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Review

Immune Checkpoint Inhibitors in Hepatocellular Cancer: Current Understanding on Mechanisms of Resistance and Biomarkers of Response to Treatment

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Hepatocellular carcinoma (HCC) is the most common primary liver malignancy worldwide and a leading cause of death worldwide. Its incidence continues to increase in the US due to hepatitis C infection and nonalcoholic steatohepatitis. Liver transplantation and resection remain the best therapeutic options for cure, but these are limited by the shortage of available organs for transplantation, diagnosis at advanced stage, and underlying chronic liver disease found in most patients with HCC. Immune checkpoint inhibitors (ICIs) have been shown to be an evolving novel treatment option in certain advanced solid tumors and have been recently approved for inoperable, advanced, and metastatic HCC. Unfortunately, a large cohort of patients with HCC fail to respond to immunotherapy. In this review, we discuss the ICIs currently approved for HCC treatment and their various mechanisms of action. We will highlight current understanding of mechanism of resistance and limitations to ICIs. Finally, we will describe emerging biomarkers of response to ICIs and address future direction on overcoming resistance to immune checkpoint therapy.

Key words: Checkpoint inhibitor; Immunotherapy; Hepatocellular carcinoma (HCC); Biomarker; Resistance

INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary tumor of the liver that most often occurs in the setting of chronic liver disease. Globally, HCC accounts for the third and seventh most common malignancy in men and women, respectively, and is the fourth leading cause of malignancy-related death[1,2]. In the US, the death rate from HCC increased by 43% between 2000 and 2016[3]. The incidence of HCC remains on the rise and is estimated to reach 27,000 by the end of 2020[4]. Although hepatitis B virus (HBV) is the most common cause of HCC worldwide, the vast majority of HCC in the US is due to hepatitis C virus (HCV), while the incidence of HCC secondary to nonalcoholic fatty liver disease (NAFLD) is on the rise[5–9]. This steep increase in incidence and mortality in HCC has led to investigation of better treatment strategies to combat this deadly disease.

Treatment of HCC is challenging due to the complex pathophysiology of the disease. Curative intent treatment options include orthotopic liver transplantation and surgical resection for early stage disease[10]. Liver transplantation is an important therapeutic option; however, there are limitations due to the shortage of organs for transplantation. Surgical resection is another potentially curative treatment modality for early, localized disease. The majority of patients with HCC are not eligible for resection

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due to advanced disease at presentation and underlying liver dysfunction. Two landmark trials, Sorafenib HCC Assessment Randomized Protocol (SHARP) and Asia-Pacific (AP), investigated the efficacy of the multikinase inhibitor sorafenib, which led to its approval in 2007 as monotherapy in patients with Childs A cirrhosis and unresectable or metastatic HCC[11–13]. It is known that the overall survival (OS) benefit from sorafenib is much higher in patients with HCC related to HCV than in those with other underlying etiologies for HCC[14]. Despite its poor side effect profile and improvement in OS of less than 3 months compared to placebo, sorafenib remains one of the frontline systemic therapies. Lenvatinib was approved as an alternative first-line therapy as it was confirmed to be noninferior to sorafenib in the REFLECT study[15]. Multitarget tyrosine inhibitors (regorafenib and cabozantinib) and vascular endothelial growth factor (VEGF) receptor inhibitors (ramucirumab) have all been approved by the Food and Drug Administration (FDA) as single-agent second-line systemic therapy for patients who have failed sorafenib[16–19]. Despite these options, better and effective alternatives are needed to improve patient survival.

Recently, immune checkpoint inhibitors (ICIs) have emerged as alternatives for patients with adequate performance status who progress on first-line therapy. On September 22, 2017, the FDA approved nivolumab as an adjunct treatment for patients who have failed treatment with sorafenib. This was followed by approval of pembrolizumab in November 2018. These two immunotherapies, which fall under the broad category of programmed cell death protein-1 (PD-1) inhibitors, have shown some promise in the treatment of advanced HCC. Drug combination treatments have found some success in HCC as combination ipilimumab [cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibitor] and nivolumab was recently approved by the FDA in late 2019. Results of the IMbrave150 trial presented at the European Society of Medical Oncology conference in November 2019 show that atezolizumab in combination with bevacizumab was superior in prolonged OS and progression-free survival (PFS) to current standard of care, sorafenib[20]. While awaiting the final publication of the IMbrave150 study and regulatory approval, this result is exciting as this combination may soon be the frontline therapy for advanced HCC. Despite the success of ICIs, there remains a large cohort of HCC patients that do not respond to ICIs, and the challenge remains to find cellular and molecular cues that could predict which patients would benefit from these therapies[21]. In this review, we will discuss ICIs currently approved for HCC treatment and new options that are currently in development. We will highlight current understanding of mechanism of resistance and limitations to ICIs. Finally, we will describe emerging biomarkers of response to ICIs and address future directions on overcoming resistance to immune checkpoint therapy.

