

# Impact of Viral Etiologies on the Development of Novel Immunotherapy for Hepatocellular Carcinoma

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## Abstract

Hepatocellular carcinoma (HCC), is the most common type of liver cancer which is derived mostly from the background of chronic inflammation. Chronic hepatitis viral infection remains one of the most common etiologies implicated in chronic liver inflammation, cirrhosis, and HCC. With such background inflammation, immunotherapy—particularly the checkpoint inhibitors—have been tested in HCC patients with unprecedented success. However, despite the initial enthusiasm, the response rate to immunotherapy remains modest in most clinical trials (approximately 20%), with mixed reports on response rates in hepatitis viral-related HCC as compared with nonviral HCC. Given such complexity in response to immunotherapy, it is increasingly appreciated that deeper understanding of the tumor molecular features and tumor microenvironment of hepatitis viral-related HCC is crucial for the design of more effective immunotherapeutics. We discuss herein the current knowledge in tumor genomic mutational and immune landscapes as well as the ongoing immunotherapy trials in HCC with the unique focus on their viral etiologies. Based on this understanding, we also outline perspectives and rationale on the design of potential immunotherapeutic strategies in HCC patients according to their viral etiologies.

## Keywords

- ▶ hepatitis virus infection
- ▶ hepatocellular carcinoma
- ▶ immunotherapy
- ▶ tumor microenvironment
- ▶ viral-related HCC

Hepatocellular carcinoma (HCC) remains the fifth most commonly diagnosed cancer and second leading cause of cancer deaths for males worldwide.<sup>1</sup> Viral hepatitis remains one of the major etiologies for HCC, particularly hepatitis B virus (HBV) chronic infection, which has a high prevalence in Asia and Africa coinciding with the high incidence of HCC in these areas.<sup>2</sup> Currently, chronic infection with hepatitis C virus (HCV) is one of the main cause of HCC in the Western world.<sup>1</sup> Besides that, hepatitis D virus (HDV) co-infection with chronic HBV has also received increased awareness and has been shown to significantly increase the risk of HCC.<sup>3</sup> Development of HCC in the background of chronic inflammation, particularly that driven by chronic hepatitis virus infection, has been reported to be a result from an immunosuppressive and exhausted tumor microenvironment (TME). This makes viral-related HCC a potential candidate for immunotherapy, which aims to reactivate the exhausted local antitumor immunity. However, many challenges remain for

this strategy. Recent reports on clinical outcome from the immunotherapy trials, mostly involving checkpoint inhibitors (immune checkpoint blockades), have been modest.

Given the complexity and dynamic interaction between the tumor cells and immune microenvironment, this review aims to explore the current understanding on viral-related HCCs, based on its unique tumor mutational landscape versus immune microenvironment and its impact on the development of novel immunotherapeutic strategies.

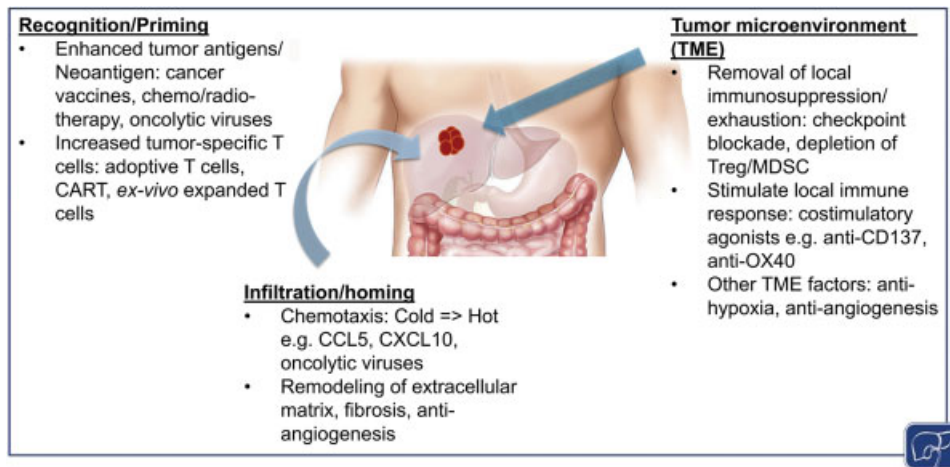
## Understanding HCC Based on Viral Etiologies

As immunotherapy is based upon the basic principle of the immune system recognizing and targeting the tumor cells as foreign, understanding both the tumor antigenicity and the immune microenvironment is essential for a successful immune-based therapy. Are the tumor antigens immunogenic in HCC? Are they being presented and recognized? Is

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**Fig. 1** Elements of a successful immunotherapeutic strategies. Three key elements of successful immunotherapeutic strategies with enhanced antitumor immunity include: (1) to enhance recognition of tumor antigens or the priming of immune system, i.e., using T-cell therapy; (2) to stimulate cytotoxic immune cells infiltration or homing to the tumor mass such as using therapies enhancing T-cell chemoattractants; and (3) to modulate the tumor microenvironment such as using check-point inhibitors to reverse the immune exhaustion in the tumor.

there sufficient tumor infiltration of cytotoxic immune cells and are they able to target and kill the tumor cells? Is the immune microenvironment conducive for immunological cytotoxicity against the tumor cells? Many of these areas are essential and can be modulated to ensure a successful immunotherapeutic strategy (► Fig. 1). As it is a complex ecosystem, tailoring a successful immunotherapy is more challenging than one would have thought. We would herein discuss these aspects of immunotherapy, starting from tumor mutational landscapes to immune microenvironment, in the highly heterogeneous HCC particularly focusing on viral etiologies.

## Mutational Landscapes in Viral-Related HCCs

As mentioned, one of the key principles of immunotherapy is the recognition of tumor antigens by the immune system as foreign.<sup>4</sup> In the case of viral-driven HCC, the recognition of viral-related antigens or the antigens derived from the viral-induced mutations could then serve as a basis for immunotherapy.

