Asian Journal of Pharmaceutical Sciences 18 (2023) 100771

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/AJPS

Review

Oncolytic virus-based hepatocellular carcinoma treatment: Current status, intravenous delivery strategies, and emerging combination therapeutic solutions

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ARTICLE INFO

Article history:
Received 3 June 2022
Revised 24 October 2022
Accepted 4 December 2022
Available online 29 December 2022

Keywords:
Oncolytic viruses (OVs)
OV intravenous delivery systems
Combination treatments
Advanced hepatocellular carcinoma (HCC)
Pexa-Vec

ABSTRACT

Current treatments for advanced hepatocellular carcinoma (HCC) have limited success in improving patients' quality of life and prolonging life expectancy. The clinical need for more efficient and safe therapies has contributed to the exploration of emerging strategies. Recently, there has been increased interest in oncolytic viruses (OVs) as a therapeutic modality for HCC. OVs undergo selective replication in cancerous tissues and kill tumor cells. Strikingly, pexastimogene devacirepvec (Pexa-Vec) was granted an orphan drug status in HCC by the U.S. Food and Drug Administration (FDA) in 2013. Meanwhile, dozens of OVs are being tested in HCC-directed clinical and preclinical trials. In this review, the pathogenesis and current therapies of HCC are outlined. Next, we summarize multiple OVs as single therapeutic agents for the treatment of HCC, which have demonstrated certain efficacy and low toxicity. Emerging carrier cell-, bioengineered cell mimetic- or nonbiological vehicle-mediated OV intravenous delivery systems in HCC therapy are described. In addition, we highlight the combination treatments between oncolytic virotherapy and other modalities. Finally, the clinical challenges and prospects of OV-based biotherapy are discussed, with the aim of continuing to develop a fascinating approach in HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related mortality worldwide [1,2]. The global annual incidence of HCC is over 500,000, with a dismal overall five-year survival rate of 5%–9% in advanced HCC. Cytotoxic chemotherapy in the clinic has low response rates without considerable impact on overall survival (OS). Given the grim current situation, it is necessary to meet the urgent demand for new therapeutic strategies against advanced HCC.

In the recent decade, tumor immunotherapies have become hot spots for the treatment of advanced HCC. Among them, oncolytic viruses (OVs) have received great attention

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Peer review under responsibility of Shenyang Pharmaceutical University.

https://doi.org/10.1016/j.ajps.2022.100771

because of their high oncolytic effect and low resistance rate. OV therapy was first proposed as an immunotherapeutic strategy in the early 20th century. However, OV-mediated oncolytic mechanisms are incompletely understood. There are three main mechanisms of action at present [3]: (i) specifically infecting tumor cells to induce cytolysis [4–6], (ii) disrupting the vascular system by inducing stromal cells in the tumor microenvironment (TME) to generate neutrophil clusters in the blood vessels at the tumor site [7], and (iii) activating antitumor immune responses [8–11]. OVs can preferentially infect tumor cells, and its microscopic mechanism may be the cascade signaling responses induced by specific proteins, including PTEN, TP53, RB1 and RAS [12]. Current evidence demonstrates that viral replication only occurs in tumor cells due to the lack of intracellular antiviral machinery [13]. Taking advantage of the conditions of tumor cells themselves, OVs can specifically recognize and infect tumor cells and utilize host resources for self-replication. After OVs cleave the tumor cells, progeny OVs are released to reinfect the nearby tumor cells and repeat the process, thereby achieving macroscopic tumor-killing effects. Furthermore, OVs can further activate the host's innate and adaptive immune responses against malignant tumors [14–16]. Antigen-presenting cells can identify and capture fragments of broken tumor cells, thereby activating $CD8^+$ and $CD4^+$ T cells to participate in immune responses [17]. Genetically engineered modifications can enhance these properties of OVs. Strikingly, pexastimogene devacirepvec (Pexa-Vec, also known as JX-594), an oncolytic and immunotherapeutic modified vaccinia virus (VV), was approved as an orphan drug for treating HCC by the U.S. Food and Drug Administration (FDA) in 2013 [18,19]. JX-594, as a leading pharmaceutical preparation, has dominated the field of OV therapy. In addition, multiple OV-related therapeutic candidates in HCC are in the clinical and preclinical stages. These trials have indicated potent antitumor effects and relatively few side effects, which is satisfying and inspiring.

In this review, we summarized the pathogenesis and current therapies of HCC. Many single OV-based therapeutic agents with particular efficacy for the treatment of HCC have been described. Moreover, the current nonbiological vehicle- and stem cell-mediated OV intravenous delivery systems in HCC therapy are outlined. We also summarized OV-based biotherapy in combination with immune checkpoint inhibitors (ICIs), molecular targeted therapy, chimeric antigen receptor-T-cell therapy, radiofrequency, and transarterial chemoembolization (TACE), which have resulted in safer and more reliable HCC therapeutics. Finally, we discuss the future challenges and perspectives of OVs in HCC therapy based on both preclinical and clinical outcomes.

2. Background of HCC

Liver cancer is the most common cancer globally and the fourth leading cause of cancer-related mortalities. HCC is the dominant type of primary hepatic cancer, accounting for 90%. Moreover, the geographic incidence of HCC varies significantly. The HCC incidence is significantly higher in less developed regions than in developed areas, such as 54.8% in Eastern Asia and 10.8% in Southeastern Asia [20,21]. HCC risk factors also differ geographically. In developing countries such as India and China, the significant risk factors are aflatoxin B1 and hepatitis B virus (HBV) [22]. Hepatitis C virus (HCV)-associated cirrhosis [23] and nonalcoholic fatty liver disease [24] are the primary risk factors in developed regions. Based on the complex and multistep tumorigenesis, there are various treatments against HCC. Unfortunately, current therapies are still unable to extend the OS in advanced HCC patients.

2.1. *Pathogenesis*

Traditionally, hepatic stem cells were recognized as the origin cells of HCC. HCC may also be initiated by mature hepatocytes or transit-amplifying populations [25]. The HCC development process is multistep and complex [26]. As the first step of HCC, low-grade dysplastic nodules gradually transform into high-grade dysplastic nodules (early HCC) and finally into advanced HCC [26].

With the emergence of next-generation sequencing technology in recent years, a deeper understanding of HCC pathogenesis has been obtained at the molecular level. HCC pathogenesis mainly includes activation of pro-oncogenes, inactivation of tumor suppressors, and abnormal activation of transduction pathways. Among the pro-oncogenes, the N-ras gene was the earliest transformation gene in HCC. Ras protein is constitutively activated via point mutations in the N-ras gene, resulting in the continuous proliferation of tumor cells. The c-myc gene was the first oncogene discovered in HCC. The enhancement or translocation of the C-myc gene induces cancer cell differentiation. The C-myc gene is overexpressed in 70% of alcohol- and virus-related liver cancers [27]. Among tumor suppressor genes, the P53 gene is highly correlated with human malignant tumors. Studies have shown that P53 gene mutation can lead to uncontrolled cell proliferation and invasion and is related to HCC staging and OS rate. Another critical tumor suppressor gene is the P16 gene. This gene acts as a brake device of the cell cycle and is involved in the negative regulation of cell proliferation. Once failed, it can lead to uncontrolled proliferation of tumor cells.

Among transduction pathways, the current hot spot in HCC research is the Wnt signaling pathway, which includes β-catenin-mediated canonical and noncanonical signaling [28]. Wnt/β-catenin signaling is a highly conserved signaling pathway that regulates homeostasis. Dysregulation of Wnt/β-catenin signaling promotes tumorigenesis. Research shows that HCC cannot be activated via mutant and wild-type β-catenin alone. The formation of HCC requires the synergy of activated β-catenin and other chemicals or carcinogenic pathways, such as AKT, MARK, and H-RAS [29]. A recent study found that HCC development could be inhibited by downregulating Wnt/β-catenin signaling *via* overexpression of miR-300, a noncoding RNA [30]. In addition, there are reports confirming that autophagy can also inhibit GPC3/Wnt/β-catenin signaling. Notably, the pathogenesis of HCC is closely related to the TME, involving epithelial-mesenchymal transition, carcinogenesis, tumor invasion, and metastasis.

2.2. Current treatments

Liver transplantation, tumor resection, and tumor ablation are currently recognized as curative therapies in HCC. TACE, as topical therapy, may achieve a long-term response. However, curative therapies are only suitable for approximately 30% of early-stage HCC patients [31]. The 5-year survival rate of curative and topical therapies is only 18.1% [32–35]. For advanced HCC patients, the opportunity for curative therapy has been lost. At this stage, systemic therapy is usually recommended. The FOLFOX4 regimen, containing oxaliplatin, was approved by the NMPA for advanced HCC. However, patients with poor liver function and poor compliance are not suitable for this regimen [32].

In addition to systemic therapy, molecular targeted therapy and immunotherapy are also effective therapeutic modalities for HCC. Among molecular targeted therapies, tyrosine kinase modulators, such as triazolopyridazine (WO2007075567), can inhibit the proliferation of tumor cells by suppressing tyrosine kinase activity. In addition, sorafenib and lenvatinib, as molecular targeted drugs, were clinically approved by the FDA due to their safety and effectiveness [36,37]. Nivolumab was approved for the second-line treatment of HCC patients after treatment with the first-line treatment drug sorafenib in the United States.

Among immunotherapies, ICIs, including programmed cell death protein-1 (PD-1), its ligand programmed cell death-ligand 1 (PD-L1), and cytotoxic T lymphocyte antigen 4 (CTLA-4), have been granted FDA approval for the treatment of advanced HCC patients [38–40]. Adoptive cell therapy (ACT) also plays a vital role in immunotherapy [41,42]. Genetically modified effector cells specifically target tumor antigens in ACT [43]. The antitumor effect of adoptive tumor-infiltrating lymphocyte (TIL) therapy is more robust than single antigen targeting therapy because TILs isolated from HCC specimens can identify multiple antigens. Clinical trials have shown that TIL therapy increases the recurrence-free survival of HCC patients after surgical resection [44]. In addition, chimeric antigen receptor T-cell (CAR-T) therapy is FDA approved for the treatment of B-cell lymphocytic leukemia, multiple myeloma, and lymphomas [45,46]. A recent phase I clinical trial also confirmed the efficacy and safety of CAR T cells targeting HLA-A*02:01+/AFP+ HCC cells (NCT03349255) [45,47]. However, although CAR-T cells have reduced toxicity to healthy tissues, the specificity of targeting antigens still needs improvement.

