The immune microenvironment and progression of immunotherapy and combination therapeutic strategies for hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) accounts for 75%-85% of all primary liver cancers and is the leading cause of cancer-related deaths. China accounts for almost half of the global incidence and deaths of HCC. The poor response of chemotherapeutics and targeted drugs may be due to the drug resistance, heterogeneity of HCC, severe chronic liver damage and cirrhosis. Restoration of the liver microenvironment changes caused by chronic injury is crucial. Immunotherapy recently seems to show promise for the treatment of HCC induced by inflammatory injury. However, the unique liver immune system and resident immune tolerance state also pose a challenge for HCC immunotherapy. Different combinations of strategies have been developed for enhancement of HCC treatment. Here, we will discuss the immune microenvironment and progression of immunotherapy and combination therapeutic strategies for HCC.

Keywords: Immune microenvironment, immunotherapy, immune checkpoint inhibitors, Chimeric antigen receptor T, hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 75%-85% of all primary liver cancers. Due to the rapid progression of HCC, the lack of effective treatment programs, and poor prognosis makes it the fourth leading cause of cancer-related deaths\(^1\). Due to regional differences in medical diagnosis and treatment, more than half of the new cases and deaths of HCC each year occur in the Asia-pacific region. Patients with early HCC in Europe and United States can be diagnosed and effectively treated in time\(^2\). More than 70% of HCC patients do not benefit from medical therapy. The vast majority of HCC patients present with an advanced stage at diagnosis, and the most effective surgical programs are often challenging to implement. In the past ten years, dozens of promising chemotherapeutics have failed the phase III trial, with only sorafenib demonstrating a low objective response rate and a slight increase in survival\(^3\). Research on targeted drugs for cell proliferation, metastasis and angiogenesis are encouraging, such as regorafenib and lenvatinib, although the overall survival rate remains dissatisfactory\(^4\). The ineffectiveness of chemotherapeutics and targeted drugs may be due to drug resistance and heterogeneity of HCC. HCC is usually accompanied by severe chronic liver damage and cirrhosis. Hence, anti-HCC drugs require a good balance of therapeutic response and drug toxicity and this often limits the application of highly active compounds with high toxicity\(^5^\text{a}\). Therefore, restoration of the liver microenvironment caused by chronic injury should be incorporated in the holistic management of HCC. In recent years, immunotherapy has been used in the treatment of various solid tumors. This was observed through the checkpoint inhibition of programmed cell death 1/programmed death ligand 1 (PD-1/PD-L1) and cell toxic T lymphocyte-associated protein 4 (CTLA-4) while improving the tumor immune microenvironment which seems to be particularly relevant for the treatment of HCC. However, the unique liver immune system and resident immune tolerance state make it different from other organs. Besides, continuous matrix remodeling of the malignant hepatocyte transformation caused by chronic inflammation and scars has created an immunosuppressive microenvironment that promotes the development of HCC, posing a challenge for HCC immunotherapy\(^7\).

THE IMMUNE MICROENVIRONMENT OF THE LIVER AND HCC

The liver has a unique immune regulation and balance mechanism. On one hand, the portal vein system is directly exposed to gastrointestinal pathogens and requires an effective immune response. On the other hand, it needs to deal with a large number of harmless blood antigens and maintain the immune tolerance of the liver\(^6\). In most cases, the liver is in a physiological immune tolerance state\(^6\). Most non-parenchymal cells, such as live sinusoidal endothelial cells (LSEC), Kupffer cells (KCs) and hepatic dendritic cells (HDC), gather in the liver sinusoids. It constitutes the physiological basis of the liver’s immunosuppressive microenvironment\(^10^,11\). LSEC has the dual functions of immune surveillance and immune tolerance. It acts as an antigen-presenting cell (APC) to present pathogens or tumor antigens\(^12\). At the same time, it inhibits the excessive responses of DC and T lymphocytes to bacterial antigens from the portal system\(^11^\text{a}-15\). KCs maintain immune tolerance by engulfing pathogenic microorganisms derived from the intestine, secreting inhibitory factors (such as IL-10 and prostaglandins) and activating the proliferation of regulatory T cells (Tregs)\(^16^\text{-}19\). Besides, HDC is also a component of the liver immune tolerance by reducing the expression of MHC II and co-stimulatory molecules\(^20\). In summary, this immune-tolerant physiological environment creates a huge obstacle to the host’s anti-tumor immunity.

The pathogenesis of HCC is characterized by destruction of the sinusoidal structure by a viral infection and inflammatory injury, impairment of immune surveillance and immune tolerance functions leading to liver cirrhosis and liver cancer\(^6^\text{a}\). The high-risk factors of HCC (hepatitis virus, alcohol, aflatoxin, etc.) drive hepatocyte DNA damage, endoplasmic reticulum stress and necrosis, which in turn leads to the formation of regenerative nodules, proliferative nodules and ultimately HCC\(^21\). HCC has abundant immune cell infiltration, which is the immune response of the host trying to clear the tumor. Unfortunately, this immune
response is often dysregulated\(^{[22]}\). Tumor-infiltrating lymphocytes (TILs) account for a high proportion of HCC\(^{[23,24]}\), but these ineffective TILs often prove to be insufficient to control tumor growth\(^{[25]}\). The increased FoxP3+ Treg may impair the effector function of CD8+ T cells, which exacerbates the immunosuppressive microenvironment in HCC and is associated with a poor prognosis\(^{[26]}\). In addition, adaptive immune cells (such as CD8+ T cells, Th17 cells and B cells) can also stimulate the development of HCC\(^{[27,28]}\). There are a large number of bone marrow-derived suppressor cells (MDSCs) and Tregs in the microenvironment of HCC, which evade immune surveillance through a variety of mechanisms, such as the expressing high-levels of SOCS3 and IL-10 to limit immune cell activation\(^{[29]}\) and secretion fibrosis factor TGF-β. This is used to build an environment of immunosuppression and drug resistance\(^{[30]}\), and directly down-regulates the expression of T cells or NK cell activation ligands (MHC class I and NKG2D, etc.)\(^{[31,32]}\). Therefore, the immunosuppressive environment of HCC is an arduous challenge to the host’s immune system, which makes immunotherapy a promising approach for HCC treatment in the future.