Hepatitis B virus is a double-stranded DNA virus which integrates with the host human genome while HCV is an enveloped single-stranded RNA virus which does not show DNA integration.<sup>5</sup> HBV viral proteins that could be expressed during its replication cycle include hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg), and hepatitis B X antigen (HBxAg).<sup>6</sup> For instance, it was previously proposed that HBV-encoded HBx protein plays multiple oncogenic role associated to hepatocarcinogenesis.<sup>7</sup> It was however, long speculated that HBV-related proteins may not be a good tumor antigen target due to the clearance of hepatitis-related proteins observed in HBV-related HCC.<sup>8,9</sup> However, recent studies have demonstrated that it is possible to target HBV-human chimeric proteins produced as a result from HBV-human genome integration.<sup>10,11</sup> In fact, recent attempts to treat HBV-related HCC by targeting HBV antigen resulted in promising benefit.<sup>12,13</sup>

On the other hand, HCV viral genomic RNA encodes for a polyprotein, which is processed by host and viral proteases into at least 10 different proteins.<sup>14</sup> There is currently no HCV vaccine available due to many challenges in vaccine development including high variability of HCV virus and the lack of suitable in vitro and in vivo models.<sup>15</sup> Henceforth, an HCV antigen targeting immunotherapy for HCV-related HCC would most likely face similar challenges. Instead, HCV infection was traditionally treated with interferon-based therapy,<sup>16</sup> until recent development of direct-acting antiviral (DAA) agents with high efficacy and safety profile.<sup>17,18</sup> However, HCV eradication was controversial,<sup>19</sup> following a series of reports on unexpected higher incidence or recurrence of HCC in DAA-treated HCV patients.<sup>20–22</sup> Although it was later concluded that patient selection may play a critical role.<sup>23</sup> There is also currently limited HDV-targeted therapy available apart from general antiviral strategy. Therefore, the most promising strategy of targeting viral-specific antigens for the treatment of HCC will be on HBV-specific antigens.

It is also not known if any of the viral-driven mutations lead to expression of immunogenic antigens which could be targeted by immunotherapy. For instance, several genes were reported to be altered due to HBV-DNA integration events such as putative cancer-related *TERT*, *MLL4*, and *CCNE1* genes.<sup>24</sup> A more recent comprehensive genomic atlas of HCC reported on two major HBV integration sites with recurrent mutations: *MLL4* and *TERT*; while HCV-related HCC displayed higher frequency of *CDKN2A* promoter silencing and *TERT* promoter mutation.<sup>25</sup> In addition, *EGF* was also implicated in HCV cirrhosis and HCV-related HCC<sup>26,27</sup> while immune-related gene *Interleukin-1 $\beta$*  (IL-1 $\beta$ ) was reported to be associated with HCC in Japanese patients with chronic HCV infection.<sup>28</sup> These studies delineate genetic alterations in hepatitis viral-related HCC; however, there were no specific studies to determine how antigenic these hepatitis viral-related mutations are.

Furthermore, it was reported and further validated that HBV-induced tumorigenesis is in fact a result from the immune-mediated liver damage<sup>29–31</sup> or dysfunction of

cytotoxic immune cells, rather than as a result of these viral-related oncogenes.<sup>32</sup> Several genome-wide association studies have also linked multiple HBV-induced genes to immune functionality such as *HLA* variants, *MICA*, *CTLA4*, and *IL-6* in the predisposition and prognosis of patients with HBV-associated HCC.<sup>33–36</sup> Likewise, HCV-induced inflammation and eventual immune dysfunction both play a major role in carcinogenesis of HCV-related HCC.<sup>37</sup> A genome-wide association study in HCV-induced HCC identified a susceptibility locus in the 5' flanking region of *MICA* that was associated with lower soluble MICA proteins levels and development of HCC from chronic HCV infection.<sup>38</sup> *MICA*<sup>38</sup> binds to NKG2D and results in the activation of NK cells and T cells.<sup>39</sup> Its downregulation in both HBV and HCV-related HCC would indicate a mechanism of hepatitis viral-induced immunosuppression. It is therefore likely that the immunosuppressive microenvironment of viral-related HCC would make a better therapeutic target as opposed to the viral-specific mutations or antigens.

Besides specific mutations, it was previously reported that total mutational burden could be an indication of response to the checkpoint blockade in multiple cancer types.<sup>40,41</sup> The total mutational burden of HCC is moderate, considerably higher than pancreatic or prostate cancer but lower than major cancers like lung cancer and melanoma, both of which respond better to the checkpoint inhibitor than HCC.<sup>42</sup> In fact it was reported that HBV-related HCC harbors lower total mean mutation rate than that of non-HBV-related HCC whereas there was no significant difference in the pattern of somatic mutations in HBV-, HCV-, and non-HBV/non-HCV-related HCC.<sup>43</sup> This may indicate a lower response rate in HBV-related HCC, which in fact was reported to be the case in CheckMate040 clinical trial using anti-PD-1 antibody nivolumab in advanced HCC (→ **Table 1**).<sup>44</sup> However, given the heterogeneous nature of HCC with multiple different molecular classifications<sup>45</sup> as well as intratumoral heterogeneity,<sup>46</sup> it remains challenging to generalize on the response to immunotherapy based on the current understanding of genomic landscape of viral-related HCC. Indeed, deeper understanding in the tumor immune microenvironment would be essential to gain better insights in this complex mechanism.

