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Exploring oncolytic virotherapy in solid tumor treatment

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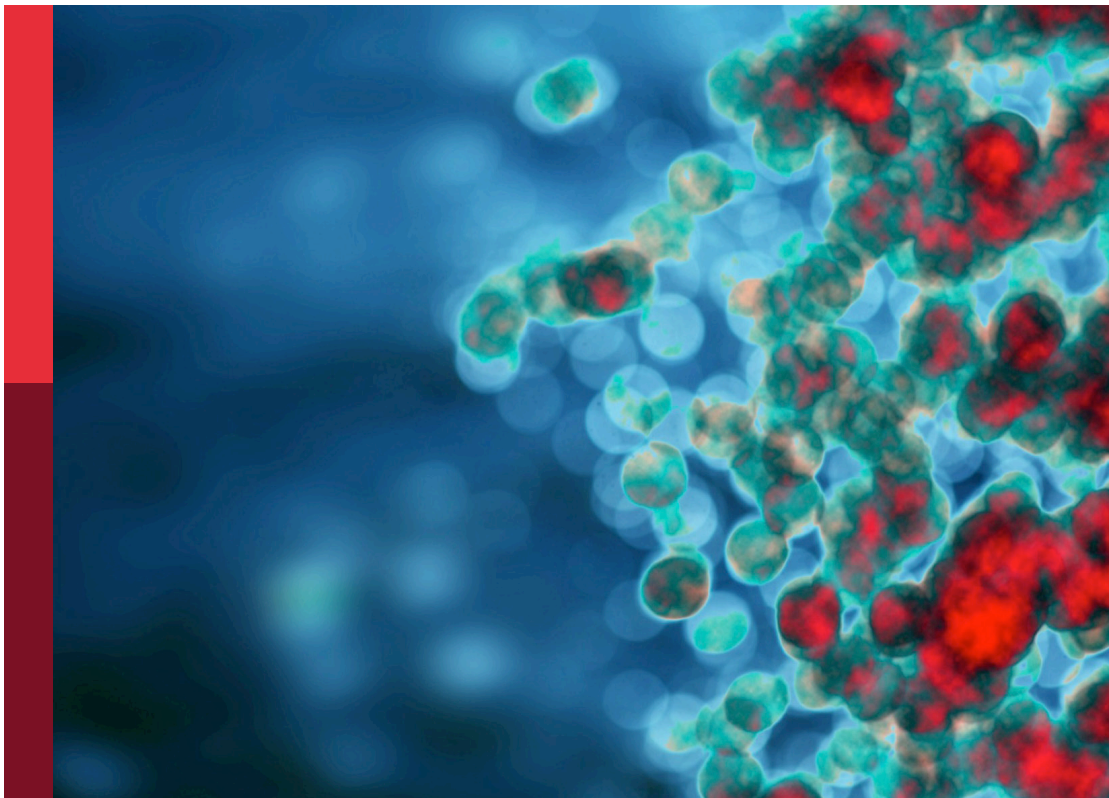

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Exploring oncolytic virotherapy in solid tumor treatment

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New hopes for the breast cancer treatment: perspectives on the oncolytic virus therapy

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Oncolytic virus (OV) therapy has emerged as a promising frontier in cancer treatment, especially for solid tumours. While immunotherapies like immune checkpoint inhibitors and CAR-T cells have demonstrated impressive results, their limitations in inducing complete tumour regression have spurred researchers to explore new approaches targeting tumours resistant to current immunotherapies. OVs, both natural and genetically engineered, selectively replicate within cancer cells, inducing their lysis while sparing normal tissues. Recent advancements in clinical research and genetic engineering have enabled the development of targeted viruses that modify the tumour microenvironment, triggering anti-tumour immune responses and exhibiting synergistic effects with other cancer therapies. Several OVs have been studied for breast cancer treatment, including adenovirus, protoparvovirus, vaccinia virus, reovirus, and herpes simplex virus type I (HSV-1). These viruses have been modified or engineered to enhance their tumour-selective replication, reduce toxicity, and improve oncolytic properties.Newer generations of OVs, such as Oncoviron and Delta-24-RGD adenovirus, exhibit heightened replication selectivity and enhanced anticancer effects, particularly in breast cancer models. Clinical trials have explored the efficacy and safety of various OVs in treating different cancers, including melanoma, nasopharyngeal carcinoma, head and neck cancer, and gynecologic malignancies. Notably, Talimogene laherparepvec (T-VEC) and Oncorine have. been approved for advanced melanoma and nasopharyngeal carcinoma, respectively. However, adverse effects have been reported in some cases, including flu-like symptoms and rare instances of severe complications such as fistula formation. Although no OV has been approved specifically for breast cancer treatment, ongoing preclinical clinical trials focus on four groups of viruses. While mild adverse effects like low-grade fever and nausea have been observed, the effectiveness of OV monotherapy in breast cancer remains insufficient. Combination strategies integrating OVs with chemotherapy, radiotherapy, or immunotherapy, show promise in improving therapeutic

outcomes. Oncolytic virus therapy holds substantial potential in breast cancer treatment, demonstrating safety in trials. Multi-approach strategies combining OVs with conventional therapies exhibit more promising therapeutic effects than monotherapy, signalling a hopeful future for OV-based breast cancer treatments.

KEYWORDS

breast cancer, immunotherapy, oncolytic virus therapy, tumour microenvironment, TME

1 Introduction

Oncolytic viruses (OVs) are the main subject of interest in multiple ongoing clinical trials in many cancer types. Effects in the field of immunotherapy for solid tumours are promising and mainly focussed on the field of immune checkpoint inhibitors and CAR-T cells (1). Despite these therapies having the potential to achieve full tumour regression, there is a number of patients whose response to the treatment is limited (2), fostering the development of new solutions targeting tumours resistant to the currently available forms of immunotherapy (3). Although the first OV gained the approval of the US Food and Drug Administration (FDA) only in 2015[1], researchers have been working on developing this form of therapy for decades (4). OV therapy is currently known as „a major breakthrough in cancer treatment" (4). What makes OVs so clinically useful is their ability to affect the cancer cells through several different mechanisms (3) as well as their selective, destructible impact only on cancer cells, without destroying physiological tissues in the human body (5, 6). For the past twenty years, many clinical trials have been carried out, with the most used OVs being adenovirus, HSV-1, reovirus, vaccinia virus, and Newcastle disease virus, and only a few of them having been approved for commercial use (7).

2 Mechanism of action

OVs may be natural or artificially engineered viruses. They can replicate in cancer cells, leading to the lysis (8). This phenomenon has been observed for years as a spontaneous tumour regression after viral infection in some patients. Recent advances in clinical research and genetic engineering enabled the development of specifically targeted viruses, performing various types of anticancer activity (8). Unlike chemotherapy and radiotherapy, OVs kill cancer cells without harm to normal tissues, which makes them a promising alternative to traditional methods of treatment. OVs have also played a significant role in cancer immunotherapy. They can modify the tumour microenvironment (TME) by triggering an antitumour immune response and having synergistic effects with other anticancer therapies (8).

Oncolytic viruses are divided into two groups, natural weak (wild-type OVs) and genetically modified virus strains. As some mutations typical for cancers, such as in *P53, RB1, PTEN, DCC, RAS*, *P16*, and *VHL* genes, impair the antiviral abilities of cells; they are often suitable targets of OV attacks. Some natural virus strains prefer tumour cells. However, their anticancer effectiveness is limited, and the pathogenicity might be challenging to control. Both the safety and performance of viruses can be increased by genetic manipulations, including gene element regulation, and inserting exogenous genes in engineered recombinant OVs. The examples of possible modifications augmenting the anti-tumour efficacy of OVs are presented in Table 1. All of them have been already considered as a potential therapy for breast cancer or its metastases.

3 Types of viruses used in oncolytic trials

Each oncolytic virus has its characteristics that imply the safety profile, influence the possible therapeutic use, and suggest which areas require modifications in a particular case (8). In this review, we decided to focus on selected oncolytic virus strains that are used in breast cancer trials in particular. Their mechanisms of tumour targeting and antitumour activity are presented in Table 2.

3.1 Adenovirus

Adenovirus is a DNA double-stranded virus that can enter the cells in a receptor-mediated way or through endolysis. Its genomic DNA is released and transferred to the nucleus, where it is replicated but not incorporated into chromosomes. The main efforts in the optimisation design of oncolytic adenoviruses concern restricting their replication selectively to cancer cells

1 Food and Drug Administration. (2024). Alphabetical List of Licensed Establishments Including Product Approval Dates as of 01-JAN-2024. https://www.fda.gov/media/76356/download [Accessed January 16, 2024].

TABLE 1 Genes expressed by the genetically engineered OVs that improve their anti-cancer efficacy.

Group	Representatives	Effect	OV examples	References
Cytokine genes	*GM-CSF, IFN, interleukin (IL-2, IL-7, IL-12, IL-15, IL-18, IL-23, IL-24)* genes	Promotion of the presentation and recognition of TAAs, activation of APCs, increase in CD4+ and CD8+ T cells, boost of the anti-tumour immune response, inhibition of tumour proliferation, metastasis, and angiogenesis	T-vec (HSV-1) OH2 (HSV-2), VG161 (HSV-1), T3011 (HSV-1), TILT-123 (AdV), Cont-VV (VV), OncoViron (AdV), Pexa-vec (VV), CG0070 (AdV), M032 (HSV-1)	(9–14)
Chemokine genes	*CCL5, CCL20, CCL21, CXCL4L1, CXCL10* genes			(15)
Immune costimulatory/ coinhibitory molecule genes	*OX40L, CD30, CD40, 4–1BB genes*	Promotion of the activation and proliferation of tumour-specific T cells	LOAd703 (AdV), Delta-24-RGDOX (AdV)	(16, 17)
Suicide genes	*HSV-TK, CD, FCU1 genes*	Transformation of some nontoxic drug precursors into cytotoxic substances (e.g. conversion of nontoxic 5-FC to toxic 5-FU and 5-FUMP by FCU1)	T601 (VV)	(16, 18)
Tumour suppressors genes	*P53, PTEN, P16, RB genes*	Enhancement of the inhibitory effect of OVs on tumour cells	HSV-P10	(19)
Pro-apoptotic genes	*Apoptin, Lactaptin, TRAIL, SMAC genes*	Apoptosis of tumour cells	p55-hTERT-HRE-TRAIL (AdV)	(20)
TAA genes	*CEA, PSA, claudin-6 genes*	Induction of systemic anti-tumour response (OVs act as vaccines)	MVvac2-CLDN6 (MV)	(21)
Anti-angiogenic genes	*endostatin, angiostatin, can stain, VEGF receptor 1-Ig fusion protein, VEGF single-chain antibody, VEGF promoter targeted transcriptional inhibitor genes*	Inhibition of tumour angiogenesis and growth	T-TSP-1 (HSV-1), HSV-Endo (HSV-1), VV encoding anti-VEGF single-chain antibody GLAF-1	(22–24)
Anti-tumour antibody genes	*anti-PD-1, Bi-specific antibody genes*	Enhancement of overall anti-tumour efficacy, maximization of local concentration of T cells at the tumour site	NG34SCFVPD-1 (HSV-1), NG-641, ICOVIR-15K-cBiTE	(25–27)
ECM-degrading enzyme genes	MMP-9, PH20 genes	Degradation of ECM components, increase of intratumoral spread of OVs	GLV-1h255 (VV), VCN-01 (AdV)	(28, 29)

together with reducing viral toxicity and enhancing their oncolytic properties.

Selective replication was achieved in ONYX-050 and H101, the prototypes for oncolytic adenoviral therapy, by deletion of the viral *E1B-55K* gene, which is essential for efficient viral replication in normal cells but not in tumour cells, where this process is regulated differently (30). Another approach is to make the adenovirus replication dependent on the enzymes highly active only in cancer cells (31). A good example is tumour-specific replication-competent adenovirus OBP-301, in which the human telomerase reverse transcriptase (the catalytic subunit of telomerase quiescent in healthy tissues) promoter element drives the gene expressions of *E1A* and *E1B*, proteins linked to the internal ribosome entry site (31). It has been shown that OBP-301 replicates effectively exclusively in human cancer cells (31).

The possible changes also include fibre viral capsid protein (32). The initial step of the adenoviral infection is the attachment of the virus to the Coxackie-adenovirus receptor (CAR). OBP- 405, a telomerase-specific replication-selective adenoviral agent, is a version of OBP-301 with a fibre modified to contain an RGD peptide that binds with high affinity to integrins on the cell surface, facilitating the CAR-independent virus entry (32). Delta-24-RGD adenovirus undergoes the 24 base pairs deletion in the E1A region that is responsible for binding the Rb protein. This deletion renders viral replication dependent on the inactivation of Rb and generates a tumour-selective, replication-competent virus that has been shown to induce an anticancer effect in some types of gliomas (33). Modification of viral hexon capsid protein into chimeric hexon with adenovirus serotype rare in nature has the potential to reduce hepatotoxicity, uptake in the liver and spleen, and innate immune response (34).

In recent years, several Oncolytic Adenoviruses (OAVs) armed with multiple regulatory elements combined, were developed. This further increased their specificity, efficacy, and ability to escape from patients' immune systems, and made them the most remarkable among all OVs (15).

Currently, attempts are made to use OAVs as vectors carrying anticancer genes, the local expression that may result in tumour suppression, without affecting other cells, ultimately resulting in OAVs' increased antitumour activity. One of the representatives of the newest generation of OAVs is OncoViron. Due to numerous modifications, its replication selectivity is regulated at both

TABLE 2 Mechanism of action of selected OVs.

Virus	Genetic material	Mechanisms of tumour targeting	Mechanisms of antitumour activity	Human pathogenicity
Adenovirus	dsDNA; does not incorporate into infected cells' chromosomes	Deletion of the gene essential for efficient viral replication in normal cells (e.g. *E1B-55K*); making replication dependent on the enzyme highly active only in cancer cells (e.g. human telomerase reverse transcriptase); modification of viral capsid protein (e.g. adding RGD peptide to a fibre capsid protein, deletion in the E1A region, creating chimeric hexon capsid protein with adenovirus serotype rare in nature)	Anticancer activity of viral structural protein; adding anticancer immunomodulatory genes (IL-12, IFN-γ and CCL5 genes)	+
Protoparvovirus	ssRNA	Natural tropism to human cancer cells (does not induce cell lysis in non-transformed cells); exploiting receptors overexpressed on cancer cells (eg. transferrin), hijacking aberrant signalling pathways (e.g. Ras pathway)	May lead to apoptotic, non-apoptotic, and lysosome-dependent cell death	–
Vaccinia virus	Large dsDNA, can be inserted with big fragments of transgenes; does not integrate into host cell's chromosomes	Knocking out the thymidine kinase	Modifying virus to express factors that activate the systemic immune response and inhibit tumour cells (eg. GM-CSF); combining features of two distinct infectious forms to help the virus evading neutralising antibodies	+ (typically very mild infection)
Reovirus	dsRNA	Replication promoted in cells with an activated RAS pathway	Increases PD-L1 expression on tumour cells; stimulates the recruitment of NK cells and reovirus-specific CD8+ T cells to the tumour site; Modifying virus to antagonize inhibitory mechanisms within the TME (e.g. mutations in viral cell attachment protein *σ1* gene)	+ (only some genera)
Herpes simplex virus type 1	dsDNA	Modifying or deleting the genes that are crucial for viral replication in normal cells (e.g. thymidine kinase, ICP34.5, ICP6, ICP47 genes)	Knocking out the ICP47 gene to help activate host antitumour immune response,	+

"+" means YES; "-" means NO.

transcriptional and translational levels. The anticancer activity of viral structural proteins, the ability to infect cancer cells and avoid the neutralising antibodies, and the adsorption by hepatocytes are enhanced, and the killing effect on cancer cells is boosted by adding three types of anticancer immunomodulatory genes (15). OncoViron showed significant anticancer effects on its own and in combination with programmed death 1 (PD-1) antibody and chimeric antigen receptor (CAR) T cells on a variety of implanted solid tumour models, including breast cancer, in immunodeficient, immunocompetent, and humanized mice (15).

3.2 Protoparvovirus

H-1PV is a small single-stranded rat RNA virus that presents natural tropism to human cancer cells but does not replicate or induce cell lysis in non-transformed cells. No pre-existing immunity to H-1PV has been found in humans that acts as its advantage over OVs based on human pathogens (15). Various factors that are overexpressed in cancer cells are known to control H-1PV nuclear transfer. The research focussed on the identification of new cellular modulators has the potential to further favour the outcome of H-1PV treatment (15). Cancer cell lines derived from multiple tumours, including brain, pancreas, lung, cervical, colorectal, and breast cancers, as well as melanoma and osteosarcoma, are indeed susceptible to H-1PV infection and oncolysis (35). H-1 PV efficiency has also been shown in haematological diseases. H-1PV may cause apoptotic, non-apoptotic, and lysosome-dependent cell death. The latter is essential in glioma cells, resistant to conventional cytotoxic agents. Besides tumour lysis, the oncosuppressive effect of H-1PV results in the stimulation of both innate and adaptive immune responses (36). H-1PV has been tested in combination with conventional treatment, epigenetic modulators, apoptosis inducers, and angiogenic and immune-modulating drugs. The potential of some other rodent protoparvoviruses as anticancer therapeutics is also currently investigated in preclinical studies (37). Overall, the combination of enhanced viral entry, exploitation of dysregulated cellular pathways, defective antiviral responses, altered cell cycle regulation, and the tumour microenvironment contributes to the selective targeting and efficient oncolysis of cancer cells by Protoparvovirus H-1PV (38).

Protoparvovirus H-1PV targets cancer cells by exploiting overexpressed receptors like the transferrin and Heparan sulphate

proteoglycan receptors, making them more susceptible to infection than non-cancerous cells (37).

Dysfunctional innate immune pathways and defective interferon responses in cancer cells create a favourable environment for viral replication and oncolysis (39).

By hijacking aberrant signalling pathways such as the Ras pathway, Protoparvovirus H-1PV facilitates its replication and spread within the tumour microenvironment, selectively killing cancer cells while sparing normal cells (40).

Factors like hypoxia, acidic pH, and immunosuppression in the tumour microenvironment enhance H-1PV replication in cancer cells while minimizing its impact on non-tumour cells (37).

Dysregulated cell cycle progression in cancer cells increases their susceptibility to viral infection and replication, with Protoparvovirus H-1PV preferring actively dividing cancer cells for infection and oncolysis (41).

3.3 Vaccinia virus

Vaccinia virus (VV) is a double-stranded DNA virus with a large genome that can be inserted with big fragments of transgenes and does not integrate into host cell chromosomes. Oncolytic VVs (OVVs) are engineered by knocking out the thymidine kinase (*TK*) gene and they can replicate exclusively in cancer cells (42). The focus of the research is to augment its oncolytic efficacy, which is naturally relatively low. Pexa-vec is an OVV that can activate the systemic immune response and inhibit tumour cells by expressing granulocyte-macrophage colony-stimulating factor (GM- CSF). It possesses features of two distinct infectious forms - intracellular mature virus (IMV) and extracellular enveloped virus (EEV). Such a characteristic allows its simultaneous intravenous and intratumoral injection, as well as evading neutralising antibodies (43). OVV has been recently used as a vector for personalised neoantigen immunotherapy against triple-negative breast cancer in a study assessing such a therapeutic approach (44).

3.4 Reovirus

Reovirus is a double-stranded RNA virus. Its replication is promoted in cells with an activated RAS pathway. The gain-of-function mutations, activating RAS signalling, are prevalent in cancers. Therefore, reovirus is a natural candidate for a therapeutic agent (45). Reovirus has oncolytic activity *in vitro* against multiple solid tumour types, including breast cancer (46). Reolysin is an unmodified wild- type oncolytic reovirus. In 2017, it received FDA approval for the treatment of metastatic breast cancer (46). Data from the Phase II trial for the treatment of advanced metastatic breast cancer showed that the combination of Reolysin and Paclitaxel significantly increased overall survival for about seven months (47). Clinical studies have demonstrated its effectiveness in combination with systemic anti-programmed cell death protein 1 (PD-1). In a murine breast cancer model, intratumoral reovirus increased PD-L1 expression on tumour cells, and combination reovirus/anti-PD-1 treatment improved survival by reducing Treg numbers and ameliorating tumour-specific cytotoxic T lymphocyte responses (48). Reovirus has also been used in combination with CD3-bispecific antibodies. Reovirus-induced IFN stimulated the recruitment of NK cells and reovirus-specific CD8+ T cells to the tumour site, while reovirus-specific effector T cells acted synergistically with CD3- bispecific antibodies, reducing the *in vivo* growth of several tumour types, including breast (46). Interestingly, this combination treatment was also effective against distant lesions that were not previously injected with reovirus. It suggests the possibility of using this therapy in case of metastatic disease (45). The advances in reovirus engineering have enabled the creation of oncolytic reoviruses that can antagonize inhibitory mechanisms within the TME. In particular, mutations in viral cell attachment protein *σ1* gene, have been incorporated to prevent proteolytic cleavage and inactivation of *σ1* by breast cancer-associated proteases (49).

3.5 Herpes simplex virus type I (HSV-1)

HSV is a DNA double-stranded neurotropic virus with a highly effective ability to infect. It is divided into two types - HSV-1 and HSV-2. The first one is commonly used for OV therapy. It has been widely used in cancer treatment. It is recognised as a potent activator of innate and adaptive immunity. Therapeutic forms of HSV-1 are obtained by modifying or deleting the genes that are crucial for viral replication in normal cells but not in tumour ones, such as thymidine kinase (*TK*), *ICP34.5* (required for viral replication in nerve cells), *ICP6* (coding the large subunit of HSV-1 ribonucleotide reductase), and *ICP47* (50–52). In addition, the knockout of *ICP47* prevents from inhibiting antigen presentation by MHC-1 and helps activate the host antitumour immune response (53). The examples of HSV-1 OVs are T-vec, with a knockout of *ICP34.5* and *ICP47*, and HSV1716, with deletion of double copies of *ICP34.5*. HSV1716 was approved for clinical trials in Europe in 1996 and has been used with satisfying results in the treatment of glioblastoma multiforme, melanoma, and head and neck squamous cell carcinoma (54). T-vec, also known as IMLYGIC or OncoVEX GM-CSF, proved to be effective and safe in a few Phase 1 clinical trials in patients with refractory breast cancer (55). It has been suggested that the retention of *ICP34.5* may be beneficial in some situations of IFN-dependent antiviral tumour status, as it can enhance the oncolytic effect and end the overall efficacy of OV. The alternative for direct deletion of the *ICP34.5* gene, proposed by researchers, is to control its expression by inserting into HSV a microRNA-responsive target element (56).

4 Discussion

4.1 Clinical use and observed adverse effects

The first OV was registered in 2004 in Latvia as a melanoma treatment. The OV is composed of the genetically unmodified Picarnoviridae family Enterovirus genus – Rigivir (57). Despite

the standard procedure in treating melanoma with surgical resection, in metastatic melanoma, oncolytic viruses are showing promising effects. Melanoma has a heterogeneous presentation and can cause distant, dermal as well as visceral metastasis. The mechanism of OVs is appropriate for this type of cancer spread, as it causes the lysis of cancer cells and the lysis of infected malignant cells (58, 59). The retrospective studies from 2015 were performed in Latvia on 79 patients with stage IB, IIA, IIB, and IIC of melanoma after surgery (54). There was no statistically significant difference in the period of time for the patients to remain disease-free, however, the overall survival was prolonged among patients treated with Rigvir (54). In this study there were no significant side effects reported (54). Additionally, previous clinical trials reported side effects that were mild, completely reversible, and not causing an interruption in treatment, such as subfebrile temperature, pain in the location of the tumour, fatigue, sleepiness, and dyspepsia (55). Despite its registration in Latvia, Georgia, and Armenia, Rigvir was discontinued in mid-2019 due to manufacturing issues and then suspended for marketing authorization (60). Currently, there is a new product on the market - Imlygic registered by FDA in 2015 for advanced melanoma (7). Talimogene laherparepvec (T-VEC; Imlygic™), is a genetically modified herpes simplex virus, type 1. This is also the first OV to be approved by the FDA[2] and EMA[3]. In the Phase III trial, 436 patients were enrolled, with unresected stage IIIB to IV melanoma but no metastases to the brain or bones. The durable response rate, overall survival, and objective response rate in patients treated with Imlygic were higher compared to the GM-CSF group (61). Adverse effects of using T-VEC were not severe, mostly presenting as flu-like mild symptoms: fatigue, chills, pyrexia, nausea, and local injection site reactions. Based on these significantly beneficial clinical results T-VEC was registered in monotherapy for curing advanced melanoma with unresectable cutaneous, subcutaneous, or nodal lesions after initial surgery by the FDA[2] and the ATGA and for the treatment of stage III and IV M1a melanoma by the EMA[3] (61–63).

In China in 2005 another OV, Oncorine, was approved by the National Medical Products Administration (NMPA) of China for treating nasopharyngeal carcinoma and advanced head and neck cancer (64). Oncorine is a genetically modified (deletion of *E1b55K*) human adenovirus type 5 called H101, which was developed by the Chinese company Sunway Biotech (65). In 2021 results of a new retrospective cohort study were published, with very promising clinical outcomes, using Oncorine as a treatment for advanced gastric carcinoma. 95 patients were divided into 3 groups (A=30, B=33, C= 32) and treated with Oncorine only (A), chemotherapy, and a combination of H101 and chemotherapy only (C). The results showed that control of lesions, progression-free survival, and overall survival were significantly higher in patients treated both with chemotherapy and Oncorine. What also was noted, is that side effects typical for chemotherapy such as nausea, vomiting, granulocytopenia, anemia, and hair loss occurred in patients from groups B and C (with no statistically significant difference between them) and more commonly than in group A. Pyrexia was observed mostly in patients from groups A and B. The study showed that Oncorine may be a therapeutic option for patients with gastric carcinoma in combination with chemotherapy (66). In 2022 the next retrospective study was carried out on the efficacy and safety of Oncorine in 29 patients with persistent, recurrent, or metastatic gynecologic malignancies: cervical cancer (22 cases), vaginal cancer (2 cases), vulvar cancer (3 cases) and ovarian cancer (2 cases). 22 patients responded to the treatment - significant tumour regression and reduction in necrotic tissue were observed as well as longer progression-free survival. Additionally, the objective response rate for radiotherapy was 72.4%, suggesting that Oncorine may increase the radiotherapy sensitivity (64). Side effects were similar to those that were previously reported, such as pyrexia, nausea, vomiting, fatigue, and pain with sometimes bleeding in the place of injection (60). There was one case reported of severe side effects - a rectovaginal fistula after Oncorine combined brachytherapy (60). Oncorine's potential in the treatment of liver cancer, MPE, and pancreatic cancer is currently being investigated.

The newest registered OV is Delytac. This is a genetically engineered replication-competent herpes simplex virus type 1, called G47Δ or teserpaturev, approved in 2021 by the Japanese Ministry of Health, Labor and Welfare and developed by Daiichi Sankyo Co (67). Of 19 patients enrolled in this trial, 13 met the primary criterion of 1-year survival and the study was terminated earlier because of high efficacy achieved (68). 3 patients developed pyrexia but also severe side effects were reported such as death, cerebral infarction, hemiplegia, syncope, urinary tract infection, postprocedural infection, and subcutaneous abscess each in 1 patient (68).

Breast cancer is the most common cancer among women in Poland and the second cause of cancer-related deaths[4]. It has a relatively good prognosis if it is diagnosed and treated in early stages. On the other hand, when it is advanced and with metastases the treatment methods are limited and mortality rises due to complications of cancer itself as well as due to the treatment-related toxicity (69)[5]. The research in OV for breast cancer is now in the clinical trials phase and it showcases very promising effects as a future cancer treatment due to its ability to target only tumour cells (70) (71). In the preclinical and clinical trials, four virus groups (from seven groups of Baltimore classification) are mostly researched: group I (double-stranded DNA viruses), group III (double-stranded RNA viruses), group

2 Food and Drug Administration. (2024). Alphabetical List of Licensed Establishments Including Product Approval Dates as of 01-JAN-2024. https://www.fda.gov/media/76356/download [Accessed January 16, 2024].

3 European Medicines Agency. (2022). Imlygic. https://www.ema.europa.eu/en/medicines/human/EPAR/imlygic [Accessed January 16, 2024]

4 Krajowy Rejest Nowotworów. Raporty. https://onkologia.org.pl/pl/raporty [Accessed January 16, 2024]

5 American Cancer Society. (2017). Breast Cancer. Facts & Figures 2017-2018. https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/breast-cancer-facts-and-figures/breast-cancer-facts-and-figures-2017-2018.pdf [Access January 16, 2024]

IV (single-stranded RNA viruses – positive-sense), and group V (single-stranded RNA viruses – negative-sense). Among these viruses there are naturally anti-neoplastic, those that are designed for tumour-selective replication, and those genetically modified to activate the immune system (71).

Currently, there is no OV registered for breast cancer treatment. Table 3 represents OVs used in clinical trials (in monotherapy and combined therapy) tested on patients with breast cancer, however, there are a number of ongoing preclinical trials focusing on a variety of viruses from those four groups.

Many preclinical trials focus on finding the OV against TNBC. TNBC is a highly aggressive cancer with a very poor response to treatment due to the lack of receptors for estrogen, progesterone, and human epidermal growth factor 2. Although the patients are currently treated with chemotherapy - the side effects are devastating, drug resistance occurs often, and the prognosis is poor. The difficulties in treatment and high heterogeneity in TNBC led to the beginning of many studies in order to find a more efficient and safer treatment (82).

Adenoviruses are the most studied OVs in breast cancer, so preclinical studies -with additional modification (antitumour and immune regulatory genes were inserted to enhance effects) - were performed also against TNBC (83). One of them is a recombinant type five adenovirus containing IL-24 gene (CNHK600-IL24). It significantly suppressed tumour growth in the nude mice model and improved survival in the metastatic model (84). Another OV which showed high efficiency against MDA-MB-435 cancer cells was G47Δ - oncolytic HSV (registered for Malignant Glioma). It presented high cytotoxicity against human breast cancer cells *in vitro* and in tumour xenografts *in vivo* (85). As for MDA-MB-231 TNBC cells VG9-IL-24 recombinant Vaccina virus presented promising effects. In the xenograft mouse model it showed efficiency in infecting and selectively killing breast cancer cells with no strong cytotoxicity to physiologic cells (86).

Recently (November 2023) a case study was published of a previously treated patient with mTNBC. The purpose was to evaluate the safety and efficiency of CHECKvacc - an oncolytic virus composed of CF33, a chimeric vaccinia poxvirus. The first intratumoral administration showed no immediate response, but later the patient underwent T-Dxd treatment and the tumour regressed significantly also disease-free survival was 10 months (87). This is just one of many examples where combined therapy shows the best effects. There is also an ongoing phase 1 clinical trial on Codalytic, this is the first codon-modified virus. In the preclinical trial, it was tested on a mouse model with implanted TNBC cells in monotherapy. After 3 weeks the tumour was reduced by 76% and the cure rate was 66% (88).

In clinical trials (listed in Table 3) the adverse effects in treating breast cancer and other types of cancer are mostly mild: flu-like symptoms such as low-grade fever, chills, nausea, and vomiting; with single severe adverse effects occurring.

Overall, OV therapy has been proven safe in many trials. Unfortunately, the inadequately effective outcomes of these trials and incomplete responses are not sufficient enough to use OVs as a monotherapy treatment for breast cancer. On the other hand, multi-approach strategies - combining OVs and chemotherapy, radiotherapy, or immunotherapy - provide better therapeutic effects and show great potential in future approaches to breast cancer treatment (69, 89, 90).

4.2 Immunological response to the oncolytic virus therapy

Cancer is not just a collection of rebellious cells. They need the support of a specific compartment of the tumour stroma called the TME. It provides many vital signals to support tumour growth and progression. We have long thought of the tumour stroma as a rather passive element of the bulky cancerous tumour, but it seems that its effects go far beyond blood supply and mechanical stabilization. It is now well established that the TME can influence almost every step of cancer growth. It plays a role in the initiation of cancer transformation, its growth, invasion, and ultimately metastasis. In rare cases, it can also induce spontaneous tumour regression.

The TME consists of cellular and non-cellular compartments. The tumour-associated stromal cell compartment contains immune cells as antigen-processing cells as macrophages (M1, M2), and antigen- presenting cells as dendritic cells (DC), and finally antigen-specific cells as T cells, both CD4 and CD8 subsets and B cells. In addition, other cells such as blood and lymphatic vessels with all their variety of cellular elements such as endothelial cells, cancer-associated fibroblasts, pericytes but also adjacent neuronal cells and adipocytes (91) play their role in the growth rate of cancer (92).

The non-cellular components of the TME are mainly matrix proteins, but also the often-neglected microbiome (93), which can be exploited by the tumour. Now that we know that the TME is involved in almost every step of cancer development and progression, it is not surprising that the development of therapies targeting the TME machinery is an emerging target for future cancer research. The cellular composition and dynamic function of the TME are not permanent but can vary greatly over time, depending on many local tissue factors reflecting the actual status of cancer growth. It depends on the tissue in which the cancer arises as well as the characteristics of the cancer cells, the stage of the tumour, and the clinical status of the patient.

TME usually reflects a normal immune response when the immune system is unable to eliminate an antigen. Under physiological conditions, the immune response initially attempts to eliminate the antigen. This situation occurs when a high burden of inflammatory cells can eliminate antigens at the site of immune action. It is associated with the presence of immunocompetent cells involved in antigen elimination, rich in CD4 Th1, T cytotoxic T, CD8, and NK cells. This situation is usually not present in TME without additional manipulations since the tumour grows when immune surveillance is impaired. If, over time, an antigen remains and the immune effort to eliminate it is useless, the immune response is redirected. It first changes to tolerate the antigen. The situation is equivalent to chronic inflammation, with an impaired ability to eliminate antigen but still control its spread. The cost is reduced immune surveillance. Finally, in the worst-case scenario, to protect the host, the immune system tries to physically isolate the source of the dangerous antigen, forming a physical barrier with

TABLE 3 Oncolytic viruses used in clinical trials (up to date, Dec 2023).

Baltimore classification	Virus group	Virus applied	Clinical outcome	Adverse reactions	References
Group I Double-stranded DNA Viruses	Adenovirus	Ad5/3-D24-GMCSF	3 out of 14 patients had tumour shrinkage or disease stabilization.	Most common: fever, fatigue, rigors, nausea, transient anemia, leukocytopenia. No serious AE is possibly related to the treatment.	(72)
		Ad5–Δ24–GMCSF	Disease stabilization in 3 of 7 patients with breast and colorectal cancer. One patient with advanced metastatic tumour, refractory to conventional therapies treated with a single round showed complete response in radiological evaluation.	Well tolerated, mostly flu-like symptoms: fever, chills, fatigue, and injection site pain.	(73, 74)
		RGD-4C (ICOVIR-7)	1 out of 3 patients presented stabilization of tumour markers, but at the endpoint nine of them showed effective response (mild, partial, non-complete).	No significant adverse effects occurred.	(75)
		ONYX-015 + etanercept	2 out of 2 patients with breast cancer showed progressive disease with a mean survival of only 125 days.	No significant AE: grade I and II fever within 24 h from administration	(76)
	Herpes simplex virus	Talimogene laherparepvec (T-VEC)	No partial or complete response was achieved but the disease stabilized in 1 patient (out of 14 with breast cancer).	The main side effects: grade 1 pyrexia with constitutional symptoms, 1 patient had grade 2 pyrexia with rigor, hypotension, and tachycardia. Other common: low-grade anorexia, nausea and vomiting, and fatigue. 2 patients developed abnormal liver function tests	(77)
		T-VEC+ neoadjuvant chemotherapy (NAC)	It was used on TNBC patients. In RCB-0 (complete pathologic response = pCR) and RCB-I (minimal residual disease) the 2-year disease-free was 89% with no recurrences. T-VEC plus NAC in TNBC may increase RCB0–1 rates.	Common AE: fevers, chills, headache, fatigue, and injection site pain. NAC as expected.	(78)
		HF10	In 6 patients with cutaneous or subcutaneous metastases from breast cancer 30% to 100% cancer cell death in histopathological evaluation.	No significant AE occurred	(79)
	Vaccinia virus	VVDD	2 out of 4 patients showed a partial antitumour response. It also showed remarkable vvDD selectivity for replication only in tumour cells.	No significant AE occurred (mostly fever, fatigue, nausea, vomiting); 1 severe adverse event - possibly related - pain in the rib 7 days after admission (no evidence of pulmonary problems).	
Group III Double-stranded RNA Virus	Reovirus	Pelareorep-wild form of reovirus	Applied on 2 breast cancer patients that resulted in partial oncolysis with 34% tumour shrinkage in one of them. Increased median overall survival (OS) in 74 advanced breast cancer patients.	Well-tolerated, grade 1-2 toxicities.	(80)
		Pelareorep and paclitaxel	74 women with previously treated metastatic breast cancer. The trial didn't meet the primary endpoint which was progression-free survival, however, the combination resulted in a significant elongation of overall survival.	Well-tolerated.	(47)
Group IV Positive sense Single-stranded RNA Virus	No clinical studies on patients				
Group V Negative-sense Single-stranded RNA Virus	Newcastle Disease Viruses	PV701	1 out of 2 patients showed stable disease for more than 6 months.	No severe AE: the most common: flu-like symptoms, and injection site reactions.	(81)

extensive fibrosis. These last two situations are also indicative of an immunosuppressive microenvironment that could in the long term be the soil for cancer initiation and progression. The typical example is the so-called scar cancer in the lung, but many others can arise on the soil of chronic inflammation, e.g. liver cancer in hepatitis B infection, lung cancer following chronic irritations such as asbestosis and silicosis, and so on. These last two situations occur in cancers, therefore TME can finally be divided into distinct separate groups currently playing a significant role in clinical outcomes and response to therapy. The pro- and anti-tumourigenic effects of tumour-infiltrating immune competent cells can profoundly determine tumour progression and the success or failure of anti-cancer therapies.

Currently, based on the immunomorphological response to immunotherapy, TME can be classified into three main groups, referred to as: immune inflamed, immune excluded, and immune desert (94, 95). However, the response to immune checkpoint blockade immunotherapy has been linked to the degree of T cell infiltration (96, 97) in tumours with high tumour mutation burden (98) and neoantigen load (99), as well as tumour antigenicity (100, 101), which led to the elucidation of four distinct TME subgroups: immune-enriched fibrotic, immune-enriched non-fibrotic, fibrotic and immune-depleted (86–88).

Oncolytic virus treatment is an emerging and promising therapy that not only directly targets tumour cells but may also modify the TME towards its more immune-eliminating rather than immunosuppressive properties. Data confirms that these types of manipulations in an experimental model increased tumour vascular permeability, host leukocyte infiltration into tumours, and ultimately, tumour inflammation (102). A combination of OV and CAR-T cell therapy may stimulate naive T cells and enhance CAR-T efficacy in mice (103). A better understanding of the complex interactions between tumour cells and their stroma determines disease progression and is critical for the rational development of effective cancer therapy.

4.3 Side effects on non-tumour cells

The application of oncolytic viruses is not without potential side effects on non-tumour cells (104). While oncolytic viruses are designed to selectively target and destroy cancer cells, they may inadvertently impact neighbouring healthy cells (105). The mechanism of cytotoxic effects on non-tumour cells may be direct but can be mediated also by immunological responses (39). The activation of the immune system by viral infection can lead to the release of pro-inflammatory cytokines and chemokines, resulting in a local inflammatory response. While inflammation is a critical component of the antitumour immune response, excessive or prolonged inflammation can cause tissue damage and exacerbate pre-existing pathological conditions. Moreover, the activation of innate immune pathways, such as toll-like receptor signalling, may trigger immune-mediated cytotoxicity against non-infected cells, contributing to bystander effects (39). The risk is put also in off-target viral replication (106).

Moreover, oncolytic viruses may alter the tumour microenvironment, impacting the function and phenotype of stromal cells, endothelial cells, and immune cells, which can influence tumour progression and treatment outcomes (107).

Integrating strategies to mitigate off-target effects, such as engineering viruses with improved tumour selectivity or combining virotherapy with immunomodulatory agents, represents a promising approach to enhance the therapeutic index of oncolytic virus-based treatments (108, 109) The successes of no histological signs of viral induced toxicity for non-tumour bearing organs have been announced for the urokinase receptor (uPAR) retargeted oncolytic measles virus in syngeneic cancer models (110), Vstat120-expressing (RAMBO) oncolytic herpes simplex virus (oHSV) (111), novel combination oncolytic adenoviral gene therapy armed with Dm-dNK and CD40L for Breast Cancer (112), or cancer-specific targeting of a conditionally replicative adenovirus using mRNA translational control (105).

4.4 Potential biomarkers of response

The emergence of OV as a promising therapeutic approach in breast cancer has generated interest in identifying predictive biomarkers for treatment response (113). Biomarkers to predict response to OV in breast cancer patients are currently lacking but are essential for selecting patients who will most likely benefit from the treatment. Moreover, the rapidly expanding combination strategies force the finding of the biomarkers to match individual patients to their most promising treatment option (114).

One of the key determinants of response to OV in breast cancer treatment appears to be the immune composition of TME (115–117). Tumours exhibiting high infiltration of tumour- infiltrating lymphocytes (TILs) demonstrate a propensity for better responses to OV. TILs are indicative of pre-existing immunity which is essential for OV efficacy. Specifically, a robust presence of CD8+ T cells within the TME indicates a potentially favourable response (118, 119). Important to predict a response are not only higher levels of TILs but also the spatial distribution of TILs and their functional state (120). Moreover, the expression of immune checkpoint molecules like PD-L1 on tumour cells can indicate the likelihood of a favourable response when OVs are combined with immune checkpoint inhibitors (119).

Tumour Mutational Burden (TMB) serves as another potential biomarker influencing treatment outcomes. Tumours with increased mutational burdens harbour more neoantigens, rendering them more susceptible to immune recognition, thus potentially enhancing the response to oncolytic viruses (98, 121).

The infectivity and replication capacity of the oncolytic virus within tumour cells represent critical aspects for predicting treatment response. Techniques monitoring viral presence or replication within the tumour tissue might serve as valuable biomarkers (83, 122).

Genetic signatures linked to viral replication, immune response pathways, or susceptibility to viral infection might also contribute to predicting response to OVs in breast cancer. An example of this

approach is the identification of realistic interferon (IFN)-mediated biomarkers to identify patients who most likely respond to virotherapy (123) as replication of OV is usually limited to cancer cells that have interferon (IFN) signalling defects. Upregulation of protein biomarkers such as IFN gamma may reflect immune induction and become an OV efficacy biomarker to improve the ability to select patients who do not exhibit resistance to virotherapy (124).

Analysing the cytokine profile within the TME provides insights into the immune response elicited by OVs. Elevated levels of specific cytokines may indicate a more favourable response to treatment. They may play a role in T-cell helper polarization in viral tolerability. The previous research described that the Th1 cytokine profile was expressed in pleural effusions of patients that responded to HSV1716 treatment for malignant pleural mesothelioma with low side effects, to be investigated as a biomarker for predictive response (125).

Serum markers such as carcinoembryonic antigen (CEA) have been also used to investigate the antitumour potential of a novel viral agent, an attenuated strain of measles virus deriving from the Edmonston vaccine lineage, genetically engineered to produce CEA against breast cancer. CEA production as the virus replicates can serve as a marker of viral gene expression (126). Therefore, CEA may serve as a low-risk method of detecting viral gene expression during treatment and could allow dose optimisation and individualization of treatment (126).

Advanced imaging techniques offer a non-invasive approach to monitor changes in the tumour microenvironment post-oncolytic virus treatment, potentially serving as a valuable tool for assessing treatment response. Examples of those may be organ-on-chip and tumour-on-chip microfluidic cell cultures (127).

Further research is imperative to establish the reliability and efficacy of these biomarkers in guiding the selection of patients likely to benefit from oncolytic virus therapy. The studies should be aimed at finding out how the ability of specific OVs to replicate in individual tumour cells is affected, and if and how it influences antitumour and antiviral action (89, 128).

4.5 Physical barriers

Several physical barriers can limit the delivery and effectiveness of the OVs and in our opinion, this topic requires a separate chapter in this review. Most of the therapies are delivered directly to the tumour. Such examples are adenovirus, poxvirus, HSV-1, measles, and reovirus which are delivered intramurally (129, 130). However, not all the tumours are available for direct delivery, because of their location. For example, the Seneca Valley Virus can be delivered to the bloodstream directly as it does not cause hemagglutination (131). Parvovirus H- 1PV can go through the blood-brain barrier and is applied in the treatment of glioblastoma multiforme (131). Another type of physical barrier is the extracellular matrix (ECM) in tumours is dense and contains impermeable for the viruses' components, such as collagen and elastic fibres. For example, in pancreatic ductal adenocarcinomas (PDACs) ECM is so dense that can lead to interstitial hypertension (130) and can form a physical barrier for the delivery of the OVs (132). Other types of physical barriers associated with the TME are necrosis, calcification, hypoxia, acidosis, and increased proteolytic activity (130).

4.6 General risks of oncolytic virus therapy

4.6.1 Immunogenicity

Immunogenicity is a critical consideration in the context of OV therapy, representing the capacity of the introduced viruses to stimulate an immune response within the host. Oncolytic viruses are designed to selectively replicate within cancer cells, triggering cell lysis and the release of tumour- associated antigens. This process is intended to provoke an immune response, engaging components such as T cells and natural killer (NK) cells. However, the effectiveness of this response depends on the ability of the immune system to recognise and mount a robust reaction against cancer cells. Successful oncolytic virus therapy relies on the activation of adaptive immune responses, particularly cytotoxic T lymphocytes (CTLs). These immune effectors play a central role in recognising and eliminating cancer cells. However, factors such as pre-existing immunity to the viral vector may influence the magnitude and efficacy of these responses (133).

4.6.2 Off-target effects

Off-target effects refer to the unintended impact of OVs on non-cancerous or healthy cells within the body. Despite the selectivity engineered into these viruses, factors such as imperfect targeting mechanisms, interactions with the host immune system, or unexpected viral behaviour may lead to unintended consequences in off-target tissues. Off-target effects may manifest as localised toxicity in tissues surrounding the site of viral administration. This can include inflammation, tissue damage, or discomfort near the treatment site. In some cases, OVs may enter the bloodstream and disseminate throughout the body, potentially affecting distant organs and tissues. Systemic off-target effects can lead to more widespread adverse events. The activation of the immune system in response to viral infection may extend beyond the tumour site, leading to immune-mediated effects in healthy tissues. This could result in autoimmune-like reactions or inflammation in non-cancerous areas. This may be especially dangerous in immunocompetent patients (134).

4.6.3 Inflammatory response

The introduction of OVs elicits an immune response aimed at recognising and eliminating foreign entities. While this response is essential for combating cancer, excessive or uncontrolled inflammatory reactions may lead to adverse effects. The inflammatory response can manifest both locally at the site of viral administration and systemically throughout the body. Locally, inflammation may cause redness, swelling, and pain at the injection

site. Systemically, the release of inflammatory mediators into the bloodstream can lead to flu-like symptoms, fever, and malaise. The delicate balance between inducing an immune response against cancer cells and minimizing collateral damage to healthy tissues is pivotal for the therapy's success. Excessive inflammation may not only compromise patient comfort but also impact the therapeutic effectiveness of oncolytic viruses by diverting the immune system's attention away from cancer cells (135).

4.6.4 Virus resistance

Virus resistance poses a significant challenge in oncolytic virus therapy, where cancer cells may develop mechanisms to evade viral infection and subsequent destruction. Cancer cells can develop resistance to oncolytic viruses through various mechanisms. These may include alterations in viral entry receptors, inhibition of viral replication, interference with the apoptotic pathways triggered by viral infection, and the evolution of antiviral immune responses within the TME (136).

4.7 Breast cancer-specific risks

Breast cancer exhibits substantial molecular and genetic heterogeneity, encompassing diverse subtypes such as luminal A/B, HER2-positive, and triple-negative breast cancer (TNBC). This heterogeneity influences disease progression, treatment response, and the overall clinical outcome. The diverse molecular landscape of breast cancer subtypes poses a challenge in designing oncolytic viruses with universal efficacy. Different subtypes may have distinct vulnerabilities and response patterns to viral infection, necessitating tailored approaches for each breast cancer subtype (90).

4.7.1 Impact on healthy breast tissue

The proximity of healthy breast tissue to cancerous lesions raises concerns about potential off-target effects. Ensuring the selectivity of OVs for cancer cells while sparing normal tissue is critical in minimizing adverse effects and enhancing the safety profile of the therapy.

4.7.2 Hormone receptor status

Hormone receptor status, including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression, further complicates the landscape of oncolytic virus therapy. Subtypes with specific hormone receptor profiles may exhibit differential responses to viral infection, necessitating a nuanced approach to treatment planning (137).

4.7.3 Combination therapies

OV therapy is often combined with other modalities such as chemotherapy, immunotherapy, or targeted therapies. Evaluating potential interactions and cumulative toxicities of these combined approaches is essential to mitigate risks and enhance therapeutic outcomes (55).

4.8 Limitations of the oncoviral therapy

OV therapy holds great potential in modern cancer therapy, however, the therapy with genetically modified viruses apart from its potential also shows limitations to overcome. One of the first obstacles is the delivery of the OVs. OVs can be administered intravenously, but it brings other barriers. OVs circulating in the bloodstream can be neutralised.

Major problems with systematic delivery are preexisting antibodies due to immunisation or previous oncolytic treatment. As Reovirus is commonly found in the environment, many people have antibodies against it which causes immunity to Reovirus and recombined OVs (138). In order to overcome the host's immunity for different types of viruses, a lot of work still needs to be performed (139). Apart from antibodies circulating in the blood, there also are factors of the complement system, which after contact with the pathogen - activate and start the protease cascade, which leads to the deposition of the membrane attack complex (140).

There is also a risk of them not being guided directly into the tumour and nonspecific uptake by the lungs, liver or spleen, so only a small payload may be delivered to the tumour (139).

The next problem of intravenous administration is collapsed vasculature in the tumour, so the penetration can be insufficient, thus, the therapeutic dose will not be met (90). Currently, most of the OVs are injected directly into the tumour. The intratumoral administration can be difficult for the operator and painful for the patient, especially if the tumour is hardly accessible, as well as if the cancer cells are in several nodes dispersed in large areas - hence, not every metastasis might be equally reached (141). As for breast cancer treatment, the intratumoral administration of the OV is difficult only if the tumour is in a hardly accessed location or has already metastasized. The most optimal way of administration has not been defined yet, and also if OV should be used in monotherapy or in combination with other available forms of therapy.

Another difficulty in administration in solid tumours apart from delivering the OV to the patient itself, is the need to reach the tumour and spread in it, which can also cause limited efficacy against cancer cells (90). First, the OVs have to overcome the physical barriers (tissues) to get to the tumour. Secondly, intratumoral hypertension (caused by abnormal lymphatic networks, vascular hyperpermeability, and dense extracellular matrix) may obstruct viral infiltration, thus, the effectiveness of the OVs. (132, 136, 142).

The tumour is constructed with a great amount of extracellular matrix. Viruses, which are passively diffusing, may not fit through the strands. Limited penetration enables further tumour regions to grow regardless of administered OV (139). To improve tumour penetration various strategies are being developed such as pretreatment with enzymes or protein effectors (for example protease and relaxin) (143).

The next obstacle in OV therapy, which has been already recognised in the ongoing clinical trials, is the inadequate effectiveness in some types of tumours, when OVs are administered in monotherapy. In this case, combined therapy seems to be the best option, as confirmed by a growing number of studies with positive

results (142). That has been reflected in a clinical trial on Oncorine, where the combination of Oncorine with chemotherapy resulted in better control of lesions, and elongated progression-free survival and overall survival in comparison to patients treated only with chemotherapy or only with Oncorine (121). Similarly, ONYX-015 with 5-fluorouracil used in clinical trials as a treatment for patients with recurrent head and neck cancer showed that after 6 months there was no progression in the tumours that responded to the OV treatment, while all of the tumours, treated only with chemotherapy, further progressing (144). ONYX-015 was also used in combination with etanercept for the treatment of patients with solid tumours. Patients with colon cancer achieved stable disease, while patients with breast cancer presented progression of the disease (76). The mechanism of OVs and drug combinations must be further understood and executed to find the best treatment solution. Currently, more research is required to present the most effective and safest treatment since most of the OV therapies are still in the early stages of development (136).

5 Future directions

Oncolytic virus therapy has emerged as a promising avenue in breast cancer treatment, showcasing remarkable potential in preclinical and early clinical trials. However, there are still challenges to overcome (Table 4).

The advent of precision medicine calls for tailoring OVs to individual patient profiles. Developing personalised viral platforms could involve genetic modifications or viral engineering to enhance tumour specificity, replication efficiency, and immune activation while mitigating adverse effects. Advancements in identifying predictive biomarkers for treatment response remain also urgent. Identifying biomarkers associated with the efficacy of OVs can aid in patient stratification, ensuring targeted therapies for individuals most likely to benefit.

TABLE 4 Challenges and future directions of developing OVs.

Challenges	Future Directions
Choosing the best treatment option for each particular patient based on tumour and patients' characteristics	Indicating which OV will be the most suitable for patients by developing personalised viral platforms
Limited efficacy of OVs in monotherapy	Exploring combination therapies with chemotherapy or immunotherapy to enhance antitumour effect.
Delivering the OVs	Research focussed on improving delivery vectors to enhance tumour - specific targeting without loss of function and skipping barriers
Poor antitumour immune response	Developing interventions regulating checkpoints, modulating cytokine profiles, stimulating adaptive immune cells

An exciting frontier remains the synergy between OVs and conventional treatments like chemotherapy, radiotherapy, and immunotherapy. Exploring combination therapies can capitalize on their complementary mechanisms, potentially amplifying therapeutic efficacy and overcoming resistance.

It is also crucial to find strategies that restore the tumour microenvironment and bolster anti- tumour immune responses. This includes interventions that regulate immune checkpoints, modulate cytokine profiles, or stimulate adaptive immune cells within the tumour milieu.

Innovations in delivery systems to ensure efficient viral dissemination and penetration into tumour sites are also imperative. Engineering improved delivery vectors that enhance tumour-specific targeting while reducing off-target effects remains an active area of research.

A glycoprotein from others that has an affinity to the receptor can be inserted directly for enveloped viruses (145). An alternative approach is to use adapters that can bind both to the OV and the receptor (146). A promising approach are genetically engineered OV, with modification including deletions in the E1B region (147), E3B gene or for the PV deleting P1 coding region (replicons), A133G mutation in cis-acting replication element (CRE) (145). Off target effects are a concern especially for adenoviruses that have a high affinity to the liver (135). It has been reported that coagulation factor X (FX) binds to Ad5-hexon and enables transduction to the liver (148). Several modifications have been developed to circumferent the issue, such as constructing FX-binding ablated adenoviruses serotype 5 vectors (149). In the recent years. Recently the 3rd generation of OV has been introduced, e.g. OV with truncated CD19 (CD19t) protein for tumour-selective delivery (150).

As for many other research areas, a fast and efficient transition from preclinical success to clinical applicability remains the clue. Streamlining regulatory pathways and conducting robust clinical trials are essential steps towards obtaining approvals for OV therapies in breast cancer treatment.

The progress requires caring about the treatment's long-term safety profiles. Establishing comprehensive monitoring mechanisms for potential adverse effects post-treatment is essential for ensuring patient safety and treatment optimisation.

The collaboration between researchers, clinicians, and pharmaceutical entities can facilitate resource-sharing, accelerate discoveries, and promote a collective effort towards advancing OV therapy. The future of oncolytic virus therapy in breast cancer treatment holds immense promise. Realizing these potential demands concerted interdisciplinary efforts, innovative strategies, and a commitment to translational research to revolutionize breast cancer management.

Author contributions

HCh: Supervision, Writing – original draft, Writing – review & editing. AŚ: Writing – original draft, Writing – review & editing, Supervision. MM: Writing – original draft, Writing – review &

editing. MBo: Writing – original draft, Writing – review & editing. MBr: Writing – original draft, Writing – review & editing. GD: Writing – original draft, Writing – review & editing. PD: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. MW: Writing – original draft, Writing – review & editing.