3. OV-based HCC treatment

The liver is considered to be an immune-tolerant organ. Hepatic immune tolerance is caused by the interactions between peripheral leukocytes and resident hepatocytes, poor activation of T cells, the upregulation of immune checkpoints and an immunosuppressive microenvironment induced by chronic inflammation [48]. Thus, immunotherapies, including ICIs, angiogenesis inhibitors and anti-vascular endothelial growth factor (VEGF), are ineffective for more than 75% of HCC patients, despite these advances in immunotherapies. OVs have been a breakthrough following ICIs in immunotherapy. In addition to powerful and specific oncolysis of OVs, lysed tumor cells can release new tumor antigens, viral pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) to promote antitumor immune responses. Some studies have found that enhancement of antitumor immune activity achieves elimination of untreated tumors and maintains long-term immunity in combination therapy of OVs and ICIs or cytokines [49,50]. We know that natural and engineered OVs can specifically target, replicate, and kill tumor cells without damaging normal cells [51,52]. According to previous evidence, the safety of OVs has been confirmed in humans and can be controlled by specific antiviral preparations [53]. Currently, two OVs have been approved for clinical therapy. Recombinant human adenovirus type 5 (Ankerui, H101) was approved by NMPA to combine with chemotherapy for nasopharyngeal carcinoma in 2005 [54]. In addition, talimogene laherparepvec (T-Vec), the second-generation herpes simplex virus type I (HSV-1), was approved by the FDA for the treatment of inoperable melanoma in 2015 and approved in Europe in 2016 [55,56]. In addition, genetically engineered OVs have been gaining extensive attention in HCC treatment. Some OVs have been used in the preclinical and clinical treatment stages in HCC, including DNA viruses such as adenovirus (Ad), herpes simplex virus (HSV), and VV, and RNA viruses such as vesicular stomatitis virus (VSV), reovirus (Reo), influenza virus (IV), Newcastle disease virus (NDV), and M1 virus (Table 1). In the application of these genes, their pathogenicity is reduced by deleting virulence genes. Tumor targeting and antitumor ability are enhanced by genetic engineering, such as the insertion or integration of cytokine genes for activating antitumor immune responses. The advantages of RNA viruses include low pathogenicity and high oncolysis. The tumor-selective mechanisms of OVs are different from each other. The interferon system and RAS expression of tumor cells may be critical in HSV and Reo. The deletion of the thymidine kinase (TK) gene of VVs and host p53 mutation contribute to selective replication in tumor cells. In RNA viruses, the sensitivity of VSV to HCV-infected HCC is increased, and the Reo-associated immune response can inhibit HCV replication. In addition, intravenous administration of HSV-1 requires more effective delivery strategies, or it will be rapidly cleared by existing antiviral antibodies against HSV-1. Thus, the choice of OV therapy (OVT) for HCC depends on the characteristics of each OV.

3.1. Herpes simplex virus (HSV)

HSV belongs to a subfamily of the herpesvirus family. HSV consists of two serotypes: HSV-1 and HSV-2. Its oncolytic advantages are as follows: (a) large genome size (152 kb) to accommodate multiple functional exogenous genes, (b) strong replication ability, and (c) lack of host gene mutation. It is a double-stranded DNA virus with neurotropism. The high infection of HSV makes it a superior OV in the therapy of cancers, including HCC. However, because it has multiple double copies of the loci, genetic modification is difficult. Moreover, HSV causes the host to generate a strong immune response, which increases the difficulty of administration. Thus, most of its administration is intratumoural injection.

Table 1 – Preclinical and clinical studies of representative OVs monotherapy.

Virus family	Virus name	Virus Modification	Function	Target	Administration	Phases	Ref/ NCT	Status
HSV	Ld0-GFP	Two syncytial mutations:gKsyn1 (Ala-to-Val at position 40) and gB (Glu-to-Asp at postion 816)	Form large syncytia	Subcutaneous HuH-7-Luc, KYN-2-Luc,HepG2-Luc female BALB/c nu/nu mice HCC model	*i.v.*	–	[60]	–
HSV	T01	Deletion of c34.5 and a47; the LacZ gene replaces the ICP6 gene	Enhance antitumor immune responses	HuH-7, KYN-2, PLC/PRF/5, HepG2, Hepa1–6;subcutaneous Hepa1–6 C57BL/6 mice HCC model	i.t.	–	[57]	–
HSV	HSV-1-RP2	No description	Generate antitumor immune response	HCC patients	i.t.	Phase I	NCT00669136	Terminated
Ad	GP73-SphK1sR-Ad5	Integrate GP73 promoter and SphK1 into Ad5	Suppress tumor proliferation and promote apoptosis	Huh7; subcutaneous Huh7 female BALB/C nude mice model	i.t.	–	[67]	–
Ad	Ad5-PC	No description	Suppress $CD8^+$ T Cells	HCC-LM3, H22, Hepa1–6; H22 HCC ascites murine model, subcutaneous Hepa1–6 C57BL/6 mice and subcutaneous LM3 NCG mice HCC model	i.t.	–	[68]	–
Ad	ADV-HSV-TK	Contain HSV-tk gene	Promote necrosis and apoptosis	HCC patients	i.t.	Phase I	NCT00844623	Completed
VV	GLV-2b372	Insert TurboFP635 gene expression cassette into TK locus	Enhance tumor selectivity antitumor activity	SNU-449, Huh-7, Hep G2; subcutaneous Huh-7 female athymic nude mice HCC model	i.t.	–	[75]	–
VV	CVV	Delete TK gene and insert GFP instead	Enhance tumor selectivity antitumor activity	HepG2,SNU354,SNU449, and Sk-Hep-1; subcutaneous Sk-Hep-1 mouse HCC metastatic model	*i.p.*	–	[76]	–
VV	JX-594	Disruption of TK gene and insertion of hGM-CSF and bacterial β-galactosidase transgenes	Induce replication, tumor vascular targeting and tumor-specific immunity	Advanced HCC patients	Transdermal injection	Phase I	NCT00629759	Completed
VV	JX-594	Same as above	Same as above	HCC patients	i.t.	–	[72]/ NCT00554372	–
VSV	rVSV-GFP	Generate by reverse genetics and encode GFP	Monitor oncolytic potential of VSV against HCC	Hep 3B, Hep G2, McA-RH7777; subcutaneous McA-RH7777 rat HCC model	i.t.	–	[81]	–

(*continued on next page*)

Table 1 (*continued*)

Virus family	Virus name	Virus Modification	Function	Target	Administration	Phases	Ref/ NCT	Status
VSV	rVSV(MΔ51)-M3	Deletion of MΔ51, digest cDNA VSV and obtain M protein gene, ligate MΔ51 into digested cDNA clone	Inhibite cellular inflammatory responses	McA-RH7777; McA-RH7777 rat multifocal HCC lesions model	Hepatic artery infusion	–	[83]	–
Reovirus	Reo	No modificated gene	Elicit innate inflammatory responses	Subcutaneous Huh7 and Huh7-JFH1 SCID mice HCC model	*i.t.*	–	[88]	–
IV	delNS1-GM-CSF	Partial deletion of NS, GM-CSF inserted into influenza nonstructural protein 1 gene	Enhance selective cytotoxicity and replication	MDCK,A549, SMCC7721, HepG2; subcutaneous HepG2 nude mice HCC model	*i.t.*	–	[89]	–
IV	rFlu-CTLA4	Clone heavy chain and light chain of CTLA4 into downstream of PB1and PA	Enhance oncolytic efficacy and safety	HepG2, SMMC7721, and MHCC97L,H22; subcutaneous H22 mouse hepatocarcinoma model, H22 HCC ascites mouse model	*i.t.*	–	[90]	–
NDV	NDV-HK84	–	Enhance oncolytic efficacy and safety	SK-HEP-1, Hep3B, subcutaneous SK-HEP-1-Luc nude mice HCC model	*i.t.*	–	[94]	–
NDV	L289A (rNDV/F3aa)	Insert LacZ gene into Xbal restriction site	Tumor-specific syncytia formation and necrosis	HepG2, Huh7, McA-RH7777; McA-RH7777 male Buffalo rats multifocal HCC lesions model	Hepatic artery infusion	–	[5]	–
M1 virus	M1	No modificated gene	Potent oncolytic efficacy, high tumor tropism, and inducing apoptosis	Huh-7, Hep3B; subcutaneous Hep3B, PLC female BALB/c-nu/nu mice HCC model	*i.t./i.v.*	–	[96]	–

HSV has been used as a single agent in preclinical practice in HCC models, achieving significant antitumor efficacy [57,58]. For example, Ld0-GFP was reported as a novel HSV-1-based vector targeting HCC [59]. The potent oncolytic effect of Ld0-GFP was restricted to HCC cells without affecting normal cells. Along with directly killing tumor cells, the antitumor effect of Ld0-GFP was achieved by fusing into large syncytia and inducing immunogenic cell death. Ld0-GFP has two main syncytia mutations. The mutation of gksyn1 is Ala-to-Val at position 40, and the mutation of gB is Glu-to-Asp at position 816, which enhances the oncolytic efficiency by forming large syncytia. The mutated syncytia accelerate the fusion of Ld0-GFP in infected cells. The syncytial mutation of OVs did not occur in replication-limited normal cells, ensuring the safety of Ld0-GFP [60]. It has proven effective in preclinical HCC models, including subcutaneous xenograft, syngeneic HCC, and orthotopic HCC models (Fig. 1) [60].

T-01 is the third generation HSV-1. The ICP6 gene is replaced by the LacZ gene, and the c34.5 and a47 loci are deleted in T-01. Deletion of the a47 gene could enhance viral replication and promote antitumor immune responses. Meanwhile, it can partially restore MHC class I expression in virus-infected cells, activating lymphocytes and reducing the lysis of natural killer cells [57]. Severe liver dysfunction is described in HCC patients receiving chemotherapy. HCC patients often have HBV and/or HCV infections and liver cirrhosis in the clinic. Fortunately, no safety issues with T-01 have been identified thus far. Preclinical experiments confirmed that the growth of human hepatoblastoma and human HCC cells in HCC mouse models was effectively inhibited by T-01 [57].

In a phase I study (ClinicalTrials.org: NCT04336241), HSV-1-RP2 was identified as HSV-1 via genetic modification. It was evaluated for destroying tumor cells and generating an antitumor immune response by expressing an anti-CTLA-4 antibody. The trial recruited 36 subjects with solid cancers, including HCC, but no results have yet been reported.

3.2. *Oncolytic adenovirus (Ad)*

Ad is a double-stranded DNA virus with a genome size of approximately 36 kb [61]. It is usually used in gene therapy due to its low pathogenicity and ease of genome modification. The application of serotype 5 Ad (Ad5) is the most extensive [62]. The E1 and E3 genes in Ad5 are critical to replication and immune evasion. They are deleted to ensure the safety of recombinant Ad and efficient delivery. The p53 gene is mutated and inactivated in 40%–50% of HCC cases. The tumor suppressor gene p53 can arrest the host cell cycle in S phase, thereby preventing viral replication. E1B-deleted Ad may also replicate in HCC cells with p53 gene mutation, eventually leading to the lysis of tumor cells [63]. The E1B-deleted Ad5 is being evaluated in some clinical studies on unresectable HCC, but no results have yet been reported. However, Ad itself has the ability to infect quiescent and dividing cells because host P53 is inactivated by the E1B protein of Ad [64]. Furthermore, its transfection efficiency is high, and it can effectively produce high-titer virus ions.

Bai et al. constructed a new type of Ad named GP73-SphK1 sR-Ad5. This serotype integrated with the sphingosine kinase 1 short hairpin RNA (SphK1 sR) and Golgi protein73 (GP73) promoter [65]. The addition of SphK1 sR and GP73 to GP73-SphK1 sR-Ad5 significantly enhanced tumor cell proliferation, invasion, and migration [66]. The experimental results demonstrated that GP73-SphK1 sR-Ad5 specifically suppressed HCC progression and promoted the apoptosis of HCC cells [67]. Zhang et al. constructed a replication-competent Ad5-PC that produced a bispecific fusion protein containing PD-1 and CD137 L extracellular domains [68]. Ad5-PC successfully promoted the production of IFN-γ and activated $CD8^+$ T cells at a high level. The antitumor immune response was induced by activating $CD8^+$ T cells and increasing IFN-γ release [69]. In tumor-associated ascites and HCC models, the cure rates for HCC *via* intraperitoneal administration and intratumoral injection of Ad5-PC can reach 70% and 60%, respectively [68].