## Tumor Immune Microenvironment

The critical role of immune microenvironment in HCC progression or disease prognosis was previously discussed.<sup>42,47–49</sup> Most of the immunoprofiling of TME in viral-related HCC was performed compared to healthy livers or the non-HCC chronic viral-infected livers.<sup>50</sup> For instance, Treg accumulated in TME of HBV-related HCC, as compared with nontumor and healthy liver controls, has been shown to associate with impaired CD8+ T cells, disease progression, and poor survival in HCC patients.<sup>51</sup> In fact, the role of Treg in maintaining immune tolerance during chronic HBV infection has been previously shown<sup>52</sup> and its accumulation was linked to HBV-induced TGF- $\beta$ -miR-34a-CCL22 signaling pathway.<sup>53</sup> Dysfunctional CD8+ T cells with upregulation of multiple exhaustion

markers such as PD-1, Tim-3 or Lag-3 were also previously described in chronic HBV infection<sup>54</sup> or HBV-related HCC.<sup>55,56</sup> It was reported that HCV-specific CD8+ T cells express various inhibitory receptors, including CTLA-4 and PD-1 with enhanced exhaustion phenotype that could be reactivated by PD-1/PD-L1 blockade.<sup>57,58</sup> Whereas, the immunosuppressive function of Treg could be fine-tuned by OX40 in HCV-infected liver tissues.<sup>59</sup>

Dysfunction of other lymphocytes such as the reduced cytotoxicity of NK cells was also reported during chronic hepatitis viral infection.<sup>60</sup> It was previously speculated that NK is more dysfunctional in chronic HBV as compared with chronic HCV infection.<sup>61</sup> However, MICA mutation, which is important for T and NK cells activation,<sup>34,38</sup> was detected in both HBV and HCV-related HCC. In fact, NK cells impairment was previously shown in both HBV/HCV-related HCC,<sup>62</sup> rendering NK cell therapy an attractive option for viral-related HCCs (→ **Fig. 2**). As for myeloid subsets, the resident macrophages in liver, the kupffer cells, were known as one of the important immune cells involved in multistep antiviral immunity and disease pathogenesis during HBV and HCV infection.<sup>63</sup> It involves a cascade of events with IL-6 and TNF $\alpha$ -driven chronic inflammation and compensatory proliferation as well as oxidative stress which eventually drives the process of carcinogenesis.<sup>64,65</sup> Such viral-driven, chronic, and unresolved inflammation is in fact a well-recognized mechanism in HCC development and progression.<sup>66</sup> In fact not only the inflammatory functions of these macrophages, the tolerogenic and suppressive M2 macrophages were also shown to be important in tumor progression and invasiveness via CCL22-mediated epithelial-mesenchymal transition.<sup>67</sup> More importantly, it was also shown that macrophages could impair T-cells immunity via enhanced PD-L1 expression in HCC tumors.<sup>68</sup>

A recent high dimensional immunoprofiling of TME with cytometry by time-of-flight comparing HBV-related versus nonviral hepatitis-related HCC demonstrated higher density of intratumoral immunosuppressive Treg and T<sub>RM</sub> in HBV-related HCC.<sup>69</sup> This is consistent with previous studies that have identified higher Treg and T<sub>RM</sub> numbers in chronic HBV infection.<sup>70,71</sup> The same study also reported relatively lower proportions of CD244 expressing cytotoxic NK cells in HBV-related HCC compared with nonviral-related HCC.<sup>69</sup> Indeed, reduced CD244 has been previously reported to associate with NK cells dysfunction and immune tolerant in chronic HBV infection.<sup>72</sup> Most importantly, PD-1 expression was enhanced in both Treg and T<sub>RM</sub> in HBV-related HCC, consistent with a virus-induced immunosuppressive or exhausted TME.<sup>69</sup> In contrast, others however, described no significant difference in PD-L1 and LAG-3 expression comparing uninfected (14/29 patients) versus virus-infected (HBV: 1/29 and HCV: 14/29 patients, respectively) HCC.<sup>40</sup> Given the low number of HBV-HCC case in this study, it is hard to conclude if PD-1/PD-L1 pathways were indeed affected. A bigger cohort study will be needed to distil the differences in immune landscapes of viral versus nonviral HCC.

Overall, chronic viral hepatitis induced a chronic but yet dysfunctional, liver inflammation which eventually led to the

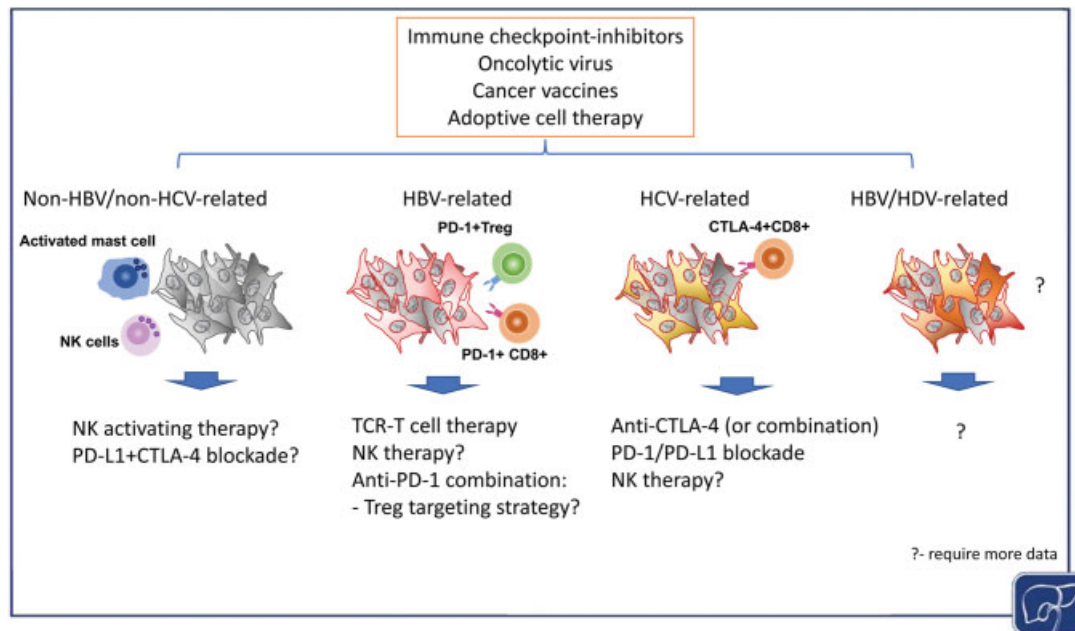
**Table 1** Major clinical trials using immunotherapy in HCC and their analyses according to viral etiologies

