike other solid tumors, there was no marked association identified between the levels of tumor cell PD-L1 expression and responses to nivolumab in HCC, reported by earlier Keynote-224 and CheckMate-040 studies (5, 11, 12). Currently, there are no validated biomarkers for HCC immunotherapy (13).

Recent sub-analysis of the CheckMate-040 study between intent-to-treat (ITT) overall population and an Asian cohort with prior sorafenib failure showed that treatment responses of Asian patients were similar to those of the overall population (14). This disappointing performance of nivolumab monotherapy may be attributed to the immune heterogeneity of HCC (15, 16). However, there is a lack of real-world clinical data demonstrating the heterogeneous responses to nivolumab. This study aims to evaluate the efficacy and safety of nivolumab monotherapy by performing retrospective analyses of patient data. The data was collected from HCC patients attending three university-affiliated hospitals in Korea where HBV infection is endemic. Specifically, we focused on the responses to nivolumab monotherapy, for every tumor in a single patient, to identify the factors associated with the heterogeneous responses to this treatment.

METHODS

Study Design and Population

The Institutional Review Board of The Catholic University of Korea approved this study (Xc20RIDI0015), which was carried out in accordance with the Declaration of Helsinki. Data was collected between October 2016 and November 2019 from 33 consecutive patients treated at three university-affiliated hospitals in Korea. Among the enrolled patients, 31 patients were enrolled between February 2018 and November 2019. All patients had a verified diagnosis of unresectable HCC by updated international guidelines (17, 18) and were treated with nivolumab. Experienced hepatologists reviewed the patients' medical data. The survival data of the patients continued to be followed-up until February 2020. Survival was determined to be from the point of commencing nivolumab treatment until the final follow-up or until the patient died, regardless of the cause. The inclusion criteria were a diagnosis of the inoperable HCC treated with nivolumab. Albumin–Bilirubin (ALBI) grade (19) was calculated to determine the liver reserve of patients treated with nivolumab.

Nivolumab Treatment and Response Evaluation

Each patient received an intravenously delivered dose of 3 mg/kg nivolumab (OPDIVO®, Bristol-Myers Squibb) every two weeks. Every 4 to 8 weeks during treatment, full blood counts were performed, and a number of markers were evaluated including alpha-fetoprotein (AFP), alanine aminotransferase (ALT), bilirubin, and prothrombin time. Nivolumab was administered according to the recommended dose and safety information. Where necessary, doctors would adjust the treatment schedules. Toxicities of nivolumab were diagnosed and managed as previously described (20, 21).

Using the modified Response Evaluation Criteria in Solid Tumors (mRECIST) tool, two independent radiologists assessed the response to the treatment, as described elsewhere (3). A maximum of two lesions per organ and five in total were chosen for the evaluation of the treatment responses by mRECIST (3, 22). Extrahepatic tumors exhibiting enhanced contrast were considered as target lesions, whereas macroscopic vascular invasions were regarded to be non-target lesions. The mRECIST tool categorizes a complete response (CR) when the intratumoural arterial enhancement disappears from all tumors. A partial response (PR) is defined when the sum of the diameters of enhanced lesions are reduced by no <30%. However, if the sum of the diameters of enhancing lesions increases by 20% or more, the disease is categorized as progressive (PD). Disease states not categorized part of PD and PR, were determined to be stable (SD) (3, 22). The sum of CR, PR and SD rates formed the disease control rate (DCR). The response evaluation was conducted regularly, between two and four nivolumab-treatment cycles.

Statistical Analysis

For the statistical analyses, SPSS version 26 software (IBM Corp., Armonk, NY, USA) was used. A chi-square test was used to analyse the two groups' categorical variables, and an independent *t*-test was conducted to evaluate the continuous variables. Univariate and multivariate analyses were performed

TABLE 1 | Clinical parameters of study patients.

Clinical parameters	*n* = 33
Median age (range)	57 (37–79)
Sex (male), *n* (%)	25 (75.8)
HBsAg-positivity, *n* (%)	29 (87.9)
Anti-HCV-positivity, *n* (%)	1 (3)
Median tumor size, cm	3.5
<5 cm, *n* (%)	21 (64)
≥5 cm, *n* (%)	12 (36)
Multiple tumors, *n* (%)	33 (100)
Portal vein tumor thrombosis, *n* (%)	10 (30)
Extrahepatic metastasis, *n* (%)	26 (79)
BCLC stage B/C, *n* (%)	4/29 (12/88)
Median AFP (range), ng/mL	665 (1.3–160000)
<1000 ng/mL, *n* (%)	17 (52)
≥1000 ng/mL, *n* (%)	16 (49)
Child–Pugh score	
5, *n* (%)	20 (61)
6, *n* (%)	6 (18)
7, *n* (%)	7 (21)
ALBI grade 1/2/3, *n* (%)	15/18/0 (45/55/0)
Prior therapy to nivolumab, *n* (%)	
Surgical resection	12 (36)
TACE / TARE	26 (79)
HAIC	5 (15)
Sorafenib	31 (94)
Regorafenib	13 (39)
Lenvatinib	2 (6)
Post nivolumab treatment, *n* (%)	
No treatment	21 (64)
Resection	1 (3)
TACE	2 (6)
Radiation therapy	3 (9)
Regorafenib	2 (6)
Cabozantinib	2 (6)
HAIC	1 (3)
Systemic chemotherapy	2 (6)
Best responses to nivolumab	
Complete response	2 (6)
Partial response	4 (12)
Stable disease	4 (12)
Progressive disease	19 (58)
Not assessed	4 (12)

AFP, alpha fetoprotein; ALBI grade, albumin-bilirubin grade; BCLC stage, Barcelona-Clinic liver cancer stage; HAIC, hepatic arterial infusion chemotherapy; HBsAg, hepatitis B surface antigen; HCV, hepatitis C; RT, radiotherapy; TACE, transarterial chemoembolization; TARE, transarterial radioembolization.

to establish prognostic factors of OS. For the univariate analyses, such as survival probabilities, the Kaplan-Meier method and log-rank tests were used. Factors that were significant in the univariate analysis at $P < 0.05$ were advanced to the multivariate analysis, which was undertaken using a Cox regression model.

RESULTS

Study Cohort Demographics

As indicated in **Table 1**, the study involved a total of 33 patients, 25 of whom were male (76%). Ages ranged from 37-79 years, with a median of 57 years. A majority of patients (88%) had been assigned stage C on the Barcelona Clinic Liver Cancer (BCLC) staging system, with a median tumor size of 3.5 cm. Extrahepatic metastases were reported in 26 patients (79%), and portal vein tumor thrombosis were detected in 10 patients (30%). The most prevalent underlying liver diseases was chronic HBV infection, which affected 29 individuals (88%). A majority of participants (79%) were classified as Child–Pugh class A at the time of enrolment, and 15 patients (45%) were ALBI grade 1. The median level of AFP was 665 ng/mL (normal range: < 8.1 ng/mL), and the level of 17 patients (52%) were above 1000 ng/mL. Most of the enrolled patients (94%) underwent sorafenib treatment, and 13 patients (39%) patients underwent regorafenib treatment prior to the nivolumab therapy. Prior to the systemic therapy, most patients had undergone local–regional therapies such as trans-arterial chemoembolization or hepatic arterial infusion chemotherapy. Eleven patients received further treatments after nivolumab, which included cabozantinib and regorafenib. In this cohort, there was no evidence of a high incidence of immunotherapy-related adverse events. Grade 3 toxicities occurred in one patient, who developed pneumonitis after 5 cycles of nivolumab treatment.

Treatment Responses to Nivolumab

In this study, the nivolumab monotherapy was administered for 2 to 160 weeks, with a median of 8 weeks, and the number of treatment cycles varied from 1 to 78, with a median to 3 cycles. In a best response evaluation after nivolumab administration, 2 patients exhibited a CR according to the mRECIST (**Table 1**). Four patients displayed a PR and 4 patients displayed a SD. However, 4 patients did not undergo imaging for the response evaluation. The objective response rate was 18% among all the patients that enrolled in this study, and 24% among patients with evaluable images. The median duration of treatment responses by nivolumab was 13.3 months. The disease control rate was 30% in our cohort. A waterfall plot describes the marked reductions in target lesions from baseline tumor burden in patients with objective responses to nivolumab monotherapy (**Figure 1**).

Factors Associated With the Overall Survival

The median follow-up period after initiation of treatment was 12.5 months, and the OS ranged from 2.3 to 175.1 weeks (median: 26.4 weeks). At the time when data analysis was performed (February 2020), 18 of the 33 patients (55%) had died from causes such as tumor progression, variceal bleeding, or fatal systemic infection. **Figure 2A** indicates a significantly better OS for individuals with controlled diseases (CR + PR + SD) than for those who displayed PD (log rank test, $P < 0.001$). **Figure 2B** indicates a significantly better OS for individuals with a maximal tumor size of < 5 cm ($P = 0.002$), although AFP level did not have a significant impact on the patient survival (**Figure 2C**).

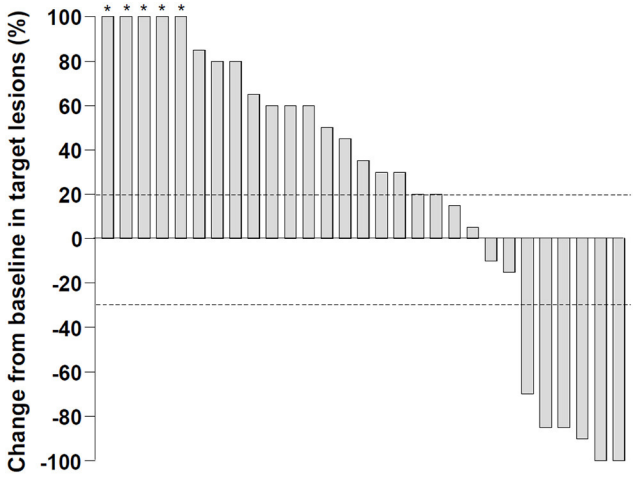

FIGURE 1 | Changes in target lesions from the baseline after nivolumab monotherapy. Dashed lines represent a 20% increase or a 30% reduction. The percentage changes more than 100% were truncated to 100% (asterisks) .

Figure 2D indicates a better OS for individuals with ALBI grade 1 than for those with grade 2 with $P = 0.004$. Patients with Child–Pugh score 5 also showed superior OS to those with score 6 (**Figure 2E**, $P = 0.035$).

The prognostic factors for OS after nivolumab treatment are presented in **Table 2**. These parameters were subjected to univariate analyses initially, and tumor size / ALBI grade were included in a subsequent multivariate Cox regression model. The favorable prognostic factors for OS were a tumor size <5 cm, the assignment of ALBI grade 1, and Child-Pugh score 5 in univariate analyses. In multivariate analysis, tumor size < 5 cm (HR = 0.269; $P = 0.034$) and ALBI grade 1 (HR = 0.312; $P = 0.04$) were both significant factors for OS.

Differential Responses to Nivolumab Among Multiple Tumors in Each Patient

Due to the previous demonstration that maximal tumor size is a critical factor of OS of patients with nivolumab treatment, we measured response of each tumor among multiple tumors in a single patient. Heterogeneous responses were detected in 6 patients among the 33 enrolled patients (18%) (**Table 3**). For patient #2, the different tumor responses between lung (1.2 cm) and peritoneal metastasis (4.3 cm) and the heterogeneous responses to nivolumab treatment was also noted even within the single peritoneal metastatic nodule. This case was previously reported by our group (16). In the peritoneal metastatic nodule, metastatic HCC with partial necrosis was present with viable tumor cells with various types of tumor-infiltrating immune cells, suggesting the immune heterogeneity within a single tumor when it exhibits a considerable size. **Figure 3** shows the imaging findings of patient #6 after 4 cycles of nivolumab. Intrahepatic infiltrative tumor (**Figure 3A**) showed slight increase in its extent in hepatobiliary phase of primovist-enhanced magnetic resonance imaging, while metastatic nodules in lung showed dramatic responses after nivolumab (**Figure 3B**).

DISCUSSION

This is the first report in Korea demonstrating the potential predictors of OS in patients treated with nivolumab for unresectable HCC. In this study, we investigated the safety, efficacy, and the potential predictors of OS in nivolumab monotherapy for unresectable HCC in an endemic area of HBV infection. Moreover, we demonstrated the striking heterogeneous responses to nivolumab monotherapy in a single patient with multiple tumors according to each tumor size. The median OS of the participating patients (26.4 weeks) was longer than the recently conducted real-world study in Europe (34 enrolled patients, OS: 7.5 weeks), which included many patients with poor liver function (41% with Child Pugh class B) (23), but shorter than the Taiwanese real-world study (92 patients with nivolumab treatment and 3 patients with pembrolizumab treatment, OS: 11.9 months) (24). As expected, it was also shorter than that of the ITT analysis comprising an Asian cohort of CheckMate-040 (14.9 months) (14). As nivolumab is approved only in patients with previous sorafenib failure in Korea and not reimbursed by the government insurance system, Korean HCC patients receive nivolumab treatment as the last possible option for the advanced HCC. Furthermore, shorter follow-up duration may also have affected the shorter OS of our real-world data than those of previous clinical trial data.

In our study, maximal tumor diameter was the significant pre-treatment factor that affected the OS in multivariate analyses. Previous report demonstrated that the ratio of T-cell invigoration to tumor burden ratio correlates with the response to pembrolizumab in melanoma patients (25, 26). Other clinical studies showed that tumor size is an independent factor for OS in melanoma and non-small cell lung cancer patients treated with nivolumab or pembrolizumab (27–29). In HCC, very recent data using multi-omics approaches demonstrated the significant heterogeneity of tumor cells in HCC, while the heterogeneity of immune microenvironment was not as dramatic (15). In line with these reports, differential responses to nivolumab among multiple tumors in a patient with HCC can be understood. Heterogeneous responses to nivolumab among multiple metastatic tumors were also demonstrated in melanoma (30) and non-small cell lung cancer patients (31). This heterogeneity can be explained by innate or acquired resistance of tumor cells when a considerable tumor burden exists. In this report, we observed that each tumor size in a patient with multiple tumors may be associated with the heterogeneous responses to nivolumab. In HCC, immune heterogeneity may be applied to the larger tumors that may contain the higher number of resistant clones to immune checkpoint inhibitors. To overcome this heterogeneity, there have been studies investigating the possible synergic benefits for advance HCC of combination therapy (32). Lenvatinib plus pembrolizumab or bevacizumab plus atezolizumab has demonstrated promising objective response rates (ORRs). A study at the European Society for Medical Oncology Asia Congress 2019 showed significant improvements of atezolizumab and bevacizumab over sorafenib

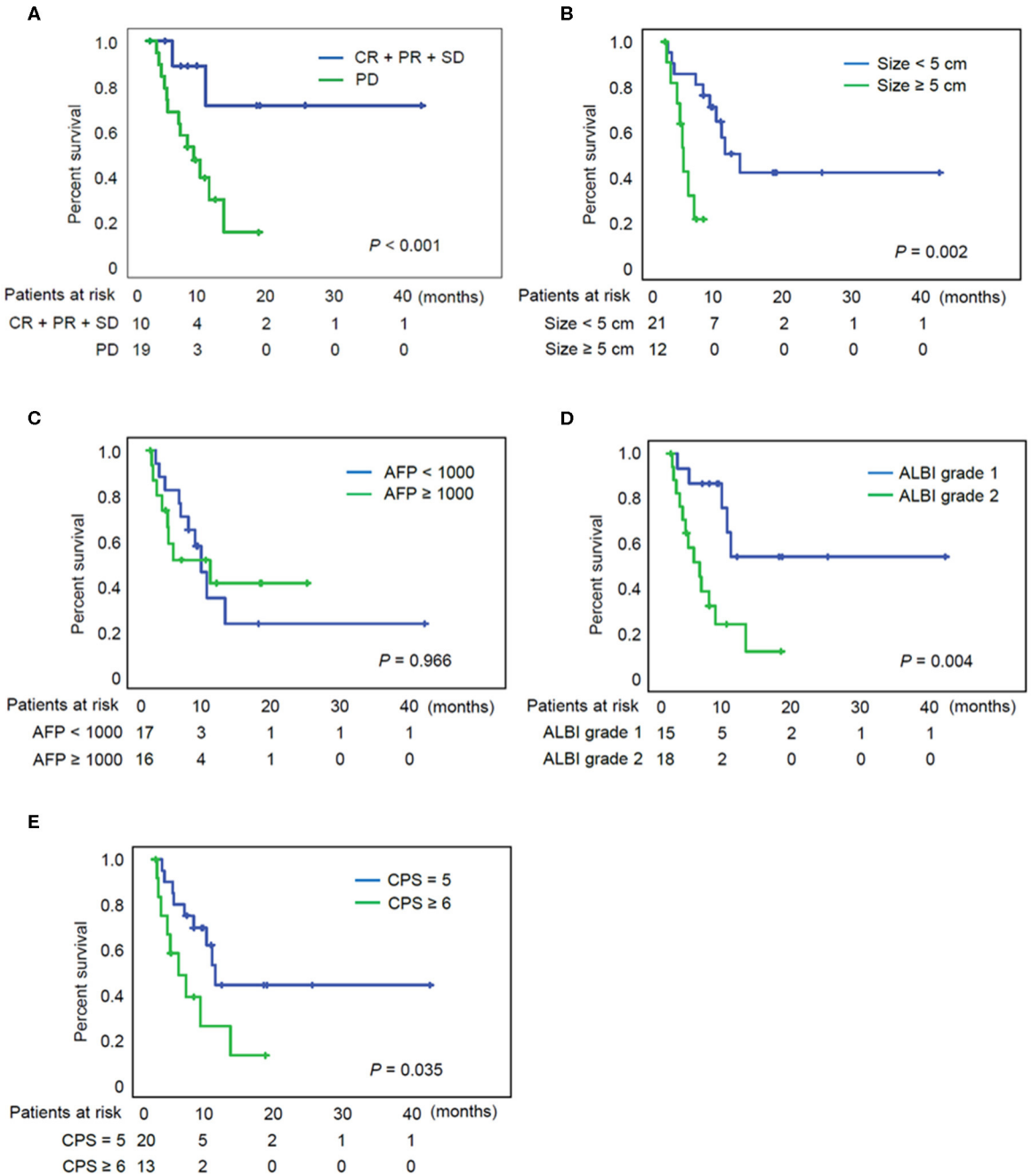

FIGURE 2 | Overall survival of patients according to the various clinical parameters. **(A)** Overall survival of patients according to the tumor response. **(B)** Overall survival of patients according to the tumor size. **(C)** Overall survival of patients according to the AFP. **(D)** Overall survival of patients according to the ALBI grade. **(E)** Overall survival of patients according to the CPS. AFP, alpha fetoprotein; ALBI grade, albumin-bilirubin grade; CPS, Child-Pugh score; CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

in OS and recurrence-free survival for unresectable HCC (phase 3 IMbrave 150) (32). This suggests that resistance to immune checkpoint inhibitors can be overcome by the combination with anti-angiogenic drugs or tyrosine-kinase inhibitors.

Patients in the real-world cohorts are typically more heterogeneous than those recruited into clinical trials. It is currently still unclear whether nivolumab offers any OS benefit to patients with decreased liver function. There was also a limited treatment effect to these patients in this study. The data from our cohort confirmed the ALBI grade as an independent survival predictor in patients undergoing nivolumab treatment. The results of our survival analysis indicate that ALBI grade 1 is an independent factor for the favorable survival. This is in accord with the previous study demonstrating the

TABLE 2 | Factors associated with overall survival in 33 patients treated with nivolumab monotherapy.

Characteristics		Univariate			Multivariate		
		HR	95% CI	*P*	HR	95% CI	*P*
Age	<60 vs. ≥60	2.065	0.734–5.807	0.169			
Sex	Male vs. Female	0.457	0.151–1.384	0.166			
Tumor size, cm	**<5 vs. ≥5**	**0.181**	**0.056–0.586**	**0.004**	**0.269**	**0.080-0.906**	**0.034**
PVTT	Yes vs. No	1.868	0.695–5.020	0.215			
AFP, ng/mL	<1000 vs. ≥1000	0.980	0.382–2.511	0.966			
Child–Pugh score	5 vs. 6	0.380	0.150–0.966	0.042			
ALBI grade	**Grade 1 vs. 2**	**0.236**	**0.083–0.675**	**0.007**	**0.312**	**0.103-0.949**	**0.040**

AFP, alpha fetoprotein; AFP, alpha fetoprotein; ALBI grade, albumin-bilirubin grade; CI, confidence interval; HR, hazard ratio. Significant factors in multivariate analysis are in bold characters.

TABLE 3 | Patients with heterogeneous responses to nivolumab.

	Intrahepatic tumor		Extrahepatic tumor−1			Extrahepatic tumor−2			Overall response
Pt.	**Size (cm)**	**Response**	**Location**	**Size (cm)**	**Response**	**Location**	**Size (cm)**	**Response**	
#1	No tumor		Lung	4.2	10% ↑	Lung	2.6	60% ↓	PR
#2	No tumor		Peritoneum	4.3	13% ↑	Lung	1.2	80% ↓	PR
#3	1.4	5%↓	Lung	1.8	10% ↑	Lung	1.2	30% ↓	SD
#4	3.3	100% ↑	Lung	1.2	70% ↓	Lung	1	80% ↓	SD
#5	3.3	25%↓	Peritoneum	1.0	200%↑	Lung	1	100%↑	PD
#6	7.2	5% ↑	Lung	2.1	55% ↓	Lung	1.9	62% ↓	PR

PD, progressive disease; PR, partial response; SD, stable disease.

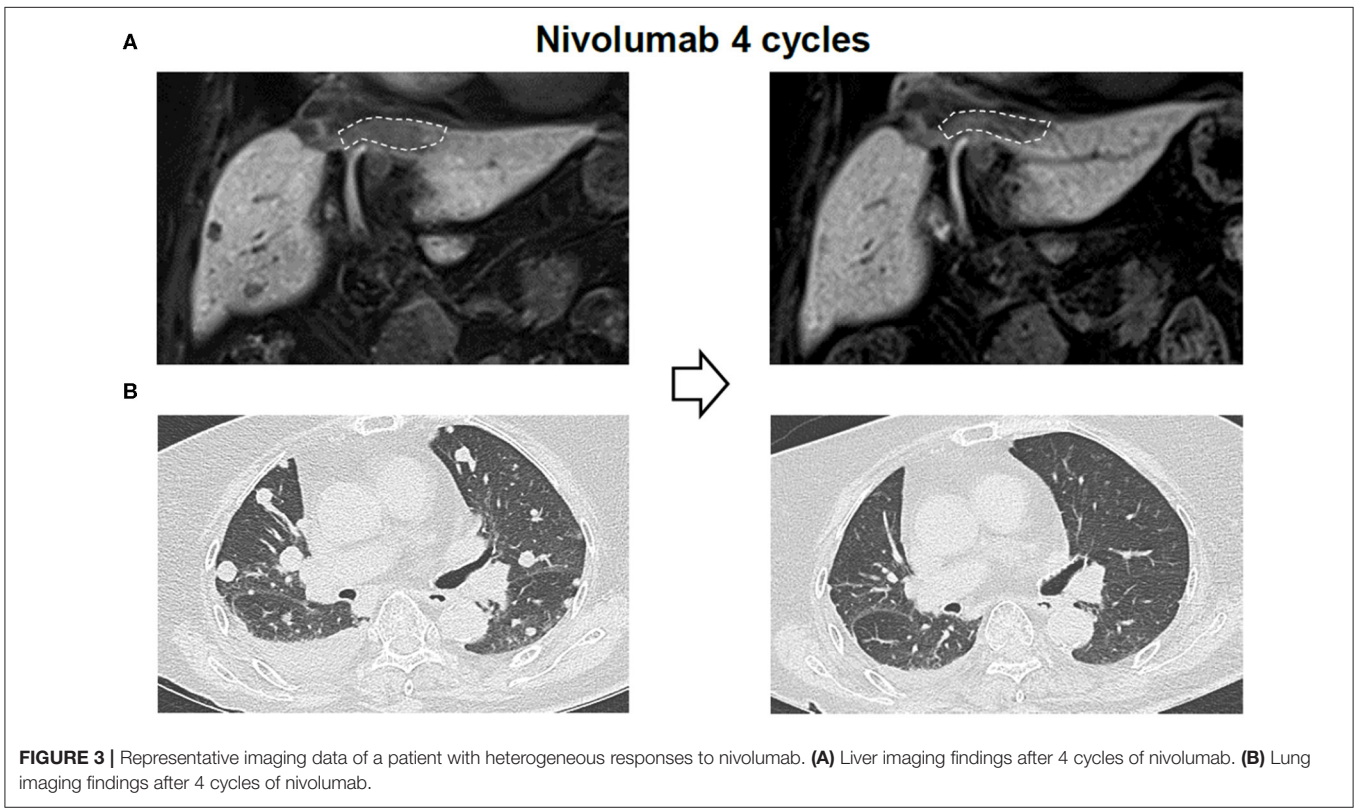

FIGURE 3 | Representative imaging data of a patient with heterogeneous responses to nivolumab. **(A)** Liver imaging findings after 4 cycles of nivolumab. **(B)** Lung imaging findings after 4 cycles of nivolumab.

survival-predictable ability of the ALBI grade at the time of sorafenib discontinuation (33).

This study has a number of limitations. This was a small-sized retrospective study that used patients only from three facilities. Such a small number of the cohort is not sufficient to validate the safety and efficacy of the drug. Moreover, regular tumor reassessment by clinical and imaging evaluation would have decreased the observation bias. Lastly, since liver biopsy was not performed routinely before nivolumab treatment, the molecular biomarkers for nivolumab responses were not studied.

In conclusion, our study demonstrates that nivolumab monotherapy is clinically efficacious in treating unresectable HCC in an endemic area of HBV infection. Maximal tumor diameter and the indicator of liver function (ALBI grade) were significant factors in multivariate analyses that predicted the OS of HCC patients treated with nivolumab monotherapy. Future prospective study is required to overcome the probable heterogeneous efficacy of nivolumab monotherapy according to each tumor size.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Catholic University of Korea (Xc20RIDI0015). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PS: study design, data collection, data analysis, data interpretation, article, and article approval. JL, SKL, HL, HY, HN, and SWL: data collection. JJ, SB, JC, NH, and SY: data interpretation and article approval. All authors contributed to the article and approved the submitted version.

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METHODS
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Efficacy and Safety of SBRT Combined With Camrelizumab and Apatinib in HCC Patients With PVTT: Study Protocol of a Randomized Controlled Trial

Yue Hu[1], Tingting Qin[2], Shuang Li[3], Tao Zhang[1] and Jun Xue[1]**

[1] Cancer Center, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2] Department of Epidemiology and Statistics, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [3] Department of Oncology, The First People's Hospital of Jingzhou, Jingzhou, China

Background: Hepatocellular carcinoma (HCC) patients with portal vein tumor thrombosis (PVTT) has poor prognosis. Sorafenib/lenvatinib is recommended as the first-line therapy in these patients currently, with unsatisfactory response and survival benefit reported. Radiotherapy (RT) is increasingly utilized in advanced HCC and is considered an alternative option for HCC patients with PVTT. Combined treatment of RT and locoregional treatments such as transarterial chemoembolization shows promising results. However, the efficacy and safety for combined treatment of RT and systemic therapy have not been reported and thus warrant further studies. This prospective clinic trial aims at evaluating the efficacy and safety of stereotactic body RT (SBRT) combined with camrelizumab and apatinib in HCC patients with PVTT.

Methods: This multicenter, open-label, randomized controlled trial will enroll 264 HCC patients with PVTT who have not received systemic therapy previously. Stratification of patients will be based on the presence or absence of extrahepatic metastasis and level of AFP (AFP $\geq$ 400 or <400 ng/mL) and randomly assigned 1:1 to study and control groups. Patients in study group will receive SBRT (95% PTV 36–40 Gy/6–8 Gy), camrelizumab (200 mg every 3 weeks), and apatinib (250 mg every day), and patients in control group will receive camrelizumab (200 mg every 2 weeks) and apatinib (250 mg every day). Patients will be followed up for 1.5 to 3.5 years since the start of therapy. We will use overall survival as the primary endpoint and progression-free survival, objective response rate, disease control rate, adverse events, and quality of life as the secondary endpoints.

Discussion: This study will be the first randomized controlled trial to assess the efficacy and safety of SBRT combined with camrelizumab and apatinib for HCC patients with PVTT. The results may help establish a new standard first-line therapy for these patients.

Trial Registration: Chinese Clinical Trial Registration No. ChiCTR1900027102.

Date of Registration: October 31, 2019.

Keywords: HCC, PVTT, SBRT, camrelizumab, apatinib

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Edited by:
Weijia Fang,
Zhejiang University, China

Reviewed by:
Shi Ming,
Sun Yat-sen University Cancer Center (SYSUCC), China
Andrea Casadei Gardini,
University Hospital of Modena, Italy

****Correspondence:***
Tao Zhang
taozhangxh@hust.edu.cn
Jun Xue
xjunion@126.com

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INTRODUCTION

Liver cancer imposes a heavy disease burden and is the fourth leading cause of cancer-related mortality globally (1). Hepatocellular carcinoma (HCC) accounts for more than 80% of liver cancer (2). Almost 85% of HCCs occur in developing countries, especially in Eastern Asia and sub-Saharan Africa with am incidence rate of more than 20 per 100,000 individuals (2, 3).

Hepatocellular carcinoma is likely to invade the adjacent vasculature. Portal vein tumor thrombosis (PVTT) is the main form of macrovascular invasion with a prevalence rate ranging from 44 to 62% at autopsy (4). HCC patients with PVTT have an extremely poor prognosis, with overall survival (OS) as low as 2.7 to 4 months if untreated (5).

Currently, the treatment strategies for HCC patients with PVTT are still debated. The Barcelona Clinic Liver Cancer staging and management system considers HCC with PVTT at least advanced HCC (stage C). The standard care recommended for advanced HCC patients is systemic therapy, with the oral kinase inhibitors sorafenib or lenvatinib as the first-line treatment, and regorafenib or Nivolumab as the second-line therapy (5–7). In addition, multiple efforts have been invested in surgery, transarterial chemoembolization (TACE), radiotherapy (RT), and various combinations. The combined therapy strategies show promising results, however, we need prospective studies to validate the efficacy among HCCs with PVTT (4, 5).

Recently, the safety and efficacy of the combined treatment with programmed cell death 1 (PD-1)/PD-1 ligand (PD-L1) inhibitors plus molecular targeted medicine were demonstrated. A phase I clinic trial (NCT02942329) assessing the safety and efficacy of camrelizumab (anti–PD-1 monotherapy) combined with apatinib (VEGFR2 inhibitor) as a second line, or later treatment in advanced HCC showed that the objective response rate (ORR), disease control rate (DCR), and median time to response were 50.0% [95% confidence interval (CI) = 24.7–75.4%], 93.8% (95% CI = 69.8–99.8%), and 3.4 months (range, 1.4–9.7 months) (8). Besides, an earlier interim analysis of a phase Ib clinical trial (KEYNOTE 524 and NCT03006926) on the combined treatment of pembrolizumab plus lenvatinib in HCC patients showed that the ORR was 42.3% (95% CI = 23.4–63.1%), and the estimated median duration of progression-free survival (PFS) was 9.69 months (95% CI = 5.55 to not evaluable) (9, 10). The US Food and Drug Administration approved pembrolizumab plus lenvatinib as a potential first-line therapy for advanced unresectable HCC patients who were not eligible to locoregional therapy (9, 11).

Recent advances in RT technology had shown that external beam RT was an effective and safe alternative treatment for HCC patients, and its role was evolving from a palliative tool to a curative one. A randomized clinic trial compared the combined therapy of TACE and RT with sorafenib alone in untreated HCC patients with macroscopic vascular invasion. The results showed the TACE plus RT group had a significantly better prognosis than the sorafenib group, with median time to progression (31.0 vs. 11.7 weeks; $P < 0.001$) and OS (55.0 vs. 43.0 weeks; $P = 0.04$), respectively (12). Another randomized study compared neoadjuvant RT plus hepatectomy with hepatectomy alone in resectable HCC patients with PVTT. The results showed that the OS rates and the disease-free survival rates of the RT plus hepatectomy group were significantly higher than those of the hepatectomy alone group (13).

Although promising results had been demonstrated for the use of RT combined with locoregional treatments in HCC with PVTT, the efficacy and safety of RT with systemic therapy had not been reported. Thus, we attempt to perform a randomized controlled study to assess the efficacy and safety of stereotactic body RT (SBRT) combined with camrelizumab and apatinib as first-line therapy for HCC patients with PVTT.

Abbreviations: 95% CI, 95% confidence interval; AEs, Adverse events; CTV, Clinical tumor volume; DCR, Disease control rate; HCC, Hepatocellular carcinoma; MRI, Magnetic resonance imaging; NCI-CTCAE, National Cancer Institute–Common Terminology Criteria for Adverse Events; ORR, Objective response rate; OS, Overall survival; PFS, Progression-free survival; PVTT, Portal vein tumor thrombosis; QOL, Quality of life; RT, Radiotherapy; SBRT, Stereotactic body radiotherapy; TACE, Transarterial chemoembolization; ULN, Upper limits of normal.

METHODS AND ANALYSIS

Study Design

This multicenter, open-label, randomized controlled trial will be conducted in 11 hospitals in China. The study has been authorized by the ethics committee of each center, and all the patients will provide written informed consent before registration. The trial has been registered in Chinese Clinical Trial Registry with number ChiCTR1900027102.

The PVTT diagnosis was made on typical radiological pattern identified on ultrasound, contrast-enhanced ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and/or histopathology findings (14). PVTT was classified into five groups based on Cheng's classification: I0: microscopic tumor thrombus; I: tumor thrombus located in segmental or sectoral branches of the portal vein; II: right- or left-side branch of portal vein; III: main trunk of the portal vein; and IV: superior mesenteric vein (14, 15).

Recruitment started in January 2020 and is estimated to continue until December 2021. Eligible HCC patients with PVTT are randomly allocated 1:1 to receive either camrelizumab, apatinib plus SBRT, or camrelizumab plus apatinib (**Figure 1**). Randomization will be performed using a minimization method with the following stratification factors: the presence or absence of extrahepatic metastases and level of AFP (AFP $\geq$ 400 ng/mL or <400 ng/mL). Monitoring will be carried out in this trial.

We will use OS as the primary endpoint and PFS, ORR, DCR, adverse events (AEs), and quality of life (QOL) as the secondary endpoints.

Selection of Subjects

Eligibility Criteria

Patients with HCC diagnosed by histopathology or clinical criteria of European Association for the Study of Liver guidelines will be screened using the following inclusion and exclusion criteria (6). Noted that, in this study, tumor burden will not be

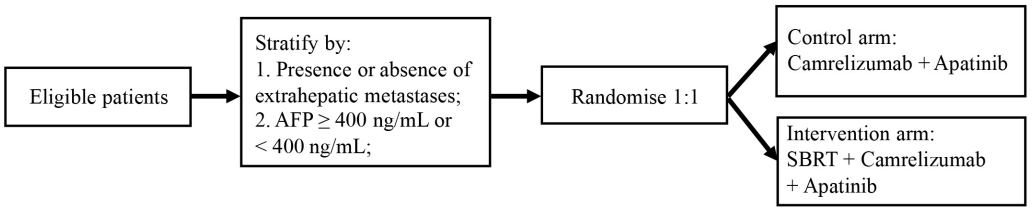

FIGURE 1 | The study design of SBRT combined with camrelizumab and apatinib in HCC patients with PVTT.

considered; i.e., diffuse or multiple tumors invade both lobe of the liver, or huge tumor more than 10 cm in diameter; as long as they meet the following criteria, the patient could be enrolled.

Inclusion Criteria

- Patients willing to participate in the study and give written informed consent;
- patients aged ≥ 18 years;
- Cheng's type II/III/IV PVTT;
- patients with recurrent HCC after locoregional treatment, such as hepatectomy, RT, TACE, hepatic artery perfusion, and radiofrequency ablation, which has been accomplished at least 4 weeks before the baseline imaging scan, and the grade of toxic reactions (except for hair loss) caused by the locoregional treatment should recover to less than 1 according to the National Cancer Institute–Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 5.0;
- patients did not receive any systemic therapy previously;
- at least one HCC lesion which can be accurately measured on CT or MR images with at least one dimension ≥ 10 mm;
- ECOG performance status ≤ 1;
- Child–Pugh A;
- adequate hematological function: absolute neutrophil count ≥ 1.5×10^9/L, hemoglobin ≥ 90 g/L, and platelet count ≥ 75×10^9/L;
- adequate kidney function: creatinine < 1.5 × upper limit of normal (ULN), creatinine clearance rate > 50 mL/min;
- adequate hepatic function: albumin ≥ 29 g/L, total bilirubin ≤ 1.5 × ULN; alkaline phosphatase (AKP), aspartate transaminase, or alanine transaminase ≤ 5 × ULN; and
- patients with HBV infection will be enrolled if the HBV-DNA is less than 500 IU/mL or 2,500 copies/mL, and the patients receive at least 14 days of anti-HBV treatment before enrollment.

Exclusion Criteria

- Patients with cholangiocarcinoma, sarcomatoid HCC, mixed cell carcinoma, fibrolamellar cell carcinoma, or a history of other cancer in the past 5 years;
- patients who have moderate or severe ascites with clinical symptoms (i.e., those who need therapeutic puncture and drainage), or uncontrolled pleural effusion or pericardial effusion;
- patients with severe gastrointestinal bleeding, gastrointestinal perforation, or intestinal obstruction, or who were unable to swallow within 6 months before enrollment;
- patients with severe infection;
- patients with a history of embolism, cerebral infarction, or lung infarction;
- patients with a history of uncontrolled or unstable angina, uncontrolled hypertension, arrhythmias, cardiac insufficiency, congestive heart failure, or cardiac infarction occurring less than 6 months before registration;
- patients with interstitial pneumonia, interstitial lung disease, autoimmune diseases, innate, or acquired immune deficiency;
- systemic treatment with steroids or strong CYP3A4/CYP2C19 inducer or inhibitors within 14 days before enrollment;
- a history of serious drug allergy to monotherapy or targeted therapy;
- women who are pregnant or intend to become pregnant, or men whose partner is considering pregnancy; and
- patients who are currently enrolled in other investigational therapeutic drug or device studies.

Interventional Methods

Immunotherapy

Each cycle of the treatment will be 6 weeks. A fixed dose of 200 mg camrelizumab will be administered intravenously every 3 weeks. In the study group, the first dose of camrelizumab will be given within 7 days after; in the control group, subjects will receive camrelizumab on day 1 of cycle 1. Camrelizumab administration will continue until intolerable toxicity or disease progression occurs. If AE, laboratory test abnormality or intercurrent disease happens, camrelizumab treatment will be delayed, but the dosage cannot be reduced. Treatment interruption will be allowed for no more than 12 weeks (either continuously or intermittingly); otherwise, the patients will be withdrawn from the study. Patients with progressive disease will continue the treatment if the investigator estimates they may still get clinical benefit and will be reevaluated after each cycle (6 weeks).

Targeted Therapy

The dose of oral apatinib will be 250 mg once daily. Similarly, the first dose of apatinib will be given within 7 days after SBRT for the patients of study arm and will be given on day 1 of the first cycle

for the patients of control arm. Dosage modifications (first dose reduction: 200 mg, 5 days on, 2 days off; second dose reduction: 200 mg, every 2 days) or treatment suspension will be allowed, resulting from grade 2 non-hematologic, or grade 3 hematologic toxicity. Dosage modifications could be made twice in every cycle (6 weeks). However, once the dose reduces, it could not be re-escalated.

Apatinib will be discontinued when the toxicity is still intolerable through two dose modifications.

For both camrelizumab and apatinib, treatment cycles will be continued until unacceptable toxicity or disease progression occurs or patient's request for withdrawal from the study. After discontinuation of apatinib, subjects will be allowed to continue to receive camrelizumab, and *vice versa*.

Radiotherapy

Stereotactic body RT will be started within 1 week after enrollment for the patients of study group. 4DCT will be used for treatment planning and evaluating the tumor motion. The gross tumor volume (GTV), defined by contrast-enhanced CT or MRI, will encompass the tumor and PVTT if this can meet the dose-volume constraints for the organs at risk. Otherwise, only PVTT will be regarded as the GTV (16). The clinical tumor volume (CTV) is produced by expanding GTV with 5 to 10 mm. The planning target volume (PTV) is constructed by adding 5 to 10 mm to CTV in all directions. A total of 36 to 40 Gy for the PTV with the fraction size of 6 to 8 Gy will be administered by using 6-MV x-rays with a linear accelerator at five fractions per week (Varian Medical Systems).

The radiation dose volume constraints for organs at risk are as follows: for liver, total spared volume (V_{total}-$V_{15\ \text{Gy}}$) should be more than 700 mL and/or $V_{15\ \text{Gy}}$ should be less than 1/3 V_{total}; for spinal cord, $V_{1\ \text{mL}}$ should be less than 15 Gy; for stomach and small bowel, $V_{1\ \text{mL}}$ should be less than 5 Gy; for duodenum, $V_{1\ \text{mL}}$ should be less than 25 Gy; for kidneys, 1/3 V_{total} should be less than 15 Gy (17).

Assessment

Tumor Response Assessment

Baseline radiological scan will be performed within 28 days prior to the first study treatment. During the treatment period, imaging assessment for efficacy will be conducted every 6 weeks. Baseline and each subsequent assessment must follow the same radiological procedures, which include chest CT and abdomen and pelvis MRI. Brain MRI is also required at baseline, but is not necessary during subsequent tumor assessment if tumor was not detected initially. RECIST v1.1 is utilized for assessment of treatment response. At the discretion of investigators, radiological scans should be repeated at any time if progression is suspected. Patients who discontinue study treatment without progression (e.g., AEs) will be followed for tumor assessments until the patients experience progression, withdraw consent, die, or until the study terminates, whichever occurs first. Patients who continue camrelizumab/apatinib treatment beyond radiographic disease progression will be monitored with a follow-up scan at least 6 weeks later or at the next scheduled tumor assessment. For patients who continue treatment based on investigator assessment of clinical benefit, tumor assessment will continue until treatment discontinuation.

Quality of Life

Two questionnaire [the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire C30 (EORTC-QLQ-C30) and the European Organization for Research and Treatment of Cancer Hepatocellular Carcinoma Quality of Life Questionnaire 18 (EORTC-QLQ-HCC18)] will be utilized to assess the QOL scores at baseline and each follow-up (18).

Follow-Up

A safety follow-up visit is required for each patient after treatment discontinuation. Patients who show progression will have the safety follow-up at the last follow-up visit when the response assessment shows progression and results in treatment discontinuation. Patients who discontinue treatment for any reason need to return to the clinic for a safety visit within 30 ± 7 days since their last treatment in the case of a treatment-emergent AE.

After study treatment discontinuation, every patient will be followed up for study drug–related serious AEs (SAEs) and survival status. These follow-ups will start 3 months after the safety visit and be done every 3 months until loss to follow-up, death, consent withdrawal, or trial termination by the investigator. Information of survival status and subsequent antitumor treatment will be collected via telephone calls, patient medical records, and/or clinic visits.

Sample Size

Sample size of the study is calculated using log-rank test. The estimated OS is 11 months for the study arm and 7 months for the control arm (8, 19–21). The estimated study duration will be 3.5 years, including 2 years of recruitment, and 1.5 years of follow-up. The upper limit of 95% CI of hazard ratio is 0.636 (two-sided) with 5% probability errors and 80% power. Thus, the total sample size required is 264 (132 per arm) after taking into account of a 5% dropout rate.

Statistical Analysis

Statistical analyses will be performed with SAS software (version 9.3; SAS Institute, Cary, NC, United States). $P < 0.05$ will be interpreted as significant, and we will be able to reject the null hypothesis. Kaplan–Meier curves will be plotted to compare OS and PFS between the two groups by means of log-rank test. Duration of overall response (DOR) and ORR will be calculated based on binomial distributions using two-sample Cochran–Mantel–Haenszel method. Independent-sample *t* test or Wilcoxon rank-sum test will be applied to compare QOL score between both groups. Descriptive statistics will be analyzed for safety data.

Outcome Definitions

- Overall survival: duration from randomization to death (regardless the cause);
- Progression-free survival: duration from randomization to progression or death whichever is earlier;

- Objective response rate: percentage of patients with a complete response (CR) or partial response (PR; using RECIST 1.1) and for a minimum duration, usually measured from the of treatment initiation to disease progression;
- Disease control rate: the proportion of patients who achieve CR, PR, or stable disease (SD);
- Adverse events: AEs will be record based on NCI-CTCAE Version 5.0;
- Quality of life: QOL scores will be measured according to the QLQ-C30 and QLQ-HCC 18 questionnaire.

ANTICIPATED RESULTS

Overall survival will be used as a primary endpoint, and PFS, ORR, DCR, AEs, and QOL will be as secondary endpoints. We expect that the OS, PFS, ORR, and DCR of the study group will be significantly better than those of the control group, but there will be no difference for AEs and QOL.

DISCUSSION

Portal vein tumor thrombosis is one of the most ominous prognostic factors in HCC. Not only may tumor thrombus cause intrahepatic tumor dissemination, but it can also rapidly decrease blood flow to the liver, resulting in portal hypertension and deterioration of liver function reserve. This, in turn, may reduce tolerance to treatment (4, 14). Although varied therapeutic patterns have been recommended for HCC patients with PVTT, the optimal treatment is still undetermined.

Because HCC tumor thrombus progresses very quickly, fast lessen tumor thrombus volume will be helpful for the following treatment of the primary cancer lesion. It has demonstrated that ORR is changing from 39 to 62% for RT in HCC patients with PVTT (12, 22–24). Although RT alone could achieve a high locoregional tumor control rate, combining systemic treatments with RT seems necessary because of the failure outside the radiation field (12, 25). Proper tumor oxygenation is good for enhancing the RT efficacy, so improvement of tumor hypoxia has been explored using antiangiogenic agents. Besides, combined immunotherapy with RT could have independent antitumor efficacy and may induce the radiation abscopal effect. A well-known mechanism for the enhancement of antitumor immune response is that RT could recruit immune cells and induce the death of immunogenic cell (26). Previous studies showed us a synergistic effect between antiangiogenic agents and immunotherapy (camrelizumab plus apatinib or pembrolizumab plus lenvatinib) (8, 9). PD-1/PD-L1 blockade could sensitize tumors to antiangiogenic treatment and increase its efficacy, and the latter could also facilitate PD-1/PD-L1 therapy because induction of intratumoral high endothelial venules will enhance infiltration and activity of cytotoxic T lymphocytes (26).

However, prospective studies investigating these observations are scarce. In this clinic trial, we sought to evaluate the efficacy and safety of combination SBRT with camrelizumab and apatinib in the HCC patients with PVTT. If the anticipated results above could be achieved, it would provide strong evidence of a new first-line therapy in HCC patients with PVTT.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by China Ethics Committee of Registering Clinical Trials. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JX and TZ devised the study concept and design. YH drafted the manuscript. TQ was responsible for the statistics. SL, TQ, and YH have made substantial contribution to the study protocol. JX, TZ, SL, TQ, and YH reviewed and approved the final version of this article. All authors contributed to the article and approved the submitted version.

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Clinical Outcomes and Prognosis Factors of Nivolumab Plus Chemotherapy or Multitarget Tyrosine Kinase Inhibitor in Multi-Line Therapy for Recurrent Hepatitis B Virus-Related Hepatocellular Carcinoma: A Retrospective Analysis

Chao Chen[1†], Li An[2†], Ying Cheng[1], Xianwen Luo[1], Zixiong Li[1] and Xiufeng Liu[1*]*

[1] Department of Medical Oncology of PLA Cancer Center, Jinling Hospital, Nanjing, China, [2] Department of Gerontology, The Affiliated Zhongda Hospital of Southeast University, Nanjing, China

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Edited by:
Weijia Fang,
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Li Xu,
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***Correspondence:**
Zixiong Li
lzx1989@126.com
Xiufeng Liu
njjloncologylxf@163.com

† These authors have contributed equally to this work

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Background: This study investigates the potential predictors of nivolumab plus chemotherapy or multitarget tyrosine kinase inhibitor (TKI) treatment response in patients with recurrent hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC).

Methods: Patients with recurrent hepatitis B virus-related HCC who underwent nivolumab plus chemotherapy or TKI treatment between July 2017 and June 2019 at Jinling Hospital in China were retrospectively evaluated and included in this study. These patients also had both complete medical charts and follow-up data available. Overall survival (OS) and progression-free survival (PFS) were calculated from the date of nivolumab initiation. Survival data were compared using log-rank tests, and the associations of patient characteristics with survival were estimated using Cox regression models.

Results: A total of 22 HCC patients were included in this cohort and constituted the basis for this analysis. Twenty progressed cases (91%) and 16 deaths (73%) were identified at a median follow-up of 8.8 months (range 1–25). The median OS from the time of nivolumab initiation was 10.7 months (95% CI, 0.8–20.6 months), with a median PFS of 5.1 months (95% CI, 3.1–7.0 months). The patients were divided into two risk groups according to a nomogram built by age, Eastern Cooperative Oncology Group (ECOG) status, hepatectomy status, and transarterial chemoembolization (TACE) use. The median PFS was 8.2 ± 2.8 months in the low-risk group compared with 1.9 ± 0.4 months in the high-risk group ($p = 0.0018$). The median OS was estimated as 16.8 ± 4.9 months for low-risk patients vs. 8.6 ± 3.5 months for high-risk patients ($p = 0.13$).

Conclusion: Nivolumab combined with chemotherapy or TKI treatment is effective in patients with recurrent hepatitis B virus-related HCC. It is observed that previous TACE treatment is associated with a better PFS, and worse PFS in those patients who received hepatectomy. Prospective studies are warranted to evaluate the effects of nivolumab combined chemotherapy or TKI on recurrent hepatitis B virus-related HCC.

Keywords: hepatocellular carcinoma, hepatitis B virus, nivolumab, chemotherapy, tyrosine kinase inhibitor, programmed cell death protein 1

INTRODUCTION

Hepatocellular carcinoma (HCC) is currently ranked as the third leading cause of cancer-related mortality worldwide (1). In China, HCC has become the second leading cause of cancer-related deaths. The HCC mortality rate has been increasing, particularly in males aged 45 to 74 years old with chronic hepatitis B and hepatitis C viral infection, over recent decades (2). Hepatic resection remains the mainstay for curative treatment of HCC (3). However, long-term outcomes after resection remain unsatisfactory, with a high rate of recurrence of up to 60 to 70% within 5 years (1, 4, 5). Although guidelines have been published in the management of primary HCC, the management of recurrent HCC remains poorly defined.

The inhibition of both programmed cell death protein 1 (PD-1)/PD-1 ligand (PD-L1) and/or CTLA-4 signaling pathways by monoclonal antibodies (MAbs) to release the antitumor activity of preexisting tumor-specific T-cell immunity has initiated a new era for immunotherapy in oncology. Immune checkpoint inhibitors (ICPIs) (anti-PD-1 MAb such as nivolumab or pembrolizumab; anti-PD-L1 MAb such as atezolizumab, durvalumab, or avelumab; and CTLA-4 inhibitors such as ipilimumab or tremelimumab) have demonstrated a survival benefit and/or durable disease control in several advanced cancers. Nivolumab is an immune checkpoint inhibitor that blocks the interaction between programmed cell death protein 1 (PD-1) and its ligand PD-L1. Nivolumab has confirmed efficacy for the treatment of various tumor types (6–13). It is a fully human immunoglobulin (IgG4) monoclonal antibody inhibitor of PD-1 receptor, which has received accelerated US FDA approval in 2017 for advanced HCC patients who previously received sorafenib. Its safety and efficacy have been confirmed in the extensive cohort study of HCC patients, CheckMate 040 (NCT01658878). Nivolumab was associated with an improved median overall survival (OS) from 14.7 to 16.4 months in a randomized phase III study as the first-line setting of advanced HCC (NCT02576509) (14). Nivolumab has shown clinical antitumor activity in patients with advanced HCC. Nevertheless, studies have shown that compared to Sorafenib, OS and ORR improvement is seen in single Nivolumab treatment, but OS benefit cannot be concluded from these data. A prospective, randomized, controlled, international multicentered Phase III hepatocellular carcinoma study (EACH Study) initiated by our research team in 2013 showed that the median OS in the FOLFOX4 group, dominated by Oxaliplatin, had a median OS of 6.40 months, with an effective rate of 8.15%, and this study provides a new treatment option for patients with advanced hepatocellular carcinoma (15). To further improve the effectiveness of treatment in patients with liver cancer, nivolumab combined chemotherapy or multitargeted tyrosine kinase for hepatocellular carcinoma later line therapy was tried.

Many trials are underway to expand its application in different populations, as well as in combination approaches (16).

Consequently, we conducted a retrospective study to describe the clinical outcomes of nivolumab combined with chemotherapy or TKI treatment in patients with recurrent hepatitis B virus (HBV)-related HCC.

METHODS

Patients

A total of 22 patients with recurrent hepatitis B virus-related HCC who started nivolumab treatment between July 2017 and June 2019 at Jinling Hospital in China were included in this study. We used the following inclusion criteria: All patients were recurrent hepatitis B virus-related HCC, aged 18 to 75 years, disease progression after sorafenib and lenvatinib in first-line treatment, Eastern Cooperative Oncology Group performance status (ECOG PS) 1–3, tumor base diameter >10 mm, and expected survival longer than 3 months, treatment period <3 months of nivolumab, or patients without follow-up.

Baseline and follow-up clinical data were collected retrospectively. The study was presented to, and approved by, the ethics committee of the hospital.

Nivolumab Plus Chemotherapy or TKI Treatment

Patients received 3 mg/kg intravenous nivolumab every 3 weeks until disease progression, combined with TKIs (three patients treated with Sorafenib, eight patients treated with lenvatinib, and four patients treated with Regorafenib) in 15 patients and chemotherapy (oxaliplatin plus fluorouracil/leucovorin) in seven patients.