**THE STRATEGIES OF HCC IMMUNOTHERAPY**

According to the immunological basis of HCC, we divide current HCC immunotherapy into four categories, including immune checkpoint inhibitors, oncolytic virus therapy, HCC vaccine and chimeric antigen receptor T (CAR-T) cell therapy [Figure 1]. Due to the destruction of the HCC sinusoidal structure, it is difficult for LSEC and HDC to complete the antigen presentation process. Therefore, the specific DC vaccine is obtained by impinging tumor-associated antigen (TAA) or tumor lysate into DC *in vitro*. It activates cytotoxic T lymphocytes (CTLs) through major histocompatibility complex (MHC) class II-TCR antigen presentation and CD40/CD80/CD86-CD28 interaction. CTLs recognize and destroy tumor cells containing HCC-related antigens on MHC class I molecules. In addition to blocking the antigen presentation process, cancer cells will evade CTLs by upregulating immune checkpoint ligands, such as PD-L1 binding to the PD-1 receptor on the surface of CTLs to exhaust it, and CTLA-4 blocking the interaction between CD40/CD80/CD86 and CD28. Therefore, antibodies against PD-L1/PD-1 and CTLA-4 are used for immune checkpoint inhibitor therapy of HCC. The other alternative is more direct, by cloning *in vitro* chimeric antigen receptor T cells that can target specific antigen genes [such as Glypican 3 (GPC3) or alpha-fetoprotein (AFP)] related antigens to directly kill tumor cells. Finally, genetically engineered
oncolytic virus therapy can also selectively replicated in tumors, killing cancer cells while stimulating antigen presentation and adaptive anti-tumor immune responses.

**IMMUNE CHECKPOINT INHIBITORS**

Tumor cells express a variety of immunosuppressive ligands on their surface, which bind to the indicated inhibitory receptors of activated T cells involved in the anti-tumor response. This process in turn reduces the intensity of the anti-tumor immune response, thereby evading immune surveillance\[32\]. Drugs that block these immunosuppressive targets to eliminate tumor immune escape are called immune checkpoint inhibitors (ICI). PD-1 is a member of the CD28 superfamily and is expressed on the surface of T cells and B cells. Its activation will lead to the phosphorylation of ITSM (Immunoreceptor tyrosine-based switch motif) in the cytoplasm of the cell, inhibiting energy metabolism in T cells, thereby hindering cell cloning proliferation and secretion of cytokines. In order to avoid the killing of T cells, tumor cells highly express PD-L1 and release the PD-L1 into the peripheral blood, which causes the exhaustion of T cells and the loss of tumor antigen presentation ability of myeloid immune cells\[34\]. Therefore, targeted inhibition of the interaction of PD-1 and PD-L1 is of great significance for the treatment of HCC.

Nivolumab, as the first PD-1 targeted drug to be used in clinical practice, was initially used in the treatment of melanoma, and its objective response rate and one-year survival rate were 40.0% and 72.9%, respectively\[35\]. Subsequently, Nivolumab was tried to treat advanced HCC. Among 144 HCC patients, 20% showed a good response to nivolumab, and 3 of them achieved complete remission (CR), highlighting the potential of Nivolumab to treat advanced HCC\[36\]. Another anti-PD-1 targeted drug Pembrolizumab has also shown effectiveness in the treatment of advanced HCC, with an objective response rate and a one-year survival rate of 17% and 54%\[37\]. In fact, a single ICI is not satisfactory for the treatment of advanced HCC. The current ICI therapy is mostly performed in a variety of combinations (for example, anti-PD-L1 antibody plus anti-CTLA-4 antibody), which is more effective than a single agent. In the absence of targetable lymphocytes in the tumor microenvironment, inhibition of PD-1/PD-L1 cannot stimulate cancer immunity, and inhibition of the CTLA-4 can cause CD8 + T cells to proliferate in the lymph nodes and infiltrate the tumor tissue, thereby enhancing the efficacy of anti-tumor. In fact, combination therapy of molecularly targeted drugs and immune checkpoint inhibitors has received considerable attention. For example, immunosuppressive cytokines that cause the immunosuppressive liver environment of patients with liver cancer, such as interleukin (IL)-10, transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF) molecular targeted drugs\[38,39\]. Table 1 shows the ongoing use of ICI in combination with various interventions (such as kinase inhibitors, cytokine or receptor inhibitors, and embolotherapy).