Study name	Phase	Target	Treatments	Estimated enrolment <sup>a</sup>	Overall response rate (ORR) <sup>b</sup>			
					All patients	HBV	HCV	Nonviral
NCT01658878 (CheckMate040)	I/II	PD-1	Nivolumab	214	20% (42/214 patients)	14% (7/51 patients)	20% (10/50 patients)	21% (12/57 uninfected progressor); 23% (13/56 uninfected untreated/intolerant)
NCT01658878 (CheckMate040)	I/II	PD-1	Nivolumab (Asian cohort analysis)	85 (Asian cohort)	15% (13/85 patients)	13% (6/47 patients)	14% (2/14 patients)	21% (5/24 patients)
NCT01658878 (CheckMate040)	I/II	PD-1	Nivolumab (Asian cohort analysis)	182 (Intent-to-treat cohort)	14.3% (26/182 patients)	12% (7/58 patients)	20% (7/35 patients)	13% (12/89 patients)
NCT01658878 (CheckMate040)	I/II	PD-1 + CTLA-4	Nivolumab + Ipilimumab	148	<sup>a</sup> Arm A: 32% (16/50 patients); Arm B: 31% (15/49 patients); Arm C: 31% (15/49 patients).	TBA	TBA	TBA
NCT02576509 (CheckMate459)	III	PD-1	Nivolumab vs. Sorafenib	726	Sorafenib-experienced: ORR 19% and Sorafenib-naive: ORR 24%. OS: <i>p</i> = 0.0752; HR = 0.85 [95% CI: 0.72–1.02]	TBA	TBA	TBA
NCT02702414 (Keynote224)	II	PD-1	Pembrolizumab	104	17% (18/104 patients)	13% (5/39 patients)	20% (13/64 patients)	20% (13/64 patients)
NCT02702401 (Keynote240)	III	PD-1	Pembrolizumab vs. placebo	413	16.9% (70/413 patients)	TBA	TBA	TBA
NCT01693562	I/II	PD-L1	Durvalumab	39 (HCC only)	10% (4/40 patients)	0% (0/10 patients)	25% (2/8 patients)	9.1% (2/22 patients)
NCT01008358	II	CTLA-4	Tremelimumab	20	17.6% (3/17 all HCV patients)	NA	17.6% (3/17 patients)	NA
NCT02519348	I/II	PD-L1 + CTLA-4	Durvalumab + Tremelimumab	545	15% (6/40 patients)	0% (0/11)	0% (0/9 patients)	30% (6/20 patients)
NCT03298451 (HIMALAYA)	III	PD-L1 + CTLA-4	Durvalumab + Tremelimumab	1,310	TBA	TBA	TBA	TBA
NCT03755791 (COSMIC-312)	III	PD-L1 + TKI	Cabozantinib + Atezolizumab	740	TBA	TBA	TBA	TBA
NCT03434379 (IMbrave150)	III	PD-L1 + VEGF inhibitor	Atezolizumab + Bevacizumab	480	TBA	TBA	TBA	TBA

Abbreviations: CTLA-4, cytotoxic T-lymphocyte-associated protein 4; HCC, hepatocellular carcinoma; HR, hazard ratio; OS, overall survival; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor.

<sup>a</sup>Based on information listed in ClinicalTrials.gov.

<sup>b</sup>Arm A: Nivolumab 1 mg/kg + Ipilimumab 3 mg/kg Q3W (four doses); Arm B: Nivolumab 3 mg/kg + Ipilimumab 1 mg/kg Q3W (four doses), each followed by Nivolumab 240 mg Q2W, or Arm C: Nivolumab 3 mg/kg Q2W + Ipilimumab 1 mg/kg Q6W.



**Fig. 2** Immunotherapy in hepatocellular carcinoma (HCC) with perspectives on viral hepatitis-related HCC. General immunotherapies tested in viral- and nonviral-related HCCs include immune checkpoint-inhibitors, oncolytic virus, cancer vaccines, and adoptive cell therapy with various success. The previously reported microenvironments of viral-related and nonviral-related HCC were summarized based on enriched immune-subsets. Suggested immunotherapies were listed below based on the current knowledge on their respective microenvironments even though more data are required in some of these areas (marked with a question mark).

development of HCC. The resulting immune exhaustion and multiple layers of immune dysregulation provide unique opportunity for immunotherapeutic intervention. Despite this, the complex immune landscapes and interaction between different immune subsets hindered the success of one single immunotherapeutic agent. This is indeed reflected in the seemingly lower response rate of HBV-related HCC to checkpoint blockade (► **Table 1**). Furthermore, given the complex etiopathologies of HCC including alcohol-induced and nonalcoholic fatty liver disease (NAFLD), most of which also induce a chronic inflammation in the liver, it will hence remain challenging to clearly define the immune landscapes of HCC according to its etiologies.

### Potential Novel Immunotherapies Based on Viral Etiologies in HCC

With the success of phase II clinical trials using checkpoint inhibitor anti-PD-1 in HCC (CheckMate040 and Keynote224), there has been increasing interest and use of immunotherapy in HCC.<sup>44,73</sup> Despite the fact that phase III Checkmate459 and Keynote240 did not reach predetermined endpoints, the clinical benefits of immunotherapy in HCC were well accepted in the field. Several immunotherapeutic strategies for HCC treatment, in particular the combination checkpoint-inhibitor immunotherapy, are currently under various phases of ongoing clinical trials. Besides that, several other immunotherapies such as adoptive cell therapy and cancer vaccine are also in different phases of development. We will discuss these immunotherapies in the light of hepatitis-related HCC, based on the limited data available.