In addition, there was a phase I study (ClinicalTrials.org: NCT00844623) that evaluated intratumoral injection of defective Ad containing TK of HSV and intravenous injection of ganciclovir in advanced HCC patients. The treated tumors were stable in 60% of HCC patients. Intratumoral necrosis was observed in two HCC patients who received increasing doses. One of them survived for 26 months. The efficacy and good safety of the treatment were established in a clinical trial.

3.3. *Oncolytic vaccinia virus (VV)*

VV is a member of the orthopoxvirus genus in the family Poxviridae. It is a double-stranded DNA virus with a genome size of ~190 kb [70]. The biological characteristics of VV are as follows: (a) VV quickly replicates in cancer cells and lyses them. The virus particles are released from infected cancer cells after 8 h and can destroy the infected cells after 48–72 h [71]. (b) In this process, DNA from the virus is not integrated into the host's genome. (c) VV enters target cells through several membrane fusion pathways. (d) The VV-produced extracellular enveloped particles integrate with complement regulatory proteins from host cells into their own envelopes, resulting in passage through the blood flow barrier [72]. (e) VV particles are relatively stable and can be kept in dry powder form, which is easy to transport and use in clinical applications [73,74]. However, 50% of the 200 viral genes encoded by VV have unclear functions, which increases the unpredictable risk in the practical application of VV. Recently, recombinant VVs have made significant advances in the function of VVs, such as the special modification of VVs, VVs armed by immunostimulatory molecules or specific tumor-associated antigens or other novel antigens.

GLV-2b372 is a luminescent recombinant VV based on a wild-type Listeria virus. The experimental results showed that the infectivity of GLV-2b372 in HCC cells was concentration- and time dependent. The virus efficiently replicates in four human HCC cell lines [75]. As another evolutionarily engineered VV, CVV was developed to replicate selectively and lyse tumor cells. It can specifically track and regress metastatic tumor cells *via* deletion or inactivation of the viral TK gene (Fig. 2) [76]. In the metastatic HCC model, CVV attenuated the migration and metastasis of HCC cells and inactivated the expression of EMT markers.

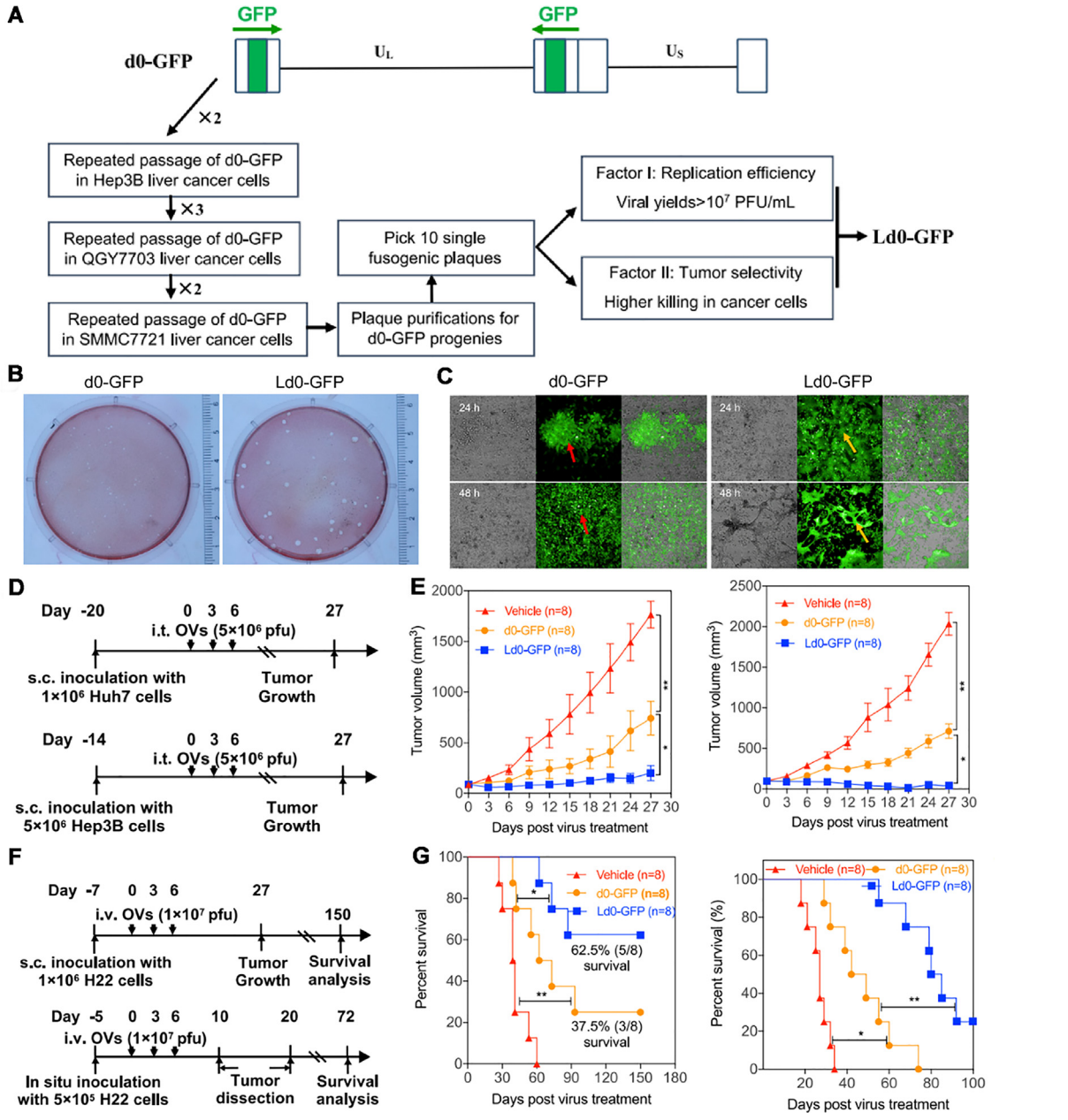

Fig. 1 – Schematic diagram of Ld0-GFP for HCC treatment. (A) Route map of generating fusogenic d0-GFP progenies and assessing viral replication efficiency and cell-killing ability. (B) Plaque assays of Ld0-GFP and d0-GFP virus on SMMC7721 monolayers. 200 PFU per dish for each virus in this assay. (C) Viral replication assays were performed on SMMC7721 cells. Ld0-GFP and d0-GFP replication patterns were monitored at 24 or 48 h after virus infection. Red arrows indicate virus-infected cells and regular plaques. Orange arrows indicate virus-infected cells and fusogenic plaques. (D) Treatment scheme. s.c., subcutaneous. (E) Growth of vehicle-, d0-GFP- or Ld0-GFP-treated Huh7 or Hep3B xenografts in nude mice. (F) Treatment scheme. i.v., intravenous. (G) Long-term survival of BALB/c mice bearing H22 tumors and orthotopic HCC model bearing mouse H22 HCC *in situ*, after receiving vehicle-, d0-GFP- or Ld0-GFP-treatment. Reprinted with permission from [60]. Copyright 2019 The Authors.

Pexa-Vec, a recombinant oncolytic VV, is used to activate specific antitumor immune responses by inducing the generation of granulocyte-macrophage colony-stimulating factor (GM-CSF) and lysis of tumor cells [74,77]. It is a VV with the destruction of the TK gene. TK activity is high in tumor cancers. This is necessary for VV replication. Thus, JX-594 has high tumor selectivity and replication ability within tumor cells. JX-594 has undergone multiple liver cancer clinical trials and is the most promising oncolytic VV used in clinical research [78]. In phase I clinical trials, HCC patients showed a positive response to intratumoral administration of JX-594 (ClinicalTrials.org: NCT00629759). A subsequent randomized phase II clinical trial (ClinicalTrials.org: NCT00554372) studied the feasibility of a low or high dose of JX-594 to treat

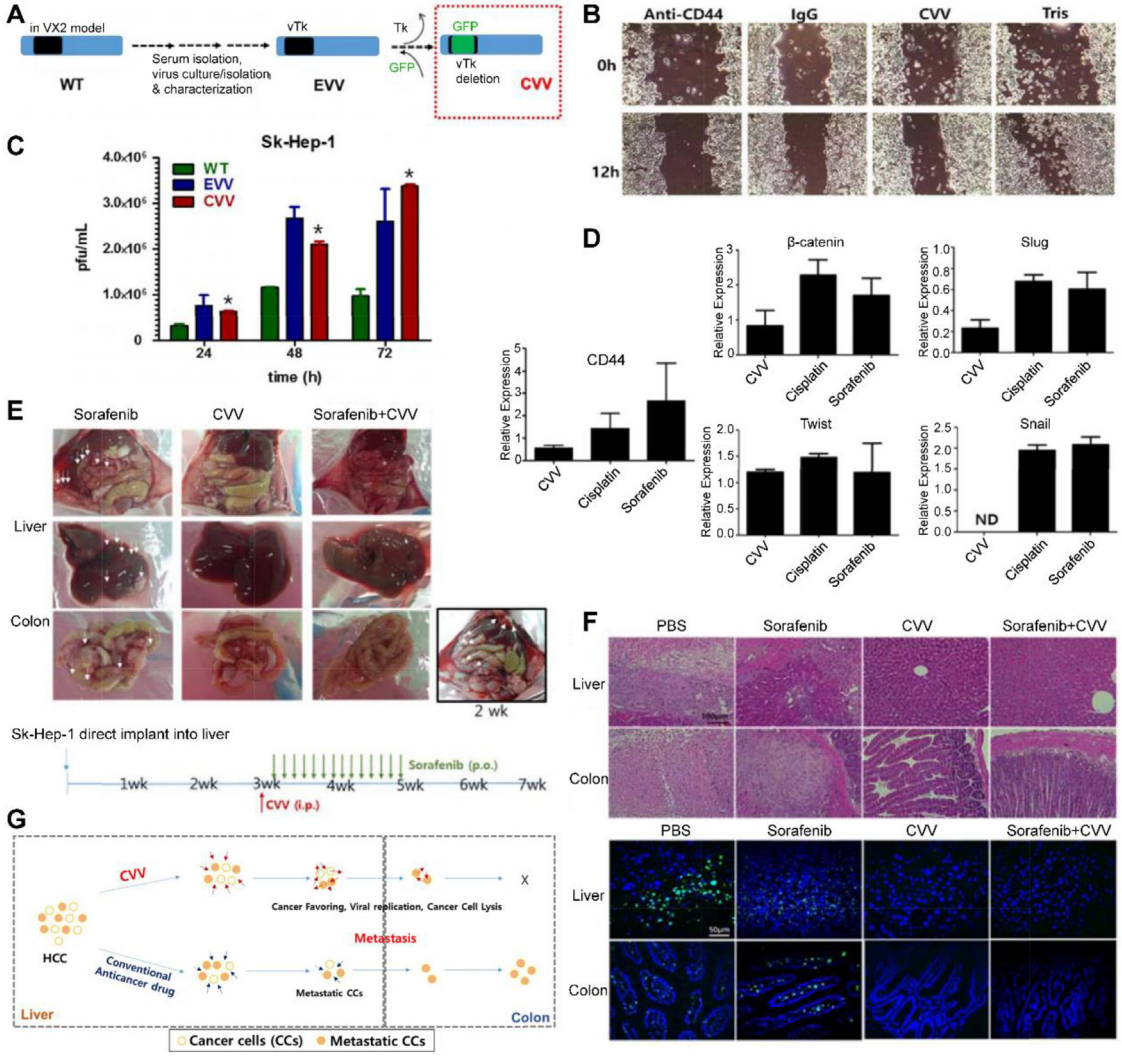

Fig. 2 – Schematic diagram of CVV for the metastatic HCC to colon treatment. (A) Constructing CVV from WT and EVV. (B) The migration of Sk-Hep-1 after the treatment of anti-CD44 or CVV. (C) Detection of Viral replication of WT, EVV and CVV. (D) The expression of CD44 and the EMT markers in CVV-treated and Cisplatin or Sorafenib-treated Sk-Hep-1 cells. (E) Metastasis of HCC to colon with the CVV or/and Sorafenib treatment. (F) Hematoxylin and eosin staining and Tunnel assay. (G) Schematic illustration of CVV targeting and killing the metastatic HCCs to colon and the restrictive effects of anticancer drug. Reprinted with permission from [76]. Copyright 2014 Elsevier.