Efficacy Assessment

Tumor response was evaluated using computed tomography or magnetic resonance imaging every two cycles (6 weeks) according to Response Evaluation Criteria in Solid Tumors (RECIST) guidelines, version 1.1. The efficacy was divided into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). The overall response rate (RR) was calculated by CR + PR, and the disease control rate

TABLE 1 | Demographic patient characteristics.

Characteristic *N* = 22	No. (%)
Median age (range), years	53 (36–71)
<63	17 (77%)
≥63	5 (23%)
Sex	
Male	19 (86%)
Female	3 (14%)
ECOG performance status (range)	1–3
<2	17 (77%)
≥2	5 (23%)
AFP, (range) ng/ml	1–68,368
<40	10 (45%)
≥40	12 (55%)
HBV-DNA, (range) copies/ml	0–7,300
<215	15 (68%)
≥215	7 (32%)
Child-Pugh	
A	13 (59%)
B	9 (41%)
Hepatectomy	
Yes	8 (36%)
No	14 (64%)
TACE	
Yes	11 (50%)
No	11 (50%)
Number of nivolumab cycles (range)	1–28
<9	13 (59%)
≥9	9 (41%)
Single dose of nivolumab, (range) mg	100–240
<200	3 (14%)
≥200	19 (86%)
Combined treatment	
Targeted therapy	15 (68%)
Chemotherapy	7 (32%)

(DCR) was calculated by CR + PR + SD. Adverse events were evaluated based on the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 4.0.

Statistical Analysis

The patients were divided according to clinically meaningful cutoff values of factors using the ROC method. Survival curves were calculated by the Kaplan–Meier method, and univariate analysis was performed using the log-rank test. Factors with $p < 0.1$ on the univariate analysis were entered into the multivariate Cox regression model. Values of significance were set at $p = 0.05$. All analyses were performed using SPSS 21.0.

RESULTS

Patient Characteristics

Patients' baseline and treatment information are shown in **Table 1**. The median age of the patients was 53 years (range: 36–71), and 19 of the 22 patients (86%) were male. The ECOG grade was 0–1 in 17 patients (77%) and 2–3 in 5 patients (23%). Thirteen patients (59%) were classified as Child–Pugh class A, and nine (41%) were classified as Child–Pugh class B. Eight (36%) patients underwent hepatectomy. Transarterial chemoembolization (TACE) was administered in 11 patients (50%).

Clinical Outcome of Nivolumab Plus Chemotherapy or TKI Treatment

Complete imaging data of 21 patients were obtained. Patients were evaluated by spiral CT every 6 weeks: 1 (4.8%) case for CR, 2 (9.5%) cases for PR, 10 (47.6%) cases for SD, 8 (38%) cases for PD (including 1 case of hyper-progressive disease, HPD), RR 14.3%, DCR 61.9%. Tumor shrinkage is noted in **Figure 1**.

There were 20 cases of progression (91%) and 16 cases of deaths (73%) at a median follow-up of 8.8 months (range 1–25). The median OS from the time of nivolumab initiation was 10.7 months (95% CI, 0.8–20.6 months), with a median PFS of 5.1 months (95% CI, 3.1–7.0 months). In the univariate analysis, the following variables were found to be associated with prognosis: age, Child–Pugh grade, ECOG status, hepatectomy, TACE, and the number of nivolumab cycles (**Table 2**). The multivariate analysis retained the following independent prognostic factors for PFS: age [hazard ratio (HR) 0.12, $p = 0.044$], hepatectomy (HR 13.1, $p = 0.009$), TACE (HR 0.09, $p = 0.004$), and number of nivolumab cycles (HR 0.09, $p = 0.010$). In the multivariate analysis, only Child–Pugh grade was an independent predictor of OS (HR, 0.20, 95% CI 0.05 to 0.81, $p = 0.024$).

We also divided the patients into two risk groups according to a nomogram (**Figure 2**) built by age, ECOG status, hepatectomy status, and TACE use. The median time of PFS was 8.2 ± 2.8 months in the low-risk group compared with 1.9 ± 0.4 months in the high-risk group ($p = 0.0018$; **Figure 3A**). The median time of OS was estimated at 16.8 ± 4.9 months for low-risk patients vs. 8.6 ± 3.5 months for high-risk patients ($p = 0.13$; **Figure 3B**).

Adverse Events

Treatment-related AEs of any grade were less frequent. The majority of select AEs were grades 1 to 2, and the main adverse reactions included six cases of anorexia (27.3%), five cases of diarrhea (22.7%), four cases of hypothyroidism (18.2%), and two cases of hypophysitis (9.1%). The most frequently reported any-grade, treatment-related, select AE categories with nivolumab treatment were hypothyroidism (18.2%), diarrhea (9%), and hypophysitis (9%). Any grade and grade 3 or greater treatment-related serious events were reported in 4.5% and 1 of 22 patients, respectively. There is 4.5% of serious AE in upper gastrointestinal bleeding (**Table 3**). The immune-related adverse events (irAEs) are shown in **Table 4**.

DISCUSSION

To our knowledge, this retrospective study is the first analysis of the efficacy of nivolumab treatment in patients with recurrent HBV-related HCC, which is a growing population with a poor prognosis in China. More than 70% of Chinese patients with

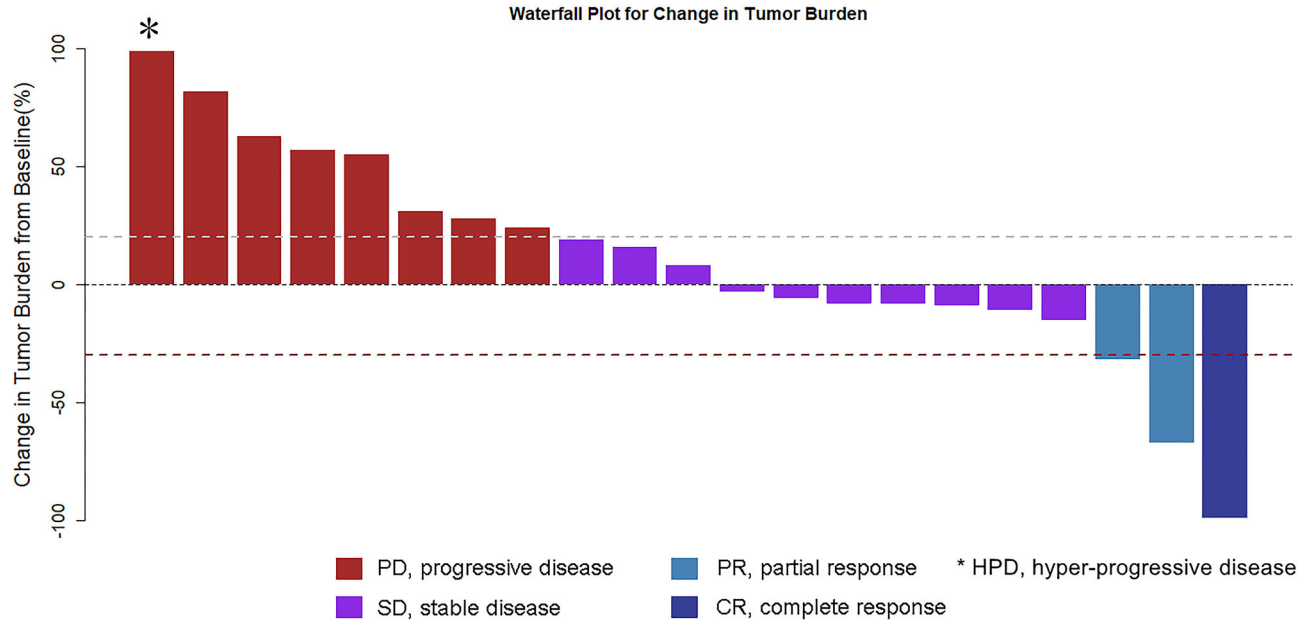

FIGURE 1 | Waterfall plot for the best percentage change in target lesion size.

TABLE 2 | Survival analysis of nivolumab treatment in patients with advanced/relapsed hepatitis B virus-related hepatocellular carcinoma.

	Parameter	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*p*	HR (95% CI)	*p*
PFS	Gender (Male vs. Female)	0.50 (0.14–1.80)	0.286	0.35 (0.05–2.50)	0.294
	Age (≥63 vs. <63)	0.19 (0.04–0.84)	0.029	0.12 (0.02–0.94)	0.044
	HBV-DNA (≥215 vs. <215)	2.24 (0.84–5.93)	0.105	1.03 (0.34–3.07)	0.964
	AFP (≥40 vs. <40)	1.09 (0.42–2.84)	0.863	3.72 (0.48–28.54)	0.207
	Child-Pugh (A vs. B)	0.43 (0.16–1.13)	0.085	4.12 (0.76–22.40)	0.101
	ECOG (≥2 vs. <2)	2.87 (0.95–8.67)	0.061	6.88 (0.94–50.22)	0.057
	Hepatectomy (Yes vs. No)	2.85 (1.01–8.03)	0.048	13.10 (1.92–89.35)	0.009
	TACE (Yes vs. No)	0.42 (0.16–1.13)	0.085	0.09 (0.02–0.46)	0.004
	Number of nivolumab cycles (≥9 vs. <9)	0.28 (0.10–0.80)	0.017	0.09 (0.01–0.56)	0.010
	Single dose of nivolumab (≥200 vs. <200)	1.97 (0.45–8.65)	0.371	0.57 (0.06–5.42)	0.624
	Combined treatment (targeted therapy vs. chemo)	0.60 (0.21–1.74)	0.345	0.54 (0.11–2.61)	0.446
OS	Gender (Male vs. Female)	0.68 (0.14–3.21)	0.625	1.99 (0.27–10.31)	0.424
	Age (≥63 vs. <63)	0.17 (0.02–1.31)	0.089	0.16 (0.02–1.35)	0.092
	HBV-DNA (≥215 vs. <215)	2.21 (0.76–6.42)	0.143	1.62 (0.52–5.01)	0.406
	AFP (≥40 vs. <40)	1.60 (0.54–4.82)	0.398	2.33 (0.56–9.78)	0.248
	Child-Pugh (A vs. B)	0.21 (0.06–0.74)	0.015	0.20 (0.05–0.81)	0.024
	ECOG (≥2 vs. <2)	2.72 (0.77–9.69)	0.122	0.66 (0.13–3.32)	0.618
	Hepatectomy (Yes vs. No)	1.87 (0.61–5.81)	0.276	2.17 (0.59–7.95)	0.241
	TACE (Yes vs. No)	0.55 (0.19–1.61)	0.273	1.19 (0.30–4.75)	0.802
	Number of nivolumab cycles (≥9 vs. <9)	0.26 (0.07–0.93)	0.039	0.44 (0.12–1.61)	0.214
	Single dose of nivolumab (≥200 vs. <200)	4.21 (0.54–32.96)	0.171	0.80 (0.06–10.35)	0.863
	Combined treatment (targeted therapy vs. chemo)	1.02 (0.31–3.32)	0.970	1.03 (0.23–4.70)	0.968

HCC have HBV infection, whereas the majority of patients with HCC in Western developed countries have HCV infection (17). HCC patients with HBV infection are more prone to develop progressive diseases and have a poorer prognosis than HCC patients with HCV infection (18). The results showed that the median OS in this cohort was 10.7 months, which is higher

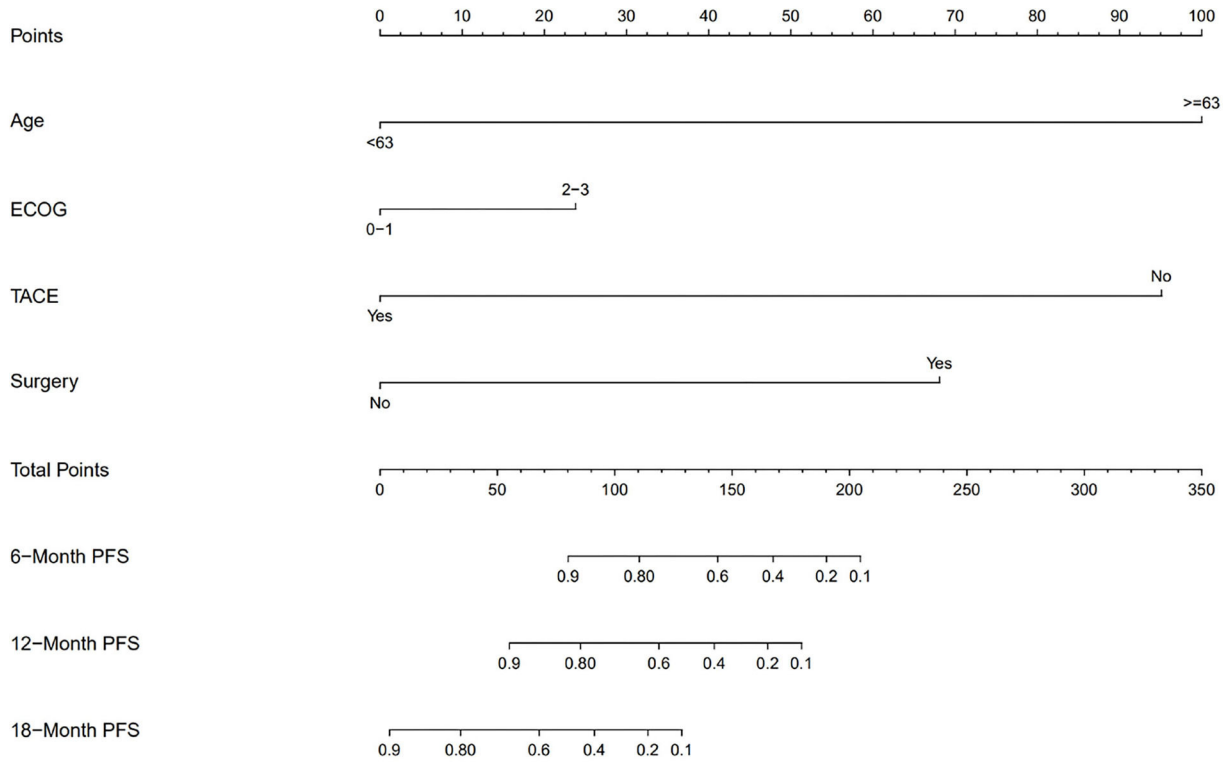

FIGURE 2 | Nomogram for predicting the progression-free survival (PFS).

than that in patients with recurrent HCC who received liver transplantation and were ineligible for surgical intervention; among these patients, median OS was reported to be 5 months (19). Eastern and Western HCC are highly heterogeneous; HCC patients in China are mainly hepatitis B virus-related, while in the CheckMate 459 study group of patients from China, only a total of 87 cases are mainly hepatitis B virus-related, with more non-hepatitis patients. Therefore, these patients are relatively less effective (15). This study showed that nivolumab displayed antitumor activity in recurrent HCC patients, even for a population with HBV infection.

Although immune checkpoint inhibitors can enhance the intrinsic tumor-suppressive microenvironment of the liver, another antitumor therapy is needed as a combination to enhance the induction of T-cell responses. Such combination treatment could result in a dramatic improvement in efficacy and clinical outcome in patients with HCC (20). Combinatorial therapies include checkpoint blockade immunotherapy with chemotherapy, targeted therapies, surgery, radiation therapy, or newer immunotherapies.

In our study, a nomogram was developed to predict the prognosis of patients with recurrent HCC based on four significant factors: age, ECOG status, hepatectomy status, and TACE use. Several studies have shown that the duration of survival is somewhat shorter in elderly patients than in younger patients (21, 22). In recurrent or metastatic squamous cell carcinoma of the head and neck, nivolumab resulted in a higher median OS in patients under 65 years old than in patients ≥65 years old (8.2 vs. 6.9 months) (23). ECOG has a significant influence on survival and facilitates physician selection of certain treatments (24). Nivolumab led to shorter OS in patients with previously treated advanced squamous non-small-cell lung cancer with ECOG PS 2 vs. 0–1 (25). Multiple overlapping signaling pathways are involved in liver regeneration and hepatocarcinogenesis, including Wnt/β-catenin and Notch (26). These signaling pathways play an important role in regulating the crosstalk between the different compartments of the tumor microenvironment (27, 28), which has been observed to correlate with the response to checkpoint blocking antibodies (29, 30). The Notch signaling pathway suppresses tumor-infiltrating CD8+ T-cell activity (31). A low level of tumor-infiltrating CD8+ T cells might be a promising prognostic factor of HCC, especially for Asian patients (32). Patients with HCC had a higher proportion of CD4 (+) CD25 (+) Tregs in peripheral blood (33). The proportion of Tregs in patients who were in stable condition or were improving after TACE decreased significantly, whereas the proportion of Tregs in patients who deteriorated increased significantly after TACE (33). Treg-induced inhibition of IFN-γ secretion can be partially blocked by PD-1 antibodies specifically in HCC patients (34). The nomogram was used to identify HCC patients who benefited from nivolumab combined chemotherapy or TKI treatment. Patients with low risk showed a significantly improved PFS (45% at 1 year, $P = 0.0018$) and a trend of improved OS (57% at 1 year, $P = 0.13$). In this study, no

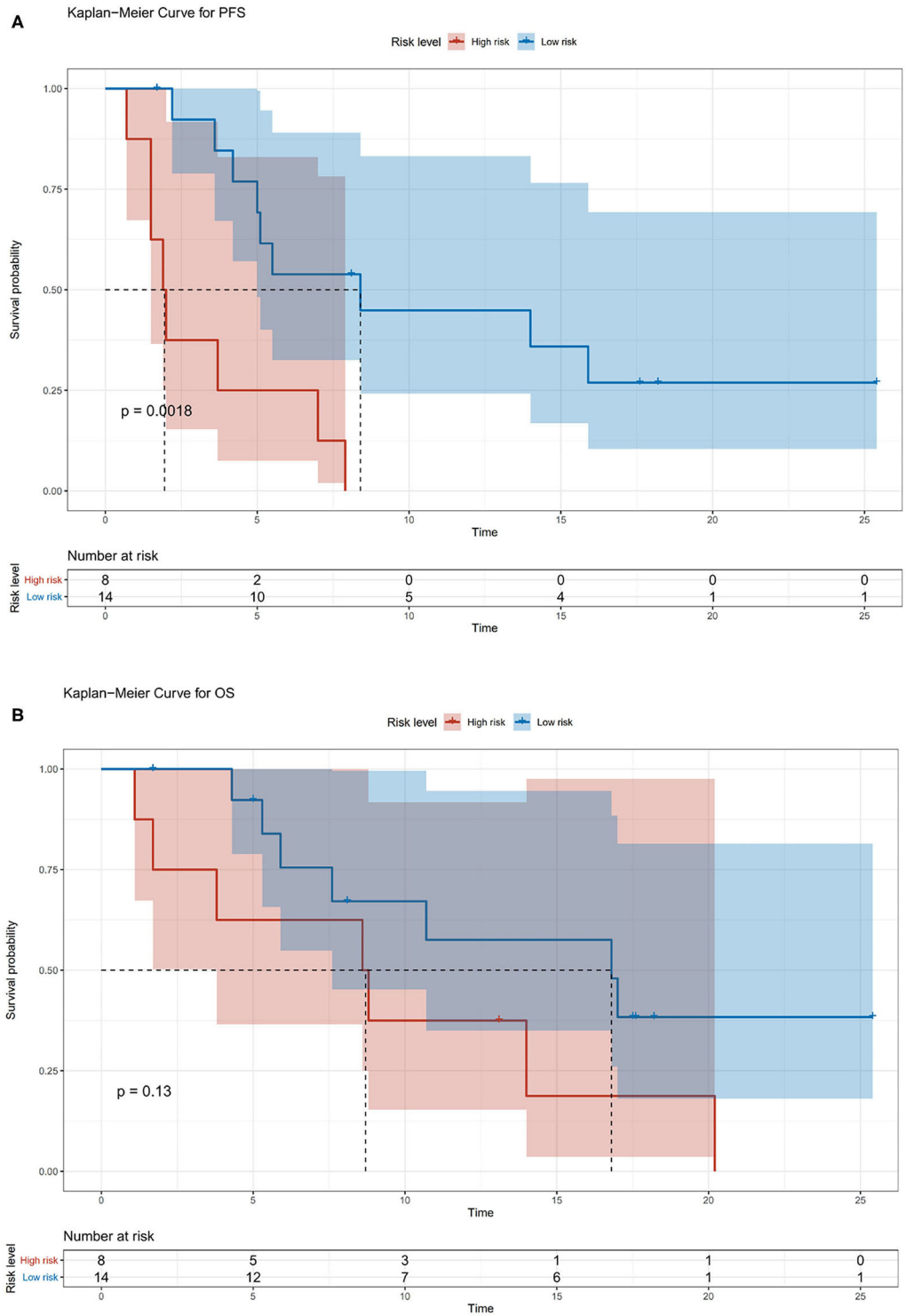

FIGURE 3 | (A) Kaplan–Meier survival curves for risk stratification in the cohort PFS. **(B)** Kaplan–Meier survival curves for risk stratification in the cohort overall survival (OS).

adverse dermatologic events were recorded, which is possibly due to the limited cases, and combined therapy might reduce the dermal toxicity.

The limitations of our study include, but are not limited to, the retrospective study and a small number of patients who were enrolled. According to multivariate analysis, due to

TABLE 3 | Common adverse events of clinical and laboratory abnormalities.

Adverse event	Grade 1/2		Grade 3/4		Patients, *n* (%)
	Chemotherapy	TKIs treatment	Chemotherapy	TKIs treatment	
Non-hematologic					
Hypertension	0	1	0	0	1 (4.5%)
Mucositis	0	1	0	0	1 (4.5%)
Hypothyroidism	1	2	0	1	4 (18.2%)
Fatigue	1	2	0	0	1 (4.5%)
Diarrhea	1	4	0	0	5 (22.7%)
Upper gastrointestinal bleeding	0	0	0	1	1 (4.5%)
Hypophysitis	1	1	0	0	2 (9.1%)
Anorexia	3	3	0	0	6 (27.3%)
Albuminuria	0	1	0	0	1 (4.5%)
Hyperbilirubinemia	0	1	0	0	1 (4.5%)
Hematologic					
Hemoglobin	0	0	0	1	1 (4.5%)
Leukocyte	0	0	1	0	1 (4.5%)
Platelets	0	0	1	0	1 (4.5%)

TABLE 4 | Immune-related adverse events.

Adverse event	Grade 1/2		Grade 3/4		Patients, *n* (%)
	Chemotherapy	TKIs treatment	Chemotherapy	TKIs treatment	
Mucositis	0	1	0	0	1 (4.5%)
Hypothyroidism	1	2	0	1	4 (18.2%)
Fatigue	1	2	0	0	1 (4.5%)
Diarrhea	1	1	0	0	2 (9.1%)
Hypophysitis	1	1	0	0	2 (9.1%)
Anorexia	1	1	0	0	2 (9.1%)
Albuminuria	0	1	0	0	1 (4.5%)
Hyperbilirubinemia	0	1	0	0	1 (4.5%)

the small sample size, several factors were associated with PFS with wide CIs, which reflected something not proper about the analysis methods. While RCTs remain the gold standard by which we base our treatment decisions, our retrospective analyses only provide important hypothesis-generating data, from which future practice-changing prospective trials can be built. The presence of tumor-infiltrating lymphocytes, expression of PD-L1, and tumor mutation burden within the liver were not tested to assess their roles in the response to immune checkpoint inhibition with nivolumab in our study. Larger and prospective clinical studies are needed to determine the most effective duration of immunotherapy combined TKI therapy and the best predictive biomarkers of response and to correlate the response. Combination therapy with checkpoint blockade is being investigated across a diverse range of tumor types and settings, including phase three trials (ClinicalTrials.gov numbers NCT01658878, NCT03439891, NCT03211416, NCT03418922, NCT03006926, NCT03347292, NCT01658878, NCT03299946, and NCT03289533).

In conclusion, nivolumab combined chemotherapy or TKI treatment is effective for patients with recurrent hepatitis B virus-related hepatocellular carcinoma; however, further research efforts are essential to confirm our data.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Jinling Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Comprehensive Study of Tumor Immune Microenvironment and Relevant Genes in Hepatocellular Carcinoma Identifies Potential Prognostic Significance

Wenbiao Chen[1], *Xujun Zhang*[1], *Kefan Bi*[1], *Hetong Zhou*[1], *Jia Xu*[1], *Yong Dai*[2]* and *Hongyan Diao*[1]*

[1] State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Disease, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, School of Medicine, The First Affiliated Hospital, Zhejiang University, Hangzhou, China, [2] Department of Clinical Medical Research Center, The Second Clinical Medical College of Jinan University, The First Affiliated Hospital Southern University of Science and Technology, Shenzhen People's Hospital, Shenzhen, China

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Fudan University, China

***Correspondence:**
Hongyan Diao
diaohy@zju.edu.cn
Yong Dai
daiyong22@aliyun.com

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Background: The tumor immune microenvironment (TIME) is an external immune system that regulates tumorigenesis. However, cellular interactions involving the TIME in hepatocellular carcinoma (HCC) are poorly characterized.

Methods: In this study, we used multidimensional bioinformatic methods to comprehensively analyze cellular TIME characteristics in 735 HCC patients. Additionally, we explored associations involving TIME molecular subtypes and gene types and clinicopathological features to construct a prognostic signature.

Results: Based on their characteristics, we classified TIME and gene signatures into three phenotypes (TIME T1–3) and two gene clusters (Gene G1–2), respectively. Further analysis revealed that Gene G1 was associated with immune activation and surveillance and included $CD8^+$ T cells, natural killer cell activation, and activated $CD4^+$ memory T cells. In contrast, Gene G2 was characterized by increased M0 macrophage and regulatory T cell levels. After calculation of principal component algorithms, a TIME score (TS) model, including 78 differentially expressed genes, was constructed based on TIME phenotypes and gene clusters. Furthermore, we observed that the Gene G2 cluster was characterized by high TS, and Gene G1 was characterized by low TS, which correlated with poor and favorable prognosis of HCC, respectively. Correlation analysis showed that TS had a positive association with several clinicopathologic signatures **[**such as grade, stage, tumor (T), and node (N)] and known somatic gene mutations (such as *TP53* and *CTNNB1*). The prognostic value of the TS model was verified using external data sets.

Conclusion: We constructed a TS model based on differentially expressed genes and involving immune phenotypes and demonstrated that the TS model is an effective prognostic biomarker and predictor for HCC patients.

Keywords: tumor immune microenvironment, gene, prognostic signature, immune activation, hepatocellular carcinoma

INTRODUCTION

A tumor is a neoplasm caused by gene mutations and adaptation of resultant mutant cells to the microenvironment (1). The tumor immune microenvironment (TIME) is a complex and dynamic network system composed of immune cells, stromal cells, and immune matrix, and it is associated with tumorigenesis (2). Previous studies report that TIME plays an immune surveillance role by inhibiting tumor proliferation and preventing escape of tumor cells from immune system regulation (3), whereas some studies report that TIME could regulate the occurrence and development of tumors (4). More recently, studies have shifted to better understanding the association between TIME and tumorigenesis. Genomic analysis is a standard approach for studying the structure, function, evolution, and effects of genomes on organisms (5). Several methods have been established to act as a bridge between gene expression and immune cell components. Applying CIBERSORT, a computational method for predicting cell composition in tumor transcriptomes, may help map prognostic genes and leukocyte subsets within and across cancers, elucidate the effect of tumor heterogeneity on cancer prognosis, and identify diagnostic and therapeutic biomarker targets (6). xCell is also the usual method to calculate cell subsets of TIMEs from transcriptomes, which helps to understand the complex cellular heterogeneity in tumor tissues, improve existing treatments, identify predictive biomarkers, and develop new treatment strategies (7). Additionally, several studies have demonstrated that TIME regulates host and immune cell populations and, thus, can be used for tumor prognosis (8, 9). Notably, the immunosuppressive effect of TIME on tumors is regulated by immune cell components, such as T and B lymphocytes, macrophages, natural killer cells, and dendritic cells. However, changes to immune cell components, especially regulatory T cells and macrophages, promote tumor progression (10). These cell populations offer immunotherapeutic strategies and diagnostic and prognostic biomarkers for many solid tumor types, such as lung cancer, hepatocellular carcinoma (HCC), and breast and gastric cancers (11–14).

HCC is the leading cause of cancer-related morbidity and mortality worldwide, and most incidences are associated with cirrhosis related to chronic hepatitis virus infection (15). Currently, it is believed that immune escape contributes to the development of HCC caused by viral hepatitis infection—particularly hepatitis B virus (16). The liver is a key immune organ that plays a protective role by promoting immune tolerance. However, changes in immune tolerance signals or escape from immune surveillance in pathological conditions leads to HCC development (17). In addition, immunosuppressive cancer environments adversely affect innate and adaptive immunity function, resulting in HCC progression and metastasis (18). The TIME of the liver is a homeostatic system governed by effective regulatory mechanisms. However, ineffective TIME mechanisms, such as an imbalance involving immunosuppressive cell subsets, tumor signaling–mediated immune response enhancement, and antitumor immune fatigue, contribute to tumor progression (19). TIME-related immune cells, such as tumor-associated macrophages, tumor-associated neutrophils, tumor-infiltrating lymphocytes, regulatory T cells, CD8+ cytotoxic T lymphocytes, and natural killer cells, have been implicated in HCC pathogenesis. Moreover, TIME-based targets for HCC immunotherapy guide and improve the efficacy of various cancer therapies, particularly those that work by enhancing host antitumor immune responses (20). Immunotherapeutic approaches targeting immune checkpoints have been extensively studied to improve HCC immunotherapy effectiveness. Excessive immunomodulation, angiogenesis, inflammation, and communication between tumor cells and extracellular matrix can be targeted for HCC immunotherapy development (19). Previous studies report that TIME is important in the prediction of survival outcomes and in the evaluation of therapeutic efficacy (8, 9). However, immunomodulatory factors associated with HCC TIMEs have not been fully explored. Notably, the development of bioinformatics tools could facilitate efficient prediction of the composition of and change in TIMEs in tumors (21). Therefore, several studies have used bioinformatic tools to explore the clinical significance of TIME, the association of TIME and tumorigenesis, and the effect of immunotherapy on TIME (22, 23). However, the cellular and molecular features of TIME and their correlation with clinicopathological signatures in HCC have not been explored. The aim of this study, therefore, was to characterize TIME immune factors and explore their role in HCC.

In this study, gene expression data were retrieved from public databases and used to analyze 22 TIME immune cell components in 735 HCC patients. Furthermore, three immune phenotypes (TIME T1–3) were identified based on TIME to further evaluate associations among immune phenotypes, genomic characteristics (Gene G1–2), prognosis, and clinical features. We developed a TIME score (TS) model with good prognostic potential to be used as an immune biomarker for HCC (**Supplementary Figure 1**). Analysis of TIME landscape features may help in better understanding the role of immune factors in HCC TIME and provide new HCC immune biomarker and immunotherapy approaches.

METHODS

Data Sources and Preparation

We searched public databases for gene expression data and clinical information regarding HCC patients. Six cohort data sets from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases were downloaded. RNA-seq data of 424 HCC patients were downloaded from TCGA using the GDC API programmatic interface. Microarray data set GSE15654 containing data for 216 HCC patients, GSE76427 for 96 HCC patients, GSE14520 for 247 HCC patients and 241 normal controls, GSE36376 for 240 HCC patients and 193 normal controls, and GSE25097 for 269 HCC patients and 243 normal controls were downloaded from the GEO database. All samples from TCGA, GSE14520, GSE36376, and GSE25097 were randomly divided into training and validation sets. The RNA-seq read data from TCGA were preprocessed as follows: (1) HCC

samples without clinical data and with overall survival (OS) <30 days were removed. (2) Normal tissue data were eliminated. (3) Genecode V22 annotation was used to transfer RNA-seq read data from fragments per kilobase million (FPKM) to transcripts per million (TPM). The distribution of TPM data was more similar to that of the microarray data than to the FPKM data. (4) Genes with a TPM expression value of 0 and that appeared in more than half of the samples were excluded. Microarray data from GEO were preprocessed as follows: (1) Normal tissue data were excluded, and thus, only primary tumor data were retained. (2) HCC samples without clinical data and OS <30 days were excluded. (3) The Bioconductor R package was used to map the chip probe to human gene SYMBOL.

Calculation of Immune Cells in Time

The distribution of immune cells in TIME in HCC vs. normal control tissues was estimated using the CIBERSORT algorithm. Scores of each human immune cell in the three cohort data sets were calculated using the LM22 gene signature as a reference (the permutation parameter was 1,000) (6). The CIBERSORT algorithm is an anticonvolution support vector regression algorithm. This algorithm uses a set of minimum gene expression values (for 547 genes) to represent each cell type as a reference to infer the proportion of cell types in the data of a large number of tumor samples with mixed cell types. In addition, CIBERSORT can precisely and sensitively differentiate between 22 different human immune cells based on gene expression data. Some of these include T cells, macrophages, neutrophils, dendritic cells, B cells, and natural killer cells. Gene expression profiles were prepared using a standard annotation file, and the data were uploaded to the CIBERSORT website (http://cibersort.stanford.edu/), where the algorithm was executed using the LM22 gene signature and 1,000 permutations.

Consensus Clustering of TIME-Infiltrating Cells

Unsupervised clustering of TCGA samples and tumor TIME-infiltrating cells was performed using the ConsensusClusterPlus algorithm based on the value obtained from TIME calculations. Euclidean distance calculation of similarity measures between clusters and K-means of unsupervised clustering were used to estimate the number of TIME clusters (24). The optimal number of clusters was determined by the cumulative distribution function (CDF) and the delta area and analyzed using the ConsensusClusterPlus R package with 1,000 repeats.

Differential Gene Expression, Identification, and Clustering

Associations involving genes and TIME-infiltrating cells were explored by first dividing the genes into clusters based on the TIME-infiltrating cells. The DEseq2 tool was used to classify genes that were significantly differentially expressed and related to the TIME cluster in TCGA. Next, differentially expressed genes were selected by excluding genes with an expression value of 0 in >50% of samples. Furthermore, the non-negative matrix factorization (NMF) algorithm was used to perform unsupervised clustering (25). NMF is an effective method for identifying different molecular patterns and enabling class discovery, especially for biological information from cancer-related microarray data. In this study, we used the standard "Brunet" pattern for NMF analysis with 50 iterations (26). We set the number of clustering K-means from 2 to 10, determined the average contour width of the common member matrix through the NMF R package, and set the minimum member of each cluster to 10. The optimal clustering number was determined according to cophenetic, dispersion, and silhouette indicators.

Construction of TIME Score Model

Before construction of the TIME score model, we identified common differentially expressed genes among the TIME clusters by dimensionality reduction. These genes were first subjected to univariate Cox analysis, after which a random forest algorithm was used to evaluate the importance of the genes using the R package (27). The random variable Mtry parameter was set for each partition, and the value with the lowest error rate was selected as the optimal Mtry value of the random Forest algorithm. Subsequently, we picked Ntree parameters according to the random Forest plot, and genes with cumulative importance >95% were chosen as candidates. Next, the K-means algorithm was used for cluster analysis through the ConsensusClusterPlus R package. Further, the Psych R package was applied to conduct principal component analysis (PCA). PCA uses dimensionality reduction technology to reduce multiple variables into a few principal components, which can reflect most attributes of the original variables (28). For each gene signature in the groups, 100 repeats were performed to obtain the optimal principal component numbers (PCs). The respective PC scores were calculated, and principal component 1 (PC1) scores of each cluster were selected as the signature score. Subsequently, Cox multivariate regression analysis was used to construct a prognosis risk model for each group. A TIME score = $\sum$PC1*β formula was used to define the TIME score model, in which β is the multivariate regression coefficient of each group, and PC1 is the score of each group.

Statistical Analysis

A forest plot was created using the Forest plot R package, based on univariate Cox regression analysis results of each data set. A univariate Cox proportional hazard risk regression model was used to calculate univariate risk ratio. The statistical significance of normally and non-normally distributed data was calculated using Student's *t*-test, and two independent variables were analyzed using Wilcoxon's sign rank test. Non-parametric testing of three or more sets of data was performed using Kruskal–Wallis tests. The least absolute shrinkage and selection (LASSO) and random-forest analyses were used to select suitable immune cell fractions. These immune cell risk scores were used to construct diagnostic models based on the coefficients of each selected marker through a logistic regression algorithm. HCC patients were assigned to high- and low-risk groups using the median value or were adjusted by Z-scores such that >0 and <0 were defined as high- and low-risk groups, respectively. The Kaplan–Meier (KM) method was used to plot survival curves for estimating survival rates of patients, and statistical differences

among means were compared using the log-rank test. Immune and stromal scores of each sample were calculated using the ESTIMATE tool employing the R package. Receiver operating characteristic (ROC) curves, which were generated with Package pROC, were used to determine the sensitivity and specificity of the KM analysis. A diagram showing the association between TIME scores and gene biology was developed using the Corrplot R package. NetworkD3 R packages were used to construct an alluvial diagram of TIME clusters with different gene clusters and survival outcomes. ComplexHeatmap R packages were used to depict the mutational landscape of genes. HCC patients were classified into high- and-low risk groups based on median TIME scores for survival analysis. The limma R package was used to analyze differential expression of TIME cluster genes, and functional enrichment was performed using the cluster profile R package. All statistical analyses in this study were conducted using either the R package or SPSS software, and $P < 0.05$ was considered statistically significant.

RESULTS

Identification of TIME-Infiltrating Cells and Classification of TIME Phenotypes

Analysis of the TIME-infiltrating cell component by the CIBERSORT algorithm revealed 22 immune cell classifications. These included B cells, T cells, natural killer (NK) cells, macrophages, and dendritic cells (DCs). Correlation analysis further grouped the 22 categories into four groups (**Figure 1A**). These four groups were positively correlated, implying communication among the 22 immune cell types. Furthermore, we carried out univariate Cox analysis to test the prognostic factor of the 22 immune cell types. Forest plots showed that follicular helper T cells ($P = 0.038$) and M0 macrophages ($P = 0.008$) were unfavorable prognostic markers [hazard ratio (HR) >1], whereas CD8+ T cells ($P = 0.021$) and resting CD4+ memory T cells ($P = 0.031$) were favorable prognostic markers (HR < 1) (**Supplementary Figure 2A**). We performed unsupervised clustering of 735 tumors from three HCC cohorts with TIME-matched cell expression profiles (**Supplementary Figure 3A**). The clustering results revealed three phenotypes (TIME T1–3) of TIME-infiltrating cells based on optimal K = 3 and verification of CDF and delta area (**Figure 1B**, **Supplementary Figures 3B,C**). Additionally, we observed that TIME T1 was characterized by high levels of regulatory T cells (Tregs) and M0 macrophages. TIME T2 was primarily associated with CD8+ T cells and activated CD4+ memory T cells, and TIME T3 was characterized by resting CD4+ memory T cells, resting DCs, and activated NK cells. To verify the value of infiltrating immune cells as biomarkers for HCC, we compared the composition of infiltrating immune cells between HCC and normal tissue in 4 data sets (TCGA, GSE14520, GSE36376, GSE25097) to understand their distribution and roles as potential HCC biomarkers. We identified significant differences in the composition of immune-infiltrating cells between HCC and normal tissue across the four data sets. Notably, Treg and M0 macrophage numbers were significantly higher in HCC tissue compared with normal tissue, and CD8+ T and resting CD4+ memory T cell levels were significantly lower in HCC tissue (**Supplementary Figure 4A**). The distribution of infiltrating immune cells in HCC tissue across the clinical features showed that key immune cells, including M0 macrophages, resting CD4+ memory T cells, M1 macrophages, activated NK cells, and CD8+ T cells constituted the majority of such cells (**Supplementary Figure 4B**). In addition, we analyzed associations involving key immune cells and clinical features (tumor-node- metastasis (TNM), stage, and grade). Apart from no statistical significance in some analyses, M0 macrophage and Treg scores were higher in advanced pathological stages (**Supplementary Figures 5A,B**). In contrast, resting CD4+ memory T cells and CD8+ T cell scores decreased in advanced pathological stages (**Supplementary Figures 5C,D**). These results reveal the components of immune infiltrating cells in HCC and indicate that Tregs, M0 macrophages, CD8+ T cells, resting CD4+ memory T cells, and activated CD4+ memory T cells are key biomarkers in HCC.

KM survival analysis based on the three phenotypes identified revealed that TIME T1 was associated with poor prognosis, whereas TIME T2 and TIME T3 exhibited favorable HCC prognosis ($P < 0.0001$) (**Figure 1C**). The distribution of TIME-infiltrating cells among the three phenotypes was analyzed using the Kruskal–Wallis test (**Supplementary Figure 2B**). TIME T1 was characterized by high levels of Tregs and M0 macrophages, and the levels of M1 and M2 macrophages in TIME T1 were lower compared with the levels in TIME T2 and T3 because M1 and M2 are regarded as classically and alternatively activated macrophages, respectively. In different immune microenvironments, three types of macrophages can be activated and transformed into subsets with different molecular and functional characteristics. In addition, TIME T2 exhibited higher levels of CD8+ T cells and activated CD4+ memory T cells, and TIME T3 was characterized by high numbers of resting CD4+ memory T cells, resting DCs, and NK cell activation.

However, it is not clear whether one or several specific immune cells could be used as HCC biomarkers. Therefore, we conducted random forest (**Supplementary Figure 4C**) and LASSO (**Supplementary Figure 4D**) analysis of the 4 data sets (TCGA, GSE14520, GSE36376, GSE25097). The two analysis methods revealed 8 possible HCC markers (Tregs, M0 macrophages, CD8+ T cells, resting CD4+ memory T cells, activated CD4+ memory T, activated NK cells, activated mast cells, and T cell follicular helpers). Furthermore, a diagnostic model based on the risk score involving these immune cells was constructed using a logistic regression method. The results show that the risk scores for HCC patients are significantly higher than those for normal controls among the four data sets (**Supplementary Figure 4E**). ROC analysis verified the high accuracy of the diagnostic model based on such immune cell risk scores to distinguish HCC patients from normal controls (**Supplementary Figures 4F,G**). In summary, our results illustrate that TIME-infiltrating cells and phenotypes with different patterns of immune cellular components could be used as potential HCC prognostic biomarkers.

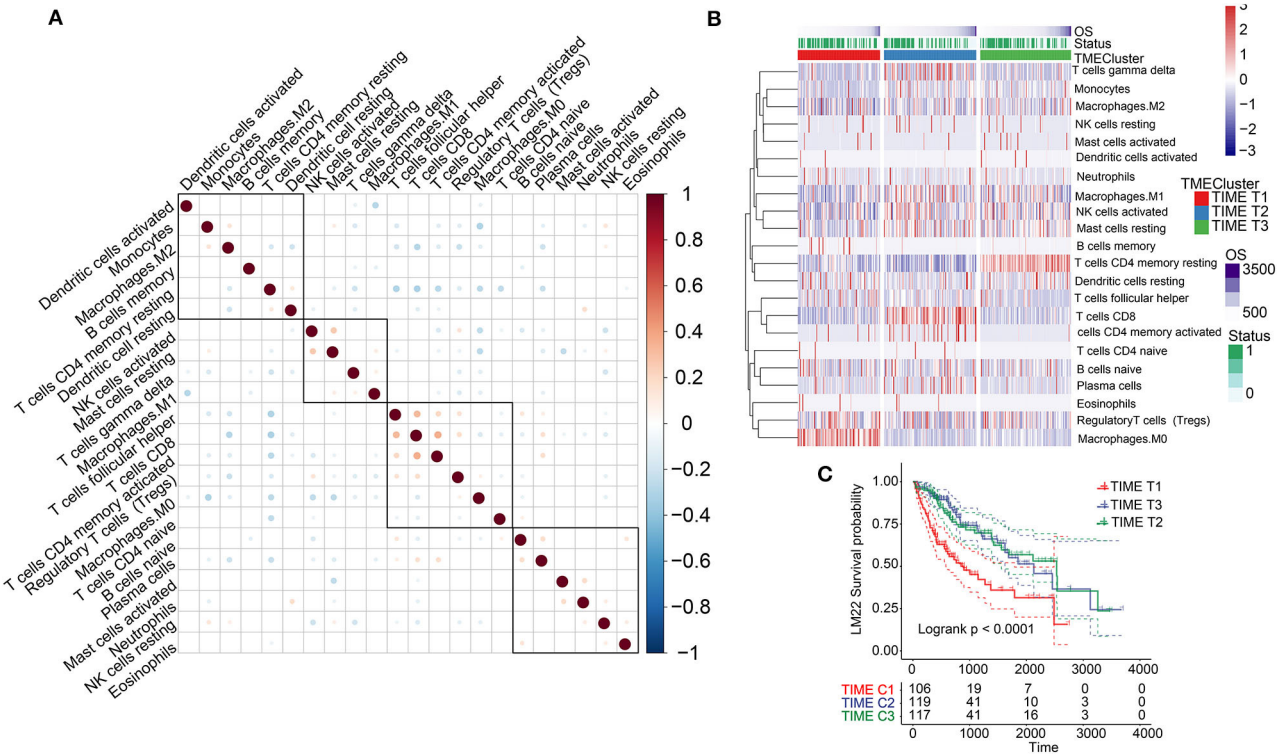

FIGURE 1 | TIME-infiltrating cells and classification of TIME. **(A)** Correlations among 22 types of immune cells in TIME. Brown and blue nodes represent positive and negative correlations, respectively. The larger the node, the stronger the correlation. **(B)** Heat map illustrating results of unsupervised clustering based on TIME phenotypes. **(C)** Kaplan–Meier analysis for three TIME phenotypes.

Identification of Gene Clusters and Analysis of Biological Function

Significant differences in patient prognosis involving TIME T1 and TIME T2/T3 were observed. Therefore, we analyzed differentially expressed genes (DEGs) between TIME T1 and TIME T2 and TIME T1 and TIME T3. In total, we identified 432 DEGs between TIME T1 and TIME T2 and TIME T1 and TIME T3 (**Supplementary Figure 6A**). After being screened by the NMF algorithm (**Supplementary Figure 3D**), the 432 DEGs were reduced to 365 and classified into two clusters (Gene G1–2) based on the optimal K = 2 (**Supplementary Figure 6B**). Unsupervised clustering analysis of the 365 DEGs grouped HCC patients into two classes (**Figure 2A**). We observed that most Gene G1 members were associated with TIME T2/T3 and were characterized by low risk, and most Gene G2 members were associated with TIME T1 and were characterized by high risk. KM analysis showed that Gene G1 and Gene G2 were associated with good and poor prognoses, respectively ($P < 0.0001$) (**Figure 2B**). We used the alluvial diagram to illustrate relationships involving the three phenotypes (TIME T1–3) and the two clusters (Gene G1–2) as well as their living status (**Supplementary Figure 6C**). Notably, the distribution of TIME-infiltrating cells among the two gene clusters (**Figure 2C**) was consistent with the three phenotypes (**Supplementary Figure 2B**). These findings indicate that Gene G2 is characterized by high levels of Tregs and M0 macrophages, and Gene G1 is characterized by CD8+ T cells and activated CD4+ memory T cells, resting CD4+ memory T cells, resting DCs, and NK cells activation. In summary, classification of patients based on genomic clusters is consistent with TIME phenotype groups.

Gene G1 and Gene G2 represented significant differences in the distribution of TIME-infiltrating cells and prognosis; therefore, we further investigated differences in cellular biological functions involving these genes. We conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis using biological pathways. We determined that Gene G1 is associated with immune processes, such as T cell receptor signaling pathways, Th1 and Th2 cell differentiation, immune system function, and complement activation. In contrast, most members of Gene G2 are involved in tumorigenesis processes, including the P53 signaling pathway, PI3K-Akt signaling, hepatocellular carcinoma, and apoptosis (**Supplementary Figures 7A,B**). Therefore, we constructed a network of genes and pathways that revealed a regulatory relationship between immune-related pathways in Gene G1 and tumorigenesis-related pathways in Gene G2, and these pathways interacted through overlapping genes (**Supplementary Figures 7C,D**). The results reveal that Gene G1 and Gene G2 are associated with immune and tumorigenesis functions, respectively. Therefore, these findings may explain the favorable prognosis of Gene G1 and the poor prognosis of Gene G2 cases.

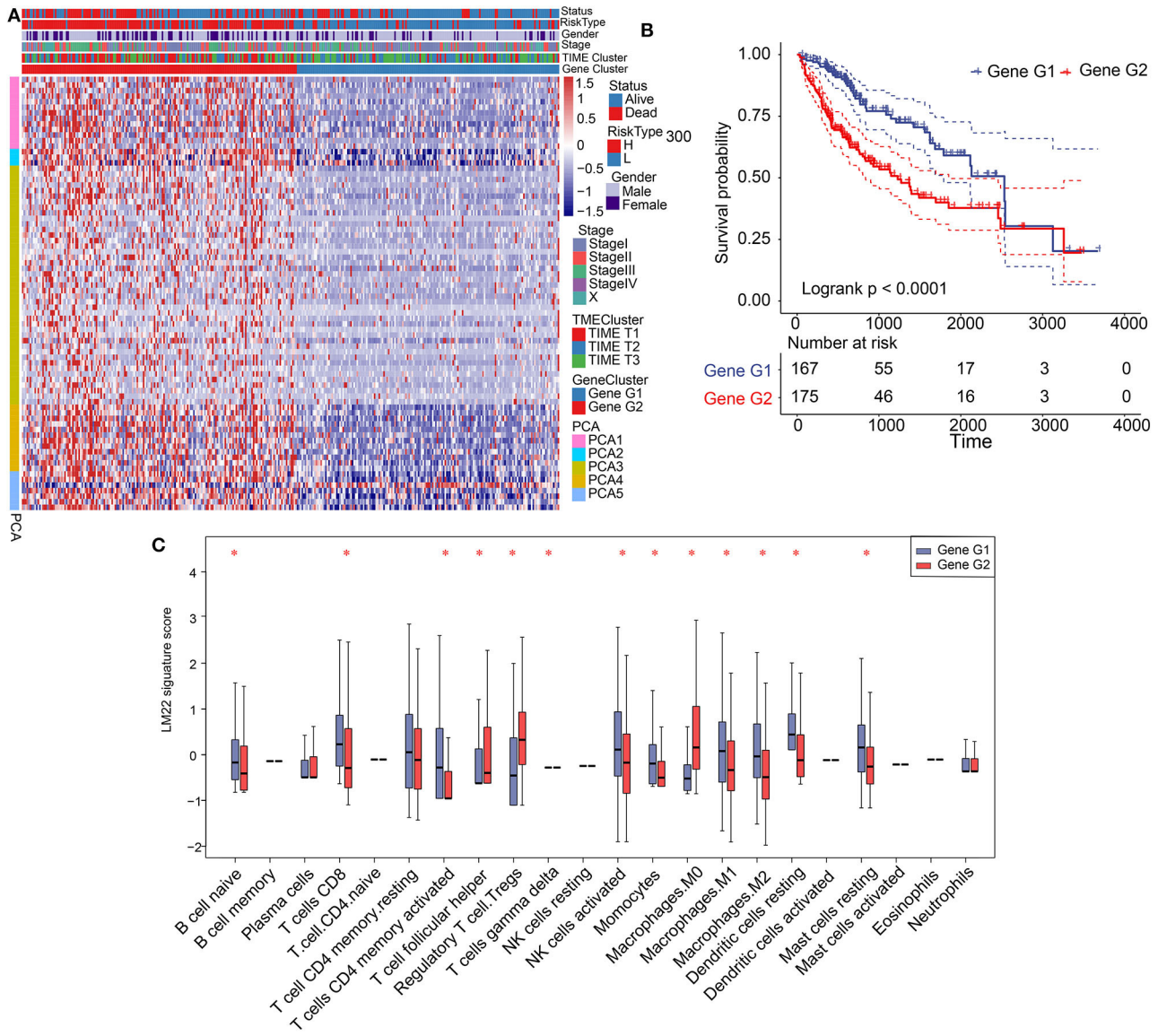

FIGURE 2 | Construction of gene clusters and association between TIME signature and TIME gene patterns. **(A)** Heat map showing results of unsupervised clustering based on gene cluster classification. **(B)** Kaplan–Meier analysis of 2 gene clusters. **(C)** Distribution and expression of 22 types of immune cells in the 2 gene clusters. *$P < 0.05$.