### Engineered T-Cell Therapy

T cell engineered to express chimeric antigen receptors (CARs) or autologous T cells expanded and engineered ex vivo with specific targeted tumor antigen(s) have been used for the treatment of HCC. For instance, there are several ongoing phase I or phase I/II trials using CAR-T cells directed against GPC-3, CEA, or Mucin 1 in various solid tumors including HCC.<sup>74</sup> It was also shown that T cell could respond to HLA-A2-restricted tumor antigens such as NY-ESO-1 and SSX-2.<sup>75</sup> T-cell therapy targeting HCC-specific antigen such as  $\alpha$ -fetoprotein (AFP) was explored previously without much success (NCT03349255). Indeed, most of the tumor-specific T cells had low affinities and expressed higher PD-1 T-cell exhaustion marker.<sup>75</sup> The highly heterogeneous nature of HCC cells also suggests that immunotherapy targeting a single tumor antigen would likely not be successful.

On the other hand, targeting HBV-related antigen in HBV-related HCC is challenging as chronic HBV infection often results in HBV proteins clearance<sup>8,9</sup> and HBV-DNA integration or insertion into host genome leads to defective or small fragments of HBV antigens expression.<sup>76,77</sup> HBV-specific T-cell responses were rarely detected in HCC patients<sup>75</sup> and HBV therapeutic vaccine composed of HBsAg with a proprietary adjuvant showed little success in clearing chronic HBV infection.<sup>78</sup> This, however, could be controversial and it was demonstrated that HBV can actually be treated with ex vivo expanded autologous T cells genetically modified to express HBsAg-specific T-cell receptor.<sup>12</sup> Even though it was demonstrated in just one case with end-stage metastatic HCC that expressed HBsAg who also received transplanted non-HBV liver, the team has confirmed its feasibility, safety as



well as significant reduction in HBsAg levels.<sup>12</sup> In fact, most of the HBV-DNA integration is incomplete and the insertion of viral DNA into the human genome results in the expression of HBV-human chimeric proteins.<sup>10,11</sup> A recent study by Tan et al demonstrated a proof of principle that T-cell therapy could be tailored to target antigens from integrated HBV as a personalized TCR T-cell immunotherapy for HBV-related HCC.<sup>13</sup> In this study, it was shown that HBV-related HCC tumor cells expressed antigenic epitopes translated from the short integrated HBV mRNAs, that could be detected by and resulting in T-cell activation.<sup>13</sup> Autologous T cells were engineered to express the selected TCRs specific for epitopes of HBV-DNA from metastases and were adoptively transferred to two HCC patients with recurrence after liver transplantation. This personalized T-cell therapy is proven to be safe in both patients with one patient showing reduction in volume of metastases during the treatment.<sup>13</sup>

For chronic HCV infection, a CAR-T against HCV targeting the HCV/E2 glycoprotein was previously constructed to control HCV infection and it was capable of secreting antiviral and pro-inflammatory cytokines and lysing HCV-infected hepatocytes in vitro.<sup>79</sup> However, there is currently no clinical trial with such HCV-targeting CAR-T therapy and the efficacy in a clinical setting for HCV-related HCC remains to be tested. Therefore, there is a stronger current evidence support T-cell therapy in HBV-related HCC but not in HCV-related or nonviral-related HCC (►Fig. 2).

### Other Adoptive Cell Therapies

Other forms of adoptive cell therapies (ACT) have been explored as immunotherapies for HCC and most of these therapies are not targeted or specific to any particular tumor antigens in HCC. For instance, treatment in 150 HCC patients with adoptive immunotherapy using autologous TILs, expanded and activated with IL-2 in vitro, demonstrated improved recurrence-free survival (RFS) after resection.<sup>80</sup> However, the technical challenge of isolating TILs as well as lack of specificity of the whole population of TILs without selection may limit the success of such approach.

Alternatively, cytokine-induced killer (CIK) cells, a heterogeneous cytotoxic immune population including CD8+ T cells, NK cells, and CD3 + CD56+ NKT cells, were also tested on HCC patients. CIK cells are expanded from autologous peripheral blood mononuclear cells stimulated with a cocktail of cytokines inclusive of IL-1, IL-2, IFN $\gamma$ , and anti-CD3 antibody. It was first demonstrated to be safe with lower recurrence rate and improved RFS in 76 treated versus 74 control HCC patients.<sup>80</sup> Subsequently two other randomized phase III trials using CIK therapy have been shown to provide longer median time to recurrence<sup>81</sup> or improved RFS and overall survival (OS).<sup>82</sup>

Another ACT using NK cells has also been explored as HCC immunotherapy, based on the rationale that NK cells are dysfunctional in HCC and tumor-infiltration with activated NK cells is associated with superior HCC patient survival.<sup>47,60,83</sup> There is only one complete feasibility and safety study using adoptive NK cells extracted from cadaveric donor

liver perfusate for liver transplant recipients with HCC, which demonstrated reduced recurrence rate.<sup>84</sup> Several other NK cell therapy trials in HCC are currently ongoing (clinicaltrials.gov). The latest development of engineered NK cells or CAR-NK cells providing tumor specificity has promising future for NK cell therapy for cancers including HCC.<sup>85</sup> Given the lower density of NK cells in HBV-related HCC compared with NBNC-HCC<sup>69</sup> and the impairment of NK cells in viral-related HCC,<sup>62</sup> it remains to be determined if adoptive NK therapy could actually enhance the clinical outcome in both HBV- and HCV-related HCCs (►Fig. 2). Last but not least, NK cell activating strategies such as using IL-12, IL-15, or type I interferons, would be worth exploring in nonviral-related HCC, given they were reported to be relatively more functional and abundant compared with the viral-HCC<sup>62,69</sup> (►Fig. 2).