30 HCC patients by intratumor injection [72]. In the trial, the intrahepatic response rate was 62%. The OS of the high-dose and low-dose administration groups was 14.1 months and 6.7 months, respectively [79]. These results verified that JX-594 has a great therapeutic effect in HCC therapy [72].

A recombinant oncolytic VV derived from the VV strain in China Tian Tan was constructed. It has a stronger attenuating ability. The recombinant VV armed by interleukin-24 (IL-24) can induce apoptosis of virious tumor cells without obvious toxicity to normal cells [80]. IL-24, as a pro-apoptotic and growth-inhibiting gene, can inhibit tumor growth, invasion, angiogenesis and metastasis. IL-24 delivery through replication-defective Ad or liposomes lacks tumor-specific targeting. This study confirmed that the expression of the tumor suppressor gene IL-24 mediated by this recombinant VV can directly lyse tumor cells and selectively induce apoptosis of HCC cells [53].

3.4. *Vesicular stomatitis virus (VSV)*

VSV is a negative-strand RNA virus with an envelope with an inherent ability to infect cancer cells [81]. Compared with other OVs, the replication speed of VSV is fast (8–10 h in tumor cells) [82], and antitumor effects can be anticipated within a few hours after administration. However, it can infect normal cells, and the oncolytic effects and immune response caused by VSV are also lower. Therefore, advanced genetic modification strategies are needed to improve the tumor selectivity and immunogenicity of VSV. Recent studies have used a recombinant VSV expressing the green fluorescent protein (GFP) gene (rVSV-GFP). In

immunocompetent orthotopic HCC models, intratumoral injection of rVSV GFP can achieve efficient and specific replication in HCC tissues, inhibit tumor growth, and prolong the survival of rats [81]. In addition, viral replication is restricted to the tumor tissues, and no collateral damage occurs in the surrounding normal liver tissues. However, the oncolytic ability of rVSV (MΔ51) is attenuated by host-induced inflammatory responses. M3 is a high-affinity chemokine binding protein derived from murine gammaherpesvirus-68. rVSV (MΔ51)-M3, a recombinant vector with M3 protein expression, can evade the host's antiviral inflammatory responses. This increases viral titers in tumor tissues and enhances the oncolytic effect. In rat models of HCC, the recombinant vector rVSV(M51)-M3 is administered through the hepatic artery to treat multifocal lesions, ultimately reaching a 50% cure rate [83].

In addition, Jeong Moon et al. found that the HCV core protein Hep3B-core can downregulate the STAT1-HDAC4 signaling pathway in primary liver cancer with HCV infection. The susceptibility of cells expressing Hep3B-core to VSV was increased, which provided a proper intracellular environment for VSV replication with decreasing HDCA4 protein expression [84]. Therefore, VSV was proposed as an effective treatment strategy for HCV-infected HCC.

3.5. *Reovirus (Reo)*

Reo is a double-stranded RNA virus [85]. Due to the unstable genome of Reo RNA, its genetic modifications are prone to pathogenic gene mutations. Reoxygenation is also not fully understood. It only replicates in cells with abnormal cellular pathways, especially in cancer cells with abnormal RAS pathways, eventually causing cancer cells to lyse. In addition, it is difficult for Reo to genetically modify. Wild-type Reo itself has significant selective oncolytic properties and has been used in clinical trials [86]. Recently, many studies have confirmed that Reo can rupture tumor cells to release tumor antigens and increase the release of inflammatory cytokines and chemokines to promote antitumor immune responses [87]. Clinical-grade oncolytic Reo induced innate immune activation in preclinical HCC models. HCV replication was effectively inhibited by the Reo-associated immune response *in vitro* and *in vivo* [88]. Thus, Reo is recommended as a complementary therapy in HCV-caused HCC. Additionally, the innate immune responses induced by Reo are suitable for treating other oncogenic virus-associated cancers, such as HBV-infected HCC.

3.6. *Influenza virus (IV)*

IV belongs to the Orthomyxoviridae family. It is a segmented single-stranded, negative-stranded RNA virus with an envelope. Due to reverse genetics technology, the IV genome is easy to manipulate, avoiding potential DNA safety problems. This offers hope for its use as an effective cancer therapeutic. However, researchers have not yet fully grasped the biological characteristics of IV, including the immune phenomenon of IV and the production of neutralizing antibodies in the host. Therefore, further exploration of IV is needed.

Influenza A virus delNS1-GM-CSF is a recombinant virus vector derived from PR8 influenza. The NS part of the viral vector was deleted, and the GM-CSF gene was inserted into the nonstructural protein-1 lesion of influenza. The delNS1-GM-CSF virus has selective cytotoxicity in a panel of HCC cell lines. Tumor growth can be suppressed by intratumoral injection of delNS1-GM-CSF in a dose-dependent manner in HCC xenograft mouse models [89]. The antitumor effect of delNS1-GM-CSF was also confirmed in clinical HCC samples, demonstrating that it is an effective oncolytic agent for the treatment of HCC.

RFlu-CTLA-4, a recombinant IV, was constructed by encoding the CTLA-4 heavy chain at PB1 lesions and the CTLA-4 light chain at PA lesions. *In vitro* and *in vivo* studies found that it had a higher oncolytic effect than delNS1-GM-CSF. In addition, the rFlu-CTLA-4 virus, expressing an immune checkpoint blocker targeting CTLA-4, could selectively kill multiple HCC cell lines and did not damage normal cell lines. In a mouse HCC model, the growth of HCC was inhibited, and the OS of mice was extended following rFluor-CTLA-4 intratumoral injection [90].

3.7. *Newcastle disease virus (NDV)*

NDV is an unsegmented negative-strand RNA virus with an envelope and belongs to the family Paramyxoviridae. The natural hosts of NDV are birds, but NDV can enter the cells by binding to sialic acid residues in the cancer cells of humans and rodents. Compared with HSV, VV or Ad, its virulence and pathogenicity are all low in humans [91]. The RNA replication of NDV in normal cells can increase the expression of RPK, which activates the cellular IFN signaling pathway, causing an antiviral response. The expression of RPK in tumors is delayed, allowing NDV to evade IFN surveillance and replicate in large quantities, thereby killing tumor cells [92]. Therefore, the biosafety of NDV is certain. NDV-HK84 is a wild-type OV. A study found that NDV-HK84 could inhibit tumor growth and had an oncolytic effect by activating IFN signaling *in vitro* and *in vivo* HCC models [93,94]. The applications of genetically engineered NDV in HCC treatment have been investigated for years. An NDV vector with the L289A mutation in the F gene lesion was constructed to enhance oncolytic activity by modifying the F3aa protein. It was named rNDV/F3aa (L289A). In Buffalo rats with multifocal orthotopic HCC, the recombinant vector was administered *via* the hepatic artery, which induced the formation and necrosis of specific tumor syncytia [5]. The adjacent liver tissue had no toxic reaction, and the survival time of tumor-bearing rats treated with L289A was significantly extended.

3.8. *M1 virus*

M1 virus was isolated from China's Hainan Island in the 1960s and belongs to the togavirus family [95]. The genome of M1 is 11.7 kb nucleotides in length. M1 virus induces the apoptosis of tumor cells by activating a severe ER stress response. Yan et al. found that the M1 virus derived from mosquitoes can kill liver cancer cells specifically and has evident oncolytic activity and tumor tropism [96]. Notably, M1 virus can specifically

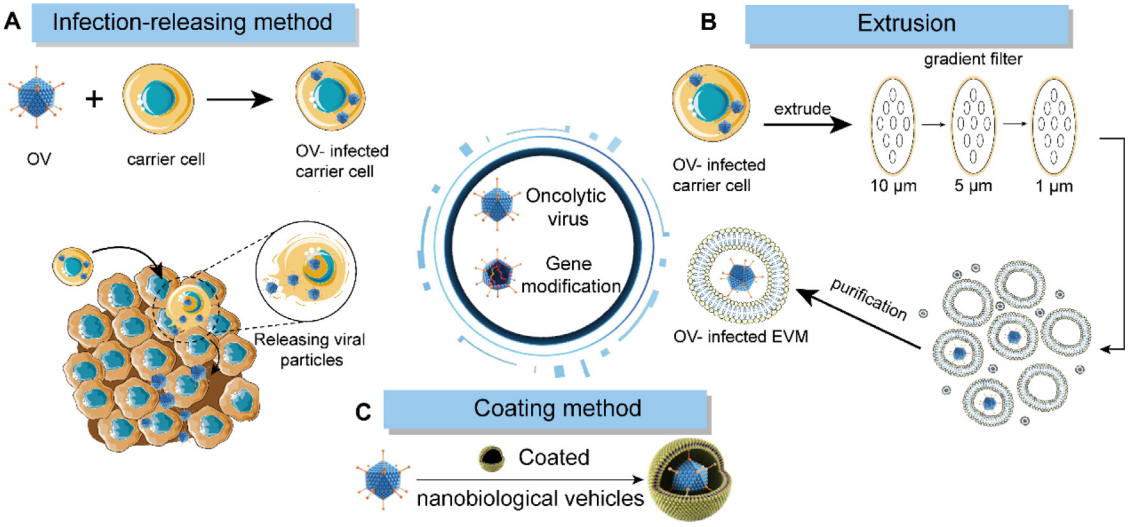

Fig. 3 – A schematic diagram of optimized OV delivery strategies. (A) Infection-releasing method about OV cellular carriers. (B) Extrusion about bioengineered cell mimetics. (C) Coating method about nonbiological vehicle.

infect various tumor cells and cause replication-related tumor cell death but not normal cells. Evidence from a study shows that the growth of tumors is inhibited by intravenous injection of M1 in an HCC nude mouse model with Hep3B xenografts. In addition, this virus can deactivate targeted tumor cells with a deficient zinc finger antiviral protein (ZAP) signaling pathway. During experiments in primate cynomolgus monkeys, no clinical manifestations, biochemical, immune, imaging, or toxicity evidence were found, verifying a certain degree of safety of the M1 virus.