Establishment of TIME Score Model and Analysis of Clinical Signature Associations

We performed dimension reduction to reduce redundant genes. A total of 117 DEGs were identified after univariate Cox analysis. Analysis of 117 DEGs using the random forest algorithm (**Supplementary Figure 3E**) identified 78 DEGs. Analysis of the biological functions of the 78 DEGs by Gene Ontology (GO) indicated that these genes are involved in cell differentiation, cell–cell junction, inflammatory responses, and antibiotic responses (**Supplementary Figure 8A**). KEGG pathway analysis of the 78 genes enriched in the immune system indicated HCC, Th1, and Th2 cell differentiation; immune responses; innate complement; and Toll-like receptor signaling pathways (**Supplementary Figure 8B**). These results show that the 78 DEGs are implicated in tumorigenesis and immune responses. Based on clustering analysis, the 78 DEGs were classified into five groups, which we assigned the signatures 1–5 (S1–5). There were 13, 3, 43, 12, and 7 DEGs in the S1, S2, S3, S4, and S5 groups, respectively (**Figure 3A**). Among these, S2 was a high-expression group, S1 and S3 were low-expression groups, and S4 and S5 were intermediate expression level groups. A heat map of the 78 DEGs is presented in **Figure 3B**, which is consistent with the clustering plot. Furthermore, we carried out PCA analysis to construct a TIME score model according to the PC1 scores of each group. In addition, we constructed a prognostic score model, which we termed the TS score model. On comparing

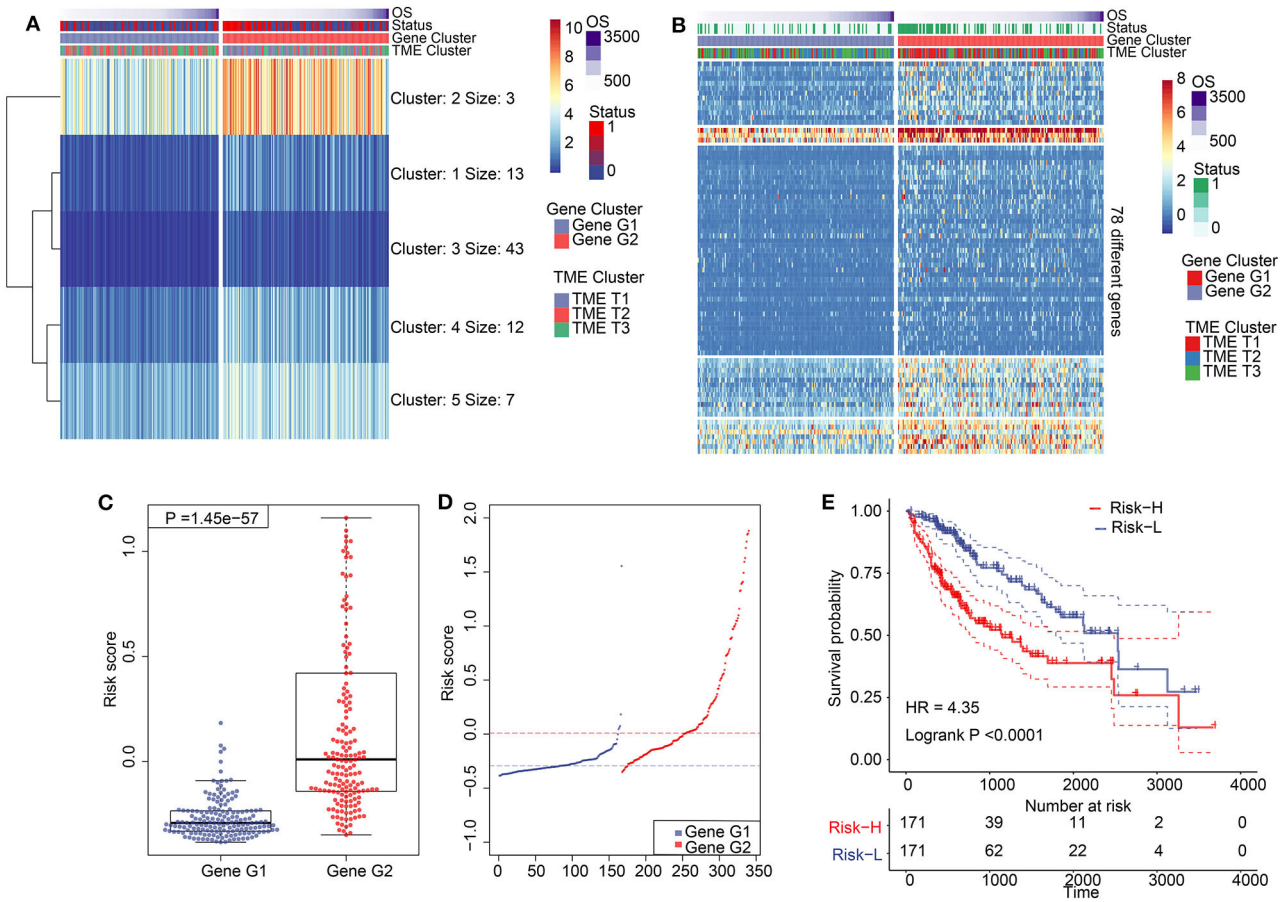

FIGURE 3 | Construction of TIME score model and its characteristics. **(A)** K-means clustering results of 78 differentially expressed genes. **(B)** A clustering heat map of 78 differentially expressed genes. **(C)** Comparation of TS between Gene G1 and Gene G2. **(D)** Distribution of TS between Gene G1 and Gene G2. **(E)** Kaplan–Meier analysis for high TS and low TS.

Gene G1 and Gene G2, we found that the TS score of Gene G2 was significantly higher than that of Gene G1 (**Figures 3C,D**). In addition, we performed ESTIMATE algorithm processing to compare stromal and immune scores across TIME1–3 and observed significant increases in stromal and immune scores in TIME1. Although no statistical significance was observed, the ESTIMATE score for TIME1 was higher than that for TIME1 and TIME2 (**Supplementary Figure 9**). HCC patients were assigned to a high TS or low TS score using a median value (−0.185). High and low TS scores were associated with poor and good prognosis, respectively ($P < 0.0001$) (**Figure 3E**). These results were consistent with the KM analysis of gene clusters (**Figure 2B**), in which Gene G2 indicated poor prognosis compared with Gene G1. We further analyzed the association between TS scores and clinical signatures, and the results showed that the grade, tumor (T), node (N), and stage classifications exhibited significantly different TS scores ($P < 0.05$) (**Figures 4A,D,F,G**). However, we did not observe any clinical significance between metastasis (M), gender, and age ($P > 0.05$) (**Figures 4B,C,E**). To study the role of immune factors involving TS scores, we investigated potential associations between TS scores and previously studied immune genes (14). In this analysis, immune-activated genes (*TBX21*, *CXCL9*, *GZMA*, *GZMB*, *PRF1*, *IFNG*, *TBX2*, *TNF*, and *CD8A*), immune checkpoint genes (*PDCD1*, *CTLA4*, *LAG3*, *PDCD1LG2*, *CD274*, and *HAVCR2*), and transforming growth factor/epithelial-mesenchymal transition genes (TGF/EMT) (*VIM*, *ACTA2*, *COL4A1*, *TGFBR2*, *ZEB1*, *CLDN3*, *SMAD9*, and *TWIST1*) were used. The results reveal differences in gene-expression patterns between different gene clusters, TS scores, and TIME phenotypes (**Supplementary Figure 10A**). However, we found that TS scores were closely associated with immune genes. Furthermore, we explored the correlation between known signatures [EMT, immune checkpoints, tumorigenesis, biological processes (cell cycle, angiogenesis, mismatch repair)] and TS scores to describe the function of our TS score model. We observed that high TS scores were associated with tumorigenic features, such as apoptosis, cell cycle, DNA replication, mismatch repair, and WNT targeting. On the other hand, low TS scores were associated with factors implicated in immune activation, including CD8+ T effector, antigen-processing machinery, and immune checkpoint steps (**Figure 4H**). Furthermore, when the TS model was tested

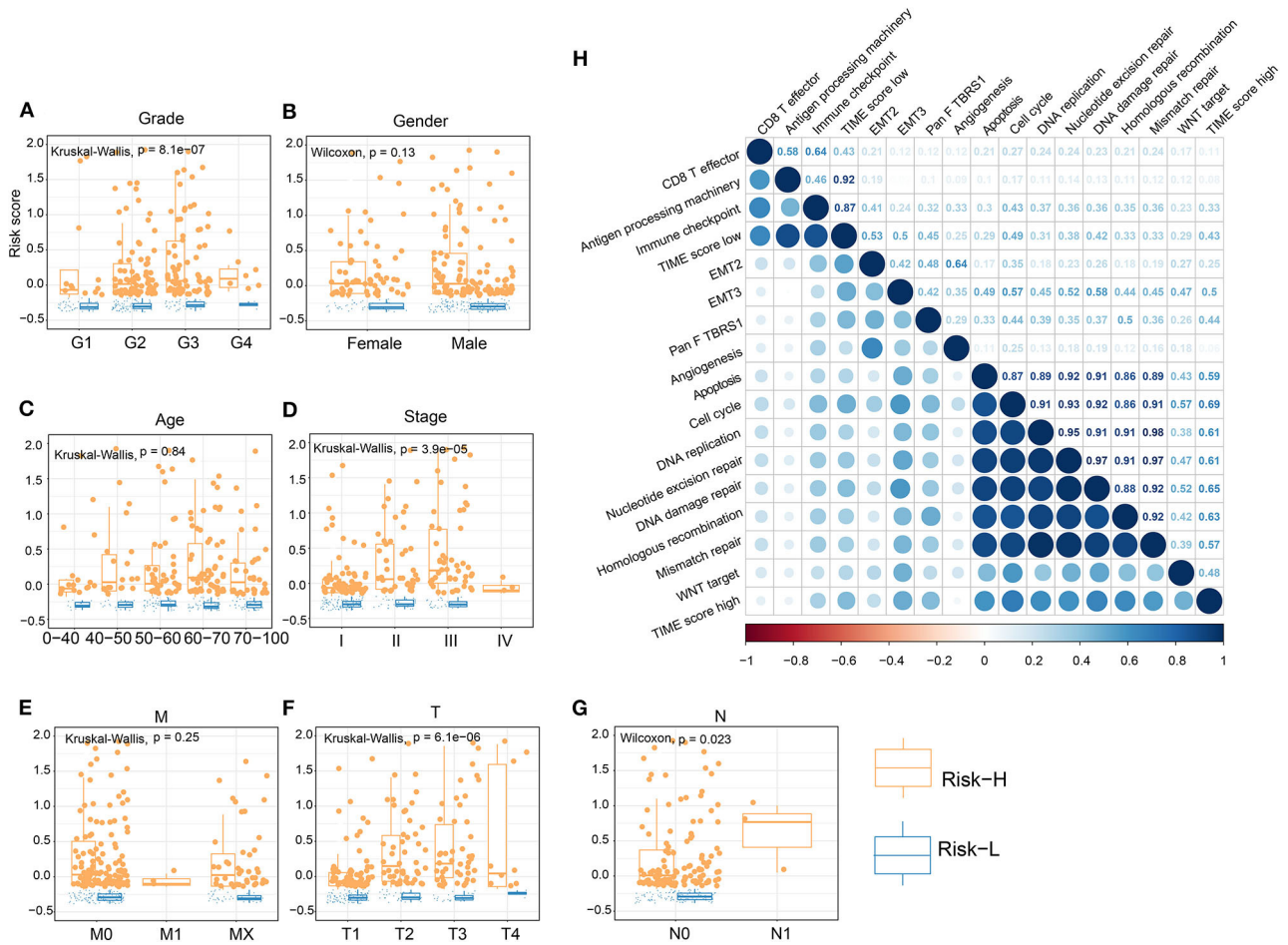

FIGURE 4 | Association of TS with clinical characteristics and biological signatures. **(A–G)** Association of TS with clinical characteristics, including grade, gender, age, stage, M, T, and N. **(H)** Association involving TS and known biological signatures. (J) Forest plot showing results of multivariate Cox analysis for TS and clinical characteristics.

as a variable signature by Cox regression, the forest plot showed that the TS model was an independent HR prognostic factor, with a more substantial HR value than other clinical signatures (**Supplementary Figure 10B**). All of these results demonstrate that the TS model is a robust feature and can, therefore, be used to predict patient HCC prognosis. Furthermore, these findings reveal that the TS model is associated with several clinical signatures.

Comparison Between TS Model and Known Signatures

Having shown that the TS model is a useful prognostic biomarker, we sought to understand associations involving the identified TS model and known HCC signatures. Therefore, we analyzed the expression of immune-activated genes, immune checkpoint genes, and TGF/EMT genes in the high and low TS score categories. The results indicate that low TS scores are associated with elevated expression of immune-activated and immune checkpoint genes (**Figures 5A,B**), whereas high TS scores are characterized by high expression of TGF/EMT genes (**Figure 5C**). Additionally, we evaluated the expression of immune-activated genes, immune checkpoint genes, and TGF/EMT genes in the TS scores of TIME T1–3 and observed that the expression of immune-activated and immune checkpoint genes in TIME T2 and TIME T3 was elevated compared with that in TIME T1 (**Figures 5D,E**). In contrast, the expression of TGF/EMT genes in TIME T1 was higher than in TIME T2/T3 (**Figure 5F**). These findings suggest that low TS scores related to TIME T2/T3 are associated with immune-activated and immune checkpoint genes, which trigger immune functions to suppress tumor development. Therefore, low TS scores may represent a favorable HCC prognostic marker. However, high TS scores related to TIME T1 were associated with TGF/EMT genes, which are linked to tumorigenesis, which results in unfavorable HCC prognosis. Furthermore, we explored the distribution of known somatic mutations involving gene expression and analyzed relationships between TS scores and these genes. Using the Fisher's exact test ($P < 0.05$), we compared known somatic gene alterations exhibiting significant differences in mutation frequency between high and low TS score groups. A total

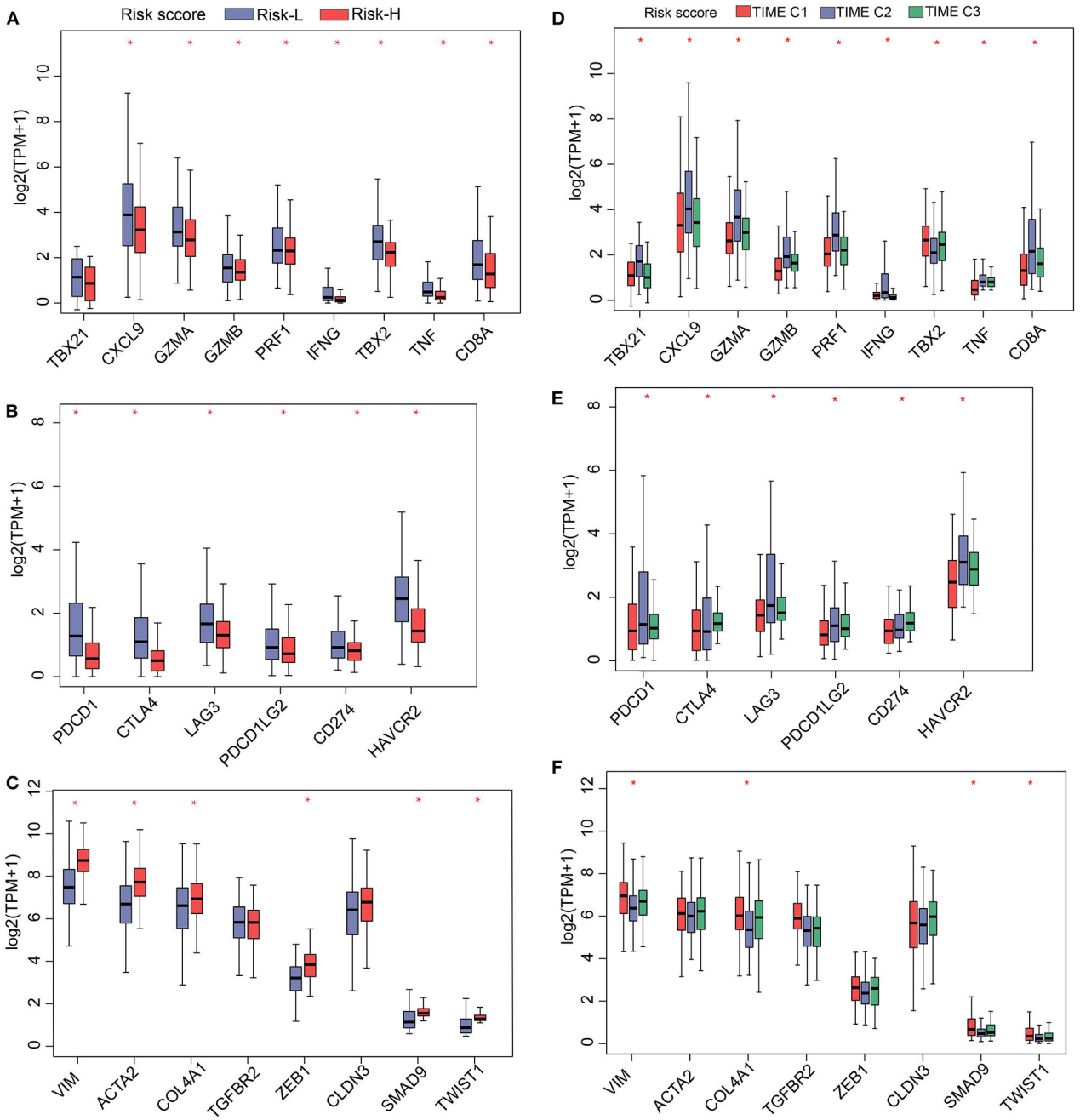

FIGURE 5 | Gene expression profiles of **(A)** immune activation, **(B)** immune checkpoint proteins, and **(C)** TGF/EMT in high and low TS. Gene-expression profiles of **(D)** immune activation, **(E)** immune checkpoint proteins, and **(F)** TGF/EMT in 3 TIME phenotypes. *$P < 0.05$.

of 49 variants were found to be associated with TS scores (**Supplementary Figure 11**). *TP53,* an anticancer gene (29), for instance, was mainly distributed in high TS scores. However, mutated *TP53* lost intrinsic cancer inhibitory function and exhibited poor patient prognosis. The *CTNNB1* gene causes cancer, and mutated *CTNNB1* was distributed in both high and low TS scores. A previous study reports that *TP53* mutation is implicated in tumor development, and *TP53* can be targeted with HCC checkpoint inhibitors for immunotherapy development (30). Other genes, such as *RB1*, *TLL1*, and *PIK3CA*, are implicated as important factors in genetic alterations in HCC (31, 32). *RB1* is one of the most significantly mutated genes in HCC and is related to proteogenomic phenotype classification and involved in distinct features in metabolic reprogramming, microenvironment dysregulation, and cell proliferation (33). Genome-wide association studies have found that *TLL1* variants

are associated with HCC after hepatitis C virus infection eradication (34). A previous study reports that blood-derived circulating tumor DNA markers, such as *PIK3CA* with frequent alteration, may be key biomarkers in diagnosis of advanced HCC and for HCC molecular diagnosis (35). This study presents a new perspective for exploring the immune mechanisms involved in immunotherapy of tumors.

Validation of the TS Model

The prognostic efficacy of the TS model was validated using the GSE15654, GSE76427, and GSE14520 data sets by KM analysis. The results indicate that a high TS score is significantly associated with poor prognosis, whereas a low TS score is significantly associated with favorable prognosis in the GSE15654 ($P = 0.03535$), GSE76427 ($P = 0.04572$), and GSE14520 ($P = 0.00273$) data sets (**Figures 6A–C**). The sensitivity of KM analysis was verified by ROC analysis. The results of ROC analysis show that the TS model is a predictive biomarker for HCC patients (GSE15654: AUC of 1 year = 0.65, 5 years = 0.64, 10 years = 0.58; GSE76427: AUC of 1 year = 0.61, 5 years = 0.70, 6 years = 0.71; GSE14520: AUC of 1 year = 0.60, 3 years = 0.67, 5 years = 0.64) (**Figures 6C–F**). These results further suggest that the TS model is an effective HCC predictor of prognostic signature and has defined replicability for different data sets

DISCUSSION

In this study, data obtained by comprehensive analysis of TIME-infiltrating cells and relevant genes were used to construct a TS model. This model accurately predicted the prognosis of HCC patients. Systematic analysis revealed that high TS scores were associated with poor prognosis, immune suppression, and tumorigenesis, whereas low TS scores were correlated with favorable prognosis, immune activation, and immune checkpoint progression. Liver cells are highly immune-tolerant. This is because immune cells in the liver form an immune-tolerance state that protects against autoimmune-induced damage. Carcinogenic factors, such as persistent viral infection, compromise immune tolerance or balance rendering immune cells unable to clear carcinogenic factors (17, 36). In the early stages of tumor growth, immune suppression decreases immune surveillance (37). Thus, the primary target of tumor immunotherapies, such as PD-1/PD-L1, is to activate and restore immune function for optimal ablation of tumor cells (38). In low-TS groups, our results show that immune activation correlates with better prognosis, suggesting that immune activation inhibits HCC tumorigenesis. This is consistent with a previous study in which key genes and tumor-associated leukocytes were identified to predict the prognosis of cancer patients and their responses to targeted therapy (39). However, the significance of our study involves not only the analyzed composition of infiltrating immune cells in HCC and classified HCC patients based on molecular phenotypes, but also systematically associated TIME phenotypes and gene clusters with genomic characteristics and clinical and pathologic features. In so doing, we identified biomarkers with potential clinical application. These biomarkers were used to construct a TS model that could predict the prognosis of HCC patients.

Analysis of TIME-infiltrating cells and phenotypes reveale that M0 macrophages were unfavorable factors assigned to TIME T1, whereas CD8+ T cells and CD4+ T cells were favorable factors assigned to TIME T2/T3. These results are consistent with those from previous research in which T cells and macrophages are reported to inhibit and promote HCC, respectively (40). M0 macrophages are undifferentiated macrophages with the potential to transform into specific subtypes of macrophages (41). Different subtypes of liver macrophages exhibit diverse ontogeny, differentiation, and function, especially Kupffer cells and tumor-associated macrophages (TAMs) (42). TAMs play an important role in the occurrence, development, invasion, metastasis, immune evasion, and angiogenesis in HCC (43). Kupffer cells enhance virus-mediated inflammation, causing liver cirrhosis and HCC (44). Liver macrophages exhibit highly variable phenotypes that are modulated by signals derived from the liver microenvironment (42). We hypothesized that M0 macrophages may stimulate the production of TAM and Kupffer cells in the presence of carcinogenic factors and, thus, promote inflammation and suppress immunity leading to HCC development. Compared to normal tissues, M0, M1, and M2 macrophage levels were generally higher in HCC cells. Macrophages are classically polarized into activated macrophages (M1) and alternatively activated macrophages (M2) under the stimulation of different immune microenvironments (45). The induction of M1 from M0 macrophages and the mutual transformation of M1 and M2 macrophages modulates tumorigenesis (46). Our research reveals that enrichment of macrophages in HCC predicts poor prognosis. T cells (CD8+ T cells and CD4+ T cells) are the key immune cells that kill tumor cells by activating the immune system (47). For this reason, novel immunotherapies, such as PD-1 and PD-L1, have been designed to modulate the activity of T cells. The role of PD-1/PD-L1 is to block the binding of tumor cells and T cells, allowing guardian T cells to identify and eliminate tumor cells (48). Activation of T cells in TIME inhibits tumor cells, and this may explain why CD8+ T cells and CD4+ T cells in TIME T2/T3 were associated with good prognosis.

Integrated analysis identified our TS model to be a prognostic biomarker associated with previously studied immune genes. In line with prior studies, upregulation of genes associated with immune activation and immune checkpoint proteins correlates with better prognosis, whereas upregulation of genes associated with TGF/EMT correlates with poorer prognosis (14, 49). In this study, we find that low TS reflects good prognosis, and high TS indicates poor prognosis, suggesting that the TS model is a robust prognostic biomarker. Further analysis of TS scores revealed that elevated TS was accompanied with tumorigenesis signatures, such as cell cycle, DNA replication, mismatch repair, and WNT targeting, whereas low TS was characterized by activation of CD8+ T cell effector and antigen-processing machinery. These results are in agreement with the prevailing knowledge that pathological division of cells is the basis of tumorigenesis (50) and that CD8+ T cells can kill

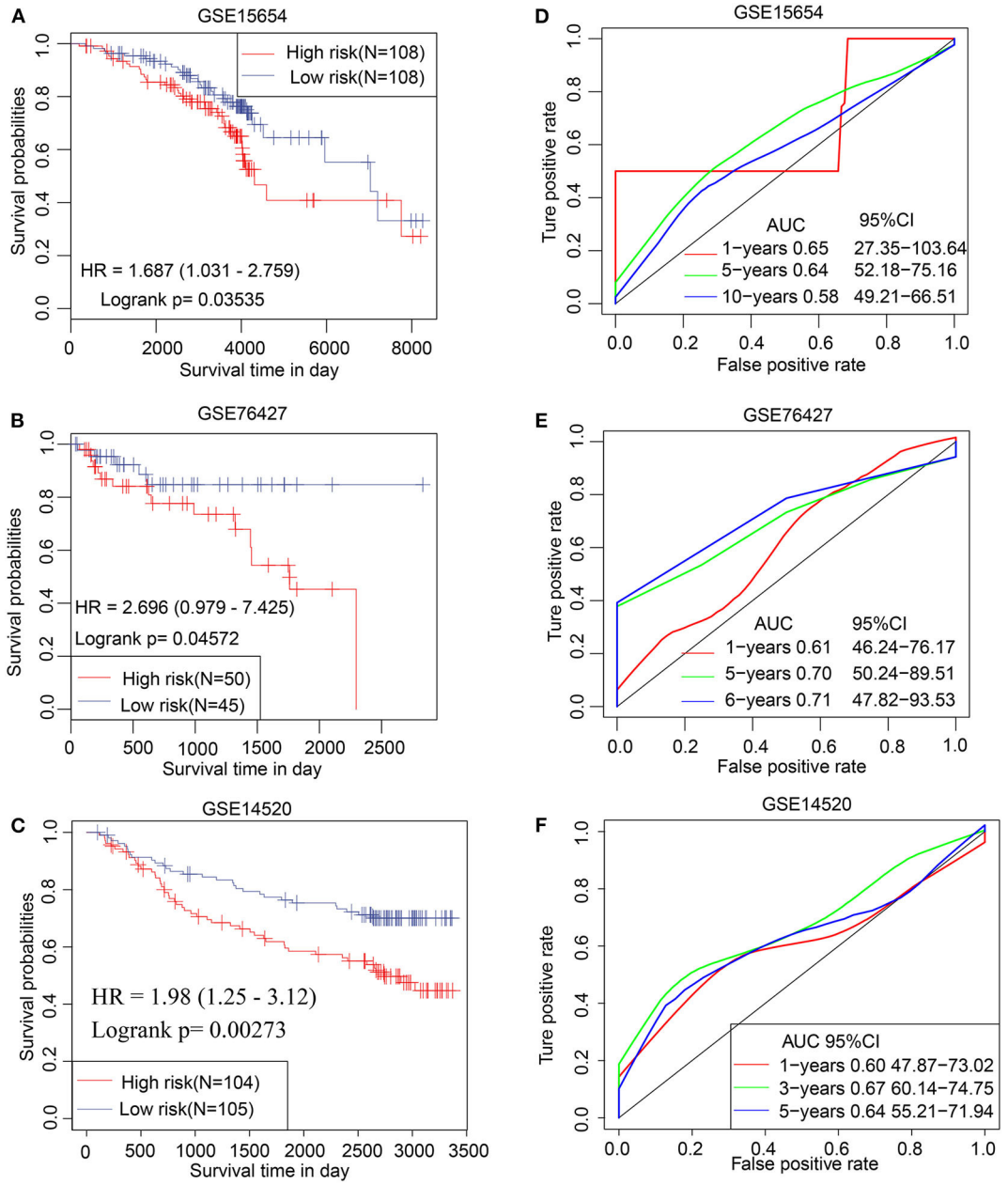

FIGURE 6 | Validation of TS model involving 3 independent data sets. Kaplan–Meier analysis for high and low TS in **(A)** GSE15654, **(B)** GSE76427, and **(C)** GSE14520 data sets. ROC curves of **(D)** GSE15654, **(E)** GSE76427, and **(F)** GSE14520 illustrating the predictive value of TS.

tumor cells by facilitating antigen processing (51). In addition, we observed that our TS model was associated with several known somatic mutations, involving *TP53* and *CTNNB1*. Alterations of these somatic genes may inactivate tumor suppressor genes and cause mutations in proto-oncogenes, resulting in tumorigenesis (52). Therefore, our study contributes to the identification of immunotherapeutic targets aimed at inhibiting pathways involved in tumorigenesis.

Compared with previous studies regarding TIME and HCC (53), this investigation was performed using a large number of HCC samples. Moreover, unlike previous studies (54), which focused only on the function of immune cells in TIME, we comprehensively mapped the landscape of interactions involving TIME-infiltrating cells, genes, and clinicopathological features. Using bioinformatics algorithms, we constructed a TS model and assessed the association between the TS model and clinicopathological features. We find that the TS model is significantly associated with grade, T, N, and, stage. Moreover, we find that the prognostic value of the TS model is superior to that of other clinical signatures.

Previous studies find a correlation between clinicopathological classification and immune response, and this implies that an immune response–related signature can be used for clinicopathological classification (55). Yutaka et al. analyze the immune microenvironment of HCC tissues and intratumor heterogeneity. They observe that several immune subtypes are associated with poor differentiation of HCC (55). In a study by Sia et al., HCC is subcategorized into 2 subclasses based on immune-specific characteristics; adaptive and exhausted immune responses. Notably, the exhausted immune subclass exhibited immunosuppression due to overexpression of TGF-1-regulated genes, which led to poor prognosis (40). Our study provides a better understanding of the TIME, upon which general histological/molecular classification of HCC based on TIME, can be achieved.

CONCLUSIONS

In conclusion, this study reveals that immune characteristics of TIME modulate the pathogenesis of HCC. A TS model was constructed based on TIME phenotypes and gene clusters, which exhibited robust prognostic predictive value for HCC patients. We also reveal promising candidate immune-based biomarkers for diagnosis, prognosis, and immunotherapy in HCC.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The study was approved by the Clinical Research Ethics Committee of College of Medicine, Zhejiang University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HD: research design and funding acquisition. WC, XZ, KB, YD, HZ, and JX: acquisition, interpretation, and analyses of data. WC, XZ, KB, and HZ: manufacture of figures. WC: writing of manuscript and article language modification. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.554165/full#supplementary-material

Supplementary Figure 1 | A flow chart of the study.

Supplementary Figure 2 | (A) Forest plot illustrating results of univariate Cox analysis for the 22 types of immune cells investigated. **(B)** Heat map showing results of unsupervised clustering based on TIME phenotypes.

Supplementary Figure 3 | Algorithms used to classify TIME phenotypes. **(A)** Consensus matrix of TIME-infiltrating cells for each K (2–5) with the corresponding heat maps. **(B)** CDF analysis for consensus cluster analysis. **(C)** Delta area curves for consensus cluster analysis. **(D)** Algorithms used to classify non-negative matrix factorization of gene clusters. **(E)** Distribution of random forest error rates across tree parameters. **(F)** Multidimensional scaling plot for Gene G1 and Gene G2 data. **(G)** Random Forest plot for significant DEGs with mean decrease in gini index (blue) and mean decreased accuracy (red).

Supplementary Figure 4 | Comparison of immune cells in HCC and normal control samples. **(A)** Comparison of immune cell fraction differences in HCC and normal tissues. **(B)** Distribution of immune cell fraction across the clinical characteristics of HCC tissues. **(C)** A random forest plot showing the multidimensional scale plot of adjacent matrix. **(D)** LASSO regression model illustrating misclassification errors across different quantitative variables. **(E)** Comparison of immune scores between HCC and normal tissue control samples. ROC analysis of the diagnostic model in **(F)** training and **(G)** validation set.

Supplementary Figure 5 | Association involving key immune cells and clinical features. Comparison of immune scores of **(A)** M0 macrophages, **(B)** Tregs, **(C)** resting CD4+ memory T cells, and **(D)** CD8+ T cells across TNM, stage, and grade.

Supplementary Figure 6 | (A) Venn diagram illustrating intersection of differentially expressed genes across TIME T1 and TIME T2 and TIME T1 and TIME T3. **(B)** Consistency matrix heat map of NMF algorithm. **(C)** An alluvial diagram showing the association between 3 TIME phenotypes and 2 gene clusters.

Supplementary Figure 7 | Biological function of genes in 2 gene clusters. The main KEGG pathways of **(A)** Gene G1 and **(B)** Gene G2. Relationship network of genes and pathways in **(C)** Gene G1 and **(D)** Gene G2.

Supplementary Figure 8 | (A) GO and **(B)** KEGG analyses of the 78 identified differentially expressed genes.

Supplementary Figure 9 | Comparison of **(A)** stromal, **(B)** immune, and **(C)** ESTIMATE scores across TIME1–3 groups.

Supplementary Figure 10 | (A) Heat map showing genes associated with immune activation, immune checkpoint proteins, and TGF/EMT. **(B)** A Forest plot showing results of multivariate Cox analysis for TS and clinical characteristics.

Supplementary Figure 11 | Association between TS and somatic mutations.

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Ablation Reboots the Response in Advanced Hepatocellular Carcinoma With Stable or Atypical Response During PD-1 Therapy: A Proof-of-Concept Study

Ning Lyu[1†], Yanan Kong[2†], Xiaoxian Li[3†], Luwen Mu[4], Haijing Deng[1], Huiming Chen[1], Meng He[1], Jinfa Lai[1], Jibin Li[5], Hailin Tang[6], Youen Lin[7] and Ming Zhao[1]*

[1] Liver Cancer Study and Service Group, Department of Minimally Invasive Interventional Radiology, Sun Yat-sen University Cancer Center, Guangzhou, China, [2] State Key Laboratory of Oncology in South China, Department of Breast Oncology, Sun Yat-sen University Cancer Center, Guangzhou, China, [3] Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China, [4] Department of Vascular Interventional Radiology, Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, [5] Department of Clinical Research, Sun Yat-sen University Cancer Center, Guangzhou, China, [6] State Key Laboratory of Oncology in South China, Sun Yat-sen University Cancer Center, Guangzhou, China, [7] Department of Interventional Radiology, Jieyang Affiliated Hospital, Sun Yat-sen University, Jieyang, China

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Weijia Fang,
Zhejiang University, China

Reviewed by:
Yuan Yin,
Affiliated Hospital of Jiangnan University, China
Mehmet Akce,
Emory University, United States

***Correspondence:**
Ming Zhao
zhaoming@sysucc.org.cn

[†]These authors have contributed equally to this work

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Background: The anti-programmed cell death protein-1 (PD-1) inhibitor is one of the second-line therapies for advanced hepatocellular carcinoma (HCC) after sorafenib failure. The goal of this study is to evaluate the feasibility and safety of ablation on the tumor in patients with advanced HCC who had stable disease or atypical response during single anti-PD-1 therapy after sorafenib failure. Atypical response defined as mixed responses in different lesions of the same individual (e.g., active or stable lesions mixed with progressive lesions).

Patients and Methods: This proof-of-concept clinical trial enrolled 50 patients treated with an anti-PD-1 inhibitor of nivolumab or pembrolizumab monotherapy between July 2015 and Nov 2017. Thirty-three cases with stable disease or atypical response to anti-PD-1 inhibitor received subtotal thermal ablation. The safety and the response of ablation during anti-PD-1 therapy were evaluated. The survival was estimated by the Kaplan-Meier curve.

Results: Of all 50 patients treated with anti-PD-1 therapy, the rate of response, stable disease, atypical and typical progression were 10% ($n = 5$), 42% ($n = 21$) 32% ($n = 16$), and 12% ($n = 6$), respectively. Additional ablation improved efficacy with tolerable toxicity, and the response rate was increased from 10 to 24% (12/50). The median time to progression, progression-free survival, and overall survival was 6.1 months (95%CI, 2.6–11.2), 5 months (95%CI, 2.9–7.1), and 16.9 months (95%CI, 7.7–26.1), respectively.

Conclusions: This proof-of-concept trial suggested that additional ablation may increase the objective response rate with tolerated toxicity and achieved a relatively better median survival, in advanced HCC patients who had stable or atypical progressive

diseases during anti-PD-1 therapy, which may provide a potentially promising strategy to treat advanced HCC.

Trial registration number: ClinicalTrials.gov identifier: NCT03939975.

Keywords: hepatocellular carcinoma, anti-PD-1 mAbs, thermal ablation, nivolumab, pembrolizumab

INTRODUCTION

Hepatocellular carcinoma (HCC) in advanced stage (Barcelona Clinic Liver Cancer stage-C) is the most frequently diagnosed status, with limited treatment options and high mortality rate (1). Current available treatment for advanced HCC, including atezolizumab plus bevacizumab regimen, multikinase inhibitors (sorafenib, lenvatinib, cabozantinib, and regorafenib), human monoclonal antibodies (ramucirumab), and immune checkpoint inhibitors (nivolumab, pembrolizumab,, and nivolumab plus ipilimumab) have been proven to improve the survivals of patients with advanced HCC by a series of clinical trials (2–7). However, due to the molecular heterogeneity and limited response, the benefits are modest with an extend survival of only a few weeks in second-line treatments, and the progression is still commonly seen.

In recent years, great progress has been made in the field of cancer immunotherapy and encouraging clinical results on many malignancies such as Hodgkin's disease, melanoma, and non-small cell lung cancer and so on raising hopes again for the treatments of advanced HCC (8). Two programmed cell death protein-1 (PD-1) immune checkpoint inhibitors, nivolumab and pembrolizumab, have been approved in second-line setting following sorafenib failure (9, 10). However, not as expected, clinical trials showed that only a small subset, ∼17–20% of participants with advanced HCC could respond to monotherapy of anti-PD-1 inhibitor (9, 10). This might be associated with the highly immunosuppressive tumor milieu in advanced HCC (11–13). Researches revealed that a multiplicity of membrane-linked inhibitory molecules [PD-1, cytotoxic T-lymphocyte-associated protein [CTLA]-4, thymocyte selection-associated high mobility group box protein [TOX]] and soluble factors (indoleamine 2,3-dioxygenase, arginase-1, adenosine, and others) involved in the suppression, leading to the exhaustion of antitumor response by T-lymphocytes, finally (8, 14).

Locoregional therapies that are commonly used in HCC have been demonstrated the advantage of boosting the tumor-specific T-cell response by exposing neo-tumor-associated antigens via necrosis of the HCC cells (15–22). We hypothesized that loco-therapies might enhance the response to anti-PD-1 monotherapy, especially in non-sensitive tumors (23, 24). In this proof-of-concept clinical trial, patients with advanced HCC who received single anti-PD-1 inhibitor after sorafenib failure and had a response of stable disease or atypical progression (defined as mixed responses in different lesions of the same individual) were enrolled. We mainly focused on whether the application of subtotal thermal ablation could improve the antitumor response of anti-PD-1 monotherapy.

Abbreviations: AEs, adverse events; CI, confidence interval; CTLA, cytotoxic T-lymphocyte-associated protein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, hazard ratio; MWA, microwave ablation; OS overall survival; ORR, objective response rate; PD-1, programmed cell death protein-1; PFS, progression-free survival; RECIST, Response Evaluation Criteria in Solid Tumors; RFA, radiofrequency ablation; TGK, tumor growth kinetics; TOX, thymocyte selection-associated high mobility group box protein; TTP, time to tumor progression.

METHODS

Participants

This proof-of-concept clinical trial was performed at three hospitals in China with approval of the ethical committee of each participating institution, and all participants provided informed consent. Eligible patients had a pathological diagnosis of HCC by either surgical resection tissue or core needle biopsy and had an advanced stage of a disease that previously received sorafenib or with unacceptable toxicity of sorafenib. Patients with previous organ transplantation, immunodeficient disease, or those who were given immunosuppressive therapies were excluded. Other eligibility criteria included: Child-Pugh A or B7 classification; Eastern Cooperative Oncology Group performance status score 0–2; adequate bone marrow (leukocyte count $>3.0 \times 10^9$/L, hemoglobin >8.0 g/L, and platelet count $>60 \times 10^9$/L), liver (alanine aminotransferase and aspartate aminotransferase <200 IU/mL), renal (creatinine <1.5 times the upper limit of the normal range), and coagulation (international normalized ratio <2.3) function.

Anti-PD-1 Therapy and Ablation Combination Procedures

Nivolumab or pembrolizumab intravenously would be administrated for up to 3 years or until at least 12 months of disease control, intolerable toxicity, or typical disease progression. Nivolumab was given a dose of 3 mg/kg every 2 weeks. Pembrolizumab was given a dose of 3 mg/kg every 3 weeks.

The radiological response was evaluated every 6–8 weeks, as identified by the immune-related Response Evaluation Criteria in Solid Tumors (RECIST) (25). In brief, the cutoff values of complete response (disappearance of all lesions), partial response (≥30% decrease of the sum of the longest diameters of target lesions from baseline) and progressive disease (≥20% increase from baseline) by RECIST were used. Progressive diseases were divided into two categories: typical progression and atypical progression. Atypical progression was the context of distinct responses occurring in different lesions in the same patient (e.g., active or stable lesions mixed with progressive lesions). Patients with stable diseases or atypical progression to anti-PD-1 monotherapy would be additionally treated with subtotal

TABLE 1 | Baseline characteristics.

	n = 50
Age † (years)	51 (19–74)
Gender	
Male	46 (92)
Etiology	
Hepatitis B virus	46 (92)
Hepatitis C virus	0 (0)
Others	4 (8)
Child-Pugh class/score	
A	46 (92)
B	4 (8)
ECOG performance status	
0	16 (32)
1	34 (68)
A-fetoprotein level † (ng/ml)	269.5 (0.97–12.1 × 10^4+)
>400 ng/ml	23 (46)
<400 ng/ml	27 (54)
No. of Tumor	
≤5	10 (20)
>5, ≤ 10	15 (30)
>10	25 (50)
Portal invasion	
Absent	30 (60)
Present	20 (40)
Extrahepatic metastases	
Absent	13 (26)
Present	37 (74)
Lung	27 (54)
Lymph node	12 (24)
Bone	5 (10)
Adrenal gland	3 (6)
Portal invasion or extrahepatic metastases	
Absent	5 (10)
Present	45 (90)
Previous treatment	
Surgical resection	27 (54)
Thermal ablation	22 (44)
TACE	29 (58)
HAIC	17 (34)
Sorafenib	50 (100)
Lenvatinib	5 (10)
Regorafenib	1 (2)
Radiotherapy	3 (6)
Recent treatment	
Therapy	
Sorafenib	28 (56)
HAIC	12 (24)
TACE	6 (12)
Lenvatinib	4 (8)
Reason for discontinuation	
Disease progression	41 (82)
Toxicity	9 (18)

Data are n (%), unless otherwise indicated.
†*Data are expressed medians. Numbers in parentheses are ranges.*
ECOG, Eastern Cooperative Oncology Group; HAIC, hepatic arterial infusion of chemotherapy; TACE, transarterial chemoembolization.

thermal ablation along with immunotherapy; and for those who with no lesions eligible for ablation, immunotherapy would be given solely. Patients with complete or partial responses would also keep on going with immunotherapy. Others with typical progression would stop immunotherapy.

Subtotal radiofrequency ablation (RFA) or microwave ablation (MWA) would be performed with computed tomography guidance within 10–14 days of radiological assessment and be followed by immunotherapy within 3–7 days. The anti-PD-1 inhibitor should be as same as those performed before ablation. Subtotal ablation defined as that up to two lesions (either intrahepatic or extrahepatic) was adequately ablated in one treated procedure, leaving most of the other lesions untreated. The lesion chosen for ablation was treated with curative intent and selected with consideration of minimizing technical risks, such as avoiding damage of large vessels, gastrointestinal tracts, among other structures. For patients assessed with atypical progression after 3 months of ablation, repeated subtotal ablation was allowed. Details of computed tomography-guided RFA or MWA were described in the **Supplementary Methods** (26, 27).

Safety and Efficacy

Safety evaluation was done continuously during immunotherapy and up to 90 days after the last dose by using the Common Terminology Criteria for Adverse Events (version 4.0). Complications related to ablation procedure were assessed during the next (0–24 h) and periprocedural (1–30 days) period and reported according to the Society of Interventional Radiology Classification System for Complications (28). Efficacy included an objective response (includes complete and partial response), duration of response, and disease control (Includes complete and partial response, and stable disease for at least 3 months).

Outcomes

The primary objective was the feasibility of systemic anti-PD-1 therapy in combination with loco-ablation in patients with advanced HCC for which anti-PD-1 monotherapy could not achieve a satisfactory response. The study mainly involved two aspects of feasibility: safety and efficacy. Secondary objectives were the time to tumor progression (TTP; time from the first dose of anti-PD-1 drug until the first typical disease progression), progression-free survival (PFS; time from first day of immunotherapy to first typical disease progression, or death, which occurred earlier) and overall survival (OS; time

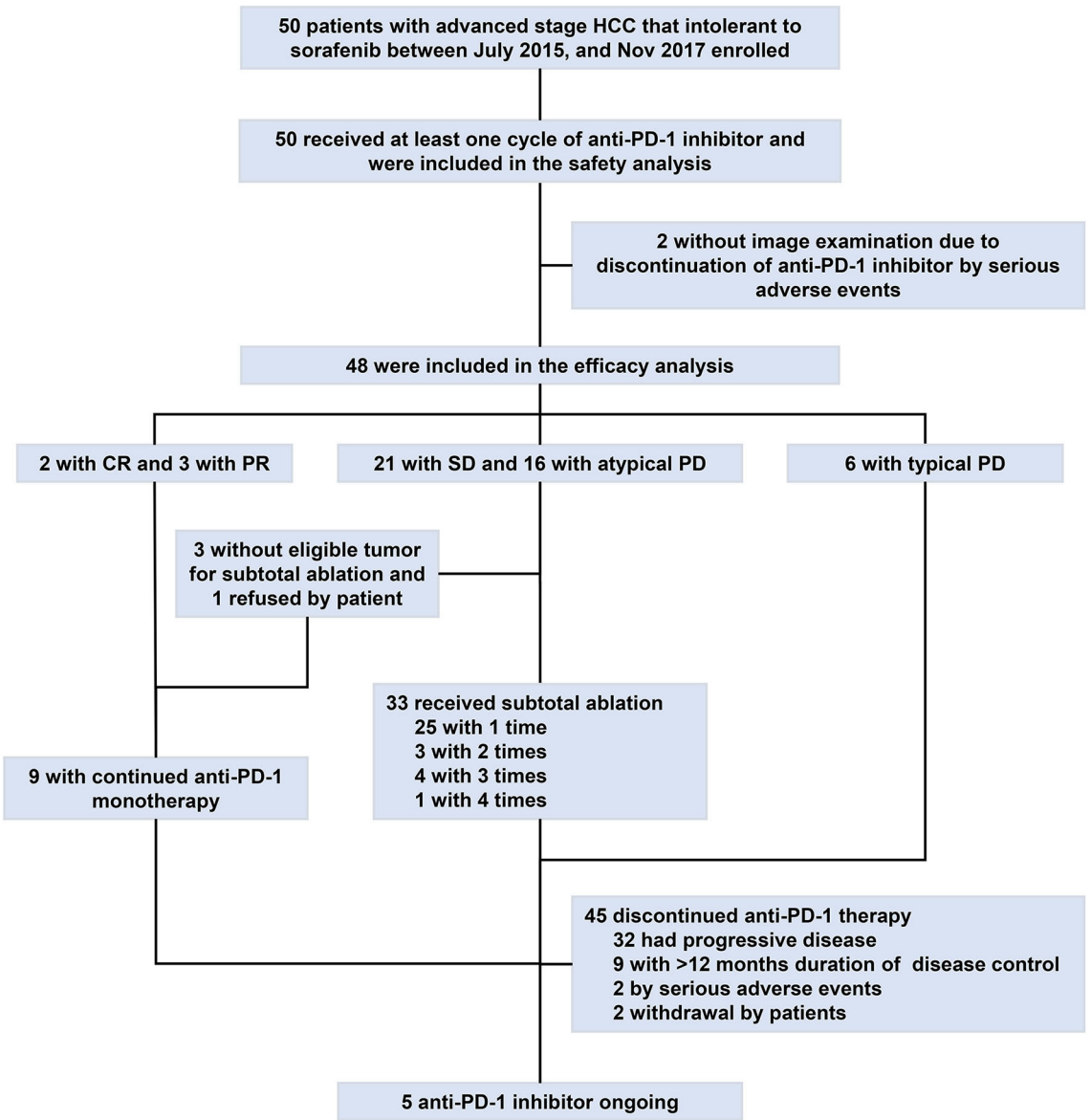

FIGURE 1 | Study profile flow. CR, complete response; HCC, hepatocellular carcinoma; PD-1, programmed cell death protein-1; PD, progressive disease; PR, partial response; SD, stable disease.

from first immunotherapy to death of any cause). Patients were followed up for survival every 4–6 months. An exploratory objective was the tumor growth kinetics (TGK) before and during immunotherapy. The method of TGK calculation was recorded in the **Supplementary Methods**.

Statistics

A sample size of about 50 subjects was chosen for the study to provide a reasonably reliable estimate of efficacy and sufficient safety or complications follow up. Baseline characteristics and adverse events (AEs) were summarized with descriptive statistics. Safety was assessed in all enrolled patients who received at least one dose of anti-PD-1 inhibitor. Duration of response, TTP, PFS, and OS were estimated by the Kaplan-Meier curve and reported along with 95% confidence interval (CI). Data were analyzed with SPSS version 25.0. All data of this study have been recorded at the study center (number RDDA2017000320). The ClinicalTrials.gov identifier number was NCT03939975.

RESULTS

Patient Characteristics

Between July 2015, and Nov 2017, fifty patients were enrolled in the study treated with an anti-PD-1 monotherapy. Two patients had drug discontinuation by serious AEs before the first

TABLE 2 | Treatment-related adverse events.

	n = 50		
Anti-PD-1 inhibitor-related AEs	**Any grade**	**Grade 1–2**	**Grade 3–4**
Discontinued due to AEs	4 (8)	1 (2)	3 (6)
Fatigue	17 (34)	17 (34)	0
Transaminitis	10 (20)	10 (20)	0
Fever	8 (16)	8 (16)	0
Diarrhea	6 (12)	6 (12)	0
Pneumonitis	5 (10)	4 (8)	1 (2)
Hyperbilirubinemia	4 (8)	2 (4)	2 (4)
Hypothyroidism	4 (8)	3 (6)	1 (2)
Pruritus	4 (8)	4 (8)	0
Rash	4 (8)	4 (8)	0
Hyperthyroidism	3 (6)	3 (6)	0
Hypoalbuminemia	3 (6)	3 (6)	0
Hypoleukemia	3 (6)	2 (4)	1 (2)
Thrombocytopenia	3 (6)	2 (4)	1 (2)
Prolactin increase	2 (4)	2 (4)	0
Alopecia	1 (2)	1 (2)	0
Anemia	1 (2)	1 (2)	0
Appetite decrease	1 (2)	1 (2)	0
Creatinine increase	1 (2)	1 (2)	0
Diabetic metabolic decompensation	1 (2)	1 (2)	0
Nausea	1 (2)	1 (2)	0
	***n* = 47 ***		
Ablation-related complications	**Any**	**Grade A-B**	**Grade C-D**
Discontinued due to complications	0	0	0
Pain	47 (100)	41 (87.2)	6 (12.8)
Transaminitis	19 (40.4)	10 (21.3)	9 (19.1)
Vomiting	22 (46.8)	22 (46.8)	0
Constipation	13 (27.7)	13 (27.7)	0
Fever	11 (23.4)	11 (23.4)	0
Intraabdominal hemorrhage	11 (23.4)	9 (19.1)	2 (4.3)
Pneumothorax	7 (14.9)	5 (10.6)	2 (4.3)
Pleural effusion	9 (19.1)	7 (14.9)	2 (4.3)
Bile duct pneumatosis	6 (12.8)	6 (12.8)	0

Data are n (%), unless otherwise indicated.
**A total of 47 times of ablation procedures were performed in 33 patients.*
AEs, adverse events; PD-1, programmed cell death protein-1.

TABLE 3 | Response to anti-PD-1 monotherapy or combined therapy.

Response	**Anti-PD-1 (*n* = 50)**	**Anti-PD-1 + ablation (*n* = 50)**
BEST RESPONSE		
Complete response	2 (4%)	4 (8%)
Partial response	3 (6%)	8 (16%)
Stable disease	21 (42%)	22 (44%)
Progressive disease	22 (44%)	14 (28%)
Not assessable	2 (4%)	2 (4%)
Objective response †	5 (10%)	12 (24%)
Disease control ‡	-	30 (60%)
Median DOR, months (95% CI) ‖	-	21.4 (14.7–28.1)
Median TTP, months (95% CI)	-	6.1 (2.6–11.2)
Median PFS, months (95% CI)	-	5 (2.9–7.1)
Median OS, months (95% CI)	-	16.9 (7.7–26.1)

Data are n (%), unless otherwise indicated.
† Includes complete response and partial response.
‡ Includes complete response, partial response and stable disease for at least 3 months.
‖ Assessed in patients with complete responses or partial responses.
CI, confidence interval; DOR, duration of response; OS, overall survival; PD-1, programmed cell death protein-1; PFS, progression-free survival; TTP, time to progression.

image examination and were assessed for safety only. Thirty-seven patients had stable or atypical progressive diseases to anti-PD-1 monotherapy; three of the 37 patients had no tumors suitable for ablation, and another one patient declined to undergo ablation treatment; thus, a total of 33 patients were treated with additional ablation.

Patients baseline characteristics in the study were summarized in **Table 1**. Either macrovascular invasion or extrahepatic metastases were present in 45 (90%) patients. All the patients were heavily pretreated by multiple therapies and had experiences of receiving sorafenib. In terms of the most recent treatment ahead of anti-PD-1 therapy, 28 (56%) of the 50 patients were treated with sorafenib, 12 (24%) with arterial infusion chemotherapy of oxaliplatin and fluorouracil, 6 (12%) with TACE, and 4 (8%) with lenvatinib; 41 (82%) patients had discontinued such therapies due to disease progression, and nine (18%) patients had discontinued due to treatment-related toxicities or technical factors (includes six by sorafenib, two by TACE, and 1 by lenvatinib). The median time interval between recent therapy stopping and anti-PD-1 therapy commencement was 1.9 months (range, 1.1–3.2).

Thirty-three (66%) of the 50 patients experienced with ablation, among whom, eight (24.2%) patients experienced two or more times of ablation due to repeated atypical disease progression included 3 (9.1%) experienced two times, 4 (12.1%) experienced three times, and 1 (3%) experienced four times. With a median follow-up of 17.9 months (range, 4.6–41.6) by Mar 31, 2019, 47 (94%) of the 50 patients discontinued immunotherapy. The median duration of immunotherapy was 6.5 months (range, 1.6–32.4). Most patients discontinued immunotherapy due to disease progression ($n = 32$; 64%) or duration of disease control longer than 12 months ($n = 9$; 18%) (**Figure 1**).