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Glossary

5-FC	5-fluorocytosine
5-FU	5-fluorouracil
5-FUMP	5-fluorouridine monophosphate
AdV	Adenovirus
APCs	Antigen Presenting Cells
CCL	chemokine (C-C motif) ligand
CD	cytosine Deaminase
CEA	carcinoembryonic antigen
CTL	cytotoxic T lymphocyte
CXCL	chemokine (C-X-C motif) ligand
ECM	extracellular matrix
EEV	extracellular enveloped virus
ER	estrogen receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HER2	human epidermal growth factor receptor 2
HSV-1	herpes simplex virus type I
HSV-10	herpes simplex virus type 10
HSV-2	herpes simplex virus type 2
IFN	interferon
IL	interleukin
IMV	intracellular mature virus
MMP-9	matrix metallopeptidase 9
MV	Measles virus
NK	natural killer
NMPA	National Medical Products Administration
OAV	oncolytic adenoviruses
OS	overall survival
OV	oncolytic virus
OVV	oncolytic vaccinia virus
PD-1	programmed cell death protein 1
PDAC	pancreatic ductal adenocarcinoma
PH20	hyaluronidase 5
PR	progesterone receptor
PSA	prostate-specific antigen
PTEN	phosphatase and tensin homolog
SMAC	second mitochondria-derived activator of caspases
T-VEC	Talimogene laherparepvec
TAAs	Tumour-Associated Antigens
TIL	tumour-infiltrating lymphocyte
TK	thymidine kinase
TMB	Tumour Mutational Burden
TME	tumour microenvironment
TNBC	triple-negative breast cancer
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	vascular endothelial growth factor
VV	vaccinia virus

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Patient-derived tumoroids and proteomic signatures: tools for early drug discovery

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Onco-virotherapy is an emergent treatment for cancer based on viral vectors. The therapeutic activity is based on two different mechanisms including tumor-specific oncolysis and immunostimulatory properties. In this study, we evaluated onco-virotherapy *in vitro* responses on immunocompetent non-small cell lung cancer (NSCLC) patient-derived tumoroids (PDTs) and healthy organoids. PDTs are accurate tools to predict patient's clinical responses at the *in vitro* stage. We showed that onco-virotherapy could exert specific antitumoral effects by producing a higher number of viral particles in PDTs than in healthy organoids. In the present work, we used multiplex protein screening, based on proximity extension assay to highlight different response profiles. Our results pointed to the increase of proteins implied in T cell activation, such as IFN-γ following onco-virotherapy treatment. Based on our observation, oncolytic viruses-based therapy responders are dependent on several factors: a high PD-L1 expression, which is a biomarker of greater immune response under immunotherapies, and the number of viral particles present in tumor tissue, which is dependent to the metabolic state of tumoral cells. Herein, we highlight the use of PDTs as an alternative *in vitro* model to assess patient-specific responses to onco-virotherapy at the early stage of the preclinical phases.

KEYWORDS

onco-virotherapy, Anti-cancer Therapy, immuno-oncology, non-small-cell lung cancer, patient-derived tumoroids, proteomic

1 Introduction

Preclinical research has developed an outgrowing interest in the development of human-based models. Indeed, there is a consciousness that current animal models don't accurately recapitulate human features such as anatomy, physiological barriers, receptor panels, physiopathology mechanisms, and especially, immune responses (1, 2). Conventional

oncology models mainly comprise murine models and present a low predictive value of human responses (1). For example, in patient-derived xenografts (PDXs) models, the main limitations are (i) human cells that are progressively replaced by stromal murine cells, (ii) the lack of immune system, and (iii) the lack of tumor cell interactions with human-relevant stroma, that represent a functional tumoral microenvironment (TME) (3). Even though murine models contain a functional immune system, there's a poor clinical prediction of human immune responses. These current oncology models fail to detect the risk of drug inefficiency and safety issues. Nearly half of the drug candidates fail in clinical phases due to the lack of efficacy (4). Regarding the lack of safety issues detection in preclinical studies, they represent 30% of candidate drug failure (4). These clinical issues lead to a high attrition rate, reaching 95% in 2021 for all therapeutic areas (5, 6). In oncology, the attrition rate is 2 to 4 times more important compared to other therapeutic areas, as reported from 1979 to 2014 (7).

To better predict therapeutic effects at the early stages of drug discovery, researchers are putting efforts on human-based models to propose a predictive and relevant preclinical model that could complement current animal models (8). Different strategies have been developed to bridge these inter-species differences and increase the link between preclinical and clinical phases. In the last decade, organoids and organ-on-chips have been widely described (9). Furthermore, the latest amendment of the Food and Drug Administration (FDA) supports the use of alternative *in vitro* models when animal models are not required, matching with the 3Rs approach (10). Organoids are described as self-organizing 3D structures that mimic a specific organ. To better reflect the primary tumoral tissue derived from patients, patient-derived tumoroids (PDTs) were developed. For example, in lung cancer, numerous models of PDTs have been developed. Yet, some limitations, such as the lack of stromal and immune cells in the TME, can be noted (11).

We have previously developed a vascularized immunocompetent model of patient-derived tumoral organoids that modeled the immune part of the TME by introducing immune cells derived from peripheral blood. Co-culture with peripheral blood mononuclear cells (PBMCs) was used to assess T cell infiltration within PDTs after a treatment (12). However, PBMCs are less predictive compared to tumor-infiltrated immune cells. As tumor-infiltrated immune cells are in contact with tumoral cells, a predictive value of these cells has been assigned to immunotherapy responses (13). For example, for non-responders to immunotherapy, an increased proportion of myeloid-derived suppressor cells (MDSCs), and a decreased proportion of natural killer (NK) cells, and monocytes in the tumor site constitute an immunosuppressive microenvironment. As for responders to immunotherapy, the presence of high T-cell immunoglobulin and mucin-domain containing 3 (TIM-3), lymphocyte-antigen gene 3 (LAG-3), and programmed death-1 (PD-1) on immune cells within the stroma were reported in patients with better survival (14). Furthermore, they recapitulate T cell activation and tumor-killing to immunotherapy treatments (15, 16). We have evolved toward a model of PDTs that could preserve the immune cells from the primary tissue (17). Therefore, we could evaluate our PDTs and patient-derived healthy organoids (PDHOs) response to onco-virotherapy. Onco-virotherapy is based on using viral vectors that were genetically modified to present oncolysis and immuno-stimulation properties, commonly named "oncolytic viruses". In this context, we have assessed two oncolytic viruses. First, an oncolytic vaccinia virus (VACV) that was engineered to express GM-CSF, a cytokine that favors the induction of cytotoxic immune responses mediated by T lymphocytes (18, 19). Another oncolytic virus, that doesn't encode for GM-CSF, was used as a control (20). We will refer to VACV for the control, and VACV GM-CSF+ along the article. We assessed both oncolytic viruses for their ability to induce viral oncolysis and immune cells responses in our *in vitro* patient-derived model. To investigate the immune cell pathways, we used proteomics to help decipher proteins involved in promoting immune responses at the level of individual patients.

2 Methods

2.1 Lung tumor and healthy tissue engineering

Human specimens were obtained by surgical resections at Hôpitaux Universitaires de Strasbourg, France. Their tumoral status was confirmed by anatomopathological analysis. The anatomopathologist confirmed the presence of a tumor on mirror samples based on morphology studies with a hematoxylin & eosin staining (Supp. Data Sheet Figure 1). Healthy lung tissue samples were obtained from the peri-tumoral tissue of the same donor. Patients' informed consents were managed by the Centre de Ressources Biologiques (CRB). For information, lung cancer patients that did not present a driver mutation were selected for this study.

After the reception, human specimens were washed in PBS to remove blood excess, then enzymatically digested using the tumor Dissociation Kit, Human (ref. 130-095-929, Miltenyi Biotech) with the gentleMACS^{TM} Octo dissociator with heaters. This kit was optimized for facilitating the maintenance of tumor-infiltrating lymphocytes while preserving important cell surface epitopes. The content of the C tube (ref. 130-093-237, Miltenyi Biotech) is filtered through a 70µm cell strainers. Strained cells were centrifuged, and pellets were resuspended in 1mL lysis buffer (ref. R7757-100mL, Sigma) to remove the remaining blood cells. A second centrifugation was performed, and the left cells were resuspended in 1mL DMEM-F12 (ref. BE12719F, Lonza).

2.2 Generation of patient-derived tumoroids and healthy organoids

As described before in our previous studies, for the formation of patient-derived tumoroids (PDTs) and healthy organoids (PDHOs) in a matrix-free condition, supportive cells such as adipose-tissue-derived microvessels (ad-MVs) were added (12). They were prepared according to the protocol from the supplier Advanced Solutions® and the previous studies.

PDTs and PDHOs were prepared according to a mix of 5000 patients' cells and 5000 ad-MVs (within 1 PDT or PDHO). Cells' suspension was then diluted in cell culture media which is composed of a mix of DMEM high glucose (ref. 41966-029, Gibco), RPMI (ref. 10101-145, Sigma) and TexMACS™ media (ref. 130-097-196, Miltenyi Biotech). This cell culture media was supplemented with different growth factors to improve patient 's cells *in vitro* culture, maintenance of tumor-infiltrating immune cells and ad-MVs (details in Table 1).

After that, 200µL of this cell suspension was transferred on a ULA U bottom 96 wells plate (ref. 174929, Thermofisher) to form PDTs and PDHOs. The medium was changed every 3-4 days by replacing IL-2 with two other interleukins: IL-7 at 155UI/mL (ref. 130-095-361, Miltenyi Biotech) and IL-15 at 290UI/mL (ref. 130-095-762, Miltenyi Biotech).

2.3 Histology and immunohistochemistry

Primary tissue samples were fixed in 10% neutral-buffered formalin (ref. HT501128-4L, Sigma-Aldrich) and processed for histologic examination including hematoxylin and eosin (H&E) (Supp. Data Sheet Figure 1). 5µm thick tissue sections from selected paraffin blocks for each specimen were used for immunohistochemical analysis. PDTs and PDHOs were fixed in 4% PFA (ref. 416250397, Roti-Histofix®) for 1 hr at room temperature (RT) and embedded in Histogel specimen processing gel (ref. HG-4000-012, ThermoFisher Scientific) before dehydration in the Pearl. After paraffin inclusion, the blocks were sectioned on the Leica microtome at the thickness of 5µm. Then, IHC staining were performed on these sectioned slides using the LEICA Bond-II system with Bond Polymer Refine Detection based on the Novolink-polymer (ref. 7161, Leica), which is a horseradish peroxidase (HRP)-based polymer conjugated fluorescent dye that labels anti-rabbit antibody. The first steps of IHC are the dewaxing and the antigen unmasking (either High pH buffer or citrate buffer during 20min). Saturation of endogenous peroxydases with 10min of incubation in 3% H2O2 (ref. H1009, Sigma) and blocking step with goat serum for 30min (ref. G6767, Sigma) were performed. Primary antibodies were incubated for 1 hr at RT (Table 2). Then, after 1 hr of incubation with the primary antibody, secondary antibodies [or post-primary which is a rabbit anti-mouse IgG (ref. 7161, Leica)] and tertiary antibody (Novolink-polymer which is an anti-rabbit Poly-HRP) were incubated during 30 min at RT sequentially (ref. 7161, Leica). The post-primary was applied for primary mouse antibodies only. The final step was the signal amplification based on TSA using Perkin Elmer kit (ref. SAT701B, Perkin Elmer), for 10min at RT. Cell nucleus were stained with DAPI (dilution 1:10000 in PBS; ref. B2883, Sigma) during 10min at RT. The washing steps were performed between each step with Bond wash solution 1X (ref. AR9590, Leica). Images were captured using the fluorescence microscope Nikon Eclipse 90.

The value of dilution is based on the concentration of the antibody in the stock solution.

H&E staining was also performed on paraffin sections that were prior deparaffinized in xylene (ref. 185566, Honeywell) for 5 min twice and in different ethanol solutions (3 min twice in ethanol 100%, 5 min in ethanol 70%, and 5 min in ethanol 30%). Colorations with hematoxylin (ref. HHS-16, Sigma) for 3 min and eosin (ref. 318906, Sigma; diluted in distilled water at 1/50) for 30 sec were done. Differentiation for 2 min with ethanol 80% was done, followed by dehydration with ethanol 100% for 2 min and xylene for 2 min. Mounting was done with the Eukitt (ref. 045798, D. Dutscher).

PDTs and PDHOs were fixed for IHC staining of CD4, CD8 and CD20 in 4% PFA (ref. 416250397, Roti-Histofix®) for 1 hr at room temperature (RT) and embedded in Histogel specimen processing gel (ref. HG-4000-012, ThermoFisher Scientific) before dehydration in the Pearl. After paraffin inclusion, the blocks were sectioned on the Leica microtome at the size of 5µm. Then, IHC stainings were performed on these sectioned slides (Supp. Data Sheet Figure 7). IHC staining was performed on the LEICA Bond-II system using Bond Polymer Refine Detection based on the Novolink-polymer (ref. 7161, Leica), which is a horseradish peroxidase (HRP)-based polymer conjugated fluorescent dye that labels anti-rabbit antibody. The first steps of IHC are the dewaxing and the antigen unmasking (either High pH buffer or citrate buffer during 20 min). Saturation of endogenous peroxydases with 10 min of incubation in 3% H2O2 (ref. H1009, Sigma) and blocking step with goat serum for 30 min (ref. G6767, Sigma) were performed.

TABLE 1 PDTs and PDHOs culture media composition.

Product	References	Final concentration
Fetal Bovine Serum	10101-145, Sigma	10% of the final volume
Gentamycin	G1272, Sigma	1% of the final volume
B27 50X	17504-044	1X
VEGF-165	H9166-10µg	50ng/mL
HGF	SRP6014-10µg	30ng/mL
FGF-2	130-093-839-10µg	20ng/mL
EGF	GF316-500µg	100ng/mL
IL-2	130-097-744, Miltenyi Biotech	20UI/mL

TABLE 2 List of primary antibodies used in immunohistochemistry assays.

Target	Host species	References	Dilution
TTF-1	Rabbit	Ab76013, Abcam	1:250
Ki-67	Rabbit	LS-B13463-100 LSBio	1:5000
CK7	Mouse	BSH-2018-100, Nordic Biosite	1:300
MUC1	Mouse	NCL-MUC1-CORE, Novocastra	1:200
CD45	Rabbit	13917S, Cell Signaling	1:250
PD-L1	Rabbit	13684, Cell Signaling	1:200

The first primary antibody (anti-CD4) was incubated for 1 hr at RT. Then, after 1 hr of incubation with the primary antibody (Table 3), post-primary, a rabbit anti-mouse IgG (ref. 7161, Leica) was incubated for 30 min, and a tertiary antibody (Novolinkpolymer which is an anti-rabbit Poly-HRP) was then incubated during 30 min at RT (ref. 7161, Leica). The final step was the signal amplification based on TSA using Perkin Elmer kit (ref. FITC FP1018, Perkin Elmer), for 10 min at RT. A prior step to eliminate the first primary antibody and to inhibit the enzymes was used with the Linblock reagent (ref. RAG0149UK, Linaris). It was incubated twice for 2 min at RT, before saturation of endogenous peroxydases and blocking step with goat serum. The second primary antibody (anti-CD8) was incubated for 1 hr at RT, followed by the post-primary and tertiary antibody as described before. The following step was the signal amplification based on TSA using Perkin Elmer kit (ref. Cy5 FP1117, Perkin Elmer), for 10 min at RT. Then, the linblock reagent was used a second time to perform the staining of anti-CD20 and the protocol was the same, except that the signal amplification was based on TSA with the fluorescent dye Cy3 (ref. FP1046). At the end of the protocol, cell nuclei were stained with DAPI (dilution 1:10000 in PBS; ref. B2883, Sigma) during 10 min at RT. The washing steps were performed between each step with Bond wash solution 1X (ref. AR9590, Leica). Images were captured using the fluorescence microscope Nikon Eclipse 90.

2.4 Preparation of oncolytic virus solutions

VACV or TG6002 is a replication-competent Copenhagen Strain vaccinia (20). The complete DNA sequence of vaccinia virus: thymidine kinase gene-inactivated, ribonucleotide reductase gene-inactivated.

VACV GM-CSF+ or JX-594 is a replication-competent Wyeth strain vaccinia, thymidine kinase gene-inactivated (18, 19).

The oncolytic viruses were amplified using chicken embryo fibroblasts and purified using tangential flow filtration (TFF). Briefly, the crude harvest containing infected cells and culture supernatant and conserved at -20°C, was thawed at room temperature and the viral suspension was homogenized using a homogenizing mixer equipped with an in-line chamber. Large cellular debris were then eliminated by depth filtration using filters of 5 µm pore size. The clarified viral suspension was subsequently concentrated and diafiltered in a formulation buffer by using filtration and 0,2 µm pore size hollow fibers. Finally, the purified virus was further concentrated using the same tangential flow filtration system and aliquoted before storage at -80°C until use.

TABLE 3 List of primary antibodies for IHC staining of immune cells.

Target	Host species	References	Dilution
CD4	Mouse	NCL-CD4-368, Novocastra	1:75
CD8	Mouse	M-7103, DAKO	1:2500
CD20	Mouse	M-0755, DAKO	1:5000

The viral titer of the purified material was then measured using plaque assay on Vero cells (described in section 2.6.).

Oncolytic viruses were prepared according to a multiplicity of infection (MOI) of 0,1. This means that one viral particle is applied to ten cells. Mock is the condition containing only DMEM media. PDTs and PDHOs were incubated with oncolytic viruses for 96 hrs.

2.5 Immunofluorescence

PDTs and PDHOs were fixed in 4% PFA (ref. 416250397, Roti-Histofix®) for 24 hrs at 4°C. The day after, PDTs and PDHOs were incubated with PBS-Triton X100 0,25% (ref. X100, Sigma) during 30 min at RT then with PBS-BSA 5% (ref. A9647-100G, Sigma) during 4h at RT. Primary antibody (Table 4) was prepared in PBS-BSA 5% and incubated for 3 days at 4°C.

Four PBS-washings were performed at 30 min intervals (at RT), including one overnight washing at 4°C. Then, secondary antibody (Table 5) was diluted in PBS-BSA 5% and incubated for 24 hrs at 4°C.

Three PBS-washings were performed at 30min intervals (at RT), and then, DAPI (ref. B-2883, Sigma; Dilution 1:2500) was added to PDTs and PDHOs for 1 hr at RT. PBS then replaced DAPI until acquisition on confocal microscopy LSM Zeiss 800.

2.6 Titration and infection of Vero cells

Prior to titration, supernatants, and PDTs or PDHOs were harvested in duplicate for each condition and stored at -80°C before use.

The day of titration, samples were thawed in water bath at 37°C and then refrozen in dry ice. Samples were thawed and refrozen alternatively at least three times to lyse the PDTs and PDHOs. Vero cells (ATCC CCL-81^{TM}) were split and seeded at 5.10^4 cells/well in a six wells-plate. Cell culture media was DMEM (ref. 41966029, Gibco), supplemented with 1% gentamycin and 10% FBS. The day after, serial dilutions (E^{-1} to E^{-6}) of supernatants comprising lysed PDTs and PDHOs were prepared. Dilutions were done in PBS (ref. D8537-500mL, Sigma) supplemented with 1% FBS and 1% of cations [Mg $(CH_3COO)_2$ (Cf = 10g/L) and $CaCl_2$ $2(H_2O)$ (Cf= 10g/L)]. These different solutions were then incubated for three days at

TABLE 4 Primary Antibody used in Immunofluorescence assay.

Target	Host species	References	Dilution
Vaccinia Virus	Mouse	Monoclonal DMAB4487, Creative Diagnostic	1:300

TABLE 5 Secondary Antibody used in Immunofluorescence assay.

Target	Host species	References	Dilution
Anti-mouse FITC	Goat	A21121, Invitrogen	1:200

37°C. Solutions containing viral particles produce some lysis zones on Vero cells, which are also called viral plaques. These viral plaques are counted after three days of incubation by using the neutral red solution 10% (ref. N2889-100mL, Merck-Millipore) diluted in DMEM media with 20% agarose (Cf = 50g/L) (ref. A9045-250g, Sigma).

2.7 Proteomics

2.7.1 Protein extraction

Organoids from the same condition were pooled (Total 6) in an Eppendorf and rinsed with PBS. The PBS was removed, and the organoids were frozen at -80°C before extraction. Radio immunoprecipitation assay (RIPA) lysis buffer (ref. 89901, ThermoScientific) supplemented with Phosstop (ref. 04 906 837 001, Roche) and cOmplete tablets (ref. 4693116001) were used to lyse the organoids under sonication to perform protein extraction. The amount of protein was quantified using the DC protein assay kit (ref. 500-0116, Bio-Rad) according to the supplier's recommendations.

2.7.2 Preparation of supernatants

Supernatants from PDTs and PDHOs, mock and infected ones, from 6 different wells were pooled in an Eppendorf. The supernatants containing onco-virotherapy treatment were filtered through a 0,1µm filter and stored at -80°C until shipment to Olink Proteomics®, Uppsala, Sweden.

2.7.3 Olink proteomics

Lysates obtained from PDTs and PDHOs or supernatants (for the secretome analysis), mock and infected ones, were sent to Olink Proteomics®, Uppsala, Sweden. The panel Olink Target 96 Immuno-Oncology was performed using the proximity extension assay (PEA) technology. The assay is based on dual recognition of each protein by a pair of antibodies that link to the same protein in the samples to analyze. These antibodies are coupled with a complementary single DNA oligo strand that hybridize when in a close proximity once fixed on the protein target. The double strand is extended via a first PCR reaction to amplify the signal and generate amplicons to detect and quantify by qPCR targets. Proteins' concentrations are normalized starting from Ct values to NPX arbitrary units on an inverted log 2 scale. This normalized protein expression (NPX) is obtained by subtracting the Ct values obtained for each protein from a control Ct value constituted by the extension control, spiked in the same concentration in each well. First, the Ct value of the extension control, for a single sample, is subtracted from the Ct value of each analyte tested in a panel. This results in a delta Ct. This first step adjusts for any well-to-well technical variation. In the second step, the data is related to a known standard by subtracting the median IPC (Inter Plate Controls) Ct values of an analyte from the delta Ct of the same analyte, producing the delta delta Ct. This step here reduces inter plate variation. And finally, a correction factor determined during the validation of the Target 96 kit is used to invert the scale. The delta delta Ct for each analyte is subtracted from the Correction factor which then generates the NPX values. This inversion makes the NPX values more intuitive for data interpretation.

2.7.4 Bioinformatics analyses

Data were analyzed using the R-script and the R package of OlinkAnalyze.

KEGG Analysis: Gene set enrichment analyses (GSEA) were performed with the function olink_pathway_enrichment from OlinkAnalyze package using Kegg pathways.

2.7.5 Proteins' level measurements in lysates

GM-CSF (ref PPX-03-MXKA49V) was analyzed via Procarta Plex technology. Procarta Plex designed this kit using magnetic bead technology. Experimental steps were performed according to the supplier protocol. Data was analyzed using GraphPad Prims 9. The statistical method used was a paired two tailed Student's t-test to determine changes in GM-CSF proteins under onco-virotherapy treatment. n represents the number of patients used for each assay and a significance threshold of $p<0,05$ was used to determine a significant effect of onco-virotherapy treatment.

3 Results

3.1 Oncolytic viruses VACV and VACV GM-CSF+ present tumor specificity and are dependent of tumoral cells' metabolic activity

In our study, we generated PDTs and PDHOs from lung adenocarcinoma and the healthy counterpart of the tumor respectively (refer Method section: lung tumor and healthy tissue engineering). They were cultured according to the methods described in previous research (12). PDTs and PDHOs were infected with a multiplicity of infection (MOI) of 0,1 to evaluate the permissivity of patient' s cells to oncolytic viruses, for 96 hrs (Figure 1A). We assessed main lung adenocarcinoma marker, TTF-1 (21) on primary tumor tissues (Figure 1B) and primary healthy tissue (Supp. Data Sheet Figure 2), then verified its expression on respective PDTs and PDHOs. Other main biomarkers of lung adenocarcinoma such as cytokeratin 7 (CK7) and mucin-1 (MUC1) were also assessed by IHC on PDTs and PDHOs. Globally, PDTs could maintain the expression of these three biomarkers, and more cells were stained in PDTs than in PDHOs (Supp. Data Sheet Figures 3, 4).

The biomarker Ki-67 defines a rapid cell division and high metabolic activity; we then evaluated the biomarker Ki-67 in concomitance with TTF-1. We can observe that patients 22T 441, and 23T 37 (both in stages IIB) were the ones presenting more Ki-67 expression in primary tumor tissues and PDTs (Supplementary Table 1; Figure 1B). The quantification of viral particles produced in cells was evaluated with the plaque assay. We can observe that the number of viral particles increases when the biomarker Ki-67 is predominant generally (Figure 1C). This biomarker was more expressed for patients 22T 441 and 23T 37.

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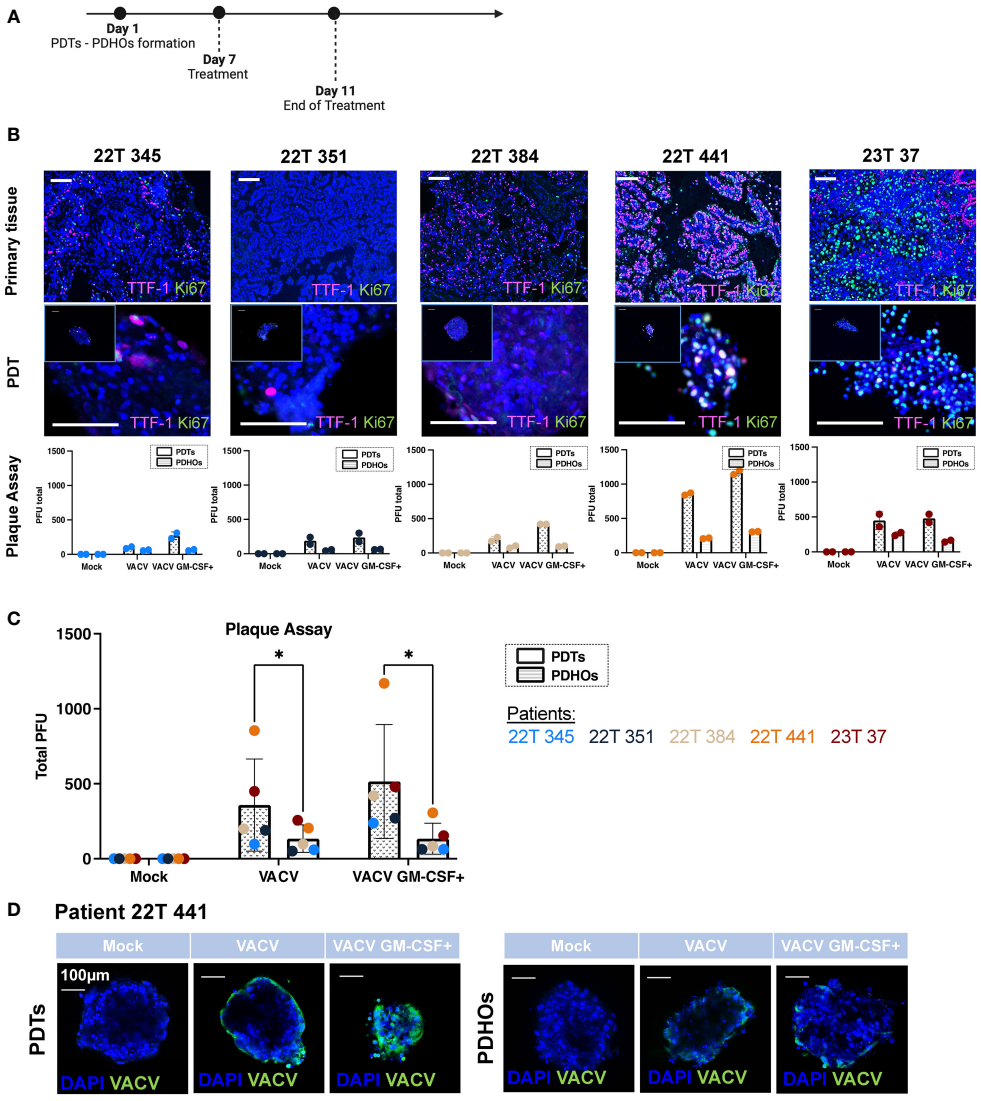

FIGURE 1
Oncolytic viruses VACV and VACV GM-CSF+ present tumor specificity and are dependent of tumoral cells' metabolic activity. **(A)** Workflow of PDTs and PDHOs oncolytic viruses' treatment. **(B)** IF of TTF-I and Ki67 biomarkers on primary tissue and PDT's paraffin slides. Scale bar 100µm. Quantification of viral particles within PDTs and PDHOs with the plaque assay based on the individual experiments (in duplicate). **(C)** Quantification of viral particles within PDTs and PDHOs with the plaque assay based on the average of technical values. P-values were calculated using an unpaired t-test with Holm-Šídák method. *p < 0,05. **(D)** IF of anti-vaccinia virus (VACV) on PDTs and PDHOs infected with VACV and VACV GM-CSF+ (e.g., patient 22T 441). Scale bar 100µm.

Furthermore, the number of viral particles was more important in PDTs than in PDHOs. This observation is consistent as we observe low expression of Ki-67 in healthy primary tissues and matched PDHOs (Supp. Data Sheet Figure 2).

The presence of vaccinia virus particles can be further confirmed with immunofluorescence using an antibody targeting vaccinia virus (Figure 1D; Supp. Data Sheet Figure 5). For organoids from patient 22T 441, we could observe a positive staining for vaccinia virus with both oncolytic viruses. On PDTs, VACV infected on the periphery whereas VACV GM-CSF+ infected cells with more spreading (Figure 1D). On PDHOs, VACV and VACV GM-CSF+ infected fewer cells compared to PDTs. This observation supports onco-virotherapy specificity for tumoral cells.

Viral replication is known to be supported by the host cellular metabolic state within the TME. We could point out that not all tumor-derived cells were metabolically active. Few co-staining of biomarkers Ki-67 and TTF-1 was observed in both type of samples. As both oncolytic viruses were deleted for enzymes involved in VACV DNA synthesis, this makes them rely heavily on the pool of these enzymes present in the target tumoral cells to support their proliferation (20). Contrary to what we might think, not all live tumoral cells are highly proliferative. These low metabolic states and quiescent states were already described in solid tumors (22). And this lack of metabolic activity could hinder oncolytic viruses' efficiency. In this part, results supported specific replication of both oncolytic viruses within PDTs.

3.2 An immunocompetent model of PDTs and PDHOs to evaluate immunostimulatory properties of oncolytic viruses

Prior to performing proteomics to evaluate immunostimulatory properties of oncolytic viruses, we checked the maintenance of primary tumor immune cells within PDTs and PDHOs. We have optimized the cell culture media to maintain them along the culture (Figure 2; Supp. Data Sheet Figures 6, 7). These immune cell subpopulations from the primary tissue are likely to have a better predictive value of prognosis and therapeutic responses (23). Maintaining these tumor-infiltrating immune cells in our 3D model was more relevant to reflect the tumor immune microenvironment better. We used the biomarker CD45 to assess the maintenance of tumor-infiltrating immune cells within PDTs, PDHOs, and their corresponding primary tissues (Figure 2; Supp. Data Sheet Figures 6, 7). We could observe CD45 biomarker expression in both type of samples (tumor tissues and PDTs). Two patients 22T 384 and 23T 37 presented more immune cells in their primary tumoral tissues, and this was also reflected on their PDTs. Then, healthy primary tissues presented fewer immune cells compared to the tumoral primary tissue. This was also observed on PDHOs compared to PDTs (Supp. Data Sheet Figure 6). As for patients 22T 345 and 22T 351, no CD45 expression was observed in corresponding PDTs. In patient 22T 351, we even observed a loss of CD45 expression, this could be due to the difficulty of maintaining immune cells *in vitro*.

Immune or tumoral cells can express PD-L1 to reduce antitumor immunity and inhibit T cell activation. The knowledge of PD-L1 tumoral cells expression is important as this ligand has emerged as a potential biomarker to predict responses to immunotherapy (24, 25). Then, we assessed PD-L1 biomarker on PDTs as well and, we could find the expression of PD-L1 in PDTs (Figure 2). When there's no colocalization of PD-L1 with CD45, we suggest that it is expressed by tumoral cells. This was the case for all patients except patient 22T 345 whose PD-L1 expression is 0%. In contrast, PDTs from patient 22T 441 presented more PD-L1 expression, which is up to 60% in clinical features (Supplementary Table 1).

These PDTs and PDHOs could maintain immune cells from the primary tissue. Hence, from these PDT and PDHO model, we could assess immune-related effects following oncolytic viruses' treatment.

3.3 Analysis of intracellular proteins with multiplex protein screening showed downregulation of proteins involved in tumoral progression in PDTs

The use of genomics on fresh tumor tissues or formalin-fixed paraffin-embedded (FFPE) blocks has permitted to have insights on

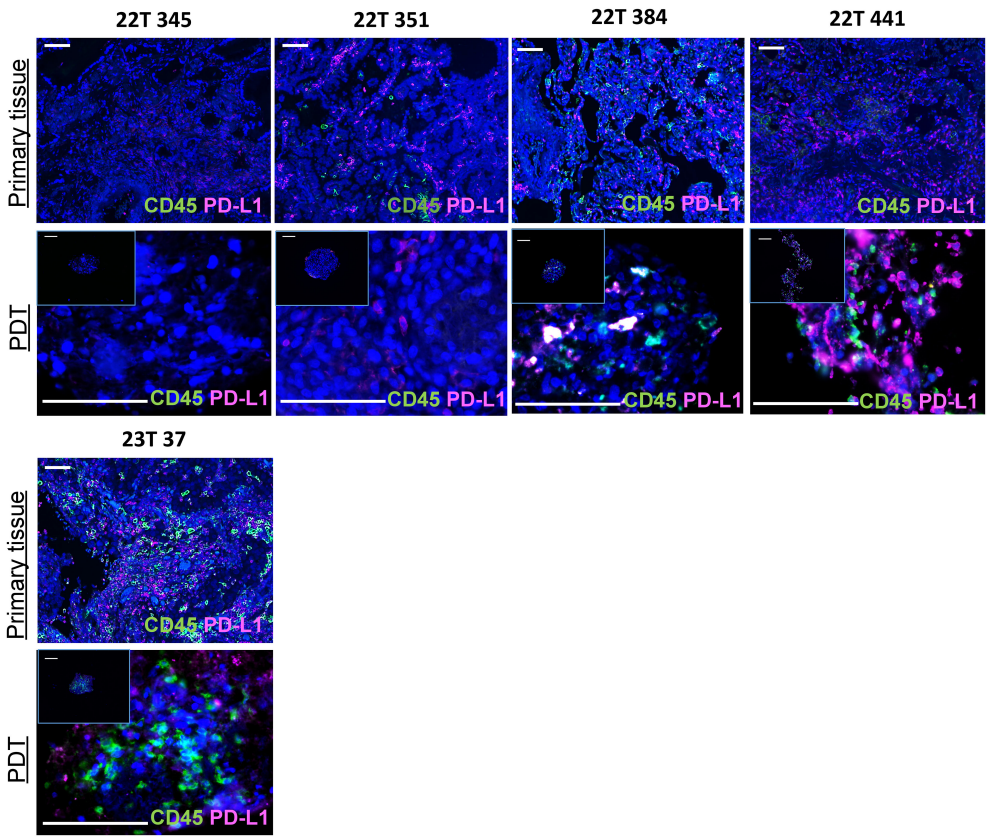

FIGURE 2
An immunocompetent model of PDTs and PDHOs to evaluate immunostimulatory properties of oncolytic viruses. Biomarkers CD45 and PD-L1 expression were analyzed on patients' primary tissues and patients' derived tumoroids. Scale bar 100µm.

genetic variants that predict responses to drugs (26, 27). The cancer genome can be completed by having a look on the protein products from these genes (intracellular and membranous proteins) (28). These proteins represent most of the human proteome besides secreted proteins. They are the molecules mostly responsible for cell growth and cancer progression (28). To better understand oncolytic viruses' effect at the protein level, we measured proteins relative expression with the proximity extension assay (PEA) technology. This technology is supported by the Olink platform (Olink Target 96 Immuno-Oncology). We mainly investigated the immune responses at the intracellular proteins (Figure 3A).

To investigate oncolytic viruses' global effect on the five patients, specifically selected for their different histology, proteomic data can be summarized under a Volcano Plot representation (Figures 3B, C). This data representation permits an overview of differentially expressed proteins on PDTs and PDHOs, either treated by VACV or VACV GM-CSF+. After both oncolytic viruses' treatment, levels of IFN-γ were upregulated (Figures 3B, C). This upregulation is a sign of antiviral responses in the TME (29). Then, both oncolytic viruses mainly presented a downregulation of proteins involved in tumoral cell progression and survival, such as interleukin-8 (IL-8), C-C motif chemokine ligand 20 (CCL20), and galectin-1 (Gal-1) (Figures 3B, C). IL-8 (30) is a chemokine known for its pro-inflammatory and pro-angiogenic effects in NSCLC. CCL20 (31) is an oncogenic chemokine favoring tumoral progression, and Gal-1 (32) is known to favor adhesion, proliferation, and metastatic processes of tumoral cells. On PDTs, VACV GM-CSF+ could induce downregulation of other proteins involved in tumoral progression, such as CX3CL1 and CXCL12 chemokines. Regarding the effect of oncolytic viruses on PDHOs, we could note a downregulation of the TNFRSF21 (33) protein, mainly involved in the negative regulation of T lymphocytes (Figures 3B, C). Besides the downregulation of pro-tumorigenic effects, oncolytic viruses can elicit immune responses.

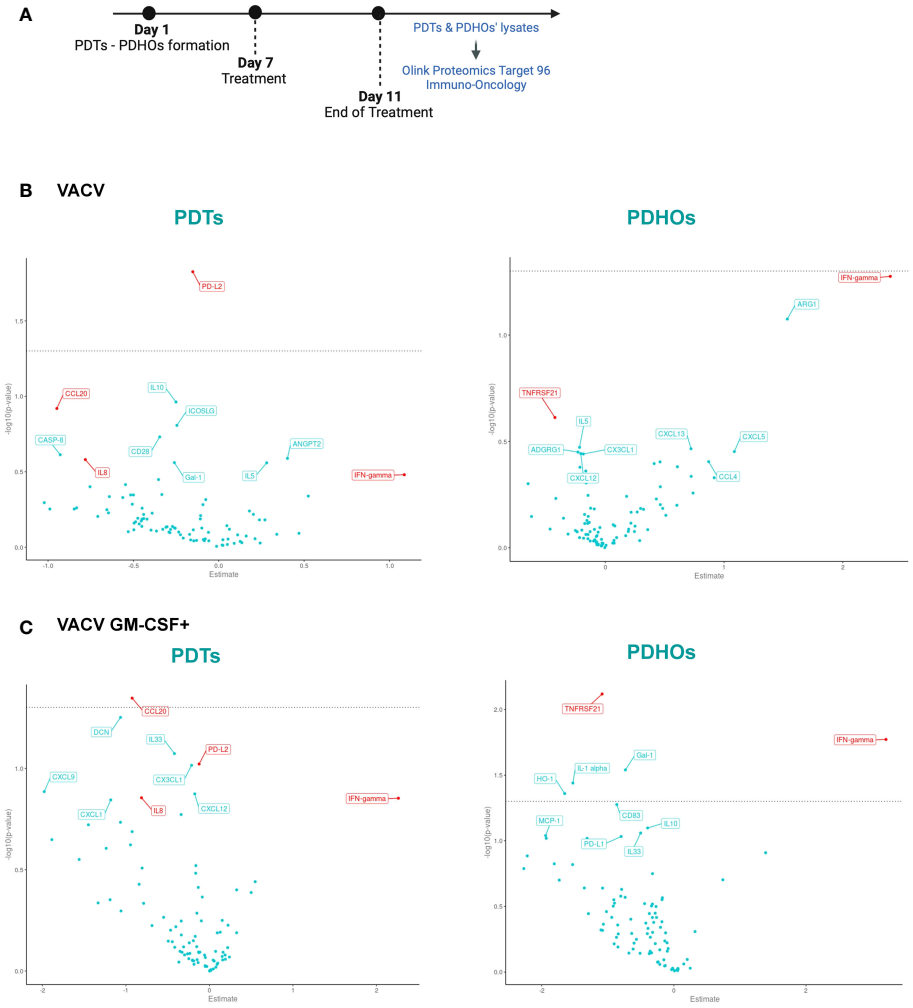

FIGURE 3
Volcano Plot showed some downregulation of proteins involved in tumoral progression in PDTs. **(A)** Workflow of PDTs and PDHO to proteomic' analysis. **(B)** Volcano plots of differentially expressed proteins are shown for the VACV versus mock n=5. **(C)** Volcano plots of differentially expressed proteins are shown for the VACV GM-CSF+ versus mock n=5 (red genes: proteins found in top 10 upon VACV and VACV GM-CSF+ treatment in either healthy or tumoral samples).

3.4 Analysis of intracellular proteins with multiplex protein screening showed immunostimulatory effects in PDTs

To assess the global effect of viral infection on patients' PDTs, a gene set enrichment analysis using KEGG pathways was also done to study the pathway significantly enriched in our comparisons infected versus mock (Supplementary Table 2, Supp. Data Sheet Figure 8). The main pathway enriched by both oncolytic viruses was the chemokines pathway. Among the enriched chemokines, we can note the decrease of CCL20 expression observed previously and CCL4 (34) and that promote cancer progression. We can also note a decrease expression of chemokine implied in decreased activity of CD8+ T cells such as CXCL5 (35) by both oncolytic viruses (Supp. Data Sheet Figure 8).

The heatmap permits to cluster the proteins' levels by the nature of the samples (tumor or healthy), patients' individuality and treatments' conditions. The proteins' levels were defined according to the normalized protein expression values (NPX) on a $\log_2$ scale. Oncolytic viruses' effects varied from one patient to another.

If looked into details, PDTs derived from patients 22T 345 and 22T 351 showed some lower protein z-score than other patients. This observation was following clinical features which are a low PD-L1 expression and the absence of CD45 expression (Supplementary Table 1). These features reflect a cold tumor (36) (Figure 2; Supplementary Table 1). Patient 22T 345 presented a slight immunomodulatory effect under VACV and VACV GM-CSF+ (Figure 4). Indeed, we could observe that the CD27 (37) protein was 1,5 to 2-fold higher in PDTs treated with both oncolytic viruses.

In contrast with these two latter patients, PDTs derived from patients 23T 37 and 22T 441 could demonstrate some immunomodulatory responses, mostly under VACV GM-CSF+ treatment. We will describe each case individually and briefly. In PDTs from patient 22T 441, we could observe an increase of IFN-γ (38), which is also a cytokine mediated by cytotoxic T cells in addition to be linked to antiviral responses. As for patient 23T 37, we observed more immune-mediated cytotoxicity effect. Indeed, we observed that proteins involved in NK-cell mediated cytotoxicity were 2-fold higher in PDTs VACV GM-CSF+ treated, with the increase of proteins Natural Cytotoxicity Triggering Receptor (NCR1) (39) that mediates MHC non-restricted cytotoxicity, and Killer cell immunoglobulin-like receptor (KIR3DL1) (40) which is the ligand for the surface protein KLRD1, involved in NK cell signaling. We can also note a slight increase of IFN-γ (38), TNF-α (41) and FASLG that participate to effector functions of NK and T lymphocytes. Then, we could observe a slight increase of Cytotoxic and Regulatory T cells Molecule (CRTAM) (42) protein involved in CD8+ T cells response (Figure 4; Supp. Data Sheet Figure 8). In summary, this patient has more response to oncolytic virotherapy. This patient presented a PD-L1 expression > 90% regarding its clinical criteria. As mentioned previously, PD-L1 expression level is among the biomarkers to define a response to immunotherapy. This suggests to integrate PD-L1 expression with tumor immune cells infiltrate to refine onco-virotherapy selection for patients (43) (Figure 4, framed in pink). Indeed, PD-L1 expression and a high T-cell infiltration are part of features to define a "hot" tumor which is mostly effective to immunotherapies (44).

In general, VACV GM-CSF+ presented more immunomodulatory effects than VACV mainly because of the GM-CSF transgene which might have potentiated an antitumoral immune response. The encoded transgene GM-CSF was mostly released in PDTs infected by VACV GM-CSF+ than in VACV (Supp. Data Sheet Figure 9).

As the research for biomarkers often relies on the secreted proteins, we also studied the proteomic signature in the secretome of two other patients 22T 31 and 22T 67 according to the same treatment workflow (Supplementary Table 3, Supp. Data Sheet Figure 10A). Indeed, biological fluids such as blood samples are used to predict outcomes on patients under ICIs treatment (45). Clinical biomarkers such as TTF-1, Ki-67, CD45 and PD-L1 were assessed in the same way as other patients (Supp. Data Sheet Figures 10B, C). Our experimental design permits us to establish the signature of secreted proteins after VACV and VACV GM-CSF+ treatments under a heatmap representation (Supp. Data Sheet Figure 10D). From PDTs of patient 22T 31, we can note that more change was seen at the level of secreted proteins. Due to its higher clinical relevance, we analyzed in greater detail the secreted proteins under VACV GM-CSF+ infection. We could observe the increase of some proteins involved in T cells activation such as CD28 (46), CD40L (47), CD70 (37), CD244 (48), CRTAM (42) and IL12 receptor subunit beta 1 (IL12RB1) (49). Also, NK-cell mediated cytotoxicity was observed with the increase of proteins NCR1 (39), KIR3DL1 (40), and Killer cell lectin-like receptor subfamily D member 1 (KLRD1) (50) or CD94 which is a surface protein involved in NK cell signaling (Supp. Data Sheet Figure 10D, proteins marked with a pink star). PDTs from patient 22T 67 showed less response (similarly to the intracellular proteins' signature), we only observed a slight increase of IL2 (51), IL5 (52), and KIR3DL1 (40) (Supp. Data Sheet Figure 10D, proteins marked with a green star). Interestingly, we also observed a decrease of lymphocyte-activation gene 3 (LAG3) protein (53), which is involved in the TCR inhibition of T cells. Its inhibition is beneficial to an anti-tumoral effect. Overall, different profiles of drug sensitivity were observed; this could be explained with patients' heterogeneity which is a hurdle to drugs efficiency (54).

As observed with the intracellular proteins, the immunomodulatory response induced by oncolytic viruses, especially VACV GM-CSF+, was also reflected with the secreted proteins. This demonstrates the relevance of combining the study of secreted and intracellular proteins to map the expression of the proteins under treatment. Thus, we have shown the usefulness of proteomics with tumoral organoids to bring more insights into onco-virotherapy responses. The use of proteomics gives multiple readouts in protein profiling and permits to stratify responders from non-responders.

4 Discussion

We have demonstrated how to maintain main features of a tumor niche, including tumoral cells and tumor-infiltrating immune cells within our PDTs and PDHOs models. Under our experimental protocol, we observed that our PDTs composition was reliable to tissues' one. However, for some patients (e.g. 22T 351), we could observe some discrepancies between the primary tissue and matched PDTs. The loss of CD45 expression in PDTs compared to primary tissue could be explained by the difficulty of maintaining immune

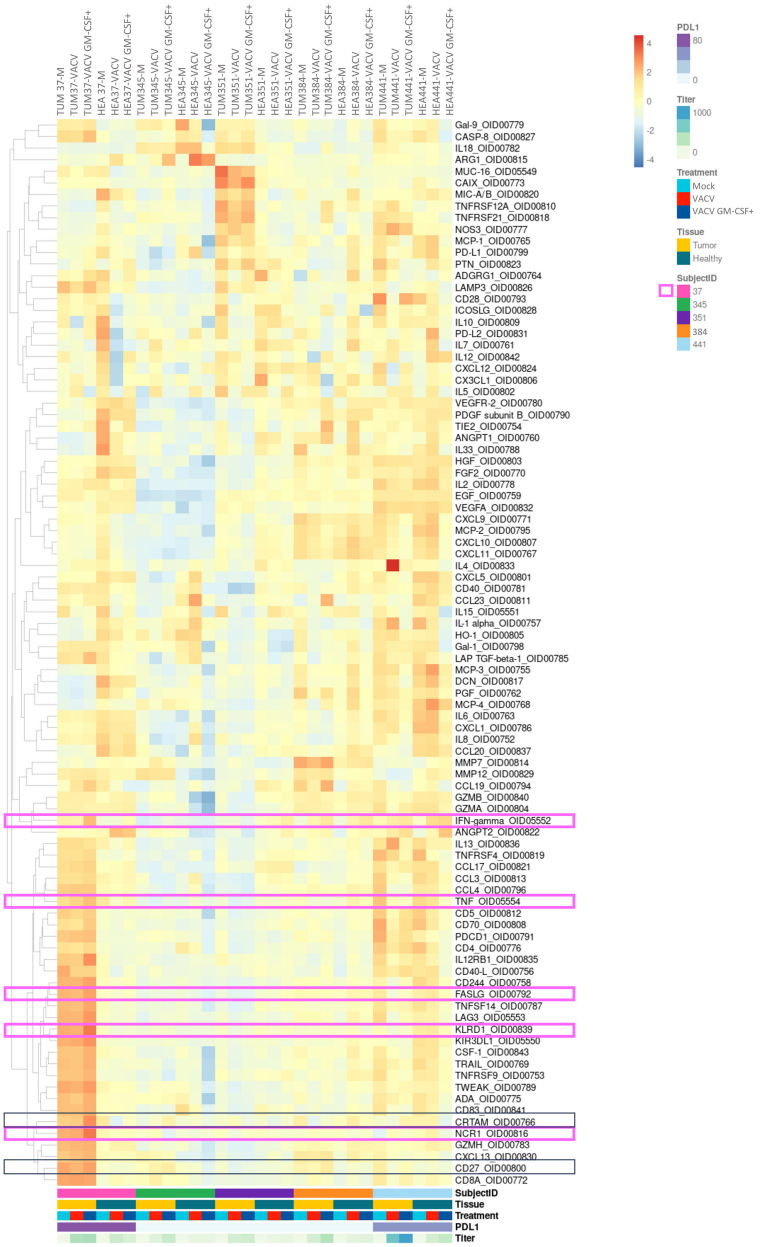

FIGURE 4
Analysis of intracellular proteins showed some immunomodulatory effects of oncolytic viruses in PDTs and PDHOs. (Heatmap showing the expression of detectable proteins based on a z-score and on a clustering cluster_rows=FALSE.) n=5.

cells *in vitro* (15). Furthermore, differences between the organoids and primary tissues have already been described before, this could be due to the cell culture conditions and the *in vitro* growth of primary cells (55). In parallel to PDTs, we also generated PDHOs from the healthy counterpart of the tumor to assess safety-related issues. We have confirmed their non-malignancy phenotype by histology with the decreased expression of lung adenocarcinoma-associated biomarkers. Results as a whole show the ability of tumoroids and organoids to represent a potential tool for drug discovery.

The great asset of oncolytic viruses is their tumor-specificity effect (56). Then, the integration of healthy organoids was relevant to confirm their tumor-specificity and safety profile. Currently, most of the data regarding oncolytic viruses' efficiency comes from murine models and clinical data. These data mainly concentrated on the therapy's final effect, which was mostly the tumor site's regression, however, this input could not add significant insight into biological responses. To increase the readouts, PDTs and PDHOs permit studying oncolytic viruses' permissivity into human tumor cells in complementary to animal models. We could study the conceptual mechanisms of oncolytic viruses, including infection, replication ability, immune cell response activation, and GM-CSF transgene delivery. Unfortunately, our findings didn't reach statistical significance due to following reasons: (i) the small number of patients samples, (ii) the substantial inactivation of genes implied in DNA synthesis in the genome of oncolytic viruses (making

them dependent on tumoral cells), and (iii) a hot tumor seems to be a predisposing factor of onco-virotherapy efficiency.

Indeed, we have studied the oncolytic viruses' effect on a subset of seven patients (five on lysates and two on supernatants). The construct of the oncolytic virus's genome had an impact as VACV GM-CSF+ presented more immunomodulatory effect than VACV. One primary mechanism of Transgene's attenuated oncolytic viruses is to replicate precisely in metabolically active cells. Our results show that not all tumoral cells, positive for lung-adenocarcinoma markers, expressed Ki-67. Moreover, it has been reported that 80% of tumoral cells are in a quiescent state within the tumor (22). We also noticed that a high PD-L1 expression (90-100%) is beneficial to onco-virotherapy efficiency. Indeed, in most of the patients analyzed, especially for patient 23T 37, we could observe a slight increase of proteins involved in NK and T cells activation including NCR1, KIR3DL1, CD27, CRTAM, FASLG, IFN-γ and CXCL13.

Altogether, there is a question of balance between the payload of oncolytic viruses, the patients immunophenotype and tumoral cells metabolic activity among other parameters (56). Based on these observations, we have a trend of patients' profile of responders to onco-virotherapy. Thus, we suggest screening tumoral cells' metabolic activity using the biomarker Ki-67 associated to a seahorse analysis for example. Also, the assessment of PD-L1 expression and immune-inflamed tumor using spatial transcriptomic before oncolytic viruses' administration at clinical phases can help to predict oncovirotherapy' effect. Next steps will be to consolidate our studies toward the immune compartment of our 3D model by studying the maintenance of native infiltrating immune cells in matched PDTs using single-cell sequencing as Neal et al. (57). did.

Once well defined, PDTs and PDHOs could be applied to study the impact of other payloads, such as cytokines and chemokines with a profile that leads to Th1-type immune response mediated by NK, CD4+, and CD8+ T cells (58). These observations can be further complemented on more complex ex vivo models such as patient-derived explants (59), and animal models, to strengthen the use of tumoroids in drug discovery.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving humans were approved by Ethics Committee of Grand Est, France. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

HL: Formal analysis, Methodology, Writing – original draft, Writing – review & editing. JD: Formal analysis, Software, Visualization, Writing – review & editing. PC: Formal analysis, Methodology, Writing – review & editing. AL: Methodology, Writing – review & editing. GH: Writing – review & editing. VL: Resources, Writing – review & editing. SJ: Formal analysis, Methodology, Writing – review & editing. JB: Conceptualization, Methodology, Writing – review & editing. NB: Conceptualization, Methodology, Supervision, Writing – review & editing. EQ: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

HL, JD, SJ, JB and EQ were employed by Transgene S.A.. PC and AL were employed by Olink.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1379613/full#supplementary-material

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The investigation of oncolytic viruses in the field of cancer therapy

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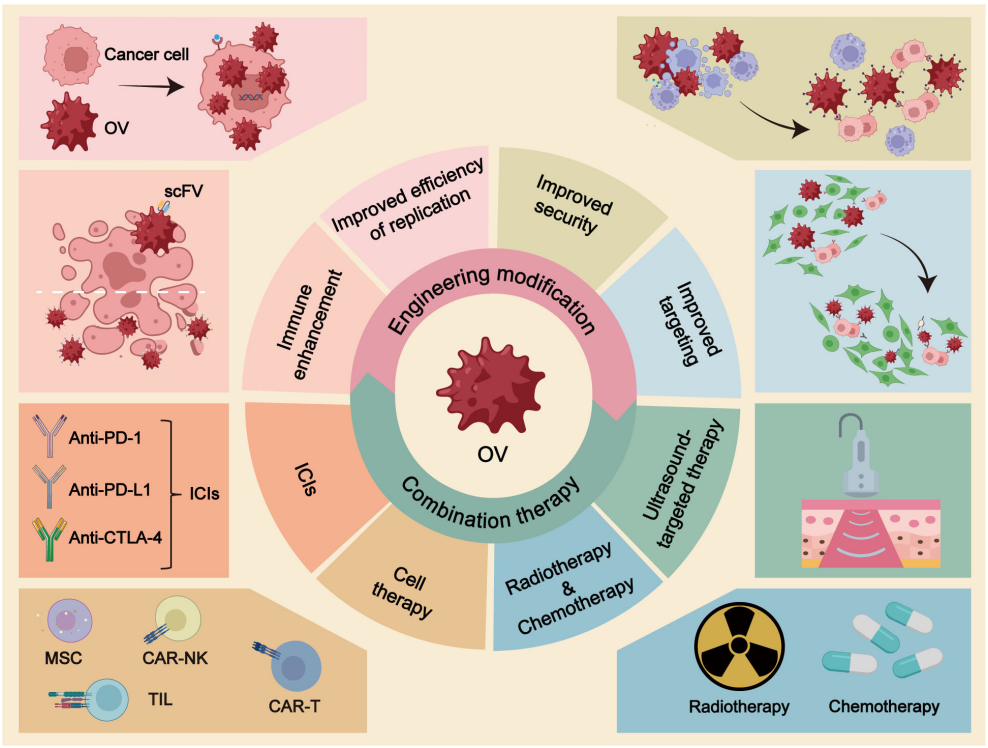
Oncolytic viruses (OVs) have emerged as a potential strategy for tumor treatment due to their ability to selectively replicate in tumor cells, induce apoptosis, and stimulate immune responses. However, the therapeutic efficacy of single OVs is limited by the complexity and immunosuppressive nature of the tumor microenvironment (TME). To overcome these challenges, engineering OVs has become an important research direction. This review focuses on engineering methods and multi-modal combination therapies for OVs aimed at addressing delivery barriers, viral phagocytosis, and antiviral immunity in tumor therapy. The engineering approaches discussed include enhancing *in vivo* immune response, improving replication efficiency within the tumor cells, enhancing safety profiles, and improving targeting capabilities. In addition, this review describes the potential mechanisms of OVs combined with radiotherapy, chemotherapy, cell therapy and immune checkpoint inhibitors (ICIs), and summarizes the data of ongoing clinical trials. By continuously optimizing engineering strategies and combination therapy programs, we can achieve improved treatment outcomes and quality of life for cancer patients.