4. Systemic delivery of OVs for HCC therapy

OVs are safe and feasible in HCC therapy. However, local administration of OVs, including hepatic artery infusion and intratumoral injection, has corresponding limitations for the treatment of advanced tumors. First, there is a certain degree of density and pressure inside the tumor mass, which makes it difficult to inject OVs into the tumor. Second, many cases cannot be performed by intratumoral injection, such as lesions located in deep organs and malignant tumors that are too diffuse. Finally, the compliance of patients with intratumoral or hepatic artery injection is poor, especially for those requiring multiple consecutive administrations. Furthermore, it is almost ineffective for metastatic deposits in patients with advanced tumors. Significant progress has been made in the development of OVs over the years [55], but the most superior delivery model of OVs for cancer therapy remains controversial. Theoretically, partial systemic metastases or micrometastases cannot be detected by current diagnostic techniques. Thus, the intravenous administration of OVs appears to be the most ideal delivery strategy. OV intravenous injection overcomes some critical limits of local administrations and has particularly unique advantages. Patient compliance is also improved via the convenience and feasibility of intravenous injection. Intravenous systemic delivery of OVs can simultaneously treat the primary tumor and metastatic deposits. However, OV therapy *via* intravenous administration cannot achieve a satisfactory therapeutic effect. The most critical reason is the rapid clearance of OVs during circulation due to virus-neutralizing antibodies, complement, anti-viral cytokines, tissue-resident macrophages, and nonspecific uptake of tissues such as lung, liver, and spleen. The suboptimal OVs could escape from the vascular compartment. The clinical application of OVs has been hindered by their low enrichment in the tumor region [97]. Researchers have been working on overcoming these obstacles over the last decade. Optimizing the intravenous delivery of OVs has become a hotspot. The aim of these OV systemic delivery strategies is to circumvent antiviral immunity, including OV cellular carriers, bioengineered cell mimetics, and nonbiological vehicles (Fig. 3). By optimizing OV delivery strategies, the viral circulation time is extended, and tumor targeting is increased, thereby enhancing antitumor efficacy. These OV delivery strategies may reduce the inactivation of OVs and change viral tropism. The safety of OVT was determined during the experiment. Among them, cell-based carriers have been considered to be the most promising delivery strategy for OVT in recent years [98]. OVs are loaded by carrier cells *ex vivo*. Then, the OV-infected carrier cells are injected intravenously. Consequently, they migrate into tumor sites, where the virions are released. Cell-free viral particles are neutralized by antiviral antibodies in the bloodstream (Fig. 3A). Several potent strategies have also been tested at the preclinical level in HCC treatment. In bioengineered cell mimetics, taking extracellular vesicle mimetics (EVMs) as an example, EVM drug-loaded technology is relatively mature. OV-infected tool cells are placed in a series of extruder devices (pore sizes of 10 μm, 5 μm, and 1 μm). Then, they were purified by centrifugation with an iodixanol density gradient, finally forming OV-loaded EVM

(Fig. 3B). Aside from bioengineered cell mimetics, wrapping OVs with nonbiological vehicles is also widely used in the field of OVT (Fig. 3C). They have special advantages, such as a well-established preparation method, high feasibility of systemic delivery models designed by researchers, and realization of industrial production.

4.1. *Carrier cell-mediated OV systemic delivery system for HCC therapy*

There are two purposes to serve carrier cells as systemic-administered carriers. First, certain cell types can enhance the tumor tropism of OVs. Second, these cells can protect OVs from neutralizing antibodies. OVs can be successfully loaded into certain cells *ex vivo*, and the biodistribution and pharmacodynamics of OVs can be improved by the application of carrier cells in mouse models. Carrier cells can internalize OVs or attach virions to their cell membranes. The duration of cell viability is maintained long enough to distribute in blood circulation and deliver OVs to the tumor [99]. Mesenchymal stem cells (MSCs) are the most investigated carrier cells for OVs [100,101]. The immunosuppressive features of MSCs may be a reason [100], which can reduce the antiviral immune response. Other types of carrier cells, including monocytes/macrophages and lymphocytes, have also been studied. However, among them, T lymphocytes are seldom used as carrier cells of OVs for HCC treatment. T lymphocytes are also potential carrier cells of OVs because they are free in the blood circulation and can home to tumor regions. In addition, T lymphocytes with cytotoxic effectors can synergistically achieve antitumor therapy. However, any immunosuppressive TME can cause T lymphocytes to be excluded from the tumor [102]. Thus, T lymphocytes as carrier cells may be used in tumors without the features of immune deserts. This may be a potential reason why they have seldom been studied as OV carrier cells for HCC treatment.

4.1.1. *Stem cell-mediated OV systemic delivery system for HCC therapy*

MSCs are multipotent nonhematopoietic cells with high self-renewal capacity, multilineage multidifferentiation potential, and specific markers on their surface [103,104]. MSCs are widely used in regenerative medicine and have proven safe and feasible [105]. MSCs have obvious tumor targeting and can migrate and implant into tumor sites. Thus, they can be ideal carriers for targeted antitumor therapy. Yoon et al. constructed MSC-encapsulating Ads to lyse HCC cells under normal and hypoxic conditions, effectively inhibiting tumor cell metastasis. In animal experiments, MSCs can specifically deliver OVs to tumors, which prolongs the circulation time of virions and reduces the emergence of potential risks of systemic administration [106]. Importantly, it was found that the recombinant oncolytic Ad can inhibit the growth of liver transplanted tumors and prolong the survival period of mice. In addition, measles immunization generated by HCC patients was able to inhibit the replication and spread of the measles virus. MSCs infected with replication-competent measles virus can deliver the virus to target tumor sites, alleviating virus sequestration and neutralization by host immune factors. In orthotopic HCC implantation in mice, the anticancer efficacy of measles virus infection with human bone marrow-derived mesenchymal stem cells (BM-hMSCs) was evaluated. After MV-containing MSCs were injected into the tail vein, BM-hMSCs homed to HCC tumors and inhibited tumor growth [107].

The OV delivery rate is still low for metastatic tumors, and OVs cannot directly enter the targeted regions. Systemically administered Ad at a high dose can cause a prominent systemic toxic effect that is rapidly cleared before the viral vector reaches metastatic tumors [108]. Human umbilical cord Wharton's jelly (WJ)-derived mesenchymal stem cells (HUMSCs), in addition to the common features of MSCs, enhance tumor-specific targeting [109,110]. Intravenously administered HUMSCs can target malignant tumors and micromigrate into metastatic tumors [111,112]. HUMSCs can supply E1A proteins, which are necessary for the replication of Ads. E1A-engineered HUMSCs were used to deliver replication-defective adenoviral vectors, which selectively migrated and aggregated into metastatic tumors and released adenoviral particles to kill tumor cells. It was also observed that Ad-loaded E1A-engineered HUMSCs supported Ad replication and promoted more virus particles to infect tumor cells [113]. Establishing a novel targeted therapy system based on HUMSCs has significant advantages in treating metastatic tumors.

4.1.2. *Monocyte/macrophage-mediated OV systemic delivery system for HCC therapy*

The biodistribution and pharmacokinetics of OVs have been demonstrated to be improved by using carrier cells. However, the type of carrier cells to execute the function is debatable. As mentioned above, MSCs serve as ideal carrier cells and have been employed in preclinical studies on HCC. Monocytes are rarely used as OV carrier cells. The infiltration of tumor-associated macrophages is an important feature of the TME and is recruited to tumor tissue by cytokines and chemokines [114]. We can recover a large number of monocytes from autologous peripheral blood. Furthermore, monocytes can differentiate into macrophages. Theoretically, monocytes may become ideal carrier cells. Hamster/human monocyte/macrophage cell lines (HM-1/mono-Mac-6) were investigated as Ad carrier cells in an athymic nu/nu mouse model with the HuH-7-cell line [115]. Ad-mediated tumor transduction via systemic administration was correspondingly improved by monocytes. Because the tumor targeting of monocytes was modest, the majority was still enriched in the liver. The homing of the liver and tumor was simultaneously increased by a larger quantity of Mono-Mac-6.

4.1.3. *Natural killer (NK) cell derivative-mediated OV systemic delivery system for HCC therapy*

NK cells, as innate immune cells, can prevent the invasion of pathogens and transformation of malignant tumors. The NK-92 cell line, as an NK-cell derivative, is derived from NK cells of patients with human malignant non-Hodgkin's lymphoma. Native Ad5 knobs replaced by Ad37 knobs can enhance the tropism of OVs. The knob is an important nexus of Ad5 combined with CAR on the surface of the tumor cell. There is a wide expression of CAR on a variety of tumor cells.

This recombinant Ad, Ad5/Ad37 has a higher transduction efficiency into NK-92 cells. NK-92-infected Ad5/Ad37 showed stronger oncolytic effects in HCC Hep3B and HepG2 cell lines [116].

4.2. *Bioengineered cell mimetic-mediated OV systemic delivery system for HCC therapy*

Natural extracellular vehicles are another ideal carrier. They can hide OVs from the immune system and gain multiple pathways into tumor cells. However, the most obvious limitation of their application is the low yield [117]. The acquisition of EVM is easier than that of natural extracellular vehicles. Moreover, their functions are similar to those of natural extracellular vehicles. For example, PD-1-expressing recombinant Ads (Ad5-P) infecting 293T cells expressing VSV-G protein were harvested. Then, EVM-coated Ad5-P (EVM/VSV-G Ad5-P) was collected *via* density gradient centrifugation. The infectivity of the encapsulated Ad5 is enhanced in cancer cells with low expression of the Ad receptor, and the virus can escape the neutralization of anti-Ad5 neutralizing antibodies. Ad-associated antibodies can be resisted due to the encapsulation of viruses [118]. The *in vivo* experiments confirmed that EVM/VSV-G Ad5-P could persistently express PD-1, enhancing the antitumor immune response. Survival was prolonged in an ascitic HCC mouse model [119]. During therapy, neutralizing antibodies against VSV-G were generated. The titer of anti-VSV-G antibodies was raised following an increase in the viral dose administration. Thus, the therapeutic window needs careful consideration due to the existence of neutralizing antibodies. The therapeutic technology of EVM encapsulation showed its advantages and clinical significance.

In addition, nanovesicles from bioengineered donor cells were constructed by Lv et al. [17]. Ads were encapsulated by these nanovesicles, forming a systemic delivery system (Ads@BCMN). The Ads were protected from neutralization of anti-Ads antibodies through systemic administration of Ads@BCMNs. This delivery system enhanced the antitumor effects of Ads in HepG2 and Huh7 cells (liver cancer cell lines), while Ads@BCMN did not increase cytotoxicity in HUVECs or LO2 cells (normal cell lines). Sodium/taurocholate cotransporting polypeptide (NTCP) specifically interacted with preS1, a bioactive ligand for NTCP. In the test, preS1 was designed to modify nanovesicles (BCMNs-preS1), which promoted cellular uptake. The targeted delivery efficiency of the nanovesicles was significantly improved in HepG2-NTCP-bearing nude mice. Mouse survival was prolonged by this delivery system (Fig. 4). The bioengineered nanovesicle delivery strategy could become a valuable technology for the clinical therapy of HCC.

4.3. *Nonbiological vehicle-mediated OV systemic delivery system for HCC therapy*

4.3.1. *Polymer-based OV systemic delivery system for HCC therapy*

Apart from encapsulating OVs with stem cell-mediated biocarrier materials, nonbiological vehicle-mediated carrier materials are also applied in the systemic delivery of OVs. For example, polyethylene glycol (PEG) or N-[2-hydroxypropyl] methacrylamide and the negatively charged Ads surface capsid are coassembled by electrostatic interactions [120,121]. In addition, positively charged poly(amidoamine) (PAMAM) dendrimers with primary amino groups at the end were used to coat Ad serotype 5 hexon proteins with negatively charged amino acids on the outer surface through electrostatic interactions [122]. In a mouse model of liver cancer xenografts, systemic administration of coated Ads significantly delayed tumor growth and prolonged the survival rate compared to uncoated Ads [123].