Safety

Treatment-related AEs for both anti-PD-1 inhibitor and ablation therapy were recorded in **Table 2**. At least one anti-PD-1 inhibitor-related toxicity has occurred in 41 (82%) of the 50 patients and, among those, 7 (14%) were as serious AEs. AEs of any grade that occurred in at least 10% of patients were fatigue in 17 (34%) patients, transaminitis in 10 (20%) patients, fever in 8 (16%) patients, diarrhea in 6 (12%) patients, and pneumonitis in 5 (10%) patients. The most frequent serious AEs was hyperbilirubinemia in two (4%) patients. No cases of fulminant increases of the hepatitis B virus (HBV) were recorded. Four (8%)

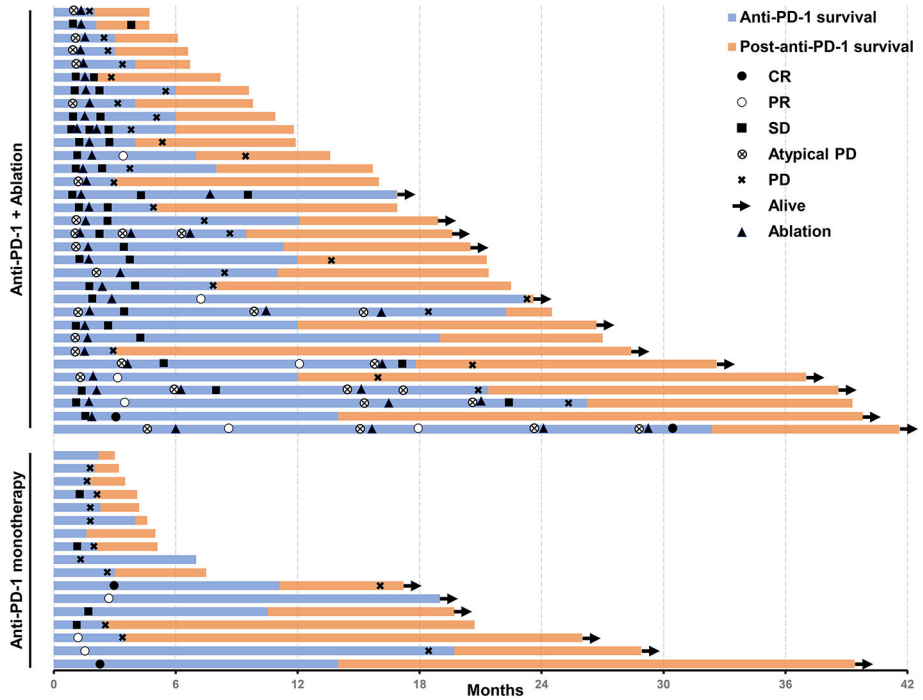

FIGURE 2 | Swimmer's plot shows the time of response, time of ablation, the survival of patients treated with an anti-PD-1 inhibitor in the combination of thermal ablation or anti-PD-1 monotherapy, post-discontinuation of anti-PD-1 treatment survival, and current status. Assessed in a total of 50 patients. PD-1, programmed cell death protein-1.

of the 50 patients had to discontinue immunotherapy due to AEs. One patient experienced grade four pneumonitis, which occurred after the third dose of pembrolizumab and died after 4 weeks of immunotherapy discontinuation. One patient discontinued pembrolizumab because of grade 4 of hyperbilirubinemia. One patient discontinued nivolumab because of grade 3 of thyroid dysfunction. One patient developed slowly increased creatinine level (max of 2.3 mg/dL) and discontinued pembrolizumab with a total of 14 doses but remained tumor control for 19.7 months by the date cutoff.

A total of 47 times of ablation procedure was performed in the 33 patients treated with combined therapy. No ablation-related severe complications (Grade E) or death (Grade F) were recorded within 30 days of the ablation procedure. There was also no immunotherapy interruption directly attributable to the ablation procedure. Most of the ablation-related complications were common in routine clinical practice and managed as per the standard of care (**Table 2**). Transaminase increase (Grade C) was the most frequent major complication occurred in 9 (19.1%) of the 47 ablation sessions.

Efficacy

An objective response was detected in five (10%) of the 50 patients who were treated with anti-PD-1 monotherapy. Twenty-one (42%) patients had stable diseases, 16 (32%) patients had atypical progressive diseases, and 6 (12%) had typical progressive diseases. Two patients (4%) died before the first image examination due to serious AEs. Thirty-seven patients (includes 21 with stable diseases and 16 with atypical progressive diseases) were preliminary candidates for thermal ablation; three of the 37 candidates could not be treated because they did not have eligible tumors for ablation and one candidate declined to receive ablation. Ultimately, ablation was performed in 33 patients, and the technical success rate was 100%. Seven (21.2%) of the 33 patients were recorded improved efficacy by combined therapy included 2 (6.1%) with a complete response and 5 (15.1%) with partial response. Thus, the objective response rate (ORR) of the 50 patients was increased to 24% (12 in 50 patients) by treating with the combined therapy. The best changes from baseline in sizes of the targeted lesions were shown in **Supplementary Figure 1**. At data cutoff, 5 (41.7%) responders were ongoing, and the median duration of response of the 12 responders was 21.4 months (95%CI, 14.7–28.1). Disease control was detected in 30 (60%) of the 50 patients with combined therapy (**Table 3**). **Figure 2** showed efficacy and survival for the participants on the study in addition to the response to treatment, time of ablation, and duration of immunotherapy. **Figure 3** described the images of radiological examinations and subtotal ablation, and target tumor growth kinetics, and alpha-fetoprotein dynamics of a patient who achieved a durable response to combined therapy. **Figure 4** summarized the clinical events of a patient who treated with a continuous immunotherapy in the combination of multiple sessions of ablation.

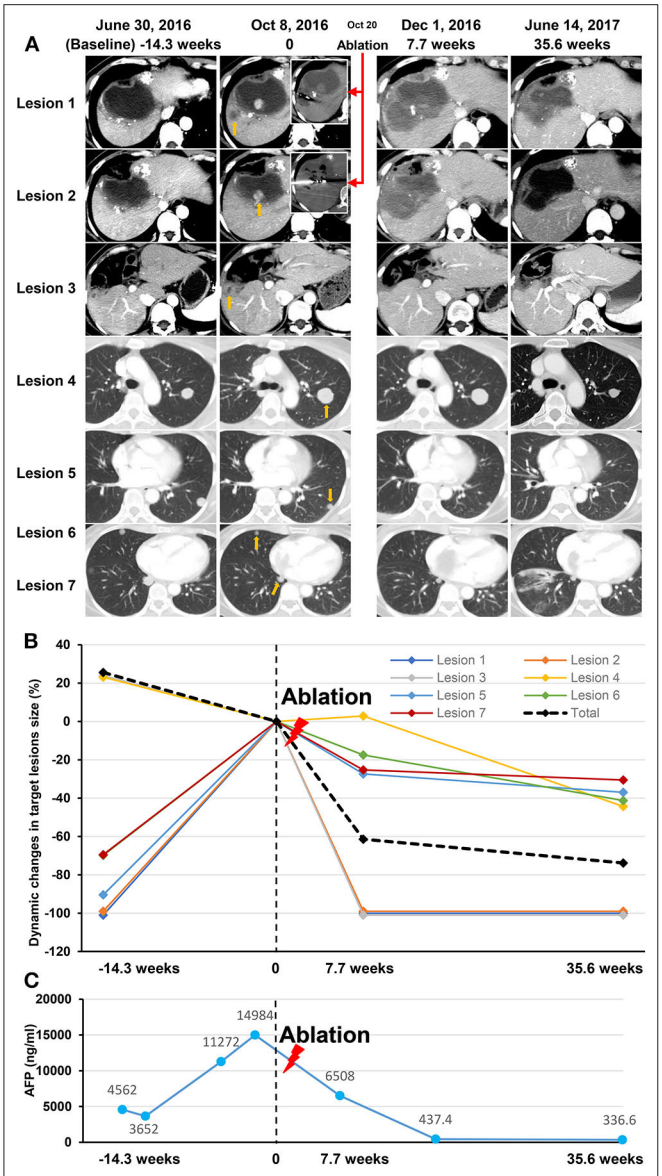

FIGURE 3 | Data of a participant treated with an anti-PD-1 inhibitor in the combination of subtotal thermal ablation. The patient had a diagnosis of recurrent intrahepatic tumors with tumor thrombus invading both inferior vena cava and right atrium, and multiple lung metastases. After receiving 14.3 weeks of anti-PD-1 inhibitor of pembrolizumab, an atypical progression was assessed on image examination on October 8, 2016 (time of 0) as that intrahepatic tumors (Lesion 1, 2, and 3; yellow arrow), vascular invasions (no showing), and part of the lung lesions progressed (a representative example as Lesion 4; yellow arrow), but the other part of lung metastases (representative examples as Lesion 5, 6, and 7; yellow arrow) regressed. Two progressive lesions (Lesion 1 and Lesion 2) in the liver was selected for subtotal thermal ablation (red arrows), and consequently, the leaving intrahepatic tumor (Lesion 3) disappeared, and all the lung metastases regressed due to the combination of pembrolizumab and ablation therapy. Stable disease was recorded on December 1, 2016 (7.7 weeks), and a partial response was achieved on June 14, 2017 (35.6 weeks). Pembrolizumab infusion was lasted for 17.8 months and discontinued because of more than 12 months of ongoing disease control. At last follow-up, the patient was still alive with a progression-free survival of 21.4 months and overall survival of 32.6 months. **(A)** The Images of seven lesions at baseline, response assessment to anti-PD-1 monotherapy,

(Continued)

FIGURE 3 | ablation procedure, and post-ablation assessment. **(B)** Dynamic changes in the size of the seven lesions before and after thermal ablation (red lighting). **(C)** The dynamic curve of the serum AFP level before and after thermal ablation (red lighting). AFP, alfa-fetoprotein; PD-1, programmed cell death protein-1.

Outcomes

Forty-one (82%) of the 50 patients had disease progression or died until the last follow-up. The median TTP was 6.1 months (95%CI, 2.6–11.2), and the median PFS was 5 months (95%CI, 2.9–7.1). Thirty-two (64%) patients had died, and the median OS was 16.9 months (95%CI, 7.7–26.1) (**Supplementary Figure 2**). The estimated 6-, 12-, and 24-months PFS rates of the 50 patients were 44, 34, and 11.9%, respectively. The estimated 6-, 12-, and 24-months OS rates were 78, 56, and 35.9%, respectively. The median PFS [16.4 months [95% CI, 7.1–25.7] vs. 2.6 months [2.2–3.0]; hazard ratio [HR], 0.181 [95% CI, 0.9–0.364]; $P < 0.001$] and median OS [27 months [11.5–42.5] vs. 6.6 months [5.3–7.9]; 0.228 [0.109–0.478]; $P < 0.001$] was significantly longer in patients with disease control (lasted at least 3 months) compared with those who without (**Supplementary Figure 3**).

In the exploratory analysis, we compared TGK on the last treatment ahead of anti-PD-1 treatment and TGK on anti-PD-1 treatment. Forty-one patients had tumors that were evaluable for TGK calculation both on last treatment and immunotherapy, among them, 4 (9.8%) patients had $TGK_R \geq 2$, 2 (4.9%) patients had TGK_R between 1 and 2, 21 (51.2%) patients had TGK_R between 0 and 1, and 14 (34.1%) patients had $TGK_R < 0$ (**Supplementary Figure 4**). At date cutoff, 3 of the four patients with $TGK_R \geq 2$ had died and had a poor OS of 3.5, 4.7, and 6.7 months, respectively; another one patient switched to receiving lenvatinib and was still alive with a survival of 28.4 months.

DISCUSSION

This proof-of-concept study investigated the feasibility and safety of the combination of anti-PD-1 inhibitors and thermal ablation in appropriate lesions of patients with advanced HCC after sorafenib failure. We found that in patients who had stable or atypical progressive diseases during immunotherapy, additional ablation could increase the ORR with tolerated toxicity and achieved a relatively better median survival, indicating that ablation may stimulate and enhance the antitumor immunity of anti-PD-1 therapy.

Nivolumab and pembrolizumab were acceleratedly approved to be used in the second line treatment for advanced HCC after sorafenib failure in recent 2 years (29). CheckMate-040 proved that the ORR of Nivolumab was 15–20% in advanced HCC (9) and Keynote-240 reported an ORR of 18.3% for pembrolizumab (30). In our study, we observed an ORR of only 10% in patients who received nivolumab or pembrolizumab, which might be attributed to two reasons, firstly, CheckMate 040 and Keynote-240 were purely second line studies and post sorafenib while in our study all patients were exposed to sorafenib but sorafenib was not the only proceeding therapy prior to anti-PD-1 antibody;

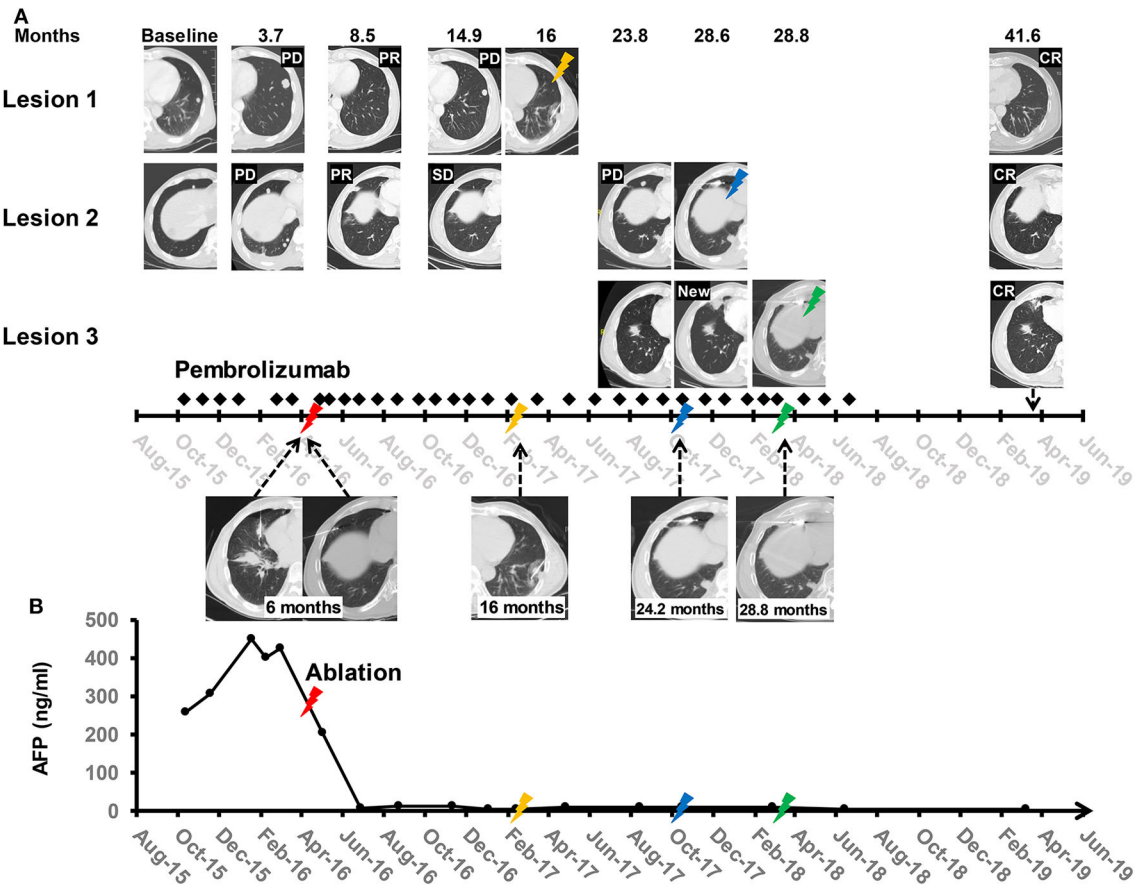

FIGURE 4 | Clinical events of a participant who treated with a continuous anti-PD-1 inhibitor of pembrolizumab in the combination of multiple sessions of thermal ablation. The patient was enrolled due to progressive lung metastases to sorafenib and had an atypical response (stable lesions with progressive lesions) to pembrolizumab monotherapy after 6 months of anti-PD-1 inhibitor initiated. Then the first subtotal ablation (red lighting) was performed, and the size of two targeted lesions (Lesion 1 and 2) shrunk obviously after 2.5 months of ablation. The duration of response of Lesion 1 and Lesion 2 since the first ablation was 14.9 months and 23.8 months, respectively. Lesion 1 (yellow lighting) and Lesion 2 (blue lighting) were ultimately ablated due to tumor progression. The fourth session of ablation (green lighting) was done for a new tumor (Lesion 3), which occurred at 28.6 months from baseline. A total of 33 doses of pembrolizumab was infused with a duration of 32.4 months. At date cutoff, the patient had a complete response to anti-PD-1 inhibitor in the combination of ablation, with a level of serum alfa-fetoprotein in the normal range, and progression-free survival of 41.6 months. **(A)** The middle panel shows the timeline of treatments, including pembrolizumab (black rhombus) and ablation (lightning). Upper panels show CT images of three representative lesions at baseline, course of treatment, and last follow-up since initiation of the pembrolizumab. The lower panel shows CT images of the four sessions of ablation. CR, complete response; PD-1, programmed cell death protein-1; PD, progressive disease; PR, partial response; SD, stable disease. **(B)** The dynamic curve of the serum AFP level. AFP, alfa-fetoprotein.

Secondly, the etiology of population-92% patients in this study had HBV infection, while the percentage in Checkmate-040 and Keynote-240 were 23.8 and 25.9%, respectively. The subgroup analysis of Checkmate-040 showed that the ORR in HBV-infection population was 7%, which was near to ours.

Ablation is one approach of loco-therapies and commonly used for HCC (31, 32). In recent years there has been an increasing wariness that loco-therapies may eliminate not only tumors but also have additional systemic effects (16, 33). Some studies described the immunological "abscopal effects" induced by loco-therapies and a range of cytokine and chemokine changed following various ablative procedures, suggesting that once the immune response is triggered the effects could be potentially amplified by immunotherapy (16, 17, 34, 35). Our study confirmed the hypothesis and found additional ablation enhanced the antitumor effects of anti-PD-1 inhibitors and increased the response rate. Repeated ablations were also proved feasible and safe. Moreover, the efficacy was not limited in the lesion which treated with ablation but observed in the outside zone, indicating that the systemic effects brought by ablation indeed exist. Shi L et al. demonstrated that in liver metastases from colorectal cancer, tumor quickly overcame T-cell-mediated immune responses which were triggered by RFA of one tumor initially by inhibiting the function of $CD8^+$ and $CD4^+$ T-cells, driving a shift to higher regulatory T-cell to effector T-cell ratio, and upregulating PD-L1/PD-1 expression (19). For MWA, broad analysis of circulating cytokines proved that the production of IL-12, a Th1 cytokine, is enhanced after MWA however the secretion of Th2 cytokines IL-4 and IL-10 is inhibited, leading to

a positive antitumor response. (36) PD-L1-PD-1 axis might play a critical role in ablation-induced antitumor immune responses, which need to be further validated in advanced HCC (24, 37).

A recent study conducted by Greten et al. firstly reported that a combination of tremelimumab, an anti-CTLA-4 inhibitor with ablation in heavily pretreated post-sorafenib population was feasible and resulted in objective tumor responses outside of the ablated zone (38). However, all the patients in that study were treated with ablation, unselectively, leading to a significant question that whether the ablation or tremelimumab itself or both account for the antitumor effects. Our study may give some reference to this question. Although all 50 patients received immunotherapy, those who would be treated with ablation depend on the response to immunotherapy. Eleven patients were excluded, including 5 (10%) patients with objective response to anti-PD-1 monotherapy and 6 (12%) with typical progressive diseases, in which situation we regarded ablation not necessary. Finally, 33 patients with stable or atypical progressive diseases during anti-PD-1 monotherapy underwent ablation. This selectivity of the population is significant to judge the value of loco-therapies during immunotherapy (39, 40). Our study found that 7 (21.2%) of the 33 patients were recorded improved efficacy including 2 (6.1%) with a complete response and 5 (15.1%) with partial response. The ORR of all 50 patients was increased from 10 to 24% after treated with the combined therapy, indicating that ablation combined with the immunotherapy is feasible in patients who had stable or atypical progressive diseases during anti-PD-1 monotherapy.

There are still some limitations in our study. Firstly, the sample size is not large enough that may lead to bias; Secondly, the population enrolled in this study was mainly with HBV-infection, accounting for 92% of all patients, leading to the results valuable in part of patients with advanced HCC; Thirdly, in our study, all patients were exposed to sorafenib but not all received anti-PD-1 antibody immediately post sorafenib failure and 44% of patients received systemic therapies more than sorafenib, which we think it should be noticed.

In conclusion, this proof-of-concept trial suggested that additional thermal ablation combined with anti-PD-1 inhibitors increased the response rate and improved survival in patients with advanced HCC after sorafenib failure who had a stable or atypical progressive disease during anti-PD-1 monotherapy, which may provide a potentially promising strategy to treat advanced HCC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethical Committees of Sun Yat-Sen University Cancer Center, Third Affiliated Hospital of Sun Yat-Sen University, and Jieyang Affiliated Hospital, Sun Yat-Sen University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MZ, NL, YK, LM, and JLi conceived and designed the study. NL, LM, XL, HC, YL, JLa, MH, and HD collected the data. NL, YK, LM, XL, JLi, HT, and MZ analyzed and interpreted the data. All authors were involved in the drafting, review, and approval of the report and the decision to submit for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.580241/full#supplementary-material

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MINI REVIEW
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The Potential Gut Microbiota-Mediated Treatment Options for Liver Cancer

Chunye Zhang[1], Ming Yang[2] and Aaron C. Ericsson[1,3]**

[1] Department of Veterinary Pathobiology, University of Missouri, Columbia, MO, United States, [2] Department of Surgery, University of Missouri, Columbia, MO, United States, [3] University of Missouri Metagenomics Center, University of Missouri, Columbia, MO, United States

Primary liver cancer is one of the leading causes of cancer death worldwide. Surgical and non-surgical treatments are optional for liver cancer therapy based on the cancer stage. Accumulating studies show that the gut–liver axis influences the progression of liver diseases, including liver inflammation, fibrosis, cirrhosis, and cancer. However, the role of gut microbiota and their derived components and metabolites in liver cancer remains to be further clarified. In this review, we discuss the roles of gut microbiota and specific bacterial species in HCC and the strategies to modulate gut microbiota to improve antitumor therapy. Given the limitation of current treatments, gut microbiota-mediated therapy is a potential option for HCC treatment, including fiber diet and vegetable diet, antimicrobials, probiotics, and pharmaceutical inhibitors. Also, gut microbiota can be used as a marker for early diagnosis of HCC. HCC occurs dependent on various environmental and genetic factors, including diet and sex. Furthermore, gut microbiota impacts the immunotherapy of HCC treatment. Therefore, a better understanding of the role of the gut–liver axis in liver cancer is critically important to improve therapeutic efficacy.

Keywords: liver cancer, treatments, sex, gut microbiota, clinical trials

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Zhejiang University, China

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Xue Wang,
Jiangnan University, China
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Anhui Medical University, China

****Correspondence:***
Aaron C. Ericsson
ericssona@missouri.edu
Ming Yang
yangmin@health.missouri.edu

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INTRODUCTION

Liver cancer is the fourth leading cause of cancer death worldwide (1). In the United States, there will be approximately 42,030 new cases of primary liver cancer and intrahepatic bile duct cancer and 31,780 deaths due to these cancers in 2019, according to the American Cancer Society's estimate[1]. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer (2), and the incidence of HCC is predicted to rise continually in the next decade (3). HCC typically results from chronic liver disease (4), and the main risk factors causing HCC are hepatitis B or C viruses, alcohol abuse, non-alcoholic fatty liver disease (NAFLD), diabetes, and other metabolic and genetic diseases (5, 6). Early diagnosis of HCC in patients is critically important for treatment with good outcomes (7). Unfortunately, the determination of HCC is often made in advanced disease stages, which are frequently accompanied by liver dysfunction or failure (2).

There are multiple options available for HCC treatment, including surgical resection and non-surgical therapies (8). HCC treatment options selectively depend on the stage of the disease, liver

[1]https://www.cancer.org/cancer/liver-cancer/about/what-is-key-statistics.html

function, and cost of treatment. Even though the survival of patients with HCC is prolonged, recurrence remains a major issue for HCC treatment. In the past few years, new molecular targeting agents have been approved for systemic treatment by the United States Food and Drug Administration (FDA) (9, 10). In 2019, the FDA approved cabozantinib (Cabomeyx, Exelixis, Inc.) treatment in HCC patients as the second-line[2]. Cabozantinib is a multi-tyrosine kinase inhibitor primarily targeting tyrosine-protein kinase Met (c-MET), vascular endothelial growth factor receptor 2 (VEGFR2), and tyrosine kinase receptors AXL and RET, which was initially approved to treat medullary thyroid cancer or advanced renal cell carcinoma (RCC) (11, 12). Given the complex pathogenesis of HCC, current therapies still fail to meet the needs of patients.

Gut microbiota and gut microbiota-derived products have been shown to play important roles in the pathogenesis of HCC and its therapy. For instance, lipoteichoic acid (LTA, a Gram-negative bacterial cell wall component) and deoxycholic acid (DCA, a secondary bile acid produced by bacteria) collaboratively induced the expression of prostaglandin-endoperoxide synthase 2 or cyclooxygenase-2 (COX-2) through Toll-like receptor 2 (TLR-2) in senescent hepatic stellate cells (HSCs) to enlarge prostaglandin E_2 (PGE_2)-mediated inhibition of antitumor immunity, resulting in HCC progression (13). It has been reported that gut microbiota-derived products can modulate hepatic inflammation and immunity to impact non-alcoholic steatohepatitis (NASH) and virus-induced HCC progression (14). HCC patients who are responsive to anti-programmed cell death protein 1 (PD-1) immunotherapy had higher taxa richness in fecal samples compared to non-responders (15). In addition, *Akkermansia muciniphila* and *Ruminococcaceae* spp. are enriched species in responder patients, while *Proteobacteria* increased in non-responders.

In this review, we first summarize current therapies for liver cancer. Then, we discuss the potential roles of gut microbiota in liver cancer and gut microbiota-mediated treatment and diagnosis for liver cancer, specifically focusing on the shift of gut microbiota in HCC development and treatment.

CURRENT THERAPIES FOR LIVER CANCER

Currently, there are several treatment options for liver cancer, but the selection is highly dependent on the cancer stage and remaining liver health (16, 17). Surgical resection is one of the major curative treatment options for the primary liver tumor or metastatic liver tumor (18, 19). However, surgical treatment requires to be performed in the early stage of liver cancer with a low potential incidence of metastasis. When surgical resection is not an option, minimally invasive local therapies such as radiofrequency ablation (RFA), microwave ablation (MWA), high-intensity focused ultrasound (HIFU), and irreversible electroporation (IRE) become treatable options for both primary and metastatic liver tumors (20, 21). For widespread liver cancer, chemotherapy, immunotherapy, and targeted therapy may be preferable. For example, sorafenib, a multi-kinase inhibitor with anti-proliferative and anti-angiogenic effects, has represented the primary treatment for advanced HCC for a long time (22). It was the only FDA-approved systemic therapeutic agent for HCC treatment until the recent approval of five new agents. In newly approved agents, lenvatinib is optional in the first-line treatment, while regorafenib, nivolumab, pembrolizumab, and cabozantinib are used as second-line therapies (9). All of these treatment options could be applied according to the stage and size of liver tumor. The treatment options for liver cancer are listed in **Table 1**.

Cancer recurrence and therapeutic resistance are the main issues that reduce the survival outcomes of cancer patients (23). In this situation, combination therapy, treatment with two or more therapeutic agents or options, is helpful for good outcomes. For example, doxorubicin is a commonly used chemotherapy drug with trans-arterial chemoembolization (TACE) in HCC treatment (24). Tremelimumab, an immune checkpoint blocker, in combination with tumor ablation, is beneficial for patients with advanced HCC and viral infection as it can improve the infiltration of $CD8^+$ T cells and reduce viral load (25).

THE ROLES OF GUT MICROBIOTA IN LIVER CANCER

The liver is directly exposed to gut microbial components and metabolites via the liver portal vein (26). Increasing studies show that the gut–liver axis influences the progression of liver diseases such as liver inflammation, fibrosis, cirrhosis, and cancer (27, 28). For instance, high-alcohol-producing bacterium *Klebsiella pneumoniae* is implicated in the pathogenesis of NAFLD in human patients, evidenced by oral gavage of a clinically isolated strain causing NAFLD in mice (29). Cirrhotic patients with or without HCC had a higher abundance of genera *Lactobacillus* and *Bacteroides* with LDA scores larger than 4.0, whereas healthy controls had a higher abundance of *Akkermansia* and *Methanobrevibacter* (30). Additionally, HCC patients possessed relatively greater abundance of *Bacteroides* and *Ruminococcaceae* and lower abundance of *Bifidobacterium* compared with cirrhotic patients without HCC.

Gut microbiota impacts liver cancer by modulating different factors, including bile acids, immune checkpoint inhibitors, and Toll-like receptors (TLRs), among others.

Bile Acids

Bile acids (BAs) consist of primary and secondary bile acids. Primary BAs such as cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized in hepatocytes from cholesterol, while secondary BAs such as deoxycholic acid (DCA) and lithocholic acid (LCA) are synthesized by the intestinal bacteria using the primary BAs (31, 32). While BAs play pivotal roles in glucose metabolism (33) and vitamin and lipid absorption (34), an overabundance of BAs can cause hepatocyte DNA damage to promote carcinogenesis by promoting the alteration of tumor

[2]https://www.fda.gov/drugs/fda-approves-cabozantinib-hepatocellular-carcinoma

TABLE 1 | Current treatment options for liver cancer.

Treatments	Conditions	Examples	References
Surgical therapy	Surgical resection is an option for patients with early-stage HCC and preserved liver function. Surgical resection is commonly applied in solitary tumors ≤5 cm in size or ≤3 cm without gross vascular invasion and portal hypertension. Liver transplantation is a curative therapy for end stage liver disease.	Surgical resection, liver transplantation.	(19, 86–88)
Ablation	Ablation is a therapy to locally destroy the tumor cells with heat, rapid cooling, etc. It is applied in scattered small liver tumors. It is an effective treatment for patients with advanced primary or secondary liver tumors.	Radiofrequency ablation (RFA), microwave ablation (MWA).	(89–91)
Embolization therapy	An effective therapy for unresectable tumors by blocking or reducing the tumor blood circulation. Gene embolization selectively transfers viruses or vector embolized with cytokines (e.g., TNF-α and IFN-γ) or p53 genes.	Transarterial embolization (TAE) Transarterial chemoembolization (TACE)	(92–94)
Radiation therapy	High-energy rays or beams of intense energy are used to kill cancer cells. It can offer local treatment for unresectable HCC, but may not be a good option for some patients whose liver has been greatly damaged by diseases such as hepatitis or cirrhosis.	Photon-based intensity-modulated radiation therapy (IMRT), three-dimensional conformal radiotherapy (3D-CRT).	(95–97)
Targeted therapy	Medicines that specifically target some proteins can reach almost all parts of the body, which makes them potentially useful against cancers with metastasis. It is optional for tumors that are not very sensitive to chemotherapy.	Tyrosine kinase inhibitors: sorafenib (Nexavar) and cabozantinib (Cabometyx).	(98, 99)
Immunotherapy	Immunotherapy uses the self-immune system to fight cancer. However, cancer cells sometimes use certain checkpoints to avoid being attacked by the immune system. By blocking immune checkpoint protein PD-1, the drugs can improve the immune response against cancer cells. This treatment can shrink or slow tumor growth.	Pembrolizumab (Keytruda) and nivolumab (Opdivo).	(100–102)
Chemotherapy	Antitumor medicines to kill fast-growing cancer cells are an option for people whose liver cancer cannot be treated with surgery and is not responsive to local therapies such as ablation or embolization, or targeted therapy. Medicines for chemotherapy and targeted treatment can reach almost all parts of the body.	Oxaliplatin (Eloxatin), mitoxantrone (Novantrone).	(103, 104)

suppressor genes and oncogenes (34). Ma et al. reported that the conversion of primary to secondary BAs impacted the infiltration of hepatic natural killer T cells (NKT cells), which controlled the progression of liver cancer in mouse (35). The accumulation of hepatic $CXCR6^+$ NKT cells was mediated by the expression of CXCL16 in liver sinusoidal endothelial cells (LSECs). In human samples, the presence of primary bile acid CDCA was positively correlated with CXCL16 expression, with which the expression of secondary bile acid GLCA was inversely correlated (36). The bile acid biotransformation was influenced by gut microbial community (37), such as bacterial species *Clostridium* (35). These findings indicate that modulating gut microbiota can change the components of BAs to improve antitumor immunity. Furthermore, BA receptors, farnesoid X receptor (FXR), and G protein-coupled bile acid receptor 1 (TGR5) are the potential regulators for BA homeostasis and carcinogenic effects in liver cancer (34).

Immune Checkpoints

Immune checkpoint inhibitors are promising treatable options for HCC treatment or applied as an adjunct therapy (38). Cancer development is associated with immune suppression since cancer cells can activate different immune checkpoint pathways to inhibit antitumor therapies (39). Antibodies or inhibitors that block cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), PD-1, programmed cell death 1 ligand 1 (PD-L1), and CD24 show promising therapeutic effects on cancer treatment (39–41). Tremelimumab, a monoclonal antibody that blocks CTLA-4, was first tested in patients with HCC and hepatitis C virus infection (42, 43). The results indicated that tremelimumab treatment showed not only anti-HCC effect but also enhanced anti-HCV immunity.

Further clinical trials demonstrated the reliable adjunct antitumor effect of tremelimumab with the combination of subtotal RFA or chemoablation in patients with advanced HCC (25). The combination of anti-PD-1/PD-L1 with anti-CTLA-4 antibodies and the synergistic application of immune checkpoint inhibitors with other antitumor therapies are being evaluated at different stages of clinical trials. The results suggest that an anti-PD-1 antibody in combination with locoregional therapy or other targeted therapy is an effective treatment for HCC (44, 45). Immune checkpoint inhibitors have been shown to prolong the survival time in HCC patients (46). Therefore, Nivolumab, a monoclonal antibody that blocks the PD-1 receptor on T cells, was approved by the United States FDA for liver cancer treatment in 2017. Pembrolizumab (Keytruda), another immune checkpoint inhibitor for PD-1, was approved by the United States FDA for HCC treatment in 2018.

Importantly, increasing evidence shows that gut microbiota influences the efficacy of immune checkpoint antibodies, as antibiotic treatment can diminish their effectiveness by depletion of gut microbiome, while the presence of specific gut microbes increases this efficacy (47). Clinical studies have shown that some of the bacterial species enhanced the efficacy of immune checkpoint therapy (48), such as the effect of *Bacteroides caccae* on anti-CTLA-4 and anti-PD-1 in melanoma (49), and the impact of *A. muciniphila* on anti-PD-1 in non-small-cell lung carcinoma (NSCLC) and renal cell carcinoma (RCC) (50). Therefore, modulating gut microbial components to improve the antitumor

effect of immune checkpoint inhibitors is a potential strategy for HCC treatment.

TLRs

Toll-like receptors are the most well-studied family of pattern recognition receptors (PRRs) (51). TLRs can recognize pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) like tumor-derived antigens to activate the innate immune responses (52, 53). Gut dysbiosis, the disruption of the balance of gut microbiome, impacts the hepatic immune response through the gut-derived components like LPS and unmethylated CpG DNA, which can activate the TLR-signaling pathway (54). Even though the role of TLRs varies in different cancers (55), a series of studies have shown that targeting TLRs is a promising strategy for cancer immunotherapy (56, 57). In the liver, TLR4 and TLR9 play essential roles in the liver inflammation–fibrosis–cancer axis, as TLR4$^{-/-}$ or TLR9$^{-/-}$ *Tak1ΔHep* mice experience reduced spontaneous HCC development compared to *Tak1ΔHep* mice (58). Clinical investigations also show TLR4, the ligand of Gram-negative bacteria membrane component lipopolysaccharide (LPS) that plays a pathogenic role in chronic inflammation, a causative factor in human HCC (59). The expression of TLR9, the ligand of which is unmethylated CpG DNA in bacteria or viruses, has been positively associated with human colorectal cancer and liver metastasis (60). Thus, modulating gut microbiota to change TLR activity may serve as a therapeutic strategy for HCC therapy.

Modulation of Gut Microbiota for Cancer Therapy

The composition of human gut microbiota can be modulated by various factors such as diet (61), lifestyle (62), antimicrobials (63, 64), environment (65), and diseases (66). Currently, probiotics and Fecal Microbiome Transplantation (FMT) are being investigated in cancer treatment as an adjuvant strategy to increase the efficacy of chemotherapy and immunotherapy (67). There are 80 recruiting or completed microbiota study trials associated with liver diseases on the website ClinicalTrials.gov with the keywords liver disease and microbiota, including NAFLD, NASH, fatty liver disease (FLD), alcoholic liver disease (ALD), HCC, liver encephalopathy, hepatitis, liver transplantation (LT), or resection. The strategies to affect change in the gut microbiota in those trials are summarized in **Figure 1**.

Overtake of soluble dietary fiber (e.g., Pectin and Fructooligosaccharide) that can be metabolized to short-chain fatty acids (SCFAs) by gut microbiota may cause cholestasis and HCC in mice, specifically with gut overgrowth of fiber-fermenting bacteria like *Clostridium* cluster XIVa (68). The authors also showed that administration of antibiotic metronidazole reduced butyrate-producing bacteria and the incidence of HCC in TLR5 knockout (KO) mice fed soluble fiber inulin-containing diet. Another study showed that vancomycin could prevent the development of HCC by selectively depleting Gram-positive bacteria *Lachnospiraceae* (Clostridium cluster XIVa), *Ruminococcaceae*, and *Bifidobacteria*, which ferment fiber and generate secondary bile acids (69). Feeding tomato

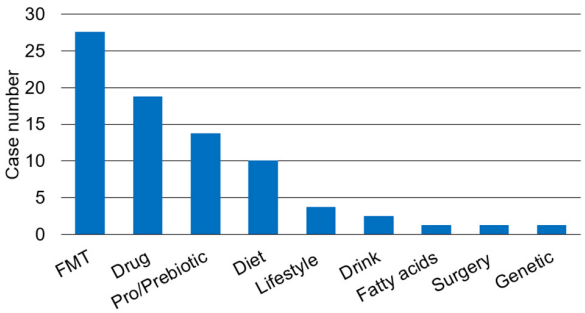

FIGURE 1 | The strategies to change gut microbiota to prevent or ameliorate liver diseases in clinical trials. There are multiple strategies to restore the balance of gut microbiota such as fecal microbiota transplantation (FMT), drug therapy such as antibiotics (e.g., rifaximin) and proton pump inhibitor, pro/prebiotic, prebiotics or probiotics, change of lifestyle, and others including diet, drink, fatty acids, surgery, and genetic modification.

powder (TP) could impede HFD plus diethylnitrosamine (DEN, injected once at 2 weeks of age)-induced HCC development in β-Carotene-15, 15′-oxygenase (BCO1), and β-carotene-9′, 10′-oxygenase (BCO2) double knockout mice (70). In addition, TP feeding altered the richness and diversity of gut microbiota, accompanying a significant decrease in the abundance of genera *Clostridium* and *Mucispirillum*. Another study reported that probiotics composed of *Lactobacillus rhamnosus* GG, viable probiotic *Escherichia coli* Nissle 1917, and heat-inactivated VSL#3 (1:1:1) could shift the gut microbiota to increase beneficial bacteria such as *Prevotella* and *Oscillibacter*, resulting in a reduction of HCC growth and Th17 cell differentiation (71). VSL#3 contains *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, and *Lactobacillus delbrueckii* subsp. Combined (synbiotic) prebiotic *B. infantis* and probiotic milk oligosaccharide treatment reverses Western diet (WD)-induced NASH in FXR knockout mice (72). Moreover, bariatric surgery, such as Roux-en-Y gastric bypass and laparoscopic sleeve gastrectomy, can induce the shift of gut microbiota to reduce obesity and weight loss (73), showing a promise in NAFLD and NASH (74). Thus, it may be a potent treatment option for early stage of NASH-HCC patients.

Gut Microbiota as a Non-invasive Biomarker for HCC

Early diagnosis of HCC comes with multiple treatment options and typically leads to good outcomes. Biomarkers including Alpha-fetoprotein (AFP), Lens culinaris agglutinin A-reactive fraction of alpha-fetoprotein (AFP-L3), and des-gamma-carboxy prothrombin (DCP) have been established as HCC-specific tumor markers (75, 76). New potential biomarkers, such as Aldo-keto reductase family 1 member 10 (AKR1B10) (77), are being investigated for the diagnosis and prognosis of HCC. Changes in the gut microbiome may also serve as biomarkers of disease as they have been associated with the progression of liver diseases, from fibrosis/cirrhosis to cancer (78, 79). For example,

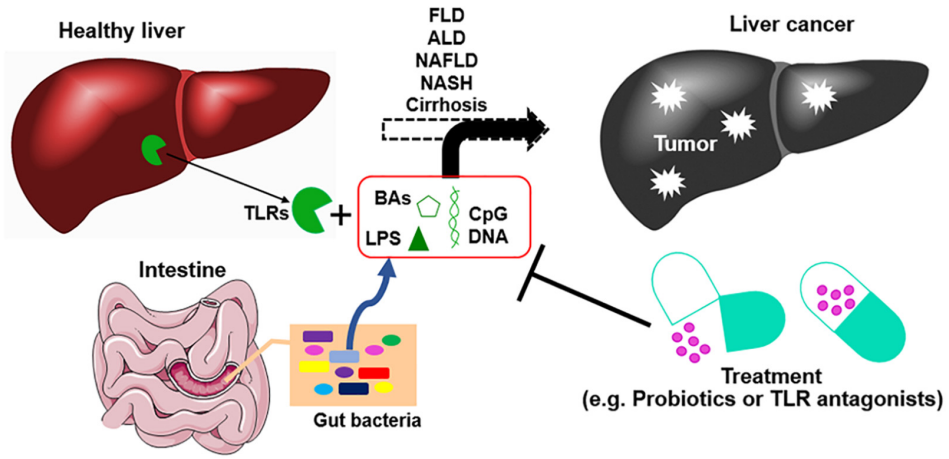

FIGURE 2 | The development of liver cancer and gut microbiota-mediated therapy. Chronic liver diseases including viral infections, fatty liver disease (FLD), alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), and cirrhosis without effective treatments can lead to liver cancer. Dysbiosis of gut microbiota promotes the progression of this process by leakage of gut microbial products such as deoxycholic acid (DCA), lipopolysaccharide (LPS), and unmethylated CpG DNA. These bacterial products promote liver inflammation, fibrosis, and cirrhosis. Modulation of gut microbiota by applying probiotics, prebiotics, and antibiotics, or using antagonists of bacterial products, can improve gut barrier and reduce the progression of the liver inflammation–fibrosis–cirrhosis–cancer axis. BAs, bile acids; TLR, Toll-like receptor.

the abundance of fecal *Enterobacteriaceae* and *Streptococcus* is increased in patients with cirrhosis, while the abundance of *Akkermansia* is reduced. In HCC patients, *Bacteroides* and *Ruminococcaceae* were increased, while *Bifidobacterium* was reduced. Further study showed that *Akkermansia* and *Bifidobacterium* were inversely correlated with inflammatory marker calprotectin (30). These results indicated that during the development of HCC, a group of bacteria are associated with different stages of disease and tumor progression. A better understanding of the association of gut microbiota with liver cancer leads to a therapy option. Potent gut microbiota-mediated liver cancer therapies are summarized in **Figure 2**.

DISCUSSION

Liver cancer is a leading cause of cancer deaths worldwide. Liver resection or transplantation is the curative treatment for HCC, but late diagnosis and lack of donor organs reduce the survival rate. Given these limitations, many non-surgical treatment options are available for advanced stages of HCC. However, the cost for some current treatments like sorafenib is relatively high, which may be associated with adverse or variable effects (80). Modulating gut microbiome is a potential option for liver cancer treatment and diagnosis. HCC occurs about three times more in men than in women (81). Therefore, sex is also another consideration when choosing gut microbiota-mediated treatment. In a streptozotocin–high-fat diet (STZ-HFD)-induced NASH-HCC murine model, male mice possessed a higher abundance of some specific genera than female mice, including *Clostridium*, *Corynebacterium*, *Bacillus*, *Desulfovibrio*, and *Rhodococcus*, which were associated with higher HCC incidence (82). Data from prospective cohort studies indicate that intake of vegetables reduces the risk of liver cancer development, especially for men (83). LT can also alter gut microbial profile. The abundance of bacteria, such as *Actinobacillus*, *Escherichia*, and *Shigella*, decreased post-LT compared to pre-LT, whereas the abundance of bacteria, such as *Micromonosporaceae*, *Desulfobacterales*, the *Sarcina* genus of *Eubacteriaceae*, and *Akkermansia* increased (84). Furthermore, features of the gut microbiota are also associated with hepatitis virus- and non-hepatitis virus-related HCC, evidenced by the fact that hepatitis B-HCC patients harbor much more pro-inflammatory bacteria such as *Escherichia*/*Shigella* and *Enterococcus*, but less amount of *Faecalibacterium*, *Ruminococcus*, and *Ruminoclostridium* relative to healthy controls (85). Therefore, precise analysis of the change of gut microbiota of each individual in the development of HCC is critically essential for modified treatment. Those recent findings suggest that microbiome-mediated therapeutic options can be applied to treat liver cancer as well as the early stage of chronic liver diseases, which may conquer the drawbacks of current therapies, such as the presence of metastasis and liver dysfunction. However, more clinical trials evaluating gut microbiota-mediated therapies are necessary to improve outcomes of HCC treatment.

AUTHOR CONTRIBUTIONS

CZ and MY conceived and wrote the manuscript. AE critically reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Immune Checkpoint Blockade Therapy for Hepatocellular Carcinoma: Clinical Challenges and Considerations

Qi Zhang[1,2,3,4], *Yiwen Chen*[1,2,3,4], *Xueli Bai*[1,2,3,4*] *and Tingbo Liang*[1,2,3,4*]

[1] *Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China,* [2] *Innovation Center for the Study of Pancreatic Diseases of Zhejiang Province, Hangzhou, China,* [3] *Zhejiang Clinical Research Center of Hepatobiliary and Pancreatic Diseases, Hangzhou, China,* [4] *Key Laboratory of Pancreatic Disease of Zhejiang Province, Hangzhou, China*

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***Correspondence:**
Xueli Bai
shirleybai@zju.edu.cn
Tingbo Liang
liangtingbo@zju.edu.cn

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Although many approaches have been developed for the treatment of hepatocellular carcinoma (HCC) that has both high incidence and high mortality especially in Asian countries, the prognosis of HCC patients is still dismal. Immunotherapy, particularly immune checkpoint inhibitors show encouraging efficacy and have already been widely applied in clinic. However, in contrast to traditional therapies, immunotherapy brings many challenges when using in a real world, including biomarker discovery, response evaluation, adverse event treatment, etc. In this review, we proposed some important and intractable issues in current clinical practice regarding the strategy of immune checkpoint blockade, collected current evidence, and discuss the critical challenges and possible approaches to a bright future.

Keywords: liver cancer, biomarker, response evaluation, adjuvant therapy, immune checkpoint inhibitor

INTRODUCTION

Although many treatment modalities including hepatic resection, liver transplantation, radiofrequency ablation, transcatheter arterial chemoembolization (TACE), and tyrosine kinase inhibitors have been widely used in clinical practice, the prognosis of hepatocellular carcinoma (HCC) is still dismal. Anti-tumoral immunotherapies especially immune checkpoint inhibitors (ICIs) show encouraging efficacy and shed light on future treatment of HCC. Currently, druggable immune checkpoints include programed cell death protein 1 (PD-1, CD279), programmed death-ligand 1 (PD-L1, CD274), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152), V-domain Ig suppressor of T cell activation (VISTA), T-cell immunoglobulin and mucin domain-3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), CD40, OX40 (CD134), and 4-1BB (CD137). Since ipilimumab got approved by the US Food and Drug administration (FDA) in 2011, several ICIs have been now used in clinical practice for many solid tumors including HCC. By activating T cells, ICIs ignite natural anti-tumoral potential of these cells and probably lead to more extensive alterations to reverse immunosuppressive tumor microenvironment. With the increasing evidence of clinical application,

more mechanisms of ICIs for cancer treatment have been revealed and are far beyond the initial understanding (1).

Early data for the clinical efficacy of ICIs in HCC were mostly from the CheckMate 040 and KEYNOTE-224 trials (both trials testing anti-PD-1 antibodies), which showed an objective response rate of 15–20% as a second-line setting (2, 3). Limited evidence of anti-PD-L1 antibody and anti-CTLA-4 antibody showed an objective response rate (ORR) of 10% and 17.6%, respectively (4, 5). Although ICIs have been frequently used for HCC treatment in the real world, no phase III trial has actually been reported. However, at least 9 phase III trials are currently ongoing, investigating the efficacy of ICIs in various clinical scenarios (**Table 1**). Among all the ICIs, nivolumab from Bristol-Myers Squibb (New York, NY, USA) and camrelizumab from Hengrui (Lianyungang, Jiangsu, China) have been approved as a second-line therapy for HCC patients who had sorafenib refractory or intolerant. However, in real world some late-stage HCC patients received ICIs beyond this indication, and various clinical trials investigating ICIs as a first-line therapy or neoadjuvant therapy are ongoing. Therefore, a considerable number of HCC patients treated by ICIs have been accumulating. Given the mild efficacy of ICIs alone for HCC, combination with other agents such as another ICI, lenvatinib, and apatinib is being explored. ICIs combined with TACE or radiotherapy is also under evaluation. These attempts demonstrate a higher response rate compared to ICI monotherapy.

Undoubtedly, ICIs will play a key role in the treatment modality of HCC. However, as a novel strategy, many critical issues in the clinical scenarios have been emerging and have confused physicians. These issues are also closely related to interpretation of the clinical trials, and thus warranted a deep discussion, which will not only improve the clinical management but also refine the design of future clinical trials.

CRITICAL ISSUES IN CLINICAL PRACTICE

Biomarker Discovery for Prediction of Efficacy of ICIs

Identification of effective biomarkers is critical for the use of ICIs; however, currently there are no ideal ones. For anti-PD-1 and anti-PD-L1 antibodies, the expression of PD-L1 in tumor sections was initially a reasonable biomarker, and the accompanied testing kit of PD-L1 expression was indispensable for approval of an anti-PD-1/PD-L1 antibody by US FDA. Indeed, a higher PD-L1 expression in tumor samples was associated with a higher ORR in the majority of cancers treated by anti-PD-1/PD-L1 antibodies. Whereas, blockade of PD-1/PD-L1 signaling in non-malignant cells was later found also clinically relevant. For instance, PD-1^{+} macrophages within tumors show compromised phagocytosis and impaired tumor immunity (6). In addition, a novel subset of PD-1^{high} regulatory B cell population in HCC was recently uncovered to suppress anti-tumor immunity *via* interleukin (IL)-10 signals (7). Other non-neoplastic cells such as monocytes and mesenchymal stromal cells that express PD-L1 were further proven to inhibit anti-tumor responses and promote cancer progression (8, 9). These results support the rationale PD-1/PD-L1 expression by stromal immune cells as a predictive biomarker for anti-PD-1 or anti-PD-L1 antibodies (10)). Even more, exosomal PD-L1 has been revealed to cause immunosuppression in tumors, and it is believed to be a possible biomarker and therapeutic target for cancer therapy (11). However, more translational studies and clinical trials are warranted for the predictive value of these potential biomarkers in patients treated with ICIs.

It needs great efforts to establish a biomarker in specific scenarios, which include the line of therapy, threshold of protein expression, type of sample (fresh or archival), type of cell staining, kit of companion diagnostic, assay of testing, and

TABLE 1 | Phase III clinical trials of immune checkpoint inhibitors for hepatocellular carcinoma (June 2020). OS, Overall survival; RFS, relapse-free survival; PFS, progression-free survival.

Study name	Treatments	Disease	Line of therapy	Primary outcome	Countries	Study start	Estimated number	Design
CheckMate 459 (NCT02576509)	Nivolumab *vs.* sorafenib	Advanced HCC	First-line	OS	Global	Nov 2015	726	Open label
CheckMate 9DX (NCT03383458)	Nivolumab *vs.* placebo	Postoperative HCC	Adjuvant	RFS	Global	Dec 2017	530	Double blinded
KEYNOTE-240 (NCT02702401)	Pembrolizumab *vs.* placebo	Refractory advanced HCC	Second-line	PFS/OS	Global	May 2016	408	Double blinded
KEYNOTE-394 (NCT03062358)	Pembrolizumab *vs.* placebo	Refractory advanced HCC	Second-line	OS	Asia	Apr 2017	330	Double blinded
KEYNOTE-937 (NCT03867084)	Pembrolizumab *vs.* placebo	Postoperative HCC	Adjuvant	RFS/OS	Global	May 2019	950	Double blinded
NCT03412773	Tislelzumab *vs.* sorafenib	Advanced HCC	First-line	OS	Global	Dec 2017	660	Non-inferiority
HIMALAYA (NCT03298451)	Durvalumab *vs.* durvalumab +tremelimumab *vs.* sorafenib	Advanced HCC	First-line	OS	Global	Oct 2017	1200	Open label
IMbrave150 (NCT03434379)	Atezolizumab+bevacizumab *vs.* sorafenib	Advanced HCC	First-line	OS	Global	Mar 2018	480	Open label
IMbrave050 (NCT04102098)	Atezolizumab+bevacizumab *vs.* active surveillance	Postoperative HCC	Adjuvant	RFS	Global	Dec 2019	662	Open label
NCT03092895	Camrelizumab	Refractory advanced HCC	Second-line	ORR	China	Apr 2017	60	Open label

particular endpoint for approval (12). For example, PD-L1 protein expression evaluated by immunohistochemistry has been reported unsuitable for prognostic or predictive of benefits from adjuvant chemotherapy in resected non-small cell lung cancer (13). The agreement between immunohistochemistry and other methods such as polymerase chain reaction is not good as shown by the CLOVER comparison study (14). Recently, the posttranslational modification especially the phosphorylation and glycosylation of PD-L1 has been paid much attention and investigators revealed that these modifications significantly affect the detection performance and therapeutic efficacy of PD-L1 antibodies (15, 16). Removal of glycosylation by suitable approaches can boost the positive rate of PD-L1 detection in tumor samples (15). Unfortunately, limited evidence has been accumulated in HCC except that PD-L1 expression by both neoplastic or intratumoral inflammatory cells is related to tumor aggressiveness and suggests clinical benefits when using ICIs targeting PD-1/PD-L1 signaling using a retrospective HCC cohort (17).