KEYWORDS

oncolytic viruses, cancer therapy, genetic engineering, combination therapy, clinical trials



GRAPHICAL ABSTRACT
OV, oncolytic virus; scFV, single-stranded fragment variable; ICIs, immune checkpoint inhibitors; MSC, mesenchymal stem cell; TILs, tumor-infiltrating lymphocytes.

1 Introduction

Oncolytic viruses (OVs) are a specific type of viruses that can selectively replicate within tumor cells, inducing apoptosis while also stimulating the immune response and preserving normal tissue from destruction (1). Over the past two decades, extensive research in genetic engineering, tumor immunology, and molecular biology has established oncolytic virus (OV) therapy as a promising approach for cancer treatment (2, 3). OVs can be categorized into two main groups: naturally occurring viruses and genetically modified viruses (4). Naturally occurring OVs include reovirus, newcastle disease virus (NDV), myxoma virus (MYXV; Poxvirus), and seneca valley virus (SVV), while most OVs have been genetically modified or serve as vectors, including measles virus (MV; Paramyxovirus), poliovirus (PV; Picornavirus), vaccinia virus (VV; Poxvirus), adenovirus (Ad), and herpes simplex virus (HSV). Genetic modifications aim to enhance the targeting specificity and safety of the OV towards tumor cells by improving selective replication and cleavage capabilities, and augmenting host anti-tumor immunity levels (1, 5, 6).

With the application of spatial transcriptomics (7), single-cell RNA sequencing (scRNA-seq), and proteomics technology in cancer research (8–10), the significance of tumor microenvironment (TME) in cancer biology has been recognized. Cancer is a complex evolutionary and ecological process involving interactions between tumor cells and TME (11). The complexity and heterogeneity of TME are closely associated with tumor growth, metastasis, and response to therapy, making it a crucial target for cancer treatment (12). Although OVs have emerged as a potential therapeutic option for cancer due to their precise targeting ability, high killing rate, dose escalation over time, and minimal side effects; however, using a single type of OV alone is insufficient to overcome the challenges posed by the immunosuppressive TME resulting in limited anti-tumor effects (13). Therefore, this review aims to summarize engineering modifications of OVs and multi-modal combination therapies that can address delivery barriers, viral phagocytosis issues, antiviral immunity responses along with other challenges faced by OV-based cancer therapy (14–16). Additionally, we will introduce clinical data from current major studies on OV.

2 Engineering modification of OVs

2.1 Enhancement of OVs immune response *in vivo*

The immune response of OVs is a crucial mechanism in tumor treatment. Enhancing the *in vivo* immune response of OVs is a complex process involving multiple aspects of optimization and strategic approaches. It has been reported that the regulation of the

following aspects can break through the immune system barrier and improve the *in vivo* immune response to OVs (1): enhancing T cell activation, polarization, and memory T cell generation (2); inhibiting cancer immune escape through cytokines and blocking immunosuppressive ligands (3); disrupting physical barriers and increasing immune cell infiltration (4); suppressing immunosuppressive cells (17). In this process, immune checkpoint molecules and various cytokines in the TME play pivotal roles.

2.1.1 Engineered OVs carrying immune checkpoint molecules

The immune checkpoint molecules play a crucial role in modulating the immune system. They function as co-stimulatory receptors present on various immune cells, transmitting inhibitory signals (18). Among the extensively studied immune checkpoint molecules are CTLA-4, TIM-3, and PD-1, which effectively regulate the immune response to prevent excessive immunological damage (19). A study conducted by Ju et al. demonstrated that OVs armed with a single-stranded fragment variable (scFv) targeting PD-1 effectively enhanced effector T cell activity in genetically engineered mice. The reported findings revealed that OVs expressing PD-1 inhibitors synergistically acted with anti-CTLA-4 or anti-TIM-3 agents to potentiate the immune response *in vivo* and consequently achieve tumor control (20) (Figure 1A).

2.1.2 Engineered OVs expressing cytokines

Genetic engineering of OVs to express specific cytokines is an effective strategy to improve the immune response of OV. A range of antitumor responses can be regulated by cytokines (21), including (1): interferons (IFNs): IFNα, IFNβ, IFNγ (2); Interleukin (ILs): IL-2, IL-12, IL-15, IL-17, IL-18 (3); chemokines: CXCL9, CXCL10 and CCL5 (4); Granulocyte-macrophage colony-stimulating factor (GM-CSF) (5); Tumor necrosis factor (TNF) (Figure 1A).

2.1.2.1 IFNs

IFN is divided into type I (IFNα and IFNβ) and type II (IFNγ), of which type II is mainly secreted by immune cells: T-helper (Th) 1 cells, natural killer cells (NK cells), etc. Expression of IFN in OVs can effectively induce tumor cell death through modulation of various pathways (22). Studies have demonstrated that oncolytic adenovirus (OAd) expressing IFN (IFN-OAd) significantly suppresses tumor growth in hamster pancreatic cancer models, leading to increased infiltration of tumor-infiltrating lymphocytes (TILs) (23). Furthermore, the incorporation of CD47 and IFNγ genes into MYXV results in the production of MYXV_IFNγ and MYXV_CD47, respectively. This dual expression strategy enhances anti-tumor immunity in a mouse melanoma model, highlighting the synergistic effects between CD47 and IFN (24). Therefore, it is

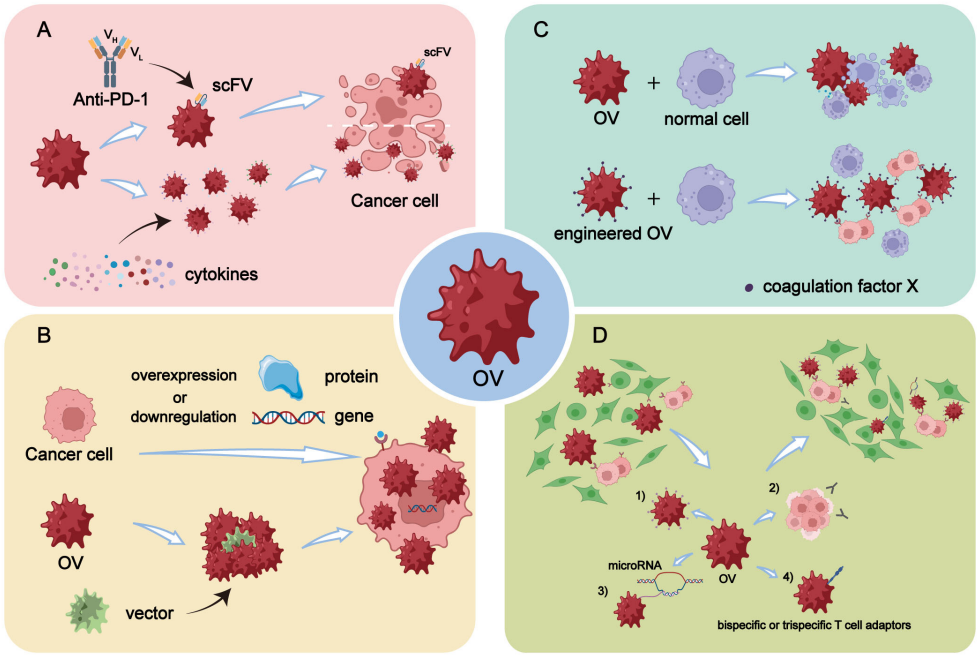


FIGURE 1
Engineering modifications of OVs. **(A)** Enhancement of *in vivo* immune response by arming OVs with scFV targeting PD-1 or by overexpressing specific cytokines through genetic engineering. **(B)** Enhancement of replication efficiency of OVs in tumor cells through genetic engineering by overexpressing or downregulating certain genes or proteins in tumor cells, or by selecting and designing more efficient virus vectors. **(C)** OVs are engineered to reduce off-target effects and damage to normal cells, making it a safer and more high-fidelity attenuated virus, thereby improving the safety profile of OV therapy. **(D)** Enhancement of tumor targeting of OVs through five main modification strategies: 1) Increasing virus affinity and binding activity to receptors overexpressed on tumor surfaces. 2) Utilizing differentiation of tumor cells to improve targeting accuracy. 3) Incorporating differentially expressed microRNAs into OVs through transgenic technology. 4) Arming OVs with bispecific or trispecific T cell engagers.

evident that direct activation of immune cells by IFNs can potentiate *in vivo* immune responses.

2.1.2.2 ILs

ILs are a class of small molecule proteins, possess the ability to both promote tumor cell growth and inhibit tumors in cancer (25). Due to their crucial role in tumor development, ILs can be incorporated into OVs for their antitumor functions. IL-2 functions as an anticancer cytokine by augmenting the activity of NK cells and cytotoxic T cells. Previous studies have demonstrated that IL-2 can be expressed in OVs such as VV, Sendai virus, Ad, and other vectors. Alternatively, IL-2 can be co-expressed with other anticancer transgenes in OVs to further enhance its immune characteristics (26). Recently, scientists developed an OAd that co-encodes TNFα and IL-2 and locally expresses them in hamster pancreatic cancer models. This approach modulates the TME by upregulating AIM2 expression and inhibiting tumor growth (27). IL-12 activates NK cells and T cells while promoting a Th1 type immune response. Previous studies have shown that engineering OAd (Ad5-ZD55-CCL5-IL12), which co-express CCL5 and IL-12, effectively increased the infiltration of chimeric antigen receptor (CAR)-T cells and TILs within tumors, resulting in potent anti-tumor effects with enhanced safety profiles (28). Additionally, treatment of colon cancer using oncolytic herpes simplex virus (oHSV) (O-HSV1211) modified to express both IL-12 and CXCL11 leads to increased infiltration of $CD8^+$ T and $CD4^+$ T cells into the tumor site (29). IL-15 functions as an upstream regulator of tumor-infiltrating $CD8^+$ T cells, and the IL-15/IL-15Rα$^+$ axis plays a crucial role in anti-tumor immunity (30). The researchers engineered a fusion protein combining IL-15 and IL-15Rα (designated OV-IL15C), which was expressed within gliomas and demonstrated the ability to enhance cytotoxicity against glioblastoma (GBM) both *in vivo* and *in vitro*, while also improving the survival of NK and $CD8^+$ T cells (31). Furthermore, expression of IL-15 within oncolytic VV (32) as well as a novel OV (SG400-E2F/IL-15) (33) also resulted in enhanced immune response and antitumor activity *in vivo*. The production of IL-18 is observed in various cell types, including activated monocytes, macrophages, and dendritic cells (DCs). IL-18 plays a crucial role as a cytokine in cancer (34). Recombinant Pseudorabies viruses (PRVs), namely rPRV-PH20 and RPRV-IL-18-gamma-PH20, were engineered using pseudorabies virus (PRV) as the vector. The results demonstrated a significant increase in the infiltration of $CD4^+$ T and $CD8^+$ T cells within tumor cells infected with recombinant PRV strains. Moreover, the oncolytic effect was superior in the treatment groups receiving rPRV-IL-18-gamma-PH20, rPRV-PH20 alone or RPRV-IL-18-gamma-PH20 compared to the control group. Notably, among these groups, the best anti-tumor effect was observed with rPRV-IL-18-γ-PH20 treatment. Overall, co-expression of PH20 with IL-18 and IFNγ enhanced systemic anti-tumor immunity mediated by IL-18 (35).

2.1.2.3 Chemokines

Chemokines are a subfamily of cytokines, produced by various cells in response to stimuli such as pathogens, drugs, or physical damage. These cells include white blood cells, endothelial cells, fibroblasts, and others. Chemokines play a crucial role in promoting cell migration throughout the body, particularly for white blood cells. They also have significant involvement in immune function regulation (36). The engineered expression of CCL5 shows promise as a method to enhance the immune response to OVs. For example, an OV called OV-Cmab-CCL5 was engineered to express CCL5 specifically within the TME. In GBM infected with OV-Cmab-CCL5, there was an increase in NK cell activity, T cell activity, and macrophage activity along with a decrease in tumor size (37). Other studies aiming to improve the immune response of OVs through engineering involve arming OAds with CXCL11 (38), overexpressing CXCR7 and CXCR4 in breast cancer cells using an armed OAd carrying CXCL12 (39), and utilizing CXCL10 as an armament for OAds (40).

2.1.2.4 GM-CSF

The incorporation of GM-CSF into OVs has demonstrated significant benefits for cancer patients. Examples of OVs utilized include, but are not limited to, oncolytic vesicular stomatitis virus (VSV) (41), oncolytic VV (42), oHSV type 1 (oHSV-1) (43), OAd (44), oncolytic Herpesvirus talimogene laherparepvec (T-VEC) (45), and oncolytic reovirus (46). Additionally, the use of ONCOS-102 encoding GM-CSF and ONCOS-204 encoding ICOSL (the ligand of inducible T-cell co-stimulator) in modified adenoviruses further enhances the function of bi-specific antibodies (BsAbs)-activated T cells within melanoma cells. Notably, the combination of ONCOS-204 and EGFRxCD3 BsAb exhibits superior ability in augmenting T cell activation and cytotoxicity compared to ONCOS-102, with ONCOS-204 particularly significantly influencing CD4+ T cell subpopulations infected with tumor cells (44).

2.2 Improve the replication efficiency of OVs in tumor cells

The enhancement of OV replication efficiency in tumor cells can be approached from two perspectives (1): Manipulation of gene or protein expression levels in tumor cells through genetic engineering techniques (2). Selection and design of more efficient viral vectors (Figure 1B).

Through genetic engineering, certain genes and proteins can be manipulated to either increase or decrease their expression levels in tumor cells. This modulation of gene expression can synergistically enhance the replication efficiency and therapeutic efficacy of OVs. For instance, in an experiment, the death domain associated protein was down-regulated, leading to increased viral replication efficiency. Additionally, overexpression of the precursor terminal protein helped overcome poor viral replication and resulted in a higher production of total viral particles (47). To address the replication defect caused by insufficient arginine succinate synthetase 1 (ASS1) expression in tumors, a series of recombinant oncolytic MYXV constructs expressing exogenous ASS1 were generated (48). Moreover, upregulation of MHC class I chain-related polypeptide

A (MICA), which serves as a ligand activating NK group 2D (NKG2D) receptor on NK cell and T cell subpopulations as an OV gene engineered transgene, was observed in tumor cells. The use of MICA-expressing oncogenic adenovirus named ICOVIR15KK-MICAMut demonstrated improved control over tumor growth compared to other viruses without MICA expression. This enhanced control is attributed to the virus's increased replication efficiency within the tumor cells, leading to a higher oncolytic activity and more robust immune-mediated tumor cell destruction (49).

Through the screening and optimization of virus strains, more efficient and tumor-selective OVs can be identified. In a clinical trial for cancer treatment using reovirus serotype 3 Dearing (T3D), the Patrick Lee laboratory strain (T3DPL type) demonstrated enhanced replication efficiency and higher oncolytic performance (50). To enhance the anti-tumor immune activity of chimeric poxvirus deVV5, a chimeric virus with thymidine kinase deletion and a suicide gene, FCU1, was generated. The deVV5-fcu1 group exhibited superior replication efficiency compared to the control group, with results indicating that it achieved the highest rate of virus production from Hep G2 liver cancer cells (51). In a study involving engineered adenoviruses, an Ad5/3 serotype chimeric vector OV was designed utilizing adenovirus type 3 (Ad3) receptors. Findings revealed that the Ad5-ΔE3-Luc group displayed greater *in vivo* replication capacity than the Ad5/3-ΔE3-Luc group. These studies have shown that modifying OAd type 3 can improve the replication efficiency of serotype chimeric Ad5/3 vectors, which should be considered in future research endeavors (52). In a recent study, expression of a new generation OAd called Ad5 KT-E1A-IL-15 (TS-2021), along with Ki67 and TGF-β2 proteins, was generated to enable selective replication in GBM cells and enhance efficacy in killing GBM tumors (53).

2.3 Enhanced safety

Since the acceptance of viruses as the cause of pathogenicity has long been widespread, the safety of OVs has also been subject to conservative debate. It has been demonstrated that OVs kill tumor cells while inadvertently attacking normal cells, akin to the side effects observed with chemotherapy (54). In response to this concern, numerous studies have shown that OVs can be engineered into attenuated viruses with enhanced targeted specificity. For instance, deletion of the γ34.5 gene prevents oHSV-1, an OV, from infecting normal neurons (55–57). The OV-containing VG161 developed by Virogin Biotech Canada Ltd. helps maintain target sensitivity to drugs like acyclovir, effectively enabling control over its virulence and safety in clinical applications —an important advantage in terms of safety (58–60). Additionally, Yiye Zhong et al. have designed an OV containing targets for neuron-specific microRNA-124 and granulocyte-macrophage colony-stimulating factor, significantly enhancing its neuronal safety while minimally impacting its replication capacity (61).

Despite the tumor cell-specific engineering, there is a potential for off-target effects and unintended toxicities resulting from genetic manipulation. Additionally, issues such as viral mutation, evolution, recombination, cytotoxic gene products, and viral transmissibility may arise (62). Based on these findings, several studies have identified certain substances that can mitigate these risks when combined with OVs. For example, Alba et al. discovered that using Ad5-hexon in conjunction with coagulation factor X (FX) facilitates liver transduction (Figure 1C). They also developed a genetically FX-bound ablative Ad5-hexon vector for symptom relief purposes (63).

2.4 Improve targeting

The ability of OVs to specifically infect tumor cells while sparing normal cells is considered a promising approach for the safe and effective treatment of cancer (64). Despite numerous clinical trials confirming the excellent targeting capability of OV therapy, there still exist certain limitations that require resolution. There are four primary modification strategies available to enhance the tumor-targeting potential of OV (Figure 1D).

The first approach is to enhance the affinity and binding activity of the virus towards the overexpressed receptor on the tumor surface. By engineering OVs to specifically recognize receptors that are upregulated in tumors, their targeting can be improved. For instance, Yang et al. demonstrated that a chimeric adenovirus composed of Ad35 knobs and axles binding to Ad5 enhances targeting and oncolytic effects across various cancers by utilizing CD46 as a differential receptor (65). On the other hand, knowledge of membrane-associated tumor-associated antigens (TAAs) enabled researchers to fully engineer a virulent OV with selective tropism for tumor cells by substituting the viral glycoproteins involved in cell entry with antibody fragments targeting specific TAAs, such as HER2, PSMA, and MSLN (66, 67). Tomer Granot et al. employed Sindbis virus (SV) vectors to deliver TAAs and enhance viral targeting. They found that SV/TAA therapy's efficacy stemmed not from direct tumor cell targeting, but from the transient expression of TAAs in lymph nodes draining the injection site. This mechanism prompted early T-cell activation, followed by a significant influx of NKG2D-expressing, antigen-specific cytotoxic CD8 T cells into the tumor. Ultimately, this led to the formation of long-lasting memory T cells, which conferred protection against rechallenge with tumor cells (68). Additionally, certain CD molecules that are overexpressed in malignant tumors serve as valuable targets for constructing targeted viral vectors to facilitate OV homing. For example, CD20-positive non-Hodgkin lymphoma (NHL) has been utilized to develop CD20-targeted MV vectors for lymphoma targeting with promising results (69). The increasing identification of tumor-specific receptors or antigens will provide more precise strategies for enhancing OV targeting.

Second, the targeting accuracy can be enhanced by leveraging the unique characteristics of tumor cells. For instance, OV can enhance its targeting selectivity by modulating genes or signaling pathways in tumor cells. Chen et al. achieved this by inhibiting the antiviral response of cells through blocking the alpha subunit of the IFN receptor using B18R (70). Additionally, overexpressing specific genes or proteins in tumor cells can also improve OV's targeting selectivity. By replacing the endogenous E1A promoter with

GOLPH2 (also known as GP73), E1B 55kD Ad deletion induces significant cytotoxic effects in prostate cancer stem cell (CSC)-like cells through GP73 overexpression and exhibits stronger oncolytic effects (71). Furthermore, armed with a full-length antibody against CD47, oHSV is capable of specifically targeting GBM and ovarian cancer (72, 73). Moreover, IL-12-carrying oHSV significantly elevates IL-12 levels within TME and the stimulates infiltration of effector T cells, NK cells, and APC into tumors to enhance anti-tumor efficacy (74).

Additionally, the integration of differentially expressed microRNAs into OVs through transgenic technology represents an alternative strategy to enhance targeting selectivity. In other words, OVs can be utilized as carriers to specifically deliver microRNAs for regulating cancer occurrence (75). MicroRNAs, which are short non-coding RNAs, play a crucial role in modulating gene expression by interfering with the translation of target mRNAs. Dysregulation of microRNAs has been implicated in tumor progression, invasion, angiogenesis, and metastasis across various types of cancer (76, 77). The OV vector demonstrates effective delivery of pre-tumor inhibition interference miRNA into tumor cells. Specifically, the interfering precursor microRNAs dissociate within the cytoplasm and undergo cleavage to generate mature microRNAs that subsequently lead to target mRNA inactivation. OAd carrying the tumor suppressor gene miR-143 induces apoptosis and reduces tumor growth by decreasing KRAS expression in HCT116 xenografts (78). Similarly, when oncolytic vesicular stomatitis virus serves as the carrier, miR-143 exhibits comparable antitumor effects in osteosarcoma cells (79). To further enhance oncolytic specificity while minimizing toxicity levels, Yang et al. have inserted miR-34a targets into both 5' untranslated region (UTR) and 3' UTR of the virus genome to develop a dual-targeting oncolytic Coxsackievirus B3 engineered variant that retains nearly complete oncolytic activity but with reduced toxicity levels (80).

Finally, the utilization of bispecific or trispecific T cell adaptors (BiTE or TriTE) molecules represents an alternative strategy for modifying OVs. BiTE is a recombinant protein consisting of two scFvs that bind to a T cell surface molecule and a malignant cell antigen, respectively, and arming OVs with a BiTE overcomes their extremely short serum half-life, while the next-generation TriTE includes three domains, such as CD3 × dual tumor antigens or tumor antigen × CD3/CD28.This technique involves linking two distinct ScFV antibodies, enabling each fragment to bind to both the surface of T cells and malignant cells. Consequently, this approach reduces the potential for immune escape due to antigen loss and minimizes side effects associated with targeted detumescence, ultimately improving tumor selectivity (81). Chen et al. demonstrated that CS1-NKG2D bispecial antibodies facilitate the augmentation of immune synapses between $CS1^+$ multiple myeloma (MM) cells and $NKG2D^+$ cytolytic innate as well as antigen-specific effector cells. As a result, these immune cells are activated, leading to improved clearance of multiple myeloma (82). Several other OVs carrying bifspecificity antibodies have exhibited distal effects through T-cell-mediated activation and tumolysis. Moreover, FAP and EGFR have been shown to enhance T cell activation and accumulation at the tumor site, thereby increasing anti-tumor efficacy (83–85). Furthermore, OV-encoded bispecific antibodies also promote T cell infiltration into the TME while exhibiting antitumor activity by enhancing T cell activation and cytokine production. Immune cold tumors, characterized by a lack of immune cell infiltration and activity, are typically resistant to immunotherapies. By promoting T cell infiltration and activation, OV-encoded bispecific antibodies help convert these immune cold tumors into immune hot tumors, which have a higher presence of active immune cells and are more responsive to immunotherapeutic interventions (86, 87).

3 Combination therapy

3.1 OV combined with chemotherapy

Chemotherapy, as a primary modality for cancer treatment, induces DNA damage by inhibiting processes such as DNA synthesis, mitosis, and cell division. Recent clinical trials have demonstrated the potential synergistic effect of combining chemotherapy with OVs, offering a promising alternative strategy in cancer therapy.

Cyclophosphamide (CTX) is an alkylating chemotherapeutic agent and was the pioneering drug to be combined with OVs. CTX undergoes metabolic conversion into cytotoxic substances within tumor cells, thereby inducing tumor cell death. Moreover, it also functions as an immunosuppressive agent, impacting both innate and adaptive immunity in the body. Research has demonstrated that early-stage low-dose CTX combined with OAd therapy can induce TH-1 immunity by reducing regulatory T cells (Treg cells), transforming the TME from a "cold" state to a "hot" state, and enhancing anti-tumor efficacy (88) (Figure 2). Talimogene laherparepvec (T-VEC) is an oncolytic virus hypothesized to enhance the response of triple-negative breast cancer (TNBC) to neoadjuvant chemotherapy (NAC). The rationale for combining T-VEC with chemotherapy stems from the observation that TNBC tumors with significant pre-existing lymphocytic infiltration respond more favorably to neoadjuvant therapy. Preclinical studies have shown a synergistic effect between oncolytic viruses and chemotherapy, further supporting this combination approach. In a Phase II clinical trial of T-VEC combined with NAC in TNBC, this combination therapy was found to improve the response rate of TNBC tumors injected with T-VEC during NAC. This provides a theoretical foundation for further investigation of T-VEC plus NAC for TNBC treatment (89). Temozolomide (TMZ), another alkylating agent and immunomodulator, is extensively employed in treating various solid tumors such as glioma and melanoma. TMZ has been shown to enhance replication and tumor lysis effects of OAds in lung cancer cell lines but not non-cancerous cells; this augmented antitumor activity may partly result from autophagy induction in these lung cancer cells (90). Additionally, gemcitabine (GCB), a nucleoside analogue antimetabolite antitumor agent, is widely used alone or in combination with other anticancer agents across multiple cancers (91). In one study, researchers utilized replicative adenovirus-mediated double suicide gene therapy(Ad 5-DS) alongside standard intravenous GCB at recommended

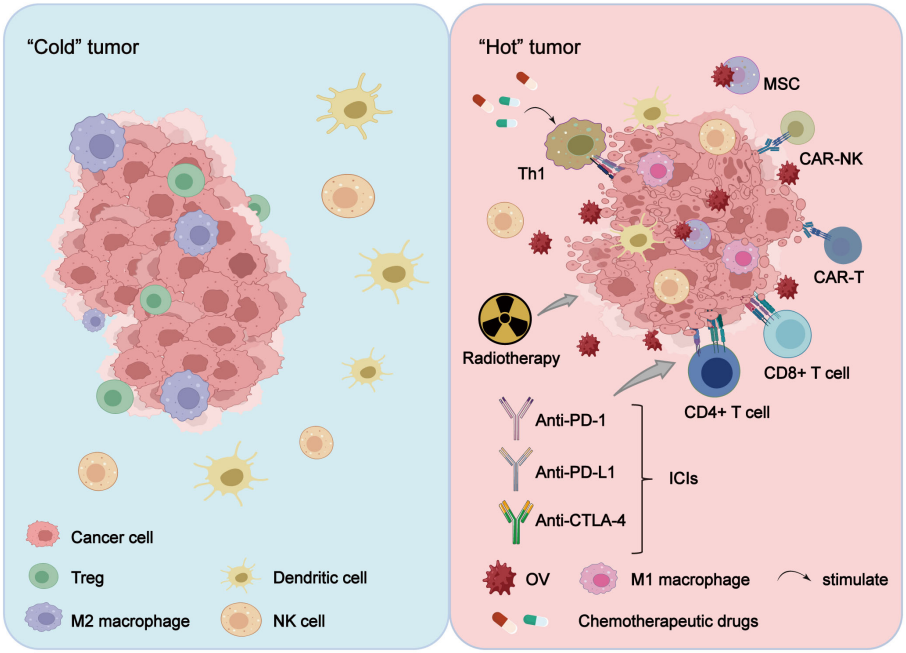


FIGURE 2
OV combination therapy reshapes the TME. Treatment of tumors with OVs alone or in combination with radiotherapy, chemotherapy, cell therapy and ICIs alters the tumor immune microenvironment, transitioning it from a "cold tumor" to a "hot tumor," with the reshaping effect more pronounced in combination therapy. Furthermore, OV combination therapy reduces the tumor infiltration of immunosuppressive cells (including Treg cells and M2-polarized macrophages), while enhancing the proliferation of activated immune cells (such as CAR-T cells, CAR-NK cells, TILs, NK cells, M1-polarized macrophages, and DCs), thereby exerting a stronger anti-tumor effect.

dosage levels; this approach proved safe and well tolerated among patients (92). These studies indicate the paramount importance of comprehending the interplay between OVs and anti-tumor chemotherapy drugs in advancing the development of combination therapy for cancer treatment.

However, subsequent studies have revealed that chemotherapy may exert a detrimental impact on the efficacy of oHSV immunoviral therapy. TMZ chemotherapy currently represents the standard treatment for GBM; however, when TMZ is combined with G47Δ-IL 12 to treat *in situ* tumor-bearing mice, it nullifies the beneficial effects of G47Δ-IL 12 and adversely affects intratumor T cells, macrophages, and spleen cells (93). Meanwhile, there remains limited clinical evidence supporting the combination of OV and chemotherapy; thus further experiments and clinical investigations are warranted to validate its effectiveness and safety.

3.2 OV combined with radiotherapy

Radiotherapy is the most efficacious cytotoxic modality for localized solid tumors (94). Its fundamental principle lies in irradiating the DNA of tumor cells, inducing DNA damage and impeding their indefinite proliferation until demise. It primarily encompasses alpha, beta, gamma rays, as well as diverse forms of X-rays. Radiotherapy is frequently employed in conjunction with chemotherapy to enhance patient survival (95). Nevertheless, the potent adverse effects of chemoradiotherapy and the heterogeneity of therapeutic efficacy are compelling researchers to explore novel combination therapies.

The combination of OV and radiotherapy not only exhibits a synergistic effect but has also demonstrated improved therapeutic efficacy in numerous studies. GBM, the most prevalent primary malignant brain tumor (96), is considered a "cold tumor" in immunology due to limited lymphocyte infiltration and unresponsiveness to current immunotherapies. Therefore, researchers are actively exploring novel techniques to convert "cold" tumors into "hot" tumors, thereby paving new avenues for cancer treatment (97) (Figure 2). One study demonstrated that GBM mice treated solely with OVs achieved a curative rate of 13.3 percent, while those treated with radiation alone had a curative rate of 21.4 percent. However, when the two therapies were combined, mice with brain cancer exhibited an impressive curative rate of up to 66.7 percent. The efficacy of combination therapy is further highlighted by the prolonged survival time observed in these mice. The median survival time for the control group (PBS group) was only 29 days, which increased to 39.5 days in the radiotherapy group and 41 days in the viral therapy group. Remarkably, when radiotherapy was combined with viral therapy, the median survival time exceeded 76 days. Further investigation revealed that this remarkable effect of combination therapy can be attributed to its significant increase in $CD3^+$ cell count and proportion of $CD3^+$ T/$CD8^+$ T and $CD8^+$ T/Treg cells in mice (98). Additionally, combining OVs with radiotherapy may enhance the "distant site effect" of radiotherapy (regression of unirradiated metastases at a

distance from the irradiated site) (99), possibly due to radiotherapy's ability to promote OV replication and increase cancer cell vulnerability (100). Moreover, OVs can augment immune checkpoint inhibitors' effectiveness through interaction with radiotherapy. A study involving NDV demonstrated that combining NDV with radiotherapy and PD-1 antibody resulted in prolonged mouse survival and significantly inhibited tumor growth compared to groups treated solely with PD-1 antibody or combinations of PD-1 antibody/NDV or NDV/radiotherapy (101).

The current research on the combination of OV and radiotherapy is limited. However, existing studies have demonstrated significant potential in this combined therapy, which is expected to enhance the efficacy and safety of tumor treatment, offering hope to more patients.

3.3 OV combined with cell therapy

3.3.1 OV combined with CAR-T

In recent years, the application of CAR-T cell therapy has demonstrated remarkable efficacy in cases where conventional cancer treatments are ineffective, particularly for untreatable blood system cancers such as leukemia, myeloma, and non-Hodgkin B-cell lymphoma (102). This approach selectively targets and eliminates tumor cells, leading to significant breakthroughs. Furthermore, there have been increasing clinical trials utilizing CAR-T cells for the treatment of solid tumors, with notable progress achieved in certain types of solid tumors. For instance, GBM exhibits high expression levels of IL-13Rα2 while normal brain cells show lower expression levels. This characteristic makes IL-13Rα2 a promising target for CAR-T cell therapy against GBM cancer. Brown et al. (NCT02208362) administered multiple infusions of IL-13Rα2-CAR-T cells directly into the resected tumor cavity via intracranial administration and observed regression of all intracranial and spinal tumors lasting 7.5 months (103). Additionally, a Phase I clinical study (NCT03182816) demonstrated the safety and feasibility of treating patients with advanced relapsed/refractory non-small cell lung cancer (NSCLC) using epidermal growth factor receptor (EGFR) CAR-T cells produced by the piggyBac transposon system instead of viral systems (104). However, despite these advancements in CAR-T cell therapy for solid tumors, several challenges and complications still exist including tumor heterogeneity, antigen escape by tumor cells, transportation limitations faced by CAR-T cells at the tumor site as well as invasion and expansion difficulties within the TME itself (105).

Notably, the combination strategy of OVs with CAR-T cell therapy holds great promise for enhancing the efficacy of CAR-T cells in solid tumors and overcoming associated challenges. Currently, there are four OVs approved worldwide for cancer treatment, among which T-VEC is the only OV approved by the Food and Drug Administration (FDA) that has demonstrated favorable safety and efficacy in clinical trials (100). Furthermore, successful CAR-T cell products already exist in the market, providing a strong foundation for combining OV and CAR-T therapy. Additionally, OVs have the ability to transform a "cold" tumor into a "hot" one. In a "cold" tumor, immunosuppressive cells like Treg cells and M2-polarized macrophages infiltrate surrounding tissues extensively while immune cell infiltration is insufficient and their function is suppressed. This allows tumor cells to evade attacks from the immune system. Conversely, in a "hot" tumor characterized by abundant infiltration of active immune cells associated with high response rates to immunotherapy (81), OVs can reshape such an environment effectively (Figure 2).

Secondly, numerous preclinical studies have demonstrated various enhancement effects achieved through combining CAR-T cells with OVs. For instance (1): Enhanced transport and persistence of CAR-T cells: Scientists infected DS CAR-T cells with VSV and reovirus as delivery vehicles to target tumors; these OVs replicated within tumor cells leading to expansion of DS CAR-T cell population while causing rupture of tumor cells. Moreover, systemic stimulation by reovirus reactivated virus-specific CAR-T cells resulting in long-term remission lasting over 60 days in six out of seven mice tested; this approach also increased *in vivo* persistence of CAR-T-cells significantly (106) (2). As previously mentioned, OVs armed with multiple cytokines or chemokines have been engineered to enhance the therapeutic efficacy of CAR-T cell therapy. These include TNFα (107), IL-21 (108), IL-7 (109), CXCL11 (110), among others. Genetically modified OVs can produce a broader range of chemokines that promote the infiltration of cytotoxic T cells, DCs, macrophages, and other immune cells into the TME for improved anti-tumor effects (111). Wang et al. evaluated the use of CXCL11-armed OAds to augment CAR-T cell infiltration in GL261 GBM models and reprogram the immunosuppressive TME. This approach resulted in increased infiltration of $CD8^+$ T lymphocytes, NK cells, and M1-polarized macrophages while reducing myeloid suppressor cells (MDSCs), Tregs, and M2-polarized macrophages. The study demonstrated that combining CXCL11 with oAd within the tumor environment led to a sustained anti-tumor response (38) (3). OV-mediated targeted delivery of surface antigens in tumor cells (112). Anthony K Park and colleagues developed an oncolytic VV that expresses a non-signal-intercepting CD19 protein (CD19t), enabling targeted delivery of CD19t to the surface of solid tumor cells. This viral infection induces antigen-specific CD19-CAR-T cell-mediated antitumor activity, leading to both viral release from dying tumor cells and expansion of CD19t expression in the tumor (113). Additionally, an oHSV (oHSV T3011) was engineered to deliver truncated CD19 and BCMA double antigens in combination with either CD19-specific CAR-T ($CAR\text{-}T^{CD19}$) or BCMA-specific CAR-T ($CAR\text{-}T^{BCMA}$) cell therapy, resulting in a synergistic antitumor response. oHSV T3011 is a recombinant herpes OV expressing IL-12 and anti-PD-1 antibodies, which helps improve the inhibitory TME and enhances overall anti-tumor activity (114).

It is worth noting that there exist antagonistic mechanisms in the combination therapy of OV and CAR-T. The VSV-IFNβ induces the release of type I interferon, which subsequently up-regulates inhibitory receptors LAG3, PD-1, and TIM3. This effect is particularly pronounced in transduced cells and correlates with the expression level of CAR. Therefore, when used in conjunction with CAR-T therapy, IFNβ may impede the antitumor activity of CAR-T cells by stimulating the CAR signaling pathway to enhance CAR

expression and modulating inhibitory receptor expression to restrict the active state of CAR-T cells (115).

3.3.2 OV combined with CAR-NK

NK cells, an integral part of the innate immune system, possess a distinct cytotoxic mechanism compared to adaptive T lymphocytes and can directly eliminate target cells without prior antigen sensitization (116). NK cells express a diverse array of activating and inhibitory receptors that regulate their activity. Activating receptors include NCR, CD16, NKG2D, DNAM1, and signaling lymphocyte activation molecule (SLAM), while inhibitory receptors comprise immunoglobulin-like receptors (KIRs), NKG2A, and leukocyte immunoglobulin-like receptors (LILRs) (117, 118). These receptor families play a crucial role in modulating the immune response of NK cells towards tumors. The emergence of CAR technology has demonstrated significant potential in cancer immunotherapy by enhancing the recognition and elimination capabilities of immune cells (102, 119). Currently, there are five CAR-T cell therapies approved by the U.S. FDA for treating B-cell-derived lymphoma, leukemia, as well as hematological malignancies such as multiple myeloma (120). However, due to the limitations of CAR-T cells in the treatment of solid tumors, such as their inability to infiltrate tumor tissue, lack of suitable targets, and associated toxicity (102), it is imperative to discover novel strategies and technical approaches to overcome these challenges and enhance the efficacy and feasibility of CAR-T cell therapy for solid tumors. CAR-NK cell therapy may serve as a promising alternative. In contrast to CAR-T cell therapy, NK cells can be derived from various sources including peripheral blood, umbilical cord blood, induced pluripotent stem cells, and NK cell lines (121). Therefore, CAR-NK cells can be produced on a large scale with significantly reduced treatment time. Moreover, unlike CAR-T cells, CAR-NK cells are not restricted by histocompatibility complex (MHC) on the surface of target cells and exhibit a broader spectrum of anti-tumor effects. A Phase 1 and 2 clinical trial (NCT03056339) involving the injection of CD19 CAR-NK cells into 11 patients with relapsed or refractory CD19-positive cancers demonstrated that this therapy was effective in most patients without any apparent secretion of inflammatory cytokines such as IL-1 or IL-6. Furthermore, no association was observed between this therapy and the development of cytokine release syndrome, neurotoxicity or graft-versus-host disease (122).

Based on the remarkable efficacy of combination therapy using CAR-T cells and OVs, researchers have proposed combining OVs with CAR-NK cells. As tumor cells infected with OVs dissolve and rupture, they express ligands related to cell stress such as MICA/B proteins and ULBP family proteins, thereby increasing the recognition targets for CAR-NK cells. This leads to more effective removal of residual tumor cells and a more comprehensive clearance effect (119). Xilin Chen et al. found that EGFR was highly expressed on the surface of breast cancer cells, and using EGFR-CAR-NK-92 cells alone or in combination with oHSV-1 resulted in significant killing of cancer cells. The combination produced a more effective killing effect than monotherapy and significantly extended survival time in tumor-bearing mice, making it an effective treatment for breast cancer brain metastases (123). Recently, multiple GBM cell lines infected with herpes simplex type I virus (OV-IL15C) expressing human IL-15/IL-15Rα sushi domain fusion protein secreted soluble IL-15/IL-15Ra complex to improve the survival rate of NK and $CD8^+$ T cells. When combined with EGFR-CAR-NK cells, this increased persistence of CAR-NK cell activity synergistically suppressed tumor growth and significantly improved survival rates (31).

3.3.3 OV combined with TILs

TILs typically comprise clusters of T cells and B cells (124, 125), and the type and persistence of these immune cells within the tumor are associated with the prognosis of cancer patients (124). The findings of studies have demonstrated that treatment of tumors with OVs had a favorable impact on both TIL infiltration and activity, thereby influencing tumor progression. The combination of OV with TIL may yield enduring antitumor effects by enhancing TIL activity. For instance, modified OVs based on OX40L and IL-12 represent a promising therapeutic approach for solid tumors. By infecting tumor cells, this particular OV can provide the necessary signals for activating T cells while transforming tumor cells into artificial antigen-presenting cells (126). Consequently, it not only induces T cell activation but also stimulates their cytotoxic function. Notably, significant tumor regression as well as long-term immune memory effects were observed when combined with TIL in tumor models (126). This suggests that this approach holds potential for persistent and effective control over solid tumor growth and metastasis. With a further comprehensive understanding of the relevant experiments, we can gain a deeper understanding of how OX40L and IL-12 based modified OVs mechanistically inhibit solid tumor growth while optimizing curative rates. Ultimately, this prospective strategy offers new hope in cancer management field as it could become an integral component of future personalized cancer management strategies (126). Another study demonstrated that the combination of OAd encoding human IL-2 (hIL2) and TNFα, along with TILs, exhibited prolonged efficacy, increased the frequency of both CD4 and CD8 TILs *in vivo*, and augmented splenocyte proliferation ex vivo, suggesting that the cytokines were important for T cell persistence and proliferation, significantly enhancing the effectiveness of TIL therapy (127). TNFα and IL-2 are incorporated into OAds to selectively infect cancer cells through tumor-specific promoters and knob protein exchange, thereby enhancing cancer cell entry (128). Moreover, utilizing TILs as carriers to deliver the virus to tumors can augment both the concentration and efficacy of the virus within the tumor site (128).

At the same time, OV can exert a stronger anti-tumor effect by increasing TIL infiltration and enhancing TIL function.

3.3.3.1 Increased TIL infiltration

Engineered OVs have the potential to enhance the infiltration of TILs during disease treatment. Genetically modified herpetic virus type 1 (HSV-1) G207 has been utilized in pediatric patients with high-grade glioma for therapeutic purposes. By inducing an immune response and attracting cells through G207 infection, it is possible to convert “cold” tumors into “hot” tumors, thereby

increasing the quantity of TILs and improving treatment efficacy (129) (Figure 2). In the context of GBM treatment using G47Δ, a third-generation oncolytic HSV-1 with triple mutations, a significant augmentation in $CD4^+$ and $CD8^+$ lymphocyte populations was observed as they rapidly infiltrated into tumor tissue. The sustained increase in these lymphocytes not only persisted over time but also exhibited a strong correlation with enhanced treatment outcomes (130). By genetically modifying the tumor-soluble bovine pox virus to express IL-7 and IL-12, it is possible to enhance the sensitivity of anti-PD-1 and CTLA4 antibody therapy. This modification also leads to an increase in the expression of major histocompatibility complex II (MHC II) in antigen-presenting cells, thereby altering the immune status and systemic immune response within the TME. Consequently, there is an augmented infiltration of $CD8^+$ T cells, $CD4^+$ T cells, NKT cells, and NK cells into the tumor site (131). The introduction of adenovirus-mediated n-terminal gasdermin domain expression induces pyroptosis in tumor cells while recruiting TILs into the brain. This process enhances their infiltration and subsequently improves anti-tumor efficacy (132). Delta-24-RGD OAd directly lyses tumor cells and activates anti-tumor immune responses, promoting invasion by T cells (133). OBP-502 is a telomerase-specific OAd that releases immunogenic cell death molecules such as adenosine triphosphate (ATP) and high mobility group box 1 protein (HMGB1) upon treatment. This release recruits $CD8^+$ lymphocytes while inhibiting Foxp3 positive lymphocyte infiltration into tumors, resulting in antitumor effects (134). OVs modified with glycosylation -PEGX can improve selective infection and killing ability against tumor cells. Additionally, they enhance infiltration of T cells and NK cells, thus enhancing anti-tumor immune responses (135). Treatment with oncolytic HSV-1 results in regression of lymphoma-guided tumors accompanied by significant invasion of antigen-specific $CD8^+$ T cells (136). In addition, MSC-mediated delivery of OAds to osteosarcoma leads to increased infiltration of TILs (137).

3.3.3.2 Enhancement of TIL function

The use of OV or modified OV treatment for corresponding diseases may facilitate the augmentation of TIL activation, metabolic capacity, and durable anti-tumor response. Researchers have genetically engineered the OV to incorporate humanized PD-1 single-chain antibodies (hPD-1scFv) in order to enhance its impact on TILs (20). Modified OV therapy has demonstrated an enhanced anti-tumor effect on $CD8^+$ T cells, leading to increased infiltration of effector $CD8^+$ T cells into tumors and establishment of memory $CD8^+$ T cells, while concurrently reducing associated depletion of $CD8^+$ T cells (20). The expression of leptin by engineered OVs within tumor cells can promote metabolic reprogramming of TILs, thereby enhancing their metabolic activity and facilitating disease treatment (138). Recently, it has been reported that oHSV can reshape the immune microenvironment in pancreatic ductal adenocarcinoma (PDAC) by augmenting immune activity. Through utilization of scRNA-seq and multicolor fluorescence activated cell sorting analysis techniques, researchers observed a significant reduction in tumor-associated macrophages (TAMs), particularly anti-inflammatory macrophages, following oHSV treatment. Additionally, there was an increase in the proportion of TILs including activated cytotoxic $CD8^+$ T cells and Th1 cells (139). Tumor cells infected with CCL5-modified OVs were able to produce CCL5 without compromising infectivity, thereby promoting NK cell accumulation and augmenting the therapeutic efficacy (140). Vv-scfv-tigitt, an engineered OV carrying ICIs, has been demonstrated to induce T cell infiltration and enhance $CD8^+$ T cell activation in tumor models, leading to the establishment of long-term immunity (141). The CD40L-armed oncolytic HSV enhances the cytotoxicity of T cells and promotes the activation of DCs and T cells in the TME by inducing the expression of TAAs and enhancing the immunogenicity of tumor cells. This approach shows potential as a therapeutic strategy for PDAC (142). The OX40L-armed OV (OV-mOX40L) reduces the number of $Foxp3^+$ Tregs and activates $CD4^+$ and $CD8^+$ T cells through interaction with OX40L. Additionally, it decreases exhausted CTLs while promoting t cell activation, leading to increased release of inflammatory cytokines such as IFNγ. Consequently, this transforms the immunosuppressive TME into a more immunologically active state (143). Combined treatment with an OV and anti-PD-1 significantly increases levels of $CD8^+$ and $CD4^+$ T cells, activates the central immune system, and enhances therapeutic efficacy (144). Adenoviruses have potential as an immunotherapy tool for stimulating TIL activity by delivering TNFα and IL-2. The results suggest that adenovirus can reshape cytokine responses and activate TILs in the TME, thereby improving their antitumor reactivity (145).

3.3.4 MSCs are used as vectors to transport OVs

The utilization of OVs for disease treatment may elicit an immune response, thereby impeding viral spread and infection, consequently diminishing treatment efficacy. Moreover, due to the absence of specific targeting in virus administration, non-target tissues may be susceptible to infection, resulting in adverse reactions and toxicity. Simultaneously, pre-existing immune tolerance can hinder inter-tumoral migration of the virus, posing a challenge for treating metastatic diseases as both injected and distant tumors need to be targeted (146). To overcome these limitations associated with OV administration, researchers are actively engaged in a series of exploratory investigations.

MSCs possess low immunogenicity, inherent tumor tropism, multi-lineage differentiation potential, excellent migratory capacity (147), homing ability, and other therapeutic properties. These innate characteristics make them ideal candidates for drug delivery and OV vectors (148, 149). Utilizing MSCs as carriers of OVs for tumor therapy can enhance viral delivery efficiency, augment the antitumor effect of viruses on cancer cells, enable precise drug targeting, and mitigate systemic side effects (150).

The researchers improved the targeting ability of MSCs and modulated the drug release time to enhance the efficacy of OAds, enabling them to function as a factory and vector for OAds. They also evaluated tumor bioavailability after MSC injection. This approach significantly increased viral production, tumor targeting, timely viral release at the tumor site, and the antitumor

efficacy of the oncolytic adenovirus. These findings indicate that engineered MSCs can substantially boost the antitumor effects of oncolytic viruses without compromising safety, potentially expanding the clinical applicability of oncolytic adenoviruses (151). In a mouse model of pulmonary melanoma, MSCs were utilized to deliver an IL-15-carrying tumor-lytic MYXV construct, resulting in sustained viral presence and increased infiltration of NK cells and $CD8^+$ T cells. This approach transformed the tumor into a "hot tumor" and induced significant regression (152) (Figure 2). Another study encapsulated CF33 within NSCs to enhance its delivery in a cisplatin-resistant peritoneal ovarian metastasis model, providing a more efficient alternative compared to conventional delivery methods (153). The MYXV, carrying the LIGHT (TNFSF14) gene, was pre-loaded into adipose-derived mesenchymal stem cells (ADSCs) and utilized for the treatment of pancreatic cancer in mice. The findings demonstrated that when combined with carrier cells, the virus could be efficiently delivered to pancreatic cancer lesions, enabling cell survival while effectively eliminating pancreatic cancer cells. This resulted in tumor regression and prolonged survival time in treated mice (154). Furthermore, compared to traditional OV treatment for colorectal cancer, combination therapy employing MSCs as carriers and prodrug activation exhibited superior therapeutic efficacy and safety. It also possessed tumor specificity and innovative advantages through prodrug activation (155). Therefore, utilizing MSCs as carriers for transporting OVs presents a novel approach to tumor virotherapy with promising application prospects.

3.4 OV combined with ICIs

ICIs are a form of immunotherapy that has garnered significant attention in recent years for their potential in tumor treatment by targeting and inhibiting immune checkpoints, such as CTLA-4 and PD-1, to activate the immune response (156). However, studies have indicated that ICI may not be suitable for all patients, with some experiencing severe adverse reactions during treatment (157). Only a minority of patients achieve favorable disease control following ICI therapy. Furthermore, ICI showed no efficacy against immunologically "cold" tumors, characterized by low levels of TILs (158). Consequently, numerous researchers are actively exploring substances capable of inducing the conversion of "cold" tumors into "hot" tumors when used alongside ICI therapy to combat the disease.

OVs have been demonstrated in numerous studies to elicit anti-tumor immune responses, augment the efficacy of existing cancer therapies, and modulate unresponsive TME, thereby converting "cold" tumors into "hot" tumors and enhancing their sensitivity to checkpoint blockade immunotherapy (159) (Figure 2). Consequently, OVs serve as an ideal adjunct to ICIs. Sachin R Jhawar et al. investigated the effectiveness of this combination therapy using *in vitro* mouse models, human cancer cell lines, and murine skin cancer models. Following initial treatment with OV and radiotherapy, ICIs were subsequently administered to establish a triple therapy comprising OV, radiotherapy, and ICI. The results revealed that this triple therapy effectively suppressed tumor growth and prolonged survival. In addition, the researchers reported that a PD-1 refractory patient with squamous cell carcinoma of the skin received a longer period of disease control and survival after triple therapy with OV, radiotherapy, and ICI, and the tumor did not show significant progression for 44 months. The mechanism of the above results is that OV combined with radiotherapy and ICI, can not only transform immunologically "cold" tumors into "hot" tumors, but also improve the infiltration of CD8+ T cells (160). ONCOS-102 is a highly engineered Ad vector that has undergone extensive preclinical investigations in recent years (161) and has advanced to Phase I clinical trial stage (NCT03003676) which used in combination with the ICI pembrolizumab. The Phase I trial, which enrolled 12 patients with advanced or unresectable solid tumors, demonstrated that ONCOS-102 exhibited no dose-limiting toxicity and reached the maximum tolerated dose at the tested level, as compared to the pre-treatment dosage. Analysis of tumor biopsies following combination therapy revealed a significant increase in infiltration of $CD3^+$ T cells (5.9-fold) and $CD8^+$ T cells (4.0-fold). Among the 10 patients evaluated by PET/CT scans at 3 months, disease control was observed in 4 patients (40%), with a median overall survival of 9.3 months (162).

In addition to demonstrating efficacy, numerous studies have substantiated the safety of combining OVs with ICIs. In a study conducted by Targovax ASA et al., where ONCOS-102 was combined with pembrolizumab for treating PD-1-resistant advanced melanoma patients, treatment tolerance was well-established. Out of the 20 patients involved, objective response was achieved in seven cases along with regression of lesions at non-injection sites - indicating systemic antitumor effects resulting from local administration of ONCOS-102. Sequential biopsies performed on injected tumors showed substantial infiltration of $CD8^+$ T cells and $CD4^+$ T cells post-administration of ONCOS-102 injections. Therefore, these findings suggest that further investigation into the combination therapy involving ONCOS-102 and PD-1 inhibitors holds promise for PD-1-resistant melanoma treatment (163). Professor Gelareh Zadeh's research team from the University of Toronto in Canada published their phase I/II clinical study results in Nature Medicine, showing that combining OV therapy DNX-2401 with pabolizumab for recurrent GBM treatment resulted in a 52.7% one-year survival rate, and some patients even survived after 60 months of treatment. Two patients achieved complete response (CR) and three patients achieved partial response (PR). With an ORR of 10.4%(90% CI:4.2-20.7%) in the intention-to-treat population and 11.9% in patients with the maximum trial dose (declared dose), this combination regimen is expected to become a novel treatment option for recurrent GBM (164). In addition, Hemminki's team recently reported on two OVs expressing TNFα and IL-2, respectively. In melanoma experiments conducted on mice, they found that when combined with anti-PD-1 antibodies, the virus significantly increased $CD8^+$ T cell numbers compared to using only the virus alone; furthermore, combining OV with ICIs significantly inhibited tumor development and prolonged survival time compared to using only the virus alone or ICIs alone. Interestingly, combining NDV with anti-CTLA-4 antibody also showed synergistic effects in mouse tumor models by

increasing $CD8^+$ T cell infiltration while inhibiting tumor growth and prolonging survival time (165). T-VEC is a genetically engineered oHSV-1 (166). In a single-center, single-arm, Phase II study, 24 resectable patients with stage IIIB-IVM1a melanoma who received intrafocal T-VEC injection and systemic nebuliuzumab had a major pathological complete response rate of up to 45%. The main mechanism is that the combination of T-VEC and ICI changes the infiltration of immune cells, transforming "cold" tumors into "hot" tumors, thus enhancing the immune response (167). In an interim report on another clinical trial that has begun studying C-REV in combination with the PD-1 inhibitor nivolumab (NCT03259425) in patients with resectable stage IIIB, IIIC, or IVM1a melanoma, Patients treated with the combination of C-REV and nivolumab showed higher T cell infiltration than patients treated alone in previous clinical trials (168).

In summary, the combined application of OV and ICIs has yielded remarkable results by enhancing lymphocyte infiltration and effectively prolonging survival. These findings strongly support the notion that OVs serve as ideal adjuvant therapies for ICIs.

3.5 OV Combined with ultrasound-targeted therapy

Ultrasound-targeted therapy is a method that uses the physical properties of ultrasound to precisely locate and treat tumors. Its main principle involves the cavitation and thermal effects of ultrasound to disrupt tumor tissue while using acoustic radiation force to enhance microbubble-mediated ultrasound-targeted drug delivery systems. This improves the concentration of drugs at the tumor site and enhances therapeutic efficacy. Additionally, ultrasound can temporarily increase the permeability of tumor vasculature, promoting the penetration of drugs or gene carriers, thereby further enhancing treatment efficiency (169). Due to its non-invasive nature, precise targeting, and low side effects, ultrasound-targeted therapy has shown great potential in treating various solid tumors (170–172).

The combination of ultrasound-targeted therapy with OVs opens new avenues for cancer treatment. OVs can selectively infect and kill tumor cells, while ultrasound-targeted technology can enhance the infection efficiency and distribution precision of OVs (173). For instance, Bazan-Peregrino et al. studied how ultrasound-induced cavitation improves the extravasation and distribution of a potent breast cancer-selective oncolytic adenovirus, AdEHE2F-Luc, to tumor areas distant from blood vessels. Inertial cavitation was found to be more effective than stable cavitation in enhancing the delivery, distribution, and efficacy of the oncolytic virus (174). Moreover, using microbubble carriers to load OVs and employing ultrasound-guided targeted delivery ensures efficient release and infection of OVs at the tumor site. Greco et al. demonstrated that ultrasound-targeted microbubbles/Ad.mda-7 (a replication-incompetent adenovirus expressing melanoma differentiation–associated gene-7/interleukin-24) significantly reduced tumor burden in xenografted nude mice. The microbubbles burst under ultrasound, releasing OVs directly into tumor cells and enhancing the oncolytic effect (175). Additionally, the mechanical action of ultrasound can increase the permeability of tumor cell membranes, enhancing OV entry and broader intratumoral spread. For example, Okunaga et al. found that ultrasound increased the efficiency of HSV-1 infection in human squamous cell carcinoma cells and tumors in nude mice, potentially enhancing the antitumor effect of oncolytic HSV-1 in head and neck cancer treatment (176).