ICIs: DISCOVERY AND EVOLUTION

Immune checkpoints are membrane-bound molecules expressed in different cells types such as natural killer (NK) cells, dendritic cells (DC), tumor-associated macrophages, monocytes, and myeloid-derived suppressor cells (MDSC) including B and T cells[22]. These immune checkpoint proteins apply a physiologic break that prevent activation of these cells, limiting widespread off-target tissue damage. The intensity of immune response and activation of cytotoxic immune response depends on the balance between costimulatory signals and immune checkpoints[23,24]. It has been found that immune checkpoint proteins can be dysregulated by tumors as an important mechanism of immune resistance[25]. The discovery of cancer therapy by inhibition of this negative immune regulation led to the 2018 Nobel Prize in Physiology or Medicine to be jointly awarded to Drs. James P. Allison and Tasuku Honjo[26]. In turn, T cells have been the major focus of immune checkpoint therapy because of three major reasons: their capacity for selective recognition of peptides derived from proteins in all cellular compartments; their ability to directly kill antigen-expressing cells through cytotoxic CD8+ T cells; and, finally, their capacity to mount diverse immune responses through CD4+ helper T cells, which link adaptative and innate immunity[25]. The immune checkpoints most commonly studied in human cancers are CTLA-4, PD-1/programmed death-ligand-1 (PD-L1), lymphocyte activation gene 3 (LAG-3), T-cell membrane protein 3 (TIM-3), and B- and T-lymphocyte attenuator (BTLA). These molecules and their functions have been well described in the literature[25,27–30]. In this review, we will focus on the two major classes (PD-1/PD-L1 and CTLA-4) that have been studied in HCC.

ICIs IN HCC

The success of ICIs in a number of malignancies have opened the prospects of ICIs as a potential immunotherapeutic strategy for treating HCC[29]. The liver possesses a unique immune biology that allows for the use of checkpoint therapy. First, HCC arises in the background of chronically inflamed livers. Patients with chronic inflamed liver disease have been shown to overexpress PD-1 in the intrahepatic lymphocytes, while the ligands, PD-L1 and PD-L2, were found to be overexpressed in Kupffer cells, liver sinusoidal endothelial cells, and leukocytes[31,32]. Additionally, the liver has an abundance of Kupffer cells, DC, and naive T cells that are prone to dysregulation in cytokine secretion, antigen and immune checkpoint expression in the local immune microenvironment[29,33,34]. This upregulation of checkpoint proteins in the liver makes ICIs a plausible option for

the treatment of HCC as checkpoint inhibitors have been developed to block these inhibitory molecules expressed on the surface of these cells, thereby generating antitumor activity.

There are two classes of ICIs that are currently being used clinically or are part of active investigation in the treatment of advanced HCC. These immunotherapies belong to the CTLA-4 and PD-1/PD-L1 inhibition pathway. Monoclonal antibodies against CTLA-4 and PD-1/PD-L1 have been used successfully in the treatment of advanced melanoma, renal cell carcinoma, non-small cell lung cancer, and others including microsatellite instability-high (MSI-H) colorectal cancers[35–40]. Checkpoint inhibitors are indicated for use as second-line treatment of HCC in patients who have failed first-line sorafenib. There are ongoing trials looking at ICIs either as monotherapy or combination therapy for first-line treatment of advanced HCC.

Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4)

CTLA-4 is an intracellular protein in resting T cells. When the T-cell receptor is activated by CD28, CTLA-4 is translocated to the cell surface. Once CTLA-4 is expressed on the surface of T cells, it binds to CD80 and CD86, preventing the binding of CD28 to these critical costimulatory molecules, mediating inhibitory signals to the T cell resulting in arrest of both proliferation and activation[36,41,42]. CTLA-4 signaling also promotes tumor development by inhibiting the binding of antigen presentation by antigen-presenting cells[42]. Furthermore, CTLA-4 signaling may also stimulate the expression of immune regulatory cytokine such as transforming growth factor- (TGF-)[43]. CTLA-4 is thought to affect Treg activation and differentiation as this receptor is constitutively expressed in Tregs. This is further supported by the fact that blockade of CTLA-4 or Treg-specific knockout inhibits their ability to regulate both antitumor activity and autoimmunity[44,45].