Surface modification with bioactive polymers can improve the selectivity of OVs and prevent their inactivation. OVs were coated with a polygalactosyl-b-agmatyl diblock copolymer (Gal32-b-Agm29), which has a high affinity for the asialoglycoprotein receptor (ASGPR) on the surface of hepatocytes [124]. The coated oncolytic Ads had strong infectivity and great anticancer properties in the HepG2 liver cancer cell line with high ASGPR expression. However, it had a poor therapeutic response in the ASPGR-negative A549 cell line. In contrast, naked oncolytic Ads had similar antitumor effects in the two cell lines. Galactosylated polymers enhanced OV entry into HCC cells. They also increased the release of ATP and HMGB1 and promoted viral infectivity and antitumor effects (Fig. 5) [124].

With the rise of nanotechnology, the *in vivo* performance of OVs was further improved. The nanotechnology-based preparation of OVs is a promising antitumor treatment strategy. Gold nanoparticles modified with 2 kDa PEG were attached to oncolytic Ads to generate Ads-gold-PEG. These complexes constructed by nanotechnology reduce binding to blood components and significantly enhance ultrasound-mediated transport levels and distances while maintaining their natural biological activity [125]. In a mouse model of HCC, it was confirmed that Ads-gold-PEG had improved antitumor efficacy [126]. The potent oncolytic agent is being tested for clinical investigation.

4.3.2. *Biomineralization-based OV systemic delivery system for HCC therapy*

The efficacy of Ads for local HCC gene therapy has been confirmed [67,68]. However, the disadvantages of Ad systemic delivery include antiviral immune response, innate immunity and nonspecific isolation caused by some organs. However, biomineralization can enhance virus-mediated gene transfer, protect OVs from hepatic sequestration and reduce the innate immune response. The Fox example, ZD55-IL-24, is engineered Ads expressing IL-24 [127,128]. An efficient delivery system for ZD55-IL-24 (PLC-Ads) was constructed through coprecipitation of calcium phosphate and ZD55-IL-24, lipoidization and PEGylation. PLC-Ads prolonged circulation times through intravenous administration and did not show any cytotoxicity in QSG-7701 and L02 cells (normal human liver cells). The delivery system with a self-carrying neutralizing antibody resisted *in vivo* neutralization in BALB/c mice and successfully achieved targeted delivery without systemic toxicity in Huh-7-bearing BALB/c nude mice (Fig. 6) [129]. In addition, recent clinical trials on ONYX-015 have shown the therapeutic effect of the Ads for local cancer [130]. Given their immune neutralization and tumor off-

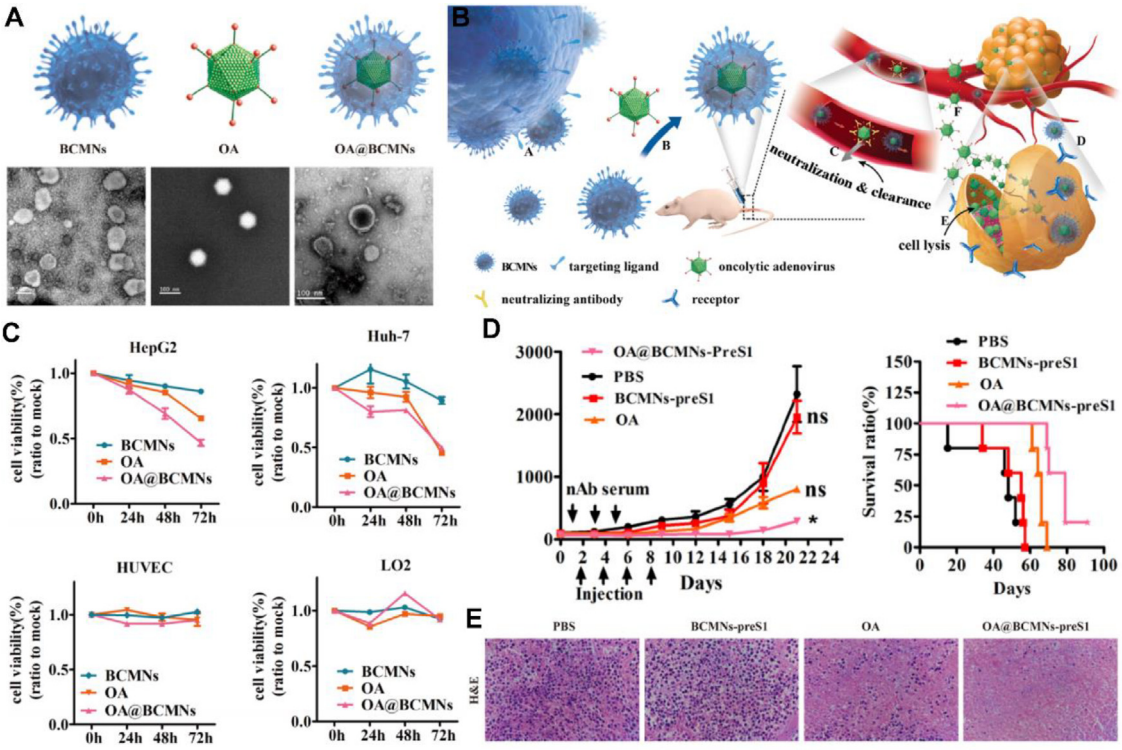

Fig. 4 – Schematic diagram of OA@BCMNs-preS1, Ads@BCMNs-preS1 for HCC treatment. (A) TEM images negatively stained with uranyl acetate in different group. (B) Design and mechanism of Ads@BCMNs. (C) Detection of cell viability by the MTT assay. (D) Growth curves of tumor and percent survival of HepG2-NTCP bearing nude mice after different treatments. (E) H&E staining of tumor sections. All the panels were magnified 400-fold. Reprinted with permission from [17]. Copyright 2019 American Chemical Society.

target phenomenon, Kong et al. constructed one systemic delivery system (Ads-Trail@SiO2) [131]. The Ads-encoding trail gene was encapsulated by silica. The experimental results suggested that compared with native Ads, the virus biodistribution and antitumor efficacy of Ads-Trail@SiO_2 were significantly improved in Hep-G2 tumor-bearing nude mice. Moreover, Ads-Trail@SiO_2 could prevent neutralizing antibodies from recognizing and eliminating the virus *in vivo*.

5. OV-based combination therapy for the treatment of HCC

5.1. *OVT combined with ICIs*

The antitumor mechanism of OVs is not entirely understood. Activating antitumor immune responses is key to OVT, and the mechanism of action is very complicated. OVs can selectively infect tumor cells and subsequently cause local antiviral inflammation, resulting in the induction of a severe host immune response. OV-infected tumor cells have highly immunogenic characteristics. After OVs infect tumor cells, IFN-γ, Toll-like receptor agonists, cell-derived DAMPs [132–134], and viral PAMPs are released to activate the antitumor immune response [135]. This further eliminates uninfected tumor cells locally and remotely. However, when the initial immune response is robust, the antitumor activity of effector immune cells is inhibited by various immunoregulatory factors, such as PD-1 and CTLA-4. This results in difficulty obtaining a sustained antitumor immune response. Once OVs are cleared by the host's antiviral immune response, the immunosuppressive TME is restored, leading to tumor recurrence. This is a crucial reason why OVs cannot achieve satisfactory antitumor effects. Many intratumorally upregulated immune checkpoints produce an immunosuppressive TME, leading to tumor immune escape [136–138]. ICIs can inhibit tumor immunosuppressive signaling pathways and expose tumor cells to be recognized by the host immune system. The combination of anticancer therapeutic modalities would be a promising antitumor strategy. A summary of OVT combined with ICI in clinical and preclinical trials is discussed below (Table 2).

5.1.1. *Preclinical and clinical trials of HSV combined with ICIs*

OVH-aMPD-1, a new type of HSV, can target the PD-1/PD-L1 pathway [139]. Recombinant OVH was constructed by replacing both copies of the ICP34.5 and ICP0 coding sequences with the eGFP gene and replacing the ICP27 core promoter with a core human telomerase reverse transcriptase (hTERT) promoter. OVH-aMPD-1 was constructed in the backbone of OVH, in which two copies of the eGFP coding sequence were replaced by a gene encoding aMPD-1 scFv. In C57BL/6 mouse liver cancer models carrying Hepa1–6 cells, OVH-aMPD-1 induced strong upregulation of PD-L1 in

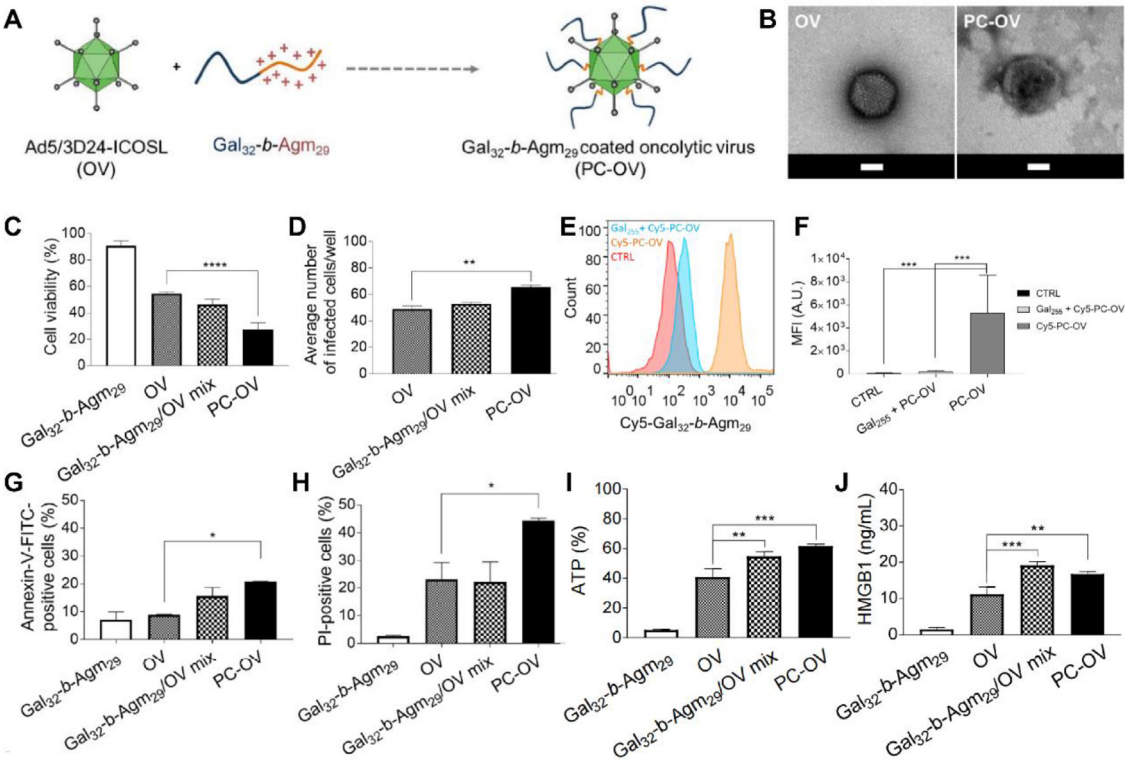

Fig. 5 – Schematic diagram of Gal32-b-Agm29 coated OV for HCC treatment. (A) Schematic diagram of the formation of Gal32-b-Agm29 coated OV complex. (B) The imaging of PC–OV by electron microscopy. (C) The cytotoxicity and (D) the quantification of infected cells assay in Gal32-b-Agm29, OV, Gal32-b-Agm29/OV mix and PC–OV treatment groups. (E) The cellular uptake of Cy5-Gal32-b-Agm29 assay by flow cytometry in each group. (F) The median fluorescence intensity in each group. (G) Early apoptotic and (H) late apoptotic/ necrosis after 48 h of treatment in HepG2. (I) Extracellular ATP after 48 h of treatment and (J) extracellular HMGB1 after 72 h of treatment in the supernatant of HepG2. Reprinted with permission from [124]. Copyright 2021 The Authors.

cancer cells. OVH-induced interferon release further increased the expression of PD-L1 in tumor cells. scFv-containing OVH-aMPD-1 could release immunogenic DAMPs, promote DC antigen cross-presentation, enhance the tumor antigen-specific T-cell response and function, and improve local and systemic tumor control in a mouse liver cancer model. In addition, the percentage of intratumoral CD155þ granulocytic-MDSCs and CD155þ monocytic-MDSCs was significantly higher in tumor cells under the combination therapy of OVH-aMPD-1 and anti-TIGIT antibody. The combination therapy blocked the immune checkpoints (simultaneous administration for the first time), highlighting the advantages of synergy [139]. OVH-aMPD-1 and anti-TIGIT antibody combination therapy can manage and control aggressive tumors locally and systemically for a long duration.