This combined therapy strategy not only improves the targeting and therapeutic efficacy of OVs but also reduces their distribution in normal tissues, thereby minimizing adverse effects. Various targeting ligands incorporated into acoustically active materials, such as nanoparticles (170, 177), polymeric micelles, and liposomes (178), contribute to this effect. Therefore, the future application of ultrasound-targeted technology combined with OVs promises to become an efficient, precise, and comprehensive cancer treatment strategy, offering new hope for cancer patients.

4 Clinical trials

In recent years, OV genetic engineering therapy has demonstrated significant potential in the field of tumor treatment. Researchers are utilizing genetically modified viruses, such as MV and HSV, to develop precise methods for selectively eliminating tumor cells while preserving normal cells. We present a comprehensive overview of major clinical trials involving engineered OVs to explore their potential applications in oncology therapy (Table 1). For instance, an embryonic MV (MV-CEA) expressing recombinant carcinoembryonic antigen (CEA) and an oncolytic MV (MV-NIS) encoding a thyroid sodium-iodine cotransporter were employed in a clinical trial for ovarian and peritoneal carcinoma (NCT00408590). These studies aimed to assess the safety and optimal dosage of engineered viral therapy for progressive, recurrent, or refractory tumors. Another clinical trial focused on recurrent brain cancer (NCT00028158), where engineered herpesvirus G207 was directly injected into the brain and administered bedside after surgical removal to evaluate its safety, therapeutic efficacy, and novel treatment possibilities for patients with brain cancer. Additionally, recent clinical research has primarily focused on evaluating the safety and efficacy of the engineered oncolytic virus injection R130 (a modified HSV-1 containing the gene coding for anti-CD3 scFv/CD86/PD1/HSV2-US11) in patients with recurrent/refractory cervical and endometrial cancers (NCT05812677). In summary, these clinical trials underscore the potential of engineered OVs as a promising strategy in oncology, highlighting their safety, efficacy, and innovative therapeutic applications.

At the same time, the clinical research on the combination of OVs with other drugs for tumor treatment has demonstrated a robust trend. These studies have investigated the feasibility and safety of combining OVs with immunotherapy drugs, ICIs, etc., aiming to enhance the efficacy of tumor treatment and potentially overcome resistance to conventional and immunotherapies (Table 2). For instance, a study (NCT02977156) aimed to assess the feasibility and safety of combining anti-CTLA-4 therapy with intratumoral injection of Pexa-Vec, an OV. This combination

TABLE 1 Major clinical trials of genetically engineered OVs.

Start time	Engineered OVs	Enhancements and modifications in genetically engineered OVs	Cancer type	Purpose of the study	Phase	Status	Clinical trial number
2004	MV-NIS	oncolytic MV encoding thyroidal sodium iodide symporter	Ovarian cancer, primary peritoneal cavity cancer	Side effects and optimal dosage	I	Completed	NCT00408590
2001	G207	G207 has been modified from the herpes virus that causes cold sores (called herpes simplex virus type 1 or HSV-1)	Astrocytoma, glioblastoma	Safety and efficacy assessments	Ib/II	Completed	NCT00028158
2017	rQNestin34.5v.2	rQNestin34.5v.2 is a genetically engineered HSV-1 virus	Brain cancer (cancernaplastic oligodendroglioma of brain), astrocytoma	Safety assessment and determination of appropriate dose	I	Completed	NCT03152318
2013	MV-NIS	oncolytic MV encoding thyroidal sodium iodide symporter.	Head and neck squamous cell carcinoma, breast cancer stage IV	Side effects and optimal dosage	I	Completed	NCT01846091
2017	MV-NIS	oncolytic MV encoding thyroidal sodium iodide symporter.	Metastatic malignant peripheral nerve sheath tumor, recurrent malignant peripheral nerve sheath tumor	Side effects and optimal dosage	I	Recruiting	NCT02700230

MV-NIS, oncolytic measles virus encoding thyroidal sodium iodide.

sought to improve antitumor effects by inducing virus-mediated tumor cell death and release of tumor antigens, as well as recruiting/maturing/activating antigen-presenting cells through GM-CSF induction while blocking/depleting Tregs via anti-CTLA-4. Furthermore, recent clinical trials have been initiated to explore the potential of OV combination therapies. The NCT06196671 trial aims to evaluate the efficacy of an oncolytic virus combined with a PD-1 inhibitor in patients with advanced pancreatic cancer. Additionally, the NCT06346808 trial is designed to explore the safety and efficacy of combining an oncolytic virus with a PD-1 inhibitor and chemotherapy as preoperative therapy for patients with borderline resectable and locally advanced pancreatic cancer.

TABLE 2 Major clinical trials of OV combination therapy.

Start time	OVs	Combination drugs	Cancer type	Purpose of the study	Phase	Status	Clinical trial number
2017	Pexa-Vec	IT ipilimumab (anti-CTLA4 Ab)	Metastatic tumor, advanced tumor	Feasibility, safety and anti-tumor effects after combination therapy	I	Completed	NCT02977156
2021	OVV-01	pembrolizumab (anti-PD-1 monoclonal antibody) or atezolizumab	Neoplasms	Evaluation of safety, tolerability, and efficacy after combination therapy	I	Recruiting	NCT04787003
2019	OH2	HX008 (an anti-PD-1 antibody)	Gastrointestinal cancer, solid tumor	Evaluation of safety and efficacy after combination therapy	I/II	Recruiting	NCT03866525
2021	RT-01	Nivolumab (ICIs)	Advanced solid tumor	Evaluation of safety, tolerability and preliminary efficacy after combination therapy	I	Current recruitment status is unknown	NCT05122572
2012	CGTG-102	low-dose oral cyclophosphamide	Malignant solid tumor	Safety and recommended dose after combination therapy	I	Completed	NCT01598129
2013	DNX 2401	TMZ	Recurrent tumor, glioblastoma multiforme	Evaluation of safety, tolerability, and toxicity after combination therapy	I	Completed	NCT01956734

(Continued)

TABLE 2 Continued

Start time	OVs	Combination drugs	Cancer type	Purpose of the study	Phase	Status	Clinical trial number
2017	Pexa-Vec (JX-594)	Tremelimumab	Colorectal neoplasms, colorectal cancer, refractory cancer	Evaluation of safety, tolerability and feasibility after combination therapy	I/II	Completed	NCT03206073
2022	H101	Camrelizumab (PD-1 inhibitors)	Bladder cancer	Safety and efficacy assessment after combination therapy	II	Recruiting	NCT05564897
2020	CAdVEC	HER2 specific CAR-T cells	Advanced HER2 positive solid tumors	Safety and efficacy assessment after receiving specific T cells after intratumoral CAdVEC injection	I	Recruiting	NCT03740256
2023	H101	PD-1 inhibitors	Advanced malignant pleural mesothelioma	The efficacy and safety of patients with malignant pleural mesothelioma resistant to advanced PD-1 inhibitors after combination therapy	Observational	Recruiting	NCT06031636
2012	GL-ONC 1	CDDP (radiation therapy and cisplatin)	Cancer of head and neck	Safety and tolerability after combination therapy	I	Completed	NCT01584284
2024	TILT-123	Pembrolizumab	Locally advanced, unresectable, refractory and/or metastatic solid tumors	Safety, tolerability, and preliminary antitumor efficacy after combination therapy	I/II	Recruiting	NCT06265025
2020	LOAd 703	Atezolizumab	Malignant melanoma	Evaluation of safety and efficacy after combination therapy	I/II	Completed	NCT04123470
	HF10	Ipilimumab	Malignant melanoma	Whether combination therapy is effective in patients with stage IIIB, IIIC, or stage IV unresectable or metastatic melanoma	II	Completed	NCT02272855
2006	MV-NIS	Cyclophosphamide	Recurrent plasma cell myeloma, refractory plasma cell myeloma	Side effects and optimal dose after combination therapy	I/II	Completed	NCT00450814
2017	NIS	Cyclophosphamide, Ipilimumab and nivolumab or cemiplimab	Multiple myeloma, acute myeloid leukemia and T-cell lymphoma	Optimal dose and side effects after combination therapy	I	Recruiting	NCT03017820

MV-NIS, oncolytic measles virus encoding thyroidal sodium iodide symporter; NIS, VSV-hIFNbeta-sodium iodide symporter; Pexa-Vec, pexastimogene devacirepvec; TMZ, Temozolomide; HER2, human epidermal growth factor receptor.

In summary, these clinical trials underscore the promising potential of OV combination therapies in enhancing tumor treatment efficacy and overcoming therapeutic resistance, particularly through the integration of ICI or chemotherapy strategies.

Taken collectively, these clinical studies unveil the potential of OV genetic engineering therapy in the treatment of tumors. By precisely targeting tumor cells and minimizing impact on normal tissue, these studies offer novel avenues for future cancer treatments and instill hope in patients. However, further validation through additional studies is required to advance the safety and efficacy of these treatments in clinical practice and thus benefit a larger population of cancer patients. Simultaneously, these studies furnish valuable data for combining OVs with other drugs to treat tumors, underscoring the potential of this treatment strategy to enhance therapeutic efficacy and overcome drug resistance. Nonetheless, further research and clinical trials are necessary to validate these preliminary findings and determine the optimal course of treatment.

5 Conclusion and discussion

OV therapy is an innovative approach for cancer treatment, utilizing viruses to infect tumor cells and induce their death in order to inhibit tumor growth. OVs gene engineering therapy has gained significant attention and research as a potential strategy for treating tumors. This paper provides a comprehensive review and analysis of the engineering modifications, combination therapies, and clinical research involving OVs, aiming to explore its prospects and challenges in tumor therapy.

We have observed that in addition to the aforementioned four strategies, engineering OVs also possess various approaches for enhancing the therapeutic efficacy against tumors. For instance, by utilizing specific functional proteins or enzymes, it is possible to augment the antitumor effect. This finding holds promising implications for the potential utilization of engineered OVs in cancer immunotherapy. However, it is important to note that extensive theoretical research support as well as rigorous animal

experiments and clinical trials are still required to further develop and validate this approach.

At the same time, in combination therapy, the combination of OVs with chemotherapy does not consistently yield positive results and may have a detrimental impact on tumor immunoviral therapy. Furthermore, there is limited research on OV combined with chemoradiotherapy; however, existing studies demonstrate its significant potential. This suggests that we can potentially mitigate the side effects of chemoradiotherapy through engineering modifications of OVs and achieve enhanced synergistic effects. Additionally, we are concerned about potential antagonistic mechanisms between OVs and CAR-T therapy based on preclinical studies. Consequently, further investigation into their interaction is warranted in order to optimize this combination therapy regimen. Furthermore, TILs play a pivotal role in this context as well. OV therapy not only directly eliminates tumor cells when combined with TILs but also activates TILs and enhances their immune response against tumors. This enhanced immune response contributes to improvements in the TME by increasing T cell infiltration and activity, ultimately bolstering the immune system's ability to combat tumors. In the realm of ultrasound-targeted therapy, while microbubble inertial cavitation can significantly enhance the delivery efficiency of drugs or gene carriers, it also presents some inevitable collateral damage, such as microvascular leakage, capillary damage, and erythrocyte extravasation leading to local edema and inflammation. Therefore, before ultrasound-mediated OV delivery can progress to clinical trials, further research is necessary to optimize this technology and minimize its side effects. Despite being in the early stages with limited studies, ultrasound-mediated MB delivery combined with OVs has shown considerable potential, not only for OVs but also for other viral therapies, significantly enhancing therapeutic outcomes and overcoming known barriers.

In summary, OV therapy represents a promising and innovative approach for treating tumors. Through ongoing refinement of engineering strategies, exploration of combination therapies, and clinical studies, we can further enhance the safety, efficacy, and targeting capabilities of OVs to improve treatment outcomes and quality of life for cancer patients. Future clinical applications of OV combination therapies hold significant promise. The potential for synergistic effects, particularly with chemoradiotherapy, offers new avenues for overcoming resistance and achieving more durable responses. However, challenges such as understanding the complex interactions between OVs and immune cells, as well as managing potential antagonistic effects with CAR-T cells, require meticulous research. Prospective studies must focus on optimizing dosing regimens, sequencing of therapies, and patient selection criteria to maximize benefits while minimizing risks. Moreover, the integration of advanced genetic engineering techniques could enhance OV specificity and reduce off-target effects, paving the way for personalized cancer therapies. Despite these advancements, potential obstacles in the clinical environment include regulatory hurdles, high development costs, and the need for large-scale manufacturing capabilities. Addressing these challenges will be critical for the successful translation of OV therapies from bench to bedside. The development of standardized protocols and robust clinical trials will be essential to establish the therapeutic efficacy and safety profile of these innovative treatments. Through continued interdisciplinary collaboration and technological advancements, the future of OV combination therapies appears promising, with the potential to significantly improve cancer treatment.

Author contributions

ZY: Writing – review & editing, Writing – original draft, Visualization, Investigation, Conceptualization. YPZ: Writing – review & editing, Writing – original draft, Visualization, Investigation, Conceptualization. XW: Writing – review & editing, Writing – original draft, Investigation. XYW: Writing – review & editing, Writing – original draft, Investigation. SR: Writing – review & editing, Writing – original draft, Investigation. XH: Writing – review & editing, Writing – original draft, Investigation. JHS: Writing – review & editing, Visualization. AZ: Writing – review & editing. SG: Writing – review & editing. YC: Writing – review & editing. SD: Writing – review & editing. XuW: Writing – review & editing. ML: Writing – review & editing. FD: Writing – review & editing. YSZ: Writing – review & editing. JS: Writing – review & editing. ZW: Supervision, Conceptualization, Writing – review & editing. ZX: Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Progression of oncolytic virus in liver cancer treatment

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The liver plays a crucrial role in detoxification, metabolism, and nutrient storage. Because liver cancer ranks among the top three leading causes of death globally, there is an urgent need for developing treatment strategies for liver cancer. Although traditional approaches such as radiation, chemotherapy, surgical removal, and transplantation are widely practiced, the number of patients with liver cancer continues to increase rapidly each year. Some novel therapeutics for liver cancer have been studied for many years. In the past decade, oncolytic therapy has emerged, in which viruses selectively infect and destroy cancer cells while sparing normal cells. However, oncolytic virotherapy for liver cancer remains relatively obscure due to the aggressive nature of the disease and the limited effectiveness of treatment. To keep pace with the latest developments in oncolytic tumor therapy for liver cancer, this review summarizes basic science studies and clinical trials conducted within 5 years, focusing on the efficacy and safety profiles of the five most commonly used oncolytic viruses: herpes simplex virus, adenovirus, influenza virus, vaccinia virus, and coxsackievirus.

KEYWORDS

HCC, oncolytic virus, influenza virus, herpes simplex virus, adenovirus

1 Introduction

As of 2020, liver cancer stands as the third leading cause of mortality worldwide, claiming the lives of 830,200 individuals annually (1). The number of liver cancer diagnoses and deaths is projected to increase by 55% from 2020 to 2040 (2). In the United States alone, liver cancer incurs an annual cost of $454.9 million, averaging $32,907 per patient. This includes the cost of healthcare and loss of productivity due to liver disease. Hepatocellular carcinoma (HCC) is one of the most common types of liver cancer, accounting for 90% of primary liver cancers (3). Without adequate treatment, patients infected with viruses that cause hepatitis can

progress into chronic liver disease, predisposing them to HCC. Other risk factors include alcohol abuse, obesity, fatty liver, and diabetes (4). Diagnosis of HCC follows the Barcelona Clinic Liver Center (BCLC) strategy, which guides treatment decisions at different disease stages (5). In the early stages (BCLC 0-A), treatment primarily includes surgical resection and ablation (6). For intermediate cases (BCLC B), conventional transarterial chemoembolization improved survival rate (7). As the disease progresses to advanced stages (BCLC C), patients manifest cancer-related symptoms, prompting the utilization of sorafenib, a tyrosine kinase inhibitor, approved by the Food and Drug Administration (8). However, in the terminal stage (BCLC D), therapeutic options become severely limited. Liver transplantation emerges as a potentially viable intervention, but its scientific efficacy remains unproven (9). Traditional liver cancer treatments, such as immunotherapies, and transarterial chemoembolization, have not shown great effectiveness due to the immune tolerance of the liver, and not all patients are eligible for these treatments (6). Thus, new therapeutic methods are urgently needed.

Cancer is the leading cause of death in every country in the world (10). Since 1921, when cancer cells first appeared, humans have sought treatments to improve survival rates. Since entering the 21st century, genetic engineering technology has made continuous progress and its application in medicine has been greatly developed, among which oncolytic virus therapy stands out among many cancer treatment methods (11). Oncolytic viruses (OVs) are genetically engineered viruses that specifically fight cancer cells. It can recognize and infect different cells in the tumor environment, replicate in tumor cells through different regulatory mechanisms, lyse tumor cells, and be released from tumor cells to further infect surrounding tumor cells; while in normal cells, oncolytic The virus is cleared by the body's immune system without affecting its normal growth (12). Since the discovery of using OVs to treat cancer cells, preclinical studies and clinical trials have employed OVs in HCC and have demonstrated some progress (13).

The effectiveness of OVT against HCC can vary due to several factors, such as changes in receptor expression, host immune response, TME, and genetic alterations (14). Commonly used virus vectors for HCC OVT include HSV, ADV type 5, IV, oncolytic VV, and COX-A, etc. This review summarizes preclinical studies from 2022 to 2024 and clinical trials from 2015 to 2024 to investigate the OVs in HCC treatment. Common administration routes include intravenous, intrasplenic, intratumoral, intraarterial, intrabiliary, etc (15).

2 Oncolytic viruses

2.1 Mechanisms for genetically engineered oncolytic viruses

Oncolytic viruses (OVs) have emerged as a promising approach in cancer therapy, leveraging the natural ability of viruses to selectively target and destroy tumor cells while leaving healthy ones unaffected. There are three primary mechanisms to genetically engineer OVs:

2.1.1 Type I interferon signaling pathway regulation

To achieve antitumor activity, one of the most common ways that OVs use is to downregulate the IFN signaling pathway, making tumor cells more susceptible to the OVs that will then replicate and kill the tumor cell through direct lysis (16, 17). This process is primarily driven by the susceptibility of oncolytic viruses (OVs) to interferon (IFN) and the decreased responsiveness of tumor cells to IFN. Preclinical studies using vesicular stomatitis virus for HCC showed that by IFN signal acts like a cytokine to direct the priming of virus and tumor-reactive T cells, which induces oncolysis and host immune response (18).

2.1.2 Tumor-specific promoters

Tumor or tissue-specific gene promoters are engineered into the OVs to selectively transcribe targeted gene sequences. This allows for rapid replication within tumor cells while limiting replication in normal cells (19). Conventionally, homologous recombination technique has been used. However, this method has been limited by its low efficacy and the complication of multiple steps involved (20). To solve this problem, several approaches have been used to insert tumor-specific promoters to OVs. The CRISPR-Cas9 system was introduced. By using a guide RNA to direct the Cas9 enzyme to a specific DNA site, it allows a donor DNA template containing the new promoter to be integrated via homology-directed repair. Yuan et al. showed that CRISPER-Cas9 system induces higher efficiency of homologous recombination by 3% when introducing DsRed into oADV (21). Additionally, Terada et al. used a bacterial artificial chromosome (BAC) -based model, in which the backbone of BAC can effectively exchange with the promoter of interest through sequential, site-specific recombination, to express luciferase protein by inserting various viral promoters on oHSV (22). Gateway recombination cloning was effectively used insert-expression vector, M134, GOI, and M136 with eGFP as fluorescent marker, into myxoma oncolytic virus (23). Moreover, to identify the site of insertion, transposon insertion strategy has been largely used to scan the genome nonprejuidicely. Kretschemer et al. used Tn7 transposon to find several sites for promoter-based expression insertions in the oADV genome, and those approaches have been proved to perform easily and effectively (24).

2.1.3 Gene silencing

Certain viral genes necessary for replication in normal cells, but not required by tumor cells, are deleted. This allows viruses to replicate rapidly within tumor cells with attenuated replicability in normal cells (25). Double-stranded interfering RNAs (RNAi) can guide Argonaute proteins to target tumor cell RNAs via Watson-Crick base-pairing to achieve gene silencing within the tumor.

Importantly, OVs contain genetic sequences not only for mediating replication but also for modifying the tumor immune microenvironment (TME) (16). Alterations in the TME can provoke innate and adaptive immune responses and inhibit tumor angiogenesis, leading to tumor death (26). Although this may initially limit the spread of OVs in tumor cells, the cell lysis induced by viruses and the danger-associated molecular patterns

triggered by OVs can overcome immunosuppression and promote antitumor immunity (27). To prevent the spread of OVs into healthy cells, neutralizing antibodies and cytokines produced in response to viruses initiate immune reactions. However, the clinical application of OVs in cancer therapy is challenging, particularly regarding their toxicity and pathogenicity to humans. Addressing these challenges is crucial for the broader adoption and effectiveness of OV-based cancer therapies (19).

2.2 Antitumor mechanisms of OVs

The mechanisms by which OVs effectively kill tumor cells are diverse and multifaceted (Figure 1):

2.2.1 Direct lysis

OVs overwhelm tumor cells with the production of viruses, causing direct lysis when the viral load exceeds the capacity of tumor cells to contain them (28).

2.2.2 Transgene expression

Genetically engineered OVs can express transgenes that induce cytotoxic effects, leading to tumor cell apoptosis and autophagy (29).

2.2.3 Sensitization to chemotherapy and radiation therapy

OVs sensitize tumor cells to chemotherapy and radiation therapy, enhancing their effectiveness in killing tumor cells (30).

2.2.4 Antitumoral activity

OVs stimulate an antitumoral immune response by triggering cytokine release upon detection by the host immune system. This immune response targets virus-infected tumor cells through the innate pathway, causing release of tumor-associated antigens, further enhancing immune recognition and tumor cell death (16).

2.2.5 Vasculature targeting

Some OVs are engineered to target the vasculature of tumor cells, reducing their blood supply and causing tumor regression (31).

2.2.6 Alteration of TME

OVs can modify the immunosuppressive TME created by tumor cells, increasing the infiltration of antigen-presenting cells and immune cells into the tumor. This alteration helps restore the immune balance and enhances the immune response against the tumor (32).

These mechanisms collectively contribute to the potent antitumor effects of OVs.

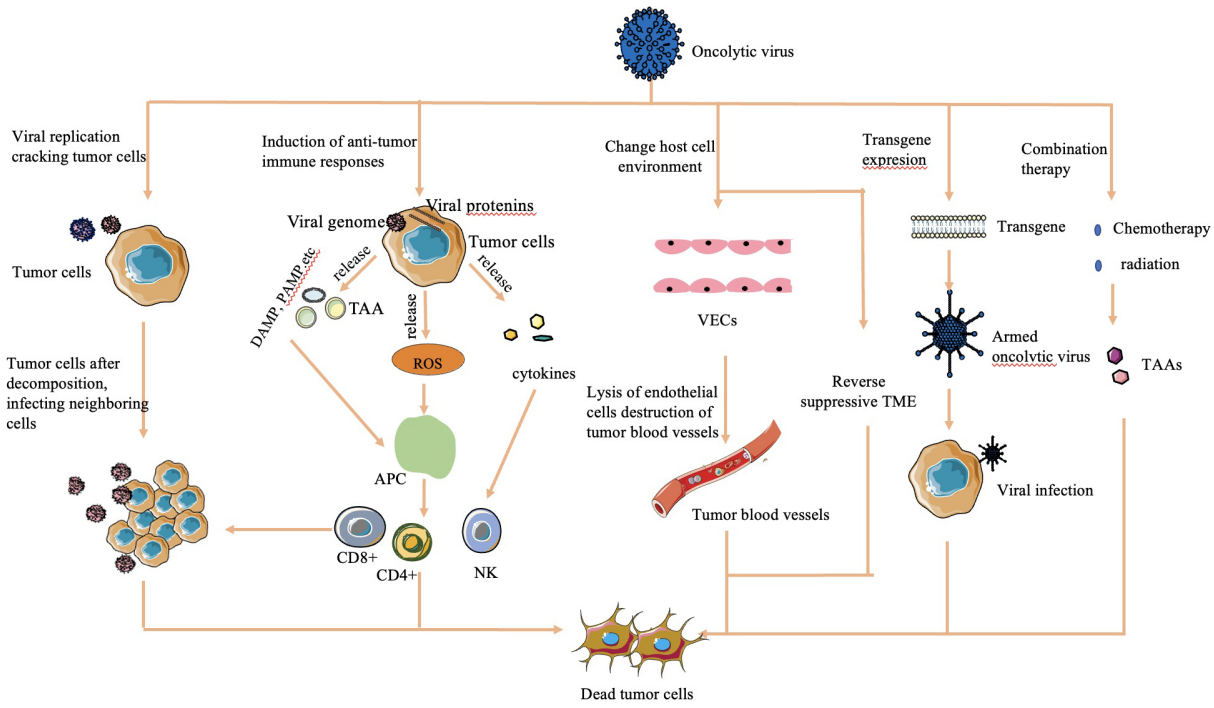

FIGURE 1
Mechanisms of cell lysis used by oncolytic viruses (16, 30, 32). The mechanism of OV cell lysis can be categorized into major categories. 1) Direct lysis due to a large volume of virus by replication. 2) Cytotoxicity by proteins encoded by the virus, which leads to tumor cell apoptosis and autophagy death. 3) Anti-tumoral immunity that leads to induction of host immune response, escape of the virus from the host response, and release of TAAs to act on adjacent sites. 4) Sensitization of chemotherapy and radiation. 5) Transgene expression through genetic engineering. 6) Change in host cell environment, including reversal of host TME and destruction of tumor blood vessels.

Furthermore, the adaptability of OVs enables their potential incorporation into multimodal approaches to cancer treatment, presenting promising opportunities for enhancing patient outcomes.

Both clinical trials and preclinical studies have demonstrated the relative safety of oncolytic virotherapy (OVT), with minimal reported adverse effects. This safety profile underscores the potential of OVT as a groundbreaking treatment for cancer. Continued research and development in this field hold promise for further enhancing the efficacy and safety of OVs as a therapeutic approach against cancer (33).

2.3 Categories of OVs

The diversity of viruses being explored for OVT highlights the breadth of research in this field. Both natural and engineered viruses show promise as potential candidates for cancer treatment. Some viruses that are used for OVT include herpes simplex virus (HSV), adenovirus (ADV) type 5, influenza virus (IV), oncolytic vaccinia virus (VV), and coxsackievirus A (COX-A), measles virus, poliovirus, retrovirus, reovirus, parvovirus H1, vesicular stomatitis virus, Newcastle Disease virus, etc. (27). In recent years, nearly all of these viruses have been investigated in both preclinical basic science studies (Table 1) and clinical trials (Table 2) for liver tumor OVT. This underscores the extensive research being conducted to evaluate the efficacy and safety of viruses in targeting and destroying tumor cells, particularly in the context of liver cancer.

Exploring the potential of multiple viruses allows researchers to identify the most effective candidates for OVT while considering safety, delivery methods, immune responses, etc. This comprehensive approach enhances our understanding of the diverse mechanisms that viruses use to exert oncolytic effects and paves the way for the development of novel and improved therapies for liver cancer and other malignancies.

In 2015, Talimogene laherparepvec (T-VEC), an HSV-1 derived JS1 OV strain, became the first and only OVT approved for clinical use by the Food and Drug Administration (34). T-VEC has been a significant development in OVT. Its approval marked a milestone in cancer treatment, particularly for melanomas. By leveraging the natural ability of HSV-1 to infect and kill cancer cells, T-VEC demonstrated promising efficacy in shrinking tumors and prompting immune responses against cancer cells.

Pexastimogene devacirepvec (Pexa-Vec), a VV with deletion of thymidine kinase, an enzyme in the DNA precursor pathway, was designed to restrict viruses to only attack tumor cells, particularly HCC cells (35, 36). Pexa-Vec expresses granulocyte-macrophage colony-stimulating factor to recruit dendritic cells through interferon cytokine expression, enhancing tumor infiltration (37).

TABLE 1 Viruses used for liver tumor or liver metastasis treatment in basic science studies.

Virus Types	Product Names	Year	Modification	Model	Ref.
HSV	humanized scFv against human PD-1 (hPD-1scFv)	2022	Insertion of humanized hPD-1 blocker gene	Mouse	(42)
Morreton Virus	MORV, University of Texas	2023	Unmodified wildtype	Mouse	(108)
ADV	Ad-GD55–α-Tim-3	2023	Inhibition of T-cell TIM-3)	Mouse, *in vitro*	(54)
Newcastle Disease Virus (NDV)	Oncolytic virus M1	2022	Unmodified wildtype	Mouse, *in vitro*	(109)
Poxvirus	CF33	2023	Deletion of *J2R* (TK) gene and addition of human sodium iodide symporter (hNIS)	Mouse, *in vitro*	(110)
VV	oncoVV-AVL	2022	Expression of gene encoding *Aphrocallistes vastus* lectin	Mouse, *in vitro*	(111)
	VvDD-IL15Rα	2022	Expression of superagonist IL-15 and erastin plus the deletion of 2 viral genes that encode thymidine kinase and vaccinia growth factor	Mouse, *in vitro*	(71)
	OncoVV-AVL	2024	Expression of gene encoding *Aphrocallistes vastus* lectin	*in vitro* Mouse	(70)
IV	rFlu-huPD1	2022	PB1 fragment encodes the heavy chain of PD-1 antibody and polymerase acid protein fragment encodes PD-1 antibody light chain.	Mouse, *in vitro*	(112)
Measles Virus	MV	2023	Unmodified wildtype	*in vitro*	(113)
Reovirus	Reo	2018	Unmodified wildtype clinical grade oncolytic orthoreovirus	Mouse, *in vitro*	(36)
Alphavirus	M1-VCPI	2017	Expression of valosin-containing protein inhibitors (VCPIs)	Mouse, *in vitro*	(114)
	SINV-GM-CSF	2024	GM-CSF carrying Sindbis virus. Mutation (G to S) at amino acid 285 in the nsp1 protein	*in vitro* Mouse	(115)
COX-A21	V937	2024	Genetically unmodified Kuykendall strain of COX-A21	Mouse, *in vitro*	(87)

TABLE 2 Viruses used for liver tumor or liver metastasis treatment in clinical trials.

Virus	OVT Product	Year	Modification of Virus	Phase	Path	Ref.
HSV	NV1020, BioReliance	2010	Deletion of UL56 internal repeat gene and UL24 gene expression.	I/II	Herpetic artery infusion	(45)
ADV	OBP-301, Oncolys Biopharma Inc.	2023	Attenuated type 5 ADV with an *hTERT* promoter	I	Intertumoral Injection (IT)	(56)
VV	Pexa-Vec, JX-594, Biotherapeutics Inc. and Transgene S.A.	2019	Inactivated thymidine kinase to express human granulocyte-macrophage colony-stimulating factor (GM-CSF) and β-galactosidase	IIb	Intravenous (IV) infusion followed by IT	(73)
	VvDD (JX-929), Jennerex Biotherapeutics	2016	Deletion of vaccinia growth factor and TK	I	IV, IT	(116)
Vesicular Stomatitis Virus	VSV-IFNβ -TYRP1, Mayo Clinic	2023	Express IFN-β and Tyrosine Related Protein 1 (TYRP1)	I	IV, IT	(117)
COX-A21	V937, Viralytics	2023	Unmodified bioselected strain of CVA21	Ib	IV	(86)
Protoparvovirus H-1	ParvOryx	2021	Unmodified	II	IV, IT	(118)

Although this phase III trial requires further optimization in HCC treatment, evidence from studies and clinical trials supports both the safety and efficacy of Pexa-Vec (36).

3 Novel finding in OVT against liver cancer

3.1 HSV

HSV is a double-stranded DNA virus (38). Its virion has four components: a DNA core, an icosapentahedral capsid, an amorphous protein coat tegument crucial for HSV infection, and a glycoprotein-bearing lipid bilayer envelope, from the inner to the outer surface (39, 40). It exists as HSV-1 and HSV-2, with HSV-2 commonly associated with sexually transmitted diseases and HSV-1 linked to infections of the oral cavity and skin. HSV-1 has been extensively used in OVT for HCC because it exhibits rapid host cell entry, efficient replication, binding to receptors broadly expressed in different types of human cells and tissues, and ability to stimulate a strong cellular and humoral immune response (41).

However, despite its potential, challenges must be addressed before HSV is widely used in clinical settings for OVT, including complexity of vector engineering, short-term stability issues, and risk of affecting normal tissue (41). Although a considerable number of preclinical studies have used HSV as the predominant OV in liver tumor treatment, in recent years, no clinical trial was successfully completed for HSV OVT targeting liver tumors. Clinical trials utilizing HSV for treating other cancers, including melanoma, lung cancer, solid tumors, breast cancers, and glioma, have been extensively investigated and have shown promising outcomes (Table 3). This indicates the potential for HSV in cancer therapy, but further research and development are needed to overcome the current challenges associated with its use in treating liver cancer.

Recent research has focused on genetically engineering a tumor-selective oncolytic HSV (oHSV) to express a human single-chain variable fragment targeting human programmed cell death 1 (PD-1) in mouse and nonhuman primate models with human liver cells implanted subcutaneously (42). PD-1, an inhibitory receptor on lymphocytes, impedes T-cell recognition and attacks upon binding to programmed death ligand 1 (PD-L1) (43). By designing a single-chain variable fragment against humanized PD-1, researchers assessed the antitumor efficacy of oHSV. The ideal PD-1 blockade candidate was selected and verified in mouse and nonhuman primate models. Results showed that mice treated with anti-PD-1-modified OV developed long-term memory of T-cell responses and reduced immunotherapy resistance.

In nonhuman primates, a humanized antibody against PD-1, called hu17D5, was constructed after library screening. hu17D5 is a single-chain antibody with better affinity to PD-1. After administering hu17D5 to nonhuman primates, a significant T-cell immune response was observed ($p < 0.01$) (42). Subsequently, an OV was engineered to express the hu17D5 gene, naming it YST-oHSV. The results demonstrated that 72 h after YST-oHSV injection, the viability of HCC cells decreased by 90%, whereas normal cells remained unaffected (42). Additionally, the antitumor activity increased after YST-oHSV injection. When YST-oHSV was injected into mice with HCC, tumor growth was significantly inhibited, leading to increased survival rates and tumor regression. YST-oHSV treatment increased CD8+ cytotoxic T-cell rejuvenation and the number of CD8+ memory T cells. YST-oHSV demonstrated great safety in nonhuman primate models, with no serious adverse effects (AEs).

Inappropriate delivery routes often limit the efficacy of oHSV in OVT. To solve this problem, surface-engineering-technique-masked oHSV with a galactose-polyethylene-glycol (PEG) polymer chain (glycosylated-PEG-oHSV) was generated to direct viruses to tumor sites and limit off-target effects, especially to the brain (44). Although glycosylated-PEG-oHSV did not affect oHSV replication, it exhibited increased specificity to the asialoglycoprotein receptor, which is selectively expressed on the surface of HCC cells, in a mouse model. This leads to enhanced tumor penetration into the center of HCC cells and reduced

TABLE 3 Clinical trials using HSV oncolytic therapy for all cancer types.

Cancer Type	Virus Product	Modifications	Year	Phase	Pathway of Delivery	Ref.
Non-Small Cell Lung Cancer	ADV/HSV-tk, Merck	Adenovirus-mediated expression of HSV thymidine kinase	2024	II	IT	(119)
Solid Tumors	HSV1716 (Seprehvir), Nationwide Children's hospital	Deletion of ICP34.5 gene and maintenance of TK expression	2019	I	IV	(120)
Primary Central Nervous System Tumors	HSV G207, Aettis, Inc., University of Alabama	Deletion of γ134.5 gene and disability of *lacZ* insertion in U_L39	2017	I	IT	(121)
Malignant Glioma	M032, Aettis, Inc., University of Alabama	Expression of IL-12	2016	I/II	IT	(122)
Recurrent Glioblastoma	CAN-3110 (rQNestin34.5v.2)	Expression of *ICP34.5* by nestin promoter	2023	I	IT	(123)
Melanoma	OrienX010	Expression of GM-CSF, deletion of ICP34.5 and ICP47, and inactivation of ICP6.	2022	Ib	IT	(124)
Soft Tissue Sarcoma of Trunk and Extremities	Talimogene laherparepvec (T-VEC)	Expression of GM-CSF and deletion of *ICP47* and *ICP 34.5* gene.	2021	Ib/II	IT	(125)
Breast Cancer	Talimogene laherparepvec (T-VEC), Amgen	Expression of GM-CSF and deletion of *ICP47* and *ICP 34.5* gene.	2021	I	IT	(126)
Malignant Pleural Mesothelioma	HSV1716, Virttu Biologics Limited	Deletion of *ICP 34.5* using strain 17+	2020	I/IIa	Intrapleural Injection (IP)	(127)
Pancreatic Cancer	HF10	Deletion of *UL43, UL49.5, UL55, UL56*, and latency-associated transcripts, and overexpression of *UL53* and *UL54*.	2018	I	IT	(128)

accumulation in non-liver organs, such as the brain and lung. Additionally, glycosylated-PEG-oHSV decreased the level of HSV-neutralizing antibodies and T cells after infection. Furthermore, it increased the release of antitumor cytokines, leading to significant infiltration into the tumor, and thereby, limiting tumor growth (44). Propidium iodide staining validated the cytotoxic effect of oHSV to induce HCC apoptosis and necrosis. The efficacy of glycosylated-PEG-oHSV is dose-dependent, with optimal efficiency at 0.2 μM.

There has been a lack of HSV OVT clinical trials in the past years; however, a phase I/II study in 2010 published in *Hum Gene Ther* showed the antimetastasis ability of HSV in liver metastasis from colorectal cancer (45). In the study, scientists engineered NV1020, wild-type HSV-1 modified with the deletion of UL24 and internal repeat UL56 genes, which confers the ability to replicate in a less harmful manner. Then, the thymidine kinase gene was introduced to allow controlled infection. NV1020 was administered to 13 patients in phase I and 19 patients in phase II via hepatic artery injection weekly over four weeks. The results showed promising outcomes, with 50% exhibiting stable disease and one patient showing partial response following chemotherapy. Median time to progression was 6.4 months, and median overall survival (OS) was 11.8 months, with a 12-month survival rate of 47.2% (45). These findings underscored the efficacy of NV1020 in stabilizing liver metastasis. Regarding safety, AEs were primarily observed within 24 h post-infusion, with no grade 3 reactions reported. Most AEs were grade 1 and 2 reactions, such as nausea and myalgia, and were effectively managed with analgesics and other supportive measures. No virus was detected in serum or saliva samples, but HSV-1 was detected on the skin of two patients during monitoring. Despite these promising results, no subsequent publications regarding further phase II/III trials have emerged. This suggests that although the initial findings were encouraging, further research may be necessary to progress to later-stage clinical trials, and ultimately, determine the broader efficacy and safety profile of NV1020 in treating liver metastases or HCC.

3.2 ADV

ADV is a nonenveloped double-stranded DNA virus characterized by an icosahedral nucleocapsid. It belongs to the Adenoviridae family and is typically isolated from human adenoids (46). ADV primarily affects children because they have lower humoral immunity than adults (47). Based on its genome structure, ADV is categorized into 52 serotypes and 7 species (A–G) (48). ADV infection manifests in various forms, such as respiratory tract infection, keratoconjunctivitis, gastrointestinal manifestations, and urinary tract infection (47). Human ADV species C type 5 has been extensively studied in OVT because it evades pre-existing immunity (49). ADV demonstrates a remarkable capacity to target tumor cells through various receptors, such as Coxsackie and ADV receptor, integrins, CD46, desmoglein-2, and sialic acid (50). Besides its high safety profile, tumor selectivity, and immunogenicity, ADV stands out as an OVT candidate for its efficient gene delivery and transient expression

(51). Specifically, ADV can infect both dividing and non-dividing cells, expanding its applicability to different tumor types, including HCC (52). ADV does not integrate its DNA into the host genome during replication, reducing the risk of insertional mutagenesis, which is a common concern with many other viruses (53).

Recent preclinical research demonstrated the modification of ADV as Ad-GD55–α-Tim-3. This engineered ADV expresses E1A, a protein for viral replication controlled by the GP73 promoter, and encodes an antibody gene of immunosuppressive T-cell immunoglobulin domain and mucin-domain molecule-3 (TIM-3) (54). TIM-3, an immune checkpoint expressed on the surface of Th1 cells to regulate macrophage activation, exhibited higher expression in HCC cells than in healthy cells. This was confirmed through immunohistochemistry and western blot analysis with a significance level of $p < 0.05$ (55). Ad-GD55–α-Tim-3 infection in HCC cells led to a decrease in pro-inflammatory cytokines, such as IL-1β and IL-6, and an increase in anti-inflammatory cytokines, such as IL-10, which fosters a less inflamed environment for viral replication. HCC cells with Ad-GD55–α-Tim-3 also showed less immunosuppressive factors, such as TGF-β and IDO, which increased the immune response of the host to target HCC cells (54). Although Ad-GD55–α-Tim-3 inhibited HCC cell growth, it did not significantly induce apoptosis compared with wild-type ADV. In a tumor xenograft HCC mouse model, treatment with Ad-GD55–α-Tim-3 resulted in higher Ki-67 antigen expression and increased CD4/CD8 cell number. Thus, Ad-GD55–α-Tim-3 inhibits tumor growth with no observed cytopathic changes in mouse organs.

In 2023, a phase I clinical trial tested an attenuated Ad5 with a human telomerase reverse transcriptase (*hTERT*) promoter. This virus, named OBP-31, maintains telomere length with expression occurring exclusively in liver cancer cells but not in healthy or differentiated cells, thereby increasing its tumor selectivity (56). This is achieved when the *hTERT* promoter interacts with an internal ribosome entry site, enhancing the replicability of OBP-301, specifically in cancer cells. OBP-301 then causes tumor cell destruction through direct lysis via viral replication and induces immune responses facilitated by the cytokines, tumor necrosis factor and IL-1 (57).

Eighteen patients with HCC were recruited, with a median time since HCC diagnosis of 3.24 years. Thirteen patients had stage C cancer according to the BCLC system, and all patients were classified as Child-Pugh class A. qPCR analysis revealed no detectable OBP-301 DNA in most patients after 24 h, and none showed positive OBP-301 DNA in blood or urine tests 14 d post administration, indicating the safety of OBP-301 in patients with HCC (56). However, no patient achieved a complete response or partial response. Fourteen patients were in the stable disease stage, whereas four were in the progressive disease stage. The mean duration of stable disease to disease control was 5.55 weeks, with a median time to progression of 8.10 weeks. The median OS was 26.00 weeks, and the average time for disease control was 4.21 weeks. CD8+ cell number increased by an average of 56.3% 4 weeks after OBP-301 injection. Overall, whereas OBP-301 demonstrated safety and elicited an immune response in patients with HCC, its efficacy in terms of disease control and survival outcomes was modest at best, suggesting the need for further investigation or combination therapies to enhance its therapeutic potential in patients with HCC.

In a recent phase I trial, ADV-5 was combined with hTERTRibozyme-expressing HSV thymidine kinase to target liver metastasis in patients with GI cancer (58). hTERTRibozyme specifically targeted hTERT, which is prominently expressed in HCC cells (59). Coupled with HSV thymidine kinase, hTERTRibozyme enhanced its cytotoxicity to HCC cells. The clinical trial involved 18 patients, with only 2 patients exhibiting stable disease after an 8-week regimen. Median progression-free survival (PFS) was 1.1 months, indicating limited clinical efficacy (58). Median OS was 6.2 months, and the maximum tolerated dose was 2×10^{12} viral proteins with higher doses failing to yield better clinical results, and no pharmacodynamic assessment was conducted. Virus DNA remained undetectable at significant levels after 72 h, with a median circulating virus half-life of 10.1 min. Due to the lack of efficacy, Ad5CRT is not ready to proceed to the next stage of clinical trials.

In addition to liver tumors, ADV has been widely used in OVT clinical trials for various other types of cancer. For instance, Ad5-yCD/mutTKSR39rep-hIL12 was used for prostate tumors (60), LOAd703 was used for pancreatic cancers (61), and Cretostimogene received the fast track and breakthrough designations for bladder cancers (62).

3.3 Oncolytic VV

VV, also known as smallpox, is a poxvirus characterized by a brick-shaped envelope and a 200-kb double-stranded DNA genome (63). Unlike many other viruses, VV does not require specific receptors for cell entry. Instead, it utilizes a protein-based entry-fusion complex or cooperates with endosomes for membrane fusion (64). Because VV does not enter the nucleus, it is easier to control its replication. VV replicates entirely within the cytoplasm of infected cells using its own DNA-encoded enzymes, avoiding competition with host cell DNA and circumventing the endomembrane system (65). Many antiviral agents can limit VV spread, including ST-246 and cidofovir (63). Cell lysis usually occurs <24 h after infection (66). VV elicits a robust T cell and antibody immune response and demonstrates a broad host cell tropism, making it a promising candidate for OVT (67). Other advantages of using VV in OVT include an efficient delivery system, stability upon intravenous administration or storage in powder or solution, and ability to encode transgenes (68).

The potent cytotoxic effect of VV was found to trigger the host immune response during HCC treatment. *Aphrocallistes vastus* lectin (AVL), a marine lectin commonly found in sponges and algae, was combined with VV to improve the cytotoxicity of VV in HCC cells through PI3K/Akt and MAPK/ERK pathways (69). Cells infected with siVV-AVL had significantly reduced viability compared with those infected with VV alone, and its antiproliferative efficacy increased progressively. VV-AVL-infected cells had 30-fold higher apoptosis than wild-type PBS control cells. Measurement of virus concentration in HCC cells

demonstrated that VV-AVL upregulated 2′-5′-oligoadenylate synthetase-like protein, thereby enhancing VV DNA replication and resulting in significantly higher virus titers. VV-AVL infection significantly increased the expression of type I interferon, notably IFN-α and IFN-β, particularly 36- and 48-h post-infection (69). This increase was mediated through phosphorylated IFN regulatory factor 3 (IRF3). VV-AVL also suppressed antiviral factors, including 2′-5′-oligoadenylate synthetase, IL enhancer binding factor 3, and phospholipid scramblase 1. Consequently, VV-AVL could replicate within the host cell by activating mammalian sterile 20-like kinase without encountering host defenses. In a mouse model, 30-d postinjection, VV-AVL significantly inhibited tumor growth. Consistently, histological examination revealed a notable presence of broken nuclei in VV-AVL-infected cells. Additionally, Zhang et al. in 2024 confirmed the effectiveness of VV-AVL in liver tumor treatment (70). They further discovered the mechanism of oncoVV-AVL, which involves reprogramming hepatocellular carcinoma (HCC) metabolism to promote reactive oxygen species (ROS). ROS, in turn, enhance the replication of oncoVV-AVL and induce tumor cell apoptosis.

In 2022, Liu et al. aimed to combine the vaccinia virus (VV) with erastin to improve its oncolytic effectiveness in liver tumor treatment (71). Erastin is a ferroptosis activator that can induce cell death in liver, colon, and ovarian cancer cells. Since both VV and erastin have been proven to inhibit tumor growth, this study investigated whether combining vvDD (VV with the deletion of thymidine kinase and vaccine growth factor) and erastin could lead to superior antitumoral activity. The results showed that although 80% of the mice exhibited inhibition of tumor growth with erastin treatment alone, the combination of erastin and vvDD (vvDD-IL15-Rα) led to a 100% reduction in tumor volume and 60% tumor cell regression (71). None of the five mice treated with the combination developed new tumors 12 days after treatment, whereas the untreated mice showed 83% new tumor growth. This indicates the immune memory provided by vvDD-IL15-Rα. Immune markers, IFN-γ and TNF-α, and immune cells, $CD86^{+}CD11c^{+}$ and dendritic cells, were also higher in the vvDD-IL15-Rα group than vvDD or erastin alone group.

A randomized phase II clinical trial conducted in 2013 investigated the oncolytic effectiveness of JX-594 (Pexa-Vec) in liver cancer treatment (72). Pexa-Vec, a vaccinia virus with inactive thymidine kinase, expresses human granulocyte-macrophage colony-stimulating factor and β-galactosidase. Low or high doses of Pexa-Vec were injected into the liver tumor on days 1, 15, and 29. The Choi response rate and intrahepatic disease control rate showed no significant differences between the injected and non-injected liver tumors at either dose. However, the survival rate was significantly higher in the injected group (14.1 months) compared to the non-injected group (6.7 months) at either dose.

In 2019, a randomized multicenter phase IIB clinical trial evaluated the effectiveness of Pexa-Vec combined with Best Supportive Care (BSC) versus BSC treatment alone in patients with HCC who had failed sorafenib therapy (73). This study highlighted the efficacy and safety of Pexa-Vec in patients with HCC. The survival rate of patients treated with Pexa-Vec + BSC (median OS: 4.2 months) did not significantly differ from those receiving BSC alone (median OS: 4.4 months). Both Pexa-Vec + BSC and BSC alone had a high likelihood of inducing AEs. Anti-β-galactosidase antibodies were detected in 56% of the patients receiving Pexa-Vec + BSC, indicating significant viral replication in HCC cells (73). Virus detection from urine or throat swabs ceased after day 8, whereas 21% of the patients had virus in rectal swab samples. ELISPOT analysis demonstrated a significant increase in T cells after Pexa-Vec injection, particularly evident after 6 weeks. The most expressed tumor antigens were MAGE-A1 and MAGE-A3, suggesting that Pexa-Vec can induce a tumor-specific T-cell immune response. Overall, whereas Pexa-Vec showed promise in inducing a tumor-specific immune response and good safety profile, it did not translate into a significant improvement in overall survival or disease control rate in this study population.

However, when comparing the effectiveness of Pexa-Vec with Sorafenib, the most commonly prescribed medication for HCC treatment, versus Pexa-Vec alone, a phase II trial showed a 62% disease control rate with Pexa-Vec alone and 59% Pexa-Vec with sorafenib (74). The Pexa-Vec was well-tolerated. The high dose of Pexa-Vec showed greater OS (14.1 months) vs the lower dose (6.7 months). Due to the higher effectiveness and safety profile of Pexa-Vec, the next stage clinical trial is warranted.

Later, a phase III clinical trial from 2015 to 2019, conducted at 142 sites in 16 countries with 459 patients, evaluated the efficacy of Pexa-Vec plus sorafenib versus sorafenib alone in HCC patients (75). The median OS was 12.7 months in Pexa-Vec plus sorafenib compared to 14.0 months in the control group. Median TTP was 2.0 months versus 4.2 months; objective response rate was 19.2% versus 20.9%; and disease control rate was 50% vs 57.3%, respectively (75). As a result, the addition of Pexa-Vec to the traditional sorafenib approach failed to demonstrate clinical benefits in treating HCC, leading to the early termination of the trial. Moreover, the safety profile was less optimal in the Pexa-Vec plus sorafenib patients, with 53.7% reporting serious AEs compared to only 35.5% in the sorafenib-only group (75).

Several factors have been proposed to explain the failure (75). First, TK1 gene was inactivated during the construction of Pexa-Vec, preventing the synthesis of thymine nucleotides essential for the replication of the OV. Additionally, sorafenib was not administered until the complication of the entire Pexa-Vec therapy, and its immunosuppressive effect, combined with the delay, allowed time for tumor growth, leading to a shorter TTP. For future trials, the time to combine Pexa-Vec with sorafenib and the dose of the virus will be needed to optimize its therapeutic potential in HCC treatment.

The potential effectiveness of VV in OVT clinical trials was also evident in solid tumors (76), colorectal carcinoma (77), head and neck cancers (78), etc.

3.4 COX-A

COX is a small, cytolytic virus belonging to the Enterovirus group of Picornaviridae family. It possesses a positive single-stranded RNA genome, lacks an envelope, and features an icosahedral capsid with surface viral proteins (79). COX is

classified into two groups: 1) coxsackievirus A (COX-A), with 23 serotypes commonly linked with hand, foot, and mouth disease and 2) coxsackievirus B (COX-B), with six serotypes often associated with myocarditis, among other conditions (80).

COX viruses, including COX-A21 and COX-B3, have been involved in OVT (81). COX-A21 stands out as a great candidate for several reasons. First, it boasts a highly specific and efficient ligand-receptor system for cellular entry. It binds decay-accelerating factor on the cell surface and requires the concurrent presence of intercellular adhesion molecule-1 for viral infection, facilitating the entry of the OVs into tumor cells (82). The replication of COX-A depends on nuclear factor κB (83). Subsequently, infected host cells undergo apoptosis induced by COX-A or a T-cell immune response. Clinical data show the safety of COX-A21, with no reported grade 3 or 4 AEs (84).

A bioselected COX-A21 strain named V937, without any modification, was used (85). V937 infects and leads to direct lysis of tumor cells that overexpresses intercellular adhesion molecule-1 (ICAM01). In the latest phase II open-label clinical trial in 2023, injection of V937 showed antitumor activity with a decrease in the size of injected and non-injected liver tumor cells metastases from melanoma. the clinical efficiency and safety of V937 were tested, with no patients reaching complete response or partial response. PFS was observed in all patients, with a median PFS of 3.7 months and a PFS rate of 9% at week 26 (86). Although V937 demonstrated relative safety in human participants, its efficacy in OVT warrants further investigation.

Later in 2024, a preclinical study further investigated the role of V937 alone versus V937 combined with pembrolizumab therapy in the treatment of HCC (87). When V937 was injected into non-contact tumor cell lines, a significant increase in IFN-α, IL-12, IFN-γ, IP-10, macrophage inflammatory protein (MIP)-1α, and IL-6 was observed. Pembrolizumab induces the expression of ICAM-1 on the surface of tumor cells, leading to increased infection and attack of V937 on HCCs, thereby establishing an antitumoral effect.

In addition to its use in liver tumors, COX-A has been mainly used for melanoma (88), and has shown some effectiveness in colorectal cancer (89), small cell lung cancer (90), etc.

3.5 IV

IV, a negative-sense single-stranded RNA virus from the Orthomyxoviridae family, exhibits a pleomorphic virion measuring 100–120 nm in diameter, encapsulated within a spherical bilayer envelope (91). IV comprises 7 serotypes, with IV A and IV B being the most commonly spread. The envelope surface of IV bears >500 spike-like projections, comprised predominantly of the glycoproteins hemagglutinin and neuraminidase in a 10 to 1 ratio (92). Upon entering the host cell, hemagglutinin undergoes activation by serine proteases. IV then integrates into the host genome, regulated by NS1, which acts as an interferon antagonist during virus replication (93).

IV can elicit a robust cytokine response, activating the adaptive immune system, and further promoting cytokine secretion. This potent ability to induce host cell death positions IV as one of the most commonly used OVs in cancer therapy (94). However, all studies remain in the realm of basic science research, with no recent clinical studies conducted.

Similar to oHSV, PD-L1 antibodies were incorporated into IV to target HCC cells (95). This oncolytic IV was identified through screening in pathogen-free chicken embryos, with all eight plasmids containing IV A/Puerto Rico/8/34 (PR8) and wild-type PR8 viral genetic materials. These plasmids were then recombined with the heavy and light chains of the PD-L1 antibody gene, named rgFlu/PD-L1. In cell culture experiments infecting normal MIHA liver cells and HCC cells, rgFlu/PD-L1 significantly reduced the viability of all tested HCC cells, with host cell survival rates decreasing as the duration and dose of infection increased. Importantly, normal MIHA cells remained unaffected, demonstrating the specificity of IV in targeting HCC cells exclusively. During infection, PD-L1 expression levels were suppressed, and apoptosis increased in rgFlu/PD-L1-treated HCC cells. In the mouse model, tumor size and weight significantly decreased compared with the control group 32-d post-injection, indicating the potential of rgFlu/PD-L1 for improving long-term survival rates (95). Safety assessments revealed negligible impact on organs, other than induced necrosis in HCC cells in the liver. The mechanism of HCC cell elimination by rgFlu/PD-L1 involved enhancing the activity and infiltration of CD8+ T cells and dendritic cells via the cyclic GMP-AMP synthase stimulator of interferon genes pathway, evidenced by the elevated levels of STING, phosphorylated STING, IRF3, phosphorylated IRF3, and TANK-binding kinase 1.

Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is an inhibitory regulator of T cells that tumors often employ to evade the immune system (96). An anti-CTLA-4 antibody was integrated into IV to evaluate its efficacy in HCC cells (93). The heavy and light chains encoding the CTLA4 antibody with PR8 IV yielded the recombinant OV named rFlu-huCTLA4 through reverse transcription. The $TCID_{50}$ was 8-9 $LogTCID_{50}$/ml. Cell viability assessments conducted 48, 72, and 96 h after rFlu-huCTLA4 injection into MIHA and HCC cell lines revealed unaffected, whereas HCC cell death increased proportionally with dose and duration of exposure. Moreover, the apoptosis rate was significantly higher in HCC cells (26.76%) than MIHA cells (3.45%) (93). In a mouse model, infection with rFlu-huCTLA4 increased the number of CD8+ T cells by 23.9%, targeting and eliminating HCC cells and CD4 + T cells by 38.7%. Liver tumor size and weight were significantly smaller compared with those in the MIHA-treated group. rFlu-huCTLA4. No virus was detected in other organs 40 d post-treatment.