CTLA-4 inhibitors (ipilimumab and tremelimumab) were introduced into clinical trials in the 2000s after preclinical models showed that inhibition of this molecular brake with an antibody could allow for T-cell activation and proliferation[41]. Clinical activity of anti-CTLA-4 therapy is more pronounced in advance metastatic melanoma with greater than 15% objective response rate lasting more than 10 years even after therapy was discontinued[36,46,47]. Tremelimumab was investigated as monotherapy for patients with HCC secondary to HCV-induced cirrhosis in a phase II clinical trial (NCT01008358)[48]. Partial response rate was observed in 17.6%, while time to progression was 6.48 months with good safety profile noted in the trial[48]. A randomized, multicenter phase III study (NCT04039607) looking at nivolumab in combination with ipilimumab compared to sorafenib or lenvatinib as first-line treatment is currently ongoing and is estimated to be completed by September 2023. Although this class of immunotherapy has not been approved for use as a single-agent therapy in HCC yet, combination of ipilimumab and nivolumab was granted priority review in November 2019 by the FDA with final approval for use granted on March 11, 2020[49]. This approval was based on positive results from CheckMate 040 (NCT01658878), which showed objective response rate of 31% in the combination group compared to 14% in the nivolumab monotherapy group[49,50]. There are ongoing trials looking at combination therapy with PD-1/PD-L1 blockade.

PD-1/PD-L1

PD-1 is an immunosuppressive receptor that is expressed on activated T cells, B cells, NK cells, Tregs, MDSC, and DC[51]. It was initially thought to be a receptor that induced cell death of activated T cell, hence the name programmed cell death protein[52]. However, it was later discovered that it is an immune checkpoint with its inhibitory function mediated by tyrosine phosphatase SHP-2[53]. PD-1 has two ligands: the first is PD-L1 (also known as CD274 or B7-H1), which is generally expressed by multiple somatic cells when exposed to proinflammatory cytokines[53]. This ligand is mainly responsible for the suppression of T-cell migration, proliferation, and secretion of cytotoxic mediators[54,55]. The second ligand is PD-L2 (CD273 or B7-DC), which is infrequently expressed in antigen-presenting cells[53]. T-cell function depends on the level of PD-1 activity[56]. Cancer cells have evolved to hijack PD-1/PD-L1 signaling by constitutively expressing PD-L1 or PD-L2 to activate PD-1 in tumor-infiltrating lymphocytes (TILs) and evade immune surveillance[57,58]. High PD-L1 expression on tumor cells has been associated with recurrence in HCC, in addition to tumor aggressiveness, and poor prognosis in patients who have never received immunotherapy[59–63].

Nivolumab and pembrolizumab are the only two PD-1 inhibitors currently approved for use in HCC treatment, although there are other PD-1 inhibitors (tislelizumab and camrelizumab) currently in clinical trials. Nivolumab was the first PD-1 inhibitor to be approved based on results of the CheckMate 040 trial (NCT10658878) that concluded on August 2016[64]. This trial was a phase I/II, dose escalation, and expansion trial of nivolumab in adults with histologically confirmed HCC with or without HCV or HBV. The authors of this study saw an objective response rate of 20% in the dose expansion phase and 15% in the dose escalation phase[64]. This trial was followed up by the CheckMate 459 trial (NCT02576509), which was a phase III multicenter study comparing the efficacy of nivolumab versus sorafenib as first-line treatment in patients with advanced HCC. The authors of this study reported that although the primary endpoint of OS was

not statistically significant (median OS of 16.4 months in nivolumab versus 14.7 months in the sorafenib group), nivolumab demonstrated clinical improvements in OS, objective response rate, and complete response rate as first-line treatment of advance HCC[65]. Pembrolizumab was approved by the FDA in November 2018 after the Keynote-224 trial, a nonrandomized phase II trial of pembrolizumab in patients with pathologically confirmed HCC who were previously treated with sorafenib and were either intolerant or showed signs of progression[66]. The trial reported overall objective response rate of 17%, stable disease in 44% of the cohort, while 33% had progressive disease[66]. This trial was followed by Keynote-240, a phase III trial of pembrolizumab as second-line therapy versus placebo for patients previously treated with sorafenib[67]. After a median follow-up of 13.8 months, OS did not meet statistical significance (13.9 months in the pembrolizumab group compared to 10.6 months in the placebo group), while the objective response rate was 17%, similar to the results from the phase II Keynote-224 trial. Various PD-L1 inhibitors (avelumab, atezolizumab, and durvalumab) are currently in clinical trials as of January 2020 as either monotherapy or combination therapy with other ICIs.