Beside PD-L1 expression, other predictive biomarkers for effectiveness of ICIs include immune cell clusters, protein expression, tumor mutational burden (TMB), and gene signatures (18, 19). Our group has divided HCC into three immunophenotypic subtypes (e.g., immunocompetent, immunodeficient, and immunosuppressive) based on their microenvironmental features using CD45 and Foxp3 expression in formalin-fixed paraffin-embedded samples, and proposed different strategies for the use of ICIs in the novel classification system (20). The clinical value of this classification is currently under investigation.

In most cancers including HCC, PD-L1 expression and TMB are independent with each other (21). TMB has been well described as a biomarker of ICIs in a variety of cancers. In HCC, the median TMB is around 4 mutations/Mb, with only approximately 5% of all samples showing a TMB higher than 10 mutations/Mb (22). It has to be noticed that the quantification of TMB is closely related to the methods and kits, which can report distinct values for the same sample. Therefore, comparison of TMB between studies adopting different approaches needs great caution and is usually meaningless. Interestingly, Chinese patients with HCC have a significantly larger part of TMB high compared to Western patients with HCC (9.3% versus 1%) (23). Although higher TMB quantified by whole-genome sequencing, whole-exome sequencing, or the next generation sequencing is positively associated with better survival in patients treated with ICI (24, 25), its clinical value in HCC is still under debate (22, 26). As a minimal invasive and convenient alternative, blood TMB presented good predicted value in some types of cancer (27, 28), but evidence is lacking for HCC. However, it has to be mentioned that although these biomarkers are used with specific cut-off values according to different assays, they are actually continuums as biological characteristics in a population of patients. Thus, the cut-off value may vary based on assays, populations, and types of diseases. Clinical validation is warranted when introducing a biomarker for evaluation of ICI treatment, and clinical interpretation should be made with caution when a biomarker is used with a specific cut-off value in ICI management.

Some potential gene alterations have been revealed to be tightly associated with tumor response to ICIs, and can serve as predictive biomarkers of therapeutic sensitivity to ICIs. Somatic mutations in *RAS* (*KRAS*, *NRAS*, and *HRAS*), *EGFR*, *TP53*, *SMO*, *DDR2*, *FGFR*, *PTCH1*, *MET*, and *PTEN* are frequent and may affect response to ICI treatment [reviewed by Wang et al. (29)]. In addition, germline gene mutations may also predict tumor response to ICIs. For instance, *JAK2* amplification or emergence of *JAK2* at the 9p24.1 site can enhance PD-L1 expression and may result in good response to ICIs (30, 31), while loss-of-function mutations in *JAK1/2* lead to acquired or primary resistance to anti-PD-1 therapy (32). Similarly, genetic alterations down-regulating the interferon signaling such as *IFNGR1*, *IFNGR2*, and *IRF1*, and amplification of genes that inhibit interferon-gamma such as *SOCS1* and *PIAS4* can weaken the efficacy of ICIs (33). These mutations are not rare and should be paid attention in clinical use of ICIs. Thus, the next generation sequencing (NGS)-based gene mutation testing is helpful for a precise choice of ICIs.

Recently, the potential roles of gut microbiota in immunotherapy for tumors have been raised, and gut microbiota may serve as a potential biomarker of ICIs. It is particularly meaningful in HCC due to the natural connection between gut and liver. Previous studies described an intestinal–microbiota–liver axis as the evidence of gut microbiota promoting chronic liver disease progression and hepatocarcinogenesis in patients with advanced liver disease (34, 35). Meanwhile, the crosstalk between microbiota and the immune system at the level of the gut is critical, and there is compelling evidence that the microbiota helps to shape the immune system (36, 37). The impact of the gut microbiota on response to ICIs has been studied since 2005 (38, 39), and increasing studies demonstrated that gut microbiota played in shaping responses to ICIs (40, 41). Routy et al. reported that patients treated with antibiotics for routine indications shortly before, during, or shortly after treatment with anti-PD-1/PD-L1 antibodies had both significantly lower PFS and OS rates compared with patients who had not received antibiotics, suggesting that primary resistance to ICIs may be attributed to abnormal gut microbiome composition (42). Several studies revealed approximately one-third of all patients undergoing anti-CTLA-4 therapy develop intestinal inflammation due to mucosal immune dysregulation (43, 44), suggesting the potential role of gut microbiota in adverse effects of ICIs. Although these investigations were mainly performed on melanoma, a study analyzed fecal samples from HCC patients and found patients responding to immunotherapy showed higher taxa richness and more gene counts than those of non-responders (45). The dynamic variation characteristics of the gut microbiome may provide promising biomarkers and early predictions of the outcomes of ICI treatment in patients with HCC, which may guide disease-monitoring and treatment decision-making. Nevertheless, gut microbiota can be influenced by many environmental, dietary, and lifestyle factors, all of which can

potentially affect the immune system and consequentially regulate the response to ICIs (36, 46). Tumor microbiome can lead to an immunosuppressive tumor microenvironment, and its diversity is correlated with overall survival (47, 48). Ablation of bacteria in animal models enhances the efficacy of ICIs by up-regulating PD-1 expression in tumor (47). Unfortunately, it is difficult to perform bacterial ablation in human patients. Therefore, both gut and tumor microbiota as a biomarker of ICI treatment are still far from clinical practice. The role of microbiota, together with other environmental, dietary, and lifestyle factors, in prediction of tumor response to ICIs can be investigated with powerful methods of molecular pathological epidemiology (46, 49).

Response Evaluation of ICI Treatment

As the use of ICI becomes increasingly available to patients, a major challenge rises, namely, the accurate determination of clinical efficacy. The World Health Organization (WHO) and the Response Evaluation Criteria in Solid Tumors (RECIST) Group have provided standard guidelines to define tumor response to therapy. Whereas these conventional criteria were developed based on data from clinical trials of cytotoxic chemotherapeutic agents for advanced malignancies. These criteria consider therapeutic success as reduction in tumor burden without any new lesions and treatment failure if early tumor growth or appearance of new lesions. Previous studies have confirmed RECIST 1.0 and 1.1 for assessment of therapeutic effectiveness for a wide range of cytotoxic chemotherapeutic agents and these response criteria have been shown to correlate with patient outcomes (50–52).

In the case of HCC, molecular-targeted therapies and therapeutic interventions are main approaches besides surgery. Previous studies have shown a poor correlation between the clinical benefit provided by molecular-targeted therapies such as sorafenib or by locoregional interventional therapies and RECIST assessments, since the antitumor activity in such situations may be presented as tumor necrosis (53, 54). The American Association for the Study of Liver Diseases (AASLD) Practice Guideline on the management of HCC issued in 2005 stated that the evaluation of the treatment response should take into account the induction of intratumoral necrotic areas in estimating the decrease in tumor load (55). The modified RECIST assessment (mRECIST) for HCC was proposed after a series of amendments (56). To be selected as a target lesion using mRECIST, an HCC lesion should meet all the following criteria: 1) The lesion can be classified as a RECIST measurable lesion (i.e., the lesion can be accurately measured in at least one dimension as 1 cm or more). 2) The lesion is suitable for repeat measurement. 3) The lesion shows intratumoral arterial enhancement on contrast-enhanced CT or MRI. In mRECIST, progression disease (PD) is defined as an increase of at least 20% in the sum of the diameters of viable (enhancing) target lesions, taking as reference the smallest sum of the diameters of viable (enhancing) target lesions recorded since treatment started and partial response (PR) is defined as at least a 30% decrease in the sum of diameters of viable (enhancement in the arterial phase) target lesions, taking as reference the baseline sum of the diameters of target lesions. The mRECIST has been validated and widely adopted in subsequent HCC studies.

Nearly all previous and current clinical trials regarding ICI still adopt RECIST 1.1 for standard of response evaluation. However, clinical practice found that in contrast to chemotherapy and targeted therapy, ICI treatment has a considerate rate of hyperprogression and pseudoprogression, leading to dramatically different decision-making based on tumor size. In a cohort of East Asian patients with HCC, up to 23% patients who received PD-1 blockade were reported to suffer from hyperprogressive disease (HPD) (57). In this real-world study, neutrophil-to-lymphocyte ratio was the only identified biomarker to predict HPD, suggesting to avoid using ICIs in such patients.

Clinical observation revealed that some patients responded to ICIs with tumor shrinkage or stable disease that was consistent with RECIST criteria; however, distinct immune-related patterns of response have been noted, including development of new lesions associated with edema and infiltration of immune cells and transient increase in the size of primary lesions (58). Delayed clinical responses such as an increase in total tumor burden were followed by significant tumor regression. Experience from patients with melanoma treated with ipilimumab indicated that the initially enlarged lesions could be infiltrated by massive inflammatory cells and necrotic tumor cells, in patients with subsequent decreased tumor burden (59, 60), which induced the definition of pseudoprogression. Pseudoprogression is defined as more than 25% increase in tumor burden at week 12 (early) or any assessment after week 12 (delayed) that was not confirmed as progressive disease at next assessment. These findings of pseudoprogression would have been classified prematurely as progressive disease by WHO or RECIST criteria and have prompted the development of the immune-related response criteria (irRC). Actually, several clinical trials reported that a few patients showed distinct immune-related patterns of treatment response that did not meet RECIST criteria (61), including the clinical trials in our center. Based on survival analysis, conventional RECIST might underestimate the benefit of pembrolizumab in approximately 15% of patients with advanced melanoma in the phase Ib KEYNOTE-001 study (61). The irRC was first proposed based on data from a phase 2 clinical trial of ICIs in patients with melanoma (58). The irRC resembles the conventional criteria for determination of overall tumor burden at baseline, which includes selection of both measurable (target/index) and non-measurable (non-target/non-index) lesions with similar standards. While in irRC, PD is defined as at least 25% increase in tumor burden compared with nadir (at any single time point) in two consecutive observations at least 4 weeks apart. The irRC has been externally validated in melanoma and non-small lung cancer from the perspective of their association with outcomes.

Since robust responses have been recorded with ICI in HCC, various clinical trials have been carried out to explore the indications and combinations of ICIs in HCC. In KEYNOTE-224, investigators compared the response evaluation results according to RECIST 1.1, mRECIST, and irRC, and found similar results among the three

criteria (3). While in this study, patients only received pembrolizumab. Whether the three criteria have a similar evaluation performance in other ICIs or in combination modality regarding ICIs especially when locoregional interventional therapies were combined is unclear. Future studies are urgently needed to validate since the evaluation criteria may deeply influence the clinical practice and judgment of results from clinical trials.

On-Target, Off-Tumor Effects for ICI Therapy

Unintended auto-immune complications can occur when the immune system is enhanced to fight cancer. Adverse events of ICI include colitis, endocrinopathies such as hypophysitis and thyroid disorders, or type 1 diabetes mellitus, hepatitis presented as increased aspartate aminotransferase concentration, alanine aminotransferase concentration, elevated bilirubin concentration, or cholestatic jaundice, pancreatitis, pneumonitis, dermatitis, and/or sarcoid-like reaction. These immune-related adverse events can occur at any stage of ICI therapy. In particular, whether previous hepatic diseases and hepatitis viruses infection increase ICI-associated hepatitis needs further study. The median onset of events typically ensues during the following time periods: varies by organ system affected with-skin-related events at 3 weeks, hepatitis at 3 to 9 weeks, gastrointestinal manifestations at 8 weeks, and endocrinopathies at 7 to 20 weeks. Immune-related adverse events can be managed according to NCCN guidelines (Version 2.2019). Almost all of these immune-related adverse events can be treated by stopping the immune-therapy and administering steroids.

Hyperprogression of HCC can be recognized as a special adverse event, and is characterized by rapid increase in tumor burden in patients treated with immune-therapy. Champiat et al. noted hyperprogression in 9% of patients treated with ICIs (62). In cases of hyperprogression, characteristics of progression include time to treatment failure less than 2 months, more than 50% increase in tumor burden compared to pre-baseline levels and more than two-fold increase in pace of tumor growth (63). The patients with HPD usually have a deteriorating clinical condition and lead to treatment failure. Sonja Kleffel et al. reported that PD-1/PD-L1 signaling has cell-intrinsic functions in tumor cells (64). It is possible that PD-1/PD-L1 blockade might affect alternative signaling pathways and accelerate tumor growth and tumorigenesis. Several genetic alterations (e.g., KRAS and STK11 mutations, MDM2, MDM4 and EGFR amplifications) have been reported to be associated with ICI-related HPD (65, 66). The rapid proliferation of PD-1$^+$ effector regulatory T cells after ICI treatment was found to promote HPD in patients with gastric cancer (67). Older age, higher metastatic burden, and previous radiation are found associated with HPD (68). Many studies have explored potential biomarkers from clinical, laboratory, and imaging angles (69). Unfortunately, there are no reliable ways to select patients with HCC who are risky for HPD before the treatment of ICIs.

Choice of Immunotherapy for Patients With HCC

Although there are many ICIs such as anti-LAG3 and anti-PD-L2 under development nowadays, only anti-PD-1, anti-PD-L1, and anti-CTLA4 antibodies have been approved for clinical use. These include three anti-PD-1 antibodies (nivolumab, pembrolizumab, cemiplimab), three anti-PD-L1 antibodies (atezolizumab, avelumab, durvalumab), and one anti-CTLA4 antibody (ipilimumab). In China, another four PD-1 antibodies (sintilimab, camrelizumab, tislelizumab, and toripalimab) were approved with specific indications. Merely nivolumab, pembrolizumab, atezolimumab, and camrelizumab have approved indications for HCC in different regions of the world. However, off-label use of these ICIs is quite common in the real world. The overall estimation of off-label use of ICIs in all cancers is between 18% and 30% (70), and HCC was once the commonest disease (more than a half) that was treated by ICIs as the off-label approach (71).

The CTLA4 and PD-1 pathways are different in human immunity, with CTLA4 regulating T cell proliferation in the early stage of immune response while PD-1 suppressing T cells in the late stage of immune response (72). CTLA4 is restricted to antigen-presenting cells and PD-1 is related to not only immune cells but also tumor cells (1). Given the differences of the two signals, combination of anti-CTLA4 and anti-PD-1/PD-L1 antibodies is reasonable and approved by US FDA in melanoma, but evidence is lacking for the combinatory use of ICIs in HCC. Since HCC cells express extensive PD-L1, strategies to block PD-1/PD-L1 signal are more acceptable than anti-CTLA4 therapy. Although *in vitro* functional assays demonstrated that currently available therapeutic PD-L1 antibodies are more superior to PD-1 antibodies in blocking PD-1/PD-L1 signaling (73), a systematic review and meta-analysis showed that anti-PD-1 antibodies were generally better than anti-PD-L1 antibodies in terms of both overall survival (HR 0.75) and progression-free survival (HR 0.73) for solid tumors (74). This may be because anti-PD-L1 antibody does not block PD-L2 induced PD-1 signal in T cells, and PD-L2 is also overexpressed and performs as a prognostic factor for HCC (75). Although some antibodies have special designs to minimize side effects and optimize efficacy, most physicians from their limited clinical observations believe that the efficacy and adverse events are similar among them since there are no head-to-head trials comparing the efficacy among these ICIs. Therefore, no evidence is available to recommend a certain ICI in HCC management.

Immunotherapy as a (Neo)Adjuvant Therapy for Resectable HCC

Rapid recurrence of HCC after curative resection or ablation is an unmet medical need. Compared to late recurrence that is sometimes believed to be independent carcinogenesis especially for patients with hepatitis B virus infection or liver cirrhosis, patients at risk for early recurrence based upon tumor characteristics may be ideal to receive immediate adjuvant immunotherapy to eliminate or control residual, perhaps radiologically occult, tumor cells. Up to date, many strategies has been explored to try to prevent or delay postoperative recurrence using TACE, sorafenib, and Huaier granule (76–78). In contrast to the weak effectiveness of TACE and

sorafenib, which target tumor-associated microvessels and/or tumor cells themselves, ICIs may be more reasonable to be applied to reduce recurrence rate after surgery or ablation because tumor cells are removed but tumor-associated antigens are exposed. Recently, the use of ICIs for adjuvant therapy has been discussed. Although there is no solid evidence to support using ICI in such clinical scenario, a randomized phase III trial (IMbrave050, NCT04102098) testing atezolizumab plus bevacizumab in patient with HCC at high risk of recurrence after curative treatment was launched. Another phase III trial (KEYNOTE-937, NCT03867084) comparing pembrolizumab and placebo as adjuvant therapy in patients with HCC and complete radiological response after surgical resection or local ablation is also recruiting participants. A clinical trial with similar design for HCC patients at high risk of recurrence is ongoing for nivolumab (CheckMate 9DX, NCT03383458). In our center, a similar clinical trial as adjuvant therapy is ongoing for toripalimab and donafenib (CISLD-8, NCT04418401). There is no standard duration of adjuvant therapy for HCC, and in existing clinical trials mentioned above, the adjuvant therapy lasts for 6 to 12 months.

Another approach to decrease the recurrence rate of HCC after curative surgery is neoadjuvant therapy. Neoadjuvant approach with immune-based therapies may prove to be successful because tumor antigens are more available before eradication of the tumor by surgery. Neoadjuvant application of pembrolizumab was safe and efficacious in patients with NSCLC (79). So far, there is no approved indications of neoadjuvant treatment of ICI. For HCC, neoadjuvant immunotherapy using ICI has just been initiated. In 2020 ASCO, a randomized phase II pilot trial evaluating nivolumab alone or nivolumab plus ipilimumab in patients with resectable HCC reached its primary endpoint of safety (NCT03222076). In this study, researchers reported a pathologic response rate of 40% (24% pCR and 16% major necrosis) for resectable HCC after preoperative immunotherapy (80). There are also several clinical trials for advanced HCC as neoadjuvant therapy that aim for down staging to reach the criteria for curative surgery. The combination of transarterial radioembolization (TARE) with nivolumab is being studied in the neoadjuvant setting (NCT03812562). Another clinical trial as neoadjuvant therapy is the combination of drug-eluting TACE and sintilimab (CISLD-5, NCT04174781). For HCC patients waiting for liver transplantation, there are no available data but there is an ongoing clinical trial testing the combination of camrelizumab and apatinib for downstaging or bridging before liver transplantation (NCT04035876). These clinical trials are highly anticipated and highlight the potentially important role of immune checkpoint blockade therapy in preoperative treatment in HCC.

Timing of Introducing Immunotherapy for Patients With Unresectable HCC

Currently only three RCTs (CheckMate-459, KEYNOTE-240, and IMbrave 150) with available results assess the clinical efficacy of ICIs compared with the standard of care in unresectable HCC patients. In first-line setting, nivolumab and atezolizumab beat sorafenib in terms of OS and ORR (81). Several trials with monotherapy of ICI or combination therapy of ICI and other treatment are ongoing (**Table 1**). Taking the advantage of similar genomic characteristics of tumor nodules in multifocal HCC patients, Huang et al. revealed that small tumors had higher immune cell infiltration and better sensitivity to anti-PD-1 therapy compared with large tumors (82). These results support early use of ICI in HCC patients without opportunity for radical resection. Intriguingly, PD-L1 expression in infiltrating macrophages rather than tumor cells was found up-regulated in patients with HCC and resistant to sorafenib treatment; additionally, circulating soluble PD-L1 was also increased (83, 84). These evidences may provide rationale for the use of PD-1/PD-L1 blockade as a second-line therapy.

Approaches to Enhance the Efficacy of ICI in Patients With HCC

The objective response rate (ORR) of ICI alone is not clinically satisfactory; thus, physicians have been investigating combination strategies to enhance the efficacy of ICIs. The US FDA has approved the combination regimen of atezolizumab and bevacizumab for advanced HCC according to a phase 1b trial, which demonstrated an ORR of 34% (RECIST1.1) and 25% of patients suffered from adverse events with grade 3 or higher (85). Another phase 1b trial testing the combinatory use of pembrolizumab and lenvatinib showed an ORR of 46% and a DCR of 92% (mRECIST) (86). Retrospective evaluation of CheckMate-040 showed that nivolumab with local-regional treatment group had an ORR of 50%, a CR of 11%, and median OS of 13.6 months, which were far better than the general results reported by the original study (2, 87). Two ICIs targeting PD-L1 and CTLA-4, respectively, are also used together in studies of advanced HCC. Durvalumab and tremelimumab combination therapy led to an ORR of 18% and a DCR of 57.5% with 20% of patients had grade 3/4 treatment-related adverse events, showing minimal enhancement of efficacy and increased risk of severe side effect compared to one ICI alone (88). Similarly, tremelimumab plus durvalumab showed an ORR of 20% and a DCR of 60% in advanced HCC with median PFS of 7.8 months (89).

Hundreds of clinical trials have been developed to test different agents including current available drugs and novel chemicals. Among these agents, anti-angiogenic molecules are currently promising and have been proved by several key trials. For instance, based on the optimal results of IMbrave 150 study, which showed reduced risk of death by 42% in the study group when compared with sorafenib, combination of atezolizumab and bevacizumab has been approved by US FDA to treat patients with unresectable or metastatic HCC (90). Other anti-angiogenic agents and multi-kinase inhibitors such as lenvatinib, ramucirumab, nintedanib, sorafenib, axitinib, and capmatinib have been under investigation in patients with HCC (91). Using mouse models, people revealed that such combination can induce high endothelial venules that promote cytotoxic T cell infiltration, activity, and consequent tumor cell destruction (92). In addition, agents targeting c-Met and TGF-β receptor I are also

being tested for their ability to enhance ICI treatment in patients with HCC (NCT02423343 and NCT02795429).

There are some other promising combination strategies that are currently explored in other solid tumors in clinical trials or preclinical studies. A DDR inhibitor AZD6738 and radiotherapy combined with anti-PD-L1 antibodies could perform better tumor growth inhibition and recurrence prevention by boosting $CD8^+$ T cell infiltration and activation in tumor microenvironment in a mouse model (93). However, these strategies are still in preclinical stage or under *in vivo* testing, and need time and luck to be translated into clinic.

PERSPECTIVES

With the increasing cases of HCC receiving ICI treatment, physicians are gaining more and more experience, while more problems are arising. Some of them are pan-cancer associated, and some of them are HCC specific. ICI is becoming a mainstay of the comprehensive management of HCC, and these clinical challenges need well-designed clinical studies to conquer. Before we get the answers, careful use of ICIs within indications or as an off-label way should balance its benefits and risks.

AUTHOR CONTRIBUTIONS

QZ and YC contributed equally to this work. QZ and TL conceived the idea. QZ and YC wrote the draft. TL and XB interpreted the clinical significance. All authors contributed to the article and approved the submitted version.

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Hepatocellular Senescence: Immunosurveillance and Future Senescence-Induced Therapy in Hepatocellular Carcinoma

Peng Liu[1,2†], Qinghe Tang[3†], Miaomiao Chen[1,2], Wenjian Chen[1,2], Yanli Lu[1,2], Zhongmin Liu[1,2] and Zhiying He[1,2*]*

[1] Institute for Regenerative Medicine, Shanghai East Hospital, School of Life Sciences and Technology, Tongji University, Shanghai, China, [2] Shanghai Institute of Stem Cell Research and Clinical Translation, Shanghai, China, [3] Department of Hepatobiliary and Pancreatic Surgery, Shanghai East Hospital, Tongji University, Shanghai, China

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***Correspondence:**
Zhiying He
zyhe@tongji.edu.cn
Zhongmin Liu
zhongmin_liu@sina.com

[†]*These authors have contributed equally to this work*

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Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide. The lack of effective targeted drugs has become a challenge on treating HCC patients. Cellular senescence is closely linked to the occurrence, development, and therapy of tumor. Induction of cellular senescence and further activation of immune surveillance provides a new strategy to develop HCC targeted drugs, that is, senescence-induced therapy for HCC. Precancerous hepatocytes or HCC cells can be induced into senescent cells, subsequently producing senescence-associated secretory phenotype (SASP) factors. SASP factors recruit and activate various types of immune cells, including T cells, NK cells, macrophages, and their subtypes, which carry out the role of immune surveillance and elimination of senescent cells, ultimately preventing the occurrence of HCC or inhibiting the progression of HCC. Specific interventions in several checkpoints of senescence-mediated therapy will make positive contributions to suppress tumorigenesis and progression of HCC, for instance, by applying small molecular compounds to induce cellular senescence or selecting cytokines/chemokines to activate immunosurveillance, supplementing adoptive immunocytes to remove senescent cells, and screening chemical drugs to induce apoptosis of senescent cells or accelerate clearance of senescent cells. These interventional checkpoints become potential chemotherapeutic targets in senescence-induced therapy for HCC. In this review, we focus on the frontiers of senescence-induced therapy and discuss senescent characteristics of hepatocytes during hepatocarcinogenesis as well as the roles and mechanisms of senescent cell induction and clearance, and cellular senescence-related immunosurveillance during the formation and progression of HCC.

Keywords: cellular senescence, hepatocellular senescence, senescence-associated secretory phenotype, immunosurveillance, senescence-induced therapy, hepatocellular carcinoma

INTRODUCTION

The incidence rate of liver cancer has been increasing along with population growth and aging in the past years. In accordance with GLOBOCAN statistics, there were about 841,080 new cases of liver cancer and 781,631 liver cancer-related deaths worldwide in 2018 (1). Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor and the third leading cause of cancer-related death globally, accounting for 75–85% of primary liver cancer cases, and it has become a serious public health issue, especially in China, where the number of annual new HCC cases and HCC-related deaths accounts for about half of the whole world's corresponding number (1–3). Among the most common cancers in China, the mortality of liver cancer ranks third in men and fourth in women respectively in 2017 (1, 3).

The occurrence and development of HCC is an irreversible process often accompanied by repeated hepatocellular injury, inflammation, necrosis, and regeneration. Up to 80% of HCC cases are associated with chronic liver injury, such as hepatitis virus infection, alcohol abuse, drug toxicity, and metabolic disorders, which gradually progress to liver fibrosis and cirrhosis, eventually resulting in HCC (1, 3–5).

Traditional treatments for HCC mainly include surgery (hepatectomy, liver transplantation, ablation, and intervention), chemotherapy, radiotherapy, and biotherapy. Among these approaches, surgery is most commonly used, but the high rate of metastasis and recurrence after operation has become the bottleneck to improve the prognosis of patients with HCC (6, 7). Currently, many new therapeutic strategies have been developed for the treatment of refractory or advanced HCC, such as molecular-targeted agents (e.g., Sorafenib and Lenvatinib), immunotherapy (immunomodulators, immune-checkpoint inhibitors, CAR-T, and HCC vaccines), and oncolytic virotherapy (6). However, the sensitivity, efficacy, and safety of these treatments are still clinically questionable, implying that the challenge of HCC therapy remains unsolved (8). For instance, intermediate- and advanced-stage HCC are not sensitive to drug therapy, which limits the clinical application of existing anti-HCC drugs (9). Although individualized treatment can be formulated according to different therapeutic methods, the five-year overall survival of HCC has not been effectively improved. Also, HCC is highly heterogeneous: analysis of a large number of HCC samples show that abnormal regulation of signaling pathways such as telomere maintenance, cell cycle control, Wnt/β-catenin signaling, chromatin modification, RTK-RAS-PI3K signaling, and oxidative stress in HCC cells (6), and the wide range of mutations or abnormal expression at the gene level. The heterogeneity in HCC greatly limits the effect of targeted drugs. Therefore, it is imperative to explore more appropriate solutions for HCC therapy.

In recent years, research on cellular senescence is gradually becoming a valuable and promising field since the correlation between age-related chronic liver diseases and senescence has been recognized (10, 11). In particular, cellular senescence is closely linked to the occurrence, development, and treatment of HCC (12). Despite much attention paid to the field, the contribution of cellular senescence to liver diseases and its precise mechanisms have not yet been clearly elaborated. Therefore, understanding the role of cellular senescence in the pathogenesis of HCC will establish theoretical and practical basis for clinical treatment of liver tumor. Of note, cellular senescence-related therapeutic strategy of HCC is gaining importance, which is achieved through the use of specific small molecular compound inducing cellular senescence, activating immunosurveillance, as well as eliminating or killing senescent precancerous hepatocytes and HCC cells in various ways. As a highly immunosuppressive tumor, the microenvironment makes it insensitive for HCC cells to respond to immune system monitoring (13, 14). Our recent study found that inducible cellular senescence showed highly effective on HCC suppression since senescent cells could remarkably activate immune surveillance and recruit multiple types of immune cells to infiltrate and remove atypical proliferative hepatocytes by the secretion of senescence-associated secretory phenotype factors (15). Taken together, this review will introduce the emerging senescence-relevant therapeutic methods of restricting HCC and discuss the future prospects and possible disadvantages of senescence-based therapy for HCC.

CELLULAR SENESCENCE AND ITS ANTITUMOR EFFECT

Characteristics of Cellular Senescence

In response to endogenous and exogenous stress, cells in various tissues may enter a state of cell cycle arrest. These cells are called senescent cells that cannot proliferate but remain metabolically active for an extended period of time (16). Abnormal telomere function in normal cells can result in replicative senescence. Harmful stimuli like oncogene overexpression, irreversible DNA damage, oxidative stress, and endoplasmic reticulum (ER) stress may individually or synergistically accelerate the senescence of normal cells (17). The transformation from normal cells to senescent cells runs through the whole life cycle of organisms and plays a key role in tissue homeostasis. Moreover, cellular senescence has been proved to be critically involved in specific physiological and pathological processes upon the conditions of stress signal stimulation, such as embryonic development, tissue repair, tumorigenesis, tumor suppression, and aging (18).

The phenotype of senescence is quite stable and persistent, showing no response to mitogenic stimulation and resistance to apoptosis, namely the ability of "senescence without death" to cause aging-related diseases (17). Senescent cells are characterized by changes in morphology and nuclear membrane, lysosome activity, and gene expressions, and present significant upregulation of cell cycle inhibitors such as p53/p21 and $p16^{INK4a}$, activation of DNA damage response, remodeling of chromatin structures, deposition of senescence-associated β-galactosidase (SA-β-Gal), and induction of senescence-associated secretory phenotype (SASP). Full-fledged senescence phenotypes are usually manifested to be enlarged and flattened, and often multinucleated (19). Aravinthan et al. reported that senescent hepatocytes with high expression of p21 exhibited much larger nuclei than non-senescent hepatocytes (20).

Except for p21 and $p16^{INK4a}$, SA-β-Gal and SASP are another two hallmarks of senescence, existing in almost all types of senescent cells. Determination of SA-β-Gal activity is the most commonly used method to detect cellular senescence (21). SASP is a generic term for all senescent cell-secreted components including a large amount of proinflammatory cytokines, chemokines, growth factors, and proteases, which contribute to senescence-related pathophysiological processes and evidently affect adjacent cells and their microenvironment in both autocrine and paracrine manners (17). Senescent cells are highly secretory and perform myriad SASP-mediated functions, the beneficial or detrimental effects of which depend on physiological context of the liver and other organs (22). These diverse activities of SASP composition include angiogenesis, activation and inhibition of cell proliferation, formation of chemoresistant niche in cancer chemotherapy, stimulation of epithelial-to-mesenchymal transition, induction of senescence, activation of inflammation and immunosurveillance, regulation of stem cell renewal and differentiation, as well as optimization of tissue repair (19, 23). Moreover, the regulation of SASP is affected by multiple pathways and molecular mechanisms. A significant proportion of SASP factors are positively regulated by p38 MAPK/MK2 signals, the DDR (DNA damage response) proteins ATM, NBS1, and CHK2 as well as the transcription factors NF-κB and C/EBP-β. In contrast, p53 negatively regulates or restrains the SASP (16). A summary of causes, characteristics, and effects of cellular senescence is shown in **Table 1** (19, 23).

The Protective Role of Cellular Senescence Against Tumorigenesis

As mentioned earlier, cellular senescence is closely related to tumorigenesis and refers to a relatively stable state where cells are irreversibly separated from cell cycle and lost the ability of proliferation due to persistent stressed injuries. Under the stimulation of carcinogenesis, usually the cells enter senescent state followed by cell cycle arrest and cell division suspension, and then tumorigenesis is inhibited (16). On the other hand, in early-phase of cancer, regulatory dysfunction occurs in senescence-related signaling pathways, making damaged cells fail to grow senescent normally and then cell cycle become uncontrolled (24). It can be seen that cellular senescence is a potential antitumor mechanism. Senescence-induced therapy for preventing oncogenesis means that artificially inducible senescent cells secrete proinflammatory SASP factors and further recruit a variety of immune cells such as T cells, NK cells, and macrophages and their subtypes to infiltrate around the lesion tissues and participate in the activation of immunosurveillance, quickly identifying and clearing senescent cells, and finally blocking tumorigenesis (13, 14, 25).

As a natural barrier for tumor inhibition, cellular senescence is regulated by p53/p21 and $p16^{INK4a}$ signaling pathways (26). Upon the stimulation of mitogenic signals or cytokines, cyclins accumulate in early G1 phase and forms a complex with CDKs (cyclin-dependent kinases). This activated complex such as cyclin E-CDK2 or cyclin D-CDK4/6 initiates the phosphorylation of RB and then promotes the release of E2F transcription factors from RB, thereby driving the expressions of genes required for cells to enter the S-phase for mitosis (27, 28). CDK inhibitors p53/p21 and $p16^{INK4a}$ can block their common downstream cyclins-CDKs-RB/E2F axis and antagonize G1-S progression by respectively targeting cyclin E-CDK2 and cyclin D-CDK4/6 complexes (19, 28).

Tumorigenesis is a long pathological process that gradually breaks the limitation of senescent mechanism. Normal cells accumulate a series of driving carcinogenic factors before becoming real cancer cells (26). Oncogene-induced senescence (OIS) is triggered by the activation of oncogene or inhibition of tumor suppressor gene (29). Due to the redundant regulation of cellular senescence pathways, cancer cells that break through the OIS limitations will still retain the response to senescent induction, which can also lead to the senescence of cancer cells after the treatment of senescent induction, namely therapy-

TABLE 1 | Summarization of cellular senescence.

Causes	Characteristics	Consequences
· Telomere shortening or dysfunction · Activation of oncogene (e.g., RAS) · Loss or inactivation of tumor suppressor (e.g., RB, PTEN, NF1, and VHL) · DNA damage · Epigenetic stress · Oxidative stress · ER stress · Proteotoxic stress · Nucleolar stress · Spindle stress · Low BubR1 · Others	· Permanent cell-cycle arrest · Apoptosis resistance · **DNA content:** 2N or 4N · **Increased:** Cell size; SA-β-gal activity; $p16^{INK4a}$; $p19^{ARF}$; ATM/R; p53/p21; $p15^{INK4b}$; $p27^{KIP1}$; ROS; p38 MAPK; DNA-SCARS; SAHF; γ-H2AX foci · **Decreased:** Telomere length; DNA synthesis (Ki67, EdU); Proliferation (CDK2/4/6) · **SASP factors:** Cytokines (IL-1α/1β, IL-6) Chemokines (IL-8, CCL2/MCP-1, CXCL1) Growth factors (bFGF, HGF, IGF, TGF-β, G-CSF) Proteases (MMP-1/3/13) · **Others:** Loss of Lamin B1 Enhanced NF-κB signaling	**In development:** · Embryonic development (e.g., morphogen gradients and changes in cellularity) · Placental angiogenesis **In adulthood:** ***Acute senescence*** · Tumor suppression · Tissue repair (e.g., wound healing) ***Chronic senescence*** · Tumorigenesis and progression · Tissue dysfunction · Aging-related degeneration or diseases (e.g., Alzheimer disease, Osteoarthritis, Type 2 diabetes, and Atherosclerosis)

ER, endoplasmic reticulum; SA-β-gal, senescence-associated β-galactosidase; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and RAD3-related protein; ROS, reactive oxygen species; p38 MAPK, p38 mitogen-activated protein kinase; DNA-SCARS, DNA segments with chromatin alterations reinforcing senescence; SAHF, senescence-associated heterochromatin foci; SASP, senescence-associated secretory phenotype.

induced senescence (TIS) (30). Many studies have achieved effective treatment of cancer by inducing cellular senescence. For example, CDK4 gene knockout could immediately induce the senescence of non-small cell lung cancer cells driven by K-Ras, thus resulting in tumor regression (31). CDK4/6 inhibitors specifically accelerate the induction of cellular senescence by inhibiting CDK4/6-induced phosphorylation of RB, subsequently activate immunosurveillance by SASP, and effectively clear senescent cancer cells including HCC cells (16, 28). Notably, as early as 2015, the specific CDK4/6 inhibitor Palbociclib has been approved by the U.S. Food and Drug Administration for clinical treatment of advanced breast cancer (32). The genotoxic drug Oxaliplatin could cause DNA damage and oxidative stress, which induced senescence in HCC cells as a form of senescence-induced therapy (33). Radiotherapy as the method of TIS can induce DNA damage, and further accelerate senescence or death of tumor cells including HCC cells under therapeutic dose (34).

Immune-checkpoint inhibitors, targeting programmed cell death-1 (PD-1), programmed cell death ligand-1 (PD-L1), or cytotoxic T-lymphocyte protein 4 (CTLA-4), can specifically block the corresponding immunosuppressive signals and exhibit potential efficacy on malignant tumor including advanced HCC (35, 36). Clinical trials have identified a manageable safety profile and durable antitumor responses of anti-PD-1 therapy in advanced HCC (6). Nonetheless, a relatively small number of responders can benefit from immune-checkpoint inhibitor monotherapy. Recently, an alternative antitumor schedule by the combination of immune-checkpoint inhibitors and CDK4/6 inhibitors has achieved clinical exploration and synergistic efficacy over the monotherapy. According to the approval numbers obtained from the ClinicalTrials.gov database, in addition to the use of single agent CDK4/6 inhibitors in anti-cancer such as anti-HCC treatment (Identifier: NCT01356628; NCT03109886; NCT02524119), several clinical trials combining CDK4/6 inhibitors (Palbociclib, Ribociclib, or Abemaciclib) with immune-checkpoint inhibitors for breast cancer (Identifier: NCT03294694; NCT02791334; NCT02779751), ovarian cancer (Identifier: NCT03294694), and non-small cell lung cancer (Identifier: NCT02779751) are ongoing or active (28).

Moreover, senescence-related immune surveillance plays an important role through SASP factors and infiltrating immunocytes such as $CD4^+$ Th1 cells and M1 macrophages, which can promote the clearance of senescent precancerous cells and restrict tumor formation. Among them, $CD4^+$ Th1 cells kill senescent cells by releasing IFNγ and TNFα, and M1 macrophages inhibit the proliferation of malignant cells and promote their apoptosis through TGFβ signal (37). It was also found that the combination of Palbociclib and Trametinib, a specific K-ras-targeted drug, could induce the senescence of lung cancer cells with K-ras mutation, and then SASP factors could activate natural killer (NK) cells, thus improving the treatment effect on lung cancer (38). In addition, the activation of hepatocellular protooncogene triggered OIS and induced $CD4^+$ T cells to participate in immune monitoring and immune clearance (13). Therefore, immunosurveillance-dependent senescence-induced therapy is a promising method for the suppression of tumorigenesis.

DEFINITION OF HEPATOCELLULAR SENESCENCE

As with other body organs, the liver undergoes a process of aging. Along with aging and upon the conditions of various stressors, such as oxidative stress or oncogene activation, there are many changes in the liver, including decrease in size and in total numbers of normal hepatocytes, decline in regenerative and metabolic capacity, and increase in proportion of polyploid and multinucleated hepatocytes. It is confirmed that the liver of aging mice accumulated polyploid or aneuploid senescent hepatocytes, which is associated with accumulation of DNA damage and activation of INK4a/ARF locus (10, 39).

Hepatocellular senescence inhibits the proliferation of damaged hepatocytes, ensures a stable arrest of proliferation and division, and further causes the alterations of microenvironment and homeostasis (40). Of note, except that more proportion of polyploid or aneuploid exists in senescent hepatocytes (10), manifestation of hepatocellular senescence is non-specific compared with other cellular senescence. Some widely used markers or common events for identifying typical hepatocellular senescence include telomere shortening or dysfunction, SA-β-Gal activity, SASP secretion, cell proliferation arrest, cell enlargement, and increased expressions of p21 and $p16^{INK4a}$. In addition, senescence-related heterochromatic foci and histone γ-H2AX foci exist in the nuclei of some senescent cells, which activate proliferative genes and respond to DNA damage stress (10, 39, 41). The authors performed the study on the mechanism of hepatocellular senescence and senescent reversion, finding that the proportion of polyploid hepatocytes increased along with aging, and the above characteristics of senescent hepatocytes following transplantation could be reversed by ploidy conversion (10).

The detailed mechanism and biological function of hepatocellular senescence in chronic liver diseases have not been fully elucidated. Enhanced oxidative stress resulting from imbalanced reactive oxygen species (ROS) is the main cause of DNA damage in senescent hepatocytes and exists in chronic liver diseases of aging individuals (42, 43). DNA damage causes the overexpression of cell cycle inhibitors and further halts the proliferation of damaged cells by inducing senescence. The liver can normally repair and regenerate if the damage is mild. However, hepatocytes with severe DNA damage lose the capacity of regeneration, and necrosis, apoptosis or senescence will occur (5, 22).

Hepatocellular senescence can cause remarkable changes in tissue homeostasis and microenvironment *via* SASP, which may serve as an antitumor role. At the early stage of chronic liver injury, hepatocellular senescence may serve a protective role by blocking the proliferation and promoting DNA repair of injured hepatocytes, which would reduce the risk of these affected cells becoming cancer cells (15), revealing that early induction of hepatocellular senescence is beneficial to the inhibition of

hepatocarcinogenesis. With the assistance of SASP, hepatocellular senescence can recruit and activate immune cells. Activated immunocytes help to clear senescent precancerous hepatocytes, namely senescence surveillance, ultimately preventing malignant transformation (13, 14). Companied with additional mutations such as p53 mutation, senescent hepatocytes contribute to invasive HCC (44). Recovery of wildtype p53 in HCC can induce the activation of immune cells and the elimination of senescent hepatoma cells (11, 45). Kang et al. found that $CD4^+$ T cells removed senescent premalignant hepatocytes in association with activated monocytes and macrophages (13), which also indicated the importance of immunosurveillance as an anti-HCC barrier in senescence-induced therapy.

THE POTENTIALLY PROTECTIVE ROLE OF HEPATOCELLULAR SENESCENCE AGAINST THE OCCURRENCE OR DEVELOPMENT OF HEPATOCELLULAR CARCINOMA

During the life span, senescence is a common biological phenomenon existing in normal somatic cells and tissues. Of note, senescence is also an unneglectable biological event in tumors (46). Senescence-based therapeutic methods can induce premature senility of cancer cells by the activation of senescence signaling pathways and subsequent SASP (11, 14, 47, 48). Previous studies provided sufficient evidence on the induction of senescence in series cancer cell lines by genetic, chemical, radioactive, as well as biological ways, which supports the consideration of senescence induction as an anti-cancer therapy (24, 49). In 5-aza-2-deoxycytidine-treated HCC cell lines, the induction of $p16^{INK4a}$ upregulation, pRB dephosphorylation, and G1 arrest was indicated by positive SA-β-Gal staining (49).

In recent years, more and more attention has been paid to the relationship between hepatocellular senescence and hepatocarcinogenesis. Accumulating evidence has gradually demonstrated that hepatocellular senescence exhibits anti-HCC effect in specific liver microenvironment. In support of this view, one study reported that inhibition of SIRT6 expression could promote the expressions of p21 and p16 through its regulation of ERK pathway, thereby inducing cellular senescence and reducing the tumorigenicity of hepatoma cells (50). In mice with the deficiency of senescence signaling pathways, hepatocytes suffering from liver injury factor CCl_4 did not appear senescent phenotypes due to the impairment and disorder of hepatocellular senescence, but turned to the characteristics of liver fibrosis and cirrhosis, finally developing into HCC (51). Our cooperative study found that DUSP16 was upregulated in HCC, which could make HCC cells escape from senescence by inhibiting p53/p21-RB and $p16^{INK4a}$-RB pathways, thus facilitating the proliferation of HCC cells (52).

Moreover, Xue et al. claimed that oncogene H-ras was highly expressed but p53 expression was inhibited in murine hepatocarcinomas with excessive proliferation of HCC cells upon transplantation into the livers of athymic mice. However, these tumors rapidly regressed following the recovery of p53 expression (11). These observations may reveal that hepatocellular senescence contributes to inhibiting oncogene-activated liver cancer. On the other hand, HCC cells driven by Myc in Tet-o-MYC mice exhibited senescent phenotypes after MYC was inhibited, and HCC regressed when p53 was expressed again (53). As another example, oncogene c-Myc downregulation and senescent induction as a result of the response to TGF-β occurred in several HCC cell lines (31), implying that senescent induction may also be linked to the inactivation of oncogene.

The progression of liver diseases is closely related to the characteristics of liver injury and the pathological phenotype or fate of hepatocytes (carcinogenesis or senescence) is determined by the degree of hepatocellular damage. The diseased liver suffering chronic and mild damage to hepatocytes can regenerate and remodel repeatedly by itself until its repair potential is exhausted, which develops into liver cancer after a long period of time. However, acute and severe liver injury as a consequence of harmful stress factors will cause senescence in most hepatocytes in a short time, even leading to fulminant liver failure (22, 54).

At present, the aspect of HCC-relevant research combining with the pathological basis of chronic liver injury is still weak due to the limitation of animal model. The authors have already introduced and applied fumarylacetoacetate hydrolase (Fah) knockout ($Fah^{-/-}$) mice (55, 56) as an ideal animal model of inducible liver injury and even HCC (10, 15) since HCC as a result of chronic liver injury in $Fah^{-/-}$ mice is highly overlapped with the genetic characteristics of human HCC (57). In line with the previous results, the authors demonstrated that $Fah^{-/-}$ mice with chronic liver injury were characterized by the inhibition of hepatocellular senescence and high rate of HCC tumorigenesis, while $Fah^{-/-}$ mice under acute liver injury were characterized by accelerated hepatocellular senescence without HCC occurrence (15). Furthermore, hepatocarcinogenesis under chronic liver injury was significantly restricted due to hepatocellular senescence following the reactivation of acute liver injury in $Fah^{-/-}$ mice (15), revealing the potential antitumor effect of inducible senescence in precancerous hepatocytes.

Of note, how immunosurveillance prevents HCC following senescence induction is still under exploration and not fully understood. Overexpression of p53 in p53 deficient HCC could cause the senescence of HCC cells again, which were further cleared by SASP-activated immune surveillance so as to restrain HCC at last (11). In another study, hepatocytes with the overexpression of Ras rapidly underwent protooncogene-induced senescence, and meanwhile secreted SASP factors to activate immune system to remove themselves, thus restricting the occurrence of HCC (13). It was demonstrated that M1 polar macrophages could promote the elimination of tumor cells (58). Besides, some studies have confirmed that senescent astrocytes expressing p53 could release regulatory factors, promote macrophages to the polarization of type M1 with antitumor effect, and contribute to the formation of antitumor microenvironment (59). Similarly, our study also proved that acute injury-reactivated hepatocellular senescence activated immunosurveillance and promoted the activation and

recruitment of NK cells and macrophages by activating $CD4^+$ Th1 cells, thus eliminating senescent precancerous hepatocytes and further inhibiting hepatocarcinogenesis in $Fah^{-/-}$ mice under chronic liver injury (15), suggesting that tumor suppression to some extent is resulting from intensive induction of senescence, and subsequent immune-mediated clearance of senescent cells. Collectively, the above hepatocellular senescence-induced immune surveillance has gradually become a potentially effective approach for HCC prevention and treatment.

THE FUNCTION AND MECHANISM OF HEPATOCELLULAR CARCINOMA INHIBITION BY HEPATOCELLULAR SENESCENCE-INDUCED IMMUNE SURVEILLANCE

As stated earlier, sufficient evidence reveals that cellular senescence plays a pivotal role in limiting tumorigenesis and development by immunomodulation (60). The occurrence and development of HCC is a complex and irreversible process, which usually goes through various stages of liver injury, liver fibrosis, liver cirrhosis, and liver cancer (61). Early interruption of either liver injury or fibrosis is the most important steps in inhibiting the progression of liver cancer.

Chronic liver injury is the initial pathological change that develops to HCC, which is typically characterized by progressive destruction and regeneration of hepatic tissues (3, 4). Since cellular senescence exhibiting permanent cell cycle arrest is a potential mechanism of tumor inhibition, significant induction of hepatocellular senescence under chronic liver injury through the treatment with DNA damage chemicals or specific cell cycle inhibitors is beneficial for limiting hepatocarcinogenesis (28, 48). In addition to small molecular reagents that induce cellular senescence, some other treatments are gradually being used, including selecting cytokines/chemokines to activate immunosurveillance, supplementing adoptive immunocytes to remove senescent cells, and screening chemical drugs such as senolytics to induce apoptosis of senescent cells or accelerate clearance of senescent cells (28, 37, 48, 62).

One of the characteristics of cellular senescence is the secretion of a variety of factors such as chemokines or cytokines, the main part of secretory components in senescent hepatocytes, which function as the important immunomodulators (63). The protective attribute of SASP is now known as senescence surveillance since it facilitates the selective clearance of precancerous and cancer cells in liver (37). Therefore, senescent cells are now thought to be antitumorigenic because they restrict tumor cell growth by permanently entering cell cycle arrest, and secretory components from senescent hepatocytes can also recruit immune cells to eliminate damaged hepatocytes under chronic liver injury (25). Generally, under non-cell-autonomous modulation of senescent hepatocyte-secreted SASP factors, a variety of immunocytes and subpopulations are recruited and activated to participate in immunosurveillance, and identify and eliminate damaged precancerous hepatocytes, and thereby preventing the formation of HCC. Hence, containing multiple SASP factors and various types of immunocytes, the tumor immune microenvironment plays a fundamental role in the regulation of senescent response in HCC (37). Th1 lymphocytes provoke senescence induction in target cells through IFNγ and TNFα release (64), and M1 polarized macrophages induce a senescence response mediated by TGFβ signaling (65). In contrast, tumor-infiltrating myeloid-derived suppressive cells (MDSCs) hinder senescence induction and spread through the secretion of interleukin 1 (IL-1) receptor antagonist within tumor microenvironment, and thereby interfering with IL-1α signaling pathway (66). Tumor-infiltrating immune subsets, such as NK cells, M1 macrophages, and Th1 cells, contribute to tumor regression by promoting the clearance of senescent premalignant hepatocytes (15). Moreover, adenovirus-delivered oncogene Ras induced hepatocellular senescence and leaded to activating immune surveillance by recruiting various immune cells (such as macrophages, $CD4^+$ Th cells, neutrophils, and NK cells) to infiltrate within the sites of damaged hepatocytes, which repressed precancerous lesions of HCC (13).

Another beneficial feature of hepatocellular senescence is its anti-fibrosis effect on the liver. The senescent process of hepatic stellate cells (HSCs) is closely related to the homeostasis of liver tissues and the formation of hepatic fibrosis. HSCs are located in the Disse space, which usually remain quiescent but become activated only when liver injury occurs. Activated HSCs contribute to fibrotic process following liver damage. The formation of fibrosis is divided into the three steps: first, activation and differentiation of HSCs to α-SMA-positive myofibroblasts; second, deposition of extracellular matrix (ECM), including infiltrative secretion of collagen and TIMP to the injured position; at last, activated HSCs undergo cellular senescence and activate immune surveillance to further eliminate senescent HSCs, or directly undergo apoptosis to be cleared (67). The process of cellular senescence often causes the activation of tumor suppressors p53 and p16. In the model of CCl_4-induced cirrhosis with deficiency of p53 or p16, Lowe et al. reported that activated HSCs could continuously deposit ECM in the absence of cellular senescence, resulting in severe liver fibrosis (51). In addition, it was also found that senescent HSCs were very helpful since they played a powerful immunomodulatory role in recruiting immune cells, such as macrophages, at the location of damaged tissues. Besides, recruited immunocytes remove senescent cells and also contribute to dissolving fibrotic lesions (51, 68, 69). Therefore, cellular senescence exhibits the function of anti-fibrosis that helps to recover injured liver tissues.

INTERVENTION STRATEGIES IN SENESCENCE-RELATED THERAPY OF HEPATOCELLULAR CARCINOMA

In accordance with the existing researches, the induction of senescent cells, the regulation of SASP, and the clearance of senescent cells are the three major senescence-targeted strategies

of HCC intervention (37, 62, 63, 70, 71). The corresponding contents are described below and summarized in **Figure 1**.

Induction of Senescent Hepatocytes by Small Molecules

As mentioned earlier, therapy-induced senescence (TIS) has become a potential antitumor scheme. Targeted therapies for HCC can be explored from the perspective of senescent induction. There are mainly two ways to induce cellular senescence: one is to induce cell replicative senescence through the intervention of telomere or telomerase; the other is to induce premature senescence through specific factors activating p53/p21-RB or $p16^{INK4a}$-RB signaling pathways, finally limiting the entry into cell cycle and blocking the ability of division and proliferation (17).

Even though the senescent signals of p53/p21 and $p16^{INK4a}$ were significantly activated following the induction of acute liver injury, which repressed chronic injury-induced hepatocarcinogenesis, it was found that acute liver injury meanwhile caused an increasing mortality of $Fah^{-/-}$ mice and poor controllability of treatment, which makes the difficulty of clinical translation. At present, development, screening, and application of specific small molecular compounds to induce cell cycle arrest for HCC prevention and therapy has been a promising field (28). For instance, lysine acetyltransferases (KATs)-catalyzed histone acetylation plays an essential role in chromatin organization and function. Chromosomal translocations of oncogenes KAT6A/B encoding for KATs were identified in a variety of cancers and KAT6A accounted for senescent suppression by regulating the suppressors of CDKN2A locus. Therefore, histone acetylation inhibitors WM-8014/1119, targeting KAT6A/B, could effectively inhibit the growth of tumor cells through inducing their senescence without causing DNA damage (72). Recent study by Wang et al. showed that cell division cycle 7-related protein kinase (CDC7) inhibitor XL413 specifically induced the senescence of HCC cells with p53 gene mutation, but exhibited no effect of senescent induction on normal cells, and therefore it could specifically eliminate HCC cells (73).