**ONCOLYTIC VIRUS THERAPY**

The oncolytic virus can specifically host in cancer cells, replicate and destroy the cell structure and hence was not initially classified as immunotherapy. Subsequent studies confirmed that oncolytic viruses could induce anti-cancer immune responses and immunogenic cancer cell death, making them a form of immunotherapy\[47\]. Compared with traditional therapies, oncolytic virus therapy is safer, has the selective specificity of host cancer cells, and continuously self-replicates to lyse cancer cells\[48\]. In the tumor microenvironment, pathogen-associated molecular patterns (PAMP) of oncolytic viruses can be recognized by pattern recognition receptors (PRR) of immune cells, such as through TLR or MDA5 activation of macrophages or dendritic cells\[49,50\]. As a secondary effect, oncolytic viruses enhance the recognition and presentation of tumor antigens, and activate the infiltration of cytotoxic T cells into tumors\[51\]. Therefore, oncolytic virus therapy is a very interesting method to overcome HCC immunosuppression. Currently, oncolytic virus therapies used for HCC include dsDNA or ssRNA viruses, such as measles vaccine virus (MeV), herpes simplex virus (HSV), adenovirus (Adenovirus) and vaccinia virus (VV), etc., which are used to engineer infection vectors\[52\]. For example, inserting the overexpression sequence of granulocyte-
| Clinical trials identifier | Target | Status | Active treatment | N     | Primary endpoints or outcomes                                      | Ref. |
|----------------------------|--------|--------|------------------|-------|---------------------------------------------------------------------|------|
| NCT03630640 PD-1           | PD-1   | Recruiting Phase 2 | Nivolumab | 50    | OS, 2 years                                                         |      |
| NCT033383458 PD-1           | PD-1   | Recruiting, Phase 3 | Nivolumab | 530   | Recurrence-free Surviva, 49 months; OS, 7 years; Time to recurrence, 49 months |      |
| NCT04161911 PD-1            | PD-1   | Completed Phase 3  | Nivolumab | 1,426 | OS, 7.75 years                                                       |      |
| NCT03222076 CTLA-4 PD-1     | PD-1   | Recruiting Phase 2 | Ipilimumab Nivolumab | 45    | AEs, 5 years                                                        |      |
| NCT033862276 CTLA-4 PD-1    | PD-1   | Recruiting, Phase I/II | Ipilimumab Nivolumab | 32    | AEs, 127 Days; Delay to surgery, 89 Days                           |      |
| NCT03510871 CTLA-4 PD-1     | PD-1   | Not yet recruiting, Phase II | Ipilimumab | 40    | The percentage of subjects with tumor shrinkage, 4 years           |      |
| NCT04310709 Multikinase PD-1 | PD-1   | Recruiting Phase II | Regorafenib Nivolumab | 42    | ORR, 6 months                                                       | [40-42] |
| NCT04170556 Multikinase PD-1 | PD-1   | Recruiting Phase II | Regorafenib Nivolumab | 60    | AEs, 24 months                                                      |      |
| NCT03299946 Multikinase PD-1 | PD-1   | Active, not recruiting, Phase I | Cabozantinib Nivolumab | 15    | AEs, 4 years                                                       |      |
| NCT03841201 Multikinase PD-1 | PD-1   | Recruiting, Phase II | Lenvatinib Nivolumab | 50    | ORR, 6 months                                                       |      |
| NCT03418922 Multikinase PD-1 | PD-1   | Active, not recruiting, Phase I | Lenvatinib Nivolumab | 30    | DLTs, 28 days                                                      |      |
| NCT03006926 Multikinase PD-1 | PD-1   | Phase 1; Active, not recruiting | Lenvatinib Pembrolizumab | 104   | AEs, 3 years; DLT, 21 days; ORR, 3 years                           |      |
| NCT02856425 Multikinase PD-1 | PD-1   | Phase 1; Recruiting | Nintedanib Pembrolizumab | 18    | MTD, 24 months                                                      |      |
| NCT02572687 PD-L1 VEGF      | PD-1   | Phase 1; Active, not recruiting | Ramucirumab MEDI4736 | 114   | DLTs, 28 days                                                      |      |
| NCT02576509 Raf-1 PD-1      | PD-1   | Active, not recruiting, Phase III | Sorafenib Nivolumab | 743   | OS, 41 months                                                      |      |
| NCT02988440 Raf1 PD-1       | PD-1   | Phase 1; Completed | PDR001 Sorafenib | 20    | AEs, 30 days; DLT, 8 weeks; DLTs, 28 days                           |      |
| NCT03893695 ALC-1 PD-1      | PD-1   | Recruiting Phase 1 | GT900001 Sorafenib | 20    | AEs, 30 days; DLT, 8 weeks; DLTs, 28 days                           |      |
| NCT03059147 PI3k PD-1       | PD-1   | Active, not recruiting, Phase II | SF1126 Nivolumab | 14    | DLTs, 56 days                                                      |      |
| NCT03655613 C-Met PD-1      | PD-1   | Recruiting Phase 1 | APL1101 Nivolumab | 119   | DLTs, 35 days                                                      |      |
| NCT02795429 PD-1+cMet       | PD-1   | Phase 1/2; Active, not recruiting | PDR001 INC280 | 90    | DLT, 42 days; ORR, 3 years                                         |      |
| NCT02423343 TGFR1 PD-1      | PD-1   | Active, not recruiting, Phase 1 Phase 2 | Galunisertib Nivolumab | 75    | MTD, 6 months                                                      |      |
| NCT0423379 PD-1 CCR2/CCR5   | PD-1   | Recruiting Phase 2 | Nivolumab BMS-81360 BMS-986253 | 50    | Primary pathologic response: 2 years; Significant tumor necrosis: 2 years |      |
| NCT030334456 Embolotherapy PD-1 | PD-1   | Recruiting, Phase II | Radioembolization Nivolumab | 40    | ORR, 8 weeks                                                       |      |
| NCT033380130 Embolotherapy PD-1 | PD-1   | Active, not recruiting, Phase 2 | Nivolumab Nivolumab SIR-Spheres | 40    | AEs, 2 years                                                       | [43,44] |
| NCT03572582 Embolotherapy PD-1 | PD-1   | Active, not recruiting, Phase 2 | Nivolumab TACE | 49    | ORR, 42 months                                                     |      |
| NCT04268888 Embolotherapy PD-1 | PD-1   | Recruiting Phase 2 | Nivolumab and TACE/TAE | 522   | OS: 2 years; TTP                                                   |      |
| NCT01658878 PD-1 Raf-1 CTLA-4 multikinase | PD-1 | Active, not recruiting, Phase I/II | Nivolumab Sorafenib Ipilimumab Cabozantinib | 1,097 | AEs, 100 days; ORR, 6 months                                      | [45,46] |

Table 1. Clinical trials of immune checkpoint inhibitors for HCC
macrophage colony-stimulating factor (GM-CSF) into the oncolytic virus sequence, GM-CSF recruits myeloid cells in the periphery to enhance the immune response in the tumor microenvironment. So far, preclinical studies for HCC oncolytic virus therapy have been very encouraging. We have compiled preclinical studies on HCC oncolytic therapy for the past ten years, as shown in Table 2.