### Oncolytic Virus Therapy

As an important organ receiving blood and nutrients from the guts and the center of detoxification, liver is known as an immune-tolerogenic organ.<sup>86</sup> It is highly tolerogenic toward gut-derived bacterial metabolites entering the liver via the portal vein<sup>87</sup> but was previously shown to illicit a robust antiviral immune response.<sup>88</sup> For instance, it was shown that HCC progression could be controlled with the TLR3 activation, a viral-related innate immune response.<sup>83</sup> Oncolytic virus therapy which show selective infection and killing of tumors cells has also been explored.<sup>89</sup> These viruses can also be engineered to express genes like GM-CSF, an immunostimulatory cytokine which could enhance antitumor immunity by stimulating antigen-presenting cells such as dendritic cells and promoting infiltration and maturation of immune cells like NK cells and T cells.<sup>90</sup> Oncolytic virus therapies were tested in preclinical trials and a few of them were tested on phase I or II clinical trials for HCC.<sup>91</sup> One of them showed that the use of JX-594, an engineered Vaccinia virus with thymidine kinase-deactivated, was well tolerated<sup>92</sup> and demonstrated promising outcome associated with high viral dose in phase II clinical trial on HCC patients.<sup>93</sup> A randomized phase III trial comparing JX-594 followed by sorafenib versus sorafenib in patients with advanced HCC (PHOCUS) (NCT02562755) started in late 2015 but was recently recommended to stop after the interim futility analysis, which predicted that the study is unlikely to meet its primary objective of overall survival by the time of the final analysis. This decision was not related to the safety of the investigational product as the therapy was generally well tolerated by the patients. Despite that, other than direct killing and immunomodulating properties, oncolytic virus could also induce tumor cell death (“immunogenic cell death”) and the release of tumor antigen which further enhances the antitumor immunity. However, given the immunosuppressive microenvironment of HCC, it is most likely that the success of oncolytic virus could be enhanced in combination with immune checkpoint blockades. Indeed, several clinical trials using the combination of oncolytic virus and immune checkpoint inhibitors are ongoing, including in advanced HCC.<sup>94</sup>

There is currently no information on whether oncolytic virus would work better or worse in viral-related HCC. However, oncolytic virus JX-594 has been shown to have an effect on suppression of hepatitis B viral genomes.<sup>95</sup>

## Cancer Vaccine

Cancer vaccines, either in the form of peptide, dendritic cell-pulsed with synthetic peptide, or RNA vectors, have been used in therapeutic setting based on the principle that immune system could recognize these cancer antigens as foreign. Cancer vaccine development in HCC has been challenging and met with limited success.<sup>96</sup> This is perhaps mostly due to the highly immunosuppressive microenvironment and highly heterogeneous HCC hampering the possibility of a single targeted cancer antigen. Several target cancer antigens for HCC include tumor-associated antigens such as NY-ESO1, glypican-3, and AFP. For instance, first HCC vaccine clinical trial against AFP showed limited clinical benefits despite detectable T-cell response.<sup>97,98</sup> Phase I cancer vaccine studies targeting GPC-3 have proven safe and achieved posttherapeutic immunogenicity; however, clinical endpoint of relapse prevention after curative treatments (surgery or radiofrequency ablation) was not achieved in phase II studies with GPC-3.<sup>99,100</sup>

The failure of single targeted cancer vaccine could be due to the heterogeneous nature of HCC. In fact, cancer vaccines based on personalized neoantigens have demonstrated promising outcome in Melanoma patients.<sup>101,102</sup> Currently, there is one ongoing therapeutic cancer vaccine single-arm clinical trial using therapeutic cancer vaccine IMA970A, a multi-peptide-based HCC vaccine composed of 16 newly discovered and overexpressed tumor-associated peptides (TUMAPs) directly identified from resected HCC tissues (clinical trial: NCT03203005). It remains to be determined if such multi-peptide cancer vaccines in HCC will be a success. However, given the immunosuppressive microenvironment of HCC, it is likely that combination with other immunotherapy will be necessary to achieve superior outcome.

Cancer vaccine based on viral antigen was designed for cancer prevention rather than therapeutic. Previous attempt to treat chronic HBV infection using HBV vaccine has failed<sup>78</sup> and the benefit of HCV eradication in HCC was controversial.<sup>20-22</sup> The benefit of targeting viral antigen in viral-related HCC is therefore uncertain. On the other hand, it was also not known if any other viral-induced mutational antigens are antigenic enough for HCC tumor killing as discussed above. Therefore, cancer vaccines specific to viral-related HCC are currently not under active development. Instead, personalized cancer vaccines based on neoantigens prediction, which was tested in melanoma,<sup>101,103</sup> could hold promising future for the treatment of HCC.

## Immune Checkpoint Blockade

Upon the FDA approval of nivolumab and pembrolizumab as second-line treatment for advanced HCC, there has been an active development of immune checkpoint blockades

(ICBs) in HCC.<sup>44,73</sup> A list of the major trials involving ICBs with viral etiologies analyses is summarized in ► **Table 1**.

### Anti-PD-1 Monoclonal Antibodies

Immune checkpoints are pathways that inhibit the immune response to maintain self-tolerance and regulate the duration and amplitude of immune responses. Immune checkpoint molecules like PD-1 or PD-L1 expression are upregulated in many cancers, exploited by tumor cells to escape immune surveillance during the cancer development. In fact, it has been previously demonstrated that higher PD-1/PD-L1 is associated with poorer progression-free and overall survival after resection of primary HCC.<sup>104</sup> Higher PD-1 expression on the CD8+ T cells is also associated with tumor progression and poor prognosis.<sup>48,105</sup> Furthermore, attempts of using anti-PD-1 as antiviral therapy for chronic HBV infection have been described to partially restore HBV-specific CD8+ T cell functions.<sup>106,107</sup> Based on this rationale, blocking PD-1/PD-L1 has since become a well-received immunotherapeutic option in HCC.