Immune-related adverse events are the side effects of the unbalanced immune system stemming from immunotherapy use, which may affect the intestine, endocrine glands, liver, and various tissues. Grade 3/4 treatment-related adverse events were reported in 25% of patients in the CheckMate 040 trial (pemphigoid, adrenal insufficiency, and liver disorder) and 24% of patients in the Keynote-224 trial (hypertransaminasemia and fatigue). One treatment-related death was reported in the Keynote-224 trial, which was associated with ulcerative esophagitis[66]. Both medications are well tolerated with few side effects and no dose-limiting toxicities similar to patients with melanoma and NSCLC treated with ICIs[68].

MECHANISMS OF RESISTANCE AND OVERCOMING RESISTANCE TO ICIs

While the mechanisms of resistance to ICIs in other cancers (melanoma, non-small cell lung cancer) are well described, there are limited data on mechanism of resistance for ICIs in HCC, probably due to its recent approval for use in HCC treatment[69–71]. These mechanisms of resistance can be categorized into tumor intrinsic and extrinsic factors. Tumor intrinsic mechanisms arise from changes in the tumor such as expression of PD-L1, downregulation of major histocompatibility complex (MHC) class I molecules and changes in oncogenic signaling pathways such as activation of -catenin signaling[25,72,73]. While more studies are needed, -catenin activation due to mutations in *CTNNB1* gene may be influencing the immune microenvironment in HCC, at least in part through modulation of nuclear factor B (NF- B) signaling pathway. A direct complex of -catenin and NF- B subunit p65 has been shown in the liver and in HCC[74]. Increased -catenin levels due to mutations [also observed as an increase in its target glutamine synthetase (GS)] was shown to enhance its association with NF- B, which in turn decreased NF- B activity in HCC cells. Further, GS-positive HCCs showed less p65 immunostaining and vice versa, suggesting that CTNNB1-mutated HCC may have decreased immune cell infiltration, at least in part due to reduced NF- B activity. Extrinsic factors arise from changes in the tumor microenvironment (TME) such as contributions from Tregs, MDSC, upregulation of coinhibitory molecules on lymphocytes, and contribution from the gut microbiome[75]. Table 1 summarizes known mechanism of resistance to ICIs. We assume that the mechanisms of resistance will be similar to those found in other tumors, but as more patients with HCC are treated with ICIs, we may uncover newer mechanisms of resistance.

Table 1. Summary of Known Resistance Mechanisms to Checkpoint Inhibitors

Categories of Resistance	Mechanism of Resistance
Tumor intrinsic factors	Downregulation of antigen processing and presentation: HLA deletion[144,145], 2 microglobulin[146]
	Downregulation of cytokines and signaling pathways: loss of *JAK1/2* function[69,147], deletion of interferon *IFNGR1/2, IRF1*[148]
	-Catenin activation (due to mutations in *CTNNB1* gene)[73]
Tumor extrinsic factors	TILs exclusion by PTEN deletion and VEGF upregulation[149]
	Expression of alternative coinhibitory checkpoint receptors like TIM-3, LAG-3, TIGIT, VISTA, and BTLA[69,126]
	Decreased TILs to Treg ratio[150–152]
	Downregulation of dendritic cell recruitment through -catenin signaling[110]
	Increased immunosuppressive cells such as MDSCs, Tregs[151,153,154]
	Epithelial-to-mesenchymal transition[155]
	Microbiome[75, 143]

BIOMARKERS FOR RESPONSE TO IMMUNE CHECKPOINT THERAPY STUDIED IN HCC

Based on published results of the clinical trials of ICIs in patients with HCC, we know that there remains a large proportion of patients who do not benefit from this class of treatment, and the challenge remains to find cellular and molecular cues that could help predict which patients would benefit from these therapies. Prognostic biomarkers of response to ICIs in various cancers have been extensively reviewed[76–79]. However, there are few studies on predictive biomarkers of response to ICI treatment in HCC owing to that fact that ICI therapy is still in its infancy in HCC. We will summarize emerging major biomarkers of response to treatment and highlight their application in HCC.