Although many preclinical research attempts have been made in oncolytic therapy in recent years, there are still very few programs that have entered the clinical stage. At present, the only HCC oncolytic virus entering clinical research is JX-549, with VV as an engineered vector. VV has the stability and efficiency of intravenous administration, is widely used in the safety of live vaccines, has the advantages of immune-inducing activity and better editability, and has become a carrier of various engineered tumor-melting viruses. The thymidine kinase gene (TK) gene of JX-594 (also known as PexaVec; Jennerex Inc.) was deleted to make it more specific for cancer cell infection. In addition, hGM-CSF and β-galactosidase were inserted to enhance its immunostimulatory activity and replication capabilities. JX-594 showed complete tumor response and systemic efficacy in a phase I clinical study. In the phase II trial, low-dose JX-594 has significant anti-cancer effect and immune activation ability, but this requires earlier interventional therapy. Currently, a large-scale 600-person multicenter Phase 3 trial is still in progress (NCT02562755). More clinical studies of HCC oncovirus are shown in Table 3.

## HCC VACCINE

Tumor vaccine is a treatment program to increase the specificity of tumor antigens, mainly antigen peptide vaccines and DCs vaccines, which are used to stimulate specific immune responses. The clinical trials of therapeutic vaccines for HCC are summarized in Table 4. At present, there are relatively few registered clinical trials for DCs vaccines in HCC, partly because of the unsatisfactory results of previous clinical trials of such vaccines. The thymidine kinase gene (TK) gene of JX-594 (also known as PexaVec; Jennerex Inc.) was deleted to make it more specific for cancer cell infection. In addition, hGM-CSF and β-galactosidase were inserted to enhance its immunostimulatory activity and replication capabilities. JX-594 showed complete tumor response and systemic efficacy in a phase I clinical study. In the phase II trial, low-dose JX-594 has significant anti-cancer effect and immune activation ability, but this requires earlier interventional therapy. Currently, a large-scale 600-person multicenter Phase 3 trial is still in progress (NCT02562755). More clinical studies of HCC oncovirus are shown in Table 3.
### Table 2. Representative Oncolytic therapy used in preclinical studies

| Virus strain | Modification | Therapeutic gene | HCC cell lines | Animal model | Dose | Ref. |
|--------------|--------------|------------------|----------------|--------------|------|------|
| Recombinant VSV-NDV, L289A | Replaced of hemaggulutinin-neuraminidase (HN) | None | HepG2 Huh7 | NOD.CB17-prkdcscid/NCrCrl (NOD-SCID). | 10^{5} TCID50/IV | [54] |
| Getal-like alphavirus, M1 | Insertion of valosin-containing protein (VCP) inhibitors | XBP1 | Hep3B | Nonhuman primate Macaca fascicularis. | 5 x 10^9 PFUs, IV | [55] |
| HSV, d0-GFP | Mutated in glycoprotein K and glycoprotein B | None | Huh7, SMMC7721, QGY7703, L-02, BEL7404, G5G7701, HCCLM3, MHHC97H, H22 | Huh7 and Hep3B xenografts BALB/c. | 1 x 10^9 PFU, IV | [56] |
| Ad5 | Insertion of Golgi protein 73 (GP73) promoter and sphingoosine kinase 1 (SphK1)-short hairpin RNA (shRNA) | SphK1 | Huh7, HL-7702 | Huh7 xenografts BALB/c nude mice. | 6 x 10^4 PFU, IT | [57] |
| Recombinant influenza viral, PR8 | Deletion of NS and insertion of hGM-CSF | hGM-CSF | MDCK, A549, SMCC7721, HepG2, LM3 xenografts BALB/c nude mice. | HepG2 xenografts BALB/c nude mice. | 2 x 10^5 PFU, IT | [56] |
| MeV, MV-Edm | None | None | CCL-63, MHCC-97H | Hep3B xenografts BALB/c nude mice. | 5 x 10^5 PFU, IT | [57] |
| Ad, Ad-sp | Insertion of Vestigial-Like Family Member 4 (VGLL4) | VGLL4 | Hep3B, Huh-7 | Hep-7 xenografts BALB/c nude mice. | 5 x 10^5 PFU, IT | [58] |
| HSV, HSV T-01 | α47 and γ44.3 loci are deleted and the LacZ gene replaces the ICP6 gene | None | HuH-7, Li-7 JHH-1, JHH2, JHH5, JHH6, JHH7, HLE, HLF, PLC/PRF/5, huH-1 | Hepa-1 xenografts BALB/c nude mice. | 2 x 10^5 PFU, IT | [59] |
| Ad, Ad-ΔB | Insertion of ING4 and TRAIL | ING4 and TRAIL | Hep3B | Hep3B xenografts BALB/c nude mice. | 1 x 10^{10} PFU, IV | [60] |
| Ad, Ad-wnt-E1A(Δ24bp)-TSLC1 | Insertion of TSLC1 | Wnt and Rb pathway | MHCC-97H, PLC/PRF/5 | Hep3B xenografts BALB/c nude mice. | 6 x 10^4 PFU, IT | [61] |
| Ad, OAV SG655-ΔGMP | Insertion of 11R-PS3 and GM-CSF | 11R-PS3 and GM-CSF | Hep3B-C, ECCG5 | ECCG5 xenografts BALB/c nude mice | Unknown | [62] |
| Ad, Ad-ΔB/TRAIl and Ad-ΔB/IL-12 | Mutated in E1A and deleted in E1B regions. Insertion of hTRAIL or hIL-12 | hTRAIL or hIL-12 | Hep3B and HuH7 | Athymic nude mice, orthotopic model | 2 x 10^5 PFU, IV | [63] |
| MeV, (Res + MeV) | Encoding of GTP as a marker gene and SCD as suicide gene | None | HepG2 and Hep3B | No animal model used | Various MOIs | [64] |
| VV, GLV-2b-372 | Deletion of TK and insertion of TurboFps35 gene | None | Huh-7, Hep G2, SNU-449, and SNU-739 | Athymic nude mice Huh-7 xenograft | 1 x 10^5 PFU, IT | [65] |
| VV, GLV-1h68 | Deletion of TK and insertion of Renilla luciferasegreen hTERT inserted upstream of the E1 gene | None | Huh-7, Hep G2, SNU-449 and SNU-739 | No animal model used | Various MOIs | [66] |
| Ad, Telomelysin | Human: Huh-7, Hep3B, PLC5, HA22T, HCC36 and HepG2 Mouse: Hepa-1c1c7 and Hepa-1-6 | Huh7 xenografts, orthotopic model | Hbx transgenic mice, orthotopic model | 1.25 x 10^5 PFU, IT | [67] |
| HSV, G47Δ | ICP47 and γ34.5-deletion | None | HepG2, HepB, SMMC-7721, BEL-7404, and BEL-7405 | Balb/c nude mice SMMC-7721, BEL-7404 xenograft | 5 x 10^5 PFU, IT | [68] |
| HSV, LC50V | Viral glycoprotein H gene linked with liver-specific apolipoprotein E (apoE)-AAT promoter. miR-122a and let-7 also inserted at 3′ UTR | miR122, miR-124a and let-7 | Huh-7, HepG2, and Hep3B | Hsd: athymic (nu/nu) mice, Hep3B xenograft | 5 x 10^5 PFU, IT | [69] |
| VV, GLV-1h68 | Deletion of TK and insertion of Renilla luciferasegreen fluorescent protein (Ruc-GFP), β-galactosidase, β-glucuronidase | None | HuH7 and PLC/PRF/5 | Athymic Nude Foxn1nu HuH7 and PLC xenografts | 5 x 10^5 PFU, IV | [70] |
Ad, SG7011\textsuperscript{MTT}