Despite promising preclinical findings, no clinical studies utilizing IV as a potential OVT for liver tumor have been publicly available in the past five years. Limited studies have shown the implication of IV in pancreatic ductal adenocarcinoma (97), lung tumors (98), etc.

4 Discussion and future directions

Researchers have used various kinds of OVs with different modifications to understand their mechanisms and test their efficacy on liver cancer. However, there is still significant room for optimizing the treatment outcomes of OVs in the future.

The route of administration for OVs should be optimized based on the stage of liver cancer and the type of OV used. For example, patients with liver metastases may not respond well if OVs are injected intratumorally due to the difficulty of injecting multiple tumors and the risk of injections near important anatomical structures such as biliary structures (99, 100). Systemic delivery, such as IV injection and hepatic arterial injection (HAI), would be more effective options as they can distribute OVs throughout the entire body, not just within the liver tumor (100). The increased survival rates had been shown in patients with liver metastases via OV treatment with HAI, compared with OV treatment intratumorally (101). However, the quantity of virus delivered over a long pathway may be compromised by neutralizing antibodies (99). Local liver tumors are more responsive to IT and intralesional injection, which helps avoid the barrier of the extracellular matrix (102). Bacterial collagenase could be used to increase OV infiltration for local tumors (103).

Despite the effectiveness and benefits of OVs in liver tumor treatment, several barriers need to be solved. First, patients with HCC often present with underlying liver cirrhosis and dysfunction, making them more susceptible to adverse effects from OVs, which can lead to liver toxicity (99). Second, the number of studies (including preclinical and clinical) specifically focused on liver OVT is limited and has not demonstrated significant clinical effectiveness of OVs (104). Third, the evaluation of antitumor activity could be improved. For instance, many studies rely solely on changes in tumor size to assess OVT effectiveness, overlooking changes in tumor density and molecular markers of tumor necrosis, such as immune cell infiltration (99).

While conventional approaches or OVT alone may not achieve superior efficacy in liver tumor treatment due to tumor heterogeneity, combining these two approaches has proven to be effective in liver cancer treatment (105). Pathways targeted by small molecular-based drugs for liver cancer treatment target sometimes overlap with those targeted by OVT, such as the EGF pathway (101). Transarterial chemoembolization (TACE) can increase tumor response during the treatment, but the antitumor effect often diminishes shortly after the treatment. However, when combined with OVs, TACE can directly deliver OVs through the blood vessels, avoiding attacks on OVs by the host immune response, which prevents a decrease in OV concentration and avoids AE on other parts of the body (105). Chemotherapy usually has limited effect on liver tumors due to the presence of resistant disease and liver toxicity. Conversely, liver cancer cells are less resistant to OVs, and OVs cause lower toxicity to the liver. Clinical research has shown increased treatment outcomes using this combined approach (106). For example, when oHSV is used with cisplatin in HCC, cytotoxicity increased in all cell lines tested (107).

5 Conclusion

The landscape of OVs in cancer treatment shows promising strides, but their application in liver cancer treatment faces a significant gap between preclinical promise and clinical validation. Although basic science studies offer encouraging insights, the lack of robust clinical evidence leaves a critical void in understanding their effectiveness in treating liver cancer. Although these viruses often demonstrate a favorable safety profile, it is crucial to recognize that this observation might be skewed by small sample size and the selective withdrawal of patients with severe illness.

To truly harness the potential of OVs in liver cancer treatment, extensive clinical investigation is imperative. Larger-scale clinical trials are necessary to provide concrete evidence of efficacy and safety in real-world patient populations. Bridging this gap between basic science research and clinical application is essential for validating OVs as an effective therapeutic option for patients with liver cancer. This journey toward clinical validation not only enhances our understanding of innovative treatments, but also holds the promise of improving outcomes for patients with liver cancer.

Author contributions

XH: Conceptualization, Data curation, Visualization, Writing – original draft, Writing – review & editing. SX: Data curation, Investigation, Writing – review & editing. YT: Resources, Writing – review & editing. SY: Resources, Writing – review & editing. SZ: Resources, Writing – review & editing. YQ: Resources, Writing – review & editing. YiL: Resources, Writing – review & editing. YoL: Resources, Writing – review & editing. YS: Conceptualization, Methodology, Writing – review & editing. PQ: Conceptualization, Methodology, Writing – review & editing.

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Conflict of interest

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Oncolytic virotherapy against lung cancer: key receptors and signaling pathways of viral entry

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Lung cancer accounts for the highest cancer-related mortality worldwide. While immunotherapies targeting anti-tumor immune responses have demonstrated efficacy in clinical practice, the demand for novel treatment modalities remains urgent. Oncolytic viruses (OVs), which selectively kill tumor cells while stimulating an anti-tumor immune response, represent a potential breakthrough in lung cancer therapy. The induction of anti-tumor immunity by OVs is central to their overall therapeutic effectiveness. Many natural receptors on the surface of cancer cells are dysregulated, providing potential entry points for OVs. Furthermore, the inherent dysregulation of some key signaling pathways in lung cancer cells promotes proliferation, progression and metastasis, which may facilitate selective viral replication. In this review, we explore the application of OVs in lung cancer by analyzing several major OVs and their corresponding entry receptors. Then, we also examine the key signaling pathways and molecules with the potential to synergize with OVs in modulating the immune tumor microenvironment. Finally, we discuss the combination and administration strategies that warrant further clinical trials for validation. Despite certain limitations, the tolerability of OVs positions virotherapy as a promising avenue in the future of lung cancer treatment.

KEYWORDS

lung cancer, oncolytic virus, virotherapy, viral entry receptors, signaling pathways

1 Introduction

1.1 Evolution of lung cancer treatment modalities

Lung cancer is among the most prevalent and deadly cancers globally, affecting countless individuals and families across various regions (1). In 2022, the International Agency for Research on Cancer (IARC) estimated 20 million new cancer cases worldwide, with lung cancer accounting for 12.4% of cases and 1.8 million deaths, the highest among

all malignant tumors (2). Lung cancer is broadly classified into two types: small cell lung cancer (SCLC), representing 15% cases, and non-small cell lung cancer (NSCLC), accounting for 85% (1, 3).

Advances in lung cancer treatment have significantly expanded therapeutic options (Figure 1). For patients with stage I-II and select stage III NSCLC, the standard surgical procedure is lobectomy and mediastinal lymph node dissection, often supplemented with adjuvant radiation therapy or chemotherapy as needed (4, 5). In advanced NSCLC or SCLC, comprehensive treatment with targeted therapy and immunotherapy have become essential (4). Early intervention is associated with improved outcomes, while the five-year survival rate for patients diagnosed at advanced stages ranges between 4% and 30%fluctuating between 4% and 30% when they are diagnosed in the middle to advanced stages (6, 7).

1.2 Oncolytic virus therapies: new options in cancer treatment

Over the past century, significant advancements have been made in lung cancer therapies. However, the biological complexity and heterogeneity of lung cancer cells present challenges for conventional treatments. Since the advent of precision medicine in the 21st century, molecular profiling of tumors and immune cells has increasingly been used to guide therapeutic decisions (8). As a form of tumor immunotherapy, viral therapy shows great potential in lung cancer treatment by selectively killing cancer cells and activating antitumor immune responses (9). The use of pathogens in cancer treatment dates back to the late 1800s, when Dr. Coley used bacterial toxins to treat solid tumors (10). Additionally, there are reports of leukemia patients achieving remission after influenza virus infections (11, 12). Renewed interest in OVs emerged during the 2021 COVID-19 pandemic, when a 61-year-old British man with advanced Hodgkin's lymphoma experienced tumor regression following COVID-19 pneumonia (13). In 2015, the Food and Drug Administration (FDA) granted approved the first and only OV therapy, T-VEC, a modified herpes simplex virus, for advanced melanoma (14). Table 1 provides a summary of currently approved OV therapies worldwide.

There are an estimated 1.5 million undiscovered viruses globally, with around 827,000 are thought believed to be capable of spilling into humans (19). The biological feature of virus determines its ability to infect host cells and replicate under permissive conditions (20). A deeper understanding of viral entry, replication, and their interactions with host immune responses has driven interest in using viruses to treat certain cancers (Tables 1, 2). Although the precise mechanisms of OV therapy are not yet fully understood, it is generally accepted that OVs exert their antitumor effects through direct oncolysis and stimulation of systemic antitumor immune responses (21).

In this review, we explore the application of viral therapy in lung cancer, focusing on key oncolytic viruses (OVs) and their entry receptors. We then highlight critical signaling pathways and molecules that may synergize with OVs to modulate the tumor immune microenvironment. Finally, we discuss combination strategies, routes of administration, and address biosafety concerns and limitations of virotherapy. In conclusion, virotherapy holds significant promise in advancing lung cancer treatment.

2 Oncolytic viruses in lung cancer treatment and entry receptors

2.1 Types of OVs in use

To date, many OVs and engineered viral vectors including adenovirus, herpesvirus, vaccinia virus, coxsackievirus, reovirus, poliovirus, Seneca Valley virus, measles virus, and etc., have progressed to early-phase clinical trials (Table 2). Currently, at least 6 oncolytic viruses are being evaluated in clinical trials for lung

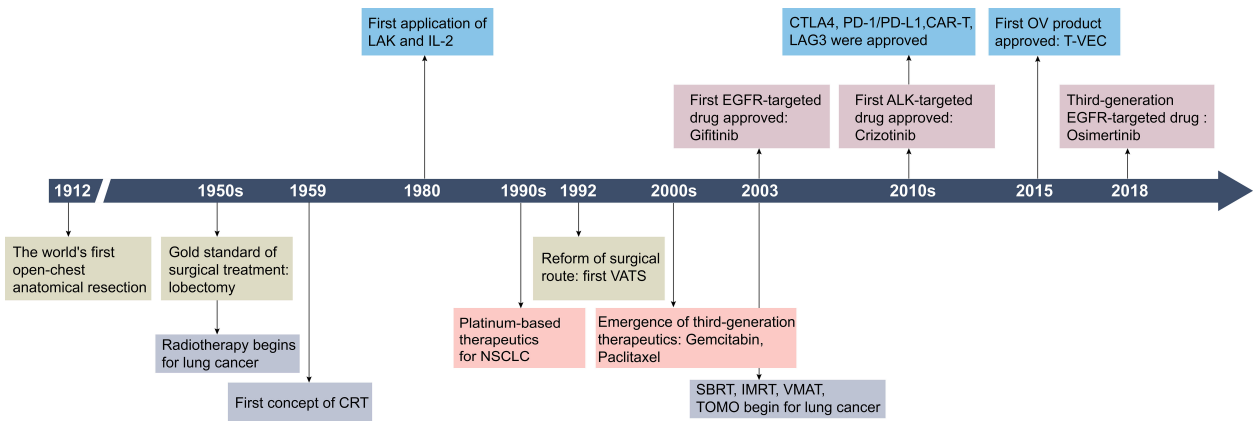

FIGURE 1
Historical timeline of key developments in lung cancer treatment strategies. CRT, 3D-comformal radiotherapy; LAK, lymphokine-activated killer cells; IL-2, interleukin-2; VATS, video-assisted thoracic surgery; SBRT, stereotactic body radiation therapy; IMRT, intensity modulated radiation therapy; VMAT, volumetric modulated arc therapy; TOMO, tomotherapy; CTLA-4, cytotoxic T lymphocyte antigen-4; CAR-T, chimeric antigen receptor T-cell immunotherapy; LAG3, lymphocyte activation gene-3. Created by Adobe Illustrator 2024.

TABLE 1 Currently approved oncolytic virus products worldwide.

Name	Virus	Country (approval Time)	Indication
Rigvir*	ECHO-7	Latvia (2004)	Stage I–II melanoma (15)
H101	AdV-5	China (2005)	Nasopharyngeal carcinoma (16)
T-VEC	HSV-1	USA (2015)	Stage IIIB–IV melanoma (17)
Delytact	HSV-1	Japan (2021)	Glioblastoma (18)

ECHO-7, wild-type echovirus type 7; AdV-5, recombinant adenovirus type 5; HSV-1, recombinant herpes simplex virus type 1; T-VEC, also known as Talimogene laherparepvec; Delytact, Teserpaturev/G47△; * Rigvir has been discontinued in 2019.

cancer (Table 3). The development of OVs for lung cancer therapy is primarily concentrated in the United States, China, and Europe, with clinical investigations limited to phase I-II trials. No successful phase III trials have been reported thus far.

2.2 Entry receptors in lung cancer as a prerequisite for the oncolytic effects

Surface receptors are the first switch that mediate viral entry and determine the viral tropism to tumors. The interaction between viral glycoproteins and host cell receptors facilitates membrane fusion and subsequent viral replication (44). Each virus has evolved specific mechanisms for genome integration, often binding to multiple receptors, while individual receptors may also be targeted by different viruses, enhancing viral infectivity from an evolutionary perspective (45) (Figure 2).

Several surface proteins are overexpressed in certain lung cancer cells, such as the coxsackie-adenovirus receptor (CAR), herpesvirus entry mediator (HVEM), and CD46. These receptors not only promote cancer cell invasion and metastasis but also serve as natural targets for OV infection (46, 47). Conversely, low receptor expression can limit the efficacy of oncolysis.

Differences in viral receptor expression provide opportunities for OVs modification. For instance, viral capsid proteins can be modified with peptide ligands or antibody fragments to target specific receptors. Adenoviral capsid fibers have been modified with an Arg-Gly-Asp (RGD) motif to bind integrins overexpressed in tumors, significantly enhancing the targeting efficiency of oncolytic adenovirus type 5 (48). However, limited data exist on the preclinical and clinical characterization of these modifications, highlighting the need for further investigation into receptor expression and associated signaling pathways in lung cancer cells to identify OVs with enhanced tropism.

2.2.1 Adenovirus

Adenovirus (Ad) is non-enveloped viruses, 90-100 nm in size, with a genome contained within an icosahedral capsid (49). There are 57 known Ad serotypes, with Ad2 and Ad5, both from subtype C, the most widely used for OVs (50, 51). Ad is considered a promising oncolytic virus due to its wide range of serotypes and receptors, high titer production, genomic stability, feasibility of genetic modification and well-characterized replication (52).

The coxsackie and adenovirus receptor (CAR) is a 46 kDa transmembrane glycoprotein in the junction adhesion molecule

TABLE 2 Examples some major oncolytic viruses in research and biological features.

Virus	Genotype	Genome length	Entry receptor	Replication site	Associated studies*	Ref
Herpesvirus	dsDNA	150kb	HVEM/nectin-1/2	Nucleus/cytoplasm	T-VEC/Delytact/OH2	(22)
Adenovirus	dsDNA	36kb	CAR/CD46/DSG2/Integrins	Nucleus/cytoplasm	H101/ONYX-015	(23–25)
Vaccinia virus	dsDNA	192kb	Receptor-mediated endocytosis	Cytoplasm	GL-ONC1/JX-594	(26–28)
Parvovirus	ssDNA	5kb	SARs	Nucleus/cytoplasm	H-1PV	(29, 30)
Reovirus	dsRNA	123kb	Receptor-mediated endocytosis/ JAM-A	Cytoplasm	Reolysin	(31)
Coxsackievirus	ss(+)RNA	7.4kb	CAR/ICAM-1/DAF/ KRM1/SCARB2	Cytoplasm	V937	(32–34)
Seneca Valley virus	ss(+)RNA	7.3kb	TEM8	Cytoplasm	SVV-001	(35, 36)
Poliovirus	ss(+)RNA	7.5kb	CD155	Cytoplasm	PVS-RIPO	(35, 37)
Newcastle disease virus	ss (–)RNA	15kb	SARs	Cytoplasm	MTH-68/H/NDV-HUJ	(38)
Measles virus	ss (–)RNA	16kb	CD46/SLAM/nectin-4	Cytoplasm	MV-NIS	(39–41)
Vesicular stomatitis virus	ss (–)RNA	11kb	LDLR	Cytoplasm	rVSV-ZEBOV	(42, 43)

dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; dsRNA, double-stranded RNA; ss(+)RNA, positive-sense single-stranded RNA; ss(-)RNA, negative-sense single-stranded RNA; HVEM, herpesvirus entry mediator; CAR, coxsackie-adenovirus receptor; DSG2, desmoglein-2; SARs, sialic acid residues; JAM-A, junctional adhesion molecule A; ICAM-1, intercellular adhesion molecule 1 or CD54; DAF, decay-accelerating factor or CD55; KRM1, Kringle-containing transmembrane protein 1; SCARB2, scavenger receptor class B member 2; TEM8, tumor endothelial marker 8; SLAM, signaling lymphocyte activity molecule; LDLR, low-density lipoprotein receptor. * In addition to the OVs products approved in Table 1, others are still in the preclinical or clinical trial stage.

TABLE 3 Current clinical trials of lung cancer-related OVs.

Virus (Submission Time)	Registration Number	Modification	Tumor type	Phase	Location
Adenovirus					
YSCH-01(2021)	NCT05180851	Recombinant L-IFN adenovirus	Lung Cancer	I	China
MEM-288(2021)	NCT05076760	Chimeric IFNβ/CD40-ligand	NSCLC	I	USA
CAdVEC(2018)	NCT03740256	Unknown*	Lung Cancer	I	USA
ADV/HSV-tk(2016)	NCT03004183	Replication-defective recombinant adenovirus vector	NSCLC	II	USA
Ad/MG1-MAGEA3 (2016)	NCT02879760	E1/E3 deletion/hMAGE-A3 insertion	NSCLC	I/II	Canada
Colo-Ad1(2014)	NCT02053220	Chimeric Ad11/3 group B	NSCLC	I	Spain
Herpesvirus					
R130(2023)	NCT05886075 NCT05961111 NCT05860374	anti-CD3 scFv/CD86/PD1/HSV2-US11 insertion	Lung Cancer	I	China
T3011(2022)	NCT05598268	Unknown*	Lung Cancer	I/II	China
Vaccinia virus					
BT-001(2021)	NCT04725331	Chimeric 4-E03/GM-CSF	NSCLC	I/II	Belgium/France
JX-594(Pexa-Vec) (2008)	NCT00625456	GM-CSF insertion, TK disruption	Lung Cancer	I	Canada/USA
Coxsackievirus					
V937(2014)	NCT02043665	None	NSCLC	I	USA/ Australia/UK
Reovirus					
REOLYSIN®(2009)	NCT00861627	None	NSCLC	II	USA
Seneca Valley Virus					
SVV-001(2006)	NCT00314925	None	Carcinoid Neuroendocrine	I	USA
Unknown*					
RT-01(2022)	NCT05205421	Unknown*	Extensive-Stage SCLC	I	China

NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; *Data not published or not retrievable. Data were collected from National Clinical Trials (https://clinicaltrials.gov/).

(JAM) family and serves as a common mediator for both coxsackieviruses B and most Ads (53) (Figure 2). CAR plays a crucial role in epithelial cell adhesion and signal transduction, as well as cancer development (44). Notably, CAR is rarely expressed on normal lung cells but shows variable levels of expression on lung cancer cells (54). H101, with E1b55K and partial E3 deleted, infects cells by the binding of the viral fiber knob with CAR (55). Deletion of E1b55K allows Ad preferentially replicate in p53-deficient cancer cells (56) and E3 genes mainly participate in host anti-virus immune response (Figure 3), however, the mechanism by which H101 selectively replicates in cancer cells remains uncharacterized. A preclinical study confirmed that the lung adenocarcinoma cell line XWLC-05 from Xuanwei highly expresses CAR by RT-PCR and immunocytochemistry staining, thereby the oncolytic adenovirus H101 is able to efficiently infect XWLC-05 and lead to oncolysis *in vivo* (57). However, the hypoxic environment of some solid tumors is often associated with CAR downregulation, and the RAS-MEK signaling pathway has also been linked to reduced CAR levels (58). Stecker et al. proposed that CAR expression in tumor cells may vary by stage and correlate with tumor aggressiveness, suggesting the need to assess CAR expression and the tumor microenvironment before selecting a viral therapy (59).

CD46, a transmembrane protein, serve as an inhibitor of complement activation and negatively regulates the complement system, as well as the primary receptor for most species B Ad types (60). While CD46 is widely expressed in normal tissues, it is often overexpressed in lung cancer, potentially due to abnormal signal transducers and activators of transcription 3 (STAT3) activation and p53 mutations (61). Additionally, CD46 also protects cancer cells from complement-mediated cell death (62). Studies have shown that CD46 is upregulated in lung adenocarcinomas (A549, Z793) more than in squamous lung cancers (QG56, NCI-H520), but the latter has a relatively higher levels of CAR (46). Other complement inhibitory proteins, such as decay-accelerating factor

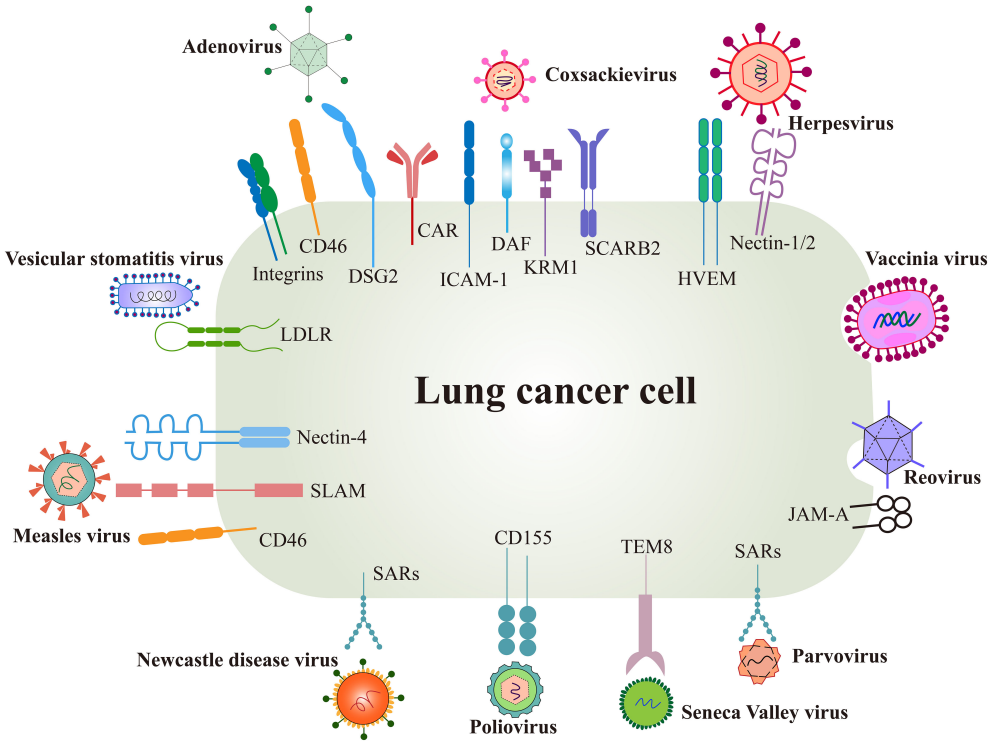

FIGURE 2
Surface receptors of oncolytic virus entry into cancer cells. OVs utilize several natural receptors to infect host cells, some of which are often overexpressed on lung cancer cells. However, more effective entry targets for OVs remain to be identified. Created using Adobe Illustrator 2024, with data referenced from published literature.

(DAF, or CD55), also play a role in protecting tumor cells from immune surveillance (63).

Desmoglein-2 (DSG2), another major receptor for Ad, a transmembrane glycoprotein belonging to the cadherin family (20). DSG2 has been shown to be involved in cell-cell adhesion and tumorigenesis, and is also overexpressed in NSCLC (64). Sun et al. analyzed lung adenocarcinoma (LUAD) patients and corresponding normal tissues to assess DSG2 expression. Combining their results with the data from TCGA and Oncomine, showed that high DSG2 expression positively correlates with tumor size, lymph node metastasis and TNM stage (65). A meta-analysis demonstrated that high DSG2 expression is associated with poor overall survival (OS) in NSCLC patients (66). Another preclinical and clinical study found that DSG2 overexpression promoted LUAD cell proliferation and migration, potentially through the regulation of EGFR and Src phosphorylation, activation of the PAK1 signaling pathway, and alterations in the tumor microenvironment (TME). DSG2 overexpression was also associated with increased resistance to the EGFR tyrosine kinase inhibitor Osimertinib. However, subsequent studies confirmed that DSG2 expression was not statistically associated with tumor size, differentiation, lymph node metastasis or stages (67), which contradicts the fundings of Sun et al.

Additionally, Ad can use integrins as entry receptor, others like SARs, CD80 and CD86 (61), MHC-I (68), etc. The receptors mentioned above are representative examples. Ad uses a variety of receptors for cell entry, lacking inherent specificity for tumor cells.

2.2.1.1 Genetic modification strategies of adenovirus in clinical trials

Ads utilize a range of receptors to enter cells, which gives them high tissue tropism but limits their specificity in targeting lung cancer cells. Ads are the most commonly used virus in tumor treatments due to various modifications for tumor cell targeting, making it a promising candidate for lung cancer oncolytic therapy (51). Similar to adenoviral engineering strategies, the basic principles of oncolytic viral modification strategies include deletion of pathogenic genes, enhancement of viral tropism and integration of immunostimulatory factors (69).

The pathogenicity of Ads is primarily linked to genes in the E region, which are activated early in the replication cycle and regulate viral replication, cell cycle control, and immune evasion (57). Consequently, in the design of oncolytic adenoviruses, the E1 region is frequently modified or deleted to reduce adenoviral pathogenicity and enhance safety, such as above mentioned H101 and YSCH-01. YSCH-01 is a kind of recombinant Ad modified in the E1A region and inserted with multifunctional anti-cancer L-IFN gene (Table 3). The L-IFN gene will induce tumor lysis and anti-tumor immunity when Ad replicates in lung cancer cells (70). An investigator-initiated trial about YSCH-01 reported an objective response rate (ORR) of 27.3%, a median progression-free survival (PFS) of 4.97 months, and a median overall survival (OS) of 8.62

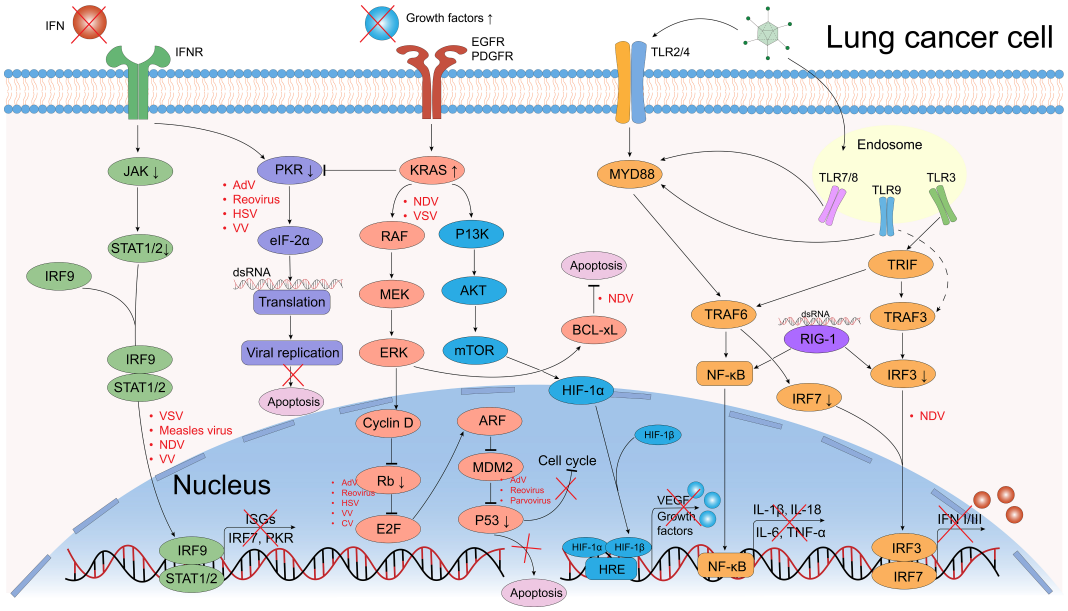

FIGURE 3
Defective antiviral responses and aberrantly activated signaling pathways OVs can use in lung cancer cells. The clearance of OVs in normal cells depends on the regulation of IFN-related signaling pathways, however, they are frequently dysregulated in cancer cells, which provide great convenience for the OVs' replication. In normal cells, when viral components are recognized by Toll-like receptors (TLRs) or retinoic acid-inducible gene 1 (RIG-1), they further activate the transcription and translation of downstream NF-κB and interferon regulatory factor (IRF) signals, causing the release of pro-inflammatory factors (IL-1β, IL-18, IL-6, TNF-α) and interferons (IFNs). IFNs bind to IFN receptors (IFNR), activating the JAK/STAT pathway, which induces interferon-stimulated genes (ISGs) expression and further IFNs production. Dysregulation of tumor suppressor genes such as p53 and Rb can promote OV replication (e.g., Ads, reovirus). The PKR pathway regulates transcription and can induce abortive apoptosis in response to viral infection. The EGFR-KRAS pathway is frequently dysregulated in lung cancer, making these cells susceptible to OVs like NDV and VSV. Similarly, the PI3K/AKT/mTOR pathway could upregulate the HIF-1α expression that promote the transduction of vascular endothelial growth factor (VEGF) and growth factors, a certain VV could target the VEGF and generates anti-angiogenic effects. Created using Adobe Illustrator 2024, with data referenced from published literature.

months. These results indicate preliminary efficacy of YSCH-01 in advanced solid tumors, including 5 lung cancer patients (71). Viral tropism is determined by the interaction between viral surface proteins and host cell receptors. In type 5 adenovirus variant VCN-01, the substitution of the heparan sulfate proteoglycan (HSG)-binding domain with an RGD motif improves infectivity and selectively targets integrins-expressing tumor cells (48, 72). Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) promotes the proliferation, differentiation, and maturation of granulocytes (e.g., neutrophils) and macrophages, enhancing immune resistance to infections and tumors (73). OVs carrying the GM-CSF gene, such as JX-594 (Table 3), destroy cancer cells through replication-dependent lysis and activation of antitumor immune responses.

2.2.2 Herpesvirus

Herpesvirus (HSV) is large enveloped dsDNA virus, with 9 known types, including the commonly studied HSV-1 and HSV-2 from the α-Herpesviridae family (74). HSV replicates in cellular nucleus without integrating into the host genome (75), a feature that enhances its safety profile as an OV.

HSV-1 glycoprotein D and B mediate viral entry by binding to specific receptors on the surface of cancer cells (76). gD serves as a ligand protein for most α-Herpesviridae receptors, which binds to 3 primary receptors: herpes virus entry medium (HVEM), nectin-1, and 3-O-sulfated heparan sulfate (3-OS-HS) (77, 78). gB is a trigger protein that is responsible for viral fusion. Reported receptors binding to gB and mediate entry including paired immunoglobulin-like type 2 receptor-α (79), myosin-9 (80), myelin-associated glycoprotein (81). However, the exact mechanisms by which gB receptors mediate viral entry remain incompletely understood and only a few studies have characterized myosin-9 and myelin-associated glycoprotein in NSCLC (82, 83).

HVEM, belonging to the tumor necrosis factor receptor (TNFR), is expressed predominantly on immune cells, and functions as a primary receptor for HSV-1 and HSV-2, excluding other α HSVs (84, 85). Notably, Ren et al. evaluated 527 NSCLC samples and 56 NSCLC cell lines, suggest that HVEM is overexpressed in NSCLC patients (positive rate 18.6% & 48.2%) with N2 lymph node metastasis or advanced stages, but its expression levels is not capable to predicting OS (86, 87). HVEM is a key immune checkpoint, interacting with B and T lymphocyte attenuator (BTLA) and CD160 (BY55) to trigger inhibitory signals (47). It is independent from PD-1/PD-L1 network and may contribute to immune evasion in lung cancer, making it a promising therapeutic target (88).

Nectin-1 is a cell adhesion protein belonging to the immunoglobulin superfamily, which functions as an entry receptor for a big part of α HSVs (89). The nectin family mainly consists of nectins 1-4, but not much research has been done on

how HSV infects cells with the help of nectin-1 or its expression in lung cancer cells. In contrast, nectin-4, a receptor for Measles virus, has demonstrated significant predictive and applied value (90). Nectin-2 also facilitates the entry of certain HSV strains and it might be used in lung cancer diagnosis since high nectin-2 expression in LUAD has been found to be associated with recurrence after surgery (91–93).

HSV-1 was the first oncolytic virus to be genetically engineered for therapeutic use. T-VEC, based on HSV-1 JS-1 strain, is modified to the deletion of neurovirulence factor ICP34.5 and ICP47 genes, also armed with GM-CSF gene, which enhancing the recruitment of APCs and immune filtration of TME (94). OH2 is a novel OV derived from HSV-2 HG52 strain, with the same modification strategy as T-VEC (95). HSV-2-based OVs for lung cancer remain in preclinical development, with no evidence distinguishing HSV-1 from HSV-2 in this context (96, 97).

2.2.3 Vaccinia virus

Vaccinia virus (VV) is a large dsDNA virus from the poxviridae family, approximately 192 kb in length, with a characteristic asymmetric brick-like complex structure (69). It can enter the host cells through membrane fusion or via receptor-mediated endocytosis in acidic environments, though the involvement of a specific host cell receptor remains unclear (26, 98). VV is considered a safe oncolytic agent, as demonstrated by its successful use in smallpox vaccines and its cytoplasmic replication, preventing host genome integration (98).

VV's tumor selectivity depends largely on the thymidine kinase (TK) gene, which supports viral replication. Since TK is overexpressed in cancer cells and minimally expressed in normal somatic cells, oncolytic VVs with TK deletions have been engineered. Additionally, VV-infected tumor cells secrete viral proteins that activate the EGFR-RAS pathway, further promoting TK synthesis and enhancing VV replication (27).

In addition, VV's large genome can accommodate substantial exogenous DNA without impairing its replication capacity (99). JX-594, the most well-known VV derivative, has been engineered with a GM-CSF gene insertion and TK deletion, showing promise for intravenous administration (73). Moreover, VV holds significant potential for future tumor vaccine development, particularly due to its ability to deliver therapeutic genes and stimulate robust immune responses (100).

2.2.4 Coxsackievirus

Coxsackievirus (CV), a member of the Picornaviridae family, is a positive-sense single-stranded RNA virus with a genome of approximately 7.4 kb, encapsulated by icosahedral capsid proteins (101). CV possesses several features that make it a promising candidate for lung cancer therapy, including multiple receptor targets, ease of genetic modification, cytoplasmic replication, and the ability to specifically target hypermutated molecular pathways in lung cancer (102).

Kirsten rat sarcoma homolog (KRAS) mutations are present in approximately 30% of NSCLC patients, with 20%-40% in LUAD and only 5% in squamous NSCLC (5, 61). CV-B3, a well-studied oncolytic virus, effectively targets KRAS-mutant LUAD cells (A549, H23, H2030) with minimal effects on normal lung epithelial and EGFR-mutant LUAD cells (H1975, PC-9, H3255, H4006) (103, 104). However, CV-B3 can cause severe viral myocarditis and pancreatitis, limiting its therapeutic potential, and further genetic modifications are needed to reduce its toxicity (105).

CVs primarily enter cells via receptors such as CAR, intercellular adhesion molecule 1 (ICAM-1), decay accelerating factor (DAF, or CD55), KRM1 and SCARB2 (scavenger receptor class B member 2, also known as lysosomal integration membrane protein-2, LIMP-2) (32, 33, 106, 107).

As mentioned above, CAR is a common receptor for Ad and CV, and lung cancer cells that express CAR on their surface would be attacked by both of them theoretically. Future studies may need to find the expression of this receptor on more lung cancer cell lines and patient-derived tumor tissue or primary cells to maximize the oncolytic effect of CV that use this receptor to infect lung cancer cells. Deng et al. found that KRAS mutations downregulate CAR expression by activating the ERK1/2 signaling pathway (103).

ICAM-1 (CD54) is an inducible glycoprotein involved in cell adhesion during immune and inflammatory responses (108). CV-A21, is the most researched recently with ICAM-1 (109). Infection with CVB upregulates ICAM-1 expression and increases production of the pro-inflammatory cytokines, including IL-6, IL-8 and TNF-α (110). NSCLC cell lines with high ICAM-1 expression are sensitive to CV-A11-mediated cytotoxicity, while DAF expression levels do not correlate with cytotoxic effects (32, 111). Preclinical and clinical trials showed that the CV-A21-based OVs product V937, which preferentially lyses ICAM-1 upregulated NSCLC cells, is currently in Phase I clinical trials, but the clinical efficacy intravenously administered V937 with pembrolizumab does not appear to be superior to that of monotherapy (34, 112).

DAF/CD55 is a co-receptor that is expressed in both lung cancer and normal lung fibroblast cell lines (113). It has been shown that DAF assists in the entry of ICAM-1 and CAR (114). DAF can act as a lower affinity attachment site, enhancing virus presentation, or as a virus binding site for subsequent higher affinity binding to ICAM-1 and CAR (63, 111). Overexpression or mutations of epidermal growth factor receptor (EGFR) can be detected in 10%-20% NSCLC patients (115). Shao et al. treated H1395 and H322M NSCLC cells with EGF for 24h, and suggest that EGFR activation increases the expression of CD55, but not CD46, upregulated CD55 expression inhibited the complement system and cytokine secretion required for $CD8^+$ T cell activation, and CD55 levels were negatively correlated with infiltration of M1 macrophages and $CD8^+$ T cells in human lung cancer specimens (n=24), which indirectly promoted tumor growth and could use predicted patient prognosis (116). Therefore, EGFR mutations may enhance CV infection by upregulating CD55, but this also contributes to immune suppression within the tumor microenvironment (TME), which may facilitate tumor progression.

KRM1 is a widespread membrane-anchoring protein located on the cell surface and in the intracellular membrane which is recently identified as an important entry receptor of a major subset of CV-As (117). Most studies on KRM1 focus on its interaction with CV-A10, yet its expression in lung cancer cells and the oncolytic effects

of KRM1-targeting CVs remain unexplored, likely due to incomplete understanding of CV-A10's infection mechanisms and pathogenesis (117, 118).

SCARB2 is a type-III transmembrane protein which mediates the translocation and reorganization of the endosomal/lysosomal compartment membranes (119). However, the expression conditions and the mechanism of how coxsackieviruses use SCARB2 to infect lung cancer cells needs to be further investigated.

2.2.5 Other OVs

Seneca Valley virus (SVV) is a ss (+) RNA virus that selectively infects and kills neuroendocrine SCLC cells (35, 36, 120). Preclinical studies showed that repeated passaging of SVV in SCLC cell cultures enhances its cytolytic activity over time, suggesting increased anti-tumor efficacy (121). Reovirus, an enveloped dsRNA virus (122), infects host cells via receptor-mediated endocytosis, primarily binding to junction adhesion molecule A (JAM-A), which is overexpressed in NSCLC and thus makes it a target for oncolytic virotherapy (31). Additional OVs, including Measles virus (MV) (123), Newcastle disease virus (NDV) (38), Vesicular stomatitis virus (VSV) (124), and Semliki Forest virus (SFV) have been explored for their potential in lung cancer therapy in preclinical settings (125).

In conclusion, OVs can target lung cancer cells by binding to overexpressed surface receptors. Different viruses show varying potential and limitations in lung cancer therapy, and optimizing viral genetic modifications and receptor selection is key to improving efficacy. Ads offer strong tumor-targeting potential due to their multiple serotypes, ease of genetic modification, and ability to use various receptors for cell entry. However, they lack tumor specificity, are prone to be cleared by immune responses, and show off-target effects, including liver accumulation *in vivo*. HSVs avoid host genome integration risk, their large genomes allow for gene insertions and effective immune activation. However, limited expression of key receptors like HVEM and Nectin-1 in lung cancer and potential neurotoxicity remain concerns for clinical application. VV have large genomes capable of carrying exogenous genes, and their cytoplasmic replication avoids integration into the host genome. Their safety is supported by widespread vaccine use, and deletion of the thymidine kinase (TK) gene enhances tumor cell selectivity. However, unidentified specific receptors limit their targeting, and replication depends on the high metabolic state of tumor cells, reducing efficacy in low-proliferation tumors. CVs can target multiple receptors and certain CVs show selective efficacy in KRAS-mutant lung adenocarcinomas. Its small genome allows easy modification, and cytoplasmic replication reduces integration risks. However, it can cause toxic effects like myocarditis and pancreatitis, requiring further modification to minimize side effects. Additionally, receptor expression (e.g., CAR, ICAM-1) varies across lung cancer types, affecting oncolytic efficacy. Other viruses, such as Seneca Valley virus (SVV), have potential for targeting specific lung cancer subtypes (e.g., small-cell lung cancer) and have shown antitumor activity in early studies. However, clinical data are scarce, mechanisms are unclear, and most are in early development, necessitating further validation of their efficacy and safety.

Future research should focus on understanding the molecular mechanisms of viral entry into tumor cells to enhance specificity and reduce toxicity. Additionally, the therapeutic potential of OVs in clinical settings remains underexplored (Table 3) and warrants extensive clinical trials to validate efficacy and safety.

2.2.6 Viral receptor expression may change after lung cancer treatment

Notably, OVs are usually used in combination with other therapies, and cancer cells often undergo molecular and phenotypic changes in response to treatment. Changes in surface receptor expression have been observed after radiotherapy or chemotherapy, though results are inconsistent across studies (126–128).

Harrington et al. assessed the combining effects of oncolytic Ad and the external beam radiotherapy, suggested that CAR and integrins were upregulated in colorectal (HCT116) and head-neck (SIHN-5B) cancer cell lines after radiation (129), but Geoerger et al. (130) reported that radiation does not upregulate CAR and integrins expression in glioma cell lines. that radiation CAR and integrins expression in glioma cell lines. Another experimental research also showed radiotherapy fails to increase CD46 receptor expression in glioblastoma for measles virus (131). Wu et al. (126) demonstrated that pre-medication of camptothecin or doxorubicin downregulated the CAR expression in tumor lines including H1299 (lung), HCT 116 (colon) and BxPC-3 (pancreas), this chemotherapy-induced downregulation was observed in patients undergoing chemoradiotherapy prior to colorectal cancer resection. However, Sakhawat et al. showed that CAR expression was enhanced by using cisplatin which improving the infection of Ad in breast cancer cells lines (127). Further research is needed to elucidate receptor expression changes in cell lines and primary tumors following neoadjuvant chemoradiotherapy, and the intracellular signaling pathways mediating these changes.

Therefore, assessing receptor expression before and after combination therapy is crucial to enhancing its clinical benefit, additional studies are required to clarify the impact of combination therapy on OVs receptor expression across different tumor types.

3 Key signaling pathways of OVs entry into lung cancer cells

Upregulation of specific surface receptors and dysregulation of key signaling pathways are critical for OVs' anti-lung cancer activity (102). Upon recognition by entry receptors, OVs selectively replicate in cancer cells and exploit dysregulated signaling pathways. In normal cells, multiple signaling pathways detect and clear viral particles, a mechanism often impaired in cancer cells (Figure 3). The first line of anti-viral response defense depends on the interferon (IFN) release producing by endosomal Toll-like receptors (TLRs) associated signaling pathways (132). TLRs, a class of pattern recognition receptors (PRRs), detect conserved pathogen-associated molecular patterns (PAMPs) (133). TLR1-10 were identified in the past decades, every TLR has capability to induce cytokines and activate different innate immune signals (133).

IFNs are classified into three main types: type-I (IFN-I), type-II (IFN-II), and type-III (IFN-III). In normal cells, IFN-I and IFN-III are induced by OVs infection (134). IFN-I is a pro-inflammatory signaling cytokine that consists of two major isoforms, IFN-α and IFN-β, that performs executing cancer immunosurveillance and promotes the remodeling of the TME, while IFN-II is the IFN-γ that is produced by activated NK cells and T cells (132). IFN-III act as an autocrine signal that triggers the production of IFN-α and IFN-λ to enhance the antiviral and antitumor activities of normal cells (135). When multiple TLRs are activated by PAMPs (including viral capsids, DNA, RNA, and viral protein products), and further triggering host cytokine signaling transduction, these factors including myeloid differentiation primary response protein (MYD88), TIR-domain-containing adapter-inducing IFNβ (TRIF), TNF receptor-associated factor (TRAF) family, interferon regulatory factor 3 (IRF3), interferon regulatory factor 7 (IRF7) and retinoic acid-inducible gene 1 (RIG-1), which in turn recruit the downstream kinases (136). IRF3 binding to IRF7 as a dimer, induces the production of IFNs and stimulates the anti-viral responses via autocrine and paracrine methods (137). These IFN-signals further lead to the phosphorylation and activation of Janus family protein kinases (JAK-STAT signaling pathway), which mediate the signals transduction and activate the transcription factor 1 (STAT1) and STAT2 (138). STAT1 and STAT2 form a multimer with IRF9, which transfers to the nucleus and ultimately induces the expression of interferon stimulated genes (ISGs), assisting in the antiviral response (137, 138).

Enhanced IFNs-release induces the activation of the downstream PKR (an intracellular protein kinase that recognizes dsRNA and other viral components) (139, 140) and initiates a cascade of events leading to the phosphorylation of eIF-2α. This phosphorylation inhibits protein synthesis, thereby suppressing further viral replication within cells (141, 142). However, it is possible that the signaling pathways of IFN and PKR are defective in certain cancer cell types, which could result in increased viral replication and impaired viral clearance. Conversely, these pathways may be more active in other cancer cell types, which could impact the therapeutic effect of oncolytic viruses (101, 143).

The mitogen-activated protein kinase (MAPK) cascade (RAS/RAF/MEK/ERK signaling pathway) is frequently dysregulated in cancer and regulates processes like apoptosis, proliferation, and motility (144, 145). The KRAS (Kirsten rat sarcoma viral oncogene) mutant accounts for almost 75% of RAS mutant cancers, which are the most common mutations in NSCLC patients (5, 146). However, despite a long history of preclinical and clinical studies, attempts to develop molecularly targeted drugs against mutations in KRAS in the past decades have ended in failure, therefore, KRAS as a molecular target has been named to be “undruggable”.

KRAS always acts as an intracellular switch, it is activated when bound to guanosine triphosphate (GTP) and inactivated by guanosine diphosphate (GDP)-bound state (147). KRAS-activation further promotes the RAF recruitment and PI3K, which facilitates the process of oncogenesis through downstream effectors (148, 149). Notably, EGFR is proposed to induce the KRAS activation through recruitment and interaction of some growth factor receptor-bound proteins, so upregulated EGFR expression may promote the KRAS activation (149). VV enters cells via receptor-mediated endocytosis, and its replication depends on EGFR-induced RAS signaling, so cancer cells with overexpression of EGFR are more susceptible to VV infection (145) (Figure 3).

Furthermore, people have been noted that certain viruses such as coxsackievirus and herpesvirus are capable of selectively targeting cancer cells that exhibit elevated RAS signaling activity. RAS-activated cancer cells fail to activate the PKR pathway, allowing viral infection and oncolysis (103, 150). MEK inhibition disrupts RAF-MEK-ERK signaling, upregulates CAR expression, and enhances adenovirus entry and oncolysis (151). The loss of CAR expression in cancer cells is at least in part mediated by the RAF-MEK-ERK transduction pathway. Restoring CAR expression on the cell surface could enhance Ad-based cancer therapies (152).

PI3K/AKT/mTOR is another RAF/MEK/ERK-independent KRAS downstream signaling pathway, which regulates the expression of hypoxia-inducible factors (HIFs) (153) (Figure 3). Jiang et al. (154) showed that NDV triggers autophagy in A549 lung cancer cells resistant to first-line therapeutics (cisplatin and paclitaxel) by targeting the PI3K/Akt/mTOR pathway. Similarly, a recombinant CV-B3 (155) have shown *in vitro* experiments that it induces apoptosis and phosphoinositide 3-kinase/Akt and mitogen-activated protein (MAP)/modulated extracellular signaling (ERK) kinase (MEK) survival signaling pathways, leading to cytotoxicity and modulation of CVB3 replication (104). BCL-xL is a therapeutic target for SCLC and NSCLC (156, 157), an anti-apoptotic protein belongs to the B cell lymphoma (BCL) family of cell survival proteins, and BCL-xL-overexpressed cancer cells permit NDV infection and viral syncytium formation required for viral spread (Figure 3) (157).

4 OVs induce systemic immune response against lung cancer

Descriptions of the receptors for several major OVs and key signaling pathways provide a general framework for lung cancer therapy. The presence of multiple natural receptors on the surface of lung cancer cells provides targets for viral entry, and defects in the antiviral response and signaling pathways of cancer cells create a microenvironment that supports viral replication, viral elements and cytokines-releasing further lead to an antitumor immune microenvironment (158). Although the specific molecular and cellular mechanisms of OVs are not fully elucidated, they can generally be described in two steps: selective killing of lung cancer cells and induction of an anti-tumor immune response (159, 160).

OVs induce the establishment of acquired immunity and turn “cold” TME into “hot” one (161) (Figure 4). OVs infect and replicate in tumor cells which induces an inflammatory response and immunogenic cell death (ICD), for instance, pyroptosis, autophagy and necroptosis are more immunogenic forms of cell death than apoptosis (162, 163). Various forms of ICD are observed following OV infection, which enhances tumor cell oncolysis (164), then a large number of damage-associated molecular patterns (DAMPs) including calreticulin, heat-shock proteins (HSPs), ATP, uric acid, high mobility group box 1 (HMGB1), pathogen-associated molecular patterns (PAMPs) including viral elements, tumor-associated

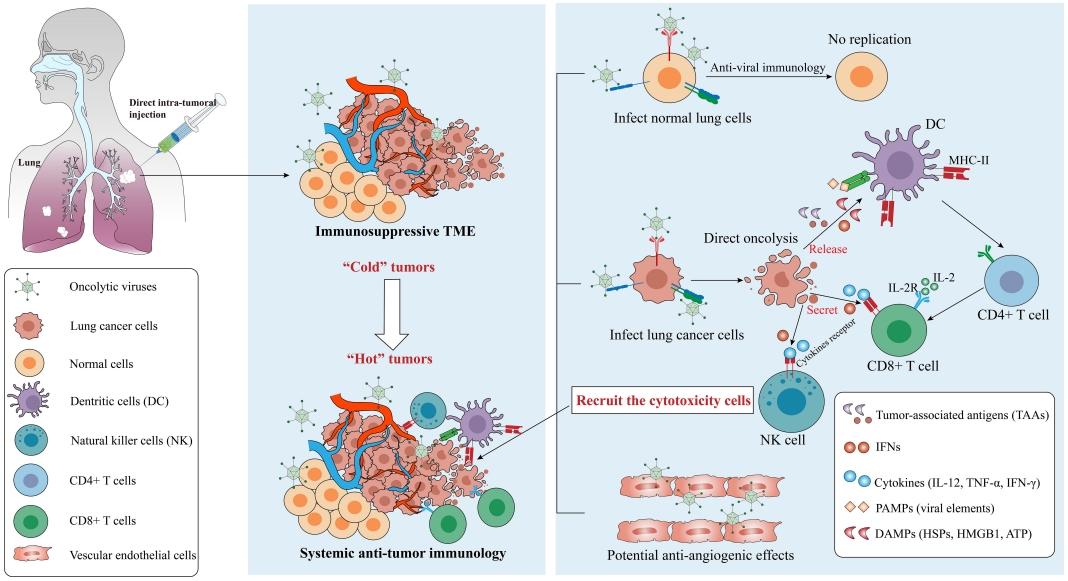

FIGURE 4
Mechanisms from "Cold" tumor becomes "Hot" tumor. Oncolytic efficacy depends on the selectively killing effect and the activation of anti-tumor immunity, and some OVs have the ability to destroy tumor vascular system. OVs can be cleared by the antiviral responses in normal cells but replicate in cancer cells, which finally leads to the recruitment of cytotoxicity cells such as $CD8^+$ T cells via several signaling pathways. Notes: PAMPs, pathogen-associated molecular patterns, include viral capsids, DNA, dsRNA/ssRNA, viral proteins; DAMPs, damage-associated molecular patterns, include HSPs, calreticulin, HMGB1, ATP, uric acid; cytokines include TNF-α, IFNs, IL-12, IL-2, etc. IL-2, interleukin-2; IL-2R, IL-2 receptor; TLR, Toll-like receptor. Created using Adobe Illustrator 2024, with data referenced from published literature.

antigens (TAAs) and cytokines (including IL-2, TNF-α, IFN-γ) are released (104, 165, 166). These factors recruit antigen-presenting cells (APCs) to the site of infection, which present antigens to T and B cells, inducing infiltration of cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, $CD4^+$ and $CD8^+$ T cells (162).

The $CD4^+$ T cells function as helpers by secreting cytokines such as IFN-γ, TNF-α, IL-2, and IL-12, which support the activation of CD8+ T cells. These cells are known as well as Th1 cells (167). Once activated, $CD8^+$ T cells exert anti-tumor effects locally or by migrating to tumor sites. Meanwhile, NK cells could be activated by IFN-I and DAMPs, and down-regulation of human major histocompatibility complex I (MHC-I) in cancer cells and increased MHC-II expression in APCs also further remodeled the inherent immune response and systemic immune status of TMEs (14, 21). Notably, in addition to the direct killing and TME-reshaping effect to tumor cells, the OVs also shows antiangiogenic effects through killing the tumoral vascular endothelial cells (168, 169), for example, VSV infects and destroys the tumor vascular system *in vivo* but leaves the normal vascular system intact (170). However, circulating OVs face the risk of clearance by neutralizing antibodies. The mechanisms balancing immune-mediated viral clearance and antitumor immune induction require further investigation.

5 Combination therapy and route of administration

Given the tumoral heterogeneity, complex genetic mutations, and the immunosuppressive TME, OV-based monotherapy often fails to achieve optimal oncolysis in lung cancer, as demonstrated in most studies (34, 171, 172). Combination strategies involve conventional lung cancer treatments, immune checkpoint inhibitors (ICIs), chimeric antigen receptor (CAR) T-cell therapy, and molecular targeted drugs. For example, H101 has demonstrated oncolytic potential in both *in vivo* and *in vitro* studies, though *in vivo* effects remain relatively mild (57), a meta-analysis shows the overall response rate (ORR) of H101 combined with chemoradiotherapy is significantly higher than those lung cancer patients treated alone, and improving both patient survival rates and quality of life (173). Sei et al. investigated the combination effects of Reovirus type 3 Dearing strain (ReoT3D) and chemotherapeutics in 9 NSCLC cell lines, the results demonstrated ReoT3D combined with paclitaxel can increase the proportion of mitotic blocked and apoptotic cells, and strong oncolytic effects on tumor killing synergized with cisplatin, gemcitabine, or vinblastine (174). A single-arm study for 37 NSCLC patients with metastatic KRAS or EGFR mutations using Reolysin (an OV product based on Reovirus type 3) in combination with paclitaxel and carboplatin, compared favorably (the median progression-free survival (PFS) and overall survival (OS) were 4 months and 13.1 months, respectively) with previous studies for the chemotherapy-alone (171). Cui et al. (54) screened a coxsackievirus B5/Faulkner strain (CV-B5) as an oncolytic virus candidate against NSCLCs (A-549, NCI-H1299, NCI-H460) through *in vivo* and *in vitro* experiments, and CV-B5/F can accelerate cell apoptosis, autophagy and endoplasmic reticulum stress in combination with DNA-dependent protein kinase (DNA-PK) or ataxia telangiectasia mutated protein (ATM) inhibitors. Collectively, pre-clinical and

clinical data are still limited and more clinical trials are needed to validate the therapeutic efficacy of OVs and different combination strategies in lung cancer patients.

The method of administration is another major challenge in the clinical application of OVs. The modes of administration of OVs include intra-tumoral (i.t.), intravenous (i.v.), intraarterial (i.a.), and even inhalation (37). Direct i.t. injection is the most studied method, offering advantages such as reduced risk of neutralization by antibodies, more targeted delivery, and localized infection, as for solid tumor in the thoracic, i.t. injections can also be performed by image-guided techniques (41). A study concerning oncolytic VV for malignant pleural effusion caused by NSCLCs, demonstrated that i.t. administration of oncolytic VV was safe and feasible, could produce local immune responses without other significant systemic symptoms (175). However, for metastatic or infiltrative tumors such as neuroendocrine tumors and leukemia, patients may require multiple injections, and OVs may struggle to reach all target tissues. Although systemic methods such as i.v. and i.a. injections are less studied, they offer the ability to treat multifocal or infiltrative tumors with the possibility of repeated administrations (176). i.v. injection may lead to immune clearance of OVs by neutralizing antibodies. However, this issue could potentially be addressed by using extracellular vesicles (EVs) to encapsulate and deliver OVs. Garofalo et al. (177) have validated that human lung cancer cell-derived EVs can be utilized to the delivery of OVs and chemotherapeutics, and its lipid membranes protect OVs from degradation by the immune system. Additionally, inhalation has been explored in viral vaccines, but few studies have investigated OV delivery via inhalation (178, 179), This limited research may be due to concerns over its lower immunogenicity. However, lung cancer usually originates from malignant transformation of bronchial epithelial cells, inhalation administration may be a specific delivery modality for the treatment.