Of note, since CDKs as downstream targets of p53/p21 and p16 signals can trigger cell cycle from G1 phase to S phase, direct inhibition of CDK activities can also induce cells to deviate from normal cell cycle and become senescent (28). Small molecule compound Palbociclib can specifically block the phosphorylation of RB protein initiated by CDK4/6, upon binding to the active site of CDK4/6, which causes cell cycle arrest in G1 phase (74). Palbociclib can induce the senescence of both tumor and normal cells *in vitro* and *in vivo* (75, 76). A study of Palbociclib treatment on a variety of human HCC cell lines suggested that cellular senescence occurred in HCC cell lines with normal RB protein function (77). Besides, the authors utilized Palbociclib as a senescent inducer to stably cause hepatocellular senescence and effectively inhibit hepatocarcinogenesis without affecting liver function in $Fah^{-/-}$ mice with chronic liver injury, showing its biological safety and feasibility in clinical application (unpublished data). Cell cycle inhibitors for potential prosenescence therapies are listed in **Table 2**.

Activation of Immunosurveillance by SASP Factors or Adoptive Immunocytes

The key point of senescence-induced therapy for HCC is that senescent hepatocytes-produced proinflammatory SASP factors recruit a variety of immunocytes to participate in immunosurveillance to further identify and eliminate senescent cells, and finally inhibit HCC (27, 48). Indeed, $CD4^+$ T cells, in the form of T helper cells, could function through monocyte/macrophage system to remove senescent cells and suppress tumor formation (82). Besides, the immune system can initiate an effective antitumor response during the development of HCC, since the progression of chemical carcinogen diethylnitrosamine (DEN)-induced liver tumor was significantly enhanced in T-cell and B-cell immune deficient mice ($Rag1^{-/-}$ mice) (83). Senescence-associated immune

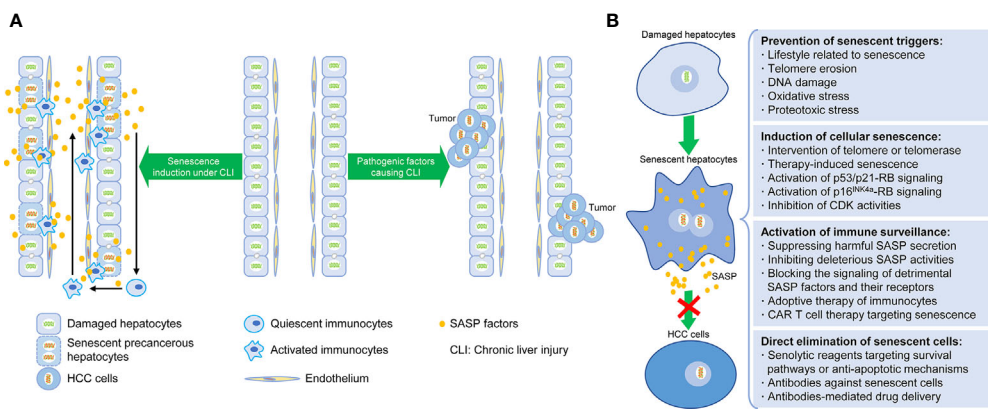

FIGURE 1 | Senescence-induced therapy for HCC. **(A)** Schematic diagram depicting the relationship between hepatocellular senescence and hepatocarcinogenesis under chronic liver injury. **(B)** Intervention strategies targeting hepatocellular senescence to inhibit hepatocarcinogenesis and extend healthy life span, including preventative steps for senescent triggers, induction and clearance of senescent hepatocytes, and activation of senescence-related immunosurveillance.

TABLE 2 | Candidate reagents for senescent cell induction.

Small molecule	Targeted protein	Reference
CVT-313, CVT-2584	CDK2	(78)
Palbociclib	CDK4 and CDK6	(79)
Ribociclib	CDK4 and CDK6	(79)
Abemaciclib	CDK4 and CDK6	(79)
Milciclib	CDKs	(6)
GRN163L	telomerase activity	(80)
Nutlin, RITA	p53-degrading ubiquitin ligase MDM2	(81)
PRIMA-1, MIRA-1	mutant p53 reactivation	(81)
WM-8014, WM-1119	histone acetyltransferases KAT6A/B	(72)
XL413	DNA-replication kinase CDC7	(73)

CDK cyclin-dependent kinase; MDM2, murine double minute 2; KAT6A/B, lysine acetyltransferase 6A/B; CDC7, cell division cycle 7-related protein kinase.

responses require the recruitment and maturation of $CCR2^+$ myeloid cells, and CCR2 ablation caused outgrowth of HCC (14). Cytotoxic T cells with chimeric antigen receptors (CAR T cells)-mediated therapy has been reported to have therapeutic potential for senescence-associated pathologies. CART T cells targeting uPAR, identified as a specific marker on senescent cell membrane, could effectively ablate senescent cells *in vitro* and *in vivo*. Of note, the therapeutic effect of uPAR-specific CAR T cells on liver fibrosis in mice with nonalcoholic fatty liver disease (NAFLD) was remarkable due to the elimination of senescent hepatocytes (84). Moreover, in $Fah^{-/-}$ mice under chronic liver injury, senescent reactivation of precancerous hepatocytes could recruit immunocytes such as M1 type macrophages, $CD4^+$ Th1 lymphocytes, and NK cells and secrete SASP factor CCL2, which played an inhibitive role against tumorigenesis (15). **Table 3** showed the types of immunocytes involved in the clearance of senescent cells in different mouse models of liver diseases.

Of note, it was found that $CD8^+$ T cells-mediated adaptive immune system played a crucial role in promoting malignant transformation of hepatocytes and the formation of HCC through lymphotoxin β signal in $Fah^{-/-}$ mice (57). Besides, SASP cytokines IL-6 and IL-8 and their signals exhibited detrimental paracrine effects of prolonged presence of senescent cells and caused tumor initiation, progression, and metastasis (63, 96). These researches demonstrate that the differences of immune microenvironment under specific conditions may exhibit the similar or opposite effect on HCC progression and emphasize that senescence-related immune surveillance should be strictly tuned to balance immune surveillance and cancer risk or amplify the net antitumor effect. For example, the development of deleterious SASP-neutralizing approaches can be considered from the following aspects: inhibiting pro-SASP signaling pathways within senescent cells, blocking the secretion of harmful SASP factors, and suppressing the activities of specific SASP factors or their receptors (**Table 3**).

TABLE 3 | Candidate reagents for SASP modulation and potential immunocytes for immunosurveillance.

Small molecule	Targeted SASP pathway	Reference
Metformin	NF-κB	(85)
UR-13756, BIRB 796	p38 MAPK/MK2	(86)
Simvastatin	Rho family GTPases	(87)
Sertraline, Rapamycin	mTOR	(73, 88)
Antibody	**Targeted SASP ligand/receptor**	**Reference**
Adalimumab/Infliximab	TNFα	(89)
Etanercept	TNFα	(90)
Canakinumab	IL-1β	(91)
Rilonacept	IL-1α and IL-1β	(92)
Anakinra	IL-1R	(93)
Siltuximab	IL-6	(94)
Tocilizumab	IL-6R	(95)
Immunocyte	**Animal model**	**Reference**
monocyte-derived macrophages and $CD4^+$ Th1 cells	$Nras^{G12V}$-transfected mouse model of HCC	(13)
$CD4^+$ T cells, monocytes, and macrophages	liver-specific MYC oncogene transgenic mouse model of HCC	(82)
NK cells	$p53^{-/-}$; $INK4a^{-/-}ARF^{-/-}$ mouse model of CCl_4-induced hepatic fibrosis	(51)
neutrophil cells, NK cells, and macrophages	$p53^{-/-}$ mouse model of HCC	(11)
$CD4^+$ Th1 cells, NK cells, and macrophages	$Fah^{-/-}$ mouse model of HCC under chronic liver injury	(15)
Senolytic CAR T cells	mouse model of CCl_4 or NASH-induced hepatic fibrosis	(84)

NF-κB nuclear factor kappa B; MAPK, mitogen-activated protein kinase; MK2, MAPKAP kinase-2; mTOR, mechanistic target of rapamycin kinase; TNFα, tumor necrosis factor α; IL-1/6, interleukin-1/6; IL–1/6R, interleukin–1/6 receptor; HCC, hepatocellular carcinoma; Th1 cells, T helper 1-type cells; NK cells, natural killer cells; CAR T cells, chimeric antigen receptor T cells; INK4a, p16 or cyclin-dependent kinase inhibitor 2A; ARF, ADP ribosylation factor; Fah, fumarylacetoacetate hydrolase; CCl_4, carbon tetrachloride; NASH, non-alcoholic steatohepatitis.

Elimination of Senescent Hepatocytes by Senolytic Drugs

In addition to the above-mentioned immune surveillance-based strategies, senescent cell elimination is another way of senescence-targeted HCC therapy. In view of the fact that the secretory phenotypes of inducible senescent cells may bring about potential adverse effects in the process of HCC development, such as persistent senescence-caused formation of tumor microenvironment, the removal of existing senescent cells by direct killing through pharmacological intervention, namely senotherapies, is a straightforward means of anti-HCC (18, 97). As discovered by researchers from Mayo Clinic, the advantages of currently screened senolytic drugs, such as ABT-263 (Navitoclax) (98), Dasatinib (99), Quercetin (99), DRI-FOXO4 (100), UBX0101 (101), and AP20187 (102), are that they can selectively target senescent cell anti-apoptotic pathways (SCAPs), accelerate senescent cell apoptosis, and specifically eliminate senescent cells. Senescent cells often release a series of anti-apoptotic signals to promote survival, such as ephrins (EFNB1 and EFNB3)/dependence receptors, PI3K/AKT, Bcl-2 (Bcl-xl, Bcl-2, and Bcl-w), p53/FOXO4/p21/Serpins (PAI-1 and PAI-2), HIF-1α, and HSP90 (23, 62). These signal factors can enhance the ability of anti-apoptosis in senescent cells and further lead to local inflammation, and even cause dysfunction of normal tissue function. ABT-263 is a pan-inhibitor of Bcl-2 family, which can selectively eliminate

senescent cells with high expressions of Bcl-2, Bcl-XL, and Bcl-W. Dasatinib (D), a small molecule broad-spectrum inhibitor of Src family protein tyrosine kinases, can induce the senescence of epithelial and adipocytes. Quercetin (Q), belonging to bioflavonoids and antioxidants, can target Bcl-2, HIF-1 α, PI3K, and p21. The combination of D and Q (D + Q) has the potential to act on multiple SCAP targets and selectively promote the clearance of senescent cells by accelerating apoptosis in multiple tissues (99, 103). The regimen of ABT-263 or D + Q suppressed tumor progression by eliminating senescent HSCs that promoted tumor growth in the liver of hepatocyte-specific $FBP1^{-/-}$ mice (104). The small molecule AP20187 could induce apoptosis through FKBP-Casp8 fusion protein dimerization in an aging-related model of INK-ATTAC mice (105), which leaded to the elimination of $p16^{INK4a}$-positive cells. It was also used to clear senescent cells to reverse age-dependent hepatic steatosis. This method was equally successful, compared to D + Q therapy, in inhibiting senescence and reducing liver fat accumulation induced by high-fat diet in mice (102). Currently, as searched in the ClinicalTrials.gov database, senolytic drugs are being tested in human clinical trials for the treatment of osteoarthritis (Identifier: NCT04210986), idiopathic pulmonary fibrosis (Identifier: NCT02874989), and chronic kidney disease (Identifier: NCT02848131) (99, 106, 107). However, senolytic drug therapy can cause extra-target effects in addition to exhibiting targeting functions of senescent cell clearance. For example, senolytic drugs targeting Bcl-2 family members have toxic effects on some immunocytes and platelets, and may cause thrombocytopenia and lymphopenia (97). Therefore, increasing the selectivity of these compounds by targeting more specific mechanisms of senescence may reduce the toxicity.

Except for senolytics, Cai et al. reported β-galactosidase-targeted prodrug SSK1 as a new anti-senescence compound. SSK1 has no toxic effect, but it can be metabolized and activated to be toxic molecule by activity-enhanced SA-β-Gal in senescent cells, subsequently inducing senescent cell death and reversing hepatic fibrosis and other senescent phenotypes of aging mice with no effect on normal cells (108). In the study by Wang et al., sertraline, a drug used in clinical treatment of depression, could specifically promote the apoptosis of CDC7 inhibitor-induced senescent hepatoma cells through downregulating mechanistic target of rapamycin (mTOR) signaling pathway (73). Results from animal models and clinical samples of HCC demonstrated that the combination of CDC7 inhibitor and mTOR inhibitor could significantly inhibit the progression of HCC, and its antitumor effect was significantly better than that of non-specific multi-target drug Sorafenib (73). In terms of antitumor effect, the model of first induction and subsequent elimination of senescent tumor cells maybe more effective than single one of the two anti-HCC methods. The small molecules that have been reported for the use of senescent cell clearance are listed in **Table 4**. In summary, promoting the clearance of senescent hepatocytes may have the potential to become an innovative approach for the prevention or treatment of HCC since there are few reports on this aspect, especially in HCC under chronic liver injury.

TABLE 4 | Candidate reagents for senescent cell clearance.

Small molecule	Targeted pro-survival protein/ pathway	Reference
ABT-737	Bcl-XL, Bcl-W	(109)
ABT-263 (Navitoclax)	Bcl-2, Bcl-XL, and Bcl-W	(98, 110)
A1331852, A1155463	Bcl-XL	(111)
Dasatinib	RTKs	(99)
Quercetin	PI3K/Akt, Bcl-2, HIF-1α, and p21	(99)
DRI-FOXO4	disruption of p53/FOXO4 interaction	(100)
UBX0101	MDM2	(101)
AP20187	dimerization of FKBP-fused Casp8	(102)
SSK1	SA-β-Gal and p38 MAPK	(108)
17-DMAG (Alvespimycin)	HSP90	(112)
Fisetin	PI3K/Akt	(111)
Phloretin	glucose and fatty acid metabolism	(113)
Panobinostat	HDACs	(114)
Cytochalasin B	AMPK and autophagy	(113)
Etomoxir	AMPK and autophagy	(113)
Sodium oxamate	AMPK and autophagy	(113)

Bcl-2 B cell lymphoma 2; Bcl-XL, B cell lymphoma XL; Bcl-W, B cell lymphoma W; RTKs, tyrosine kinase receptors; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; Akt, PKB or protein kinase B; HIF-1α, hypoxia inducible factor-1α; DRI-FOXO4, d-retro-inverso peptide-forkhead box protein O4; MDM2, murine double minute 2; FKBP, FK506-binding protein; Casp8, Caspase 8; SSK1, senescence specific killing compound 1; SA-β-Gal, senescence-associated β-galactosidase; MAPK, mitogen-activated protein kinase; HSP90, heat shock protein 90; HDACs, histone deacetylase inhibitors; AMPK, adenosine monophosphate (AMP),-activated protein kinase.

DUAL EFFECTS OF HEPATOCELLULAR SENESCENCE ON THE OCCURRENCE AND DEVELOPMENT OF HEPATOCELLULAR CARCINOMA

There are two opposite views explaining the biological effect of cellular senescence. Cellular senescence is considered as a natural mechanism of anti-cancer since senescence can cause arrest of cell division and proliferation, indicating that senescence may be a beneficial event in the body (27, 48). On the other side, cellular senescence may lead to the decline of regenerative ability of tissues or organs (62). Thus, it may impede functional and organizational renewal and thus becomes a deleterious process in the body. Along with the increase of age, accumulative senescent cells may lead to aging process, organ dysfunction, and even aging-related diseases such as cancer, stroke, atherosclerosis, type 2 diabetes, Alzheimer's disease, cataract, and osteoporosis (18, 62). In the future, how to balance the two aspects of cellular senescence so that we can better utilize its antitumor effect in the prevention and treatment of HCC while avoid its detrimental aspect, is worth to be studied in depth.

Recent literature reported that the degeneration of immune surveillance and clearance system in the elderly is one of the important causes of cancer formation (115). Along with aging, not only the accumulation of mutated cells undergoing irreversible injury, but also the metabolic abnormalities and functional decline of immune system in the organism will increase the risk of cancer (62). Therefore, aging-caused weakening of immune surveillance may be another important factor for tumorigenesis. Indeed, compared with young mice, chronic and slight liver injury in one-year-old $Fah^{-/-}$ mice will

spontaneously develop HCC (116), which warrants further investigation of the changes in the type, proportion, and metabolism of immunocytes such as $CD4^+$ Th cells, macrophages, and NK cells.

The correlation between senescent cells and immune system is mediated by SASP. Although SASP-regulated immune response plays a crucial role in the prevention and treatment of HCC, it is quite a complex process since SASP factors have highly dynamic changes in expression and composition over the period of senescence, which depends on the mode of senescence induction, the cell type, the duration of senescence, and active signaling cascades. In different situations, immune signaling system following the induction of hepatocellular senescence exhibits the distinct effects on the progression of HCC (117, 118). As mentioned above, under early chronic liver injury, hepatocellular senescence plays a protective role against hepatocarcinogenesis *via* immunosurveillance mechanism. However, in the late stage of chronic liver injury, single and scattered HCC nodules appear. Existing senescent cells secrete SASP factors including proinflammatory cytokine/chemokines such as IL-1, IL-6, and IL-8 at an abnormal level, which can promote a large number of transformations from non-senescent cells to senescent cells in a paracrine manner (117). It is possible that chronic liver injury causes progressive and repetitive liver destruction and regeneration, which leads to the accumulation of shorter telomeres in hepatocytes and further results in accumulative hepatocellular senescence (119). In this case, the microenvironment created by SASP secretory factors is more suitable for the survival of hepatoma cells, eventually promoting the formation and development of HCC (117).

Recently, using $Fah^{-/-}$ mouse model of HCC, the authors demonstrated that Palbociclib-induced hepatocellular senescence effectively restricted the occurrence of HCC following Palbociclib administration in the early-phase, but surprisingly enhanced the development of HCC in the late period of drug treatment. The next findings revealed that hepatocellular senescence at early precancerous stage could eliminate atypical hyperplastic hepatocytes and restrain hepatocarcinogenesis through SASP-activated immunosurveillance. However, in HCC development period, compared with early stage, the emergence of much more senescent hepatocytes may secrete a large amount of SASP factors, which caused the changes in tumor microenvironment and accelerated HCC progression (unpublished data).

Hence, induction of tumor cellular senescence is a double-edged sword, which may not only inhibit the occurrence of tumor, but also accelerate the development of tumor, especially in aging body (16, 120–122). Administration of small molecule compounds targeting cellular senescence induction or SASP modulation could become rather complicated. In the future, the investigation of mechanism on cellular senescence regulating the occurrence and development of HCC, the exploration of scheme on inducing hepatocellular senescence, and the construction of favorable antitumor microenvironment will become new research hotspots. In addition, it is also important that more attention should be paid to the choice of optimum medication time and clinical safety assessment of small molecule compounds inducing or inhibiting hepatocellular senescence.

CONCLUSION AND FUTURE PROSPECTS

In recent years, cellular senescence has aroused great interest in the research of HCC. It can be mediated by various pathways and molecules, just like cell death. Cell cycle suppressor-induced senescence may possess the antitumor mechanism on atypical hyperplastic hepatocytes, which highlights its clinical significance. On the other hand, inducible or spontaneous senescence observed in HCC will help to explore new methods of HCC prevention and treatment. The following is the summary points:

1. Senescence is characterized by a number of phenotypes and closely involved in the pathogenesis of age-related diseases including HCC (**Table 1**). Established senescence markers such as $p16^{INK4a}$, p21, and SA-β-Gal can be used to identify senescent cells, but these markers are representative rather than specific. Hopefully, more reliable biomarkers could be investigated to selectively identify and target senescent cell subtypes with harmful secretory phenotypes while maintaining other subtypes with beneficial secretory phenotypes for the suppression of tumor occurrence and development. Meanwhile, screening and examination of circulating SASP factors may be helpful to evaluate the efficacy of senescence-targeting therapy.
2. SASP entails continuous secretion of various proinflammatory factors and have highly dynamic changes in expression and composition over the senescent process. Heterogeneity of SASP can cause both beneficial and deleterious effects on age-related diseases including HCC, each of which depends on the physiological and pathological context in different periods of diseased organs including liver.
3. Hepatocellular senescence is regarded as a stress response that inhibits liver tumorigenesis early in lifespan, but it may become a basic harmful process along with the age growth that drives the accumulation of persistent age-relevant pathologies (e.g., local or systemic inflammation, impaired regenerative capacity, and weakened immune surveillance) and subsequent emergence of hepatoma cells failing to enter cell cycle arrest late in lifespan, and even fuels advanced and recurrent liver cancer. Accordingly, it can also be considered that transient presence of senescent hepatocytes may be beneficial while their chronic presence may be detrimental.
4. The positive functions of hepatocellular senescence and accompanying SASP contribute to proposing new clinical strategies of HCC-targeted therapy, namely senescence-induced therapy in HCC, for the purpose of reinforcing tumor suppressive growth arrest, stimulating immune clearance of senescent hepatocytes, and optimizing the repair of injured liver tissues through specific interventions in several checkpoints of senescence-mediated therapy.

However, there are still issues to be solved in the knowledge of complex roles of cellular senescence and senescence-related

anti-HCC therapies in both the occurrence and development of HCC. Future problems are listed below:

1. More intensive search and screening for small molecular compounds that can selectively target the induction or removal of senescent cells with harmful secretory phenotypes, or selectively modulate the SASP. Of note, these reagents inhibiting purely deleterious senescent cells or SASP factors would avoid to disrupt beneficial functions of senescence and proinflammatory processes if they exist and can be screened.
2. More comprehensive understanding of the reason why senescent cells increase along with age and play the role of promoting tumorigenesis during the process of tumor late in lifespan, despite the ability to eliminate themselves through immune system.
3. More comprehensive understanding regarding the optimal situation where senescent cells are beneficial and participate in tumor suppression, tissue repair, and regeneration. Also, cellular senescence induction should be strictly tuned to amplify the net antitumor effect of senescence.
4. More exploration of combining other pharmacological treatment strategies and senescence-induced therapies to enhance the antitumor effect of hepatocellular senescence in HCC, such as the combination of immune-checkpoint inhibitors and CDK4/6 inhibitors, or senescence-inducing drugs and proapoptotic senolytic reagents.

Despite the close relationship of cellular senescence abnormality and HCC pathogenesis, the regulatory roles of senescence pathways in HCC have not been full clarified. It is anticipated that senescence study will attract more attention in the future and provide more promising methods and experience for the aim of translating these senescence-associated therapies to clinical applications. Further elucidation on the molecular mechanisms of cellular senescence and senescence-associated immunotherapy will enable us to make therapeutic options more accurately in the prevention and treatment of HCC.

AUTHOR CONTRIBUTIONS

PL wrote the original version of the manuscript. QT contributed to the modification of the main sections. MC provided the suggestions for the content on hepatocellular senescence and senescence-induced therapy. WC contributed to the editing of the section on SASP and senescence-related immunosurveillance. YL offered the assistance for the section on the induction and clearance of senescent cells. ZL reviewed the manuscript and contributed to valuable academic advice. ZH provided the guidance and supervision for conceptual proposal and design, and contributed heavily to the writing and revision of the manuscript in its present form. All authors contributed to the article and approved the submitted version.

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REVIEW
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Immune Checkpoint Inhibitors in the Treatment of HCC

*Clelia Donisi[1], Marco Puzzoni[1], Pina Ziranu[1], Eleonora Lai[1], Stefano Mariani[1], Giorgio Saba[1], Valentino Impera[1,2], Marco Dubois[1], Mara Persano[1], Marco Migliari[1], Andrea Pretta[1,2], Nicole Liscia[1,2], Giorgio Astara[1] and Mario Scartozzi[1]**

[1] Medical Oncology Unit, University Hospital and University of Cagliari, Cagliari, Italy, [2] Medical Oncology Unit, Sapienza University of Rome, Rome, Italy

Hepatocellular carcinoma (HCC) is the typical inflammation-induced neoplasia. It often prospers where a chronic liver disease persists, thus leading a strong rationale for immune therapy. Several immune-based treatments, including immune checkpoint inhibitors (ICI), cytokines, adoptive cell transfer, and vaccines, have been tested in the treatment of HCC. In this review, we summarize the role of the ICI in HCC patients in various sets of treatment. As for advanced HCC, the anti-Programmed cell Death protein 1 (PD1) antibodies and the anti-Cytotoxic T-Lymphocyte Antigen *4* (CTLA-4) antibodies have been examined in patients with enthusiastic results in phase I-II-III studies. Overall, this led the Food and Drug Administration (FDA) to approve pembrolizumab, nivolumab, and nivolumab + ipilimumab in the second-line setting. The anti- Programmed Death-Ligand 1 (PDL-1) antibodies have also been evaluated. Thanks to the results obtained from phase III IMbrave study, atezolizumab + bevacizumab is now the standard of care in the first-line advanced setting of HCC. As for localized HCC, the putative immunological effect of locoregional therapies led to evaluate the combination strategy with ICI. This way, chemoembolization, ablation with radiofrequency, and radioembolization combined with ICI are currently under study. Likewise, the study of adjuvant immunotherapy following surgical resection is underway. In addition, the different ICI has been studied in combination with other ICI as well as with multikinase inhibitors and anti-angiogenesis monoclonal antibody. The evidence available suggests that combining systemic therapies and locoregional treatments with ICI may represent an effective strategy in this context.

Keywords: Hepatocellular carcinoma, immune checkpoint inhibitors, atezolizumab, pembrolizumab, nivolumab

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Sant'Orsola-Malpighi Polyclinic, Italy
Anuhya Kommalapati,
MedStar Washington Hospital Center, United States

***Correspondence:**
Mario Scartozzi
marioscartozzi@gmail.com

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INTRODUCTION

Hepatocellular Carcinoma (HCC) claims to be 90% of primary liver cancer and represents the second cause of death due to malignancy in males (1). The triggers most likely involved in cancer development are chronic infections by Hepatitis B or C viruses, diabetes, aflatoxin-B1 (AFB1) exposure, obesity, alcohol abuse, nonalcoholic steatohepatitis (NASH), nonalcoholic fatty liver disease (NAFLD), and metabolic syndrome (2–11).

Indeed, chronic inflammation boosts the tumor immunogenicity and induces hepatocellular DNA damage, genetic and epigenetic mutations. Furthermore, chronic inflammation allows to

escape the host immune surveillance in cooperation with an immunosuppressive surrounding (2–8).

The impairment of various immune components promotes tumorigenesis. The liver immune milieu consists of an assortment of innate and adaptive immune cells that undergo alterations that promote cancer development and progression. Immune checkpoints are involved in the inhibition of T- or natural killer cell activation as well as in the initiation and preservation of tumor immune tolerance. B and T cells, natural killer cells, dendritic cells, tumor-associated macrophages, monocytes, and myeloid-derived suppressor cells express on their surface immune-checkpoints and their ligands. The most well-known of them are cytotoxic T-lymphocyte protein 4 (CTLA-4), which promotes immunosuppression, and programmed cell death protein 1 (PD-1) that leads to the T-cell exhaustion status, which inhibits T-cell multiplication and release of cytotoxic mediators (2–8).

In a physiological state, antigens are presented to CD4+ T cells that consequently promote the activity of CD8+ T cells. Thus, leading to an upregulation of CTLA-4 and PD-1. Consequently, the immune checkpoints prevent hyperactivation of the immune response. That way, the tolerogenic environment of the liver is preserved. Therefore, HCC is an immunogenic tumor that builds-up in an immune-suppressed microenvironment. In the setting of chronic inflammation, the cancer develops and flourishes thanks to the recruitment of regulatory T cells (Treg), myeloid-derived suppressor cells (MDSCs), and the upregulation of immune checkpoints, CTLA-4 and PD-1. PD-1 binding its ligand PD-L1 prevents TCR signaling, blocks T cell proliferation, and induces the exhaustion of T cells. Tregs constitutively express CTLA-4 and preclude the immune response through it. CTLA-4 binds CD80/CD86, competing with CD28, and blocks activation of the T cells. It appears clear that the inhibition of immune checkpoints avoids immune exhaustion, reduces Treg activity, and leads to the reactivation of the anticancer immune response (2–5). Thus, immune-checkpoint inhibitors (ICI) seem to be promising treatment strategies (**Figure 1**).

IMMUNE CHECKPOINT INHIBITORS IN ADVANCED HCC

The systemic therapies for patients with HCC in advanced and intermediate stage, according to Barcelona Clinic Liver Cancer (BCLC), refractory to locoregional therapy was limited to sorafenib for a long time (12). Instead, since 2017, several

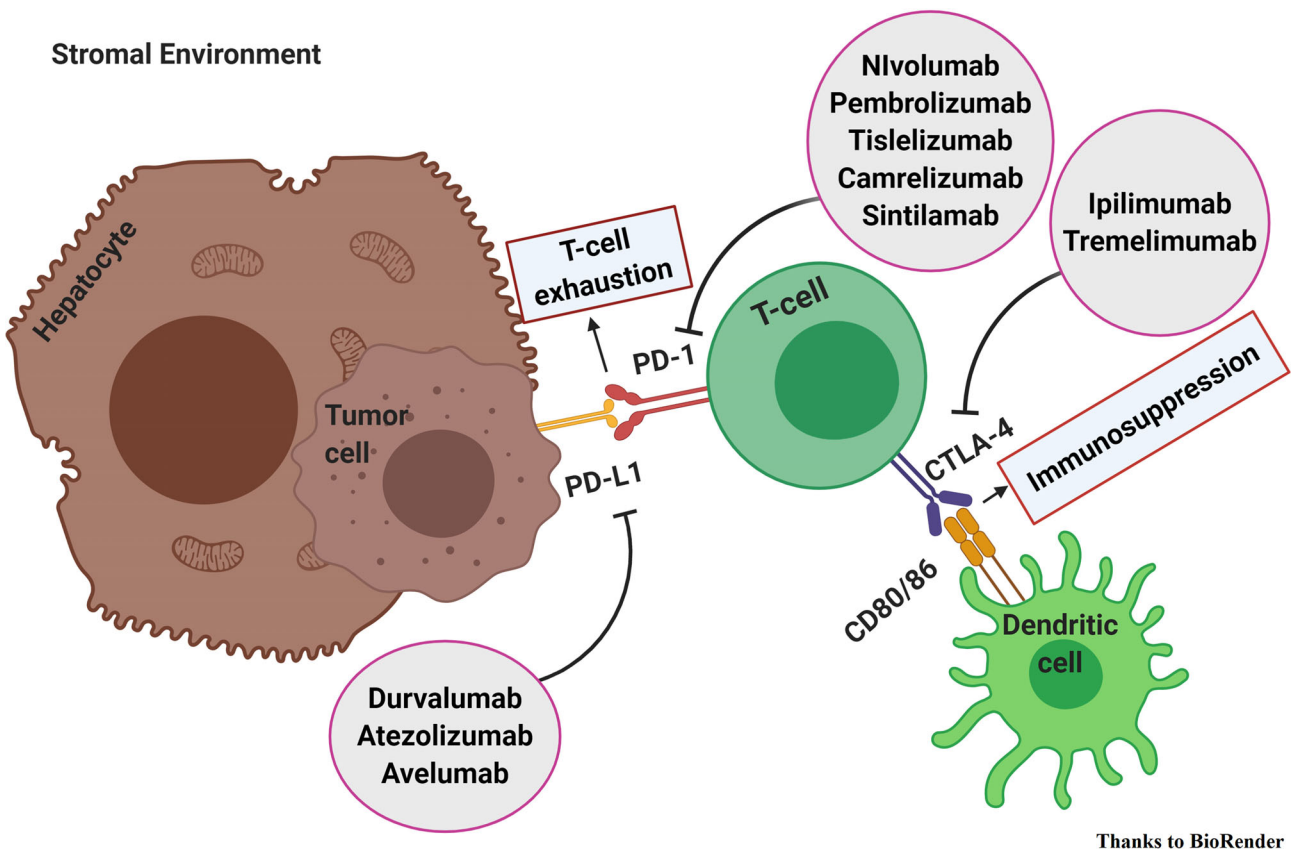

FIGURE 1 | Immune checkpoint inhibitors in hepatocellular carcinoma. PD-1 binding its ligand PD-L1 prevents TCR signaling, blocks T cell proliferation, and induces the exhaustion of T cells. CTLA-4 binds CD80/CD86 and blocks activation of the T cells. The inhibition of immune checkpoints avoids immune exhaustion, reduces Treg activity, and leads to the reactivation of the anticancer immune response.

effective systemic therapies have been recommended by the Food and Drug Administration (FDA), thus turning treatment decision making into a challenge. Several TKIs are now available for first-line [sorafenib (13–22), lenvatinib (23, 24)], second and third-line treatment [regorafenib (25), cabozantinib (26)]. Also, a monoclonal antibody [ramucirumab (27)] is available for second-line treatment. In addition, two anti-PD-1 antibodies [nivolumab (28) and pembrolizumab (29)] and the combination anti-PD1 + anti-CTLA4 [nivolumab + ipilimumab (30)] received FDA's accelerated approval. On the whole, the anti-angiogenesis remains a cardinal point for treatment, whereas the ICI, including anti-PD-1, anti-PDL-1, and anti-CTLA-4, are becoming increasingly important in the therapeutic scenario.

As for anti-CTLA-4, tremelimumab (31) has been evaluated in a phase II, in a non-controlled, open-label, multicenter clinical trial, in patients with HCC not amenable to locoregional treatment and chronic hepatitis C. Tremelimumab showed a good safety profile along with encouraging outcomes in terms of RR (17.6%), disease control rate (DCR) (76,4%) and time to progression (TTP) (6.48 months).

On this basis, tremelimumab, in combination with durvalumab, has been evaluated. A randomized phase II trial (NCT02519348) has been examined tremelimumab and durvalumab as single-agent as well in combination with two different dosage regimens (tremelimumab 300 + durvalumab vs tremelimumab 75 + durvalumab) in advanced HCC patients. A safety profile along with an antitumor activity were demonstrated in the preliminary results, especially for the tremelimumab 300 + durvalumab regimen. Grade 3/4 adverse events were reported in 28.9% of patients (tremelimumab 300 + durvalumab, 35.1%; tremelimumab 75 + durvalumab, 25.6%; durvalumab, 19.8%; tremelimumab, 42%). The ORR observed were the following: 22.7% for tremelimumab 300 + durvalumab; 9.5% for tremelimumab + durvalumab; 9.6% for durvalumab, and 7.2% for tremelimumab (32).

As a result, the data from the phase III Himalaya trial (33) are expected to assess the efficacy of tremelimumab + durvalumab versus sorafenib in the first-line setting of HCC patients not susceptible to locoregional therapy.

As regards anti-PD-1, nivolumab and pembrolizumab have been investigated in phase II (CheckMate 040 and Keynote 224, respectively) and phase III studies (CheckMate 459 and Keynote 240, respectively).

The CheckMate 040 phase I/II non-comparative study evaluated nivolumab in patients with unresectable HCC with or without previous treatment with sorafenib. The phase II study showed a promising ORR of 20% with a median extent response of 9.9 months along with a manageable safety profile. The 9-month overall survival (OS) rate was 74%. On this basis, the FDA speeded up the acceptance of nivolumab for HCC pretreated with sorafenib (29). Conversely, the CheckMate 459 trial phase III study (34) failed to demonstrate improved OS with nivolumab versus sorafenib in this setting. Although the results obtained are impressive, showing improvements in survival and response rate along with a lack of adverse events, they were not statistically significant. Median overall survival was 16.4 months for nivolumab and 14.7 for sorafenib [Hazard Ratio (HR): 0.85 p 0.0752], the ORR was 15% for nivolumab and 7% for sorafenib. Also, nivolumab has been assessed in combination with ipilimumab in the Cohort 4 of Checkmate 040 (30). The ORR was 31% with a median duration of response (DOR) of 17 months; DCR was 49%, and 24 months OS rate was 40%.

Based on these impressive results, the FDA recommended the combination of nivolumab and ipilimumab for HCC patients previously treated with sorafenib.

As regards of pembrolizumab, it has been evaluated in the phase II Keynote 224, non-randomized, multicentre, open-label, in HCC BCLC B-C patients pre-treated with sorafenib. Pembrolizumab demonstrated a manageable safety profile along with antineoplastic activity with an ORR of 17%. On this basis, pembrolizumab received FDA's accelerated approval, and it has been evaluated versus placebo in pre-treated advanced HCC patients in phase III randomized, placebo-controlled Keynote 240 (35). Pembrolizumab improved OS (13.9 months vs 10.6 months HR: 0.78 p: 0.0238), progression free survival (PFS) (3.0 months vs 2.8 months HR: 0.77 p: 0.022) and ORR (16.9% vs 2.2%) with durable responses (DoR 13.8 months) vs placebo. The study, however, was negative. The outcome measures OS and PFS, although impressive, did not achieve statistical significance. Regarding anti-PDL-1, atezolizumab has been tested as first-line treatment in combination with bevacizumab in the phase Ib GO30140 Study (*NCT02715531*). Patients included in arm A received atezolizumab + bevacizumab IV every three weeks, whereas patients included in arm F were randomized 1:1 and took atezolizumab-bevacizumab (F1) or single-agent atezolizumab (F2). In arm A, the ORR (primary endpoint) was 36%, with 76% of responses still ongoing. In arm F, the primary endpoint was PFS. A statistically significant improvement in median PFS was reached with the combination therapy respect to single-agent atezolizumab (F1: 5.6 versus F2: 3.4 months, HR 0.55, 80% confidence interval (CI), 0.40–0.74, P = 0.0108). As for safety, another one primary endpoint for both arms, any-grade treatment-related adverse events (TRAEs) were 68% in arm F1 and 41% in arm F2 (36).

Another crucial study that represents a turning point in the treatment of HCC was the phase III IMbrave 150 Study. In this randomized, open-label trial, advanced HCC patients were randomized 2:1 to receive atezolizumab + bevacizumab or sorafenib until loss of clinical benefit or unacceptable toxicity. Co-primary endpoints were OS and PFS by independent review facility (IRF)-assessed response evaluation criteria in solid tumors (RECIST) 1.1, whereas key secondary endpoints were IRF-ORR per RECIST 1.1 and IRF-ORR per HCC modified RECIST (mRECIST). The primary data analysis showed the achievement of both co-primary endpoints: in the intent-to-treat (ITT) population, at a median follow-up of 8.6 months, OS HR was 0.58 (95% CI, 0.42, 0.79; P = 0.0006) and PFS HR was 0.59 (95% CI, 0.47, 0.76; P < 0.0001) in the atezolizumab plus bevacizumab arm vs the control arm. ORR was 27% in patients receiving atezolizumab and bevacizumab vs 12% in patients receiving sorafenib (P < 0.0001) per IRF RECIST 1.1 and 33 vs 13% (P < 0.0001) per IRF HCC mRECIST for

experimental arm vs control arm, respectively. Median treatment duration was of 7.4 months for atezolizumab, 6.9 for bevacizumab, and 2.8 for sorafenib. Moreover, the association of atezolizumab and bevacizumab was well tolerated and procrastinated time to deterioration (TTD) of the quality of life (QoL) of the patients [median TTD, 11.2 vs 3.6 mo; HR, 0.63 (95% CI: 0.46, 0.85)], physical functioning [median TTD, 13.1 vs 4.9 mo; HR, 0.53 (95% CI: 0.39, 0.73)], and role functioning [median TTD, 9.1 vs 3.6 mo; HR, 0.62 (95% CI: 0.46, 0.84)] compared with sorafenib. Furthermore, the combination therapy postponed TTD in patient-reported symptoms (loss of appetite, fatigue, pain, diarrhea) and led to meaningful clinical symptoms deterioration in a lower proportion of patients. Based on this data, atezolizumab + bevacizumab was approved as the first-line standard of care in advanced HCC (37, 38).

IMMUNE CHECKPOINT INHIBITORS IN LOCALIZED HCC

Hepatic resection (HR), liver transplantation (LT), and ablation (39) are treatments with curative intent in HCC, according to the EASL clinical practice guidelines (40).

To date, no therapy has proven to be effective in the adjuvant setting (41, 42). Nonetheless, the promising results of immunotherapy in advanced HCC have led to a growing interest in the adjuvant setting too. It is well-known that the liver has an immune suppressive microenvironment to avoid autoimmune phenomena (43, 44). However, in patients with HCC, persistent inflammatory state upregulates the expression of PD-1 (45) and PD-L1 (46), leading to CD8+ T-cells apoptosis and a decrease of their action against tumor cells (47, 48). Moreover, this effect relates to a poor prognosis and a considerable aggressiveness of the tumor and promotes postoperative recurrences in HCC patients (49–52). An increased PD-1 and PD-L1 expression could provide the rationale for the employment of both PD-1 and PD-L1 ICI as adjuvant treatment in HCC.

Adjuvant Immunotherapy with ICI is currently under investigation in HCC patients who underwent loco-regional treatment and are at high risk of recurrence. Unfortunately, no published randomized trials are yet available.

Nivolumab, an anti-PD-1 monoclonal antibody (mAb), is being assessed in a phase III, multicenter, randomized, double-blind CheckMate 9DX trial (NCT03383458). The study has an estimated enrollment of 530 HCC patients who will randomly receive either nivolumab (arm A) or placebo (arm B) (53).

Pembrolizumab, an anti-PD-1 mAb, is now being studied in a phase III, multicenter, randomized, double-blinded, two-arm study Keynote-937 (NCT03867084). Participants (estimated enrollment: 950 patients) will receive intravenous (IV) pembrolizumab if assigned to arm A, and IV placebo if assigned to arm B.

Durvalumab, an anti-PD-L1 mAb, alone or combined with bevacizumab, is under examination in a phase III, randomized, double-blind, placebo-controlled, multicenter study, EMERALD-2 (NCT03847428), in the same HCC high-risk population of the abovementioned studies. Patients randomized to arm A will receive IV durvalumab plus IV bevacizumab; arm B patients will receive durvalumab plus placebo, and arm C subjects will be assigned two placebos. The estimated enrollment is of 888 participants.

Atezolizumab, an anti-PD-L1 mAb, is under evaluation in association with bevacizumab in phase III, multicenter, randomized, open-label IMbrave050 study (NCT04102098). Patients will be randomly allocated to arm A to receive IV atezolizumab plus IV bevacizumab or to arm B to active surveillance. The study estimates to enroll 662 participants.

Toripalimab, an anti-PD-1 mAb, is under study in a phase II/III, randomized, double-blind, placebo- controlled study, the JUPITER 04 trial (NCT03859128). The estimated 530 participants enrolled will be treated with toripalimab if assigned to arm A, whereas they will not receive it if assigned to arm B.

The primary outcome of these trials is the measure of the recurrence-free survival (RFS), except for Keynote-937, which will consider both RFS and OS. However, it is significant to specify that EMERALD-2 will evaluate only the RFS for arm B versus arm C as primary endpoint, while the RFS for arm A versus arm C represented the secondary endpoint.

Loco-regional treatments in HCC are used in patients with early-stage (0-A BSCL staging) who are not eligible for surgical treatment or transplant, or in patients with advanced-stage (B-C BSCL) not amenable to kinase-inhibitor drugs (Sorafenib or Regorafenib). The most used local procedures are transarterial chemoembolization (TACE) (54–59), radiofrequency ablation (RFA) (60), stereotactic body radiotherapy (SBRT) (61, 62), transarterial radioembolization, and embolization *via* microspheres loaded with yttrium-90 (Y-90) (63–65).

These loco-regional treatments allow to release a high quantity of tumor antigens through the destruction of the tumor cells. For this reason, the effectiveness of their combination with the ICI has been investigated with encouraging results (66).

The results of two studies are currently available. In the study conducted by Duffy et al., 32 patients were started on tremelimumab therapy at two dose levels every four weeks for six administrations total, then followed by 3-monthly infusions until they matched up off-treatment. On the 36th day, subtotal radiofrequency ablation or chemoablation were performed. Of the 19 evaluable patients, 5 (26,3%) reached a firm partial response. Six-week tumor biopsies displayed an increase in CD8+ T cells in patients who presented a clinical benefit alone. For this refractory HCC population, six and twelve- month probabilities of tumor progression-free survival were 57,1 and 33,1%, respectively, with a median time to tumor progression of 7,4 months. The mOS was 12,3 months (67).

Furthermore, the phase II trial by Zao et al. (NCT03939975) assessed the response of 50 HCC patients who progressed to a first-line with sorafenib and started a second-line treatment with anti-PD1 (pembrolizumab or nivolumab). Of these, 33 patients

underwent subtotal thermal ablation because the disease did not progress or had an atypical response to anti-PD-1 inhibitor. Additional ablation ameliorated effectiveness with acceptable toxicity, and the RR rose from 10 to 24% (12/50). The median time to progression (MTP), PFS, and OS was 6.1, 5, and 16.9 months, respectively (68).

Currently, there are several trials underway to evaluate which combination is more useful and could allow us to get the best results in terms of ORR.

The combination of ICI with stereotactic radiotherapy (SBRT) is still under study. In particular, the phase II/III trial NCT04167293 (ISBRT01) is evaluating this type of local treatment in association with sintilimab (a monoclonal antibody anti-PD1) in an advanced stage of HCC. Another study is NCT03380130 (NASIR-HCC), a phase II clinical trial that is investigating nivolumab combination in the same patient settings. While phase II study NCT03316872 is studying SBRT combined with pembrolizumab.

The role of TACE in combined therapy is also under study. In phase II trial IMMUTACE (NCT03572582), the procedure is associated with nivolumab administration in patients affected by intermediate-stage hepatocellular carcinoma. Moreover, in the phase II study TRIPLET (NCT04191889), the association of TACE with apatinib plus camrelizumab is under investigation in patients with C staged HCC, in BCLC classification. Even the phase II trial LEAP-012 (NCT04246177) is evaluating TACE combined with the administration of lenvatinib and pembrolizumab. In addition to the classic TACE (c-TACE), a variant is the drug-eluting bead transarterial chemoembolization (DEB-TACE). This type of procedure is also under investigation in combination with ICI, such as durvalumab and tremelimumab (NCT03638141) or nivolumab (NCT03143270).

A recent phase II study, NCT03259867 (TATE-PD1), involves the use of trans-arterial tirapazamine embolization (TATE) in patients with advanced HCC or other malignancies, simultaneously treated with nivolumab or pembrolizumab. The results of this new procedure are particularly interesting.

Considering radioembolization with yttrium 90 (Y90-RE), the results of a phase II, non-randomized trial (NCT03033446), and analyzing the combination with nivolumab in Asian advanced HCC patients, were recently presented. It enrolled 40 patients with a median follow-up of 16.4 months, and 36 patients were assessed. The combination of nivolumab plus Y90-RE resulted in an encouraging ORR of 31% (95%CI 16,4–48,1%), median PFS of 4.6 months (95%CI 2.3–4.8 months), and mOS of 15.1 months (95%CI 7.8–NE) (69). Furthermore, other trials are currently investigating Y90-RE in combination with nivolumab (NCT02837029) or pembrolizumab (NCT03099564).

In addition to the trials involving a single loco-regional procedure, several combination trials compare different methods. Among these, there is the phase III study NCT03949231 that confronts the hepatic artery infusion with the vein infusion of toripalimab (monoclonal anti-PD1 Ab) in patients with (BCLC) C-stage hepatocellular carcinoma. Furthermore, the phase II study NCT02821754 is estimating differences between chemoembolization (TACE), radiofrequency ablation (RFA), and cryoablation (CA) in patients with HCC and biliary tract cancer treated with tremelimumab and durvalumab. Another comparison study is the phase II trial NCT03753659, in which patients with early HCC received pembrolizumab and then underwent RFA versus Microwave Ablation (MWA).

IMMUNE CHECKPOINT INHIBITORS + TYROSINE-KINASE INHIBITORS (TKI)

HCC has a less dense vasculature with abnormal leaky and fragile tumor vessels, which lead to interstitial hypertension, tumor hypoxia, and necrosis (70–74). Hypoxia can, in turn, stimulate the angiogenic process, the tumor growth (71, 73, 75, 76), and may recruit immunosuppressive cells (77). Indeed, there is a complex bidirectional relationship between angiogenesis and immunity (78–88).

In particular, vascular endothelial growth factor (VEGF), in association with other pro-angiogenic determinants in the tumor microenvironment (TME), may down-regulate intercellular adhesion molecule 1 (ICAM-1) or vascular cell adhesion protein 1 (VCAM-1), repress T cell trafficking and dendritic cell (DC) maturation (77, 89). Moreover, the VEGF-A and pro-inflammatory cytokines cause Fas ligand (FasL) expression by tumor endothelial cells that gain the capacity to put CD8+ T cells but not T-reg cells to death (90). VEGF also increases PD-1 expression of tumor-infiltrating CD8+ T-cells (79). Also, PD-L1 expression is strongly dependent on transcriptional regulation of hypoxia-inducible factor 1-alpha (79, 91). Therefore, the blockade of the angiogenesis pathway might modify the immune TME, up-regulating CD8+ T-cells, and down-regulating immunosuppressor cells. That way, ICIs may improve the effectiveness of anti-angiogenic drugs inducing antibody-related cytotoxicity on endothelial cells. As a result, the destruction of the malignancy's vasculature was obtained (92).

A Phase 1b study evaluated the safety and effectiveness of the association of durvalumab with ramucirumab, an anti-VEGF receptor-2 (VEGFR-2) IgG1 mAb, in different cohorts of advanced pre-treated cancer patients, including one cohort of 28 HCC subjects (NCT02572687). In the HCC cohort, ORR was 11%, but in patients that had "high" PD-L1 expression (≥25% of tumor cells or immune cells) achieved 18%. No significant differences in median PFS were observed accordingly to PD-L1 expression (4.4 in overall patients and 5.6 months in patients with high PD-L1 expression) as well as in mOS (10.7 and 16.5 months, respectively). Hypertension (17.9%), anemia (21.4%), and fatigue (10.7%) were the most frequent 3/4 TRAEs reported. Grade 3/4 TRAEs of interest reported in >5% of patients were hypertension, bleeding events (10.7%), and venous thromboembolic events (7.1%) for ramucirumab and lipase (10.6%) and AST increase (17.9%). Globally, the combination of durvalumab and ramucirumab did not show new safety signals and suggested potential anti-tumor activity, especially in the case of high PD-L1 expression. Further results are expected (93).

A multicenter, open-label, phase I/II dose-escalation and expansion study is assessing the harmlessness and benefit of MGD013, an anti-PD-1/anti-LAG-3 Dual-Affinity Re-Targeting (DART) protein in monotherapy and in combination with brivanib, a selective dual inhibitor of VEGFR and fibroblast growth factor receptors (FGFR) in advanced liver cancer patients (phase I- dose escalation also included intrahepatic cholangiocarcinoma) (NCT04212221).

Most TKIs have a remarkable anti-angiogenic effect through the inhibition of the VEGFRs (70) and have an immune-modulatory role as immune effectors involved in the TME and antigen presentation process (82). The association with ICI opens to the exploration of new treatment combinations to improve the anti-tumor immune response (94, 95).

Sorafenib is a multi-target TKI, approved since 2007 for first-line treatment of HCC, which can block the RAS, VEGFR, platelet-derived growth factor receptor (PDGFR), fms related tyrosine kinase 3 (FLT3), and KIT kinases, inducing apoptosis and blocking cell proliferation, migration, and cancer angiogenesis (96). Among the explored mechanisms of resistance to sorafenib in HCC, Liu et al. reported PD-L1 and DNA methyltransferases contribution (97). Currently, TKI and anti- PD-1 mAbs combination therapies were under study as first-line treatment for advanced HCC. In particular, the association with nivolumab is being assessed in a phase II, multicenter pilot trial in advanced HCC patients not eligible for surgery (NCT03439891). This trial will estimate the maximum tolerated dose, the safety, and ORR of the combination of sorafenib and nivolumab, along with the DOR, PFS, OS, peripheral and tumor immune cell profiling, PD-L1 expression, and alpha-fetoprotein (AFP) response (98).

A phase Ib/II study is evaluating sorafenib and pembrolizumab combination therapy in advanced HCC (NCT03211416). The primary endpoint is RR; secondary endpoints are safety, OS, and PFS. Moreover, the study will compare in blood and cancer samples the pre-treatment quantity of immunosuppressive cells and the functional activity of effector T cells post-treatment (99). Another phase Ib of dose-escalation and dose-expansion study is assessing the safety and tolerability of the combination of sorafenib with spartalizumab, an anti-PD-1 mAb, in advanced HCC (NCT02988440).

Lenvatinib is a small multi-TKI which works against VEGFR-1,-2, and -3, FGFR-1,-2,-3, and -4, PDGFRα, KIT, and (RET), approved on August 2018 by FDA for first-line treatment of unresectable HCC (100). Some ongoing clinical trials are studying its association with ICI.

The association between sorafenib and nivolumab is under evaluation in advanced HCC patients in two trials. In particular, a Japanese phase Ib trial aims to assess the tolerability and safety of this combination. Its secondary endpoints include OS, PFS, ORR, DOR, DCR, TTP, clinical Benefit Rate (CBR), and pharmacokinetics (PK) (NCT03418922). On the other hand, an exploratory, open-label, single-arm, multicenter phase II study evaluates the effectiveness and feasibility (as determined by safety and tolerability) of first-line sorafenib combined with nivolumab in patients with multinodular, advanced stage HCC. Primary endpoints are ORR, safety, and tolerability; secondary endpoints are TTP, PFS, OS, and translational research that consists of correlation of biomarkers potentially associated with clinical efficacy (NCT03841201-IMMUNIB).