Two previously completed key phase I/II clinical studies in HCC using anti-PD-1 monoclonal antibodies, nivolumab, and pembrolizumab are CheckMate040 and Keynote224, respectively.<sup>44,73</sup> CheckMate040, a phase I/II, open-label, noncomparative, dose escalation and expansion trials using nivolumab for the treatment of HCC patients previously treated with sorafenib, yielded an objective response rate (ORR) of 20% and disease control rate of 64%.<sup>44</sup> Interestingly, it was reported that the ORR of HBV-positive cases was lower (14%) compared with HCV-positive (20%) and nonviral-related cases (21–23%) (► **Table 1**).<sup>44</sup> A subsequent Asian cohort analysis demonstrated an ORR of 15% with less difference across viral etiologies (► **Table 1**: HBV-related ORR: 12–13% vs. HCV-related ORR: 14–20% or nonviral-related ORR: 13–21%).<sup>108</sup> The third arm of analysis from CheckMate 040 using nivolumab and ipilimumab combination therapy has first been announced in Asco 2019<sup>109</sup> and the analysis according to viral etiologies will be available in the near future. On the other hand, the open label phase II Keynote224 trial that assessed the efficacy and safety of pembrolizumab in patients with advanced HCC previously treated with sorafenib had demonstrated an ORR of 17% and disease control rate of 62%.<sup>73</sup> Analysis based on hepatitis viral-related HCC: both HBV- (21% of the total cohort) and HCV- (25% of the total cohort) related cases again demonstrated a lower response rate in viral-related cases (ORR of 13%) versus the uninfected cases (ORR of 20%)<sup>73</sup> (► **Table 1**). 15 or 25% of the patients experienced greater than grade 3 treatment-related adverse events such as rash, pruritus, and increase in liver enzymes such as aspartate aminotransferase increase and alanine aminotransferase (ALT) from the Keynote224 and CheckMate040 trial, respectively.<sup>44,73</sup> The rate of adverse events was as high as 37% in nivolumab and ipilimumab combination therapy.<sup>109</sup> It is however, not known if HCC patients with viral hepatitis may experience more adverse events.

The complete reports from two phase III trials involving nivolumab (CheckMate459, NCT02576509) and pembrolizumab (Keynote240, NCT02702401) are still pending despite announcement on the failure to meet predetermined end

points of OS. As announced by Bristol-Myers Squibb, CheckMate459 evaluating nivolumab versus sorafenib as a first-line treatment in patients with unresectable HCC did not meet its prespecified primary endpoint of OS (hazard ratio [HR] = 0.85 [95% CI: 0.72–1.02];  $p = 0.0752$ , **Table 1**) despite showing a clear trend toward improved OS by nivolumab compared with sorafenib, a current standard of care. The ORR according to mRECIST was reported to be around 20% similar to the outcome from CheckMate040. Keynote240, on the other hand, is a phase III, randomized, double-blind trial evaluating pembrolizumab compared with placebo in patients with advanced HCC who were previously treated with systemic therapy. According to announcement in ASCO 2019, this trial too did not meet the predetermined primary end points of improved OS (HR: 0.78; one-sided  $p = 0.0238$ ) and PFS (HR: 0.78; one-sided  $p = 0.0209$ ).<sup>110</sup> The ORR was however 16.9% for pembrolizumab versus 2.2% for placebo.<sup>110</sup> The analysis based on viral-etiological factors from these two phase III trials is not known at the moment, even though one would not suspect much difference compared with the results from the earlier completed phase I/II trials.

The lack of significant difference or even the inferior response rate in HBV-related versus nonviral or HCV-related HCC may be explained by several studies of TME in HCC. Our previous study comparing the TME of HBV-related versus nonviral-related HCC showed higher expression of PD-1 in HBV-related HCC.<sup>69</sup> As PD-1 expression on CD8+ T cells was reported to be predictive of PD-1 blockade response in nonsmall cell lung cancer,<sup>111</sup> one would expect a better response rate in patients with HBV-related HCC. Indeed, tumor-specific T cells were detectable in HCC patients but exist in exhausted state which could potentially be reinvigorated with checkpoint inhibitor.<sup>75,112</sup> However, it is also important to note that the TME of HBV-HCC is enriched with Treg,<sup>69</sup> which was previously reported to exhibit immunosuppressive phenotypes leading to disease progression and in some cases even promote hyperprogression of cancer upon PD-1/PD-L1 blockade.<sup>113,114</sup> It therefore remains to be confirmed if a combination therapy targeting both PD-1 and Treg<sup>115</sup> simultaneously would enhance the clinical response in HBV-related HCC particularly (**Fig. 2**). Also, given the complex TME of HCC, it would also be interesting to see the possibility of further stratification of viral-related HCC to several more distinctive TMEs or molecular subgroups for better design of future immunotherapeutic strategies.

### Anti-PD-L1 Monoclonal Antibodies

There are several anti-PD-L1 monoclonal antibodies: avelumab, durvalumab, and atezolizumab currently on clinical trials in advanced HCC. One phase II study of avelumab (NCT03389126) as monotherapy and another phase I study with combination of avelumab and axitinib, a small molecule tyrosine kinase inhibitor (NCT03289533), were registered and both are pending patients' recruitment. There were more trials involving the use of durvalumab, particularly a phase I/II trial in solid tumors including 39 HCC patients demonstrated a 10% ORR with better response in HCV-

