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# The advances in immunotherapy for hepatocellular carcinoma

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## Abstract

Hepatocellular carcinoma (HCC) is one of the malignant tumors with higher incidence and mortality worldwide. Recently, significant progress has been made in uncovering immunotherapy in HCC, for instance programmed death-1, cytotoxic T-lymphocyte antigen 4, chimeric antigen receptor T-cell therapy, T cell receptor T cell therapy, dendritic cell vaccine, and cytokine-induced killer cells. This paper reviews the advances in immunotherapy and focuses on the results of many of preclinical studies and clinical trials in the field, as well as some of the promising therapeutic strategies for HCC in the future.

**Keywords:** HCC, PD-1, PD-L1, CTLA-4, CAR-T, T cell receptor, DC, CIK

## INTRODUCTION

Hepatocellular carcinoma (HCC) was predicted to have the sixth highest incidence and the second highest mortality of malignant tumors worldwide in 2018[1]. The risk factors for HCC are closely related with lifestyle, chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, fatty liver disease, and cirrhosis[2-4]. The management of HCC involves a multidisciplinary team approach, considering not only the tumor stage and patient complications but also the seriousness of damaged liver function, as most HCC treatments can aggravate the severity of disease[5]. Although surgical resection remains the cornerstone of HCC therapy, limitations are caused by high recurrence rates after surgery because HCC is often diagnosed at advanced stage[6]. Liver transplantation (LT) is the optimal treatment means for early-stage HCC, but limitations of LT are caused by organ shortage, tumor recurrences, and low-ratio eligibility. Comprehensive therapies for advanced HCC patients, such as radiotherapy, chemotherapy, interventional therapy, and targeted therapy, have been developed, but the 5-year survival rate remains low[7].

Cancer immunotherapy was selected as its annual breakthrough in Science journal in 2013. Following the advancements of immunotherapy in solid tumors over the last few years, as shown by the results of immune checkpoint inhibitors (ICIs) in lung cancer, renal cell cancer and melanoma[8], in recent years, ICIs with anti-CTLA-4 antibodies and anti-PD-1/PD-1L antibodies have been utilized to treat advanced melanoma[9]. In 2018, because of the achievements in the treatment of cancer with ICIs of CTLA-4 and PD-1/PD-L1, James P. Allison and Tasuku Honjo were awarded the Nobel prize.

Chimeric antigen receptor T-cell (CAR-T) immunotherapy has become more popular in the last decade as an antitumor therapy. Anti-CD19 CAR-T cell was approved by the FDA for treatment of subjects up to 25 years of age with B-cell acute lymphoblastic leukemia in 2017[10]. This article mainly summarizes the advances in ICIs and cellular immunotherapy for HCC.

## PD-1/PD-L1

PD-1 is expressed on a subset of thymocytes and is upregulated on activated T cell, B cell, and myeloid cells[11]. Two ligands for PD-1 were identified in 2000 and 2001 and named PD-L1 and programmed death ligand 2 (PD-L2), respectively[12,13]. PD-L1 is mainly expressed on stationary T cells, B cells, DC, and hepatoma cells, while PD-L2 is only expressed on DC and macrophages[14-16]. In theory, the interaction of PD-1 and PD-L1 expressed on immature T cells can interfere with activation. Similarly, if PD-L1 is highly expressed on tumor cell, the ligand receptor interactions between tumor cells and activated T cells triggers the immunosuppressive response, leading to immune tolerance[17]. It provides a theoretical basis for the treatment of PD-L1 in HCC.

It has been demonstrated that PD-L1 is overexpressed in HCC tissues; however, the results are controversial with respect to PD-L1 as predictive biomarkers for HCC[18]. Several studies have reported that the higher PD-L1 expression on tumor cell in HCC patients were related with worse prognosis and tumor recurrence, and the studies also showed PD-L1 expression on macrophages was associated with favorable survival rate[19-23]. However, two studies suggested that the expression of PD-L1 was not significantly correlated with survival outcomes in HCC[24,25]. Both soluble PD-1 (sPD-1) and soluble PD-L1 (sPD-L1) were prognostic factors with opposite prognostic values for HCC patients, while sPD-1 and sPD-L1 were not significantly related with PD-L1 expression in tumor[26]. However, two studies suggested plasma sPD-1 was associated with HBV activity and increased risk of HCC[27,28].