Regarding the association of lenvatinib with pembrolizumab, preliminary data from a phase Ib study analyzing this combination in first-line setting for advanced HCC (NCT03006926) reported an ORR of 42.3%, and a median PFS of 9.69 months (95% CI 5.55–not evaluable). The most frequent any-grade TRAEs were decreased appetite and hypertension (53.3% each), diarrhea (43.3%), and fatigue (40%). The most common grade ≥3 TRAEs described were hypertension (16.7%), aspartate aminotransferase (AST) increment (16.7%), neutropenia (13.3%), and hyponatremia (10.0%). Eight patients had severe adverse events (SAEs) (26.7%), and 16.7% discontinued lenvatinib and or pembrolizumab due to TRAEs, but side effects were controlled (101).

Based on these results, the phase III multicenter, randomized, double-blinded, active-controlled, LEAP-002 trial (NCT03713593) is testing the effectiveness and safety of lenvatinib and pembrolizumab combination therapy versus lenvatinib combined with placebo as first-line treatment in advanced HCC Child-Pugh class A patients. This trial estimates to randomize 750 patients approximately. The primary endpoints are OS and PFS, whereas secondary endpoints include ORR, DOR, DCR, TTP, adverse events, and PK (102). Also, a single-arm phase IIb study is assessing lenvatinib and pembrolizumab combination therapy as second-line treatment in patients with unresectable hepatobiliary tumors, including the analysis of potential biomarkers of response (NCT03895970).

Regorafenib is a multi-target TKI that actively suppresses VEGFR-1,-2,-3, PDGFR, TIE-2, fibroblast growth factor receptor 1 (FGFR1), KIT (CD117), RET, and B-Raf (103). It is under evaluation in combination with ICI in two ongoing studies.

A multicenter, non-randomized, open-label, dose-escalation, phase Ib study is assessing the harmlessness and tolerability of the association of regorafenib and pembrolizumab as first-line treatment for patients with advanced HCC (NCT03347292). Moreover, the study aims to explore the anti-tumor activity of this combination and to determine blood/tissue biomarkers related to the tumor activity, status or response.

The REGOMUNE trial (NCT03475953) is a multicenter phase I/II trial which is estimating the combination of regorafenib and avelumab in solid tumors, including HCC, after at least one previous line of systemic therapy. Phase I will establish the recommended phase II dose (RP2D), whereas phase II will assess the efficacy and safety of the drugs combination.

Cabozantinib is a TKI targeting VEGFR-2, c-MET, AXL, RET and FLT-3 (100, 104). One cohort of the Checkmate040 phase I/II trial (NCT01658878) is assessing the potential synergistic activity of cabozantinib combined with nivolumab, with or without ipilimumab, in Child-Pugh A advanced HCC patients; primary endpoints are safety and ORR (29, 105, 106).

A phase Ib, open-label trial will explore the safety, tolerability, preliminary efficacy, and PK of cabozantinib combined with

atezolizumab in advanced HCC patients (NCT03170960). In the dose-escalation phase (3 + 3 design), a recommended dose for cabozantinib and atezolizumab combination therapy will be determined. In the expansion phase, 18 cohorts will be recruited at the recommended dose of cabozantinib and atezolizumab, comprising one cohort of advanced systemic-treatment naïve HCC. The primary objective is the ORR for each cohort (107).

The phase III COSMIC-312 trial (NCT03755791) is appraising cabozantinib plus atezolizumab versus sorafenib in the first-line setting in advanced HCC patients, Child-Pugh A. Patients will be randomized in a 2:1:1 ratio to take cabozantinib plus atezolizumab, sorafenib, or single-agent cabozantinib. The study has two primary endpoints: compare OS and PFS for cabozantinib + atezolizumab versus sorafenib; the secondary endpoint is PFS for cabozantinib versus sorafenib (108).

The open-label, single-arm, CAMILLA trial is a phase Ib study of cabozantinib and durvalumab combination therapy in pretreated patients with advanced HCC (NCT03539822). The study intends to examine the safety and tolerability and display preliminary data on effectiveness (109).

Axitinib is a TKI selective for VEGFR-1/2/3. VEGF Liver 100 (NCT03289533) is a Phase Ib study assessing the feasibility of the combination of avelumab plus axitinib in treatment-naive patients with HCC in terms of harmlessness and effectiveness. Provisory results of the analysis showed an ORR of 13.6% based on RECIST 1.1 and 31.8% based on mRECIST criteria. mPFS was 5.5 and 3.8 months, according to RECIST and mRECIST, respectively. Tumor shrinkage was reported in 68.2% of patients by RECIST and 72.7% of patients by mRECIST. OS data were still immature. The most common grade 3 TRAEs were hypertension (50.0%) and hand-foot syndrome (22.7%); no grade 4/5 TRAEs were mentioned. Immune-related AEs (irAEs) occurring in ≥10% of patients were hypothyroidism (31.8%) and hyperthyroidism (13.6%). None of irAEs were grade ≥3. No treatment discontinuations due to TRAEs or irAEs were registered. Thus, safety and efficacy results were promising, but further follow-up is required (110).

Apatinib is an impressive TKI inhibitor of VEGFR-2, c-Kit, c-Src, and PDGFR. An open-label, dose-escalation (phase Ia) and expansion study (phase Ib) evaluated the safety and efficacy of the camrelizumab, an anti-PD-1 mAb, and apatinib combination therapy in advanced HCC patients (NCT02942329). The main goals were harmlessness and tolerability and RP2D determination. A grade 3 TRAE was reported in 60.6%. Hypertension (15.2%) and elevated AST (15.2%) were the most common. Results showed that camrelizumab and apatinib combination had a feasible safety profile and activity against cancer cells in HCC patients (111). The phase II, single-arm, RESCUE study (NCT03463876) is preliminary exploring the efficacy and safety of the combination of apatinib and camrelizumab regimen as second-line treatment in advanced HCC; the primary endpoint is ORR.

Currently, is ongoing a randomized, open-label, international, multicenter, phase III trial of camrelizumab plus apatinib versus sorafenib in first-line setting in patients with unresectable HCC that did not receive systemic treatment in the past (NCT03764293). The co-primary endpoints are OS and PFS.

IMMUNE CHECKPOINT INHIBITORS + C-MET INHIBITORS

The MET/HGF pathway stimulate cellular proliferation, survival, and invasion and progression in HCC and has been associated with TKI resistance (112–114). A phase Ib/II, open-label, multicenter study is assessing the association of capmatinib (INC280), a selective oral c-MET recently developed in HCC, and spartalizumab versus spartalizumab single-agent in advanced HCC patients, progressing after sorafenib (NCT02795429).

Another phase I/II dose-escalation, and expansion study is testing bozitinib, a c-MET inhibitor, combined with genolimzumab, an anti-PD-1 mAb, after first-line treatment for locally advanced or unresectable HCC not pretreated with a PD-1 inhibitor or a c-MET inhibitor (NCT03655613).

IMMUNE CHECKPOINT INHIBITORS + FGFR INHIBITORS

Another promising approach is represented by the association of ICI with inhibitors of the fibroblast growth factor 19 (FGF19)/FGF receptor 4 (FGFR4) pathway (115). The alteration of the FGF19/FGFR4 signaling is a known driver of HCC carcinogenesis (116). It suppresses E-cadherin expression and promotes the expression of epithelial-to-mesenchymal transition (EMT)-related genes, leading to increased HCC cell invasion. FGF19/FGFR4 axis has been associated with poor prognosis. Moreover, FGF19 expression has been related with early relapse and shorter disease-specific recurrence in a cohort of resected HCC patients and appears implicated in sorafenib resistance (117, 118).

A Phase I/II, multicenter, open-label study is assessing the combination of oral FGF401, an FGFR4 inhibitor, with spartalizumab in refractory HCC patients harboring FGFR4 and KLB (an FGF19 co- receptor) expression and FGF401 as single-agent in other advanced solid tumors. The study is investigating the efficacy as the dose-limiting toxicity to detect the maximum tolerated dose and/or RP2D (NCT02325739).

IMMUNE CHECKPOINT INHIBITORS + TGFβ PATHWAY INHIBITORS

TGF-β contributes to cell invasion, angiogenesis, EMT, and drug resistance in HCC, as demonstrated by several preclinical findings (119–121). Moreover, TGF-β may induce *in vitro* FGFR4 expression through the extracellular-signal-regulated kinase (ERK) pathway, and its interaction with FGFR4 promotes the metastatic spread of HCC *in vivo* (122). TGF-β

TABLE 1 | Adjuvant ICI: ongoing and still recruiting clinical trials.

Drug	Trial name	Phase	Design	Endpoint	N	Start date	ClinicalTrials.gov	Status
Toripalimab	JUPITER 04	II/III	Toripalimab vs placebo	RFS	402	01/03/2019	NCT03859128	Recruiting
Nivolumab	CheckMate 9DX	III	Nivolumab vs placebo	RFS	530	18/12/2017	NCT03383458	Recruiting
Durvalumab	EMERALD-2	III	Durvalumab + bevacizumab (arm A); Durvalumab + placebo (arm B); placebo + placebo (arm C);	RFS (arm B vs arm C)	888	29/04/2019	NCT03847428	Recruiting
Pembrolizumab	KEYNOTE-937	III	Pembrolizumab vs placebo	RFS and OS	950	28/05/2019	NCT03867084	Recruiting
Atezolizumab	IMbrave050	III	Atezolizumab + bevacizumab (arm A); active surveillance (arm B);	RFS	662	31/12/2019	NCT04102098	Recruiting

TABLE 2 | Ongoing trials on loco-regional treatments of unresectable HCC.

Phase	Drugs	Procedure	Setting	NCT
III	Toripalimab	Hepatic artery versus vein infusion of Toripalimab.	(BCLC)-C-stage Hepatocellular Carcinoma (HCC)	NCT03949231
II/III	Sintilimab	Stereotactic body radiotherapy (SBRT)	Advanced hepatocellular carcinoma (HCC)	NCT04167293 (ISBRT01)
II/III	Pembrolizumab and/or ipilimumab	Trans-artery/intra-tumor infusion	Solid tumors (including hepatocellular carcinoma)	NCT03755739
II	Nivolumab	Transarterial Chemoembolization (TACE)	Intermediate Stage Hepatocellular Carcinoma	NCT03572582 (IMMUTACE)
II	tremelimumab and durvalumab	Chemoembolization (TACE), radiofrequency ablation (RFA) and cryoablation (CA)	Advanced hepatocellular carcinoma (HCC) and biliary tract carcinomas (BTC)	NCT02821754
II	Nivolumab	Y90-Radioembolization	Asians with hepatocellular carcinoma	NCT03033446
II	Nivolumab	Selective internal radiation therapy (SIRT)	Advanced hepatocellular carcinoma (HCC)	NCT03380130 (NASIR-HCC)
II	Apatinib and Camrelizumab	Chemoembolization (TACE)	C staged Hepatocellular Carcinoma in BCLC classification	NCT04191889 (TRIPLET)
II	Pembrolizumab	Radio frequency ablation (RFA), microwave ablation (MWA)	Early stage hepatocellular carcinoma (HCC)	NCT03753659
II	nivolumab or pembrolizumab	Trans-arterial Tirapazamine Embolization (TATE)	Hepatocellular carcinoma (HCC), metastatic colorectal cancer (mCRC), metastatic gastric cancer and advanced non-small cell lung cancer	NCT03259867 (TATE-PD1)
II	Durvalumab and Tremelimumab	Drug-eluting bead transarterial chemoembolization (DEB-TACE)	Newly diagnosed with hepatocellular carcinoma	NCT03638141
II	JS001 (Terepril) and Apatinib	Stereotactic body radiotherapy (SBRT)	BCLC stage C hepatocellular carcinoma (HCC) with PVTT	NCT04165174
II	PD-1 mAb and lenvatinib	Chemoembolization (TACE)	Middle and late stage (BCLC-B and BCLC-C) HCC patients	NCT04273100
II	Carrizumab and Apatinib	Radiofrequency ablation (RFA)	Advanced hepatocellular carcinoma (HCC)	NCT04150744
II	Lenvatinib and Pembrolizumab	Transarterial Chemoembolization (TACE)	Advanced hepatocellular carcinoma (HCC)	NCT04246177 (LEAP-012)
II	PD-1 mAb	TACE, SBRT	Neoadjuvant HCC	NCT03817736
II	Anti-PD-1 Antibody (IBI308)	Stereotactic body radiation therapy (SBRT)	Advanced hepatocellular carcinoma (HCC)	NCT03857815
II	Pembrolizumab	Stereotactic body radiotherapy (SBRT)	Advanced hepatocellular carcinoma (HCC)	NCT03316872
II	Sintilimab	Transarterial chemoembolization (TACE)	Advanced hepatocellular carcinoma (HCC) as first-line therapy	NCT04297280
II	Sintilimab and FOLFOX	Hepatic arterial infusion chemotherapy (TAI)	Locally advanced, potentially resectable HCC	NCT03869034
I/II	Toriplimab	Radiofrequency ablation (RFA)/ microwave ablation (MWA)	Advanced hepatocellular carcinoma (HCC)	NCT03864211
I	Nivolumab	Drug eluting bead transarterial chemoembolization (deb-TACE)	Advanced hepatocellular carcinoma (HCC)	NCT03143270
I	Sintilimab	Microwave ablation, TACE	Advanced hepatocellular carcinoma (HCC)	NCT04220944
I	ImiquimodDrug: Standard of Care PD-1 Therapy	Focused ultrasound ablation (FUSA)	Solid tumors (including hepatocellular carcinoma)	NCT04116320 (AM-003)
I	Nivolumab	Yttrium Y 90 glass microspheres	Stage III-IV hepatocellular carcinoma (HCC)	NCT02837029
I	Pembrolizumab	Y90 radioembolization	Hepatocellular carcinoma (HCC)	NCT03099564
I	Sintilimab	Radiotherapy	HCC with main portal vein tumor thrombosis	NCT04104074

TABLE 3 | Clinical Trials in Advanced HCC.

.Drug	Trial name	Phase	Design	Endpoint	N	Start date	ClinicalTrials.gov	Status
Tremelimumab		II	Tremelimumab	ORR	20	December 2008	NCT01008358	Completed
Durvalumab, tremelimumab		II	Tremelimumab; Durvalumab; Tremelimumab 300 + Durvalumab; Tremelimumab 75 + Durvalumab	Safety, tolerability, and activity	433	19/10/ 2015	NCT02519348	Active, not recruiting
Durvalumab, tremelimumab	Himalaya	III	Durvalumab vs tremelimumab + durvalumab vs sorafenib	OS	1324	11/10/ 2017	NCT03298451	Active, not recruiting
Nivolumab, ipilimumab, cabozantinib	CheckMate 040	I/II	Nivolumab; nivolumab + ipilumumab; nivolumab + cabozantinib; nivolumab + ipilimumab + cabozantinib; sorafenib	ORR	1097	26/09/ 2012	NCT01658878	Active, not recruiting
Nivolumab	CheckMate 459	III	Nivolumab vs sorafenib	OS	743	25/11/ 2015	NCT02576509	Active, not recruiting
Pembrolizumab	KEYNOTE-224	II	Pembrolizumab	ORR	104	31/05/ 2016	NCT02702414	Active, not recruiting
Pembrolizumab	KEYNOTE-240	III	Pembrolizumab vs placebo	PFS and OS	413	26/05/ 2016	NCT02702401	Active, not recruiting
Atezolizumab, bevacizumab	GO30140	Ib	Atezolizumab + bevacizumab; atezolizumab	ORR and PFS	223	06/04/ 2016	NCT02715531	Active, not recruiting
Atezolizumab	IMbrave150	III	Atezolizumab + bevacizumab vs sorafenib	OS and PFS	501	15/03/ 2018	NCT03434379	Active, not recruiting

also plays a critical role in HCC immune-tolerance. Indeed, it is secreted by Kupffer cells and liver sinusoidal endothelial cells, and it can up-regulate the Treg, and recently, Mariathasan et al. reported that TGF-β weakened tumor response to PD-L1 inhibition by contributing to exclude T cells (123–127). For these reasons, a combined approach of the TGF-β pathway and PD-1/PD-L1 inhibitors, or a managing bifunctional fusion proteins targeting both TGF-β and PD-L1, might overcome drug resistance and have a synergistic effect (128–130).

Galunisertib (LY2157299 Monohydrate) is an oral TGF-β receptor-1 (TGF-βR1) inhibitor that showed a favorable safety profile as single-agent or in combination with sorafenib (131). Currently, galunisertib is under investigation in combination with nivolumab in a phase Ib/II (dose escalation and cohort expansion) study in advanced solid tumors, including HCC with AFP ≥200 ng/ml, as second-line treatment. The main goal of this study is to estimate the harmlessness, tolerability, and effectiveness of this drug association (NCT02423343).

A phase I/Ib, open-label, multi-center, dose-escalation ongoing trial is assessing the safety and tolerability of NIS793, a novel anti-TGF-β antibody (Ab) alone or in combination with spartalizumab in advanced refractory solid tumors, including HCC (NCT02947165). The study also aims to identify recommended doses and schedules of these drugs (NIS793: every 2 or every 3 weeks; spartalizumab: every 3 or 4 weeks) for future studies.

Another promising approach for the future might be M7824 (MSB0011359C), an innovative first- in-class bifunctional fusion protein that consists of a human IgG1 anti-PD-L1 mAb (avelumab) fused to the extracellular domain of TGFβ receptor II (TGF-βRII) to act as a TGFβ "trap". Results of a phase I dose-escalation study with M7824 showed an amenable safety profile in heavily pre- treated patients with advanced solid tumors. Multiple expansion cohorts are ongoing in various tumor types (NCT02517398) (132).

IMMUNE CHECKPOINT INHIBITORS + CHEMOTHERAPY

The EACH trial, a randomized, multicenter, open-label study of palliative FOLFOX versus doxorubicin in Asian patients with advanced HCC, has led the China FDA to introduce FOLFOX4 in the clinical practice guideline (PR 8.6%, 38.6% SD, median OS 5.7 months) (133).

It has been reported that oxaliplatin can induce an anti-tumor immune response and immunogenic cell death, more specifically by activation of DCs, the enhancement of cross-priming of CD8-positive (CD8+) T cells, the stimulation of the anti-tumor CD4+ T cells phenotype, and down- regulation of MDSC and T-reg cells. Moreover, oxaliplatin promotes tumor cell death through lytic receptors/pathways, boosted serum inflammatory cytokines, and switch to pro-inflammatory status in the TME (133, 134). A Phase II, non-randomized study is assessing the combination of camrelizumab with apatinib or with chemotherapy in patients with advanced HCC (FOLFOX4) who failed or were unbearable to prior systemic therapy (NCT03092895).

FUTURE PERSPECTIVES

It is well-known that in some patients, due to the lack of tumor-infiltrating effector T cells, checkpoint inhibitors were ineffective. However, cancer vaccines seem to be able to increase effector T-cells infiltration into tumors. Therefore, a strategy combining a cancer vaccine with an immune checkpoint inhibitor may be promising. The synergistic action of the two drugs may lead to an effective antitumor immune response: whilst the vaccine raises the number of tumor-infiltrating effector T cells, the anti-PD-1 makes sure that these cells stay active (135). Hence, clinical trials are warranted.

TABLE 4 | ICI + Target Therapies Clinical trials for Advanced HCC patients.

Phase	Drugs	Molecular Target	Setting	NCT
Ib	Durvalumab + Ramucirumab	Tyrosine-kinase inhibitor	Advanced pre-treated HCC	NCT02572687
I/II	MGD013; MGD013 + brivanib	Tyrosine-kinase inhibitor	Advanced liver cancer patients	NCT04212221
II	Sorafenib + Nivolumab	Tyrosine-kinase inhibitor	1st line in Advanced HCC	NCT03439891
Ib/II	Sorafenib + Pembrolizumab	Tyrosine-kinase inhibitor	Advanced HCC	NCT03211416
Ib	Sorafenib + Spartalizumab	Tyrosine-kinase inhibitor	Advanced HCC	NCT02988440
Ib	Sorafenib + Nivolumab	Tyrosine-kinase inhibitor	Advanced HCC	NCT03418922
II	Sorafenib + Nivolumab	Tyrosine-kinase inhibitor	Advanced HCC	NCT03841201-IMMUNIB
Ib	Lenvatinib + Pembrolizumab	Tyrosine-kinase inhibitor	Advanced HCC	NCT03006926
III	Lenvatinib + Pembrolizumab vs Lenvatinib + placebo	Tyrosine-kinase inhibitor	1st line in Advanced HCC	NCT03713593 – LEAP-002
IIb	Lenvatinib + Pembrolizumab	Tyrosine-kinase inhibitor	2nd line unresectable Hepatobiliary cancers	NCT03895970
Ib	Regorafenib + Pembrolizumab	Tyrosine-kinase inhibitor	1st line in Advanced HCC	NCT03347292
I/II	Regorafenib + Avelumab	Tyrosine-kinase inhibitor	2nd line Advanced HCC	NCT03475953 -REGOMUNE
I/II	Cabozantinib + Nivolumab	Tyrosine-kinase inhibitor	Advanced HCC	NCT01658878 – CheckMate 040
Ib	Cabozantinib + Atezolizumab	Tyrosine-kinase inhibitor	Advanced HCC	NCT03170960
III	Cabozantinib + Atezolizumab	Tyrosine-kinase inhibitor	1st line in Advanced HCC	NCT03755791 - COSMIC-312
Ib	Cabozantinib + Durvalumab	Tyrosine-kinase inhibitor	Pretreated Advanced Cancer	NCT03539822 - CAMILLA
Ib	Axitinib + Avelumab	Tyrosine-kinase inhibitor	Treatment-naive HCC patients	NCT03289533 – VEGF Liver 100
Ia/Ib	Apatinib + Camrelizumab	Tyrosine-kinase inhibitor	Advanced HCC	NCT02942329
II	Apatinib + Camrelizumab	Tyrosine-kinase inhibitor	2nd line Advanced HCC	NCT03463876 - RESCUE
III	Apatinib + Camrelizumab vs Sorafenib	Tyrosine-kinase inhibitor	1st line in unresectable Advanced HCC	NCT03764293
Ib/II	Capmatinib + Spartalizumab vs Spartalizumab	c-MET inhibitor	2nd line Advanced HCC after progression to Sorafenib	NCT02795429
I/II	Bozitinib + Genolimzumab	C-MET inhibitor	2nd line for locally advanced or unresectable HCC	NCT03655613
I/II	FGF401 + Spartalizumab	FGFR inhibitor	in refractory HCC patients harboring FGFR4 and KLB	NCT02325739
Ib/II	Galunisertib + Nivolumab	TGF-βR1 inhibitor	Advanced HCC	NCT02423343
I/Ib	NIS793 vs NIS793 + Spartalizumab	Anti-TGF-β Antibody	Advanced refractory HCC	NCT02947165
I	M7824	A TGFβ "trap"	Heavily pre-treated patients with Advanced Cancer	NCT02517398
II	Apatinib + Camrelizumab vs Chemotherapy + Camrelizumab	Tyrosine-kinase inhibitor	Advanced Cancer	NCT03092895

DISCUSSION

In the last few years, several studies evaluated new drug combinations (134, 136). These new therapeutic approaches could soon make a difference.

As for the adjuvant setting, there are no available data up to now, but there are several phase III trials ongoing on various immunocheckpoint inhibitors. We will look forward to the results of these studies, which would seem to prospect the best disease control rate. If data will be statistically significant, we will make a relevant step forward. Anyhow, for now, in the localized HCC, surgery represents the standard of care (**Table 1**).

Regarding the combination of locoregional treatments and immunocheckpoint inhibitors, several phase II trials are underway. There is only a phase III trial on Toriliplimab, but no data is available yet. The unique existing data are related to a small cohort. Thus, the results are not reliable (**Table 2**).

Nonetheless, the available evidence suggests that combining systemic therapies and locoregional treatments with immune checkpoint inhibitors may represent a useful strategy in this context.

In the advanced HCC, thanks to the improvement of OS, PFS, and QoL achieved by the phase III IMbrave150 trial, the FDA approved atezolizumab + bevacizumab as first-line therapy in this setting (26).

Another drug that seems to be promising is tremelimumab, but we are looking forward to the phase III Himalaya trial results. This trial is assessing the combination of tremelimumab + durvalumab.

As for anti-PD-1, nivolumab and pembrolizumab, there are controversial results. Based on the results of phase II trials (CheckMate 040 and Keynote 224), the FDA approved nivolumab and pembrolizumab for advanced HCC. However, the phase III trials (CheckMate 459 and Keynote 240) did not match up to their primary endpoints of OS and PFS. Nonetheless, there are some aspects to take into consideration. CheckMate 040 was a non-comparative study on advanced HCC patients not all pre-treated with Sorafenib. On the other hand, CheckMate 459 compared Nivolumab with Sorafenib in the first-line setting. Although the design of the studies was different, phase III data were interesting thanks to the best tolerability of the drug in the patients, along with a positive trend in terms of response rate and overall survival. Likewise, the Keynote 224 examined the use of Pembrolizumab in 104 advanced HCC patients pre-treated with Sorafenib, whereas the Keynote 240 analyzed pembrolizumab vs placebo in 413 patients as second line treatment. Maybe a first-line setting could have different outcomes or maybe an enlarged sample of patients might have led to different results. Even so, the patients did not suffer the side effects as well as an improvement in survival and response rate. Therefore, taking in consideration the QoL of the patients the approval of these drugs was considerate.

Also, due to the promising results of the combination of nivolumab + ipilimumab, analyzed in cohort 4 in phase II CheckMate 040 trial, the FDA approved them for usage in clinical practice.

No phase III trials are ongoing, so they are warranted (**Tables 3** and **4**).

Many studies are analyzing the combination of ICI + TKI in the first-line in the metastatic setting. A few of them are phase III trials such as the LEAP-002 trial that is evaluating lenvatinib + pembrolizumab versus placebo, whereas the COSMIC-312 trial is assessing cabozantinib + atezolizumab versus sorafenib as the NCT03764293 trial camrelizumab + apatinib versus sorafenib. Their results were awaited. Other combinations of ICI with target therapies as C-Met, FGFR, and TGF-β, are understudy for the second-line in advanced HCC. However, they are still phase I or II trials. For sure, these emerging combinations represent the most promising therapies so far, on which we could rely more in the future.

Also, a combination of chemotherapy, oxaliplatin, and ICI is evaluating in phase II trials based on the role that oxaliplatin plays in promoting the action of immunotherapy.

However, it appears clear that we should opt for combining therapies over a single-agent treatment to overcome the drug-resistance. Nevertheless, in order to tailor a therapy that fits the single patient perfectly, we need to determine some specified biomarkers.

In conclusion, given the encouraging results emerging from the preliminary data of some phase I-II trials, and waiting for the results of the ongoing studies, it is possible to hope that some agents can be successfully combined in the second-line as well as in the first-line. Indeed, these new promising therapeutic options may soon change the clinical practice. Nonetheless, other clinical trials are needed to define a better treatment sequence.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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The Roles of circRNAs in Liver Cancer Immunity

Ying Tang[1,2,3†], Mei Jiang[4†], Hai-Mei Jiang[1,2,3†], Zeng Jie Ye[3], Yu-Sheng Huang[2], Xiu-Shen Li[1,2,3], Bin-Yu Qin[3], Rui-Sheng Zhou[1,3], Hua-Feng Pan[3] and Da-Yong Zheng[3,5,6*]*

[1] Department of Oncology, Institute of Tumor, Guangzhou University of Chinese Medicine, Guangzhou, China, [2] Department of Oncology, Lingnan Medical Research Center of Guangzhou University of Chinese Medicine, Guangzhou, China, [3] Department of Oncology, Guangzhou University of Chinese Medicine, Guangzhou, China, [4] Department of Oncology, The First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou University of Chinese Medicine, Guangzhou, China, [5] Department of Hepatopancreatobiliary, Cancer Center, Southern Medical University, Guangzhou, China, [6] Department of Hepatology, TCM-Integrated Hospital of Southern Medical University, Guangzhou, China

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***Correspondence:**
Hua-Feng Pan
gzphf@gzucm.edu.cn
Da-Yong Zheng
zhengdy@smu.edu.cn

[†]*These authors have contributed equally to this work*

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Circular RNAs (circRNAs) are stable covalently closed non-coding RNAs (ncRNAs). Many studies indicate that circRNAs are involved in the pathological and physiological processes of liver cancer. However, the functions of circRNAs in liver cancer immunity are less known. In this review, we summarized the functions of circRNAs in liver cancer, including proliferative, metastasis and apoptosis, liver cancer stemness, cell cycle, immune evasion, glycolysis, angiogenesis, drug resistance/sensitizer, and senescence. Immune escape is considered to be one of the hallmarks of cancer development, and circRNA participates in the immune escape of liver cancer cells by regulating natural killer (NK) cell function. CircRNAs may provide new ideas for immunotherapy in liver cancer.

Keywords: liver cancer, circRNA, immune evasion, natural killer (NK) cells, innate immunity

INTRODUCTION

Liver cancer, a disease with high mortality and poor prognosis, is one of the most common malignant tumors in the world (1). Statistics show that liver cancer ranks the fifth in cancer incidence, the second in all cancer deaths, and the third in cancer mortality (2). Liver cancer includes three major pathological types: hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC), and HCC-ICC mixed type (3). The occurrence of liver cancer is closely related to hepatitis B, hepatitis C, and non-alcoholic fatty liver disease (4–6). According to the patient's overall conditions, a range of therapies have been utilized in the liver cancer treatment, such as surgical resection, liver transplantation, immunotherapy, local ablative therapies, and systemic chemotherapy. However, liver cancer is generally detected at the late stage because the patients might not perform the clinical symptoms at the beginning. Its recurrence is approximately 50–80% after treatment within 5 years (7). A better understanding of the molecular mechanisms of liver cancer is essential to largely improve the overall prognosis and discover novel effective therapies of liver cancer.

Immune escape refers to the growth and metastasis of tumor cells through various mechanisms to avoid recognition and attack by the immune system (8). The mechanisms of immune escape are mainly related to modifications, changes in tumor cells and alterations in the tumor immune microenvironment. Through the mechanisms of modification and change, tumor cells themselves

can enhance their ability to evade immune surveillance and attack. Tumor has a highly heterogeneous structure, and tumor cells interact with many cells and factors including immune cells and immune factors to form a complex tumor immune microenvironment. The tumor microenvironment is the place where the immune system interacts with tumor cells. Natural killer (NK) cells are involved in tumor immune escape through multiple mechanisms (9–11). Various soluble factors and cytokines released by tumor cells or the tumor microenvironment reduce the activity of NK cells and their cytotoxic activity (12–14). Therefore, restoration of NK cell function is an important area of research in antitumor immunotherapy. Various strategies have been developed to restore NK cell function, including cytokine therapies, monoclonal antibodies, and adoptive cell transfer (15–18). NK cells can be divided into CD56bright and CD56dim based on the expression of CD56. Two subpopulations of CD56 are present, of which the CD56bright subpopulation can be amplified by IL-2 stimulation. The CD56bright subpopulation can be amplified by IL-2 stimulation, and about 10% of them express killer cells. Immunoglobulin-like receptor secretes synthetic TNF-associated apoptosis-inducing ligand (TRAIL). CD56dim subpopulation is insensitive to IL-2 stimulation, and 85% of CD56dim are KIR+ (19–22). In HCC, Rae1 is expressed on the surface of HCC cells, and this factor, as a ligand of NKG2D, the NK cell activation receptor, can activate NK cells and promote their anti-tumor immunity. On the other hand, the immune function of NK cells is limited, and the subsets of CD56dimNK cells in the peripheral blood of HCC patients were significantly lower than those in the healthy control group (23). CD56dimNK cells in the tumor area of HCC patients expressed fewer IFN-γ than non-CD56dimNK cells, which was associated with CD4+CD25+ Tregs *in vitro*. During hepatocarcinogenesis, changes in the microenvironment of the extracellular matrix and the secretion of TGF-β by hepatic stellate cells can inhibit the activity and function of NK cells, thus weakening their monitoring function of hepatocytes (24). TGF-β secreted by Treg can inhibit NK cell activation by down-regulating NKG2D, affecting its immune killing function against liver cancer cells (25). Studies have reported that in liver cancer, circular RNAs (circRNAs) are involved in NK cell-associated immune evasion. Targeting circRNAs to restore NK cell function may provide new directions for the treatment of liver cancer.

Non-coding RNAs (ncRNAs), without the ability to translate into protein, were seen as 'junk DNA' by scientists for years. However, an array of ncRNAs nowadays has been discovered based on advances in sequencing technologies. In addition, accumulating lines of evidence indicate that ncRNAs play major roles in the processes of carcinoma initiation, progression, and metastasis by regulating proliferation, apoptosis, and cell cycle (26, 27). Based on the length and shape of RNA molecules, the ncRNAs are divided into three types including short ncRNAs (<200 nucleotides) and long ncRNAs (lncRNAs, >200 nucleotides) and circRNAs. CircRNAs, a novel category of endogenous ncRNAs, come from non-canonical back-splicing events of precursor mRNAs (pre-mRNAs) (28). CircRNAs were originally discovered in an RNA virus in 1976 and observed in eukaryotic cells in 1979 (29–31). CircRNAs have been recognized as 'splicing noise' or aberrant byproducts for a long time because they present a covalently joined continuous loop structure without 5′caps and 3′tails (32, 33). However, high-throughput sequencing and bioinformatics algorithms have clearly shown that circRNAs are not the accidental byproducts (34–37). Besides, circRNAs have been proved to be abundant and evolutionarily conserved, and are expressed in different types of tumors (38, 39). CircRNAs can not only regulate the expression of host genes by acting as transcriptional regulators, but also serve as microRNA (miRNA) sponge to fine-tune the regulatory axis of miRNA-mRNA (40–45). It has been confirmed that circRNAs can be used as prognostic biomarkers because they have remarkably stable characteristics (46). Furthermore, studies demonstrated that circRNAs can encode hidden peptides, and serve as a new drug targets resource bank (47–50). We herein illustrated the circRNAs molecular mechanisms connected to liver cancer, offered a novel perspective and a new horizon for cancer treatment and diagnosis. CircRNAs provide new ideas for the study of immune escape in liver cancer.

BIOGENESIS OF CIRCRNAS

CircRNAs are stable RNAs that are resistant to RNase R, circRNAs are mainly produced by the pre-mRNA through backsplicing. Although backsplicing is considered as an alternative splicing, it has different molecular mechanisms from linear alternative splicing. The hypothesis of backsplicing is that the downstream splicing site is reversed, and the upstream splicing site is connected to form a closed circRNA molecule. According to the region of origin, circRNAs can be divided into three types: (a) exon–intron circRNAs (EIcircRNAs), (b) exon circRNAs (ecircRNAs), (c) Intronic RNAs (ciRNAs) (**Figure 1**) (51).

The circularization model of circRNA is divided into intron circularization and exon circularization. There are three models for the circularization of EIcircRNAs and ecircRNAs: Intron pairing, Lariat and RNA-binding protein (RBP) (**Figure 1**) (52). Intron pairing-driven circularization, which known as direct backsplicing, is achieved by direct base pairs of intron flanking complementary sequences or reverse repeats (53, 54). The main component of intron pairing-driven circularization is the cis-acting elements, which enable direct base pairing between flank introns, either as short interspersed nuclear elements or as non-repeating complementary sequences (55, 56). Lariat-driven circularization, which is known as exon-skipping, is formed during linear splicing. During the transcription, the pre-mRNA can be partially folded, which formed an RNA lariat containing a 7 nt GU-rich element adjacent to the 5′ splice site and an 11 nt C-rich element closed to the branch point site consensus motif (28, 57). In addition, the third pattern is RBP-driven circularization. Through protein-protein interactions or the dimerization of the RBPs, the splicing sites are pulled closer,

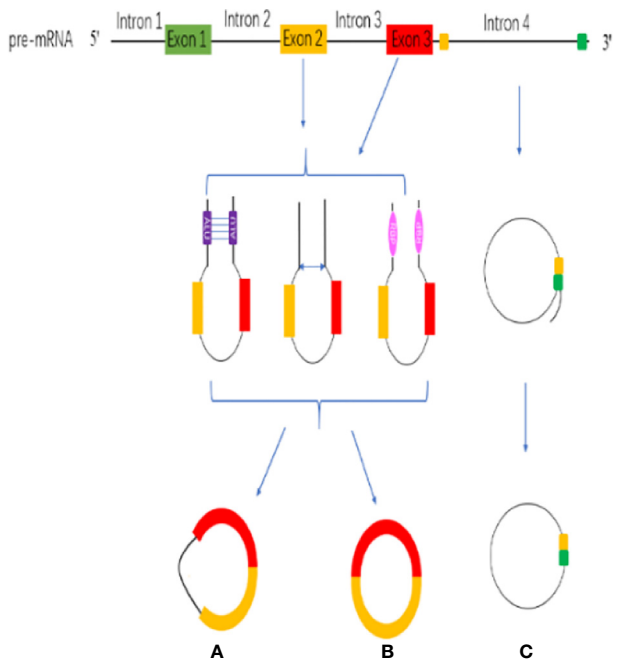

FIGURE 1 | CircRNA biogenesis. **(A)** exon–intron circRNAs (EIcircRNAs). **(B)** exon circRNAs (ecircRNAs). **(C)** Intronic RNAs (ciRNAs).

and the spliceosomes participate in the backsplicing reaction (40, 58). RBP-driven circularization is guided by two flanking intron pairs that are close to the flanking intron reverse complementary sequences (59). Above all, biogenesis of circRNA is a complicated process, and there are many regulatory details need to dig into.

FUNCTIONS OF CIRCRNAS

CircRNAs have become a hot topic in the field of ncRNA. The function of circRNAs has been extensively studied. Different types of circRNAs have different characteristics. EIcircRNAs and ciRNAs are usually located in the nucleus, and ecircRNAs are usually located in the cytoplasm. Different locations make them play different functions. The main mechanisms and biological functions of circRNAs are shown in **Figure 2** and discussed below.

TRANSCRIPTION REGULATION

A growing number of studies have shown that circRNAs play a role in regulating gene expression. CircRNAs, ciRNA, and EIciRNAs are located in the nucleus that can regulate protein expression by regulating transcription or post-transcription (59, 60). EIciRNAs can regulate transcription because they retain intronic sequences of host genes (61). For instance, circEIF3J and circPAIP2, which are located in the nucleus, can interact with U1 small nuclear ribosomal nucleoprotein (snRNP) to promote the transcription of host genes by binding to RNA polymerase II (RNA pol II) (41). Although EIciRNA and ciRNA do not have the function of miRNA sponge, they can regulate gene transcription and expression in transcription or post-transcription (**Figure 2a**).

TRANSLATION

Traditionally, the 5′ and 3′ untranslated regions (UTRs) have been regarded as the basic elements of translation in eukaryotic cells. Although circRNAs contain exons, the absence of a 5′ cap

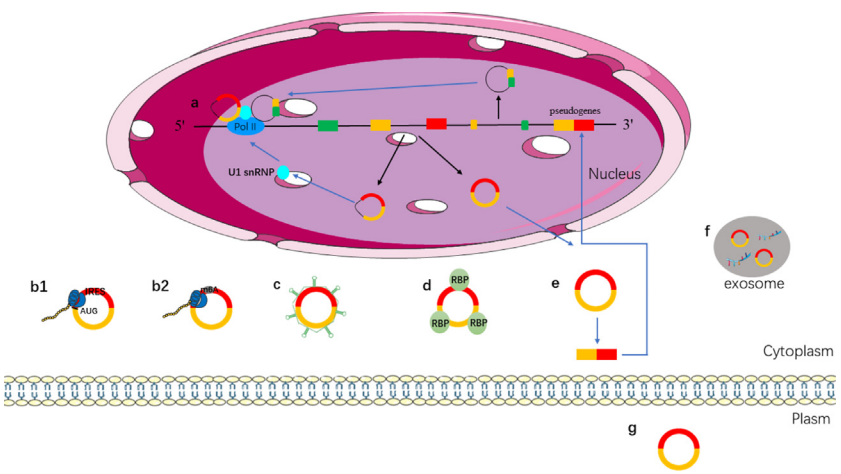

FIGURE 2 | Functions of circRNAs. **(a)** EIcircRNAs and ciRNAs can regulate gene transcription *via* binding to U1 snRNP and RNA pol II in the nucleus; **(b)** ecircRNAs exported into cytoplasm; **(b1)** IRES-mediated cap-independent translation of ecircRNAs; **(b2)** m6A-mediated cap-independent translation of ecircRNAs; **(c)** ecircRNAs act as miRNA sponges; **(d)** ecircRNAs interact with RBP motifs; **(e)** ecircRNAs can form pseudogenes; **(f)** ecircRNAs can be secreted from the cell to outside *via* exosomes; **(g)** ecircRNAs act as biomarkers.

structure and a poly A tail is considered to be ncRNA that does not encode proteins. However, an increasing number of researches have shown that some circRNAs can be translated into proteins. The researchers constructed artificial circRNAs containing an infinite reading frame to recruit 40s ribosomal subunits and translation into peptides *in vitro* (62). In 2017, Legnini et al. found that circ-ZNF609, a backsplicing product of ZNF609 exon 2, can be translated into proteins based on high-throughput phenotype screening. And, it can be translated into proteins in a splice-dependent and cap-independent manner (63).

More and more circRNAs are found to be able to translate into proteins, so how do the circRNAs initiate the translation mechanism? In some conditions, such as viral infection, mRNA translation can be initiated *via* internal ribosome entry site (IRES), which is an alternative mechanism for cap-independent translation (64, 65). IRESs can recruit ribosomes directly to initiate translation. IRES mediated translation is a widely accepted mechanism for initiating translation of circRNAs (66–68). Studies have shown that circRNA can be translated when an IRES is introduced into it (62). Both IRES and N6-methyladenosines (m6A) can drive circRNA translation (**Figure 2b**). The presence of methylated adenosine residues in m6A form is another cap-independent translation mechanism (69). Studies have shown that m6A can directly bind to eukaryotic initiation factor 3 and initiate the translation of circRNAs into proteins in human cells (69, 70).

MIRNA SPONGE

MiRNA is a type of ncRNA with a length of about 19–25 nt, which regulates the transcription of the target gene by binding to the 3′ UTR of the target gene through its seed sequence (71). Studies have shown that circRNAs contain miRNA response elements (MREs), which can competitively bind to miRNA (72). That is, circRNAs can bind to miRNA as miRNA sponges and then regulate the expression of target genes (71), such as, overexpression of circITCH can bind miR-17 and miR-224 to regulate p21 and PTEN genes to inhibit the development of breast cancer (73). CircHIPK3 inhibits the growth of cancer cells by binding to various miRNAs such as the tumor suppressor miR-124 (74, 75) (**Figure 2c**).

PROTEIN REGULATION

Some circRNAs have been shown to bind to RBP, and can isolate RBP and transfer proteins to specific subcellular sites (76). The combination of circPABPN1 and RBP (HuR) prevented the interaction between HuR and PABPN1 (mRNA) and inhibited the translation of PABPN1 (77). High expression of circANRIL can be combined with peccadillo ribosomal biogenesis factor 1 (PES1) to control ribosomal RNA maturation (78). CircAmotl1 can promote the nuclear translocation of PDK1, AKT1, STAT3, c-myc, and other proteins by interacting with RBP and regulate the expression of corresponding target genes (79–81). The above lines of evidence suggest that circRNAs can regulate the function of proteins by binding to PBP instead of a single protein (**Figure 2d**).

FORM PSEUDOGENES

Pseudogenes are typically derived from reverse-transcriptional of linear mRNA, which integration into the host genome. In the human genome, thousands of pseudogenes are found at about 10% of the gene sites (82, 83). In 2016, the research revealed for the first time that mammalian genomes contain pseudogenes derived from circRNA by establishing a new type of computing analysis process (CIRCpseudo). It revealed that mice circSATB1 source of pseudogenes can be combined with CTCF, which prompts the pseudogenes derived from circRNAs to have the potential to control gene expression. This study showed a fresh perspective on the fact that circRNAs can be inserted into the genome *via* reverse transcription to alter genomic genetic information and regulate gene expression. Furthermore, many pseudogenes derived from circRNAs have been identified by searching for non-collinear backsplicing in both mouse and human genomes (84). In mice, the reverse transcription of circRFWD2 produced pseudogenes associated with long terminal repeats. The molecular mechanism of circRNA reverse transcription remains to be further studied (**Figure 2e**).

OTHER FUNCTIONS

Exosomes are a type of vesicles with a diameter of 40–150 nm; it is released by the majority of cell types (85). Exosome contains miRNA, lncRNA, circRNA, mRNA, transcription factors, lipids, and proteins (86). Exosomes can be used for liquid biopsy to monitor the development and metastasis of tumors. CircRNAs can be transported to the extracellular *via* exosomes (**Figure 2f**) (87). It has been found that exosomes contain abundant circRNAs, and the role of exosomal circRNAs remains to be further explored. CircRNA can be secreted into the blood, saliva, and other body fluids as a biomarker for disease prediction (**Figure 2g**). CircRNA is stable in body fluids because of its properties, and it is a promising biomarker for the diagnosis of cancer (88).

THE FUNCTIONS OF CIRCRNAS IN LIVER CANCER

The high mortality, poor prognosis, and lack of effective treatment methods of liver cancer force us to search for effective therapeutic targets and better tumor biomarkers. The studies have shown that a large number of circRNAs are abnormally expressed in liver cancer, which play a regulatory role in the development of liver cancer. The expression and function of circRNA in liver cancer are shown in **Figure 3**.

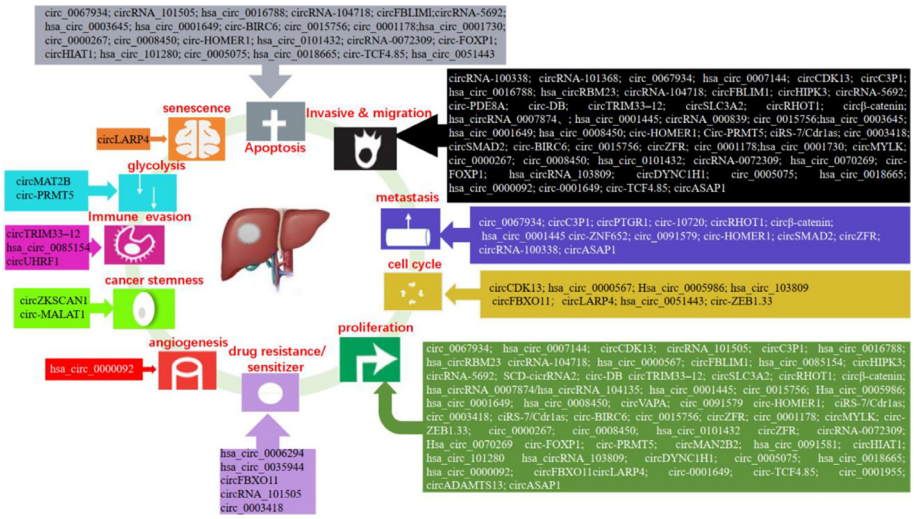

FIGURE 3 | Functions of circRNAs in liver cancer.

PROLIFERATIVE, METASTASIS, AND APOPTOSIS

Studies have shown that circRNAs can regulate the proliferation, migration, invasion, apoptosis, and metastasis of liver cancer cells. In liver cancer, hsa_circ_0000567, hsa_circ_0085154, hsa_circRNA_0007874, hsa_circ_0005986, hsa_circ_0001730, circRNA-0072309, hsa_circ_0070269, circHIAT1, circADAMTS13, ciRS-7/Cdr1as, and hsa_circ_0018665 suppressed cell proliferation (44, 89–98). While, hsa_circ_0101432, SCD-circRNA 2, circVAPA, circ_0015756, circ_0001178, circMYLK, circ-ZEB1.33, circZFR, circ-FOXP1, circMAN2B2, hsa_circ_0091581, hsa_circ_0005075, hsa_circ_101280, hsa_circ_103809, circDYNC1H1, hsa_circ_0000092, circFBXO11, circLARP4, hsa_circ_0001649, circ_0001955, and circ-TCF4.85 promoted tumor growth (99–120). Circ_0067934, hsa_circ_0007144, hsa_circRBM23, circHIPK3, circSLC3A2, circRHOT1, circβ-Catenin, circRNA-104718, circ-PRMT5, ciRS-7/Cdr1as, exosomal circ-DB, circ_0015756, circ_0091579, and circZFR enhanced cell proliferation, migration, and invasion (121–134). However, circCDK13, circC3P1, circ_0003418, circTRIM33-12, hsa_circ_0001445, and hsa_circ_0008450 inhibited cell proliferation, migration, and invasion (135–140). The study showed that circRNA_100338 increased cell invasive (141). CircRNA_000839 enhanced cell invasion and migration (142). Exosomal circPTGR1, circASAP1, and exosomal circRNA-100338 increased cell metastasis (143–145). Circ-10720 and circ-ZNF652 induced epithelial–mesenchymal transition (EMT) (146, 147). CircSMAD2 suppressed the EMT (148). And, circRNA_101368 suppressed the migration (149). The research showed that circRNA_101505 decreased cell proliferation and induced apoptosis (150). Conversely, hsa_circ_0016788, circFBLIM1, circ-BIRC6, circ_0000267, and circ_0008450 promoted cell proliferation, invasion, and suppressed the apoptosis (151–155). The research validated that circRNA_5692 suppressed the progression and invasion, induced apoptosis (156). On the contrary, hsa_circ_0003645 promoted cell migration, invasion and suppressed cell apoptosis (157). Circ-HOMER1 enhanced the proliferation, migration, invasion, and suppressed apoptosis (158). In addition, exosomal hsa_circ_0051443 enhanced cell apoptosis (159).

LIVER CANCER STEMNESS

Both cancer stem cells (CSCs) and circRNAs could affect the carcinogenesis and development of liver cancer, but there are few studies on the relationship between CSCs and circRNAs. Recent studies have found that circ-MALAT1, generated by the backsplicing of lncRNA, promoted the self-renewal of liver cancer CSCs (160). In addition, the researchers found that circZKSCAN1 can regulate the CSCs of HCC *via* Qki5/circZKSCAN1/FMRP/CCAR1/Wnt signaling axis (161). These findings revealed the role of circRNA in regulating stem cells and enrich the function of circRNA.

CELL CYCLE

An increasing number of studies have shown that circRNAs can be involved in the regulation of the cell cycle in liver cancer. For instance, hsa_circ_0000567 induced G1/S arrest in HCC cells by sponging miRNA-421 (89). Hsa_circ_0005986 suppressed the cell proliferation of HCC through promoting the G0/G1 to S phase transition (91). Circ-ZEB1.33 increased the percentage of S phase by regulating CDK6/Rb (105). Inhibition of hsa_circRNA_103809 significantly induced G1/S arrest (113).

Down-regulation of circFBXO11 induced G1/G0 arrest (116). Furthermore, exosome-derived circRNA could be involved in the regulation of the cell cycle. Such as, exosome-transmitted hsa_circ_0051443 arrested the cell cycle in HCC (159).

GLYCOLYSIS

Hepatoma cells required glycolysis to meet their proliferation needs under hypoxia conditions, and glucose reprogramming is a feature of cancers. Under the hypoxia environment, circMAT2B enhanced glycolysis of HCC *via* the miR-338-3p/PKM2 axis (162). Furthermore, circ-PRMT5 increased glycolysis of HCC by the miR-188-5p/HK2 axis (129).

ANGIOGENESIS

Cancer cells secrete the angiogenic factors that lead to the formation of abnormal vascular networks. Tumor blood vessel is the key target of tumor treatment. A recent study found that hsa_circ_0000092 promoted angiogenesis in HCC (115).

DRUG RESISTANCE/SENSITIZER

Resistance to chemotherapy is one of the causes of failure in the treatment of hepatocellular carcinoma. The research showed that circRNA_101505 inhibited cisplatin chemoresistance through miR103/Oxidored-Nitro Domain-Containing Protein 1 pathway (150). In addition, circ_0003418 sensitized HCC to cisplatin by Wnt/β-Catenin pathway (137). CircFBXO11 regulated oxaliplatin resistance through miR-605/FOXO3/ABCB1 axis in HCC (116). The expression of hsa_circ_0006294 and hsa_circ_0035944 was decreased in resistant HCC cells, and they may play a key role in sorafenib-resistant HCC cells (163). Therefore, circRNAs may provide us with a new strategy for the treatment of HCC.

SENESCENCE

Cell senescence is a defense mechanism to prevent and control cell damage and a barrier to prevent tumorigenesis. p53 and p21 are regulatory molecules in the senescence process. Research has found that circLARP4 promoted cellular senescence by regulating miR-761/RUNX3/p53/p21 signaling in HCC (117).

IMMUNE EVASION

Dysfunction of the immune system can lead to abnormal immune surveillance of liver cancer, and liver cancer cells can also act on the immune system to lead the immune escape. NK cells account for 50% of the total number of hepatic lymphocytes and are cytotoxic cells with antitumor functions mediated by the release of cytotoxic granules, FasL and TRAIL (164). NK cells do not rely on antigen presentation; this allows NK cells to target stress and damaged self-cells (165).