6 Biosafety and limitations

Oncolytic viruses, including engineered variants, have demonstrated efficacy as an anti-tumor strategy in numerous preclinical and clinical trials. However, as replicating viruses, OVs raise biological safety concerns related to their potential for replication and infection in non-target tissues. These concerns necessitate careful consideration of storage, handling, and administration protocols (180, 181). Moreover, the toxicity limits of many OVs remain incompletely assessed, and data on potential long-term effects or survival outcomes are still limited. Achieving a balance between antiviral and antitumor immunity is essential for the successful development of OVs. The immune response can restrict viral biodistribution, and OVs are susceptible to detection and inactivation by neutralizing antibodies. Thus, reducing viral toxicity while enhancing antitumor efficacy through genetic engineering and combination with immunotherapy will be crucial future directions. Second, many studies use immunocompromised mice as models, which limits the ability to study virus-immune system interactions in humans. A more rational selection of animal models is needed to better understand the tumor microenvironment (TME) and the interactions among different cellular components.

7 Conclusions

Lung cancer is an escalating global public health issue, and its therapeutic strategies are continuously evolving. The emergence of OVs offers a favorable risk-benefit ratio for lung cancer treatment. However, the molecular and cellular mechanisms underlying the oncolytic effects of OVs are not yet fully elucidated.

As previously discussed, several viruses are potential candidates for OVs, with natural receptors on the surface of lung cancer cells serving as therapeutic targets. Future research should focus on identifying more effective targets on the surface of lung cancer cells, elucidating key oncolytic signaling pathways of OVs, and further investigating the TME reshaping process.

In conclusion, while oncolytic virotherapy shows significant promise for lung cancer treatment, additional research is necessary to optimize OVs design and improve clinical efficacy. Combining OVs with other therapeutic modalities could offer a more comprehensive and effective strategy for treating lung cancer.

Author contributions

WD: Writing – original draft, Writing – review & editing, Conceptualization. YL: Investigation, Visualization, Writing – review & editing. DH: Investigation, Visualization, Writing – review & editing. MZ: Writing – review & editing. JZ: Writing – review & editing. YC: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Innovative strategies in genitourinary cancer: the role of oncolytic viruses

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Urinary tumors pose a significant health threat because of their high prevalence and recurrence rates. Despite the availability of various treatment options, many patients poorly respond to traditional therapies, highlighting the urgent need for alternative approaches. Oncolytic viruses are promising therapeutic agents. These viruses exploit the unique characteristics of cancer cells to specifically target and destroy them, thereby triggering potent antitumor immune responses. This review delves into recent advancements and future prospects of oncolytic viruses, focusing on their application in renal, bladder, and prostate cancers. By discussing practical implications and the potential of different viruses, including the cowpox virus, adenovirus, measles virus, coxsackievirus, and reovirus, we pave the way for further exploration and refinement of this exciting field.

KEYWORDS

oncolytic virus, renal cancer, bladder cancer, prostate cancer, tumor, therapy

1 Introduction

As a serious threat to human health, urological tumors require diverse treatment methods. Traditional treatment approaches, including surgery, chemotherapy, and radiotherapy, control the disease to some extent (1). Onset may be associated with smoking, obesity, insulin resistance, hypertension, and chronic kidney disease. However, issues, such as high recurrence rates and significant side effects, continue to affect the medical community and patients. For instance, renal cell carcinoma (RCC), a malignant tumor originating in the kidneys, is generally treated by surgical removal (2). However, for patients with metastatic RCC, surgery often has a limited efficacy, making adjuvant and targeted therapy crucial supplements (3). Renal cell carcinoma encompasses a range of histopathological entities, with the most common subtypes being clear cell renal cell carcinoma (80%), peroid renal cell carcinoma (13-20%), and chromophilic renal cell carcinoma (5%) (4). For localized renal cell carcinoma that has not metastasized, the

standard treatment is surgical resection, and radiofrequency ablation, cryoablation, and stereotactic ablative radiotherapy may be used when the patient has a high surgical risk, is weak, has isolated kidneys, has impaired baseline renal function, or has multiple bilateral tumors (5). Bladder cancer is the 10th most common malignancy worldwide (6). Onset is often related to factors such as smoking, air or water pollution, dietary patterns and medical conditions (7). Based on the depth of invasion, BC is divided into non-muscle-invasive bladder cancer (NMIBC) and invasive bladder cancer (MIBC). Transurethral resection of bladder tumors (TURBT) is the standard of care for NMIBC. For non-muscle-invasive bladder cancer (NMIBC), intravesical therapy (primarily BCG) plus maintenance therapy is the mainstay of treatment to prevent recurrence and progression after initial TURBT; For those patients who do not respond to BCG, additional treatment is required. For localized MIBC, optimizing care and reducing morbidity after cystectomy are important goals (8). Urothelial carcinoma (UC) is the major subtype of bladder cancer, and the first-line treatment for patients with locally advanced urothelial carcinoma is cisplatin-based chemotherapy (9). Prostate cancer affects millions of men worldwide, mainly in areas with high human development indices (10). The main contributing factors for prostate cancer include genetics, obesity, physical activity, and smoking (11). The treatment of prostate cancer (PC) is complex. Localized PC can be managed with active surveillance, radiotherapy, or prostatectomy. Androgen deprivation therapy (ADT), salvage radiotherapy, and chemotherapy are the primary treatment methods for recurrent or metastatic PC (12). Although these traditional approaches increase the patient survival and improve the quality of life, they have limitations. Surgical trauma, the toxic side effects of chemotherapy, and damage to normal tissues due to radiotherapy cannot be ignored. More importantly, these methods have relatively high recurrence rates, especially in the case of advanced or metastatic tumors, and treatment outcomes are often unsatisfactory (13).

In recent years, the rapid development of biotechnology has brought new hope to the treatment of urological tumors in the form of oncolytic viruses (OVs), representing an emerging therapeutic strategy (14, 15). OVs selectively infect and kill tumor cells by stimulating the body's own antitumor immune response to achieve therapeutic goals (16). Compared with traditional treatment methods, OVs offer a higher targeting specificity and fewer toxic side effects. More importantly, OVs activate the patient's immune system, forming long-term immune memory, thereby effectively preventing tumor recurrence and metastasis (16).

OV research can be traced back to the late 19th century when doctors observed cases of tumor regression coinciding with viral infections (17). However, because of scientific and technological constraints, this discovery could not be promptly converted into an efficacious treatment modality (17). With the rapid development of modern biotechnology, our understanding of OVs has deepened and their application in cancer treatment has gradually moved from theory to practice. It has been shown that OVs have tremendous potential for the treatment of urological tumors. Researchers have developed various targeted OV strains for different types of urological tumors including RCC (18), BC (19), and PC (20). These viral strains can replicate efficiently within tumor cells and cause cell lysis as well as activate the body's immune system by releasing tumor-associated antigens, forming a powerful antitumor immune response.

Overall, OVs provide new ideas and methods for the treatment of urological tumors. Although research in this field is in its early stages, excellent results of preclinical studies and preliminary clinical trials bring hope to patients with urological tumors (13). We believe that OVs will play an important role in the future treatment of urological tumors.

2 Overview of oncolytic viruses

OVs, as a new type of biological therapy, selectively replicate and lyse within tumor cells, causing immunogenic cell death and subsequently inducing antitumor immune responses. It is generally anti-tumor in four ways: including oncolysis, anti-tumor immunity, transgene expression, and vascular collapse (21). First, the replication of the virus in cancer cells can induce cell lysis, and the viral replication leads to a continuous increase in the viral dose, which is more lethal to the tumor, and at the same time, proteins are also produced during the viral replication process, which are also toxic to tumor cells (22). The third mechanism by which oncolytic viruses mediate tumor cell destruction is through the induction of non-specific and specific anti-tumor immunity (23). Finally, oncolytic viruses can greatly increase the sensitivity of tumor cells to chemotherapy and radiation therapy (24). Because of these advantages, we chose OVs for discussion. OV therapy has advantages, such as strong targeting, relatively few side effects, and the ability to improve the efficacy through the genetic engineering of viruses, providing new ideas for the treatment of genitourinary tumors (25). A review of 97 published OV trials reported that most OVs tested have used large deoxyribonucleic acid (DNA) viruses such as adenovirus, HSV-1, reovirus, and poxviruses (26) (Figure 1).

2.1 Vaccinia virus

VACV is an enveloped double-stranded DNA orthopoxvirus that only replicates in the cytoplasm. VACV has a large genome (~190 kb) that stably expresses at least 25 kb of exogenous therapeutic genes in a single vector (Table 1). Since the late 1980s, recombinant DNA technology has been used to explore the utility of recombinant VACV and other poxviruses as vectors for active immunization in cancer and infectious disease settings (27). VACV has natural tumor tropism and potential for systemic administration. It has a rapid replication and lysis cycle. The virus is released from infected cells within 8 h of infection and destroys infected cells within 48–72 h post-infection (28). VACV has been used as a smallpox vaccine for many years, and adverse effects have occurred less frequently (29). In terms of being used as an oncolytic virus, firstly, VACV has a certain safety, which is mainly manifested in the fact that it only replicates in the cytoplasm and does not participate in the host's genes; Second, VACVs have a natural tumor tropism, which means that they are able to localize naturally

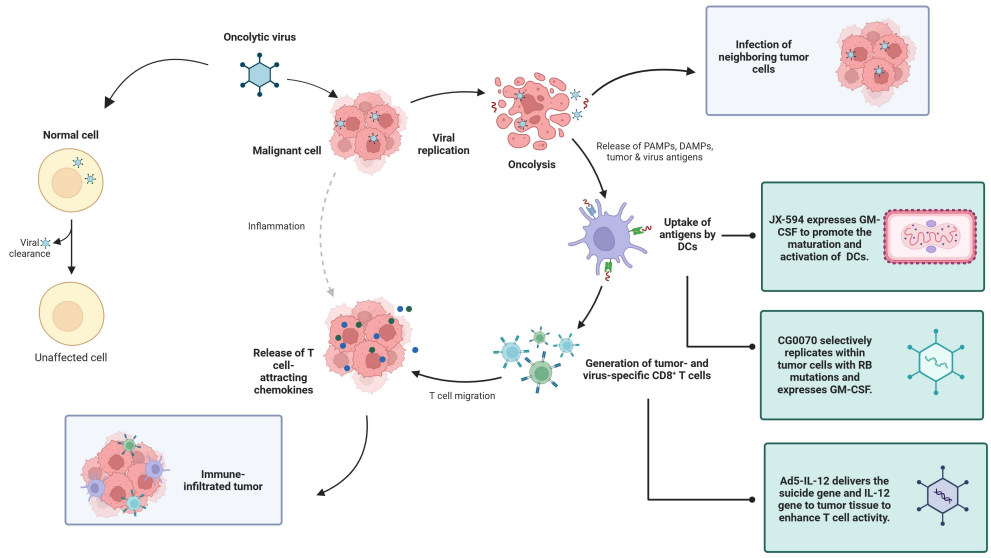

FIGURE 1
Properties of oncolytic viruses.

to tumor tissues and have the potential to do so through systemic administration. At the same time, the replication cycle of VACVs is fast and lytic, which allows them to proliferate rapidly and release rapidly after infecting host cells; In addition, a notable feature is their ability to replicate under hypoxic conditions, increasing their adaptability in the tumor microenvironment; Finally, because VACVs have no receptor restrictions on their entry into host cells, they exhibit high infectivity not only in various host species, but also in a wide range of tissue types, which facilitates their use in a variety of preclinical studies (30). At present, VV has been intensively studied in many preclinical and clinical studies, such as the NOV virus applied in colorectal cancer, which increases antitumor activity by replacing the vTk and VGF regions with TRAIL and Ang1 (31).

2.2 Encephalomyocarditis virus

The encephalomyocarditis virus (EMCV) is a single-stranded RNA picornavirus with a broad host range that infects various mammals and birds (32) (Table 1). EMCV causes sudden death, myocarditis, encephalitis, neurological disorders, and diabetes.

TABLE 1 Properties of oncolytic viruses.

Virus Type	Attribute
Smallpox virus	Enveloped double-stranded DNA virus
Enterovirus	Positive-sense single-stranded RNA virus
Measles virus	Enveloped single-stranded negative-sense RNA virus
Norovirus	Non-enveloped double-stranded RNA virus
Adenovirus	Non-enveloped double-stranded linear DNA virus

However, when EMCV infects people, it causes only mild disease (33). EMCV also can inhibit apoptosis and induce inflammatory reactions, which may play a role in tumor suppression (33). Unlike many viruses, which may not be able to overcome hypoxia-mediated inhibition of protein synthesis for viral replication, hypoxia or increased HIF activity common in solid tumors may inadvertently exacerbate EMCV replication and virulence due to various oncogenic mutations on general oxygen-sensitive pathways or a growing list of genes such as PTEN, TSC, and VHL. Although EMCV is currently primarily used in the treatment of kidney cancer, its potential as an oncolytic virus may extend far beyond kidney cancer, suggesting that it could have therapeutic potential for a variety of tumor types (34).

2.3 Measles virus

MV is an enveloped single-stranded negative-sense RNA virus belonging to the genus Morbillivirus of the family Paramyxoviridae (13) (Table 1). The anticancer properties of MV were first discovered in 1949 when wild-type MV infection led to the regression of Hodgkin's lymphoma. Oncolytic MV is an attenuated vaccine strain derived from the Edmonston-B (MV-Edm) vaccine lineage, which has been demonstrated to be safe and efficacious for cancer treatment in preclinical *in vitro* and *in vivo* studies (35). The MV receptor nectin-4 is abundantly expressed in lung, colon, ovarian, and breast cancers, making it a potential tumor marker (36). In addition to the tropism of MV to specific cell receptors, other underlying mechanisms contribute to the tumor selectivity of MV vaccine strains, such as defects in the IFN antiviral response pathway, which is often dysregulated in tumor cells to facilitate their escape from the host immune system (37). There are currently many open and recruiting MV clinical trials: such as Modified Measles Virus (NCT02962167) for Recurrent

Medulloblastoma or Recurrent ATRT, Measles Vaccine (NCT00828022) for Non-Small Cell Lung Cancer, and Progressive, Recurrent, or Refractory Ovarian Epithelial Cancer or Primary Peritoneal Cancer's Recombinant Measles Virus Vaccine (NCT00408590) and many more. Despite some clinical trials of oncolytic measles viruses, there is only one clinical trial underway involving an oncolytic measles virus expressing pro-inflammatory transgenes (38).

2.4 Reovirus

Reovirus is a non-enveloped double-stranded RNA virus that was initially isolated from the respiratory or intestinal tract of humans and animals (Table 1). However, it is not associated with any disease (except for infection in rodents and birds, generally not causing notable disease, especially in adult animals). The Reoviridae family consists of six genera among which orthoreoviruses can infect both animals and humans (14, 15).

Ras-activated tumor cells are effectively killed by reovirus, possibly due to double-stranded RNA-dependent kinase (PKR) inactivation, and efficient translation of viral proteins occurs in Ras-activated tumor cells, allowing for efficient production of progeny viruses. Reovirus was originally thought to function primarily through apoptosis (39). Apoptotic signals commonly exhibited by infected cells include IFN production and NF-κB activation, cytoplasmic dsRNA detection by PKR, retinoic acid-inducible gene I (RIG-I), or melanoma differentiation-associated protein 5 (MDA5), or inflammatory cytokines (e.g., TNF-associated apoptosis-induced ligand) in response to NF-κB and/or IRF3 signaling after σ1 and μ1 receptor binding or membrane penetration, TRAIL, which binds to surface death receptors and triggers activation of caspase-3 and -7. Blocking apoptotic caspases does not always eliminate reovirus-induced cell death, and necroptosis depends on viral dsRNA recognition and induction of type I IFN responses, as well as autophagy following acute endoplasmic reticulum (ER) stress, which have been identified as alternative modes of reovirus-induced cell death (40). Reovirus T3D is the most widely used oncolytic virus therapy (OVT). It is currently available for the treatment of glioma, ovarian, pancreatic, peritoneal, and gastric cancers, with the initial trial using reovirus as monotherapy, mostly given intravenously; There were almost no serious adverse events, and safety was demonstrated (41). Reovirus, as an OV therapeutic, has shown certain efficacy in preclinical models, but has been effective in only a minority of patients in clinical applications (40).

2.5 Adenovirus

Ad belongs to the non-enveloped virus class and contains a linear double-stranded DNA genome with a diameter of ~950 Å within a twenty-sided icosahedral capsid (19) (Table 1). Adenoviruses are relatively easy to produce in high titer and high purity, making them one of the most commonly used viral vectors for applications ranging from gene and cancer therapy to vaccine development (20). The E1A gene is the first gene to be expressed at the time of viral infection and is essential for the expression of all subsequent viral genes. Therefore, E1A deletion is commonly used to generate replication-deficient adenoviral vectors. In contrast, CRA is produced by mutating the E1A gene or replacing the native E1A promoter with a cancer-specific promoter to alter E1A expression. Because adenovirions can pack up to 105% of the length of the wild-type genome, it is common to remove certain parts of the viral gene that are necessary for virion formation, such as the E3 region, to insert the therapeutic gene into the recombinant adenovirus genome (42). At present, there are many kinds of adenoviruses that have entered clinical trials, including but not limited to bladder cancer, prostate cancer, and kidney cancer, and CG0070 has been administered intravesically to treat bladder cancer with good therapeutic results (43).

3 Application of oncolytic viruses in different genitourinary tumor treatments

3.1 Application in renal cell carcinoma treatment

RCC accounts for 3% to 5% of adult malignancies. The incidence is increasing annually in most countries, but the mortality rate is decreasing in developed countries (44). The etiology of RCC remains unclear, but it is associated with genetics, smoking, alcohol consumption, obesity, hypertension, antihypertensive drugs, and diabetes (45). Currently, treatment options for RCC mainly include targeted therapy drugs such as sorafenib, sunitinib, and pazopanib; immunotherapy drugs in combination therapy; and immune checkpoint inhibitors in combination with targeted drugs; or immune checkpoint inhibitors (46). Although drug therapy has shown good efficacy, cases of patient intolerance, significant side effects, or moderate treatment effects are known. Therefore, the development of new antitumor drugs is necessary and OVs have a great development potential as emerging treatment modality (Figure 2).

3.1.1 Vaccinia virus and renal cell carcinoma

VACV is considered to be an OV for RCC. It is generally used in combination with tumor drugs to enhance the efficacy or is modified to better target cancer cells. JX-594 is a thymidine kinase (TK) gene-inactivated oncolytic vaccinia virus expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) and lac-Z transgenes, and is the most widely used oncolytic vaccinia virus in clinical trials (47),designed to destroy cancer cells by replication-dependent cell lysis and stimulation of anti-tumor immunity (48). For example, Park et al. combined the oncolytic VACV JX-594 with programmed cell death protein-1 (PD-1) inhibitors to reshape the cellular environment into a tumor-suppressive environment, effectively reducing the primary tumor and metastatic burden and reducing liver damage (49). Similarly, they evaluated the efficacy of systemic JX-594 monotherapy versus sunitinib monotherapy in a mouse model of metastatic orthotopic

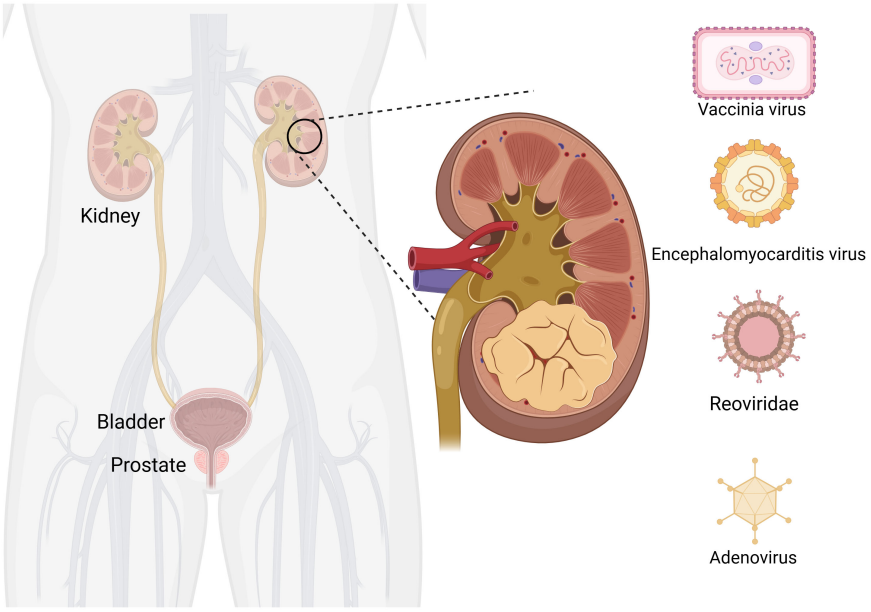

FIGURE 2
Oncolytic viruses are common in kidney cancer. Created with Biorender.com.

RCC as early as 2022. Compared with sunitinib monotherapy, systemic JX-594 monotherapy yielded significantly better treatment outcomes when cold tumors were converted to hot tumors, demonstrating better therapeutic efficacy in early and late mRCCs. Sunitinib monotherapy effectively inhibited early mRCC primary tumor growth and lung metastasis (50).

To increase the toxicity of the VACV to renal tumor cells, Fend et al. constructed a novel strain of VACV and verified its ability to inhibit tumor growth in models (64). This virus was constructed by deleting the thymidine kinase (TK) and ribonucleotide reductase (RR) genes and expressing the fusion suicide gene FCU1, which is derived from yeast cytosine deaminase and uracil phosphoribosyltransferase genes. In a xenograft mouse model, the percentage of tumor tissue necrosis in mice injected with this virus was higher than that in the control group. Currently, three oncolytic viruses derived from the cowpox virus have entered clinical application: Pexa-VEC combined with Cemiplimab is used in Phase I/II clinical trials, while JX-594 and TBio-6517 are used individually in Phase I clinical trials (Table 2).

3.1.2 Encephalomyocarditis virus and renal cell carcinoma

In recent years, a few studies were focused on the treatment of RCC using the EMCV. In 2010, Roos et al. reported that EMCV treatment rapidly reduces clear-cell RCC (CCRCC) growth (34).

TABLE 2 Clinical trials of oncolytic viruses.

Virus species	Virus Name	Type of cancer	Conbination	Mode of administration	Phase	NCT
VV	Pexa-VEC	RCC	Cemiplimab	Intravenous administration	I/II	NCT03294083
VV	JX-594	solid tumors	Null	Intravenous administration	I	NCT00625456
VV	TBio-6517	solid tumors	pembrolizumab	Subcutaneously injection	I	NCT02432963
VV	TBio-6517	solid tumors	Pembrolizumab	Intratumoral injection	1/2a	NCT04301011
VV	VET3-TGI	solid tumors	pembrolizumab	Intratumoral injection or Intravenous administration	I	NCT06444815
VV	PF-07263689	solid tumors	sasanlimab	Intravenous administration	I	NCT05061537
Ad	adenovirus-transfected DC	RCC	CIK	Not applicable	I/II	NCT01924156
Ad	GVAX	RCC	Null	Subcutaneous injection or intramuscular injection	IV	NCT00258687
Ad	ColoAd1	RCC	Null	Intravenous administration or intratumoral injection	I	NCT02053220

(Continued)

TABLE 2 Continued

Virus species	Virus Name	Type of cancer	Conbination	Mode of administration	Phase	NCT
Ad	adenovirus p53 dendritic cell vaccine SC	Progressive or recurrent metastatic cancer	Null	Subcutaneously injection	II	NCT00704938
Ad	DNX-2440	RCC	Null	Intratumoral injection	I	NCT04714983
Ad	Ad-p53	solid tumors	ICIs	Intratumoral injection	II	NCT03544723
Ad	CG0070	BC	Null	Bladder administration	I	NCT00109655
Ad	CG0070	NMIBC	Null	Bladder administration	I/II	NCT01438112
Ad	CG0070	NMIBC	Null	Not applicable	III	NCT06111235
Ad	CG0070	NMIBC	Null	Not applicable	Not applicable	NCT06443944
Ad	Ad-p53	BC	Null	Bladder injections	I	NCT00003167
VV	PANVAC	NMIBC	BCG	Not applicable	II	NCT02015104
Coxsackievirus	CVA21	NMIBC	mitomycin C	Bladder drip	I	NCT02316171
Reovirus	REOLYSIN®	MIBC	Gemcitabine and Cisplatin	Intratumoral administration	1b	NCT02723838
Ad	ETBX-071/ETBX-061/ ETBX-051	mCRPC	Null	Subcutaneously injection	I	NCT03481816
Ad	Adenovirus/PSA Vaccine	PC	ADT	Subcutaneously injection	II	NCT00583752
Ad	Adenovirus/PSA Vaccine	PC	Null	Subcutaneously injection	II	NCT00583024
Ad	ETBX-011, ETBX-061 and ETBX-051	solid tumors	Null	Subcutaneously injection	I	NCT03384316
Ad	AdNRGM	PC	CB1954	Intravenous administration	I	NCT04374240
Ad	Ad.hIL-12	PC	Null	Prostate injection	I	NCT00110526
Ad	ADV/RSV-tk	PC	Brachytherapy	Prostate injection	I/II	NCT01913106
Ad	AD5-SGE-REIC/Dkk-3	PC	Null	Prostate injection	1/2a	NCT01931046
Ad	M-VM3	PC	Null	Prostate injection	I	NCT02654938
Ad	Ad5-yCD/ mutTKSR39rep-hIL12	PC	Null	Prostate injection	I	NCT02555397
Ad	Adenoviral vector delivery of the IL-12 gene	PC	Null	Prostate injection	I	NCT00406939
Ad	M-VM3	PC	Null	Prostate injection	Ib2	NCT02844699
Ad	CV787/CG7870	PC	Null	Intravenous administration	I/II	NCT00116155
Ad	ChAdOx1.5T4-MVA.5T4	PC	Null	Bladder instillation	I	NCT02390063
Ad	Ad5-yCD/ mutTKSR39rep-ADP	PC	IRMT	Not applicable	II	NCT00583492
Ad	Ad-sig-hMUC-1/ecdCD40L	PC	Null	Subcutaneously injection	I	NCT02140996
Ad	VTP850	PC	ADT	Not applicable	I/II	NCT05617040
Ad	Ad-REIC/DKK-3	PC	Null	Prostate injection	I	NCT01197209
Ad	ORCA-010	PC	Null	Intratumoral administration	I/IIa	NCT04097002

They reported that hypoxia-inducible factor (HIF) increases the NF-κB-mediated antiapoptotic response in CCRCC and the inactivation of NF-κB weakens the toxicity of EMCV by triggering rapid apoptosis of infected cells, limiting viral replication, and leading to apoptosis of tumor cells. Immunohistochemical analysis of xenograft tumors showed that the necrotic area of tumors treated with EMCV was much larger and more prominent than that in the control group.

3.1.3 Reovirus and renal cell carcinoma

Reoviruses target tumor cells and generally do not cause notable symptoms in humans after infection; therefore, they have been widely used in tumor research. In most tumor cells, RAS is abnormally overexpressed, which promotes tumor growth and creates conditions for the oncolytic effects of the reovirus. Abnormally activated RAS signaling pathways inhibit the normal function of double-stranded RNA-dependent protein kinase (PKR), preventing its ability to inhibit virus replication by phosphorylating eukaryotic translation initiation factor 2α. This allows the viral genome to be freely transcribed and translated into tumor cells without being hindered by host defense mechanisms (51). In contrast, in non-cancerous cells, PKR can effectively recognize reoviral double-stranded RNA, dimerize rapidly, and initiate a defense response, effectively inhibiting viral replication. In addition, activation of the RAS pathway indirectly affects immune responses by promoting the activity of signaling molecules, such as phosphoinositide 3-kinase, mitogen-activated protein kinase, and extracellular signal-regulated kinase, ultimately inhibiting mRNA translation of the pattern recognition receptor RIG-1 and reducing the ability of cells to perceive and resist viral invasion. Reovirus also uses its σ3 protein as a "cloaking device" to hide its double-stranded RNA structure, further evading detection and activation by PKR, enhancing its survival in tumor cells. Lawson et al. reported that the combined treatment with VCN-01 (a proprietary genetically modified oncolytic reovirus) and cyclophosphamide improves the antitumor immune response in patients with mRCC (52). Reovirus has been shown to initiate an innate immune response characterized by the production of pro-inflammatory chemokines, including RANTES, MIP-1-α, MCP-1, KC, IP-10, and MIG, and can produce pro-inflammatory chemokines in a variety of melanoma and prostate cancer cell lines, in addition to their direct oncolytic effects (48, 53, 54). Cyclophosphamide pretreatment effectively depletes immunosuppressive regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), alleviates the immune inhibition of effector T cells, and enhances the antitumor immune response. Immunohistochemical analysis of xenograft tumors showed that the necrotic area of tumors treated with EMCV was much larger and more prominent than that in the control group.

3.1.4 Adenovirus and renal cell carcinoma

Research on Ads was primarily focused on their oncogenic properties, with limited applications in the treatment of kidney cancer. In 1983, Bernards et al. first constructed a recombinant Ad type 5 (Ad5) in which the E1b region of Ad5 was replaced with that of Ad12. Based on cell infection and analysis, they demonstrated that the recombinant virus effectively replicates in human embryonic kidney and HeLa cells (55).

Subsequently, Guse et al. modified the Ad and constructed Ad5/3-9HIF-Delta24-VEGFR-1-Ig, an oncolytic Ad with a 5/3 chimeric fiber, HRE (9HIF) driving E1 and E1A, and 24 bp deletion in VEGFR-1-Ig in the E3 region. Ad5/3-9HIF-D24-VEGFR-1-Ig exhibited good specificity and oncolytic activity against kidney cancer cells *in vitro* and demonstrated antitumor efficacy in subcutaneous *in vivo* models (56).

Dendritic cells (DCs) are the most powerful full-time antigen presenting cells (APCs) in the body, which can efficiently uptake, process, process and present antigens, immature DCs have strong migration ability, mature DCs can effectively activate naïve T cells, and are at the center of initiating, regulating and maintaining immune responses. Ad-assembled DC vaccines (DCs-CD137L/CAIX) have shown limited therapeutic efficacy in targeting antigens for kidney cancer treatment. Ding et al. combined dendritic cells with an oncolytic Ad to facilitate the entry of dendritic cells into kidney cancer cells, inducing a persistent protective effect against tumors through the generation of memory cell-mediated immune responses (57). OVs enhance the effectiveness of immunotherapy and interleukin-12 (IL12) increases the antitumor activity. Based on combination, the oncolytic Ad OAV-IL-12 was developed to enhance the immunocytotoxic effects of non-replicating Ad-based DC vaccines. Similarly, Fang et al. combined chimeric antigen receptor T (CAR-T) cells with an oncolytic Ad carrying chemokine (C-C motif) ligand 5 (CCL5) and IL12 to create Ad5-ZD55-hCCL5-hIL12 (58). It has been shown that this combination infects and replicates in renal cancer cell lines, demonstrating its ability to suppress tumor proliferation (58). The combination did not reduce but promote the therapeutic effects, prolonging the survival time of mice and increasing survival rates. A variety of adenovirus vectors, including adenovirus-transfected DCs, GVAX, ColoAd1, DNX-2440, and Ad-p53, have been applied in clinical trials for renal cell carcinoma (RCC). Among these trials, some are in Phase I, while the clinical trial for GVAX has progressed to Phase IV (Table 2).

OV therapies for kidney cancer have undergone significant developments. Early studies were focused on the genetic modification of viruses, such as VACV and Ad, to enhance their tumor targeting and apoptotic induction capabilities. In recent years, researchers have begun to explore the combined use of OVs with existing drugs such as PD-1 inhibitors and sunitinib, leveraging the synergistic effects between drugs to enhance the treatment efficacy and improve patient survival rates. For example, the combination of VACV JX-594 and PD-1 inhibitors effectively reduced the tumor burden, whereas the combination of Ad with CAR-T cells, chemokines, and cytokines promoted the therapeutic effects. These results provide new strategies for the treatment of kidney cancer. In addition to PD-1 inhibitors, the following modifications and combinations have been explored: CRISPR-Cas9 genome editing: Recent advances in genome editing technologies, such as CRISPR-Cas9, have enabled the precise modification of OV genomes to enhance their safety, targeting, and oncolytic potency (59). This approach has the potential to overcome viral resistance and improve therapeutic outcomes. Combination with chemotherapy: OVs have been combined with chemotherapeutic agents to improve treatment outcomes. For instance, CG8840 adenovirus demonstrated a synergistic antitumor effect when combined with docetaxel in bladder cancer models (22). Similar studies have shown enhanced efficacy when OVs are combined with cisplatin, gemcitabine, or paclitaxel. Nanoparticle-mediated delivery: OVs can be encapsulated within nanoparticles for targeted delivery and protection from immune

neutralization. This approach has been used to improve the stability, biodistribution, and efficacy of OVs (49) (Tables 2–4).

3.2 Application in bladder cancer treatment

Worldwide, BC ranks 10th in the incidence of malignancy. Its main causes are smoking and long-term exposure to industrial chemicals (75). The main treatment methods for non-muscle-invasive BC are surgical treatment and intravesical perfusion, which can be divided into intravesical chemotherapy and intravesical immunotherapy (76). Neoadjuvant chemotherapy combined with radical cystectomy and pelvic lymph node dissection is the mainstay (67). Although various treatment options are available for BC, there is a lack of effective treatments for incurable resectable and metastatic BC. The emergence of OVs provides new ideas for the treatment of BC (Figure 3).

3.2.1 Adenovirus and bladder cancer

The treatment efficacy of BC has been improved by partially modifying the genes of Ads or combining them with other treatment modalities. A replication-competent attenuated Ad

TABLE 3 Combination of oncolytic viruses with antitumor drugs.

Type of cancer	Virus	Virus name	Targeted drug	Reference	Mode of administration	Novel Payload
Renal cell carcinoma	VV	JX-594	ICIs	Park et al. (49)	Intraperitoneal injection	None
Renal cell carcinoma	VV	JX-595	Sunitinib	Park et al. (50)	Intraperitoneal injection	None
Renal cell carcinoma	REO	None	Sunitinib	Lawson et al. (52)	Intraperitoneal injection	None
Renal cell carcinoma	Ad	DCs-CD137L/CAIX	DC	Ding et al. (57)	Peritumorally injected	OAV-IL-12
Renal cell carcinoma	Ad	Ad5-ZD55-hCCL5-hIL12	CAR-T	Fang et al., (58)	Intratumorally administered	CCL5 and IL12
Bladder cancer	CVB	CVA21	Mitomycin C	Annels et al. (60)	Intravesical administration	None
Bladder cancer	CVB	CVA21	Pembrolizumab	Rudin et al. (61)	Intravenous administration	None
Bladder cancer	MRV	T3D-C	Protein 1 (PD-1) inhibitor	Smelser et al. (62)	Intraperitoneal injection	None
Bladder cancer	MRV	RC402 and RP116	NK cell	Lim et al. (63)	Intravesical administration	None

TABLE 4 Oncolytic virus monotherapy.

Type of cancer	Virus name	Virus	Effect	Reference
Renal cell carcinoma	VV-FCU1	VV	Inhibits orthotopic tumor growth	Fend et al. (64)
Renal cell carcinoma	EMCV	EMCV	Causes inactivation of NF-κB	Roos et al. (34)
Renal cell carcinoma	MV-GFP	MV	Antitumor effect	Miest et al. (65)
Renal cell carcinoma	Ad5	Ad	Replicate in tumors	Bernards et al. (55)
Renal cell carcinoma	Ad5/3-9HIF-Delta24-VEGFR-1-Ig	Ad	Antitumor effect	Guse et al. (56)
Renal cell carcinoma	OAV-IL-12	Ad	Enables dendritic cells to enter cancer cells	Ding et al. (57)
Renal cell carcinoma	Ad5-ZD55-hCCL5-hIL12	Ad	Inhibits tumor expansion	Fang et al., (66)
Bladder cancer	CG8840	Ad	Inhibits tumor expansion	Zhang et al. (67)
Bladder cancer	CG0070	Ad	Inhibits tumor expansion	Ramesh et al., (68)
Bladder cancer	Ad.shDCIR	Ad	Improves T cell activity	Hu et al., (69)
Bladder cancer	vAd-VEGFR-3	Ad	Antitumor effect	Hao et al., (70)
Bladder cancer	VACV	OC	Antitumor effect	Potts et al. (71)
Bladder cancer	rVV-TK-53	OC	Induce P53 expression	Fodor et al. (72)
Bladder cancer	CVA21	CVB	Inhibits tumor expansion	Annels et al. (60)
Prostate cancer	Ad5-IL-12	Ad	Inhibits tumor expansion	Nyati et al. (73)
Prostate cancer	AdKi67-C3	Ad	Inhibits tumor expansion	Fang et al., (74)

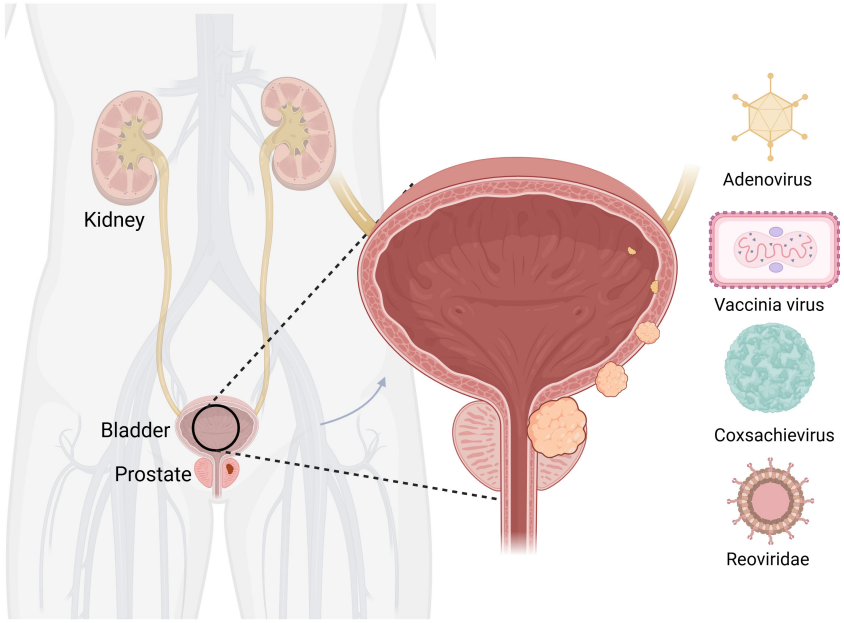

FIGURE 3
Oncolytic viruses are common in bladder cancer. Created with Biorender.com

variant, CG8840, was created by linking a DNA segment upstream of genes expressing urinary tract proteins to a promoterless firefly luciferase reporter gene. The replication capacity of the Ad variant in bladder transitional cell carcinoma (TCC) cells was assessed using the virus yield and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (68). Compared with non-bladder cells, CG8840 efficiently replicated and eliminated bladder TCC cells with high specificity. In xenograft models of human BC, both the intratumoral and intravenous administration of CG8840 significantly suppressed the tumor growth. When CG8840 was used in combination with docetaxel, a synergistic antitumor effect was observed (68). A marketed oncolytic Ad, CG0070, is used to treat BC (77). CG0070 is based on a modified Ad5 backbone and incorporates a tumor-specific promoter and granulocyte-macrophage colony-stimulating factor (GM-CSF) transgene. It operates through two main mechanisms: 1) it replicates within tumor cells, leading to tumor cell lysis and immunogenic cell death; and 2) the rupture of cancer cells releases tumor-derived antigens and GM-CSF, stimulating systemic antitumor immune responses involving host leukocytes. For the treatment of non-muscle-invasive bladder cancer (NMIBC) that does not respond to interleukin therapy, a Phase II clinical trial of CG0070 in combination with pembrolizumab has been completed. The results of the trial showed that no patients developed muscle-invasive bladder cancer (MIBC) or metastatic bladder cancer, and there were no unintended immune-related adverse effects (78). Both *in vitro* and *in vivo* studies demonstrated that CG0070 possesses selective replication, cytotoxicity, GM-CSF production, and antitumor efficacy in various BC models (77).

In addition to modifications, Ads can be combined with traditional treatment modalities, such as chemotherapy, radiotherapy, and platinum-based drugs, to enhance their effectiveness. CG0070 has been used in clinical trials for BC and NMIBC, including Phase I, Phase I/II, and Phase III (Table 2).

3.2.2 Vaccinia virus and bladder cancer

In recent years, there has been limited research on the application of VACV in the treatment of BC. In 2001, Gomella et al. delivered live virus directly to the human bladder for the first time, demonstrating that VACV can be safely administered to the bladder by recruiting lymphocytes and inducing a rapid local inflammatory response (71). To enhance the tumor-specific recognition and cell killing ability of VACV, Potts et al. mutated the F4L and J2R sites, which encodes a viral homolog of the ribonucleotide reductase small subunit (RRM2) involved in cell cycle regulation, to produce a novel oncolytic VACV (72). The tumor selectivity and cell-killing ability of VACV were validated by *in situ* inoculation of human BC cells into rat bladders. Similarly, in 2005, Fodor et al. used a recombinant VACV expressing human p53 to detect the virus's oncolytic effects its ability to induce p53 transgene-mediated death by assessing the tumor incidence, survival rate, and transgene expression in cultured mouse BC MB-49 cells and cells grown *in situ* in genetically modified mice (79).

3.2.3 Coxsackievirus and bladder cancer

The application of coxsackieviruses in BC treatment typically involves assisting Ads in effectively infecting BC cells (80). Coxsackievirus and Ad receptors (CAR) are considered to be the primary receptors for Ads and are commonly used as gene delivery vectors. The most common coxsackievirus subtype in BC is A21. Coxsackievirus A21 (CVA21) is a novel intercellular adhesion molecule-1 (ICAM-1)-targeted immunotherapeutic virus. Annels

et al. investigated the cytotoxicity induced by CVA21 in a series of human BC cell lines, revealing a sensitivity closely associated with the expression of the viral receptor ICAM-1, and studied the ability of CVA21 to induce immunogenic cell death (60). The following year, they completed a phase I trial of CVA21 oncolytic therapy for non-muscle-invasive BC (61). The results showed that, when used alone or in combination with mitomycin C, coxsackievirus led to interferon(IFN) induction, including immune checkpoint inhibitory genes (PD-L1 and LAG3) and Th1-related chemokines, as well as induced the innate activator RIG-I, genes associated with the Th1-mediated immune response, and caused significant inflammatory changes in Non-muscle-invasive bladder cancer (NMIBC) tissue biopsies (61). In 2023, Charles et al. published a study on the safety of the intravenous injection of coxsackievirus A21 (V937) alone or in combination with pembrolizumab in patients with late-stage cancer. They showed that intravenous injection of V937+pembrolizumab is safe; however, in non-small cell lung cancer and BC, its efficacy was not superior to that of previous monotherapy with pembrolizumab, although V937 could be detected in tumor tissue (81). Currently, CVA21 in combination with Mitomycin C has been used in a Phase I clinical trial for NMIBC (Table 2).

3.2.4 Reovirus and bladder cancer

The earliest discovery of the ability of bluetongue virus in Reoviridae to produce large amounts of interferon was made by stimulating animals and cell cultures (including human leukocytes) and continuous cell lines (82). In 2003, Kilani et al. first reported preclinical studies of coxsackievirus-mediated oncolysis in BC (83). Hanel et al. were the first to use coxsackievirus *in situ* in a bladder tumor model for the treatment of superficial BC, studying the ability of the virus to kill BC cells *in vitro* and inhibit tumor growth *in vivo* (62). Compared with the complications of the Bacillus Calmette-Guerin Vaccine (BCG vaccine), coxsackieviruses have fewer side effects; the tumor-free survival rate of animals treated with coxsackievirus is significantly higher than that of animals receiving immunotherapy or saline treatment (62). Smelser et al. concluded that the single intravesical administration of coxsackievirus, PD-1 inhibitor, or a combination injection resulted in a higher survival rate in mice with *in situ* bladder tumors compared with the control group (63). Similarly, Lim et al. evaluated the effect of combined treatment with natural killer (NK) cells and coxsackievirus on BC cells using an *in vitro* assay and reported the effective cytotoxicity in metastatic tumor cells (84). Coxsackievirus research started with its ability to produce interferons similar to BC. Subsequently, it was used for the killing of BC. Currently, research leans towards combining it with immunotherapy and testing the effectiveness of this combination.

Ads, VACVs, coxsackieviruses, and reoviruses have been extensively studied and used in BC treatment because of their unique biological properties. These viruses can be engineered to enhance their selectivity for recognizing and killing BC cells, thereby improving the efficacy of BC treatments. OVs yield better treatment outcomes and quality of life for patients with BC. Currently, the clinical application of oncolytic viruses for the treatment of bladder cancer remains limited, thus further research and development are needed to expand their scope of application (Tables 2–4).

3.3 Application in prostate cancer treatment

PC is the most common malignant tumor of the genitourinary system in men. The incidence of PC in China has significantly increased in recent years (75). Currently, the most recent view is that PC is mainly caused by genetic (85) and sex hormone disorders (86). The primary treatments for organ-limited and locally advanced PC include radical prostatectomy and radiotherapy. Radical resection and radiotherapy are the main treatment methods for PC recurrence after curative therapy (87). Metastatic PC is mainly mediated by androgen deprivation (88). The development of OVs has provided new solutions for the treatment of PC (Figure 4).

3.3.1 Adenovirus and prostate cancer

The use of Ads for oncolytic therapy of PC has led to some success. Ads used in the clinical treatment of PC include CV706 (89), CG7870 (90), Ad5-CD/TKrep (FGR (91), and Ad5-yCD/mutTKSR39rep-ADP (73). In addition, many drugs that use oncolytic Ads as a vector for treating PC are still in clinical research. For example, Nyati et al. used an Ad as a vector to deliver suicide and IL12 genes to tumor tissues (92). This trial entered phase I clinical trials and demonstrated a good tolerability when the replication-competent Ad5-IL-12 (Ad5-yCD/mutTKSR39rep-hIL-12) was locally administered to prostate tumors (92). Autio et al. utilized AdC68 vectors to express three selected PC-specific antigens: prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), and prostate stem cell antigen (PSCA), along with plasmid DNA (PF-06755990), monoclonal antibodies targeting cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), pembrolizumab (PF-06753388). This drug has entered phase I trials (NCT02616185). Overall, PF-06753512 a vaccine-based immunotherapy regimen (VBIR) has been declared safe similar to other immune checkpoint inhibitor combination trials; it stimulates antigen-specific immune responses in all cohorts and produces moderate antitumor activity in patients with B-cell receptor (BCR), without the use of ADT (74).

Although many clinical trials have been initiated, the use of Ad as a vector for the treatment of PC is still being investigated. Fang et al. aimed to enhance the efficacy of CAR-T cells against solid tumors. They constructed a novel recombinant oncolytic Ad controlled by the Ki67 promoter, carrying CCL5, IL12, and IFN-γ genes (named AdKi67-C3), which significantly promoted the proliferation and persistence of CAR-T cells *in vitro* and *in vivo* and established long-term antitumor immune responses (93). Gavrikova et al. overcame the shortcomings of control elements and poor infectivity using fiber modification and an androgen-independent promoter (cyclooxygenase-2, COX-2). The results of both *in vitro* and *in vivo* studies showed potent antitumor effects

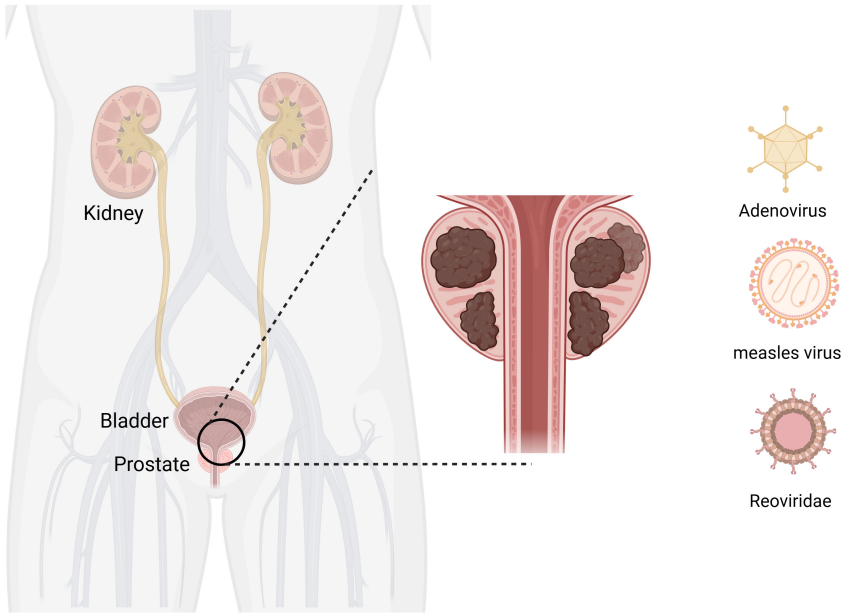

FIGURE 4
Oncolytic viruses are common in prostate cancer. Created with Biorender.com

(94). Currently, many Ads are being used in preclinical research. Various adenovirus vectors such as Adenovirus/PSA Vaccine, ETBX-011, ETBX-061, ETBX-051, AdNRGM, Ad-REIC/DKK-3, and ORCA-010 etc. have entered clinical trials for prostate cancer (PC), with trial phases ranging from Phase I to Phase II. Some trials have combined other treatment methods such as ADT, CB1954, and IRMT (Table 2). It is evident that there is a relatively large number of adenoviruses currently applied to prostate cancer.

3.3.2 Measles virus and prostate cancer

MV is less commonly used in PC. Recent research was primarily focused on the use of green synthesis-encapsulated attenuated MV to create a novel controllable targeted viral delivery system with ligand-coated surfaces (95). This synthetic virus actively targets cancer cells, protects the virus from antibody clearance, releases OVs via receptor-mediated endocytosis, achieves efficient oncolytic immunotherapy, and enhances targeting (95). Opyrchal et al. studied the effect of actin cytoskeleton regulatory factor inhibition on the oncolytic effect of the MV (96). Msaouel et al. created a MV capable of expressing a human sodium iodide symporter, enabling the virus to induce oncolysis and its use for imaging through iodine-125 (125I) uptake measurements (97).

3.3.3 Enterobacteria and prostate cancer

Enterobacteria can effectively replicate in cells with activated RAS signaling pathways. More importantly, untransformed cells are not sensitive to enterobacteria, indicating the selective infectivity of the virus. Coffey et al. made an exciting discovery: a single intratumoral injection of enterobacteria led to the regression of 65%–80% of tumors in mice (98).

The use of Ads has led to significant progress in the treatment of PC and good potential has been demonstrated in clinical trials. Currently, various Ads are used for the oncolytic therapy of PC including CV706, CG7870, Ad5-CD/TKrep (FGR), and Ad5-yCD/mutTKSR39rep-ADP. These viruses treat PC through different mechanisms such as direct destruction of cancer cells, activation of the immune system, or delivery of anticancer genes. Further research and development of these viruses will lead to more secure, effective, and personalized treatment options for patients with PC (Tables 2–4).

4 Conclusion and challenges

The use of OVs as a potential treatment modality for urological tumors has witnessed a surge in advancement and research. Widely studied OVs include VACV (99), EMCV (100), Ad (101), MV (102), coxsackievirus (103), and reovirus (104), all of which have unique properties and mechanisms of action. The core of research in this area revolves around the genetic modification of these viruses, with the aim of minimizing their adverse effects on healthy cells while maximizing their ability to specifically target and eradicate tumor cells. As a result of these efforts, several OVs have transitioned from laboratory to clinical settings, providing cancer patients novel immunotherapeutic options. For example, the oncolytic Ad CG0070, which is currently in phase II clinical trials, targets bladder tumor cells via a defective retinoblastoma pathway, providing a new solution for patients with BCG-unresponsive non-muscle-invasive BC (105). Recent clinical trials yielded promising results, highlighting the efficacy of OVs in treating urological malignancies (61, 81). This emerging modality represents a ray of hope for cancer patients, providing a potential alternative to traditional treatment methods.

With the advancements in technology and further research, the design of OVs has become increasingly refined. Oncolytic viruses

(OVs) are able to trigger cell lysis when they replicate within cancer cells, and as the virus replicates, the number of viruses increases, thus enhancing the destructive power to tumors. At the same time, proteins produced during viral replication also have toxic effects on tumor cells. In addition to directly killing tumor cells, oncolytic viruses can also function through two immune mechanisms: one is to induce a non-specific immune response, and the other is to activate a specific anti-tumor immune response. In addition, oncolytic viruses can significantly increase the sensitivity of tumor cells to chemotherapy and radiotherapy, thereby enhancing the efficacy of these traditional treatments. Precision engineering enables the targeted delivery of viruses to tumor cells, minimizing collateral damage to healthy tissues. The mode of administration of oncolytic viruses is a crucial factor influencing their efficacy and safety in treating genitourinary tumors. Oncolytic viruses can be administered via various routes, including systemic delivery (such as intravenous injection) and loco-regional delivery (such as intratumoral or intravesical injection). Systemic administration enables widespread distribution of the virus throughout the body, which can be beneficial for metastatic tumors. However, it also increases the risk of systemic toxicity. In contrast, loco-regional administration allows for targeted delivery to specific tumor sites, minimizing off-target effects and potentially enhancing the local immune response. For instance, intravesical administration of oncolytic viruses for bladder cancer has shown promising results while minimizing systemic side effects. Therefore, the choice of administration route is guided by the type and stage of the tumor, as well as the patient's overall health condition. As clinical trials progress, a deeper understanding of the underlying mechanisms and optimal treatment protocols for oncolytic viral therapies will emerge. This knowledge provides a solid foundation for the clinical application of these viruses and their integration into existing treatment paradigms. However, despite the remarkable progress achieved in OV therapy, several challenges remain: 1) Ensuring the safety of the viruses and minimizing potential adverse events remain crucial; 2) Reducing the treatment cost is essential to provide access to a wider patient population; 3) Exploring the synergistic potential of combining OVs with other therapies, such as chemotherapy or immunotherapy, holds promise for enhancing treatment outcomes. In the future, with the continuous advancement of technology and the deepening of clinical trials, the design of oncolytic viruses will be more precise, able to target tumor cells more effectively, and reduce the damage to normal cells. In addition, a better understanding of the mechanism of action of oncolytic virus therapy will help to develop personalized treatment plans to provide the most suitable treatment option for each patient. Although oncolytic virus therapy has shown great potential in the field of urological cancer treatment, it still needs interdisciplinary cooperation, continuous funding and policy support to achieve its wide clinical application. Through these efforts, oncolytic virus therapy is expected to become one of the important means of urinary cancer treatment, bringing new hope and options to patients. Ongoing research, development, and clinical applications are imperative to address these challenges and further advance the field (Table 2).

In conclusion, OV therapy is a promising new cancer treatment modality. Although several challenges remain, its prospects are promising considering continuous technological advancements and more detailed clinical investigations.

Author contributions

JZ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. KL: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft, Methodology, Writing – review & editing. SS: Writing – review & editing, Supervision. DW: Supervision, Writing – review & editing. YZ: Supervision, Writing – review & editing. LZ: Supervision, Writing – review & editing. YL: Supervision, Writing – review & editing. DJ: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. JY: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. ZZ: Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glossary

Ad	adenovirus
ADT	androgen deprivation therapy
BC	bladder cancer
BCR	B-cell receptor
CAR-T	chimeric antigen receptor T
CCRCC	clear cell renal carcinoma
COX-2	cyclooxygenase-2
CTLA-4	cytotoxic T-lymphocyte-associated antigen 4
DCs	dendritic cells
EMCV	encephalomyocarditis virus
GM-CSF	granulocyte-macrophage colony-stimulating factor
IL12	interleukin-12
MDSCs	myeloid-derived suppressor cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MV	measles virus
NK	natural killer
NMIBC	non-muscle-invasive bladder cancer
OVs	oncolytic viruses
PC	prostate cancer
PD-1	programmed cell death protein 1
PKR	double-stranded RNA-dependent protein kinase
PSA	prostate-specific antigen
PSCA	prostate stem cell antigen
PSMA	prostate-specific membrane antigen
RCC	renal cell carcinoma
RR	ribonucleotide reductase
TCC	transitional cell carcinoma
TK	thymidine kinase
TURBT	transurethral resection of bladder tumor
VACV	vaccinia virus
VBIR	vaccine-based immunotherapy regimen

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Comparison of differences in immune cells and immune microenvironment among different kinds of oncolytic virus treatments

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Oncolytic viruses are either naturally occurring or genetically engineered viruses that can activate immune cells and selectively replicate in and destroy cancer cells without damaging healthy tissues. Oncolytic virus therapy (OVT) represents an emerging treatment approach for cancer. In this review, we outline the properties of oncolytic viruses and then offer an overview of the immune cells and tumor microenvironment (TME) across various OVTs. A thorough understanding of the immunological mechanisms involved in OVTs could lead to the identification of novel and more effective therapeutic targets for cancer treatment.