Liang *et al.*[29] illustrated that inhibition of PD-1 can suppress the growth of hepatoma and promote the apoptosis of hepatoma. Increased expression levels of PD-1 were detected in peripheral blood and tumor infiltrating lymphocytes (TILs) of recurrent HCC patients[30]. Blockade of PD-1 on TILs can restore anti-tumor effects of TILs[31]. However, sPD-1/sPD-L1 was not associated with either PD-L1 expression of tumor cell or the numbers of CD4-positive TILs and CD8-positive TILs[26]. Tumor infiltrating neutrophils as a new target of immunotherapy participate in tumor progression, while the tumor microenvironment (TME) induces impaired antitumor immunity via the modulation of PD-L1 expression on tumor infiltrating neutrophils[32]. PD-L1 was positively associated with expression of CD3 and CD8 in HCC samples[23]. PD-L1 expression on macrophages is also a prognostic factor for HCC patients, and it could activate high levels of CD8(+) cytotoxic T-lymphocyte (CTL) infiltration and immune related gene expression[21].

Since immune checkpoint molecules are recognized as vital indicators of HCC progress, series of clinical trials with ICIs have been implemented to confirm their potential function for advanced HCC. Nivolumab was approved by the FDA as immunotherapy for advanced stage HCC in 2017[33]. The efficiency of nivolumab was observed in a Phase I/II non-comparative trial (CheckMate 040) of patients with HCC and prior sorafenib treatment[33]. Forty-eight patients were treated with nivolumab in a dose-escalation phase. Then, since nivolumab showed adequate safety and feasibility, 214 patients from 39 sites in 11 countries received

nivolumab in a dose-expansion phase. The objective response rates (ORR) of nivolumab were 15% in the dose-escalation phase and 20% in the dose-expansion phase, suggesting that efficacy of nivolumab is not efficient. Twelve of 48 patients had Grade 3/4 treatment-associated adverse events. Although the study was positive in favor of anti-PD-1 treatment, it is worth corroborating the efficacy of nivolumab in a therapeutic schedule. Pre-treatment of sorafenib might potentiate the therapeutic response to subsequent treatment with nivolumab. In real-life experience from three German centers, Grade 3 treatment-associated events occurred in two patients (5.9%), and the partial response rate and stable disease rate in 34 patients with advanced HCC and nivolumab treatment were 11.8% and 23.5% in line with data from the CheckMate 040 trial[34].

Pembrolizumab is also an antibody against PD-1. In a Phase II open-label non-randomized trial (KEYNOTE-224) to assess the efficacy of pembrolizumab as an alternative second-line treatment for HCC patients, the median overall survival (OS) was 12.9 months with a disease control rate of 61% and ORR of 17%[35]. Grade 3 toxicities arose in 25 (24%) of the 104 patients. Hence, pembrolizumab is temporarily approved by the FDA as a second-line therapy for advanced HCC, but it still needs to be verified by the results of more Phase III trials[36]. A Phase III randomized, double-blind trial to further assess the efficacy of pembrolizumab versus placebo in HCC patient is still ongoing (NCT02702401). A Phase II study evaluating camrelizumab for HCC patients with resistance to systemic treatment displayed ORR of 13.8% and acceptable treatment-related adverse events in Chinese advanced HCC patients[37]. In addition to monotherapy, possible multimodality therapeutic options involving ICIs are under investigation. Some research has observed that ICIs of PD-L1 in combination with sorafenib, lenvatinib, rapamycin, and histone deacetylase inhibitor may enhance therapeutic benefit[38-41].

Clinical trials of PD-1 antibodies combined with other adjuvant therapy, e.g., transarterial chemoembolization (TACE) and selective internal radiation treatment, are currently in progress. In addition, different combination regimens, which depend on understanding of the actual immune mechanisms in the various combinations, could help us select the optimal therapeutic option for advanced HCC.