T-Vec is a genetically engineered HSV-1 that can selectively replicate and kill tumor cells. The newly released progeny virus continues to infect the surrounding tumor cells [140,141]. Simultaneously, tumor antigens are presented to specific $CD8^+$ T cells to form potentially effective antitumor immunity [142]. However, T-Vec oncolytic therapy still requires a combination with systemic therapy for easy metastasis of malignant tumors. A phase Ib/II clinical trial for HCC/liver metastases (ClinicalTrials.org: NCT02509507) is underway. In this study, T-Vec was intratumorally injected into the liver tumor under ultrasound/computed tomography guidance, combined with the systemic administration of pembrolizumab MK-3475–611/Keynote-611 (Day 1 of each cycle). Its safety and antitumor efficacy in patients with liver cancer are currently under investigation.

5.1.2. *Clinical trials of VV combined with ICIs*

Pexa-Vec is a potent recombinant HCC oncolytic soybean virus used in clinical research. However, in a randomized phase IIb clinical trial (ClinicalTrials.org: NCT01387555), JX-594 as a second-line therapy did not improve the median OS of advanced HCC patients who had failed previous sorafenib treatment [143]. The median OS of patients treated with JX-594 intravenously plus best supportive care (BSC) was 4.2 months. In comparison, the median OS of BSC alone was 4.4 months [HR 1.19 (95% confidence interval: 0.78–1.80); $p = 0.428$]. There was no significant difference between the two groups [143]. Therefore, systemic OV therapy as a single agent and supplementary therapy could not achieve satisfactory outcomes for patients who failed sorafenib therapy.

A combinational therapy is critical to improving the therapeutic effectiveness of JX-594. Several clinical trials of JX-594 for HCC therapy have been carried out. A phase I/IIa

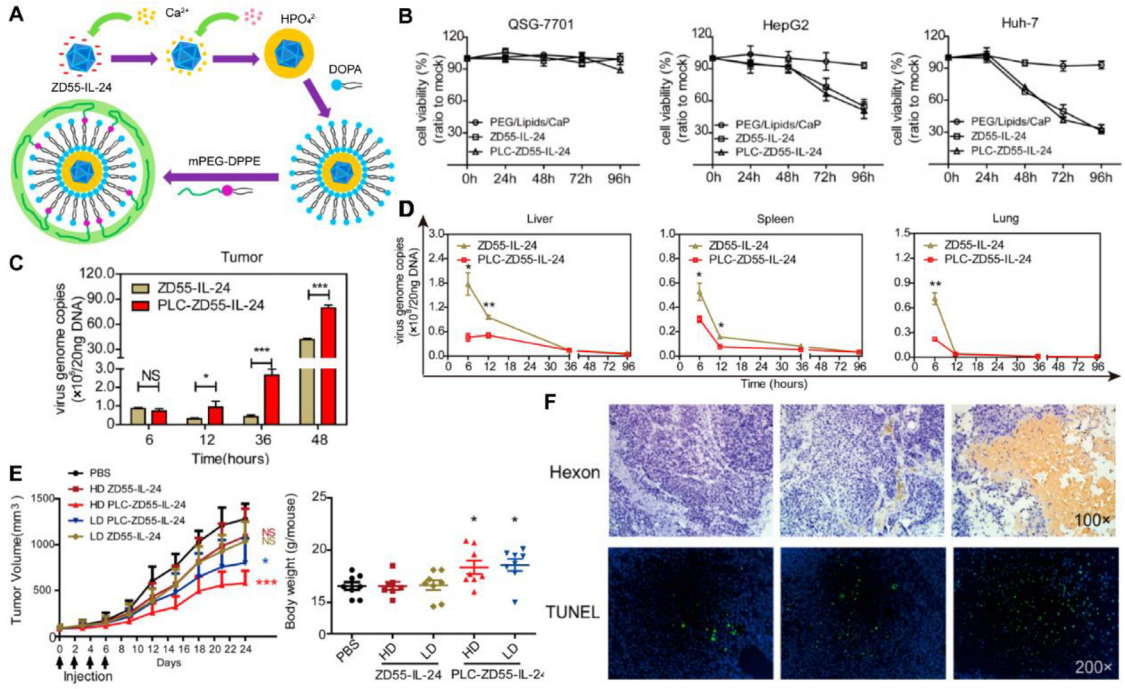

Fig. 6 – Schematic diagram of PLC-ZD55-IL-24 for HCC treatment. (A) Synthetic route of PLC-ZD55-IL-24. (B) Detection of cell viability by the MTT assay. (C, D) Viral genome copies in tumor, liver, spleen, and lung. (E) Growth curve of tumor and body weight of the mice on day 24. (F) Tumor sections were stained with Hexon and TUNEL. The upper panel (Hexon) was magnified 100-fold and the lower panel (TUNEL) was magnified 200-fold. Reprinted with permission from [129]. Copyright©2021 The Authors.

trial (ClinicalTrials.org: NCT03071094) using JX-594 combined with nivolumab as the first-line therapy for advanced HCC patients has been completed. Participants were administered JX-594 as 3 biweekly intratumoral injections and nivolumab intravenously every 2 weeks (from week 2). Twelve patients with stage C liver cancer were enrolled, including 5 completed subjects in phase I and 4 completed subjects in phase II a. The overall response rate according to RECIST 1.1 was 33.3% (9.9% to 65.1%). The risk for serious adverse events in phase I and phase IIa was 85.71% and 80.00%, respectively. Further research is needed to overcome the cytotoxicity.

5.1.3. *Clinical trials of M1 virus combined with ICIs*

Based on the promising biological activity and safety of oncolytic M1, a phase 1 clinical trial has begun (ClinicalTrials.org: NCT04665362), which uses recombinant oncolytic M1, anti-PD-1 antibodies (SHR-1210, camrelizumab), and apatinib (VEGFR2 inhibitor) combination therapy for advanced/metastatic HCC. They are not yet recruiting.

5.2. *Clinical trial of OVs combined with molecular targeted therapy*

A phase III trial (ClinicalTrials.org: NCT02562755) evaluated the survival rate of HCC patients by the combination treatment of Pexa-Vec and sorafenib *vs.* sorafenib monotherapy. In the trial, pexa-Vec, as the VV, was designed to induce an antitumor immune response through intratumoral injection followed by sorafenib at week 6. Sorafenib, a specific kinase inhibitor, has antiproliferation and antiangiogenic properties and is the standard of care for advanced HCC. However, the combination treatment of Pexa-Vec and sorafenib did not show more advantages than sorafenib monotherapy. The synergistic effects of OVT and specific kinase inhibitors need further research in HCC.

5.3. *OVT combined with CAR-T-cell therapy*

CAR-T-cell therapy is used mainly for the treatment of hematological malignancies. However, it is less satisfactory in clinical studies of solid tumors, primarily due to antigenic heterogeneity, suboptimal CAR-T-cell trafficking, and a tumor immunosuppressive environment [144]. Preclinical results showed that OVs are natural allies of CAR-T cells, and both played a synergistic role in antitumor therapy. OVs can increase CAR-T-cell infiltration and antitumor activity and kill antigen-negative tumor cells. CAR-T cells can also enhance subsequent oncolysis mediated by OVs [145].

A phase I clinical trial for solid tumors was carried out to further understand the optimal dose and safety of the combined therapy (ClinicalTrials.org: NCT03740256). In a clinical trial, special immune cells of HER2 chimeric antigen receptor-specific cytotoxic T lymphocytes (HER2-specific CAR-

Table 2 – Preclinical and clinical studies of representative OVT combined with ICIs.

Virus family	Virus name	Virus Modification	Administration	Phases	Interventions	Target	Refer/ NCT	Trials Status
HSV	OVH-aMPD-1	eGFP gene replaced ICP34.5 and ICP0, hTERT replaced ICP27	i.t.	–	Anti-TIGIT antibody	C57BL/6 mouse liver cancer models carrying Hepa1–6	[139]	Completed
HSV	T-Vec	Coding for human GM-CSF	i.t.	Phase Ib/II	Pembrolizumab	HCC/Liver Metastases patients	NCT02 509,507	Active, not recruiting
VV	Pexa-Vec (JX-594)	TK-deletion plus GM-CSF	i.t.	Phase I/IIa	Nivolumab	HCC patients	NCT03 071,094	Terminated
M1	M1-c6v1	No description	i.v.	Phase I	SHR-1210/Apatinib	Advanced/ Metastatic HCC patients	NCT04 665,362	Active, not recruiting
VV	Pexa-Vec (JX-594)	TK-deletion plus GM-CSF	i.t.	Phase III	Sorafenib	HCC patients	NCT02 562,755	Completed
Ad	CAdVEC	No description	i.t.	Phase I	HER2-specific autologous CAR-T cells	Advanced $HER2^+$ Solid Tumors patients	NCT03 740,256	Recruiting
HSV	G47D	Deletion of diploid γ134.5 and a47 gene, insertion of Escherichia coli lacZ	i.t.	–	RFA	Hep3B, SNU-398, HuH-7, HepG2, PLC/PRF/5, and Hepa1–6;BALB/c nu/nu female mice, A/J mice, and C3H female mice	[148]	Completed
HSV	T-Vec	Coding for human GM-CSF	i.t.	–	RFH	HCC patients	[150]	Completed
HSV	HSV-TK/GCV	Recombinant lentivirus-carrying HSV-TK	i.t.	–	RFH	HepG2;nu/nu mice HCC models carrying Hep2	[151]	Completed
Ad	rAd-p53	p53 expression cassette replaced E1	i.t.	–	TACE	Advanced HCC patients	[153]	Completed
Ad	H101	Deletion of E1B gene	Catheter into hepatic artery	–	TACE	HCC patients	[154]	Completed
Ad	ADV-TK	Insertion of TK gene	i.t.	–	GCV	HCC patients	[155]	Completed

T cells) were used in combination with intratumoral injection of the oncolytic adenovirus CAdVEC to treat solid tumors. Once CAdVEC infected tumor cells, an immune response was activated to attack and kill cancer cells. However, local administration of OVs was ineffective against metastatic deposits. Subsequent treatment with specific T cells could kill virus-infected tumor cells.