KEYWORDS

oncolytic virus, tumor, treatment, immune cell, tumor microenvironment

1 Introduction

Treatment options for tumors have expanded significantly in recent years, driven by an enhanced understanding of the immunologic mechanisms underlying these diseases (1, 2). These options now include traditional surgical treatments and chemoradiotherapy, as well as innovative approaches like immune checkpoint inhibitors, chimeric antigen receptor T-cells, adoptive cell immunotherapy, monoclonal antibodies, nanoantibodies, and other forms of immunotherapy (3–7). While surgical resection remains the most effective treatment for cancer and can substantially relieve symptoms, it is often not viable for patients in advanced stages of the disease. Moreover, surgery often leads to distant metastasis and local recurrence post-operatively (8). Chemoradiotherapy can decelerate cancer growth and prolong survival, however, it poses severe risks due to its damaging effects on normal cells while targeting cancer cells (9, 10). Immunotherapies, despite their potential, benefit only a select group of patients due to various immunosuppressive factors such as immune system suppression, deficiencies in cytokine types, reduced activity of tumor-infiltrating lymphocytes, impaired function of antigen-presenting cells, and

weakened effector T-cell activity (11–13). Oncolytic viruses have emerged as one of the most promising treatments to address these challenges (14). These viruses are capable of selectively replicating within tumor cells, delivering multiple eukaryotic transgene payloads, inducing immunogenic cell death, enhancing antitumor immunity, and exhibiting a safety profile that generally does not overlap with other cancer treatments (15, 16). Recent clinical trials of oncolytic virus therapy (OVT) have shown minimal severe adverse reactions, with only local injection site reactions and low-grade systemic symptoms noted (17–20). In 2005, Chinese regulators approved the first genetically modified oncolytic adenovirus for the treatment of nasopharyngeal carcinoma in combination with chemotherapy, marking the world's first oncolytic virus approved for clinical cancer treatment (21).

Oncolytic viruses, either genetically modified or naturally occurring, have the capability to activate immune cells that specifically target and destroy cancer cells while sparing healthy cells (22). These viruses selectively infect tumor cells harboring mutations associated with malignancies (23), and often have modifications in or deletions of certain viral genes essential for replication in normal cells but not in tumor cells (24). This selective infection is facilitated by the viruses' ability to bind specifically to certain receptors on tumor cells, enhanced by altering the virus's tropism (25). The viral genes are controlled by tumor-specific and/or tissue-specific gene promoters, enhancing the safety by confining viral replication to tumor cells (26, 27). Tumor cells frequently exhibit dysfunctional immune response signaling pathways, which support the virus's replication and proliferation within them (28, 29). Oncolytic viruses inhibit the production of host cellular products while enhancing viral product synthesis within the tumor cells. When oncolytic viruses lyse tumor cells, they release large quantities of tumor antigens that are then processed by antigen-presenting cells, triggering T-cells to target both infected and uninfected tumor cells (30, 31). Upon infecting tumor cells, the viruses can either directly affect immune cells or stimulate the tumor cells to produce more cytokines, thereby enhancing the immune system to eliminate any remaining tumor cells by phagocytosing them (32). Oncolytic viruses induce various cell death pathways in cancer cells, including necroptosis, pyroptosis, apoptosis, and autophagy (Figure 1) (33–36). For example, the N-terminal gasdermin domain (GSDMNT), when delivered into tumor cells via a recombinant adeno-associated virus, induces pyroptosis, offering significant therapeutic potential (34). Similarly, the oncolytic vaccinia virus, when armed with the aphrocallistes vastus lectin gene, can alter the metabolism of hepatocellular carcinoma cells, increase reactive oxygen species (ROS) production, and promote apoptosis, all contributing to its antitumor effects (37). OVT works by killing cancer cells using viruses that have a selective replication function (1, 38). There are variations among oncolytic viruses in terms of how they interact with immune cells and the tumor microenvironment (TME) during treatment (39, 40). In this paper, we provide a detailed overview of the immune cells, TME, and oncolytic viruses, and discuss the variations in TME and immune responses observed with current OVTs.

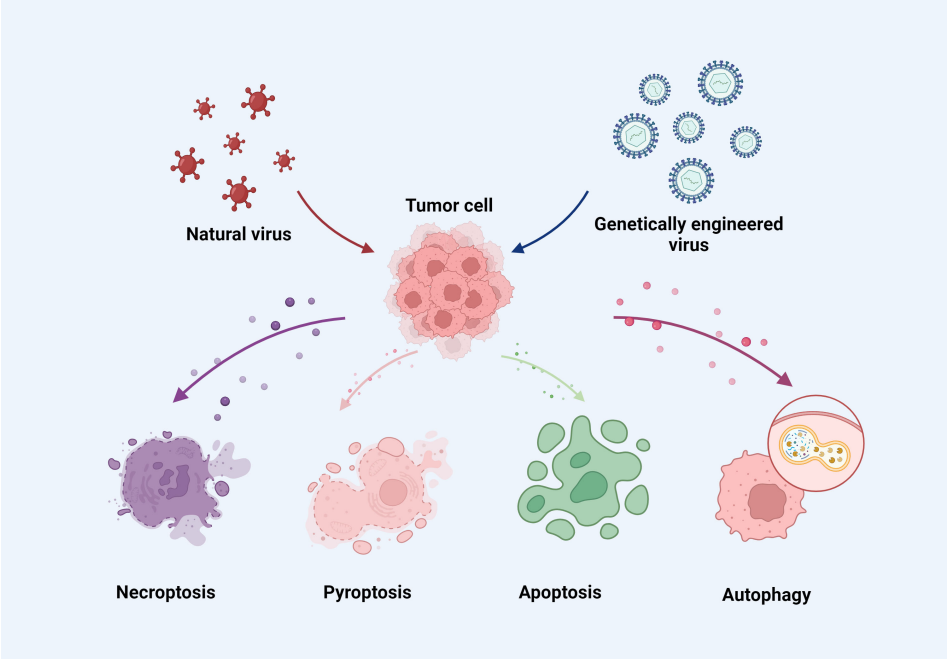

FIGURE 1
Oncolytic viruses induce necroptosis, pyroptosis, apoptosis and autophagy in cancer cells.

2 Origins and characteristics of oncolytic viruses

For nearly a century, researchers have been studying viruses. The first viruses identified were the foot-and-mouth disease virus from animals and the contagium vivium fluidum from plants, both discovered in 1898 (41, 42). Additionally, the yellow fever virus was first identified in 1901 and associated with human disease (43, 44). Advances in technology and an enhanced understanding of virus morphology and virulence have enabled ex vivo virus replication. This has facilitated the linking of several diseases, including rabies and influenza, to specific viruses (45, 46). Subsequent research has provided a detailed understanding of viral species, including their structure and biological characteristics (47). Simultaneously, researchers discovered that the virus may be utilized to cure tumors in addition to causing infectious diseases (48, 49).

Studies have documented cases of tumor regression following viral infection (49, 50). However, these remissions were typically short-lived, generally lasting only one to two months. In 1922, Levaditi and colleagues observed that the vaccinia virus could inhibit various cancers in mice and rats (51). In 1950, Pack reported remissions in patients with metastatic melanoma who were treated with the rabies virus. Additionally, patients with hematological malignancies such as leukemia or lymphoma experienced remission following infections with chickenpox or influenza (52, 53). Concurrent regressions of leukemia, Hodgkin's disease, and Burkitt's lymphoma have also been noted during measles infections, suggesting that under the right circumstances, certain viruses can target malignancies effectively without endangering the patient (54, 55). For instance, in a study of juvenile diffuse intrinsic pontine glioma treated with the oncolytic DNX-2401 virus, magnetic resonance imaging showed a reduction in tumor volume in 75% of the cases, and 66.7% of the patients achieved stable disease (56). Furthermore, a study involving 19 patients with recurrent glioblastoma treated with the oncolytic virus DNX-2401 demonstrated its safety and feasibility; notably, one patient achieved complete regression and was still alive eight years later (57).

Various viruses have been evaluated for their oncolytic properties in human tumor cell lines prior to their progressive utilization in clinical trials and other therapeutic contexts (58). In models of KRAS-mutated colorectal cancer, such as HCT116 colon cancer cells and patient-derived peripheral blood mononuclear cells, the oncolytic reovirus (pelareorep) exploits host autophagic machinery to enhance its proliferation and achieve selective oncolysis (59). In a phase 1 dose-escalation study, patients with newly diagnosed high-grade glioma who were treated with neural stem cell-delivered engineered oncolytic adenovirus showed promising safety and efficacy (60). Originally, hepatitis viruses were used to treat hematological malignancies and leukemia symptoms remitted in patients infected with adenovirus or EB virus (61). Subsequently, Alice Moore solidified the existence of an oncolytic virus in 1950 through *in vivo* tumor models and clinical and preclinical research (62). Oncolytic viruses, defined as naturally occurring or genetically modified viruses, selectively replicate in tumor cells, destroying them without harming healthy tissues and can also stimulate systemic or localized anti-tumor immunity (14, 63). These viruses target specific cell surface receptors overly expressed by cancer cells and naturally prefer cell surface proteins, enabling them to bind these receptors and penetrate the cells (64, 65). Oncolytic viruses are divided into two main categories: RNA and DNA viruses, including single-stranded (ssRNA and ssDNA) and double-stranded (dsRNA and dsDNA) viruses (Figure 2) (66).The most common RNA viruses used are measles and coxsackie virus group B, while commonly used DNA viruses include adenovirus, herpes simplex virus 1, and vaccinia virus. Except for vaccinia, DNA viruses have longer replication cycles than RNA viruses and replicate in the nuclei of infected cells (67, 68). Oncolytic viruses can also be classified into genetically engineered and naturally occurring strains. Engineered strains exhibit decreased pathogenicity, enhanced tumor expression, and increased lethality compared to wild-type strains (69). Mutations in the presence or lack of certain viral genes that are necessary for the virus to multiply in healthy cells but not in tumor cells (24). Genetically engineered oncolytic viruses are also designed to improve targeting selectivity for tumor cells and host cells with cancer-related mutations (70). Oncolytic viruses are intended to identify tumor-upregulated receptors, allowing the virus for an improved fidelity (71).To increase safety and limit viral replication to tumor cells, their principal genes are cloned into tumor-specific or tissue-specific promoters (26, 27). Besides their direct oncolytic activity of preferentially replicating within and destroying cancer cells, oncolytic viruses are highly effective in triggering immune responses against both the tumor cells and themselves. They are capable of inducing both local and systemic anti-tumor immunity, utilizing immune evasion strategies employed by cancer cells (72).

3 Tumor-associated immune cells and immune microenvironment

3.1 Tumor-associated immune microenvironment

Since the 1970s, there has been accumulating evidence that TME plays a crucial role in tumor development, either by facilitating or hindering it (73, 74). Additionally, Stephen Paget's "seed and soil theory," introduced in 1989, conceptualized the interplay between cancer cells and the TME (75, 76).There is increasing evidence that tumor progression requires the recruitment and reprogramming of adjacent normal cells. The TME consists of a complex network of exosomes, the extracellular matrix (ECM), and stromal cells. During the occurrence and progression of cancer, tumor cells interact with surrounding cells to influence the cancer's spread, proliferation, immune evasion, and chemoresistance within the TME; concurrently, these components undergo dynamic changes (77, 78). Different tumor locations and types exhibit specific TMEs, notable for their heterogeneity and dynamic changes. In colorectal cancer, stratifying patients based on

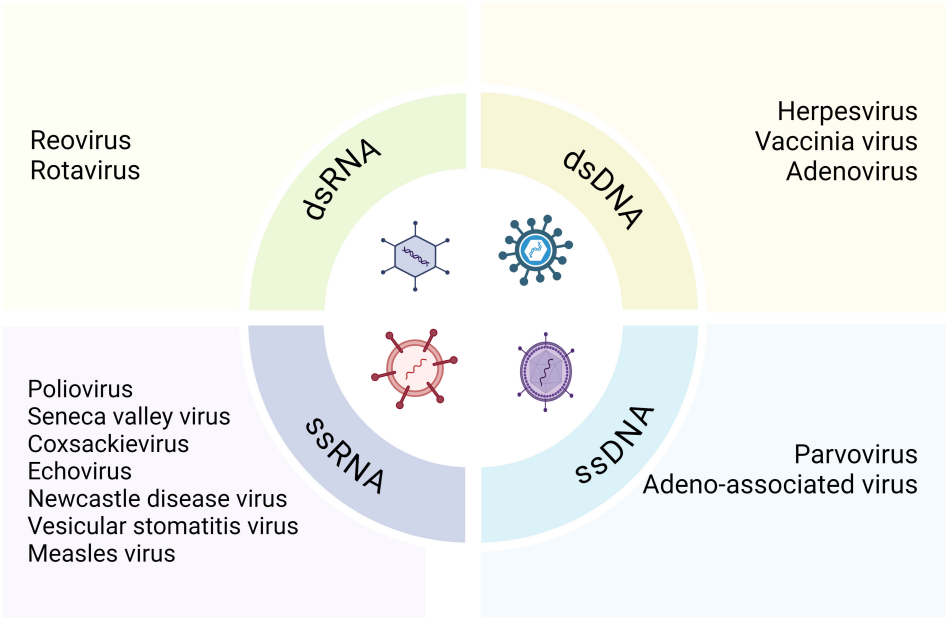

FIGURE 2
Classification of the oncolytic viruses.

the interindividual variability of the TME revealed differences in the immune evasion tactics employed by cancer cells among various TME subtypes (79). A cell-level analysis in prostate cancer samples studied the cell states associated with tumorigenesis, focusing on epithelial cell subsets, stromal cells, and the TME. This analysis found that ERG- cells show heterogeneity with luminal epithelial cells and differ from ERG+ tumor cells, potentially inducing a characteristic TME response (80).

The TME is comprised of stromal cells including cancer-associated fibroblasts (CAFs), macrophages, dendritic cells (DCs), natural killer (NK) cells, neutrophils, lymphocytes, endothelial cells, as well as chemokines, matrix metalloproteinases (MMPs), integrins, and other secreted molecules (Figure 3) (81). The TME contains various signaling molecules and pathways that contribute to immune suppression and angiogenic responses (82–84). The methyltransferase-like 3 associated with RNA N6-methyladenosine (m6A) modification is strongly activated by lactate accumulation in the TME, which enhances m6A modification in tumor-infiltrating myeloid cells (TIMs) through H3K18 lactylation. This activation of the m6A-YTHDF1/JAK1/STAT3 axis is associated with poor prognosis in colon cancer and increases the immunosuppressive capabilities of TIMs (83). Hypoxia in the TME can induce high levels of diacylglycerol kinase gamma in tumor vascular endothelial cells, which in turn can promote tumor angiogenesis and immune evasion in hepatocellular carcinoma through the ZEB2/TGF-β1 axis (84). The Food and Drug Administration's (FDA) approval of

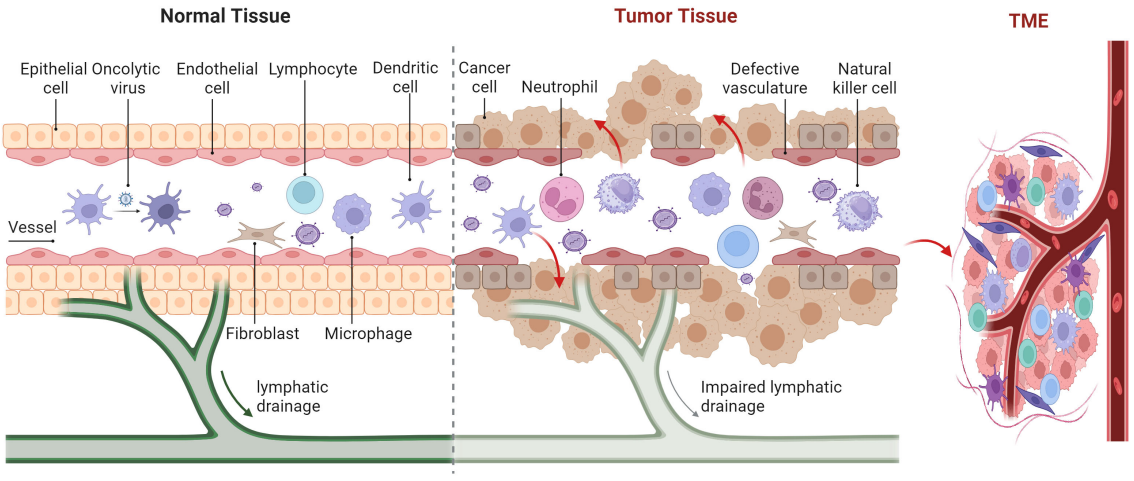

FIGURE 3
Various cells and oncolytic viruses in the tumor microenvironment.

antiangiogenic drugs and, more recently, immunological checkpoint inhibitors has reignited interest in understanding the role of the TME (85). Angiogenesis primarily depends on vascular endothelial growth factor (VEGF). Antiangiogenic drugs are those that inhibit tumor angiogenesis by targeting VEGF, its receptors, and other related molecules (86). The first antiangiogenic targeted medication to target VEGF was bevacizumab, which the FDA approved for application in 2004 (87). Clinical trials have shown that recombinant poliovirus therapy for gliomas, when combined with bevacizumab, had synergistic effects by reducing the local inflammatory response (88). Immune checkpoint inhibitors, including monoclonal antibodies targeting programmed death-1 (PD-1), programmed death-ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), counteract the mechanisms tumors use to evade immune surveillance. The first monoclonal antibody targeting CTLA-4, ipilimumab, was approved by the FDA in 2011 for treating melanoma (89). In advanced melanoma patients, combining ipilimumab with a modified oncolytic herpes simplex virus type 1 (HSV-1) demonstrated enhanced anticancer efficacy without additional harm, and these improved response rates persisted at the 5-year follow-up (90).

3.2 Tumor-associated immune cells

3.2.1 Cancer-associated fibroblasts

The TME significantly influences the survival, proliferation, migration, and even dormancy of cancer cells (91, 92). It has been established that cancer-associated fibroblasts (CAFs) play a multifaceted role in promoting tumor growth within the TME. CAFs secrete inflammatory ligands, growth factors, and extracellular matrix (ECM) proteins that support tumor growth, contribute to immune exclusion, and foster resistance to treatment (91). As a principal component of the stromal cells, CAFs are recognized for their tumor-promoting properties. However, evidence that CAFs activate the Hedgehog signaling pathway indicates that under certain conditions, CAFs might also exhibit tumor-suppressing functions (93, 94).

Studies have demonstrated that CAFs promote tumor growth through various mechanisms, such as secreting ECM proteins, inducing inflammation and neovascularization, enhancing angiogenesis, increasing the prevalence of tumor-initiating cells, and altering cancer cell signaling (95). Under specific cellular conditions, the multifunctional cytokine TGF-β can both promote and inhibit tumor growth. In pre-menopausal breast cancer, knocking down the TGF-β receptor in cancer-associated fibroblasts (CAFs) has been identified as a predictive factor that can increase the growth, proliferation, and clonogenic survival of breast cancer cells (96). In pancreatic ductal adenocarcinoma, a population of predominantly quiescent CAFs is observed among long-term survivors; those with activated CAFs are highly likely to benefit from therapeutic interventions targeting CAFs (97). In cases of nasopharyngeal carcinoma, CAFs and tumor cells can enhance neoangiogenesis by recruiting endothelial progenitor cells from the bone marrow into the tumor stroma, a process that relies on VEGF and stroma-derived factor-1. Thus, molecules related to angiogenesis may offer viable therapeutic targets in nasopharyngeal carcinom (98). In head and neck cancers (HNCs), fibroblast activation protein (FAP) is expressed by CAFs within the tumor microenvironment. Radiolabeled inhibitors targeting FAP administered to patients with HNCs have demonstrated localized uptake in tumor lesions, indicating activity above background levels (99).

3.2.2 Macrophages

Macrophages are both ubiquitous and specialized across different tissues, playing roles in tissue development, coagulation, inflammation, and every stage of wound healing (100). Macrophages are categorized into two types: immune-suppressive M2 (alternatively activated) and inflammatory M1 (classically activated) (101, 102). Macrophages are essential for immunological homeostasis because they not only play a crucial role in wound healing and tissue repair but also control immune responses through pathogen phagocytosis and antigen presentation. The tumor-promoting actions of immune-suppressive M2 macrophages are enhanced by the TME. A high level of macrophage infiltration in tumors is generally associated with a poor prognosis (103, 104). In the TME, macrophages influence epithelial cell motility, which can be exploited by tumor cells to facilitate their migration and invasion (105).

In prostate cancer, interactions between tumor cells and macrophages are known to facilitate tumor development, although the precise mechanisms remain unclear. It has been found that high-mobility group box 1 activates macrophages, which then produce IL-6. This, in turn, promotes prostate cancer progression, resistance to androgen deprivation therapy (ADT), and gankyrin expression via the STAT3 signaling pathway, creating a self-reinforcing loop. Interestingly, inhibiting the interactions within this loop in a tumor xenograft model has shown to prevent ADT resistance (106). Another study found that patients with metastatic colorectal cancer undergoing chemotherapy that included bevacizumab experienced significantly improved clinical outcomes when they had genetic variations affecting macrophage-related functions. Additionally, alterations in genes related to macrophages may predict the outcome of bevacizumab treatment depending on the KRAS status (107).

3.2.3 Dendritic cells

The innate and adaptive immune systems are bridged by DCs, which are the most potent antigen-presenting cells and are crucial for initiating the adaptive immune response (108). DCs are classified into various subtypes, such as classical DCs, plasmacytoid DCs, and monocyte-derived inflammatory DCs, based on their functional attributes. The cross-priming of tumor-specific T cells by DCs is vital for initiating and sustaining anti-tumor immunity, a process that involves dynamic interactions between different DC subtypes and the tumor (109). The specific type or subtype of the tumor, along with its unique TME, significantly influences the composition and function of DCs. DCs within the tumor are associated with improved patient survival and are capable of inducing T cell responses that enhance protective immunity and decelerate cancer progression

(110, 111). Typically, tumors manipulate their environment to ensure their survival, encountering immune-suppressive agents such as VEGF and IL-10 in the TME. These factors hinder the maturation of DCs into immunogenic cells, promoting instead the development of a tolerogenic phenotype in DCs (112, 113).

For some melanoma patients, immunization with mature monocyte-derived DCs)that were loaded with tumor antigens led to tumor regression. Additional refinement of the DC immunization protocol is required to determine which factors contribute to better clinical outcomes and enhanced anti-tumor responses (114). DC vaccines that were transfected with personalized tumor-associated antigen mRNA triggered specific CD4+ and CD8+ T cell responses in patients with advanced lung cancer or glioblastoma multiforme (GBM), and these responses were associated with favorable overall survival without significant autoimmune side effects (115). In a phase I trial, the safety and specificity of immune responses to tumor antigens were evaluated following *in situ* vaccination with autologous DCs transduced with an adenoviral vector expressing the CCL21 gene in patients with advanced non-small cell lung carcinoma (116).

3.2.4 Neutrophils

Neutrophils, constituting up to 70% of circulating leukocytes, serve as the primary line of defense against infections. Typically, they have a short lifespan, remaining in circulation for up to five days. When tissue is infected or damaged, epithelial cells emit chemokines that direct neutrophils to the affected site. Upon arrival, neutrophils deploy extracellular traps (NETs), release inflammatory cytokines, and engulf invading pathogens (117, 118). These NETs, which carry toxins and antimicrobial peptides on a chromatin backbone, serve as an additional mechanism of attack, albeit at the cost of the neutrophil's own survival (119). The phenotype of neutrophils within the TME varies depending on the type of tumor and the stage of the disease. Initially, during the early stages of tumor development, neutrophils exhibit an inflammatory behavior, but as the tumor progresses, they adopt an immunosuppressive role. Neutrophils manage inflammation by producing ROS and nitrogen species. They also support angiogenesis, tumor invasion, and the remodeling of the ECM by releasing neutrophil elastase and MMPs in the TME. These proteases break down pro-inflammatory cytokines and restructure the TME, fostering tumor growth and metastasis (120, 121).

In the early stages of lung cancer, tumor-associated neutrophils do not suppress the immune system, instead, they enhance T cell responses. This activity leads to a marked increase in costimulatory molecules on the surface of neutrophils, which in turn fosters T cell proliferation in a positive feedback loop (122). Moreover, prior studies have shown that some patients with non-small cell lung cancer exhibit significant anticancer effects after undergoing salvage chemotherapy following PD-1 inhibition. For patients who did not respond to salvage chemotherapy, both the neutrophil-to-lymphocyte ratio (NLR) and the absolute neutrophil count (ANC) increased over the course of treatment with nivolumab. An inverse relationship was observed between the response to the drug and NLR or ANC at four to six weeks (123).

3.2.5 Natural killer cells and natural killer T cells

NK cells are innate lymphoid cells recognized for their cytotoxic capabilities (124). These cells target tumor cells to inhibit the formation of primary tumors through apoptosis induced by death receptors and cytotoxic mechanisms mediated by perforin and granzyme. While NK cells are effective at eliminating circulating tumor cells, they struggle to kill cells within the TME. Tumors deploy various tactics to evade destruction by NK cells, such as surrounding themselves with collagen to trigger inhibitory NK receptors and using platelets as shields to prevent NK cell detection (125, 126). Additionally, many cytokines commonly present in the TME can effectively dampen NK cell effector functions. Natural killer T cells (NKTs) are innate-like T lymphocytes that bind to CD1d and, like conventional T cells, possess a T cell receptor and respond quickly to antigen exposure. These cells are also prevalent within the TME (127). To be more precise, NKTIs may be further classified into subtypes such as Th1-like, Th2-like, Th17-like, regulatory T-like (Treg-like), and T follicular helper (TFH)-like for type I NKTs; whereas type II NKTs can be classified as Th1-like and Th2-like. It has been observed that NKTs can switch roles in the environment, toggling between immune-suppressive and inflammatory roles. Type I NKTs generally exhibit anti-tumor properties, whereas type II NKTs tend to support tumor growth (128, 129).

In ovarian cancer patients, including those from whom cells were isolated from ascites, NK cells demonstrated effective killing of autologous tumor-associated macrophages (TAMs) that expressed low, non-protective levels of HLA class I molecules when appropriately stimulated within the complex TME (130). For achieving pathological complete responses in patients with HER2-positive breast cancer, maintaining functional T cell responses to specific antigens and enhancing NK cell efficacy during neoadjuvant chemotherapy appears crucial (131). Hypoxic TAMs, induced by the tumor, secrete chemokine ligand-21 (CCL21), which attracts and suppresses NKTs. CCL21 further impairs NKT survival and function, inhibiting NKT migration *in vitro* toward tumor-conditioned hypoxic monocytes and preventing their localization to neuroblastoma grafts in mice (132). Patients with asymptomatic myeloma who underwent combination therapy exhibited signs of NK cell activation and an activation-induced reduction in detectable iNKT cells, with the therapy proving effective in driving tumor regression and synergistically activating various innate immune cells (133).

3.2.6 Innate lymphoid cells

Five distinct cell types comprise the innate lymphoid cells (ILCs), including NK cells) (134, 135). Unlike other ILCs, which primarily produce cytokines in reaction to different stimuli, NK cells exhibit the highest cytotoxic activity within the ILC group. These cells are a crucial part of the tumor microenvironment TME (136). ILCs originate from the same lymphoid progenitor as B and T cells but are categorized as innate immune cells due to their lack of B and T cell receptors. They play a role in T cell polarization by presenting antigens and secreting cytokines (137).

Prior to transplantation, the presence of acute leukemia patient ILCs and donor ILCs expressing specific markers was associated with a reduced risk of acute graft-versus-host disease (GVHD) and therapy-induced mucositis. Consequently, the dynamics of ILC recovery and its interaction with treatment-related tissue damage influence the development of GVHD (138). ILCs exhibit a dual role in cancer contexts, showing either pro-tumor or anti-tumor effects depending on the ILC subset and the type of cancer involved. ILC1s, in particular, are an early source of IFN-γ and are generally associated with anti-tumor activity through mechanisms like macrophage activation, Th1 polarization, and enhancement of major histocompatibility complex molecules (139). In cases of metastatic colorectal cancer, the overall frequency of ILCs was markedly increased compared to healthy donors and showed an inverse relationship with Th1 immune responses (140).

4 Oncolytic viruses mediate the immune cells and the immune microenvironment

Immunosuppression in the tumor microenvironment, exacerbated by immune checkpoint inhibitors like PD-1/PD-L, diminishes effective neoantigen presentation and hinders anti-cancer T cell responses (141). An engineered oncolytic virus designed to express PD-L1 inhibitors can initiate tumor neoantigen-specific T cell responses. This approach has shown promise in enhancing endogenous T cell reactions against tumor neoantigens in patients with advanced cancers, leading to durable outcomes, including complete responses in various cancer types such as melanoma, and metastatic lung, kidney, and bladder cancers (142, 143).

Oncolytic viruses, capable of inducing anti-tumor immunity both locally and systemically, are either injected directly into the tumor or administered systemically. Once these viruses infect tumor cells, they multiply, often triggering immunogenic cell death, and spread throughout the tumor, stimulating an inflammatory response that can be modulated by macrophages and NK cells of the innate immune system (144). Armed oncolytic viruses also express immunomodulatory transgenes, enhancing immune reactions against the tumor. The immunogenic cell death and inflammation attract DCs to the tumor site, where they initiate a comprehensive immune attack on the tumor by activating T and B cells (145).

Oncolytic viruses can counteract immune evasion strategies utilized by cancer cells. These strategies often involve immuno-inhibitory receptors on tumor cells and in the surrounding microenvironment, which deactivate immune effector cells and promote the secretion of IL-10 and TGF-β. These factors can attract immunosuppressive cells, such as myeloid-derived suppressor cells and tumor-associated macrophages, to the tumor site (146, 147). Oncolytic viruses disrupt this suppressive environment through various mechanisms that alter the cytokine landscape and the composition of immune cells within the TME (148).

Furthermore, several oncolytic viruses possess the capability to express therapeutic genes or alter the function of tumor-associated endothelial cells, enhancing the recruitment of T cells into TMEs that are otherwise immune-deserted or immune-excluded.

5 Promisting oncolytic viruses in tumor therapy

5.1 Nervous system tumor

Gliomas account for approximately 81% of all central nervous system (CNS) tumors, making them the most common type of malignant brain tumor (149). These tumors are notorious for their aggressive behavior, rapid growth, therapy resistance, and generally poor prognosis. Several non-neurotoxic viruses such as parvovirus, myxoma virus, M1 virus, and Seneca Valley virus are used in treatments as they generally do not require additional modifications for safety (150). The CNS is considered immune-privileged due to the blood-brain barrier (BBB), comprised of astrocytes, pericytes, and vascular endothelial cells forming tight junctions, which typically restricts access of peripheral immune cells to the brain. Overcoming this barrier involves strategies such as direct injection into CNS tumors or using external reservoirs that interface with brain tumor sites. Notably, parvovirus can naturally cross the BBB, facilitating the entry of oncolytic viruses into the bloodstream (151). In GBM clinical studies, parvovirus H-1PV has been utilized. However, there have been relatively few *in vivo* studies on the distribution of oncolytic viruses across the CNS to evaluate viral penetration effectively (152, 153).

5.2 Digestive system tumor

Digestive system cancers (DSC), including colorectal cancer (CRC), gastric cancer, hepatocellular carcinoma, pancreatic cancer (PC), and esophageal squamous cell carcinoma, are a leading cause of cancer-related deaths worldwide. Patients with advanced DSC generally have a poor prognosis. Peritoneal metastases, which often occur due to the spread of tumor cells within the peritoneal cavity, commonly triggered by CRC, represent the second most frequent type of CRC metastasis following those in the lung and liver. Approximately 25% of CRC patients develop metastatic disease, and peritoneal metastases are associated with particularly poor outcomes and are a major cause of mortality (154). According to a study, a tumor-lysing vaccinia virus that carries GM-CSF was found to successfully prevent CRC from spreading to the peritoneum by selectively infecting and lysing peritoneal tumor cells, as well as by activating peritoneal DCs and CD8 T cells to restore peritoneal anti-cancer immunity. Various oncolytic viruses, such as vaccine viruses, reoviruses, HSV, adenovirus, oncolytic measles virus, and of virus, are being explored for treating CRC (155). In pancreatic ductal adenocarcinoma, pelareorep, an intravenously administered oncolytic reovirus, has been shown to induce a T-cell-inflamed phenotype in tumors. This treatment has

led to T-cell infiltration, increased PD-L1 expression, and active reovirus replication in the tumors of treated patients (59). For gastric cancer, HSV-based OVT has shown promise. The recombinant vaccinia virus used in these treatments has proven safe *in vivo* and demonstrates enhanced replication in tumor cells is an exciting treatment used in OVT for patients with gastric cancer. The recombinant vaccinia virus was safe *in vivo* and had a greater capacity for reproduction in tumor cells (156).

5.3 Urogenital system cancer

Prostate cancer is the second most common malignant tumor globally, with an incidence rate of 13.5% and a mortality rate of 6.7%. Current treatments include surgery, hormone therapy, chemotherapy, and radiation therapy (157). However, these treatments often fall short, especially in cases of advanced metastatic prostate cancer. Oncolytic viruses offer a promising alternative due to their high selectivity, efficiency, and low toxicity. These viruses are limited in their ability to replicate in healthy cells but can proliferate within tumor cells, inducing apoptosis and promoting viral growth. The released progeny viruses can then infect adjacent tumor cells, leading to tumor destruction. A phase II study revealed that bladder cancer patients treated with the intravesical oncolytic virus CG0070 experienced a 47% complete response rate at six months (158). Additionally, the bluetongue virus has been shown to infect and selectively lyse human hepatic and prostate cancer cells while significantly increasing the proportion of apoptotic renal cancer cells (159).

5.4 Potential clinical applications

There is an increasing body of research on oncolytic viruses that has enhanced our understanding of their role in tumor therapy. Some oncolytic viral therapies, identified as potential prognostic markers and therapeutic targets, have already been approved for clinical use or are advancing into clinical trials for tumor treatment. Currently, single-method treatments for some types of tumors have proven inadequate for achieving optimal results. Oncolytic viruses can inhibit DNA damage repair proteins, thereby minimizing the DNA damage caused by chemoradiation when used in combination with it. Furthermore, chemoradiation can promote tumor cell death, which in turn supports the replication and dissemination of oncolytic viruses. Additionally, combining immunotherapy drugs with oncolytic viruses enhances their synergistic effect, leading to more potent and sustained therapeutic outcomes. Due to their extensive mechanisms of action, oncolytic viruses are vital for intercellular communication and therapeutic applications. It has been observed that insufficient tumor cell tropism and transduction can make the therapeutic virus ineffective when administered systemically in clinical settings. To counter this, modifications to the viral surface have been employed to enhance tumor cell targeting. One such modification is the insertion of an RGD motif into the HI loop of the adenovirus fiber knob domain, which has significantly improved the virus's infection efficiency and anti-tumor efficacy, especially in CAR-negative tumor models. Additionally, using different virus types, such as Human adenovirus (HAdV)-G52, which binds to polysialic acid on target cells, can offer another layer of targeting specificity. HAdV-G52 might specifically infect cancers, such as brain and lung cancers, that express high levels of polysialic acid, although adjustments are necessary to mitigate any potential neurotropism. Through various modifications, oncolytic viruses can be tailored to enhance their safety, infection capability through tumor cellular receptors, tumor-targeting selectivity, and replication efficiency within tumor cell cytoplasm. These modifications can aid to improve viruses' immunostimulatory capacity and tissue tropism while maintaining safety and antitumor efficiency.

6 Conclusion

There are several challenges with the oncolytic viral therapy approach. Research on oncolytic viruses is still in its infancy. Many oncolytic viruses are currently being explored both theoretically and experimentally, and their potential therapeutic value remains uncertain. Additionally, there are no specific diagnostic criteria to identify patients who might benefit from oncolytic viral therapies.

In conclusion, the function of oncolytic viruses in tumor therapy and the tumor microenvironment is gradually becoming apparent. As either naturally occurring or genetically engineered agents, oncolytic viruses achieve their therapeutic effects by influencing immune cells, tumor cells, and the tumor microenvironment. This review has discussed the history and characteristics of the tumor microenvironment, immune cells, and oncolytic viruses, as well as the potential therapeutic efficacy of oncolytic viruses. These viruses activate various pathways to induce tumor cell death. A significant challenge in cancer treatment is that many oncolytic virus therapies are still in the preclinical trial stage. While a few oncolytic viruses have been approved for clinical use, transitioning these therapies into clinical applications remains a substantial hurdle. Consequently, understanding how oncolytic viruses modify the tumor microenvironment in different tumors will lead to the enhancement and development of oncolytic virus treatments for cancer. Moving forward, continued research into oncolytic viruses is essential.

Author contributions

XW: Data curation, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. SF: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

OVT	Oncolytic virus therapy
TME	Tumor microenvironment
ROS	Reactive oxygen species
ECM	Extracellular matrix
CAF	Cancer-associated fibroblast
DC	Dendritic cell
NK	Natural killer cell
NKT	Natural killer T cell
MMP	Matrix metalloproteinase
m6A	RNA N6-methyladenosine
TIM	Tumor-infiltrating myeloid cells
FDA	Food and Drug Administration
VEGF	Vascular endothelial growth factor
PD-1	Programmed death-1
PD-L1	Programmed death-ligand 1
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
HSV-1	Herpes simplex virus type 1
FAP	Fibroblast activation protein
HNC	Head and neck cancer
ADT	Androgen deprivation therapy
CCL21	Chemokine ligand-21
TAM	Tumor-associated macrophages
NLR	Neutrophil-to-lymphocyte ratio
ANC	Absolute neutrophil count
ILC	Innate lymphoid cells
GVHD	Graft-versus-host disease
CNS	Central nervous system
BBB	Blood-brain barrier
DSC	Digestive system tumor
PC	Pancreatic cancer
CRC	Colorectal cancer
HAdV	Human adenovirus

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Reverse resistance to immune checkpoint inhibitor in a patient with recurrent cardia cancer by intratumoral injection of recombinant human adenovirus type 5: a case report and literature review

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Advanced metastatic cardia cancer is an intractable malignance with poor prognosis. It is often accompanied by upper digestive tract obstruction, which seriously affects the quality of patients. Therefore, effective relief of eating obstruction is an important goal in the treatment of cardia cancer. Immune checkpoint inhibitors (ICIs) have shown significant efficacy in cardia cancer, but only a small percentage of patients will benefit from them due to immune resistance. Oncolytic viruses have been shown to enhance the efficacy of ICIs by altering the immune microenvironment. This indicates that oncolytic virus has the potential value of overcoming the immune resistance of cardia cancer. Here, we present a case with local recurrent and multiple metastatic cardia cancer accompanied by eating obstruction. After 4 cycles of chemotherapy plus ICI therapy, the patient´s metastases were significant shrink, but the recurrent carida lesion were almost unchanged. Then we implemented exploratory local injection of recombinant human adenovirus type 5(H101) into recurrent cardia lesion by painless gastroscopy. Surprisingly, the cardia lesion shrank significantly, and the eating obstruction was greatly relieved. We also observed a significant increase of infiltrated CD4+T cells in biopsy tissues after H101 treatment. Our study not only conformed the value of oncolytic viruses to reverse ICI resistance in patients with gastric cancer, but also revealed its underlying impact on immune microenvironment.

KEYWORDS

cardia cancer, immune checkpoint inhibitors, recombinant human adenovirus type 5, CD4+ T cell, CD8+ T cell, immune microenvironment

Introduction

Advanced cardia cancer is a highly aggressive and heterogeneity malignance with a poor five-year survival rate (1). Patients are often suffer from eating obstruction, which can seriously affect the patient's quality of life, which can seriously affect the patient's quality of life, resulting in poor nutrition and even shorter survival. Therefore, it is a difficult and urgent problem to be solved in clinic practice. In recent years, immune checkpoint inhibitors (ICIs) have shown significant efficacy in cardia cancer, but only a small subgroup of patients will benefit from them due to immune resistance (2–5). Therefore, overcoming immune resistance is an imperative task.

Oncolytic viruses have been shown to enhance the efficacy of ICIs by directly lysing tumor cells and altering the immune microenvironment (6, 7). However, there are few reports about the effect of oncolytic viruses on reversing immune resistance of cardia cancer. We have a patient diagnosed with metastatic cardia cancer who underwent 4 cycles of immune checkpoint inhibitor combined with chemotherapy without no shrink in cardia lesions. What's worse, the patient was suffered from the symptom of eating obstruction. However, the patient refused radiotherapy for fear of its toxic side effects. Considering the unique anti-tumor mechanism of oncolytic virus and its mild toxic side effects, we innovatively injected H101 into the cardia lesions by electronic gastroscope. Surprisingly, the cardia lesions were significantly reduced, and the parent symptom of eating obstruction were significantly relieved. The details are reported below.

Case presentation

In April 2021, a 72-year-old man was diagnosed with cardia cancer and subsequently underwent radical gastrectomy. Pathological diagnosis reported a poorly differentiated ulcerative adenocarcinoma (stage IIIB,T3N3M0). He received oral S-1 (40mg twice daily for 14 days, discontinued for 7 days, and repeated every 21 days) as adjuvant chemotherapy regimen for about 4 months, but discontinued due to significant gastrointestinal reaction.

In February 2023, he was admitted to our department with an worsen eating obstruction. Chest and abdominal computed tomography (CT) scan showed cardia recurrence and multiple metastases in liver, peritoneum, abdominal cavity and retroperitoneal lymph nodes. Sintilimab (200mg, intravenously every three weeks) combined with nanoparticle albumin-bound paclitaxel(300mg, intravenously every three weeks) was admitted as first-line treatment after recurrence. After 4 cycles of chemotherapy plus ICI therapy, the patient´s metastases were significant shrink (Figure 1), but the cardia lesions were almost unchanged. As the patient strongly requested further relief of eating obstruction, we implemented exploratory local injection of H101 into recurrent cardia lesion by painless gastroscopy with the patient's full knowledge and consent. Since June 9, 2023, H101 (5.0×10^{11} virus particles/0.5ml every time) was multipoint injected by painless gastroscopy every six weeks (8), 1 day before Sintilimab in each cycle. We observed on CT and gastroscopy images that the cardiac lesion were significantly reduced after two H101 treatment (Figure 2). Meanwhile, the patient´s eating obstruction symptom was subsequently relieved. Before and after two H101 treatment, we biopsied the cardia lesions and performed immunohistochemical staining of CD4+T cells and CD8+T cells respectively, results revealed a significant promotion of $CD4^+$ T cell infiltration after H101 treatment (Figure 3). Unfortunately, no significant infiltration of CD8+T cells was found after H101 treatment (Figure 4). Then Sintilimab combined with S-1(60mg orally twice daily for 14 days) was admitted as maintenance antitumor therapy to date. (The timeline of treatments is shown in Table 1).

Discussion

Here, We present a case involving an individual diagnosed with advanced cardia cancer who exhibited favorable responses to a combination therapy involving oncolytic virus, chemotherapy, and immunotherapy. We innovatively treated cardia cancer by local injection of oncolytic virus through gastroscopy and effectively reversed immune resistance. Following H101 treatment, the patient experienced notable reduction in cardia lesions, improvement in eating obstruction, and modest activation of the local tumor immune microenvironment, fostering infiltration of CD4+ T cells. Research in cancer immunotherapy has mainly focused on CD8+ T cells in the

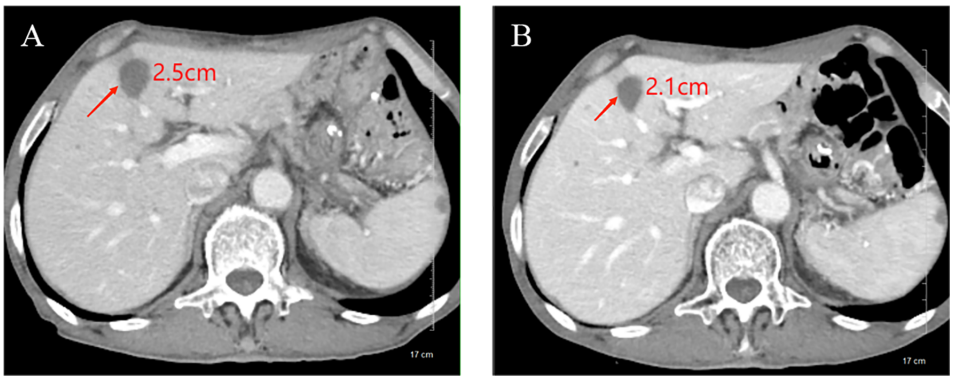


FIGURE 1
Comparison of longest diameter of liver metastases on CT images before and after chemotherapy plus ICI therapy. **(A)** Before treatment, the longest diameter of liver metastases was about 2.5cm; **(B)** After treatment, the longest diameter of liver metastases was reduced to 2.1cm.

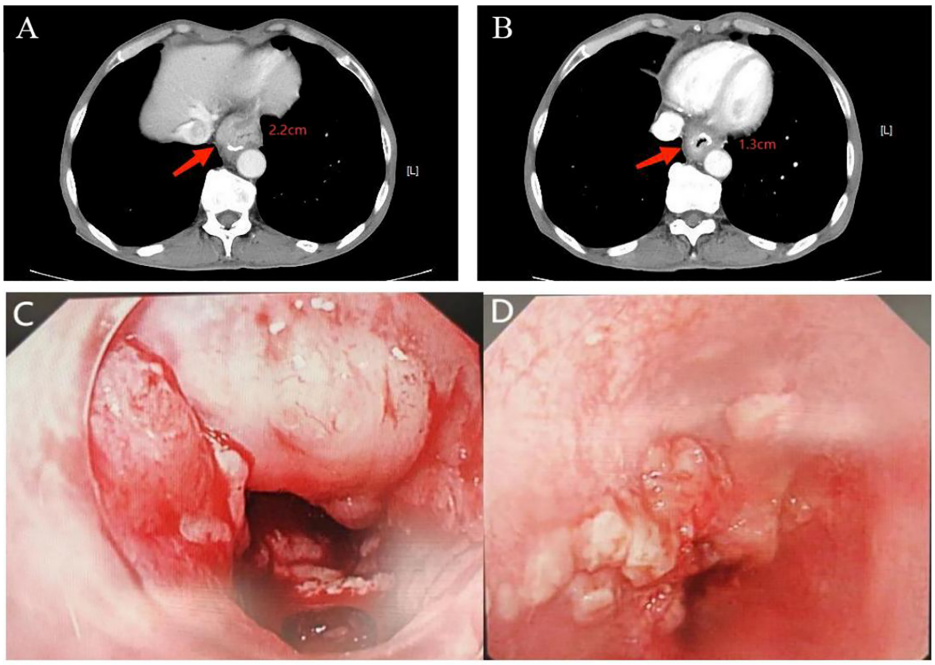


FIGURE 2
Comparison of cardia lesion thickness on CT and gastroscopy images before and after H101 treatment. **(A)** Before H101 treatment, the thickest part of the cardia lesion was about 2.2cm; **(B)** After H101 treatment, the thickest part was significantly reduced to 1.3cm; **(C)** Before H101 treatment, gastroscopy showed that the cardia lesions were large and protruding into the cavity; **(D)** After H101 treatment, gastroscopy showed that the cardia lesions were significantly reduced and the local stenosis was significantly improved.

tumor microenvironment (TME), However, it has been reported that antitumor immunity cannot be induced unless the tumor cells have the MHC class II binding neoantigens, which are recognized by CD4+ T cells. CD4+ T cells are likely to be the driving force of the cancer immunity cycle, allowing for a continuous supply of cytotoxic lymphocytes(CTLs) to the TME. Additionally, studies also have demonstrated that after oncolytic viruses infect tumor cells, they induce cell lysis and the release of tumor antigens and other immunostimulatory molecules. While this process can trigger an immune response, these antigens and stimulatory factors may predominantly recruit and activate helper T cells (CD4+ T cells) rather than cytotoxic T cells (CD8+ T cells). CD4+ T cells play a

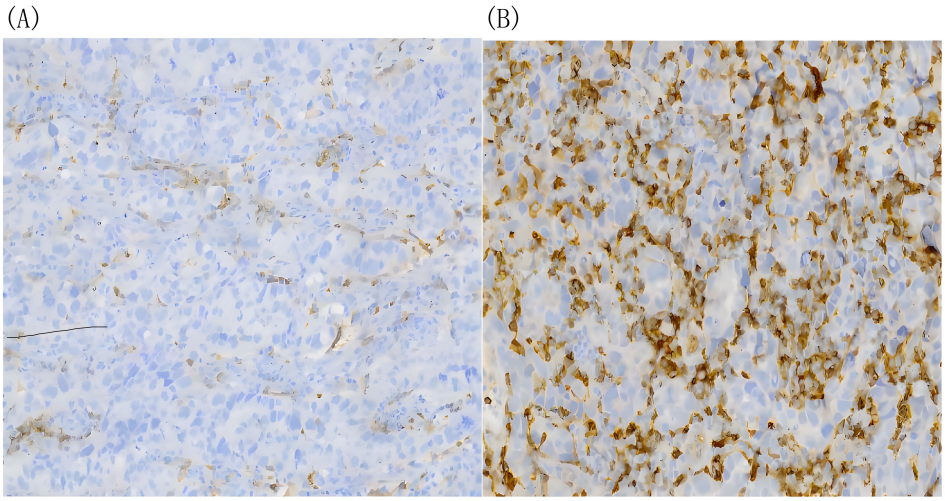


FIGURE 3
The density of infiltrating CD4+T cells tested by immunohistochemical DAB staining. **(A)** Before H101 treatment, the density of infiltrating $CD4^+$ T cells in cardia lesion is low(-);**(B)** After H101 treatment, the density of infiltrating $CD4^+$ T cells increased significantly(+++). Reagent information: Rabbit monoclonal to CD4, Product code: ab133616, Abcam (Shanghai) Trading Co. LTD. Quantitative method: The percentage of artificially counted positive cells in all cells of 10 times microscope field: Count the proportion of positive cells accurately located by IHC markers in the total cells and score (using nucleus as the cell localization standard). The standard is as follows: 25% of the total cell number is (-), 25%-50% is (+), 50-75% is (++), and more than 75% is (+++).

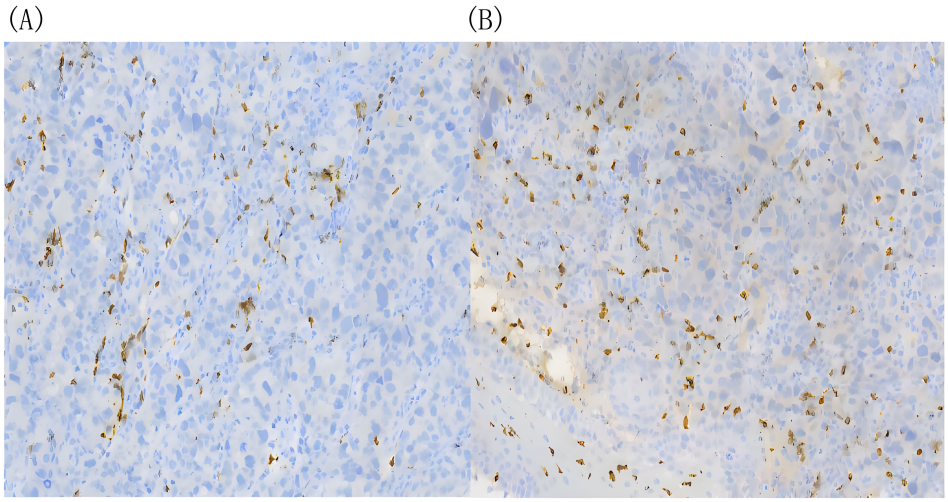


FIGURE 4
There was no significant difference in infiltrating CD8+ T cells before and after H101 treatment. **(A)** Before H101 treatment, the density of infiltrating $CD8^+$ T cells in cardia lesion is low(+);**(B)** After H101 treatment, the density of infiltrating $CD8^+$ T cells increased slightly (+). Reagent information: Rabbit monoclonal to CD8, Product code: ab217344, Abcam (Shanghai) Trading Co. LTD.

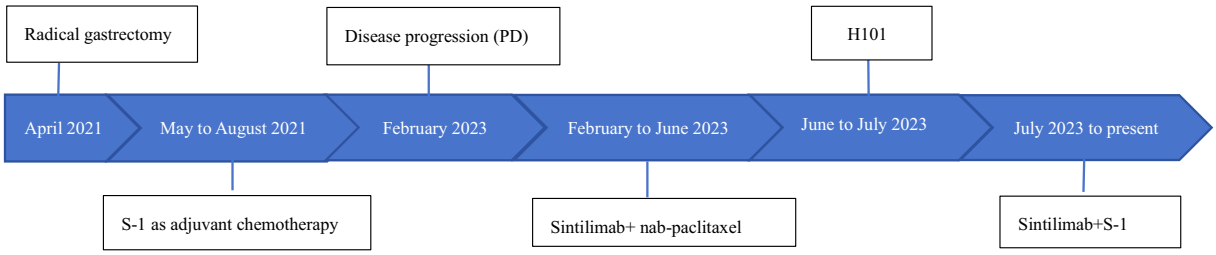
crucial role in coordinating subsequent immune responses by secreting cytokines (9–11). This conclusion is consistent with our test results. No significant infiltration of CD8+T cells was observed after H101 treatment, possibly due to the deviation of the biopsy specimen location from the injection site, and lack of enough tissue samples to fully observe. The deeper reason may be due to the special characteristics of oncolytic virus to change the TME. Moreover, subsequent to the localized administration of H101, we observed effective control over distant liver metastases, thereby further substantiating the synergistic potential of oncolytic viruses in conjunction with immunotherapy.

Oncolytic viruses (OVs) represent a widely employed strategy for localized tumor treatment. Engineered to selectively target and destroy tumor cells while sparing normal tissue, they hold promise in cancer therapy. However, their clinical application remains constrained. After the approval of the herpes virus-based drug T-VEC by the US Food and Drug Administration (FDA) in 2015, other OVs such as adenovirus, coxsackie virus, and measles virus have since entered clinical investigation (12, 13). In a recent phase II clinical study, H101 also found to trigger a proliferative burst of $CXCR6^+$ and $GZMK^+CD8^+$ T cells in malignant ascites (14). Nonetheless, OVs alone often fail to elicit robust therapeutic responses against malignant tumors, encountering obstacles like limited tumor penetration, constraints associated with local drug delivery, and pre-existing immune responses against the virus (15). These challenges underscore the need for innovative approaches to enhance the efficacy of OVs in cancer treatment.

To further enhance therapeutic efficacy, numerous genetically engineered oncolytic viruses have undergone development and clinical trials. A phase 1/2 clinical investigation demonstrated the feasibility and safety of LOAd703, an oncolytic adenovirus carrying transgenes encoding TMZ-CD40L and 4-1BBL, in combination with nab-paclitaxel and gemcitabine for treating patients with advanced pancreatic ductal adenocarcinoma (16). Additionally, efforts to diminish the extracellular matrix and facilitate oncolytic virus diffusion within tumors have led to modifications incorporating hyaluronidase or relaxin into oncolytic viruses, showing promising efficacy in preclinical studies (14, 17). In addition, to bolster dendritic cell maturation, GM-CSF fragments are integrated into viral genes to further stimulate immune activation (18).

Presently, immune checkpoint inhibitors demonstrate substantial effectiveness against advanced solid tumors (5, 19). Nonetheless, a majority of patients do not derive benefit, likely attributed to the heterogeneous nature of the tumor immune microenvironment. An expanding body of research indicates that modifying the tumor immune microenvironment holds promise for

TABLE 1 This table provides detailed information about the patient's medical history, including the timeline of treatments.



augmenting the effectiveness of immune checkpoint inhibitors and overcoming immune resistance (20, 21). Oncolytic viruses offer a compelling approach by efficiently lysing tumor cells to release antigens, inducing immunogenic cell death, fostering immune cell infiltration, and bolstering anti-tumor immunity (22). A preclinical study showed that oncolytic parapoxvirus ovis can induce GasderminE-mediated pyroptosis and activate anti-tumor immunity, adding new evidence for oncolytic viruses to stimulate anti-tumor immunity (23). A phase II trial found that H101 can trigger a proliferation burst of $CXCR6^+$ and $GZMK^+CD8^+$T cells in malignant ascites, enhancing tumor-specific T cell cytotoxicity (14). Furthermore, biopsy results from another phase II trial, following treatment with a triple-mutated, third-generation oncolytic herpes simplex virus type 1, revealed an increase in tumor-infiltrating $CD4^+/CD8^+$ lymphocytes, while the count of Foxp3+ cells remained low (24). These findings suggest the effective activation of anti-tumor immunity by oncolytic viruses.