PD-L1 Expression

This is one of the earliest and the most commonly used predictive biomarker in immunotherapy. High PD-L1 expression has been associated with improved objective response rate and survival in patients with melanoma, non-small cell lung cancer, and head and neck squamous cell lung cancer[80–82]. In fact, PD-L1 testing by immunohistochemistry has been approved by the FDA as a companion diagnostic when considering the use of anti-PD1 therapy in non-small cell lung cancer[83,84]. PD-L1 has been previously investigated in HCC prior to initiation of immune checkpoint therapy. In HCC tissues, PD-L1 is found to be expressed by both the tumor cells and macrophages[59,85]. Previous studies have shown that PD-L1 expression is generally low in the tumor (roughly 10% of tumor cells), and there is heterogeneity in PD-L1 immunohistochemical detection in HCC[84,86]. A meta-analysis study by Gu et al. surmised that higher PD-L1 levels predict poor differentiation, higher alpha-fetoprotein, vascular invasion, and poorer survival in HCC[87,88]. Finkelmeier et al. studied circulating levels of PD-L1 and concluded that a high soluble PD-L1 level may be a prognostic indicator for poor prognosis[89].

All this background evidence of PD-L1 as a prognostic biomarker was promising. However, when PD-L1 expression was evaluated in the CheckMate 040 and Keynote-224 trials, it failed to have an impact on the objective response rates to anti-PD-1 therapy[64,66,90]. This was further confirmed by a study by Feun et al., where response to anti-PD-1 had no correlation with PD-L1 tumor staining in advanced HCC[91]. However, it is worthwhile to understand why the use of PD-L1 as a biomarker failed to predict response to treatment in these clinical trials. One reason for this failure was because different assays were used at the different institutions for the detection of PD-L1 as well as varying cutoffs in assessing positive staining, thus making it hard to interpret the results[83,84,92]. In the Keynote-224 trial, two different methods were used to investigate PD-L1 expression as a potential biomarker. One method was the combined positive score (CPS), which was calculated by dividing the number of PD-L1-positive cells (tumor cells, lymphocytes, and macrophages) by the total number of viable tumor cells and multiplying by 100. The other method, tumor proportion score (TPS), was calculated by dividing the number of PD-L1-expressing tumor cells by the total number of viable tumor cells and multiplying by 100[66]. The authors of this trial found that CPS was associated with response to anti-PD-1, while TPS was not significant, suggesting that inclusion of immune cell scoring could improve predictive value of a PD-L1 immunohistochemistry assay[66]. Additionally, we know that patients with PD-L1-negative tumors also respond to PD-1/PD-L1 blockade[93]. Furthermore, PD-L1 expression is inducible and can change during the course of HCC and during treatment[94]. It has been shown that interferon- (IFN-) induces upregulation of PD-L1 expression in melanoma and ovarian cancer and is a biomarker of response to checkpoint therapy in numerous cancers[95–98]. However, this needs to be investigated in HCC.

Tumor Mutational Burden (TMB)

The landscape of biomarker discovery for checkpoint therapy is constantly evolving, and there is more recognition that interactions between the microenvironment, genetic, and systemic factors play a role in determining response to these therapies. It is reasonable to assume that, in the near future, immunotherapy selections will be based on some combination of TMB, cell surface marker, or blood markers[99,100]. TMB is defined as total number of unique mutations in the tumor exome[101]. Known causes of TMB include microsatellite instability (MSI), or DNA mismatch repair gene deficiency and somatic mutations that arise in DNA polymerase[102,103]. MSI is an FDA-approved indication for use of PD-1 checkpoint inhibitor in solid tumors like colorectal cancer, given that there is a stronger response in MSI-high tumors compared to MSI-low tumors[104]. TMB such as changes in DNA damage response genes and DNA polymerase epsilon (*POLE*) and delta (*POLD*) have been investigated as a biomarker in multiple tumor types[103,105,106]. Ang et al. performed a comprehensive genomic profiling of 755 patients with advanced HCC to evaluate the frequency of genomic biomarkers. The most commonly altered genes were *TERT* (44%), *TP53* (35%), *CTNNB1* (31%), *ARID1A* (12%), and *MYC* (12%), with median TMB for the entire cohort being four mutations per megabase[103]. Only 27 (4%) patients had POLE or POLD alterations, and there was no significant correlation between TMB and responders, progressors, or stable disease[103]. High tumor burden is hypothesized to generate elevated neoantigen expression by cancer cells that are not subject to immune tolerance, marking them as targets for clearance by the immune

system[103,107]. The reported prevalence of MSI in HCC is approximately 0.80%–3% and, together with the relatively lower median TMB and lack of DNA repair mutations, provides an explanation for why TMB has failed to predict response in the majority of HCC patients[108].