Insertion of eight copies of let-7 target sites (let7T) into the 39 untranslated region of E1A

mRNA, let-7

HepG2, Hep3B, PLC/PRF/5, and HuH7

5 × 10\textsuperscript{8} PFU, IT

[71]

VV, JX-963

Deletion of TK and VGF, insertion of hGM-CSF

None

Immunocompetent, orthotopic, NZW rabbits VX2 tumor model

Various PFU, IV

[72]

MeV: measles vaccine virus; HSV: herpes simplex virus; Ad: Adenovirus; VV: vaccinia virus; NDV: newcastle disease virus; VSV: vesicular stomatitis virus; IV: intravenous; IT: intratumoral; MOI: multiplicity of infection; PFU: plaque-forming units

### Table 3. Clinical trials of oncolytic viral therapy for HCC

| Clinical trials identifier | Status | Active treatment | n   | Primary endpoints or outcomes                      | Ref. |
|---------------------------|--------|------------------|-----|----------------------------------------------------|------|
| NCT03071094               | Active, not recruiting. Phase 1 and 2 trials | JX-594; Nivolumab | 30  | DLTs, 4 weeks; ORR, 6 months                       |      |
| NCT02562755               | Active, not recruiting. Phase 3 trials | JX-594; Sorafenib | 600 | ORR, 6 months                                      |      |
| NCT00554372               | Completed. Phase 2 trials | JX-594 | 30  | mRECIST v1.0 criterion; Cho criterion, 4 weeks     | [81] |
| NCT01387555               | Completed. Phase 2b trials | JX-594 | 129 | OS, 21 months                                      | [82] |
| NCT00629759               | Completed. Phase 1 trials | JX-594 | 14  | MTD, Safety evaluation throughout study participation |      |

Most data were obtained from findings from www.clinicaltrials.gov using the search terms “hepatocellular carcinoma” and “oncolytic”. JX-594: Recombinant vaccinia virus [Thymidine Kinase (TK)-deletion plus granulocyte-macrophage colony-stimulating factor (GM-CSF)]. DLTs: dose limiting toxicities; ORR: overall response rate; OS: overall survival; MTD: maximum tolerable dose; mRECIST: modified response evaluation criteria in solid tumors

### Table 4. Clinical trials of therapeutic vaccines for HCC

| Clinical trials identifier | Status | Active treatment | n   | Primary endpoints or outcomes                      | Ref. |
|---------------------------|--------|------------------|-----|----------------------------------------------------|------|
| NCT04248569               | Recruiting, Phase I | DNAJB1-PRKACA peptide vaccine, Nivolumab, Iplimumab | 12  | DLTs, 4 weeks; Fold change in interferon-producing DNAJB1-PRKACA-specific CDB8+ and CD4+ T cells, 12 weeks; CTCAE v4.0, 1 year |      |
| NCT03674073               | Recruiting, Phase I | Neoantigen Vaccines; Microwave Ablation Individualized anti-cancer vaccine (CRCL-AlloVax) | 24  | Registration of adverse events. 0.5 years | [84] |
| NCT02409524               | Completed, Phase II | COMBIG-DC vaccine (Ilixadencel). | 15  | Registration of adverse events, 2 years; Immunogenicity, 2 years | [85] |
| NCT01974661               | Completed, Phase I | IMA970A vaccine; CV8102 adjuvant; Cyclophosphamide | 18  | OS, 12 weeks                                      |      |
| NCT03203005               | Completed, Phase I | Alpha-fetoprotein peptide-pulsed autologous dendritic cell vaccine | 22  | Registration of adverse events, 2 years; Immunogenicity, 2 years | [86] |
| NCT00005629               | Completed, Phase I | Alpha-fetoprotein peptide-pulsed autologous dendritic cell vaccine | 6   | Safety, 1 month                                   |      |
| NCT00022334               | Completed, Phase II | Alpha-fetoprotein peptide-pulsed autologous dendritic cell vaccine | 33  | DLT and MTD, 1 year                               |      |
| NCT04147078               | Recruiting, Phase I | Personalized neoantigen DNA vaccine (GNOS-PVO2) and plasmid-encoded IL-12 (INO-9012) in combination with pembrolizumab (MK-3475) | 80  | DFS, 5 years                                      |      |
| NCT04251117               | Recruiting, Phase I | Cancer stem cell vaccine | 12  | CTCAE v5.0, 2 years                               | [87] |
| NCT02089991               | Completed, Phase II | Recombinant fowlpox-CEA(6D)/TRICOM vaccine | 40  | Adverse events, 3 months                          | [85] |
| NCT00028496               | Completed, Phase I | Receptor-ligand vaccine | 48  | DLT and MTD, 56 days                              |      |
| NCT03942328               | Recruiting, Phase I | Autologous dendritic cells and Prevnar vaccine | 26  | Adverse events, 1 year                            | [88] |
| NCT02232490               | Recruiting, Phase III | Hepcortespenisimut-L (VS) therapeutic vaccine | 120 | Changes in plasma AFP, 3 months                   |      |