## CTLA-4

CTLA-4 downregulates activation of T cells by interacting with CD80/CD86 on the surface of DCs[42]. For naive T cell activation, CD28 on T cells provides the second activation signal by binding to CD80/CD86 on DCs[43]. CTLA-4 has a greater affinity for interacting with CD80/CD86 than CD28 so that it interferes in T cell activation[44]. Various single nucleotide polymorphisms (SNP) in CTLA-4 have been well-studied. Several studies observed that polymorphism of CTLA-4 was associated with increased susceptibility to HCC and haplotypes of CTLA-4 may affect the risk of HCC[45-48].

In 2013, the first CTLA-4 blocking inhibitor in practical HCC treatment was tremelimumab, which displayed promising antitumor activity and acceptable safety[49]. In a clinical trial to validate efficacy of tremelimumab in patients with HCC and HCV infection, partial response rate and disease control rate were 17.6% and 76.4%, respectively[49]. Duffy *et al.*[50] attempted to combine tremelimumab with ablation as an expected therapeutic option for patients with advanced HCC (NCT01853618) . Five partial responses were observed in 19 patients, with median OS of 12.3 months. Tremelimumab is a human IgG2 monoclonal antibody that blocks the binding of CTLA-4 on the surface of activated T cell[51]. It has been reported that tremelimumab could induce tumor responses in a subset of patients with non-small cell lung cancer and refractory biliary tract cancer[52,53]. Tremelimumab therapy could elevate the amount of T cells in the peripheral blood and TILs, and CD4(+) PD-1(+) cells were more likely to be activated by tremelimumab[54]. An important adverse effect of tremelimumab is transaminitis, as a high proportion of reversible Grade 3/4 transaminitis was observed in both the above-mentioned studies.

Preclinical data based on series of solid tumors indicate that dual immune checkpoint blockade is synergistic and leads to higher response rates and improved treatment outcomes compared to monotherapy. Most

clinical data suggest that both CTLA-4 and PD-1/PD-L1 blockade a portion of HCC patients. Compared to CTLA-4 blockade, PD-1 and PD-L1 blockade showed relatively higher ORR, which could reach 10%-20% in advanced HCC patients. PD-1/PD-L1 blockade agents were more tolerable and less hepatoxic. Further studies for combined PD-1/PD-L1 and CTLA-4 blockades in HCC treatment are still expected, which may help to mitigate the adverse effects of the treatment. Immune checkpoint blockade in advanced HCC combined with other conventional ablative treatments, such as radiofrequency ablation (RFA) or microwave, TACE, chemotherapy, targeted medicine, or surgery would be the most promising approach for HCC patients. However, for unresectable advanced HCC, it is more appropriate to search for other combination strategies, such as the combination with multi-kinase inhibitors, vaccines, and oncolytic viruses, as well as dual inhibition of two immune checkpoint molecules.

Based on current evidence, combination therapies with CTLA-4 are now an expected direction for the immunotherapy of advanced HCC patients in the future. A Phase III study (NCT03298451) of durvalumab with or without tremelimumab *vs*. sorafenib in patients of advanced HCC enrolled about 1,350 patients and explored two treatment schedules. Given the limited data to date, further testing of this combination is ongoing in a Phase II expansion. Most ongoing clinical trials have been designed to assess the efficiency of the combination strategies.

## CAR-T CELL THERAPY

CD19 targeted CAR-T immunotherapy is an expecting therapeutic option that has shown high efficacy in treating hematologic malignancies[55]. Moreover, a great number of CAR-T cell products in solid tumors has also been investigated in preclinical and clinical studies. In 2008, Wilkie *et al.*[56] reported for the first time that MUC1 targeted CAR-T could significant delay tumor growth in solid tumor[56]. The basic principle of CAR-T cell therapy is the modification of T cells with CARs, so that they can identify tumor cells, and then the retransfusion of these CAR-T cells into the human body to fight against the target cells[57,58]. Several studies have found that GPC3-targeted CAR-T cell therapy can eliminate HCC cells in preclinical research[59-61]. GPC3 is a 70-kDa heparan oncofetal proteoglycans that is located on the tumor cell membrane[62]. It has been demonstrated that GPC3 is detected in HCC tissues with higher expression but not in normal tissues[63]. A Phase I trial (NCT02395250) of 13 Chinese GPC3-positive HCC patients illustrated the safety and preliminary efficacy of GPC3 CAR-T cells in 2017[64]. According to the patient's tolerance, the preliminary analysis showed that GPC3 targeted CAR-T combined with the lymphodepleting conditioning had a certain efficacy[64]. The pre-clinical studies for dual-targeted CAR-T cells co-expressing GPC3 CARs and GPC3-specific CAR-modified T cells fusing a soluble PD1-CH3 fusion protein showed promising results[60,61].