Signaling Pathways

Next-generation sequencing (NGS) of HCC tumors in patients treated with ICIs (either anti-PD/PD-L1 monotherapy, anti-CTLA-4 monotherapy, or combination anti-PD-1 with LAG-3, KIR, or CTLA-4) has identified alterations in Wnt/ -catenin signaling pathway to be associated with lower disease control rates, shorter median PFS, and OS[109]. WNT/ -catenin alterations may play a role in ICI response in HCC, as this signaling pathway is known to render tumors immunologically cold by way of T-lymphocyte exclusion[110]. Although the authors did not find any other pathways that correlated with responsiveness or resistance to ICI treatment, their work represents an attempt to use NGS to identify potential genetic markers that could improve current HCC treatment. Tumor cell biodiversity has been implicated in patient outcome after treatment with checkpoint inhibitors in HCC and intrahepatic cholangiocarcinoma[111]. In this study, single-cell RNA sequencing of liver cancer specimens from 19 patients treated with ICIs revealed a diverse landscape of TME. Tumors with less intratumoral diversity had a higher OS compared to highly diverse tumors[111]. Additionally, they found that T-cell dysfunction in these tumors was linked to increased VEGF expression, and combination therapy of ICIs with anti-VEGF therapy could potentially improve therapeutic outcomes. This provided the preclinical rationale for the phase III IMbrave150 trial, which compared combination atezolimumab and bevacizumab to sorafenib in patients with unresectable HCC who have not received prior systemic therapy[20]. In this study, 336 patients were treated with atezolimumab at a dose of 1,200 mg plus 15 mg/kg of bevacizumab intravenously, while the sorafenib group received 400 mg twice daily. The coprimary endpoints were OS and PFS by RECIST v1.1. With a median follow-up of 8.6 months, the trial demonstrated statistically significant and clinically meaningful improvement in both OS (median OS for the atezolimumab + bevacizumab group not reached versus 13.2 months in the sorafenib group) and PFS (median PFS of 6.8 months in the atezolimumab + bevacizumab group versus 4.3 months in the sorafenib group[20]. Grade 3–4 adverse events occurred in 57% of patients in the atezolimumab + bevacizumab group versus 55% in the sorafenib group. The safety profile of the combination was similar with known safety profile of each individual drug. This study is practice changing as this combination treatment may soon replace sorafenib as first-line treatment for advanced HCC pending regulatory approval.

Tumor-Infiltrating Lymphocytes

Intratumoral immune cell density have previously been correlated with survival in HCC. High densities of $CD3^+$ and $CD8^+$ T cells both intratumor and along the tumor margin of HCC samples were found to be significantly associated with lower recurrence rates and prolonged recurrence-free survival[112–114]. Additionally, tumor-infiltrating NK cells have also been associated with prolonged survival in HCC[115]. Similarly, higher densities of TILs have been associated with good prognosis in other types of cancers such as colorectal cancer and melanoma[116,117]. These findings suggest that both baseline and posttreatment TIL density could be important markers of response to ICI treatment. Indeed, this has led to the investigation of TILs as a biomarker of response to ICIs in numerous cancers. In patients with melanoma treated with pembrolizumab, Tumeh et al. found that presence of $CD8^+$ TILs, which also was associated with higher PD-1/PD-L1 expression, correlated with radiographic reduction in tumor size[118].

TILs have also been investigated as a biomarker of response to ICIs in HCC. Kaseb et al. found that clinical response in patients who received combination checkpoint inhibitor (nivolumab and ipilimumab) followed by surgical resection of HCC tumors correlated with an increase in $CD8^+$ T-cell infiltration and specifically with two effector T-cell clusters ($CD3^+CD8^+CD45RO^+Eomes^+$ and $CD3^+$ $CD8^+$ $CD45RO^+Eomes^+$ $CD57^+$ $CD38^{low}$ clusters)[119]. However, it is important to note that T-cell infiltration was already present in the tumor prior to treatment, likely due to chronic HCV infection. This study is currently ongoing, and final results may provide insight on the use of TILs as a biomarker. Further work is needed to clarify if background viral infection plays a role in the utility of TILs as a prognostic biomarker in HCC.