Most data were obtained from findings from www.clinicaltrials.gov using the search terms “hepatocellular carcinoma” and “vaccines”. DLTs: dose limiting toxicities; CTCAE: common terminology criteria for adverse events; OS: overall survival; MTD: maximum tolerable dose; DFS: disease-free survival
CHIMERIC ANTIGEN RECEPTOR T CELL THERAPY

In addition to immune checkpoint inhibitors, oncolytic viruses and vaccines, adoptive therapy using genetically modified T cells have also become one of the potential immunotherapy options for HCC. T cells can be engineered to express a chimeric antigen receptor (CAR), which is composed of a T cell receptor $\text{CD3}_\zeta$ chain and co-stimulatory receptors (e.g., CD28 and TNFRSF9) to form an antigen recognition domain. The antigen recognition domain endows CAR-T cells with specificity for tumor-associated antigens, which shows promise in the treatment of HCC. Besides, CAR-T cells have a strong adaptive immunity and can recognize antigens that are not present in MHC molecules. CAR-T cell therapy has been used in the preclinical treatment of a variety of solid tumors, but there are few clinical studies on HCC, and more are still in the preclinical research stage. Like the HCC vaccine, the technical difficulty lies in the choice of tumor-specific antigens. CD133 is expressed by cancer stem cells derived from various epithelial cells and is an attractive cancer treatment target. CAR-T cells targeting CD133 have shown the feasibility of treating advanced HCC, with controllable toxicity and effective activity. Glypican-3 (GPC3) is a member of the heparan sulfate glycoprotein family and belongs to a transmembrane glycoprotein. It plays an important role in cell proliferation, differentiation and metastasis. CAR-T cells targeting glypican-3 can inhibit the growth of HCC. Besides, there are HCC recognition antigens such as NKG2D and CD147 for CAR-T cell transformation. In addition, the CAR of CAR-T cells can be inserted into the expression of a variety of cytokine genes to overcome the immunosuppressive effects of the HCC microenvironment. The clinical trials of CAR-T cell therapy for liver cancer are summarized in Table 5.

THE CURRENT COMBINATION OF THERAPEUTIC STRATEGIES FOR HCC

Currently, there are many immunotherapy and other target therapy drugs approved by the Food and Drug Administration (FDA) of The United States of America (USA) for liver cancer treatment, including Atezolizumab, Avastin (Bevacizumab), Bevacizumab, Cabometyx (Cabozantinib-S-Malate), Cyramza (Ramucirumab), Keytruda (Pembrolizumab), Lenvatinib Mesylate, Lenvima (Lenvatinib Mesylate), Nexavar (Sorafenib Tosylate), Nivolumab, Opdivo (Nivolumab), Pemazyre (Pemigatinib), Pembrolizumab, Pemigatinib, Ramucirumab, Regorafenib, Sorafenib Tosylate, Stivarga (Regorafenib), Tecentriq (Atezolizumab). Single agent therapy has historically shown poor results in HCC, leading to trials of combination therapy for a more efficacious outcome. For example, the FDA has approved Opdivo (nivolumab) + Yervoy (ipilimumab) based on the CheckMate 040 trial, atezolizumab + bevacizumab for patients with advanced HCC based on the IMbrave150 (NCT03434379) study. The CheckMate 040 is a mult centered, open-labelled, multicohort, phase 1/2 study. The result showed that nivolumab + ipilimumab had manageable safety, promising objective response rate, and durable responses. The arm A regimen (4 doses nivolumab 1 mg/kg + ipilimumab 3 mg/kg every 3 weeks then nivolumab 240 mg every 2 weeks) received accelerated approval in the US based on this study. The IMbrave150a study is a global, open-labelled, phase 3 trial for patients with unresectable HCC who had not previously received systemic treatment. The study included 336 patients in the atezolizumab + bevacizumab group and 165 patients in the sorafenib group. The result showed that atezolizumab + bevacizumab resulted in better overall (overall survival at 12 months was 67.2% vs. 54.6%) and progression-free survival (6.8 months vs. 4.3 months) outcomes than sorafenib. There are many different combinations of immune checkpoint inhibitors with other different therapeutic strategies under investigation. Some of the combination clinical trials are concluded in the Table 1.