α-fetoprotein (AFP) has been used not only as a biomarker for surveillance and diagnosis of HCC, but also as a target for immunotherapy[65]. In a clinical trial of 15 HCC patients who were given a subcutaneous injection of AFP-derived peptides, 1 patient had a complete response and the disease stabilized in 8 patients[66]. AFP, an intracellular/secreted protein, can generate AFP peptide-major histocompatibility complex (MHC) complexes as targets for CAR T-cell therapy for solid tumors. Liu *et al.*[67] detected that AFP-targeted CAR-T cells showed significant antitumor capacity in a mouse model Additionally, AFP-derived vaccines can augment the activity of ICIs, leading to deterioration of HCC.

The experience from successful clinical studies of hematologic malignancies provides us with the understanding that, although selection of the specific antigen to avoid off-target or on-target/off-tumor toxicity is a primary task to be tackled, for HCC, the challenge of CAR-T is the need to ascertain a specific neoantigen and overcome the TME, gut microbiome, and HCC genomic features. Furthermore, the activation, proliferation, and persistence of CAR-T are more important for therapy. In addition, standardization in the production of CAR-T and achieving individualized treatment should be considered.

## TCR-T CELL THERAPY

TCR-T cell immunotherapy, as one of the novel and effective antitumor treatment means, has been widely studied in oncotherapy. In 2011, Parkhurst *et al.*[68] firstly reported that human carcinoembryonic antigen (CEA)-targeted TCR-T cell therapy could induce objective regression of metastatic colorectal cancer[68]. The mechanisms of TCR-T cell therapies are similar to CAR-T immunotherapy. TCR-T therapy also modifies the autologous T cells with TCR, and then retransfusion expands TCR-T cell back into the patient to recognize and eliminate tumor cell, but the mechanisms for identifying antigens are quite different from CAR-T cell therapies[57]. The specific antigens recognized by CAR-T cell are all cell membrane antigens, while TCR-T cell can identify intracellular and cell membrane antigen peptides presented by MHC molecules[69]. In HBV-related HCC, by performing the high-throughput TCR sequence of TILs in tumor and matched adjacent normal tissues, Lin *et al.*[70] found that the combination of TCR repertoire overlap and TNM stage showed a better prognostic effect for HCC than TNM stage. Qasim *et al.*[71] firstly reported an HBV-related end-stage HCC case treated with HBV surface antigen as a target for HBV-specific TCR T cell therapy in 2015. In most HBV-related HCC, HBV integrations have been observed and can result in the expression of HCC cells[72]. HCC cells comprise fragments of integrated HBV-DNA that encodes peptides, which can be identified by T cells[73]. Another trial was conducted in two advanced HCCs patients who underwent liver transplantation with HCC relapses[74]. During the one-year period of follow up, the volume of 5/6 pulmonary metastases was decreased in one patient receiving HBV-specific TCR T cell therapy[74]. Basic studies of TCR-T cells therapy with specific targets, such as HCV, AFP, and GPC, may be a promising immunotherapy strategy for HCC in the future[75-77]. With TCR-T immunotherapy, the efficacy and side effects seem to mainly depend on the quality of the specific target and the TCR structure. The primary challenge is the discovery of new targets, particularly in the promising field of neoantigens. However, it should be emphasized that neoantigens may be expressed on a subset of tumor cells due to heterogeneity of tumor cell; otherwise, it may cause immune escape.