Circulating Soluble Factors

Circulating soluble factors such as cytokines have been investigated as biomarkers of response to ICIs. Recently, Feun et al. analyzed several representative circulating biomarkers before treatment and after 60 to 90 days of treatment with pembrolizumab in unresectable HCC patients and at the time of tumor response or disease progression[91]. Using enzyme-linked immunosorbent assay (ELISA), they detected interleukin-1 (IL-1), IL-6, IL-8, IL-12, IL-18, IFN- , TGF- , IL-10, CXCL9, CCL4, CCL5, PD-L1, and PD-L2 from patient plasma. They found that low baseline levels of TGF- were significantly associated with improved OS and PFS after treatment with pembrolizumab. This study is in line with a previous study by Mariathasan et al., which shows that TGF- attenuates tumor response to PD-L1 inhibition by excluding $CD8^+$ effector T cells from the parenchyma[120]. TGF- is known to promote immunosuppression through

various mechanisms such as impaired differentiation or activation of innate and adaptive immune cells, inhibition of cytotoxic T-cell function, and impaired regulation of cytokine production[121]. Taken together, this suggests that TGF- could be used as a predictive biomarker for response to PD-1/PD-L1 blockade.

Epithelial-to-Mesenchymal Transition

The TME has been shown to play a role in the mechanism of resistance to ICIs. Epithelial-to-mesenchymal transition (EMT) has been implicated as a resistance mechanism in HCC. EMT is known to promote immune evasion of cancer cells through Snail signaling[122,123]. Ueno et al. have shown that there is an association between EMT and PD-L1 expression in extrahepatic cholangiocarcinoma[124]. Shrestha et al. analyzed 422 HCC patient samples from the The Cancer Genome Atlas (TCGA) liver cancer database and found that high expression of PD-L1 and EMT markers (vimentin and E-cadherin) was significantly correlated with poor survival[29]. In fact, drugs that inhibit both PD-L1 expression and EMT have been developed for use in non-small cell lung cancer[125]. This correlation between PD-L1 expression and EMT presents an opportunity to investigate EMT as a potential biomarker for ICI response.

OVERCOMING RESISTANCE

Despite the success of ICIs, approximately 85% of HCC patients do not respond to ICIs. Newer approaches to overcome resistance to ICIs are desperately needed. One proposed mechanism of resistance to ICIs is the overexpression of alternate immune checkpoints such as T-cell immunoglobulin, mucin domain-3 protein TIM-3, and LAG-3[126–128]. A study by Thommen et al. showed a positive association between progressive T-cell exhaustion and increased coexpression of these alternate checkpoints in non-small cell lung cancer including BTLA[129]. This work provides a rationale for combination checkpoint therapy to increase efficacy of ICI therapy. In a preclinical study, pembrolizumab in combination with lenvatinib was shown to suppress tumor-associated macrophages, regulatory T cells resulting in decrease in TGF- , IL-10, and downregulation of PD-1 and Tim3[130]. The combination of lenvatinib and pembrolizumab is on fast track designation by the FDA for the treatment of unresectable HCC. Combination therapy involving ICIs and antiangiogenic medications may work synergistically because VEGF-A inhibition increases tumor infiltration and survival of cytotoxic T lymphocytes, thereby producing a favorable microenvironment for ICIs to function[131]. Currently, combination atezoliumab and bevacizumab is also on fast track designation by the FDA for first-line treatment of HCC. Table 2 lists ongoing classes and examples of combination therapies that are being investigated in HCC cancer. In fact, it is possible that immunotherapy in HCC may move to combination triple therapy as one study has shown the efficacy of combination of PD-L1 blockade and CD137 plus OX40 (immunostimulatory agonists) against spontaneous liver cancer in transgenic mice[132]. One of the major limitations of combination therapy is significant immune-related adverse events from the treatment. Although these toxicities are rare, clinicians should always monitor for these events.