CONCLUSION AND PROSPECT

Immunotherapy is a revolution in HCC treatment. Significant responses have been observed in various tumor types with immunotherapy, especially immune checkpoint inhibitors and CAR-T cells. However, it is clear that not all HCC patients are sensitive to current immunotherapy, and even in those who do respond, the effect is difficult to last. Lots of data indicate that most HCCs are immunosuppressive
### Table 5. Clinical trials of Chimeric antigen receptor T cell therapy for liver cancer

| No. | Title                                                                 | Status         | Conditions                  | Interventions                                                                 | URL                                                                 |
|-----|-----------------------------------------------------------------------|----------------|-----------------------------|-------------------------------------------------------------------------------|----------------------------------------------------------------------|
| 1   | Study evaluating the efficacy and safety With CAR-T for liver cancer  | Unknown status | Liver neoplasms             | Biological: EPCAM-targeted CAR-T cells                                        | https://ClinicalTrials.gov/show/NCT02729493                            |
| 2   | Clinical study of ET1402L1-CAR T cells in AFP expressing hepatocellular carcinoma | Terminated     | Hepatocellular carcinoma| Biological: autologous ET1402L1-CART cells                                     | https://ClinicalTrials.gov/show/NCT03349255                            |
| 3   | T cells co-expressing a second generation glypican 3-specific chimeric antigen receptor with cytokines interleukin-21 and 15 as immunotherapy for patients with liver cancer (TEGAR) | Withdrawn      | Hepatocellular carcinoma| Genetic: TEGAR T cells[drug: cytoxan][drug: fludarabine]                      | https://ClinicalTrials.gov/show/NCT0409396848                           |
| 4   | Glypican 3-specific chimeric antigen receptor expressed in T cells for patients with pediatric solid tumors (GAP) | Recruiting     | Liver Cancer                | Genetic: GAP T cells[drug: cytoxan][drug: fludara]                            | https://ClinicalTrials.gov/show/NCT02932956                              |
| 5   | Safety and Efficacy of CEA-targeted CAR-T therapy for relapsed/refractory CEA+ cancer | Recruiting     | Solid Tumor|Lung Cancer| Biological: CEA CAR-T cells                                                   | https://ClinicalTrials.gov/show/NCT04348643                              |
| 6   | Autologous CAR-T/TCRT-T cell immunotherapy for solid malignancies     | Recruiting     | Esophagus cancer| hepatoma|glioma|gastric cancer                  | Biological: CAR-T/TCRT-T cells immunotherapy                           | https://ClinicalTrials.gov/show/NCT03941626                              |
| 7   | A Study of MG7 redirected autologous T cells for advanced MG7 positive liver metastases (MG7-CART) | Unknown status | Liver Metastases            | Biological: MG7-CART                                                          | https://ClinicalTrials.gov/show/NCT02862704                              |
| 8   | A Study of CD147-targeted CAR-T by hepatic artery infusions for very advanced hepatocellular carcinoma | Recruiting     | Advanced hepatocellular carcinoma| Biological: CD147-CART                                                         | https://ClinicalTrials.gov/show/NCT03993743                              |
| 9   | CAR-T hepatic artery infusions and Sir-Spheres for liver metastases   | Completed      | Liver Metastases            | Biological: anti-CEA CAR-T cells[Device: Sir-Spheres]                         | https://ClinicalTrials.gov/show/NCT02416466                              |
| 10  | CAR-T hepatic artery infusions or pancreatic venous infusions for CEA-expressing liver metastases or pancreatic cancer | Active, not recruiting | Liver Metastases   | Biological: anti-CEA CAR-T cells                                               | https://ClinicalTrials.gov/show/NCT02850536                              |
| 11  | Hepatic transarterial administrations of NKR-2 in patients with unresectable liver metastases from colorectal cancer | Active, not recruiting | Colon Cancer Liver Metastasis   | Biological: NKR-2 cells                                                        | https://ClinicalTrials.gov/show/NCT03370198                              |
| 12  | Dose escalation and dose expansion phase I study to assess the safety and clinical activity of multiple doses of NKR-2 administered concurrently with FOLFOX in colorectal cancer with potentially resectable liver metastases | Active, not recruiting | Colon Cancer Liver Metastasis | Biological: NKR-2 cells                                                        | https://ClinicalTrials.gov/show/NCT03310008                              |
| 13  | Interleukin-15 armored Glypican 3-specific chimeric antigen receptor expressed in T cells for pediatric solid tumors | Not yet recruiting | Liver Cancer|Rhabdomysosarcoma, et al. | Genetic: AGAR T cells[drug: cytoxan][drug: fludara]                           | https://ClinicalTrials.gov/show/NCT04377932                              |
| 14  | Treatment of relapsed and/or chemotherapy refractory advanced malignancies by CART33 | Completed      | Liver Cancer|Pancreatic Cancer, et al. | Biological: anti-CD133-CAR vector-transduced T cells                          | https://ClinicalTrials.gov/show/NCT02541370                              |
| 15  | Autologous CAR-T/TCRT-T cell immunotherapy for malignancies            | Recruiting     | Solid tumors                | Biological: CAR-T cell immunotherapy                                           | https://ClinicalTrials.gov/show/NCT03638206                              |
| 16  | A study of chimeric antigen receptor T cells combined with interventional therapy in advanced liver malignancy | Unknown status | Carcinoma, Hepatocellular|Pancreatic Cancer, et al. | Biological: CAR-T cell immunotherapy                                           | https://ClinicalTrials.gov/show/NCT02959151                              |
| 17  | A clinical research of CAR T cells targeting EpCAM positive cancer     | Recruiting     | Hepatic Carcinoma, et al.  | Biological: CAR-T cell immunotherapy                                           | https://ClinicalTrials.gov/show/NCT03013712                              |
| 18  | NKG2D-based CAR T-cells immunotherapy for patient with r/r NKG2D+ solid tumors | Not yet recruiting | Hepatocellular Carcinoma|Glioblastoma, et al. | Biological: NKG2D-based CAR T-cells                                             | https://ClinicalTrials.gov/show/NCT04270461                              |
19 GPC3-T2-CAR-T cells for immunotherapy of cancer with GPC3 expression
Recruiting Hepatocellular Carcinoma, et al. Biological: GPC3 and/or TGF-beta targeting CAR-T cells https://ClinicalTrials.gov/show/NCT03198546