## DENDRITIC CELL VACCINE

DCs are powerful antigen-presenting cells that can stimulate T cells to induce antitumor activity. The infiltration of DCs in tumor tissue was closely associated to the improved clinical prognosis in HCC patients[78,79]. In 2002, Ladhams *et al.*[80] firstly reported two patients with end-stage HCC treated with autologous DCs vaccination co-cultured with autologous HCC antigens. The efficacy of DC vaccination loaded with tumor antigens from different sources has been investigated in clinical studies. Lee *et al.*[81] reported a trial which enrolled 31 advanced HCC patients receiving DC vaccine pulsed with autologous tumor lysates in 2005. They reported that rates of partial response and stable disease were 12.9% and 54.8%, respectively. A Phase II clinical trial reported disease control rate was 28% for advanced HCC patients with DC vaccination pulsed HepG2 lysate[82]. In another study of note, El Ansary *et al.*[83], also using DC vaccine pulsed with HepG2 lysate, showed that DC vaccination could partially improve survival outcome. DC vaccination loaded with autologous tumor lysates or ex vivo HepG2 cell lysate were feasible and effective.

However, the efficacy of DCs vaccination pulsed with tumor cell lysate is not satisfactory, and thus the use of specific antigen-modified DC vaccination has been attempted. Kakumu *et al.*[84] suggested that the depressed function of DCs is associated with pathogenesis of HCC with HBV or HCV infection. Several pre-clinical studies indicated that DCs infected with AFP or HBV antigen or both were effective strategies to enhance efficacy of DC-based vaccine[85-87]. GPC3-modified DCs were potent in inducing T cell proliferation and interferon (IFN)-y production[88]. Tada *et al.*[89] reported a clinical effect was observed in one of the five patients receiving DC vaccination pulsed with AFP, GPC3, and MAGE-1 fusion proteins in 2012. Subsequently, a large sample study confirmed that the median time of progression of HCC patients with DC vaccination pulsed with AFP, GPC3 and MAGE-1 fusion proteins was longer than the control group (36.6 months *vs.* 11.8 months)[90].

DCs pulsed with Hsp70 peptide and OK-432 can enhance efficacy of vaccine inducing T cell proliferation and CTL response[91,92]. In a clinical trial using Hsp70-DC vaccination, 2/12 patients demonstrated complete response and 5/12 patients demonstrated stable disease[93]. In our previous meta-analysis, we concluded DC-based therapy could prolong the median progression free survival (PFS) time and median OS time[94].

However, the maturation of DC was closely associated with efficacy of DC immunotherapy. The stimulatory capacity of dendritic cells from HCC patients was significantly lower than dendritic cells from liver cirrhosis tissue and normal samples[95]. Meanwhile, the numbers of CD83-positive DCs in HCC specimens were significantly lower compared with liver cirrhosis samples[96]. Therefore, it is very important to improve the maturation of DC, increase antigen source, and depress TME. Various stimuli, such as tumor necrosis factor alpha, lipopolysaccharide, IFN gamma, CD40-ligand, PEG10, IL-12, EpCAM, and HCA661, can significantly increase the stimulatory capacity of DCs[97-102]. Tumor endothelial marker 8 modified DCs could stimulate antitumor immunity by disrupting tumor vasculature, and DCs loaded with specific peptide, such as FoxM1, could significantly inhibit tumor growth and metastasis[103,104]. In addition, RFA can create an antigenic source with stimuli appropriate for maturation of DCs[105]. Regulatory T cells, producing immunosuppressive cytokine IL-10, were concentrated within HCC tissue and were inducted by local TME to interfere the differentiation and maturation of DC[7]. To overcome the immunosuppressive TME, Hu *et al*.[106] introduced a promising vaccine candidate, which combine the DC/tumor cell fusion vaccine with nanoparticles of folate-modified chitosan carrying interferon-induced protein-10, which could effectively inhibit tumor cell proliferation and significantly reduce myeloid-derived suppressor cells in mouse immune organs.