Another strategy to improve efficacy of ICIs is by priming adaptive response through treatments that release tumor antigens such as the addition of radiofrequency ablation (RFA), transarterial chemoembolization (TACE), transarterial radioembolization (TARE), stereotactic body radiation therapy (SBRT), or conventional chemotherapy[51]. Adaptive immune response can be primed by vaccines

Table 2. Classes of Therapies in Combination Therapy With Checkpoint Inhibitors in Hepatocellular Carcinoma

Class of Therapies	Examples of Combinations Currently Tested	Rationale
Combination with another checkpoint inhibitor	Anti-PD-1/PD-L1 and CTLA-4; -PD-L1 and TIM-3; -PD-L1 and LAG-3	Inhibition of alternate inhibitory pathway in immune cells; increase number of activated $CD8^+$ T cells[156]
Combination with multikinase inhibitor or antiangiogenic drug	Anti-PD-1/PD-L1 with sorafenib; Anti-PD-1/PD-L1 with levantinib; Anti-PD-L1 and apatinib; Anti-PD-L1 with bevacizumab (IMbrave150)	Reduces immunosuppressive Tregs and MDSCs; antiangiogenic properties may increase tumor hypoxia and enhance expression of immune checkpoint molecules[157]
Combination with local therapy	Anti-PD-1 with TACE; Anti-CTLA-4 with RFA/TACE	Enhanced immune cell activation and recruitment[158]; upregulation of soluble PD-L1[159]
Combination with oncolytic virus	Anti-PD-1 with Pexa-Vec (JX-594)	Promotion of NK and T-cell tumor infiltration[160]
Combination with polypeptide	Anti-PD-1 with DSP-7888 (NCT03311334)	Polypeptide HCC vaccine to expand preexisting neoantigen-specific T-cell population
Combination with antibiotics	Anti-PD-1 with vancomycin and tadalafil (NCT03785210)	Oral antibiotic alters gut commensal bacteria inducing antitumor effect

that use tumor-specific peptides to increase antigen presentation[133]. In a preclinical study, Chen et al. evaluated the effect of microwave ablation of subcutaneous hepatoma followed by combination intratumoral microspheres encapsulating GM-CSF and anti-CTLA-4 administration[34]. They found that this combination therapy resulted in local eradication of tumors and, surprisingly, led to rejections of tumors following rechallenge, including distant metastasis[34]. Radiation therapy has previously been shown to have a synergist effect in combination with ICIs[134]. There are several ongoing clinical trials (NCT03143270 and NCT01853618) looking at combination ICIs with local therapy (TACE or RFA)[35]. Tremelimumab in combination with tumor RFA leads to accumulation of intratumoral $CD8^+$ T cells and is a potential treatment combination for patients with advanced HCC[35]. The results of these studies may shed light on another therapeutic option to improve the efficacy of ICIs in HCC.

T cell stimulation by delivering an oncolytic virus into the tumor can promote tumor infiltration and maturation of T and NK cells[135]. The oncolytic virus, which can sometimes be genetically modified, selectively targets and kills tumor cells in addition to stimulating the host's immune system[136,137]. In melanoma, tamilogene laherparepvec (T-VEC) was the first approved oncolytic virus to be used in combination with ipilimumab or pembrolizumab and was found to have better efficacy than with monotherapy ICI[138]. JX-594, an oncolytic poxvirus, has been tried in patients with refractory primary and metastatic liver cancer and was found to be well tolerated[139]. There is an ongoing phase I/IIa clinical trial (NCT03071094) looking at the safety and efficacy of combination nivolumab with oncolytic viral therapy (Pexa-Vec) in advanced HCC.

The importance of gut microbiota in modulating key processes of inflammation and immunity has been a focus of recent studies. Alterations in the gut microbiota have been implicated in the progression of chronic liver disease and in the development of HCC[140,141]. Some studies have highlighted the relationship between gut microbiota and response to treatment with ICIs[142,143]. Sivan et al. found that oral administration of *Bifidobacterium* in combination with anti-PD-L1 therapy improved tumor control by increasing the accumulation of $CD8^+$ T cells in melanoma[142]. It is unclear whether gut microbiota has a role in ICI response in HCC. There is an ongoing phase II trial (NCT03785210) looking at combination oral vancomycin, tadalafil, and nivolumab in patients with advanced HCC. The result of this study will hopefully shed light on the efficacy of altering the gut microbiome in patients with HCC who are on checkpoint inhibition.

FUTURE PERSPECTIVES

Since its introduction, ICI therapy has significantly changed the treatment of numerous malignancies especially in situations where there have been fewer alternatives. Despite its success in various malignancy, only very few patients with advanced HCC benefit from checkpoint inhibition. Understanding the mechanism of resistance and proper patient selection will hopefully provide better treatment results. The future of ICIs is reassuring as there are many ongoing clinical trials that could discover better biomarkers of response, combinatory treatments, and uncover newer methods of overcoming resistance to immunotherapy.

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