related cases (25%) compared with HBV-related (0%) and noninfected (9.5%) HCC<sup>116</sup> (**Table 1**). This outcome is interesting and may be consistent with previous study stating a role of PD-L1 in HCV-induced immunosuppressive TME<sup>57,58</sup> and hence supports the use of anti-PD-L1 in HCV-related HCC (**Fig. 2**). Despite that, it was also previously demonstrated that expression level of PD-L1 shows no difference between viral etiologies: HBV, HCV versus nonviral etiology in HCC.<sup>117</sup> Another phase I/II study using the combination of durvalumab and tremelimumab (anti-CTLA4 antibody) in patients with unresectable HCC (NCT02519348) is currently ongoing and the result based on only 40 patients showed a modest 15% ORR with none of the patients with HBV or HCV-infected HCC responded<sup>118</sup> (**Table 1**). It is not known why durvalumab alone showed better response rate in HCV-related HCC but durvalumab and tremelimumab combination therapy shows only response in noninfected HCC patients. Furthermore, the results from the above two trials involved very small cohort of patients and hence might be too immature to conclude the final verdict. We await with anticipation of the randomized, multicenter phase III study of durvalumab and tremelimumab as first-line treatment in patients with unresectable HCC: HIMALAYA study (NCT03298451)<sup>119</sup> (**Table 1**). The result from this large scale trial with estimated enrolment of 1,310 patients will be announced in the near future. The analysis based on the etiology of HCC (HBV vs. HCV vs. others) will be performed and it will be interesting to observe if there is any interesting difference between them.

Other two large-scale anti-PD-L1 combination phase III studies in advanced HCC include the COSMIC-312 trial with atezolizumab (Anti-PD-L1)+cabozantinib (multitargeted tyrosine kinase inhibitor) and the IMbrave150 trial with atezolizumab+bevacizumab (VEGF [vascular endothelial growth factor] inhibitor; **Table 1**). Cabozantinib is approved as a second-line treatment in previously treated patients with advanced HCC based on improved overall survival versus placebo in the phase III CELESTIAL trial.<sup>120</sup> COSMIC-312 (NCT03755791) is a multicenter, randomized, open-label, controlled phase III trial combining cabozantinib with atezolizumab for patients with advanced HCC who have not yet received systemic therapy.<sup>121</sup> IMbrave150 (NCT03434379), on the other hand, is a phase III, open-label, multicenter, randomized study to evaluate the combination of atezolizumab and bevacizumab versus sorafenib in patients with locally advanced or metastatic and/or unresectable HCC.<sup>122</sup> It is known that VEGF plays an anti-angiogenesis role which has modulatory properties on both tumor cells and the immune microenvironment.<sup>123</sup> For instance, VEGF plays a role in Treg recruitment to the tumor and hence would be a good strategy to inhibit the pathway to enhance the local antitumor immunity.<sup>124</sup> The IMBRAVE trial is currently ongoing with anticipation of promising outcome. In fact, with the increasing appreciation of immune-modulatory properties of targeted therapies, future combination of immunotherapy and targeted therapy based on strong rationale and well-studied mechanism of actions might be an upcoming trend.



### Anti-CTLA-4 Monoclonal Antibodies

Anti-CTLA4 antibody (ipilimumab) was first approved by FDA in 2011 for the treatment of melanoma following the phase III trial showing significant better overall survival compared with gp100 vaccine alone.<sup>125</sup> Following that, ipilimumab as monotherapy was compared with nivolumab monotherapy or combination therapy with nivolumab in a phase III trial in advanced melanoma patients and demonstrated superior outcome in terms of both progression-free survival and median survival in combination therapy.<sup>126</sup> This lays a foundation for combination therapy using nivolumab plus ipilimumab in HCC. The nivolumab and ipilimumab combination therapy on 148 advanced HCC patients in the third arm of analysis from CheckMate 040 was further divided to three arms with different doses and regimens. For Arm A, 50 patients were given 1 mg/kg nivolumab and 3 mg/kg ipilimumab every 3 weeks (Q3W) for four cycles, followed by 240 mg nivolumab every 2 weeks (Q2W); Arm B, 49 patients were given 3 mg/kg nivolumab and 1 mg/kg ipilimumab Q3W for four cycles, followed by 240 mg nivolumab Q2W; or Arm C, 49 patients were given 3 mg/kg nivolumab Q2W and 1 mg/kg ipilimumab every 6 weeks (Q6W). The initial result showing 31 to 32% ORR with acceptable safety profile was first announced in Asco 2019<sup>109</sup> and the analysis based on viral etiologies to this promising combination regimen will be known in the near future.

Another anti-CTLA4 antibody, tremelimumab, was previously reported in a clinical trial assessing the safety profile and antitumor as well as antiviral activity in HCV-related HCC patients (NCT01008358)<sup>127</sup> (→ **Table 1**). An ORR of 17.6% based on 17 patients was reported with enhanced tumor response as well as anti-HCV viral immunity detected.<sup>127</sup> As mentioned above, tremelimumab was also combined with durvalumab in another two more trials (NCT02519348 and NCT03298451).<sup>118,119</sup> Given the potential benefit in anti-HCV viral activity, the role of anti-CTLA4 ICB in HCV-related HCC warrants future validation (→ **Fig. 2**).

### Future Perspectives and Concluding Remarks

Chronic viral hepatitis infection remains one of the most common etiologies of HCC<sup>128</sup> which will hopefully decrease with more effective control of infection in the future. In the meantime, the incidence of nonviral-related HCC particularly that from NAFLD is on the raise.<sup>129,130</sup> Therefore, future immunotherapeutics will have to take into considerations and require deeper understanding of TME from these cases.<sup>131</sup> In fact, our recent study implicated the role of Tim-3 in nonviral-related HCC.<sup>69</sup> Despite the potential role of Tim-3 in T-cell dysfunction and disease progression in HCC,<sup>55</sup> there is by far only one trial in advanced primary liver cancer using TSR-022 (Anti-TIM-3 Antibody) in combination with TSR-042 (Anti-PD-1 Antibody) (NCT03680508) and its outcome will only be revealed in the future. With the increasing use and multiple combination strategies of immunotherapies as well as enhanced understanding the mecha-

nisms of actions with advancement in immunomonitoring technologies, the future of treating of HCC according to various etiologies might be a possible aim to achieve.

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#### Conflicts of Interest

None.

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