Liver cancer cells avoid being destroyed by immune escape. Studies showed that circRNA could be involved in immune escape. Activation receptor natural killer group 2 member D (NKG2D) and its ligands in NK cells play a crucial role in cell-mediated immune responses to cancer (166). The researchers examined the expression of NKG2D in 200 patients with HCC and showed that the number of NKG2D-positive cells in HCC tissues was significantly reduced compared to adjacent non-tumor tissues. The expression of circTRIM33-12 was positively correlated with the number of NKG2D-positive cells in HCC. The result showed that circTRIM33-12 may enhance immune function by protecting Ten eleven translocation 1 (TET1) *via* sponging miR-191 (138). TET1, one of the 2-OG-dependent dioxygenases, is involved in regulating the formation of 5-hydroxymethylcytosine (5hmC) and has been proposed to be involved in DNA demethylation process (167). The studies have indirectly linked TET1 as a tumor suppressor in HCC (168). Hsa_circ_0085154 could enhance the innate immune monitoring effect of NK cells by up-regulating UL16 binding protein 1 (ULBP1), which suggests that circRNA may play a role in tumor immunity (169). In HCC, hsa_circ_0085154 promoted ULBP1 expression and assisted NK cells to recognize target tumor cells (169). ULBP1 is an NKG2D ligand that activates receptors expressed by NK cells (170) (**Figure 4**). NKG2D is a basic activation receptor belonging to the C-type lectin-like family that is constitutively expressed on NK cells (171). The apparently invariant activation receptor NKG2D binds promiscuously to a variety of ligands, such as major histocompatibility complex class I-associated chains A and B (MICA/B) and a unique family of long 16 binding proteins (ULBPs), which are poorly expressed on healthy cells, but they are up-regulated under DNA damage (172). The up-regulation of these ligands may lead to a shift in NK cell homeostasis from inhibition to activation. The research revealed that ULBP1, one of the NKG2D ligands, was not expressed in poorly differentiated human hepatoma tissues and cell lines, but was abundantly expressed in hyperplastic abnormal nodules and well to moderately differentiated HCC cells (172). These findings provided conclusive evidences for the role of NK cells and the NKG2D receptor pathway in immune surveillance of HCC. In addition, HCC-derived exosomal circUHRF1 induced impairment of IFN-γ and TNF-α secretion in NK cells. In HCC, high level of circUHRF1 suggested poor clinical prognosis and dysfunction of NK cells. CircUHRF1 inhibited the secretion of NK cell-derived IFN-$\gamma$and TNF-α. High level of plasma exosomal circUHRF1 was associated with a decreased proportion of NK cells and decreased NK cells tumor infiltration. In addition, circUHRF1 up-regulated the expression of T cell immunoglobulin and mucin domain 3 (TIM-3) by degrading miR-449c-5p, thereby inhibiting the function of NK cells (173) (**Figure 4**). TIM-3 plays an important role in cell immunity, it was expressed in NK cells and affects cellular

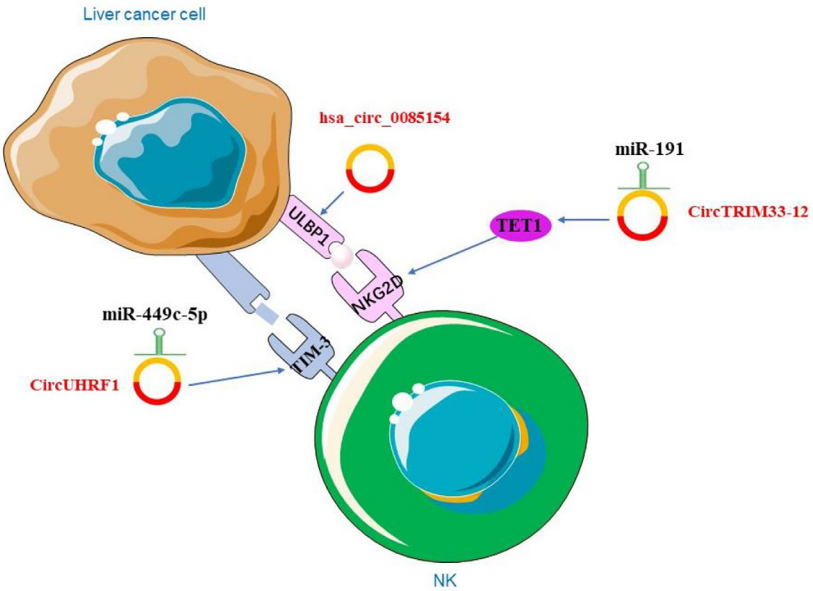

FIGURE 4 | The role of circRNAs in liver cancer immunity.

immune responses (174). In recent years, many studies have focused on the expression of TIM-3 in HCC and its mechanism (175). TIM-3 polymorphisms have been found to play an important role in the susceptibility and characteristics of HCC. The TIM-3 promoter region is associated with certain features of HCC, including lymph node metastasis and tumor stage (176). Modulation of the role of TIM-3 in innate immunity offers new directions for HCC treatment.

CONCLUSION

Structurally and mechanistically, the liver is an immune organ. It is rich in immune cells. Immune cells including dendritic cell (DC), NK cell, myeloid-derived suppressor cell (MDSC), CD8+ T cell, CD4 +T cell, regulatory T cell (Treg), T helper cell 1 (Th1), T helper cell 2 (Th2), T helper cell 17 (Th17), and tumor-associated macrophages (TAMs) (177–179). Liver cancer is one of the most common malignancies in the world. It is seriously threatening the health of Chinese people. In recent years, tumor immunotherapy is a major advance in cancer treatment, and targeted blocking of PD-1/PD-L1 immune checkpoints antibody-therapy is a milestone in the development of cancer immunotherapy. Currently, the FDA approved PD-1 antibody drug nivolumab (Opdivo) is being used in the treatment of cancer, and pembrolizumab (Keytruda) with the popular anti-cancer drug docetaxel (Sorafenib) combination for the treatment of HCC has been effective (180, 181). However, due to the primary/secondary drug resistance, immune escape, and antibody-drug effectiveness, the survival rate did not increase significantly in liver cancer. Therefore, it is of great significance to find a new way to improve the immunotherapy of liver cancer. NK cells are a new target for immunotherapy. There is a growing body of research using NK cell-related therapies to fight cancer (182, 183). NK cell-mediated immune surveillance is an important mechanism for tumor suppression. NK cells kill tumor cells through the release of perforin and granzyme and the secretion of pro-inflammatory cytokines and chemokines (184).

More and more studies have shown that circRNAs are involved in the carcinogenesis and progression of liver cancer. In this review, we summarized the functions of circRNAs in liver cancer. We found that circRNAs affect the cytotoxicity of NK cells. CircUHRF1 up-regulated TIM-3, the immune checkpoint, to inhibit the function of NK cells. Binding of TIM-3 to its ligand induces immune tolerance by depletes NK cells (185). It has been found that in tumor cells, immune checkpoints can lead to NK cell dysfunction, blocking these immune checkpoints (*e.g.* TIM-3, NKG2A, CTLA-4, PD-1, KIR2DL-1/2/3, CD96, TIGIT) can restore the function of NK cells (186). We can inhibit circUHRF1 to enhance NK cell function by down-regulating the expression of TIM-3. CircUHRF1 may provide a potential therapeutic strategy for immune checkpoints in liver cancer. More circRNAs regulating immune checkpoints are yet to be discovered, and targeting circRNAs provided a new direction for immune checkpoint therapy. Furthermore, circRNAs can affect the function of NK cells by regulating the receptor and ligand of NK cells. However, the relationship between circRNAs and other immune cells still needs further study. Understanding the mechanism of circRNAs in HCC patients is important in the design of effective immunotherapeutic protocols. Although circRNAs have shown an important role in liver cancer, many fields remain to be studied. For instance, the mechanism that

ecircRNAs transported from the nucleus to the cytoplasm? the degradation of circRNAs?

AUTHOR CONTRIBUTIONS

YT and H-MJ wrote the draft of the manuscript. ZY and Y-SH collected the data. R-SZ contributed to the discussion. D-YZ and H-FP organized the structure of the manuscript. YT and D-YZ contributed to the conception of the work. X-SL and B-YQ contributed to the revision. All authors contributed to the article and approved the submitted version.

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Immunotherapy for Hepatocellular Carcinoma: Current Limits and Prospects

Cheng Zhong[1,2†], *Yirun Li*[1,2†], *Jing Yang*[1,2], *Shengxi Jin*[1,2], *Guoqiao Chen*[1,2], *Duguang Li*[1,2], *Xiaoxiao Fan*[1,2,3*] *and Hui Lin*[1,2*]

[1] Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China, [2] Biomedical Research Center, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China, [3] State Key Laboratory of Modern Optical Instrumentations, Centre for Optical and Electromagnetic Research, College of Optical Science and Engineering, International Research Center for Advanced Photonics, Zhejiang University, Hangzhou, China

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***Correspondence:**
Hui Lin
369369@zju.edu.cn
Xiaoxiao Fan
fanxx_gs@zju.edu.cn

[†]These authors have contributed equally to this work

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Although many approaches have been used to treat hepatocellular carcinoma (HCC), the clinical benefits remain limited, particularly for late stage HCC. In recent years, studies have focused on immunotherapy for HCC. Immunotherapies have shown promising clinical outcomes in several types of cancers and potential therapeutic effects for advanced HCC. In this review, we summarize the immune tolerance and immunotherapeutic strategies for HCC as well as the main challenges of current therapeutic approaches. We also present alternative strategies for overcoming these limitations.

Keywords: immunotherapy, hepatocellular carcinoma, immune tolerance, tumor mutational burden, tumor microenvironment, epigenetic modification, tertiary lymphoid structure

INTRODUCTION

Liver cancer is the seventh most commonly diagnosed cancer and third leading cause of cancer-related death worldwide. Hepatocellular carcinoma (HCC), the most common form of liver cancer, shows high morbidity and mortality (1). The major clinical risk factor for developing HCC is liver cirrhosis. Chronic infections with hepatitis B virus and hepatitis C virus as well as long-term heavy alcohol consumption are the main causes of cirrhosis development (2).

Surgical resection, liver transplantation, and radiofrequency ablation (RFA) are widely applied in the clinical treatment of early stage HCC (Barcelona Clinic Liver Cancer [BCLC] stage A). For patients with intermediate HCC (BCLC stage B), transarterial chemoembolization is considered as the first-line treatment with a median survival of approximately 40 months (3–5). However, most patients with HCC are first diagnosed in an advanced stage (BCLC stage C). The multi-tyrosine kinase inhibitors sorafenib and regorafenib have been approved by the Food and Drug Administration as first- and second-line treatments for advanced HCC but only increase survival by less than three months (5). Although many treatment approaches have improved the clinical efficacy, patients with HCC suffer from tumor recurrence and show poor survival rates. Thus, novel therapeutic strategies are urgently needed.

Cancer immunotherapy (CIT) has rapidly developed in the past few years and has improved the survival of patients with different tumors. However, only a few patients with specific cancers, such as melanoma or Hodgkin's lymphoma, exhibit life-altering improvements with CIT. Most patients

with solid tumors still respond negatively to immune therapies. In this review, we summarize the immune tolerance and immunotherapeutic strategies for HCC, analyze the limits of current therapeutic approaches, and present alternative strategies which might overcome these limitations.

IMMUNE TOLERANCE OF HCC

The liver is constantly exposed to non-self proteins derived from nutrients or microbiota, which can trigger immune responses. Many mechanisms protect these harmless antigens from being attacked by the hepatic immune system to maintain homeostasis in the hepatic microenvironment (6). In chronic liver disease, continuous inflammation makes the liver an immunosuppressive microenvironment. Chronic hepatitis B virus and hepatitis C virus infection are the most important risk factors for HCC and are associated with 80% of HCC cases globally (7, 8), providing an immunosuppressive milieu for the initiation and progression of HCC (9). Tumor cells and the specific immune system of HCC constitute an immune-resistant microenvironment, allowing tumor tissue to evade the surveillance of the immune system and protecting the tumor tissue from immune system attack.

Tumor Cells Promote Immune Tolerance of HCC

Hepatocytes under chronic pressure gain 'driver' mutations (10), leading to growth advantages and gradually transforming them into low-grade dysplastic nodules, high-grade dysplastic nodules, early HCC, and finally advanced HCC (11). The progression of tumor cells under the selective pressure of immune system resulting in the emergence of immune-resistant tumor cells with fewer immunogenic or immunosuppressive factors is named as 'immunoediting' (12).

Tumor cells show weakened antigenicity. Tumor-associated antigens (TAAs) are antigens that are either only produced by tumor cells or overexpressed in tumors compared to in normal cells. The most studied TAAs are oncofetal antigens and cancer/testis antigens, including alpha fetoprotein (AFP), glypican-3 (GPC-3), New York esophageal squamous cell carcinoma-1, synovial sarcoma X-2, melanoma antigen gene-A, and human telomerase-reverse transcriptase, which can elicit a defensive immune response in the host. In the progression of a chronically inflamed liver and HCC, genetic and epigenetic alterations under pressure from the microenvironment transform tumor cells and deregulate the expression of TAAs. In addition to decreasing TAAs, HCC cells escape immune attack by releasing immunosuppressive cytokines, such as transforming growth factor-β and indoleamine 2, 3-dioxygenase (13, 14).

Immunosuppressive Cells in HCC

The liver prevents harmless antigens from being attacked by the hepatic immune system and thus maintains homeostasis in the hepatic microenvironment (6). However, long-lasting inflammatory and antigenic stimulation switches the immune system in the liver to an immunosuppressive status, which is exacerbated during the initiation and progression of HCC (15).

Repressive T Cells in HCC

The mechanisms of immunological tolerance for T cells in HCC including inactivation or deletion of effector T cells, mainly refers to $CD8^+$ T cells as well as priming and expansion of regulatory T cells (T_{reg} cells). The presence of tumor-infiltrating lymphocytes (TILs) is associated with a good prognosis and improved overall survival in HCC (16). Cytotoxic infiltrating $CD8^+$ T cells are the major cell type functioning to kill tumor cells. However, persistent exposure to antigens stimulates effector $CD8^+$ T cells to differentiate into exhausted $CD8^+$ T cells (17). Exhausted $CD8^+$ T cells were originally characterized by down-regulated expression of interferon gamma (IFN-γ) during chronic inflammation. Poor expression of tumor necrosis factor-β and interleukin-2 (IL-2) in exhausted $CD8^+$ T cells is also observed, resulting in impaired cytotoxic function (18). In addition to the loss of effector function, exhausted $CD8^+$ T cells express inhibitory receptors (IRs), such as programmed cell death 1 (PD-1), lymphocyte-activation gene 3, T cell immunoglobulin domain and mucin domain-containing protein 3, and cytotoxic T lymphocyte-associated antigen (CTLA)-4 (19–21). IRs are negative regulatory pathways that prevent the immune system from attacking cells indiscriminately. However, in the tumor immune system, IRs protect tumor cells from immune system attack. Persistent and elevated expression of these IRs has been observed in HCC (22, 23).

T_{reg} cells are a subpopulation of T cells that modulate the immune system and play an immune suppressive role in immune tolerance in cancer. Depletion of T_{reg} cells results in severe autoimmunity and allergies (24–26). In HCC, accumulation of intra-tumoral T_{reg} cells correlates with tumor progression and poor prognosis (27, 28). T_{reg} cell depletion can also activate an effective immune response in tumor models in animals (29, 30). T_{reg} cells express the CD4, CD25, and Foxp3 biomarkers. Foxp3 is a key regulatory gene in the development of T_{reg} cells (31). The transcription factor Foxp3 has been proposed to regulate the expression levels of immune-suppressive molecules in T_{reg} cells. Ectopic expression of Foxp3 confers Treg-like suppressive function to $CD4^+CD25^-$ T cells (32), and various molecules encoded by Foxp3-controlled genes are associated with immune suppression (33).

Myeloid Cells in HCC

There are two types of myeloid cells; marrow-derived suppressor cells (MDSCs) and tumor associated-macrophages (TAMs), which play important roles in the tumor microenvironment. MDSCs are a population of immature myeloid cells with strong immunosuppressive functions and can promote tumoral angiogenesis. MDSCs can differentiate into macrophages, granulocytes, and dendritic cells (DCs) (34). However, in the hypoxic microenvironment of HCC, tumor cells express ectonucleoside triphosphate diphosphohydrolase 2, which can convert extracellular ATP to 5′-AMP and thus prevent the differentiation of MDSCs (35). Arginine is an essential amino acid for the proliferation of $CD4^+$ and $CD8^+$ T cells. MDSCs

suppress T-cell proliferation *via* increased arginase activity, leading to arginine depletion (36). MDSCs also exert an immunosuppressive effect by inducing the differentiation of $CD4^+$ T cells into T_{reg} cells (36).

TAMs are also immunosuppressive myeloid cells. Macrophages can differentiate *via* two routes, known as macrophage polarization. Classically activated macrophages (M1) produce high levels of IL-12 and low levels of IL-10 and promote tumor initiation, whereas alternatively activated macrophages (M2) are characterized by low IL-12 and high IL-10 production and promote tumor progression. The microenvironment of HCC stimulates macrophages towards M2 polarization, which are named as TAMs (37). A previous study reported that macrophages in the early stage of HCC express high levels of major histocompatibility complex (MHC)-class II and cytokines, such as IL-1β, IL-6, IL-12, and inducible nitric oxide synthase, which suppress tumor progression. However, in advanced HCC, macrophages express M2-like molecules, including macrophage mannose receptor c1, arginase, IL-10, and transforming growth factor-β and low levels of MHC-class II, which promote tumor progression (38).

TAMs promote tumor progression through angiogenesis, tumor cell invasion, and metastasis (39). Infiltrating TAMs contribute to poor prognosis in HCC, and *in vivo* and *in vitro* experiments have shown that TAMs in HCC enhance tumor invasion by producing C-C motif chemokine 22 (40). Another study showed that TREM-1^+ TAMs in HCC induce immunosuppression by recruiting C-C chemokine receptor type 6-positive T_{reg} cells, releasing CCL20 and producing the immune checkpoint molecule PD-L1 which may endow HCC with anti-PD-L1 therapy resistance (41). Transforming growth factor-β in the HCC environment can promote TAMs to produce T-cell immunoglobulin- and mucin-domain-containing molecule-3, which can promote bone marrow-derived macrophages and peripheral monocytes to differentiate into TAMs (42). After co-culture with tumor cells, TAMs promoted the expansion of $CD44^+$ HCC stem cells by producing IL-6 and signaling *via* STAT3 (43). The $CCR2^+$ macrophage subset has pro-angiogenic properties in HCC, and inhibition of $CCR2^+$ TAMs in the fibrosis-HCC model significantly suppress angiogenic activities (44).

Hepatic Stellate Cells in HCC

Hepatic stellate cells (HSCs) are the main producers of extracellular matrix in the liver. In liver fibrosis, HSCs are activated towards a myofibroblast-like phenotype and play a key role in fibrogenesis (45). Activated HSCs produce extracellular matrix, cytokines, and growth factors to create a tumor-favoring environment in HCC (46). Activated HSCs in HCC suppress the antitumor immune response by depleting effector T cells and promoting the accumulation of immunosuppressive cells. HSCs can induce apoptosis of activated T cells through PD-L1 signaling (47, 48). Activated HSCs can convert mature peripheral blood monocytes into MDSCs (49). In murine models, HSCs can present antigens to naïve CD4+ T cells and transform activated naïve CD4+ T cells into Foxp3+ Treg cells by producing retinoic acid (50).

Liver Sinusoidal Endothelial Cells in HCC

Liver sinusoidal endothelial cells (LSECs) form a bed in the liver and receive blood from both the hepatic artery and portal veins in the hepatic parenchyma. In addition to functioning as vascular channels, LSECs play a role in the immune system. LSECs function in both pathogen recognition and antigen presentation. LSECs can cross-present antigens to $CD8^+$ T cells by taking up, processing, and transferring antigens to MHC class I. The presentation of antigens produces a tolerogenic response in naïve $CD8^+$ T cells by upregulating PD-L1 on the surface of LSECs, which bind to the PD-1 receptor expressed on naïve $CD8^+$ T cells (51). LSECs also present antigens to the MHC class II complex to activate $CD4^+$ T cells. However, because of the lack of co-stimulatory molecules, LSECs drive naïve $CD4^+$ T cells to develop into T_{reg} cells rather than into T helper cells (52). LSECs express various receptors for angiogenic factors including vascular endothelial growth factor receptors 1 and 2, Tie-2 (angiopoietin-1 receptor), and platelet-derived growth factor receptor. The interaction between these receptors and their ligands promotes the proliferation of LSECs and angiogenesis (53, 54).

IMMUNOTHERAPY FOR HCC

Immunotherapy for cancer mainly involves three approaches: vaccines, adoptive cell transfer (ACT), and immune checkpoint inhibitors (ICIs) (**Table 1**). Vaccines or ACT with genetically modified T cells target specific antigens. ICI inhibits the suppressive regulators of T cells and stimulates already present antitumor immune responses to kill tumor cells.

Vaccines

Vaccines have been widely used to prevent various diseases by providing active acquired immunity. Clinical studies using neoantigen peptide, mRNA, or DC vaccines in patients with melanoma have achieved promising results (55, 58, 67, 68). This antigen-based immunotherapy has also been tested for other tumors, such as ovarian cancer, breast cancer (56), and small-cell lung cancer (57). TAAs released from tumor lysates are considered to be optimal vaccines to activate immune response, but the low representation of the TAAs with high immunogenicity limits the clinical effect (69, 70).

Some vaccines are being evaluated for treating HCC. In a phase I trial, administration of AFP-derived peptides as an anti-tumor vaccine was explored in 15 patients with advanced HCC. The results demonstrated that the vaccine was safe and effective. The peptides stimulated the immune system to produce peptide-specific T-cell receptors (TCRs), with one patient showing a complete response and eight patients exhibiting slowing of tumor progression (71). In a phase I trial, a carcinoembryonic antigen glypican-3 (GPC3) peptide vaccine was explored for treating advanced HCC, with 30 of 31 patients (91%) showing a peptide-specific CTL response. For the clinical response among 33 patients, one patient showed a partial response and 19 had stable disease for 2 months (72). A telomerase peptide was also

TABLE 1 | Clinical trials of immunotherapy for HCC.

Therapy approaches	Phase	Agents or approaches	Population	Endpoints	Relevant finding	Reference
Vaccine	I	AFP-derived peptides Vaccines	15 patients with advanced HCC	P: safety S: immune response	No AE; CR, 1; PR, 8.	(55)
Vaccine	I	GPC3 peptide vaccine	33 patients with advanced HCC	P: safety S: immune response	No AE; PR, 1; SD, 19; GPC3-specific CTL response in 30 patients; MST in patients with CTL frequencies ≥ 50 (N=15), 12.2 months; MST in patients with CTL frequencies < 50 (N = 18), 8.5 months	(56)
Vaccine	II	cyclophosphamide and a telomerase peptide (GV1001) vaccine	40 patients with advanced HCC	P: tumor response S: TTP, TTSP, PFS, OS, safety and immune responses.	SD: 17; TTP: 57 days; TTSP: 358 days; GV1001 treatment result in a decrease of regulatory T cells.	(57)
Vaccine	I/IIa	DC vaccine	12 patients	AE, TTP and RFS	AE, no grade 3 or 4 AE; TTP, 38.4 months; the 1-, 2-, and 5-year RFS, 75%, 69% and 41.7% respectively.	(58)
CIKs	III	CIKs therapy after curative treatment (control, curative treatment without CIKs therapy)	230 patients with HCC	P: RFS S: OS, cancer-specific survival, and safety	The median time of RFS (44 months vs 30 months); AEs, (62% vs 41%), no difference in serious AEs, (7.8% vs 3.5%).	(59)
TILs	I	TILs therapy after tumor section	15 patients with HCC	P: safety	Alive 15, Tumour recurrence: 3.	(60)
ICB	I/II	Nivolumab	48 patients with advanced HCC	P: safety and tolerability for the escalation phase and RR	Grade 3/4 treatment-related adverse events, 12 (25%); treatment-related serious adverse events, 6%; RR, 20% in the dose-expansion phase; RR, 15% in the dose-escalation phase.	(61)
ICB	II	Pembrolizumab	28 patients with advanced HCC	Safety, immune response, PFS and OS	CR, 1; PR, 8; SD,4; the median PFS, 4.5 months; the median OS, 13 months;	(62)
ICB and Antiangiogenic therapy	Ib	Atezolizumab and bevacizumab vs. Atezolizumab	223 patients with unresectable hepatocellular carcinoma	Safety and PFS	PFS (5.6 months vs 3.4 months); serious AE (12% vs 3%)	(63)
ICB and Antiangiogenic therapy	III	Atezolizumab and bevacizumab vs. Sorafenib	501 patients with unresectable hepatocellular carcinoma	OS, PFS and AE	OS at 12 months (67.2% vs 54.6%); PFS (6.8 months vs 4.3 months); Grade 3 or 4 AEs (56.5% vs 55.1%)	(64)
ICB and Ablation	I/II	Tremelimumab with RFA or chemoablation	32 patients with HCC	PR, PFS and OS	PR, 26.3%; PFS at 6 months and 12 months, 57.1% and 33.1%	(65)
ICB and Cytokines	I	mogamulizumab (anti-CCR4 antibody) and nivolumab	15 patients with HCC	Safety, PFS, OS and PR	No AEs; PFS, 3.8 months; OS: 11.3 months; PR, 27%.	(66)

HCC, hepatocellular carcinoma; AFP, alpha fetoprotein; P, primary endpoint; S, secondary endpoint; AE, adverse effect; CR, complete response; PR, partial response; GPC-3, carcinoembryonic antigen glypican-3; SD, stable disease; CTL, cytotoxic T lymphocyte; OS, overall survival; MST, median survival time; PFS, progression-free survival; RFS, recurrence-free survival; TTP, time to progression; TTSP, time to symptomatic progression; RR, response rate; CIK, cytokine-induced killer cell; TIL, tumor-infiltrating lymphocyte; ICB, immune checkpoint blockade; RFA, radiofrequency ablation; CCR4, CC chemokine receptor 4.

explored as a vaccine target for the treatment of advanced HCC in a phase II trial. No patients showed a complete or partial response, and 17 patients (45.9%) had stable disease for six months (73). Currently, a multi-epitope multi-HLA peptide vaccine is being evaluated in a phase I/II clinical trial for 40 patients with early and intermediate stages of HCC (HepaVac-101-NCT03203005). The results are extremely expected. In addition, lack of high immunogenic vaccines restricts the development of vaccine. A new prediction algorithm is needed for the identification of neoantigens with high immunogenicity, which may have unique homology compared with any human self-antigen and induce vigorous immune response (74–76).

Adoptive Cell Transfer

Patients receiving ACT therapy are directly treated with autologous natural or engineered anti-tumor T cells (77). The transferred cells can divide into three types, including cytokine-induced killer (CIK) cells, TILs, and genetically modified T cells. CIK cells and TILs can enhance the overall immune response by increasing the number of immune cells, whereas the genetically modified T cells target specific antigens.

Cytokine-Induced Killer Cells

CIK cells are a mixture of cytotoxic T cells and natural killer (NK) cells separated from peripheral blood mononuclear cells and are cultured *in vitro* under treatment with cytokines such as IFN-γ, anti-CD3 antibody and IL-2 to promote their proliferation and anti-tumor activities (78). Reinfusion of expanded and activated CIK cells either alone or as a combined therapeutic strategy has been widely studied to suppress tumor progression, with some impressive results observed in metastatic colorectal cancer, myeloid leukemia, and renal cell carcinoma (79–81). Some studies investigated the efficiency of ACT with CIK cells for HCC treatment. In a Korean phase III clinical trial, CIK cells, including $CD3^+/CD56^+$ cells, $CD3^-/CD56^+$ NK cells, and $CD3^+/CD56^-$ cytotoxic T cells (82, 83), were used as an adjuvant treatment for 230 patients with HCC who had been pre-treated with other curative therapies (surgical resection, RFA, or percutaneous ethanol injection). The results showed that the adjuvant immunotherapy group with activated CIK cells had increased overall and recurrence-free survival compared with the control group without adjuvant therapy (median time of recurrence-free survival: 44 vs 30 months) (59).

Tumor-Infiltrating Lymphocytes

The presence of TILs in tumors is associated with good prognosis (60, 84). TILs are obtained from surgical tumor specimens and then cultured *in vitro* with sequential treatment with IL-2 for expansion and anti-CD3 antibody for activation. These proliferative and activated TILs are then transferred back into patients. ACT with TILs has been studied for the treatment of metastatic human papillomavirus-associated carcinomas, with clinical responses occurring in 5 of 18 (28%) patients in the cervical cancer group and 2 of 11 (18%) patients in the non-cervical cancer group (85). A phase I clinical trial confirmed the safety of ACT using TILs in patients with HCC: The toxicity and immune response of therapy with autologous TIL is being tested in an ongoing phase I clinical trial of patients with advanced HCC (ClinicalTrials.gov number: NCT01462903) (86).

Genetically Modified T Cells

Heterodimeric antibody receptors expressed on the surface of T cells are known to be tumor antigen-specific TCRs that recognize the antigenic peptide-MHC complex. The gene sequence of TCRs that recognize specific TAAs can be analyzed and introduced into autologous T cells by retroviral or lentiviral vectors (87). These proliferative and activated autologous modified TCR-expressing T cells are reinfused into patients. In response to tumor cells, the cells express the target antigen, leading to effective antitumor activity by releasing cytokines such as IFN-γ, granulocyte macrophage colony-stimulating factor, and tumor necrosis factor alpha-α and directly killing tumor cells (88). An AFP TCR with optimal affinity, function, and safety is being evaluated for its clinical efficacy in an early phase clinical trial (ClinicalTrials.gov number: NCT03971747) (89).

Despite the powerful anti-tumor function of immunotherapies based on the interaction between peptide-MHC molecules and TCRs, tumor cells can escape immune surveillance by down-regulating peptide-MHC complex expression (90). ACT with T cells engineered to express a chimeric antigen receptor (CAR) are not limited by the presentation of MHC molecules on the tumor cell surface. CAR can recognize a defined TAA on the surface of tumor cells *via* the single chain variable fragment region, which is constructed from the variable heavy and variable light sequences of a monoclonal antibody specific for TAAs. Activation signals are transferred into cells by activating the transmembrane adaptor signaling protein CD3ζ and one or more co-stimulatory molecules (CD28, CD137, or OX40). The mechanism of CAR therapy causes tumor variants, which can escape the immune surveillance through deficiencies in antigen presentation, to remain susceptible to CAR therapy (87). Other biomarkers have also been considered as targets for CAR T cell therapy. AFP is a well-known biomarker for HCC, and CAR T-cell therapy targeting the AFP-MHC complex showed robust antitumor activity in AFP-CAR T cells in a mouse xenograft model of liver cancer (91). Another attractive liver cancer-specific target is GPC3 because of its high expression in HCC but low expression in normal tissues (92). GPC3-CAR T cells efficiently eradicated GPC3+ HCC cells rather than GPC3- HCC cells. This approach showed high treatment efficiency in an HCC xenograft model with high levels of GPC3 expression and low treatment efficiency in HCC xenografts with low GPC3 expression (93). Another ACT study of GPC3-CAR T cell transfer into patient-derived HCC xenografts also revealed suppression of tumor cell growth (94).

Immune Checkpoint Inhibitors

The human immune system is in an equilibrium state. Immune checkpoints regulate immune function by suppressing immune activity, interrupting the immune response to avoid overactivation of T cells, and protecting tissues from damage caused by an excessive immune response. Immune checkpoints

in tumor tissues promote immune evasion. Most studies of immune checkpoints focused on cytotoxic T-lymphocyte antigen-4 and PD-1 with corresponding PD-L1 ligands. The ICI approach results in nonspecific immune stimulation by targeting negative regulators of T cell signaling pathways.

CTLA-4 Inhibitors

The hepatic microenvironment contains a large number of DCs, which are the major antigen-presenting cells in the liver (95). In a normal hepatic microenvironment, DCs take up foreign peptides and present them to T cells *via* the TCR (signal 1). In addition to signal 1, activation of T cells requires co-stimulatory molecules from DCs. After stimulation by the peptide-MHC complex, DCs present CD80 and CD86 to T cells and bind to the CD28 receptor on the surface of T cells (signal 2) and further promote maturation, proliferation, activation, and survival of naïve T cells. Signal 2 prevents the recognition of self-antigens, whereas the absence of such a signal leads to T cell anergy. Upon activation, T cells induce CTLA-4 to competitively bind to CD80 and CD86 with higher affinity than CD28, to prevent an excessive immune reaction (96). CTLA-4 inhibitors prevent CTLA-4 from binding to CD80 and CD86, thereby initiating signal 2, which can activate specific T cells in lymphoid organs and promote their migration into the tumor (97).

Two anti-CTLA-4 monoclonal antibodies, ipilimumab and tremelimumab, are being evaluated in clinical trials for the treatment of other tumors. Studies have shown that CTLA-4 inhibitors deplete T_{reg} cells in the tumor, leading to enhanced effector function of antigen-specific T cells in the tumor. Patients with melanoma and administered ipilimumab exhibit T_{reg} cells depletion (98, 99). However, the clinical data associated with application of CTLA-4 inhibitors alone for advanced HCC are limited. A clinical trial of tremelimumab in patients with HCC and chronic hepatitis C revealed a partial response rate of 17.6% and disease control rate of 76.4% (ClinicalTrials.gov number: NCT01008358) (100).

PD-1/PD-L1 Inhibitors

The cell surface receptor PD-1 is expressed on activated T, B, and NK cells and binds PD-L1 and PD-L2 ligands to convey co-inhibitory signals to the TCR. The PD-1 signal terminates immune responses appropriately and maintains self-tolerance by causing apoptosis of antigen-specific T cells, attenuation of TCR-mediated activation, and proliferation of T cells (101). The PD-1 signal mediates the function of T_{reg} cells by promoting their differentiation and proliferation (102). These ligands are expressed on leukocytes and tumor cells (103). PD-L1 binding results in phosphorylation of PD-1, inhibiting T cell proliferation and cytokine releasing through SHP2. SHP2 dephosphorylation results in dephosphorylation of key TCR signaling components, most notably CD28 and the ZAP70/CD3zeta signalosome (104, 105). When chronically exposed to antigens, overexpression of PD-1 in T cell induces their exhaustion.

Anti-PD-1 monoclonal antibodies, such as nivolumab and pembrolizumab, and anti-PD-L1 monoclonal antibodies, such as durvalumab and atezolizumab, have been approved for several hematologic and solid malignancies. Many clinical trials for HCC are underway. In a phase I/II of escalation trial, safety was evaluated in 48 patients treated with nivolumab, with grade ≥3 adverse events observed in 31% of patients (15 of 48), which was considered to be a manageable safety profile (61). A phase II study of the efficacy of pembrolizumab in 28 patients showed that one patient achieved a complete response and eight patients achieved partial responses (62).

LIMITS OF CURRENT IMMUNOTHERAPIES

Although immunotherapy has shown promising clinical outcomes in some tumors, including melanoma, non-small cell lung carcinoma, and urothelial carcinoma, the application in HCC faces some limitations **(Figure 1)** (106–108).

Tumor Mutational Burden

Immunotherapies are ineffective for HCC because of the low tumor mutational burden of HCC compared to that of melanoma or non-small cell lung carcinoma (109). Neoantigens are tumor-specific peptides that result from somatic mutations in cancer cells. A larger number of somatic mutations is associated with higher levels of neoantigens, and the tumor mutational burden is used to evaluate somatic mutations in cancer to give a useful estimation of the tumor neoantigen load (110, 111). Neoantigens are newly expressed antigens on the surface of tumor cells and can be recognized and presented to T cells to result in adaptive immune response activation. However, even in tumors with a high tumor mutational burden, such as those with deficiencies in DNA damage repair pathways resulting in the accumulation of DNA mutations, a high mutational load is not related to high levels of neoantigens. In fact, only a minority of mutations generates peptides that bind to MHC molecules and present on the surface of tumor cells, and fewer can be recognized by T cells (67, 112). The antigen presentation pathway in tumor cells can be inhibited by mutations in antigen presentation genes. For example, in metastatic melanomas, the loss of b2-microglobulin may result in defects in antigen presentation and escape from immune recognition (113). Not all neoantigens presented on the surface of tumor cells can drive effective antitumor immunity. A study reported that neoantigens could be expressed on either all tumor cells (clonal) or a subpopulation of tumor cells (subclonal). Tumors with a high load of clonal neoantigens show an excellent response to ICI therapy, whereas tumors with a high load of subclonal neoantigens evade immunotherapy (114) (**Figure 2**).

Tumor Microenvironment

The tumor microenvironment (TME) contains many components, including bone marrow inflammatory cells, lymphocytes, blood vessels, fibroblastic cells, and the extracellular-derived matrix composed of collagen and proteoglycans. The clinical efficacy of ICI depends on three tumor immune status characteristics. First, antigen-specific

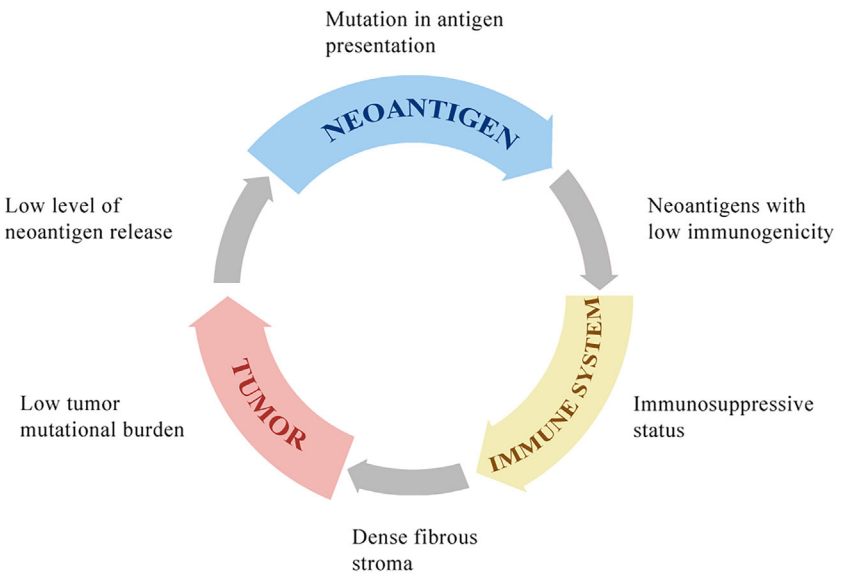

FIGURE 1 | The potential mechanisms of resistance to immunotherapies. HCC with low tumor mutational burden releases few neoantigens. The mutation in antigen presentation pathways also inhibits tumor-specific peptide presentation. Most of these neoantigens cannot drive effective anti-tumor immunity because of low immunogenicity. The immune system in the tumor microenvironment is under immunosuppressive status, with few effector $CD8^+$ T cells, many regulatory $CD4^+$ T cells, and other immunosuppressive cells, which is associated with poor clinical response to immunotherapy. The dense fibrous stroma around tumor islets inhibits immune cells' access to the tumor.

$CD8^+$ T cells must be present within the TME. Second, the resident immune cell populations in the TME must be polarized towards an immune permissive state. Third, tumor cells must have MHC class I-mediated antigen presentation and PD-1 signaling as the dominant mechanism of immune tolerance. A tumor with these characteristics is vulnerable to ICIs and named as an immune "hot" tumor. Immune "cold" tumors lack these characteristics and are associated with poor clinical response to ICI therapy (115). The absence of $CD8^+$ T cells in the TME in several tumor types has been associated with poor clinical outcomes of ICI therapy (116–118). A study of the stroma of human lung tumors showed that the stromal extracellular matrix influences the migration and positioning of T cells (119) (**Figure 2**).

EFFORTS TO ENHANCE IMMUNOTHERAPY FOR HCC

Although the immunotherapy approaches discussed previously have achieved impressive clinical efficacy in other tumors, they have failed to benefit patients with advanced HCC. The limitations of these approaches are discussed above. Here, we summarize some potential combinatorial strategies for enhancing the effects of immunotherapy for HCC.

Epigenetic Modulation

Epigenetic modification plays an important role in tumor progression, causing transcriptional aberrations in gene expression and immune function changes, which may result in a favorable TME (120). In contrast, epigenetic therapy has the potential to enhance immunotherapy for HCC by converting an immune "cold" tumor into an immune "hot" tumor (121). Epigenetic therapy can promote the expression of immunogenic antigens on the tumor surface such as cancer testis antigens (122–125). Cancer testis antigens are a group of proteins expressed on male germ cells but not in healthy adult somatic tissues and can serve as target antigens for antitumor immunotherapy (126, 127).

Epigenetic modification can regulate the composition of immune cell populations. Methylation of DNA represses genes related to effector function, proliferation, metabolic activity, and tissue homing of exhausted T cells. Chronic antigen stimulation drives $CD8^+$ effector T cells towards the exhausted phenotype, which is characterized by a series of changes in gene expression associated with alterations in methylation, leading to increased PD-1 expression and decreased CXCR3 expression (128). *De novo* DNA methylation is essential for establishing exhaustion in T cells, whereas treatment with ICI contributes to rejuvenation of exhausted T cells (128) (**Figure 3**). Azacitidine and histone deacetylase inhibitors have been shown to suppress MYC signaling, activate interferon responsiveness, and potentiate the recruitment of T cells in mouse models of non-small cell lung carcinoma (129). An EZH2 inhibitor (DZNep) and DNMT1 c (5-azacytidine) can augment anti-PD-L1 immunotherapy for HCC by increasing the release of the chemokines CXCL9 and CXCL10, which stimulate T cell trafficking into the TME. This combination therapy strategy can also upregulate the expression of cancer testis antigens New York esophageal squamous cell

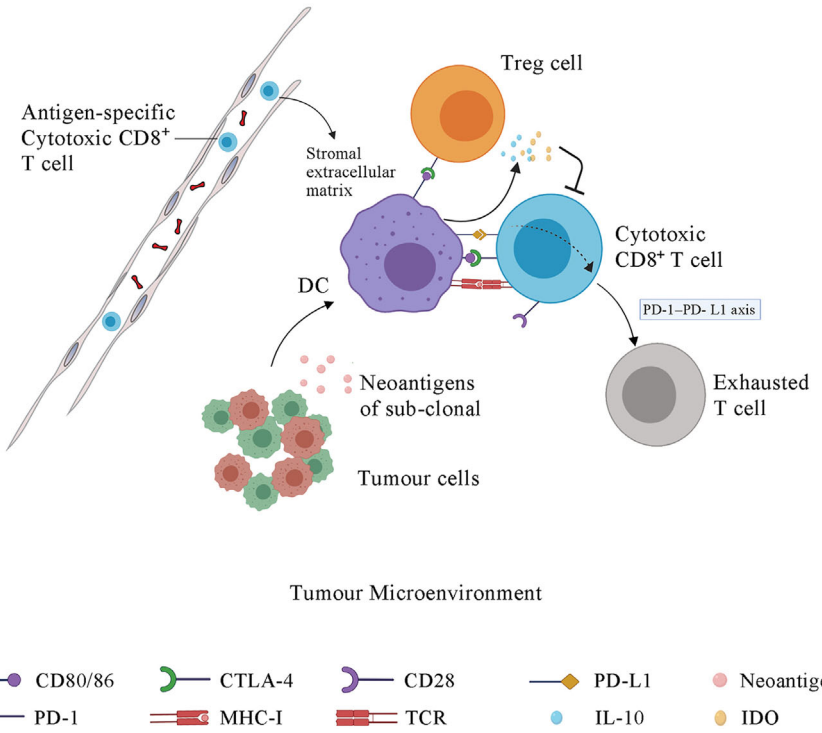

FIGURE 2 | The immune response in tumor microenvironment and the function of immune checkpoints. Some sub-clonal tumor cells release neoantigens while others do not, contributing to the immune response to only part of tumor cells and thus leading to the failure of tumor immunotherapy. Upon antigen recognition, DCs present the antigen-MHC molecules, bind to the TCR on T cell membrane and stimulate the proliferation and activation of $CD4^+$ T cells and $CD8^+$ T cells in lymph node. Then the antigen-specific cytotoxic T cells migrate to tumor microenvironment *via* blood system. The stromal extracellular matrix in tumor may prevents T cell infiltration. CTLA-4, which is the membrane receptor of activated T cells, outcompetes CD28 for binding to the CD80/86 expressed on the DC membrane, further inhibiting the signal 2, which is essential for the maturation, proliferation, activation and survival of T cells. The interaction of PD-1 and PD-L1 promotes the differentiation and proliferation of Treg cells and induces the cytotoxic $CD8^+$ T cells into an exhausted state. DCs under the influence of CLTA-4 signal and PD-1 signal release some immunosuppressive molecules, such as IL-10 and IDO, which suppress T cells activation. IDO, indoleamine 2,3-dioxygenase; PD-1, programmed cell death protein 1; PD-L1, programmed cell death 1 ligand 1; CTLA-4, cytotoxic T-lymphocyte protein 4; DC, dendritic cell; Treg cell, regulatory T cell.

carcinoma-1 and L antigen family member, which are normally expressed at low levels, as neoantigens to stimulate the adaptive immune response (130). The potential therapeutic strategy combined with epigenetic modulation has emerged in recent years and is promising for treating HCC.

Antiangiogenic Therapy

The hypoxia microenvironment stimulates tumor angiogenesis and promotes HCC development. Drugs target angiogenic pathways, including vascular endothelial growth factor (VEGF), are approved for the treatment of advanced HCC (131). Anti-VEGF therapy are widely used in HCC treatment (132). Sorafenib, a tyrosine kinase inhibitor (TKI), can disturb VEGF signaling pathway and approved for HCC treatment (133, 134). Despite survival benefits observed, the high rate of acquired resistance to sorafenib limits its use for advanced HCC treatment.

Despite of high rate of resistance to anti-VEGF drugs for HCC patients, some studies have reported that these drugs can enhance immune response. Drugs targeting VEGF-A/VEGFR-2 axis inhibited T_{reg} cells accumulation in colorectal cancer (135). A VEGFR-2 inhibitor (DC101) promoted tumor-specific $CD8^+$ T cells infiltration (136). These findings promote the combined strategies of anti-VEGF drugs and ICBs for HCC treatment. Bevacizumab is an anti-VEGF agent approved to treat metastatic colorectal cancer, glioblastoma, renal cell cancer and cervical cancer (137–139). However, the clinical efficacy of Bevacizumab for HCC treatment was less, with 13% response rates in a phase II study (140). Recent studies focus on the combination of anti-VEGF therapy and immunotherapy. In an open-label, multicenter, multiarm, phase Ib study, atezolizumab plus bevacizumab shows optional results, with longer progression-free survival compare with atezolizumab alone for patients with unresectable HCC (63). A similar result is showed in another clinical trial, the combination strategy of atezolizumab and bevacizumab for HCC treatment showed better overall and progression-free survival outcomes than sorafenib in 501 patients with unresectable HCC in a global, multicenter, open-label, phase III trial (ClinicalTrials.gov number: NCT03434379) (64). Other anti-VEGF drugs are also being evaluated in the combined therapy with immunotherapy for HCC treatment (ClinicalTrials.gov number: NCT03170960

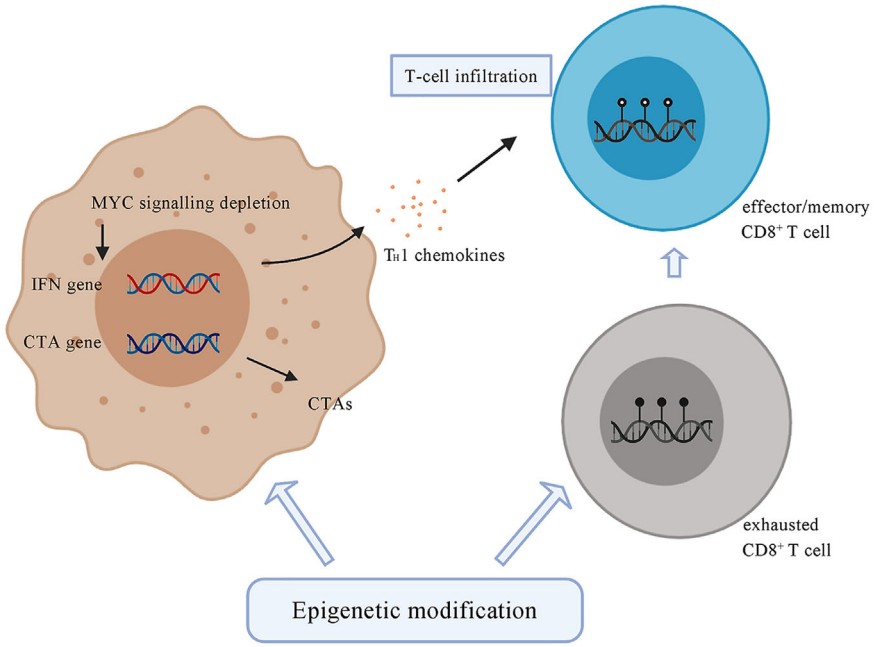

FIGURE 3 | Tumor cells under the treatment of epigenetic drugs upregulate the expression of CTAs, such as NY-ESO-1 and LAGE. Epigenetic modification contributes to the depletion of MYC signalling, activates type I interferon signalling and potentiates the recruitment of T cells. Epigenetic agents can modulate the state of $CD8^+$ T cells by transforming exhausted $CD8^+$ T cells, which are characterized by a series of changes in effector genes associated with alterations in methylation, into effector or memory $CD8^+$ T cells. CTA, cancer testis antigens; NY-ESO-1, New York Esophageal Squamous Cell Carcinoma-1; LAGE, L antigen family member.

(Cabozantinib and Atezolizumab) and NCT03006926 (Lenvatinib and Pembrolizumab)).

Inducing the Formation of Tertiary Lymphoid Structures

Although intra-tumoral infiltration by immune cells is a predictor of sensitivity to ICI treatment and many studies have focused on the role of T cell in antitumor responses, other immune cells have not been widely examined. Recently, studies revealed that the presence of intra-tumoral tertiary lymphoid structures (TLSs) improves ICI treatment of melanoma (141). TLSs are ectopic lymphoid aggregates that reflect lymphoid neogenesis occurring in non-lymphoid tissues in response to chronic inflammation, characterized by mature DCs in a T-cell zone adjacent to B-cell follicles including a germinal center (142, 143). TLSs are found in most types of cancer, with high TLS densities associated with improved clinical outcomes (144). In HCC, intra-tumoral TLSs are correlated with a decreased risk of early HCC recurrence after surgical resection, which may reflect ongoing, effective antitumor immunity (145). Therapeutic strategies to induce the formation of TLSs may enhance the antitumor immunotherapy of HCC (145). A reagent targeting LIGHT, a member of tumor necrosis factor superfamily of cytokines, can induce the formation of TLSs and can be combined with ICI to increase the number of TILs, conferring a survival benefit in mice with insulinomas (146). Other strategies aimed at stromal cells, which participate in the establishment of TLSs (147). Stromal cells derived from lymph nodes and induce TLSs cause infiltration of host immune cell subsets to suppress tumor growth *in vivo* (148).

Locoregional Therapy

Locoregional therapies such as RFA can be as efficient as surgical resection of HCC nodules (149) but patients treated with this therapy frequently experience cancer recurrence. Although it is not effective as monotherapy, locoregional therapy causes tumor cell death *via* the release of tumor antigens and stimulation antitumor immunity (150), named as immunogenic cell death (ICD). ICD may enhance the anti-tumor immune reaction through the antigens and adjuvants released during this process. ICD of tumor cells results in the release of neoantigens which may be recognized by DCs followed by activation of the adaptive immune response (151). Moreover, heat shock proteins induced by RFA have been shown to enhance the immune response by activating the natural immune response and augmenting the antigen-specific cytotoxic T-cell response (152–154). Although the effect of immune activation by locoregional therapy alone is not sufficient for treating HCC, it may be an effective adjuvant for immunotherapy (150).

Tremelimumab combined with RFA or chemoablation for advanced HCC resulted a partial response in 26.3% of patients (5 of 19), with a clear increase in $CD8^+$ T cells. Progression-free survival rates at 6 and 12 months were 57.1% and 33.1%,

respectively, and the median overall survival was 12.3 months (65).

Chemotherapy

Chemotherapeutic drugs alone for treating HCC, such as oxaliplatin, have shown limited effects on the overall survival of patients with advanced HCC. These cytotoxic drugs induce tumor cell death, which may also stimulate anti-tumor immunity by induced ICD. The cytotoxic effect also induces a decrease in the immunosuppressive cell population, such as MDSCs and T_{reg} cells (155, 156). High-dose chemotherapy, which is the proper strategy for the treatment of HCC, leads to the death of both tumor and immune cells. The suppressed immune system then loses its function and no longer targets therapy-resistant tumor cells.

Low dose metronomically administered chemotherapy can increase the ablation of immunosuppressive T_{reg} cells (156, 157), promote the maturation and activation of DCs (158, 159), and improve the activation and functionality of cytotoxic NK and $CD8^+$ T cells (160). Treatment with metronomic cyclophosphamide affected gliomas by activating anti-tumor $CD8^+$ T cell responses and immune memory in an immune-competent mouse model with implanted GL261 glioma (160). Pre-treatment with metronomic chemotherapy for HCC may enhance the effect of ICI and avoid unacceptable toxicity (161).

Cytokines

Although cytokines have multiple functions in the formation of the immune system, cytokine treatment alone as an immunotherapy for HCC is limited. IFN-α was the first immunotherapy tested in many clinical trials. Although IFN-α has anti-proliferative, immunostimulatory, and anti-angiogenesis properties (162), most trials failed to show clinical benefits (163, 164).

Overexpression of cytokine CCL5 in CTNNB1-mutant HCC cells led to the recruitment of $CD103^+$ DCs and antigen-specific $CD8^+$ T cells, which may enhance the clinical outcome of ICI therapy (165). CCR4 expressed by T_{reg} cells can suppress anti-tumor immune response. In a phase I study, the safety and efficacy of combined mogamulizumab (anti-CCR4 antibody) and nivolumab are evaluated for patients with HCC, with four (27%) tumor responses among 15 patients. During treatment, the immune system activated with population of T_{reg} cells decreased and effector $CD8^+$ cells increased (66). Although immunotherapy using cytokines alone is limited for treating HCC, the potential advantage of cytokines as adjuvants to enhance the clinical efficacy of immunotherapy is promising.

CONCLUSION

Immunotherapy as monotherapy or combined with other therapeutic strategies has demonstrated clinical efficacy. Although some patients benefit from these therapeutic approaches, most patients suffering from advanced HCC do not. Novel immunotherapy strategies are currently being evaluated.

AUTHOR CONTRIBUTIONS

Conceptualization, XF and HL. Resources, CZ. Writing – Original Draft Preparation, CZ and YL. Writing – Review and Editing, CZ and SJ. Visualization, GC and DL. Supervision, JY, XF and HL. Funding Acquisition, HL. All authors contributed to the article and approved the submitted version.

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