5.4. *OVT combined with radiofrequency ablation (RFA) or radiofrequency hyperthermia (RFH)*

RFA is an effective local treatment for HCC that can reduce the recurrence of HCC at treated lesions but not at other sites in the liver [146]. HCC tumors over 3 cm may show local recurrence after RFA treatment. Thus, new treatment strategies must control larger HCC tumors after RFA. When tumor-associated antigens are released by RFA, an effective adjuvant strategy is required to improve the immunosuppressive TME. Combining RFA with OVs has proven effective in synergizing RFA against HCC and reducing recurrence at remote sites after RFA therapy [147]. For example, G47D, a triple-mutated HSV-1, can be an effective adjuvant therapy after RFA treatment in HCC by enhancing specific antitumor immune responses [148]. Tumor growth at new lesion sites without RFA treatment can be inhibited by combination therapy with G47D and RFA. Another study showed that synergistic therapy of RFH and OVT enhances the replication efficiency of OVs and promotes apoptosis and cytolysis of HCC cells, leading to the accelerated fracture of HCC tumors [149]. T-Vec was injected into the tumor by a

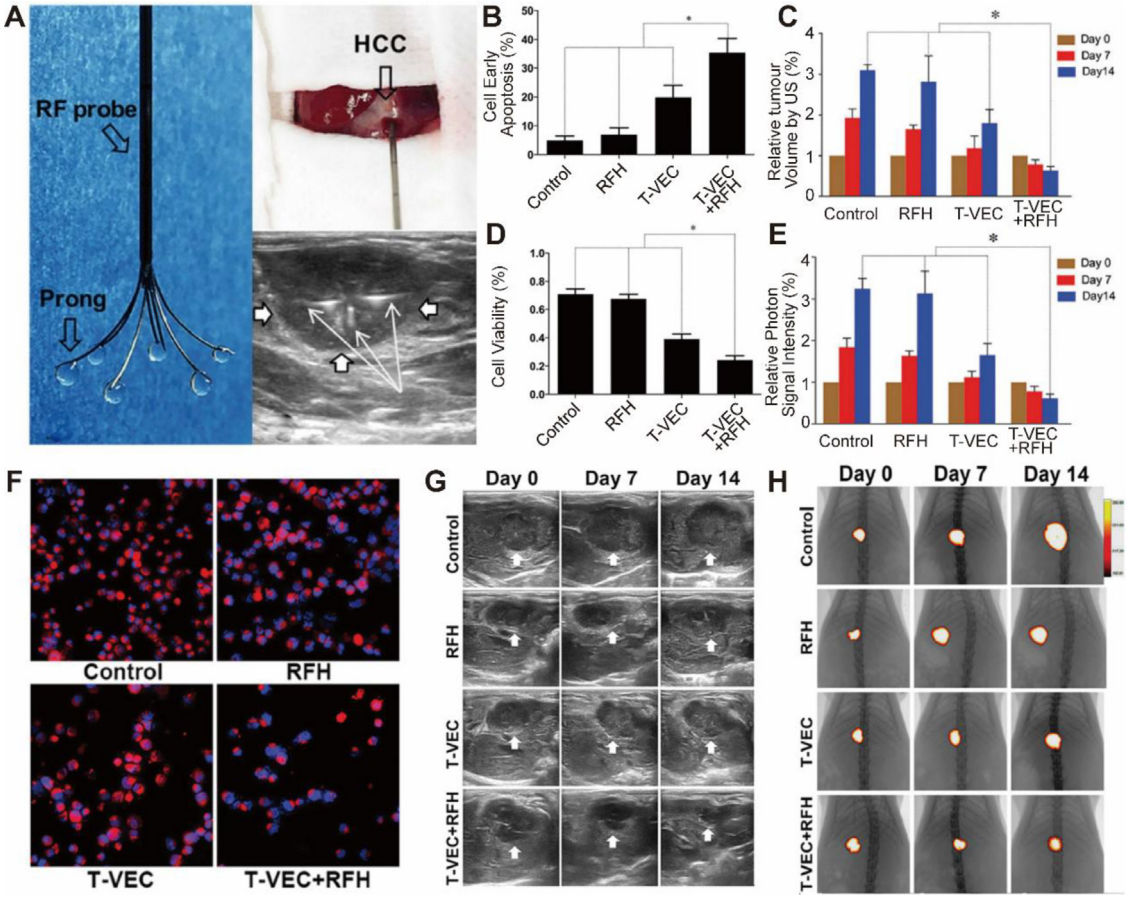

Fig. 7 – Schematic diagram of combination treatment of T-Vec and RFH for HCC. (A) The multi-functional perfusion-thermal electrode setup. The RF electrode is inserted into the center of the tumor. Ad5-PC is delivered into the margin of a rat HCC. (B, C) The cell apoptosis and viability in control, RFH, T-Vec and T-Vec+RFH treatment groups. (D, G) The tumor volume by ultrasound imaging on days 0, 7 and 14 in each group. (E, H) Optical/X-ray imaging and the value of relative phonton signal intensity on days 0, 7 and 14 in each group. (F) The number of viable cells by confocal microscopy. Reprinted with permission from [150]. Copyright©2019 The Author(s).

multifunctional perfusion-thermal radiofrequency electrode, followed by RFH. The results showed that RFH promoted the apoptosis of tumor cells. RFH can also enhance the delivery of T-Vec by intratumoral injections, thereby reducing the toxicity of T-Vec through intravenous administration. The combination treatment of RFH and OVT was confirmed to enhance the control of local tumors for larger HCC (Fig. 7) [150]. A preclinical trial verified the feasibility of the combination therapy of RFH and HSV thymidine kinase/ganciclovir (HSV-TK/GCV) in HCC treatment. The results showed that nonablative RFH as a local therapy could improve the antitumor effects of HSV-TK/GCV in an HCC xenograft mouse model [151]. The combination strategy may effectively reduce the recurrence rate of marginal tumors after local therapies such as RFH, TACE, and intratumoral gene-targeted therapy.

5.5. *OVT combined with TACE*

TACE is recognized as a standard and effective local therapy applied in unresectable intermediate-advanced HCC. The mechanism is usually that certain chemotherapeutic drugs are delivered to the tumor lesions, and the arteries feeding the lesions are blocked. The TACE therapeutic efficiency was weakened after 6 months of administration in patients with large tumors, resulting in portal vein thrombosis or tumor thrombus and poor liver function [152]. Meanwhile, TACE can induce ischemia and hypoxia of liver tissue, promoting HCC metastasis and enhancing angiogenesis *via* upregulation of VEGF.

Gene therapy is a promising treatment for HCC. HCC patients with a mutated or deleted p53 gene have a poor prognosis. RAd-p53, a recombinant Ad encoding human p53, can specifically replicate and kill tumor cells. A study demonstrated that the combination therapy of rAd-p53 and TACE could be more effective in extending the survival of HCC patients and controlling the growth of the tumor than TACE monotherapy [153]. In addition, H101, a recombinant human Ad type-5 that replicates only in p53-mutated tumor tissues, is sensitive to HCC with p53 inactivation. The combination therapy of H101 and TACE can prolong OS and benefit HCC prognosis [154].

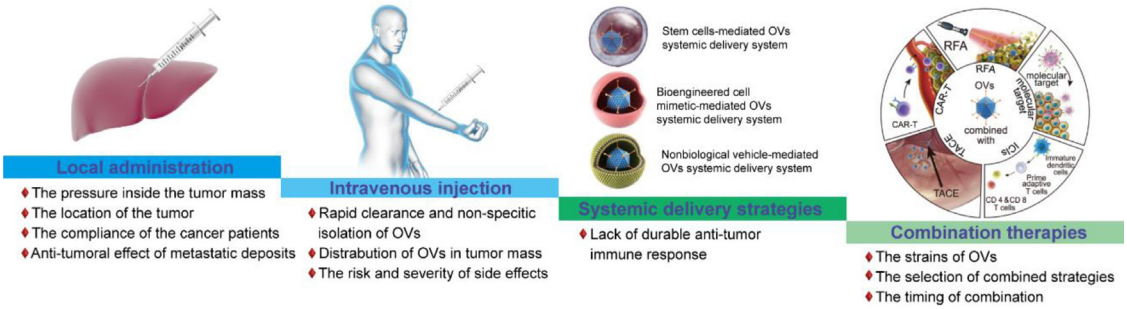

Fig. 8 – Clinical challenges and prospect of OVT.

5.6. *Other methods*

ADV-TK is a recombinant Ad coding the TK gene that serves as a suicide gene. ADV-TK can be transferred to nontoxic GCV, interfering with DNA synthesis and promoting spontaneous death in tumors. In addition, combination treatment of ADV-TK and GCV can produce the 'bystander effect'. Even if the TK gene is only transferred into a small number of tumor cells, GCV–triphosphate molecules can induce the death of untransduced tumor cells *via* gap junctions and cell-to-cell signal transduction. A previous *in vitro* study found that coexposure to ADV-TK and GCV suppressed the viability of SMMC-7721 HCC cells. Moreover, a recent study illustrated that the combination of ADV–TK and GCV influences the expression levels of cell apoptosis-associated proteins such as caspase–3, Bcl-2, and Bax in tumor tissues, thereby activating the apoptotic pathway [155].

6. Clinical challenges and prospects

OVT has been widely investigated for the treatment of advanced liver cancer. Currently, the types of OVs used in preclinical and clinical trials of liver cancer include (1) wild virus strains with natural specificity to infect tumor cells, such as the M1 virus, and (2) OVs constructed by genetically engineered deletion or insertion of related functional genes. For example, the T-01 virus with deletion of the a47 site can enhance virus replication and partially restore the expression of MHC class I molecules in infected cells, thereby stimulating lymphocytes and reducing the lysis of natural killer cells in the host; and (3) control the replication of OVs only in tumor cells by inserting tumor-specific promoters in front of the essential genes of the virus, such as the hTERT promoter and AFP promoter. OVT has achieved initial-stage achievements, but its development still faces challenges (Fig. 8).

A large amount of evidence has confirmed the regression of local tumors through OVs intratumoral injection or hepatic artery infusion, but complete tumor eradication is unachievable. This local administration of OVs has certain limitations during treatment [156], including the pressure inside the tumor tissue, the location of the tumor, and the compliance of the cancer patients. Notably, it is almost ineffective for metastatic deposits in patients with advanced tumors. OV intravenous injection overcomes some critical limits of local administration and has particularly unique advantages. Systemic administration can prevent tumor recurrence and metastasis. However, systemic OV administration also has critical disadvantages in practical applications. First, systemic administration may induce the rapid clearance and nonspecific isolation of OVs from the *in vivo* circulation. Then, the concentration and depth of OVs within tumor tissue through intravenous injection are worse than those through intratumoral injection at the same dosage. Conversely, the risk and severity of side effects are more significant. To address these fatal limits, OVT still requires effective systemic delivery strategies.

There are three main systemic delivery strategies for HCC therapy: (i) stem cell-mediated OV systemic delivery; (ii) bioengineered cell mimetic-mediated OV systemic delivery; and (iii) nonbiological vehicle-mediated OV systemic delivery systems. These new technologies can overcome neutralizing antibodies, evade liver tropism, enhance tumor targeting of OVs, and reduce innate and adaptive immune responses.

Another potential obstacle of OVT is that OVs incite a strong initial immune response but fail to generate a durable antitumor immune response. The virogenicity of OVs and the tumor antigens released after lysis of tumor cells can activate the endogenous immune system, improve the recognition of DC cells, and enhance the killing effect of $CD8^+$ T cells, ultimately resulting in simultaneous reversal of the immunosuppressive TME. However, OVT monotherapy cannot fully achieve a satisfactory antitumor effect. Combination therapy can play a synergistic antitumor role and reduce the dosage of OVs, which can achieve low toxicity. Although promising, the strains of OVs, the selection of combined strategies, and the timing of combination still need further research.

We should emphasize the exploration of the safety and antitumor effect of OVs in the future. More superior delivery strategies and combination therapy of OVs will become the direction of future research on OVT (Fig. 8).

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81700453).

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