## CIK/DC-CIK

CIK are a subset of non-MHC-restricted T lymphocytes with immune modulatory effects and a crucial role in anti-tumor immunotherapy[107]. Several studies suggested that CIK cells co-cultured with DCs can significantly enhance antitumor efficiency[108,109]. Qiu *et al*.[110] reported that alpha-Gal epitope-pulsed DC-CIK therapy remarkably prolonged the survival of patients with stage III primary HCC as compared to the controls (17.1 months *vs*. 10.1 months). In a retrospective study from 45 patients with metastatic HCC, median OS of DC-CIK immunotherapy plus ablation (32 months) or ablation (17.5 months) was higher than untreated group (3 months)[111]. In a propensity score-matched analysis, autologous CIK immunotherapy showed significantly longer RFS than the control group[112]. After 5-year follow-up, CIK immunotherapy show a significant reduction in the risk of recurrence or death[113]. The combination therapies DC-CIK with other therapeutic options, such as TACE, could improve the antitumor efficacy. Guo *et al*.[114] reported that DC-CIK therapy combined with TACE can improve the PFS but not the OS outcomes. However, TACE plus DC-CIK therapy for HCC patients is superior to TACE alone in improving median OS and PFS in a meta-analysis[115]. Zhou *et al*.[116] analyzed that clinical benefit rate of sorafenib combined with DC-CIK is higher than oral administration of sorafenib (88.6% *vs*. 41.9%) in a meta-analysis.

To enhance the therapeutic efficacy of CIK cells, several pre-clinical studies suggested that co-culture of modified DCs, such as IL24-modified DCs, AFP-modified DCs, and GPC3-modified DCs, with CIKs can significantly promote CIKs differentiation and enhance lytic activity of CIK cells[117-119]. They provided a promising DC-CIK vaccine candidate for further clinical trials of HCC patients. Indeed, CIK or DC-CIK immunotherapies from autologous or allogeneic donors have already been extensively used in solid tumor patients. In our clinical center, we have experience with more than 100 gastric cancer patients with DC-CIK immunotherapy and have found that treatment outcomes were effective, safe, and feasible for gastric cancer patients. The standardization in the preparation and criteria of indication are progressing; several clinical trials are registered and ongoing. CIK or DC-CIK immunotherapies, combined with other antitumor agents, should be considered.

## CONCLUSION AND OUTLOOK

Immunotherapies appear to be a promising treatment for advanced HCC. Multiple prospective studies are attempting to validate the therapy outcomes with PD-1/PD-L1 and/or CTLA-4 blockade. However, only a small proportion of HCC patients effectively respond to immunotherapies and much research is still needed. One of the future directions for immunotherapies is combination therapies with other ICIs, TKIs, vaccines, and oncolytic viruses, as well as conventional treatments in various stages of patients to improve the antitumor efficacy. In addition, it is the important to research how to elevate immunotherapy efficacy and ascertain the biomarkers of predictive therapeutic response to immunotherapy. For example, TMB has been used in several tumor types to predict therapeutic response to anti-PD-1 therapy. In the future, we expect to identify more predictive biomarker subsets which can be used to accurately evaluate the efficacy of immunotherapy.

CAR-T technology and its application has been hailed as a scientific breakthrough in the field of hematological tumors. Application of CAR-T therapy and TCR to treat HCC is expected to be a promising therapeutic method. The crucial challenge is the need to identify specific antigens; overcome the TME, gut microbiome, and HCC genomic features; and guard against adverse effects. Furthermore, the activation, proliferation, and persistence of CAR-T immunotherapy should be considered with the outcomes of treatments for HCC. In addition, standardization in the production of CAR-T and achieving individualized treatment should be considered.

Different modifications of DC vaccine or DC-CIK therapy, such as selection of specific antigen targets and appropriate immunologic adjuvant, may elevate the effectiveness and safety in further studies. Dendritic cells lead to an increase in the naturally occurring neoantigen-specific immune response as well as the diversity of neoantigens. The combination of DC vaccination with other immunotherapies, e.g., TCR-T, may be a novel treatment modality in the future.

Because of the heterogeneity of tumor cells and the complexity of immuno-regulatory mechanisms, multimodality therapies based on immunotherapy represent the next step in clinical antitumor efficacy, which will enable advancing the field and improving the outcomes of HCC patients.

## DECLARATIONS

### Authors' contributions

Planned and designed of the study: Li Y
Searched the literature and wrote the manuscript, performed revisions, read and approved the final manuscript for publication: Zhang F, Li Y

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that they have no conflicts of interest.

### Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.



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