20 NKG2D CAR-T (KD-025) in the treatment of relapsed or refractory NKG2DL+ tumors
Not yet recruiting Solid Tumor[Hepatocellular Carcinoma, et al.]
Drug: KD-025 CAR-T cells https://ClinicalTrials.gov/show/NCT04550663

21 GPC3-CAR-T Cells for the hepatocellular carcinoma
Not yet recruiting Hepatocellular Carcinoma Biological: GPC3-CAR-T cells https://ClinicalTrials.gov/show/NCT04506983

22 CAR-T cell immunotherapy for HCC targeting GPC3
Withdrawn GPC3 Positive Hepatocellular Carcinoma Biological: CAR-T cell immunotherapy https://ClinicalTrials.gov/show/NCT02723942

23 Clinical Study on the efficacy and safety of c-Met/PD-L1 CAR-T cell injection in the treatment of HCC
Unknown status Primary Hepatocellular Carcinoma Biological: c-Met/PD-L1 CAR-T cell injection https://ClinicalTrials.gov/show/NCT03672305

24 A study of GPC3 redirected autologous T cells for advanced HCC
Unknown status Carcinoma, Hepatocellular Carcinoma Drug: TAI-GPC3-CART cells https://ClinicalTrials.gov/show/NCT02715362

25 GPC3-targeted CAR-T cell for treating GPC3 positive advanced HCC
Recruiting Hepatocellular Carcinoma Biological: CAR-T cell immunotherapy https://ClinicalTrials.gov/show/NCT04102173

26 A Study of GPC3-targeted T cells by intratumor injection for advanced HCC (GPC3-CART)
Unknown status Carcinoma, Hepatocellular Carcinoma Drug: GPC3-CART cells https://ClinicalTrials.gov/show/NCT03130712

27 Phase I/II study of anti-Mucin1 (MUC1) CAR T cells for patients with MUC1+ advanced refractory solid tumor
Unknown status Hepatocellular Carcinoma, et al. Biological: anti-MUC1 CAR T cells https://ClinicalTrials.gov/show/NCT02587689

28 Anti-GPC3 CAR T for treating patients with advanced HCC
Completed Hepatocellular Carcinoma Biological: anti-GPC3 CAR T https://ClinicalTrials.gov/show/NCT02395250

29 Anti-GPC3 CAR-T for treating GPC3-positive advanced hepatocellular carcinoma (HCC)
Unknown status Hepatocellular Carcinoma Biological: retroviral vector-transduced autologous T cells to express anti-GPC3 CARs
Drug: fludarabine|drug: cyclophosphamide https://ClinicalTrials.gov/show/NCT03084380

30 Clinical study of redirected autologous T cells with a chimeric antigen receptor in patients with malignant tumors
Active, not recruiting Hepatocellular Carcinoma, et al. Genetic: CAR-CD19 T cell|genetic: CAR-BCMA T cell|genetic: CAR-GPC3 T cell|genetic: CAR-CLD18 T cell|drug: fludarabine|drug: cyclophosphamide https://ClinicalTrials.gov/show/NCT03302403

31 A clinical research of CAR T cells targeting CEA positive colorectal cancer (CRC)
Not yet recruiting Stage III Colorectal Cancer|Colorectal Cancer Liver Metastasis Biological: Anti-CEA-CAR T https://ClinicalTrials.gov/show/NCT04513431

32 Study of anti-CEA CAR-T + chemotherapy vs. chemotherapy alone in patients with CEA+ pancreatic cancer & liver metastases
Not yet recruiting Malignant tumor of pancreas metastatic to liver Biological: anti-CEA CAR-T cells|drug: gemcitabine|nab paclitaxel|drug: NLIR+FU|FA|drug: capcitabine https://ClinicalTrials.gov/show/NCT04037241

33 Glypican 3-specific chimeric antigen receptor expressing T cells for hepatocellular carcinoma (GLYCAR)
Recruiting Hepatocellular Carcinoma Genetic: GLYCAR T cells|drug: cytoxan|drug: fludarabine https://ClinicalTrials.gov/show/NCT02905188

34 4th generation chimeric antigen receptor T cells targeting glypican-3
Recruiting Advanced Hepatocellular Carcinoma Drug: CAR-GPC3 T cells https://ClinicalTrials.gov/show/NCT03980288

35 PD-1 antibody expressing CAR-T cells for EGFR family member positive advanced solid tumor (lung, liver and stomach)
Unknown status PD-1 Antibody|CAR-T cells|advanced solid tumor Biological: HerinCAR-PD1 cells https://ClinicalTrials.gov/show/NCT02862028

36 Chimeric antigen receptor T cells targeting glypican-3
Recruiting Hepatocellular carcinoma Biological: CAR-GPC3 T cells https://ClinicalTrials.gov/show/NCT03884751

37 A clinical study in patients with high-risk recurrent primary hepatocellular carcinoma using autologous TILs
Active, not recruiting Hepatic Carcinoma Drug: tumor infiltrating lymphocyte https://ClinicalTrials.gov/show/NCT04538313

38 CAR-GPC3 T cells in patients with refractory hepatocellular carcinoma
Completed Hepatocellular Carcinoma Genetic: CAR-GPC3 T cells https://ClinicalTrials.gov/show/NCT03146234
tumors. Therefore, ongoing research using a multifaceted approach to enhance the activity of the immune environment remain underway to enhance current immunotherapy strategies.

DECLARATIONS

Authors’ contributions
Drafted the outline of this review: Feng ZY, Xia HP
Drafted the manuscript: Feng ZY, Xu FG, Liu Y, Xu HJ, Wu FB, Chen XB, Xia HP
Finalized the manuscript: Chen XB, Xia HP

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