However, investigations into combining oncolytic viruses with immunotherapy for solid tumors predominantly reside in Phase 1/2 clinical trials, necessitating additional trials to conclusively establish the efficacy of this combination. This study underscores that oncolytic viruses, functioning as localized treatments, can manage primary lesion reduction and immune activation. Immunotherapy, serving as a systemic approach, can potentiate the cytotoxicity of immune cells and efficiently control lesions.

Conclusion

We present a case study of a patient diagnosed with cardia cancer who underwent local administration of oncolytic virus H101 following ineffective treatment with paclitaxel combined with PD-1 inhibitors. Remarkably, this approach exhibited promising efficacy and immune-stimulating effects, offering insights into the selection of subsequent-line immunotherapy for cardia cancer. This finding unveils further therapeutic avenues for exploration.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics Committee of Changzhou Cancer Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

QZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft. MX: Data curation, Investigation, Supervision, Writing – review & editing. JM: Data curation, Formal analysis, Investigation, Software, Writing – review & editing. YB: Investigation, Supervision, Validation, Writing – review & editing. CF: Software, Methodology, Writing – review & editing. QG: Formal analysis, Methodology, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Neutrophils in oncolytic virus immunotherapy

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Oncolytic viruses have emerged as a highly promising modality for cancer treatment due to their ability to replicate specifically within tumors, carry therapeutic genes, and modulate the immunosuppressive tumor microenvironment through various mechanisms. Additionally, they show potential synergy with immune checkpoint inhibitors. A study report indicates that from 2000 to 2020, 49.5% of oncolytic viruses were administered intratumorally and 35% intravenously during clinical trials. However, both administration methods face significant challenges, particularly with intravenous delivery, which encounters issues such as non-specific tissue uptake, neutralizing antibody responses, and antiviral effects mediated by various immune cells. Despite extensive research into the antiviral roles of CD8+ T cells and NK cells in oncolytic virus therapy, neutrophils—constituting approximately 50% to 70% of human peripheral blood leukocytes—have received relatively little attention. Neutrophils are the most abundant leukocyte subset in peripheral circulation, known for their phagocytic activity. Beyond their traditional roles in bacterial and fungal infections, emerging literature suggests that neutrophils also play a critical role in the body's antiviral responses. Given the gaps in understanding the role of neutrophils in oncolytic virus therapy, this article reviews current literature on this topic. It aims to provide a theoretical foundation for developing oncolytic virus-based cancer therapies and enhancing their anti-tumor efficacy in future clinical treatments.

KEYWORDS

neutrophils, oncolytic viruses, oncolytic virus immunotherapy, cancer, antiviral immune response

1 Introduction

Oncolytic viruses (OVs) have shown significant potential in cancer therapy due to their ability to selectively replicate within and lyse tumor cells, exploiting aberrant signaling pathways and the impaired antiviral defense mechanisms in tumor cells. This selective replication activates the tumor immune microenvironment (TME) while sparing healthy cells, thereby enhancing their therapeutic potential. Various OVs have been tested in clinical trials, including Adenovirus (AdV), Vesicular stomatitis virus (VSV), Measles virus

(MV), Reovirus (RV), Newcastle disease virus (NDV), Herpes simplex virus (HSV), and Vaccinia virus (VACV) (1–9).

Between 2000 and 2020, 3,233 patients received oncolytic virotherapy (OVT). This therapy not only utilizes naturally oncolytic viral vectors but also incorporates genetic modifications to reduce viral pathogenicity and enhance therapeutic efficacy. Therapeutic genes are introduced into non-essential regions of the viral genome to deliver targeted cancer therapies, promote anti-cancer activity, induce immune responses, inhibit tumor angiogenesis, and enhance radiosensitization (10). Delivery methods for OVs have evolved, with initial research focusing on direct intratumoral injection (i.t.), which has limitations for deep or metastatic tumors. As a result, intravenous (i.v.) injection has become a more viable option for targeting multiple metastatic lesions. However, challenges such as immune cell interactions in the bloodstream can affect the biological distribution of OVs and limit their efficacy.

Neutrophils, which constitute 50%-70% of peripheral blood leukocytes, are traditionally known for their role in defending against bacterial and fungal infections (11). Recent evidence indicates that neutrophils also play a significant role in antiviral responses. They can directly phagocytize viruses, release antiviral peptides like alpha-defensin Human Neutrophil Peptide-1 (HNP-1), and produce antimicrobial peptides such as Cathelicidin LL-37, which neutralize viral particles (12, 13). Emerging research suggests that neutrophils are also crucial in the context of oncolytic virus therapy. For instance, Patients with low neutrophil-to-lymphocyte ratio before treatment had significantly longer OS ($P < 0.001$) (14). In animal models, neutrophil depletion has impaired the antitumor effects of oncolytic measles virus (15), and significant neutrophil infiltration has been observed in tumor tissues during treatment with recombinant VACV in both mouse models and clinical trials (16, 17).

This paper reviews the role of neutrophils in various oncolytic virus therapies, providing a theoretical foundation to enhance the clinical application of these therapies and improve their antitumor efficacy.

2 Oncolytic viruses

2.1 Introduction to oncolytic viruses

In the early 1904s, it was serendipitously discovered that the influenza virus could be used to treat leukemia, sparking significant interest in the concept of oncolytic viruses (18). However, due to their nature as foreign pathogens, these viruses posed challenges in controlling toxicity and eliciting strong immune responses, which hindered their development and application. The advent of genetic engineering in the 1990s marked a transformative period for the oncolytic virus field. Genetic modifications enabled the development of oncolytic viruses with reduced toxicity and the ability to carry therapeutic genes targeting tumors, leading to a rapid advancement in the field (19). In 2004, the FDA approved the first oncolytic virus, and since then, numerous oncolytic viruses have entered clinical trials (20). Oncolytic viruses offer several mechanisms to specifically target and replicate within tumor cells, distinguishing them from other cancer treatments (21–23):

2.1.1 Tumor cell surface antigen overexpression

Many types of oncolytic viruses need receptor-mediated entry into cells, and tumor antigen overexpression on the surface of cancer cells enhances the tumor targeting of oncolytic viruses. Compared to normal cells, cancer cells have high expression of receptors on their surface, such as CD46, which facilitates the targeting of oncolytic viruses such as measles virus and adenovirus to cancer cells (24, 25).

2.1.2 Defective Signaling Pathways

Normal cells often have signaling pathways that inhibit viral growth that may be defective in tumor cells, allowing the virus to replicate more efficiently.

The IFN signaling pathway plays an important role in controlling normal cell growth and apoptosis, however it is deficient in tumors, which facilitates viral replication. For example, VSV has a diminished role in interferon-responsive cells and a high oncolytic role in tumor cell (26).

2.1.3 Dysregulation of tumor metabolism

Tumor cells are metabolically reprogrammed to obtain more energy to meet their rapid proliferation and invasion, such as enhanced nucleic acid metabolism, protein metabolism, and glucose metabolism, which provide benefits for viral replication (23).

2.1.4 Defective Apoptosis Pathways

Tumor cells with defective apoptosis pathways may support increased viral replication. Elevated AKT expression in tumor cells is associated with anti-apoptotic mechanisms and has been shown to facilitate the replication of some viruses. Pharmacological and genetic inhibition of PI3K (AKT upstream protein) or Akt resulted in a significant decrease in vaccinia virus production (from 80% to >/=90%) (27). (Figure 1).

This progress underscores the potential of oncolytic viruses as a targeted cancer therapy, leveraging specific vulnerabilities in tumor cells to enhance therapeutic efficacy.

2.2 Mechanism of action of oncolytic viruses

Oncolytic viruses (OVs) can mediate anti-tumor activity through several mechanisms (1): Direct Tumor Cell Killing: OVs replicate specifically within tumor cells, leading to immunogenic cell death (ICD) that directly destroys these cells. (2) Release of Tumor-Associated Molecules: The destruction of tumor cells by OVs results in the release of soluble tumor-associated antigens (TAAs), damage-associated molecular patterns (DAMPs), and pathogen-associated molecular patterns (PAMPs). These molecules can recruit and activate antigen-presenting cells (APCs), such as immature dendritic cells (DCs) and innate

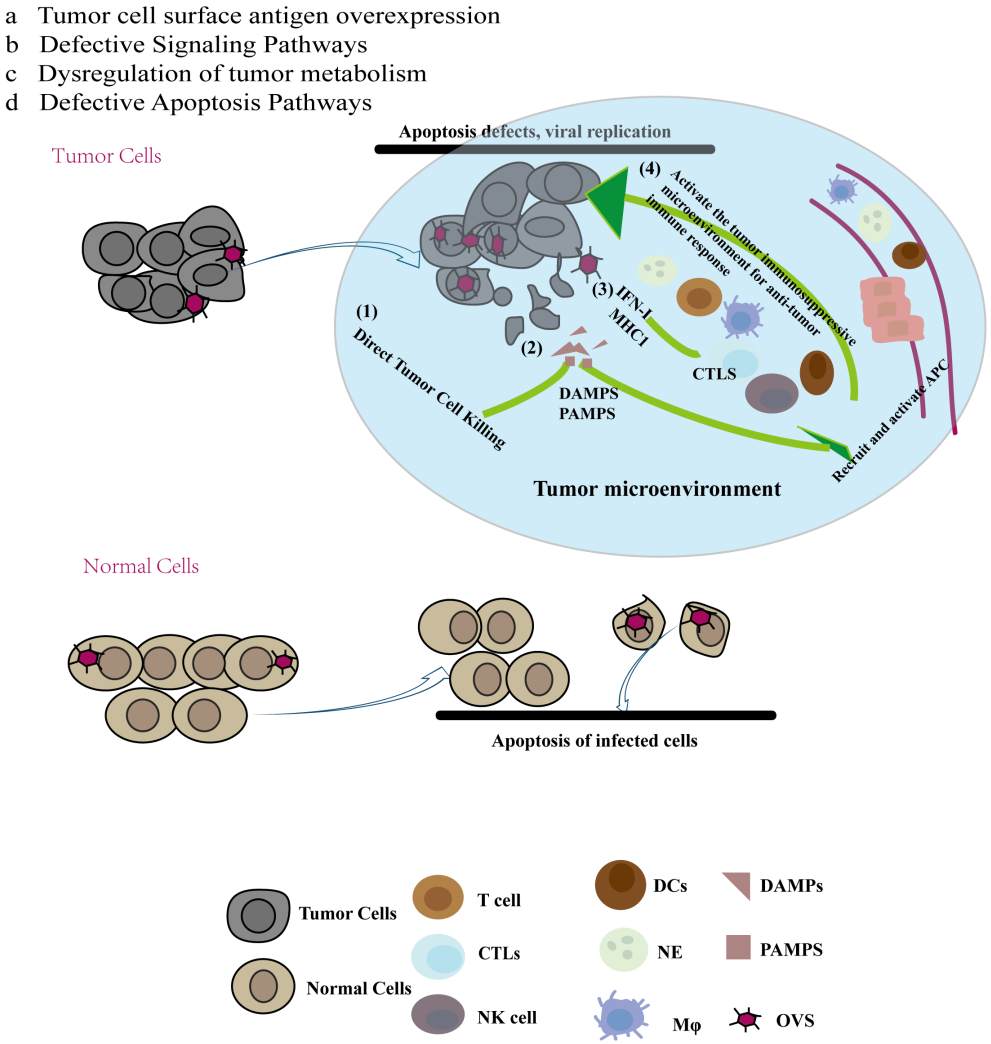

FIGURE 1
Multiple mechanisms of anti-tumor immunity of oncolytic virus (OV): Compared with normal cells, cancer cells have the specificity of abcd, and due to this specificity, oncolytic virus preferentially chooses to replicate and lyse tumor cells in tumor cells. At the same time, tumor cell lysis releases tumor antigen molecules and cell damage molecules recruit immune cells to reach the tumor site, reverse the immunosuppressive state of the tumor site, and produce anti-tumor effects.

lymphoid cells, to the site of viral infection. Immature DCs capture TAAs and migrate to regional lymph nodes, where they initiate an adaptive T cell response against the tumor. (3) Enhanced Antigen Presentation: The virus-induced release of type I interferons and chemokines boosts the levels of antigen processing and presentation factors, including the expression of MHC class I molecules. This results in the recruitment of tumor-specific CD8+ T cells (cytotoxic T lymphocytes, CTLs) and NK cells, which recognize and kill tumor cells. (4) Systemic Anti-Tumor Responses: CTLs can also target distant tumor cells, including those at metastatic sites. Furthermore, the interferon response can increase the expression of immune checkpoint molecules on tumor cells, such as programmed cell death ligand 1 (PD-L1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). This upregulation of immune checkpoints can make tumors more susceptible to checkpoint blockade therapies following oncolytic virus treatment (28). (Figure 1).

These mechanisms highlight the multifaceted approach of oncolytic viruses in targeting and destroying tumor cells while enhancing the overall anti-tumor immune response.

2.3 Challenges of oncolytic virus

Despite the multiple advantages of oncolytic viruses (OVs) over other immunotherapies, traditional administration methods, primarily intratumoral injection, remain limited in effectiveness for deep-seated or metastatic tumors. Consequently, intravenous injection has emerged as a promising alternative and has yielded some positive results (29). However, preclinical studies have highlighted several challenges associated with intravenous administration, such as non-specific tissue uptake, neutralization by antibodies, and interactions with human blood cells (30, 31). Notably, there has been limited research

on the role of neutrophils—the most abundant immune cells in peripheral blood—in relation to oncolytic viruses.

3 Neutrophils

3.1 Introduction to neutrophils

Neutrophils are the most abundant white blood cells in human peripheral blood, roughly 60% of peripheral blood leukocytes. Characterized by their multi-lobed nuclei, neutrophils originate from medullary precursors in the bone marrow. They undergo a series of developmental stages—from myeloblasts to promyelocytes, myelocytes, metamyelocytes, band neutrophils, and finally segmented neutrophils—before being released into the peripheral circulation (32, 33). This maturation process is regulated by various transcription factors, including PU.1 and CCAAT/enhancer-binding proteins (C/EBP). Neutrophil production is robust, with daily output reaching up to 5 × 10^10–10 × 10^10 cells, highlighting their crucial role in the innate immune system (34).

3.2 Neutrophils in cancer

In cancer, neutrophils have a dual role (35). They can promote tumor angiogenesis, thereby aiding tumor growth and progression. The neutrophil-to-lymphocyte ratio (NLR) serves as a marker of systemic inflammation (36, 37) and is associated with various malignancies, including metastatic gastric cancer (38), metastatic breast cancer (39), and triple-negative breast cancer (40). Clinical data also suggest that neutrophil expansion can influence immune suppression after cancer resection, facilitating immune escape and leading to poorer outcomes (41, 42). Additionally, neutrophils within tumors may undergo rapid and self-destructive cell death through NETs, with components like histones and neutrophil elastase promoting cancer cell proliferation, adhesion, migration, and metastasis (43).

However, neutrophils have phenotypic plasticity, and type I IFN polarizes tumor-associated neutrophils into anti-tumor N1 phenotypes in mice and humans (44) and TGFβ-regulated neutrophils exhibit a unique N1 profile (45).

3.3 Antiviral effect of neutrophils

Traditionally, neutrophils are recognized for their critical role in responding to bacterial and fungal infections as the first immune cells to arrive at sites of injury and infection. Their clearance mechanisms are well understood. While antiviral responses have traditionally been attributed to T cells and B cells, recent evidence reveals that neutrophils, as innate immune cells, also play a significant role in combating viral infections.

Neutrophils act as the first line of defense by engaging in various immune activities (46). They clear pathogens through interactions with other immune cells, direct phagocytosis (47), and the release of cytokines, chemokines, and antimicrobial components (48). Additionally, neutrophils can eliminate viruses through Toll-like receptor (TLR)-mediated formation of neutrophil extracellular traps (NETs). Electron microscopy, radioactivity, and fluorescence analyses have demonstrated that neutrophils exhibit phagocytic functions similar to macrophages, effectively engulfing viruses such as influenza virus (IVA), vesicular stomatitis virus (VSV), Ebola virus, Marburg virus, and hepatitis virus. This phagocytic activity initiates antiviral processes or activates innate immunity through pattern recognition receptors (PRRs) (49).

Neutrophils produce various antibacterial and antiviral substances, including myeloperoxidase (MPO), defensins (50–52), and antimicrobial peptides (53), which have been shown to possess both antibacterial and antiviral effects. NETs, extracellular structures composed of genomic DNA, histones, defensive proteins, and proteases (54), play a crucial role in trapping and inactivating viruses. This extracellular matrix, likened to a "mosquito net," captures viruses, and the granular proteins within the NETs contribute to virus inactivation (55).

Moreover, neutrophils can enhance antiviral responses by interacting with other immune cells, such as natural killer (NK) cells. Evidence suggests that neutrophils can activate adaptive immunity through CD8+ T cell activation following pathogen phagocytosis (56).

We have summarized the dual role of neutrophils in tumor and its antiviral mechanism. However, the relationship between neutrophils and a special class of viruses that are used as tumor therapeutic agents is not clear.

4 Neutrophils in oncolytic virus therapy

Genetic engineering has enabled the transformation of viruses such as AdV, VACV, HSV, MV, VSV, RV, and NDV into oncolytic virus products, enhancing their selectivity and efficacy in targeting tumors (57–62), In 2005, H101, an adenovirus-based oncolytic agent, was approved in China for the treatment of cancer patients (63). Currently, numerous oncolytic virus products are undergoing preliminary animal studies and clinical trials in the quest for improved therapeutic outcomes.

Despite the promise of virotherapy, the therapeutic effects of oncolytic viruses are not always as effective as hoped. Oncolytic viruses are designed to recognize oncogenic signaling pathways that are highly expressed in tumor cells, replicate specifically within these cells, and induce the release of tumor antigens while overcoming immunosuppression in the tumor microenvironment. Traditional administration methods, primarily intratumoral (i.t.) injection, are limited in effectiveness for deep or metastatic tumors. Consequently, intravenous (i.v.) injection is being explored as an alternative. However, i.v. delivery must navigate barriers posed by immune cells in peripheral blood before reaching the tumor site (64).

Next, we summarized the content of common oncolytic virus species and neutrophil-related content.

4.1 Oncolytic vaccinia virus and neutrophils

As a double-stranded DNA oncolytic virus from the natural poxvirus family, Vaccinia Virus (VACV) offers several advantages over other oncolytic viruses (OVs). These include its specific targeting of tumor cells, lack of specific receptors, short life cycle, robust replication in hypoxic tumor microenvironments, and lack of integration with the host cell genome, making it a strong candidate for oncolytic virus vectors (65). Understanding the interaction between VACV and the immune system, particularly neutrophils, is crucial for the development of clinically effective VACV-based OVs. Evidence suggests that both wild-type and recombinant VACV strains can induce significant neutrophil infiltration and migration. For example, recombinant VACV expressing human interleukin-1 beta (HIL-1beta) was tested in a mouse model with subcutaneously established pancreatic tumors. Intravenous injection of this recombinant VACV led to notable tumor size reduction and a significant presence of neutrophils at the tumor site, accompanied by tumor cell necrosis (16). Similarly, in a study involving recombinant VACV expressing interleukin-2 (IL-2), direct intratumoral injection resulted in neutrophil aggregation and tumor necrosis in some patients with malignant mesothelioma (17).

Modified Vaccinia Virus Ankara (MVA) has also been shown to induce leukocyte migration, especially of neutrophils. This migration is mediated by the production of chemokine receptors such as CCR1 and CXCR2 in mouse pulmonary fibroblasts and bone marrow-derived macrophages following MVA infection, independent of Toll-like receptor 2 (TLR2) signaling. These chemokines facilitate neutrophil infiltration and inflammation, enhancing the adaptive immune response induced by MVA (66). Complement component C5 (67) further contribute to neutrophil aggregation and migration. However, the precise role of neutrophils in these processes, including their ability to engulf and neutralize viruses, remains unclear.

In vitro studies using VACV labeled with ^14C and ^3H demonstrated that neutrophils can phagocytose VACV, with the virus being detected in intracellular lysosomes. This process is serum-dependent, and VACV load decreases over time due to neutrophil activity (68). In murine models, both wild-type and recombinant VACV expressing tumor necrosis factor showed that while NK cells and cytotoxic T lymphocytes (CTLs) did not significantly alter VACV levels, a dramatic and transient increase in neutrophils was observed, which limited VACV replication (69). Our research group has also found that inhibiting neutrophil function can enhance the anti-tumor efficacy of oncolytic VACV (70).

Despite their role in controlling viral replication through phagocytosis, VACV has evolved mechanisms to evade the immune response. VACV Complement Control Proteins (VCPs) bind to complement components C3 and C4, and to heparin and heparan sulfate proteoglycans on the cell surface. This interaction reduces neutrophil infiltration and decreases the effectiveness of human neutrophils and NK cells, aiding VACV in escaping immune destruction (71, 72).

Additionally, neutrophils can function as antigen-presenting cells, bridging innate and adaptive immunity. After intradermal injection of MVA, neutrophils can transport the virus from the dermis to the bone marrow, aiding in the activation of CD8+ T cells (73). Moreover, a recombinant VACV strain expressing the HIV-1 C antigen, but lacking specific viral genes (A52R, K7R, and B15R), has been shown to affect the NF-kB signaling pathway in mice. This alteration enhances the antigen- presenting capabilities of neutrophils (74, 75).

Understanding these interactions between VACV, neutrophils, and the broader immune system is essential for optimizing the therapeutic potential of oncolytic viruses.

4.2 Vesicular stomatitis virus and neutrophils

Unlike the traditional idea that OVs targets tumor cell replication and leads to tumor cell lysis, vesicular stomatitis virus (VSV) injection reduces blood flow inside the tumor by inducing apoptosis of tumor cells, but viral replication is limited. The results of tumor transcription spectroscopy showed that viral infection caused the increase of neutrophil chemokine 1(C-X-C ligand 1, CXCL1) and chemokine 5(C-X-C ligand 1, CXCL5), and induced neutrophil infiltration into infected tumor tissues. Injection of VSV after neutrophils are pre-deleted with RB6-8C5 antibodies increases the replication and spread of VSV in tumor tissue, but also eliminates apoptosis in tumor cells that are not infected with VSV. These results suggest that excess neutrophils inhibit OVs replication and transmission, but targeted recruitment of neutrophils to infected tumor sites can enhance the killing of malignant tumor cells (76).

There is also evidence that intravenous injection of VSV can not only infect tumor cells, but also directly infect and destroy the vascular system of tumors. Three-dimensional reconstruction shows that VSV-infected tumors lack blood flow in tissues compared with uninfected tumor tissues. These results demonstrate that VSV replicates in the tumor neovascularization system and spreads within the tumor mass, triggering an inflammatory response and forming thrombus, a process that forms dependent on the presence of neutrophils. After deletion of neutrophils with anti-GR-1 monoclonal antibodies, infected tumors showed significantly reduced fibrin deposition and reduced thrombosis, demonstrating that neutrophils are necessary to induce tumor perfusion loss during VSV infection of tumor tissue (77).

At the same time, there has been evidence that bone marrow, blood, lung and spleen were collected by intravenous injection of VSV with 1×10^9 PFU for 3h and 24h, and acute changes of neutrophils during infection were analyzed by flow cytometry. VSV infection resulted in rapid migration of neutrophils from bone marrow to lung accumulation. The accumulation of immature neutrophil antigen presenting potential in the spleen is also increased. In addition, infection with VSV labeled with green fluorescent protein (GFP) revealed the potential of neutrophils to acquire the protein encoded by the virus transgene. After incubating spleen cell populations with αCD3 and αCD28 *in vitro*, a significant proportion of neutrophils became GFP positive. This suggests that

neutrophils are able to take up VSV or VSV is able to infect neutrophils after VSV infection (78).

These findings offer new insights into the role of neutrophils in the antitumor activity associated with vesicular stomatitis virus (VSV). Neutrophils recruited by VSV enhance cytotoxicity against tumor cells. However, an excess of neutrophils may inhibit both the replication and dissemination of VSV. These results indicate that the involvement of neutrophils should be carefully considered in all aspects when utilizing VSV for future therapeutic applications.

4.3 Adenovirus and neutrophils

Adenovirus (Adv) is a non-enveloped, double-stranded DNA virus from the adenovirus family. It is used as a vector for vaccines against viruses like Ebola (79) and SIV (which causes AIDS in apes). Preclinical trials of the Adv-based SIV vaccine have demonstrated that it can induce a strong neutrophil response and activate neutrophils, which exhibit both phenotypic and functional changes. This activation leads to B cell activation and antibody production, primarily influenced by post-infection neutrophils, and is independent of Interleukin-10 (IL-10). Thus, neutrophils contribute to both innate and adaptive immunity in Adv vector vaccine infections (80).

Despite the potential of oncolytic Adv immunotherapy, there is a lack of specific biomarkers for its effectiveness. Elevated levels of Interleukin-8 (IL-8) in many cancers have been associated with poor outcomes in oncolytic Adv therapy. This suggests that IL-8 may influence the efficacy of oncolytic Adv therapy, IL-8 blockade together with adenovirus can influence TIL proliferation and activation when co-cultured with TANs isolated from ovarian tumors (81).

In clinical data from 290 patients treated with oncolytic Adv between 2007 and 2012, the use of Adv modified with granulocyte-macrophage colony-stimulating factor (GM-CSF) improved patient prognosis (hazard ratio (HR) 0.378, $p < 0.001$). Patients with a lower neutrophil-to-lymphocyte ratio before treatment had longer overall survival ($p < 0.001$). These findings provide insights into optimizing oncolytic Adv therapy and patient selection (14).

In addition to direct interactions between neutrophils and oncolytic Adv, neutrophil-related proteins and peptides have also been implicated. Human Neutrophil Peptides (HNPs), such as HNP-1, HNP-3, and HNP-4, have been shown to play a protective role in respiratory diseases caused by Adv. ELISA results indicated increased levels of these peptides and an associated rise in neutrophil count, suggesting an anti-Adv immune effect (82). This results in increased production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and Human Macrophage Inflammatory Protein 2 (MIP-2), enhancing the anti-tumor effects of recombinant oncolytic Adv (83).

Due to the limited literature, we have summarized some aspects of the relationship between neutrophils and oncolytic adenovirus in this content. While we did not delve into the direct interaction between neutrophils as immune cells and oncolytic adenoviruses, our summary provides valuable insights that may guide future clinical use of Adv in tumor therapy.

4.4 Herpes simplex virus and neutrophils

Herpes Simplex Virus (HSV) is a double-stranded DNA virus with several strains, such as HSV-1 and HSV-2, that exhibit oncolytic properties. Among these, HSV-1 has been the most commonly modified for oncolytic therapy. For example, Talimogene laherparepvec (T-Vec), an HSV-1 derivative, is used for treating melanoma (84). In studies involving Vaccinia Virus (VACV), neutrophils have been shown to phagocytose viruses, and similar evidence has been observed for HSV-1. Puncture biopsies and electron microscopy have demonstrated that HSV-2 virions and viral capsids can be found in neutrophils from genital infections, confirming that neutrophils phagocytose HSV-2 and play a role in limiting its replication and clearance (85, 86).

In studies of delayed hypersensitivity (DTH) after HSV-1 infection in BALB/c mice, neutrophils were among the first immune cells to arrive at the infection site (87). Their presence significantly inhibited HSV-1 replication, indicating that neutrophils are crucial in the DTH response. They are recruited to the site by human macrophage inflammatory protein-1 alpha (MIP-1α) and activated by interleukin-1α, which helps inhibit viral replication (88).

In vitro studies with neutrophils from neonates and adults co-cultured with HSV-infected Vero or CEM tumor cells showed that neutrophils significantly reduced HSV's ability to form plaques (89).

In studies involving HSV-2 carrier oncolytic viruses, such as the FusOn-H2 strain with a deleted N-terminal region of the ICP10 gene, neutrophils were found to lyse tumor cells effectively. FusOn-H2 exhibited oncolytic effects in 80% of tumor cell lines *in vitro*, and the remaining 20% resistant lines were also susceptible *in vivo*. After injecting FusOn-H2 into mouse tumors and analyzing neutrophils from the treated tissues, it was found that neutrophils in virus-infected tumors had a higher ability to lyse tumor cells compared to those in untreated tumors. These neutrophils also showed increased cell migration. This evidence underscores the potential of neutrophils to enhance the anti-tumor effects of the HSV-2 carrier oncolytic virus FusOn-H2 (90).

These findings highlight that neutrophils interact with both HSV-1 and HSV-2, contributing to antiviral immunity. However, it is important to note that HSV-1 rapidly absorbs into the skin after infecting the epidermis of mice. Treatment with anti-LY6G-specific monoclonal antibodies induces systemic neutropenia but does not affect virus replication or damage development. Instead, Gr-1(+) cells seem to limit viral replication (91). Interestingly, enzyme-linked immunosorbent assay (ELISA) revealed that HSV-1 enhances the expression of the cell death receptor Fas and its ligand FasL on neonatal neutrophils, inducing apoptosis. However, this effect was less pronounced in adult neutrophils (92).

4.5 Measles virus and neutrophils

Measles virus (MV) is a single-stranded, negative-sense RNA virus with an envelope, belonging to the Paramyxoviridae family. MV targets tumor cells through various receptors, including

lymphocyte activation molecule 150 (CD150), lymphocyte activation molecule 46 (CD46), and Nectin cell adhesion molecule 4 (NECTIN-4). Tumor cells often express higher levels of CD46 compared to healthy cells, which enhances MV's specificity for tumors. However, the widespread use of MV vaccines presents a challenge for MV-based oncolytic therapies, as the immune system can clear the virus quickly after injection (25).

Research has shown that both wild-type MV (WT-MV) and tumor-lytic vaccine strains such as MV-Vac can infect and replicate within neutrophils, resulting in increased survival of these cells post-infection. MV-Vac, in particular, activates neutrophils more effectively than WT-MV by inducing new RNA and protein synthesis. This activation stimulates the secretion of anti-tumor cytokines such as IL-8, MCP-1, and IFN-alpha, and triggers the release of TRAIL (TNF-related apoptosis-inducing ligand), enhancing the anti-tumor effect. Although neutrophils are not the sole factor influencing viral replication, they play a critical role in the anti-tumor efficacy of oncolytic MV (93). Additionally, in a mouse model with congenital immune deficiencies, subcutaneous inoculation of tumor cells followed by MV-Vac therapy demonstrated that neutrophils are crucial for tumor regression.

In studies involving two different B-cell malignancy models, a recombinant oncolytic MV expressing human granulocyte colony-stimulating factor (HG-CSF) was evaluated for its effects on the MV

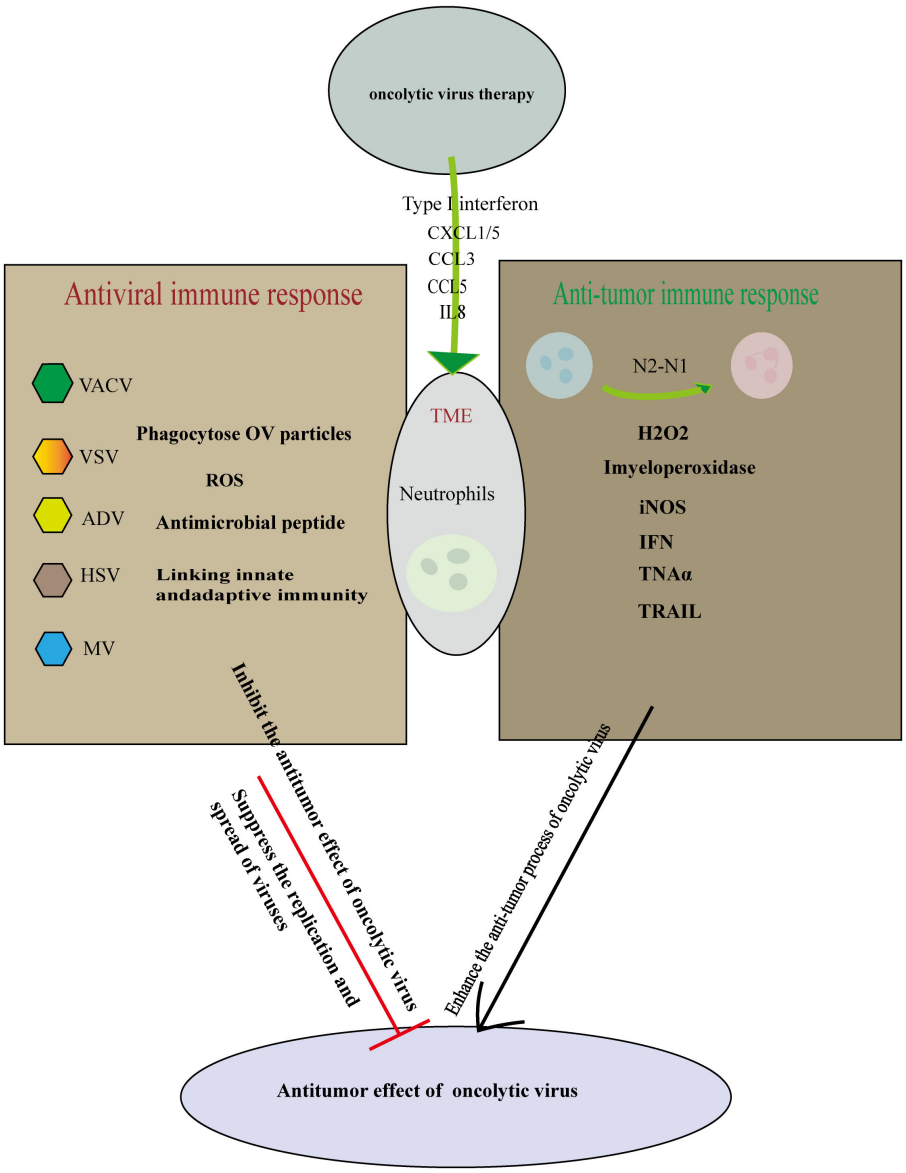

FIGURE 2
Cutting both ways: Neutrophils in oncolytic virus immunotherapy. Oncolytic virus replicates and lyses tumor cells specifically, causing immune death of tumor cells and releasing cell damage factors and tumor antigen molecules. On the one hand, the oncolytic virus infection induces tumor-associated neutrophils anti-tumor phenotype differentiation from N2-N1, Initiate anti-tumor action and mediate tumor cell killing. On the other hand neutrophils can use multiple mechanisms to perform antiviral effects. Therefore, the role of neutrophils should be considered in many aspects in the treatment of oncolytic virus, so that the synergistic anti-tumor effect of immune cells and oncolytic virus is the strongest.

oncolytic response. While simultaneous treatment with MV-hG-CSF was observed, neutropenia reduced the oncolytic effect of MV-hG-CSF in one model, specifically the Nalm-6 human acute B-lymphocytic leukemia cell line (15).

Another study involved the use of recombinant MV expressing mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) in a human lymphoid tumor model using immunodeficient mice. The study compared the effects of parental MV, ultraviolet-irradiated MV, and MV-GM-CSF. Results indicated that intratumoral injection of MV could reduce or eliminate tumor progression, with MV-GM-CSF further enhancing the oncolytic effect. This enhancement was attributed to neutrophil infiltration and the absence of NK cells and macrophages in the tumor. The strong neutrophil response was closely linked to tumor regression (94). Further research showed that recombinant MV can stimulate a potent neutrophil-mediated anti-tumor response, which is enhanced by cytokines to boost the anti-tumor activity of neutrophils (95).

These findings suggest that the role of neutrophils may vary across different models during MV-based oncolytic therapy. Besides neutrophils, Helicobacter pylori neutrophil-activating protein (NAP) also plays a significant role in treating metastatic breast cancer using MV as a vector. Recombinant MV strains, such as MV-lambda-NAP and MV-s-NAP, which secrete NAP, have been shown to improve the median survival rate of metastatic breast cancer patients. This improvement is associated with increased levels of Th1-type cytokines, which further enhance the anti-tumor effects of oncolytic MV (96, 97).

5 Conclusions and perspectives

Neutrophils have traditionally been recognized for their role in combating bacterial and fungal infections through various mechanisms. However, emerging evidence highlights their integral role in antiviral immune responses, especially as the first immune cells to arrive at the site of infection following viral exposure. Oncolytic virus (OV) therapy, a promising approach in cancer treatment, presents a unique challenge in understanding neutrophils' dual roles in antiviral and anti-tumor responses.

During OV therapy, neutrophils exhibit seemingly contradictory behaviors. While they can inhibit OV replication and engage in antiviral activities by recruiting cytokines and other immune factors, they also play a role in modulating the tumor microenvironment. The immunosuppressive nature of the tumor microenvironment can potentially be alleviated by neutrophil activation, thereby enhancing the anti-tumor effects of OVs (98) (Figure 2).

These complex interactions underscore the need for further research to reconcile these contradictory roles. Future studies should focus on finding a balance between inhibiting neutrophil activity to increase OV replication in tumor cells and subsequently activating neutrophils to counteract the immunosuppressive tumor microenvironment. Achieving this balance could optimize the anti-tumor efficacy of OVs in clinical settings.

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DZ: Writing – original draft. CZ: Writing – original draft. JS: Writing – original draft. MY: Writing – review & editing.

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Advanced progress in the genetic modification of the oncolytic HSV-1 virus

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The use of replication-competent viruses for selective tumor oncolysis while sparing normal cells marks a significant advancement in cancer treatment. HSV-1 presents several advantages that position it as a leading candidate for oncolytic virotherapies. Its large genome can accommodate insertions over 30 kb or deletions of multiple virulence genes without compromising lytic replication in tumor cells. Additionally, anti-herpes drugs can inhibit its replication during accidental infections. Importantly, HSV-1 does not integrate into the host genome and cause mutations. The HSV-1 genome can be modified through genetic engineering in two main ways: first, by reducing infectivity and toxicity to normal cells via limited replication and assembly, altered protein-virus receptor binding, and minimized immune evasion; second, by enhancing anticancer activity through disruption of tumor cell metabolism, induction of autophagy, improved immune recognition, and modification of the tumor microenvironment. In this mini-review, we systematically examine genetic modification strategies for oncolytic HSV-1 while highlighting advancements from these modifications. Certain genetic alterations have shown efficacy in improving clinical outcomes for HSV-1-based therapies. These modifications include silencing specific genes and inserting exogenous genes into the HSV-1 genome. The insertion of exogenous genes has increasingly been used to develop new oncolytic HSV-1 variants. Finally, we discuss limitations associated with oncolytic virotherapy at the conclusion of this review. As more clinical trials explore newly engineered therapies, they are likely to yield breakthroughs and promote broader adoption for cancer treatment.

KEYWORDS

oncolytic virotherapy, herpes virus 1, genetical engineering, solid tumor, genetic modification, cancer treatment

1 Introduction

Recent advancements in cancer treatment include the utilization of replication-competent viruses for selective oncolysis of tumors, known as oncolytic viruses (OVs), while sparing normal cells. This therapeutic approach can be employed either as a standalone treatment or in combination with other therapies to inhibit tumor progression (1). OVs encompass both wild-type and genetically modified variants. Genetic engineering strategies aimed at modifying these viruses involve deleting specific genes to limit toxicity to healthy cells (2, 3), inserting genes to activate the immune system, stimulate immune responses, or inhibit angiogenesis (4–6), and combinations of these strategies.

Herpes simplex virus type 1 (HSV-1) possesses a genome consisting of 152 kb of double-stranded linear DNA that encodes approximately 85 protein-coding genes, with 47 being dispensable in cell culture (7). HSV-1 has several advantages that position it as a leading candidate for oncolytic virotherapy (OVT). Notably, its genome contains two unique segments: one is the unique long (UL) segment and the other is the unique short (US) segment; each is flanked by inverted repeat (IR) elements. This genomic architecture allows for the insertion of fragments exceeding 30 kb or deletion of multiple virulence genes without compromising its lytic replication cycle within tumor cells (8, 9). Additionally, anti-herpetic drugs can inhibit HSV-1 replication in cases of accidental infection (10). Importantly, HSV-1 does not integrate into the host genome nor induce insertional mutations (7). Due to the above characteristics of HSV-1 virus, it has three advantages compared to other oncolytic viruses. First, it has a larger genome that can insert and accommodate multiple foreign genes. Additionally, the use of acyclovir can easily control HSV-1 infections in non-tumor cells. Finally, theoretically, HSV-1 has a lower likelihood of causing insertional mutagenesis in infected cells.

Currently, there are two primary directions for genetic modification of oncolytic HSV-1 (Figure 1). The first direction focuses on reducing HSV-1's infectivity and toxicity towards normal cells by limiting viral replication and assembly (3, 11–13), modifying proteins that bind viral receptors (14), and decreasing mechanisms involved in viral immune evasion (15, 16). The second direction aims to enhance HSV-1's anticancer efficacy through interference with tumor cell metabolism (17), induction of autophagy (18, 19), improvement in immune recognition processes (20–23), and alteration of the tumor microenvironment itself (6, 24, 25). Several key gene modifications related to silencing specific genes or introducing exogenous genes into the HSV-1 genome will be discussed separately.

2 Silencing genes

2.1 Gene γ134.5

To enhance the selectivity of HSV-1 for infecting epithelial-derived malignancies while minimizing the risk of infection in healthy somatic cells and preventing uncontrolled spread of HSV-1 to normal somatic cells, numerous research groups knockout the γ134.5 gene. The diploid gene γ134.5 located within the inverted terminal repeats flanking the long unique sequence of HSV-1 DNA is classified as a gamma-late or "leaky late" gene. It encodes ICP34.5, a neurovirulent protein. ICP34.5 consists of 263 amino acids organized into three main domains: the N-terminal domain, the linker region, and the C-terminal domain. Those domains are responsible for binding host proteins essential for both viral replication and immune evasion (26–29).

The principal function attributed to ICP34.5 involves enhancing viral propagation across peripheral tissues alongside central nervous systems, contributing significantly toward HSV, and inducing neurovirulence via various mechanisms including protein phosphatase I (PPI) dephosphorylating eIF2α, thus preventing shutoff from host protein synthesis while enabling continuous production (11, 30, 31). Furthermore, ICP34.5 converts proliferating cell nuclear antigen (PCNA) from repair mode back toward a replicative state, crucially initiating HSV replication (32).

Moreover, ICP34.5 inhibits antiviral signaling pathways ensuring persistent infections. ICP34.5 disrupts retinoic-acid-inducible gene I (RIG-I) signaling preventing interaction between RIG-I and mitochondrial antiviral signaling protein (MAVS), a pivotal adaptor inhibiting downstream activation IRF3 and subsequent IFN production (33). Stimulator interferon genes (STING) is another important player during antiviral responses where N-terminal domain binds/inactivates STING, thereby diminishing IRF3 activation/IFN secretion (34).

Additionally, ICP34.5 impedes autophagic processes through Beclin binding interactions specifically targeting Beclin-1 (Atg6) (35). Such engagement hinders this vital cellular defense mechanism allowing enhanced pathogenesis while blocking class II antigen presentation, further augmenting HSV virulence (36, 37).

Lastly, ICP34.5 also disrupts NF-kB activation suppressing dendritic maturation and ultimately impairing effective adaptive immunity against infection. The N-terminal domain of ICP34.5 interacts with IKKα/β, components of the IκB kinase complex, while its C-terminal domain recruits PP1α. This interaction leads to the dephosphorylation of IκB kinase, preventing the activation of NF-κB, a transcription factor that regulates genes involved in immune responses, inflammation, and cell survival (38, 39).

Through these combined mechanisms, ICP34.5 serves as a critical factor in HSV-1 pathogenesis by supporting viral replication, evading multiple immune pathways, and altering host cellular functions. However, after silent gene γ134.5, the oncolytic efficacy of HSV-1 in malignant tumors of neurological origin (e.g., glioblastoma and neurofibroma) has decreased. In clinical treatment, it is necessary to choose the oncolytic HSV-1 with silent gene γ134.5 according to the tissue source of the tumor.

2.2 Gene US11

To reduce immune evasion and subsequent uncontrolled viral infection after the injection of oncolytic HSV-1 into patients, gene US11 was selectively silenced. It can not only reduce the immune evasion of oncolytic HSV virus immunocompromised patients but

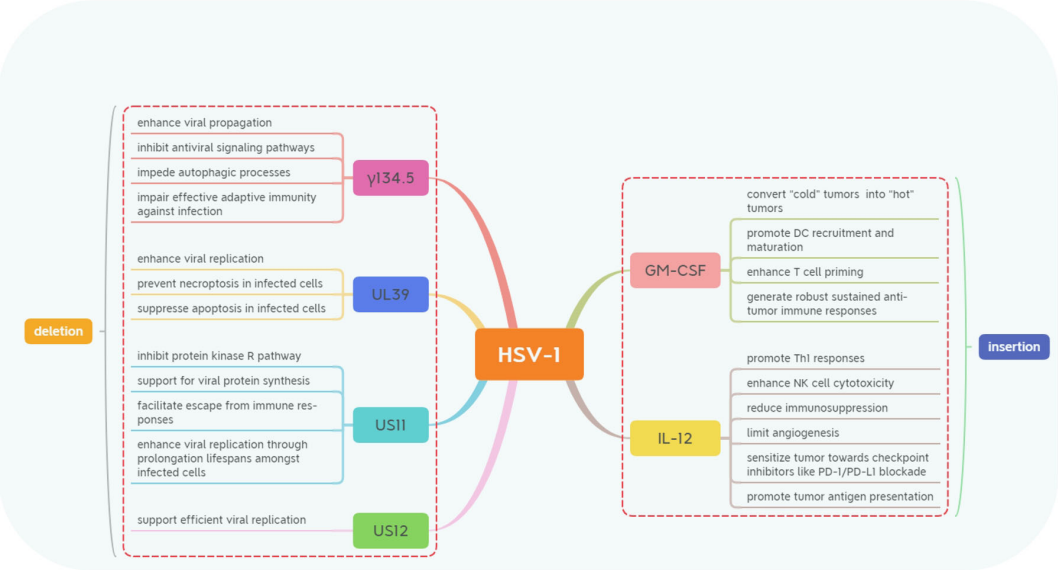

FIGURE 1
The genetic modifications in Herpes Simplex Virus-1 (HSV-1) involve deletions and insertions. These majority modifications include the deletion of genes such as γ134.5, US11, US12, and UL39 and the expression of transgenes like GM-CSF and IL-12. These strategic genetic engineering techniques are designed to enhance the oncolytic properties of HSV-1 while modulating immune responses to improve anti-tumor efficacy through various mechanisms.

also decrease the replication and spread of the virus in healthy cells. The US11 protein is a small basic phosphoprotein with a molecular weight of approximately 18 kDa. Its coding region extends from the ATG codon at residue 12,641 to the TAG stop codon at residue 12,158, resulting in a protein mass of 17,756 Da. The carboxy-terminal half contains several arginine-X-proline (R-X-P) repeats that confer RNA-binding capability (40). These repeats also harbor nucleolar import and nuclear export signals that facilitate localization within both nucleus and cytoplasm as required. Encoded by the late γ2 gene, US11 is expressed during later stages of HSV infection and performs several crucial functions enhancing HSV-1 survival within host cells (41).

Inhibition of protein kinase R (PKR) pathway alongside support for viral protein synthesis are primary functions attributed to US11. The PKR pathway becomes activated upon binding double-stranded RNA (dsRNA), leading to phosphorylation of eIF2α, an event typically halting protein synthesis as part of an antiviral response. US11 exhibits high affinity for dsRNA, allowing it to sequester this molecule away from PKR (42, 43). By obstructing PKR activation through this mechanism, US11 prevents eIF2α phosphorylation, thus sustaining viral protein synthesis. Furthermore, when expressed early during infection, US11 can partially compensate for ICP34.5's function inhibiting eIF2α phosphorylation. This redundancy enables HSV-1 to maintain ongoing translation even if ICP34.5 is absent. However, both proteins are generally necessary for full resistance against type I interferon (IFN) responses (43).

Additionally, US11 modulates various host antiviral pathways facilitating escape from immune responses by HSV-1. During late phases when levels of dsRNA peak, US11 binds/sequesters dsRNA effectively preventing MDA5/RIG-I activations, which subsequently suppress IRF3 activity along with interferons production (44, 45). Such inhibition impedes induction of ISGs establishment, hence compromising antiviral states among infected cells. Another important mechanism involves oligoadenylate synthetase (OAS) pathway activated via dsRNA binding where US11 inhibits OAS activity, blocking RNase L, thereby aiding virus evade degradation while preserving infectivity (46).

Moreover, US11 plays pivotal roles regulating cell survival pathways ultimately promoting enhanced replication through prolongation of lifespans among infected hosts. Within nuclei, US11 interacts with homeodomain-interacting protein kinase HIPK2 responding stress signals including those arising from ER-regulating cycle progression/pro-apoptotic signaling (47). By antagonizing growth-arrest-induced HIPK2, HSV-1-infected cells evade apoptosis, continuing to facilitate virion propagation (48).

Through multifaceted functionalities, US11 facilitates HSV-1 replication by preventing translational shutoff, inhibiting immunological signaling and obstructing pro-apoptotic response. By suppressing activations across PKR, OAS, MDA5, and RIG-I enable HSV-1 to evade defenses and sustain syntheses, thus augmenting survivability and pathogenicity. After the silencing of US11, the therapeutic effect of a single injection of oncolytic HSV-1 may be transient. Throughout the course of treating malignant tumors, multiple injections of oncolytic HSV-1 are required, and continuous monitoring of tumor growth is necessary to evaluate whether to administer oncolytic HSV-1 again.

2.3 Gene US12

One of the immune evasion mechanisms of HSV-1 is to inhibit antigen presentation by binding to TAP, thereby preventing cytotoxic T cells from recognizing infected cells. The protein encoded by the US12 gene is key to binding with TAP. The US12 gene (ICP47) spans residues 12,972 to 12,708 and encodes the immediate-early protein ICP47, which consists of 88 amino acids. Similar to US1 at the opposite end of the Us region, both the promoter region of ICP47 and a significant portion of its 5′-non-coding mRNA are situated within the terminal repeat (TR) sequences of HSV-1 (26). ICP47 plays a pivotal role in HSV-1's immune evasion strategy through various mechanisms and polymorphic functions during different stages of infection. It exhibits high-affinity binding to the transporter associated with antigen presentation (TAP). By occupying TAP's substrate-binding site, ICP47 inhibits viral peptide loading onto MHC class I molecules for presentation on cell surfaces to CD8+ T cells, effectively blocking cytotoxic T lymphocyte (CTL) recognition of infected cells and enabling HSV-1 to evade immune detection (14, 49).

The function of ICP47 is polymorphic as infection progresses. During early infection stages, it may impede RNA splicing, thus limiting host and viral gene expression in a tightly regulated manner. In later stages, however, ICP47 appears to facilitate viral mRNA export from the nucleus into the cytoplasm, thereby supporting efficient viral replication (50).

Deletion of US12 has been shown to enhance HSV-1's oncolytic potential and tumor-cell-killing ability alongside a stronger immune response. This deletion places US11 under immediate-early promoter control while enhancing replication efficiency in tumor cells for HSV-1 strains lacking ICP34.5, suggesting promising applications for oncolytic virotherapy (51, 52).

In summary, when ICP47 is deleted from its genome context, it can serve as an effective tool to reduce immune evasion in immunodeficient environments and tumor cells.

2.4 Gene UL39

The UL39 gene is situated within the Unique Long Region of the HSV-1 genome and plays a critical role in viral replication and in modulating physiological processes within host cells (53). To reduce the spread of oncolytic HSV-1 proliferation within tumors, gene UL39 is selected as the candidate gene to be silenced. Unlike certain other HSV-1 genes, UL39 does not generate repetitive sequences with adjacent regions of the viral genome, rendering it structurally distinct. This gene is expressed early during the HSV-1 replication cycle, prior to the entry of the viral genome into the host cell nucleus (54, 55). Its initial translation depends on transcription and translation mechanisms within host cells, enabling HSV-1 to swiftly produce essential proteins for sustained infection (55).

ICP6, which is encoded by the UL39 gene and serves as the large subunit of ribonucleotide reductase, is vital for converting ribonucleotides into deoxyribonucleotides necessary for DNA synthesis in viruses (56). Additionally, ICP6 can phosphorylate eIF2α, a key initiation factor, thereby suppressing host protein synthesis and favoring production of viral proteins over cellular functions. This mechanism facilitates enhanced viral replication within infected hosts (57).

Another significant function attributed to ICP6 involves its modulation of programmed cell death (PCD) processes in infected cells through its receptor-interacting protein-homotypic interaction motif (RHIM) (54). The RHIM domain prevents necroptosis by obstructing RIPK1-RIPK3 complex formation (receptor-interacting protein kinases 1 and 3) in human cells (58). Furthermore, it promotes aggregation of RIPK1 that subsequently undergoes degradation via aggrephagy, further diminishing necroptotic activity (11). It also inhibits RIPK1/RIPK3-dependent necroptosis in human cells. Beyond preventing necroptosis, ICP6 additionally suppresses apoptosis by directly binding to and inhibiting caspase-8. This dual inhibition strategy allows HSV-1 to circumvent major apoptotic pathways while promoting both survival and proliferation within host environments (53).

Inactivation of ICP6 through fusion with LacZ results in restricted virus propagation primarily among dividing cells, particularly tumor cells capable of supplying deoxyribonucleotides via endogenous pathways (59). This tumor-specific characteristic exhibited by mutated forms of ICP6 positions HSV-1 variants makes them promising candidates for oncolytic therapies. Although silencing the gene UL39 can limit the proliferation of oncolytic HSV-1 after injection, enhancing the safety of this oncolytic virus in clinical applications, the tumor-killing effect of this oncolytic virus is also restricted, requiring a larger dosage and multiple injections to achieve the desired effect.

3 Inserting exogenous genes

3.1 GM-CSF

Induction of immune cells to kill tumor cells is one of the key mechanisms of oncolytic virus anticancer. In addition to the immune activation of the viral particles themselves, the cytokine genes carried by oncolytic viruses can be synthesized and released in tumor cells, and this process also significantly improves the killing efficacy of immune cells to tumor cells. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a multifunctional cytokine that plays critical roles in immune modulation, serving as a bridge between hematopoiesis and immune activation (60, 61). Initially identified as a growth factor that stimulates the differentiation of bone marrow progenitor cells into granulocytes and macrophages, GM-CSF also activates various signaling pathways, including JAK/STAT, MAPK, and PI3K, through JAK2 activation, thereby influencing immune functions (62–65).

GM-CSF enhances the survival, proliferation, and differentiation of myeloid lineage cells such as neutrophils, macrophages, and dendritic cells (DCs) (66). By promoting DC maturation, GM-CSF improves antigen presentation capabilities and T-cell activation (60). To enhance the phagocytic abilities of macrophages and their anti-tumor activities, GM-CSF drives the polarization of these cells from

an M2 (anti-inflammatory) phenotype to an M1 (pro-inflammatory) phenotype (67). Furthermore, GM-CSF strengthens immune recognition of cancer cell neoantigens by fostering antigen, presenting cell generation, and elevating major histocompatibility complex (MHC) expression, thereby reinforcing the overall immune response against tumors (23, 68).

Oncolytic viruses (OVs) armed with GM-CSF lead to localized cytokine expression within the tumor microenvironment while enhancing tumor cell susceptibility to viral infection by driving these cells into the cell cycle. This effectively converts "cold" tumors characterized by low immune activity into "hot" tumors exhibiting high levels of immune activity (69). Additionally, GM-CSF-armed OVs promote DC recruitment and maturation at tumor sites, which enhances T-cell priming and generates robust anti-tumor immune responses (70). This process can also foster long-term immunological memory resulting in sustained anti-tumor effects. The first oncolytic HSV-1 armed with GM-CSF, talimogene laherparepvec (T-VEC), demonstrated significant anti-tumor efficacy leading to FDA and EMA approval for melanoma therapy (71–73). However, excessive GM-CSF release also aggravates the

TABLE 1 Genetic modification of the modified oHSV.

Aim	Target genes	Related oncolytic HSV-1	References
Enhance the potency of oncolytic viruses	HCMV IRS1	C132,C134	(81)
	HCMV TRS1	C130	(81)
	GADD34	NG34, NG34 ScFvPD-1	(82)
	MyD116	GD116	(83)
	GALV-GP R-	OncoVEX$^{GALV/CD}$	(84, 85)
	Nestin γ134.5	rQNestin34.5	(86, 87)
	angiostatin complementary	G47Δ-mAngio	(88)
Enhance the host immune response against the tumor	EphA2	C172, C170	(89)
	Flt3L	ONCR-177, G47Δ-Flt3L	(72, 90)
	IL-15	VG161	(91)
	anti-CTLA4	ONCR-177, RP2	(72)
Immunorecruitment and chemotactic infiltration	CCL2	M010,	(92)
	CCL4	ONCR-177	(72)
	CCL5	OV-Cmab-CCL5	(93)
Cooperate with PD-1 inhibitor	anti-PD-1 Fab	T3011	
	PD-L1B	VG161	(91)
	hPD-1scFv	YST-OVH, NG34 ScFvPD-1	(94, 95)
Prodrug invertase	cytochrome P450 enzyme	rRp450	(96)
	Fcy::Fur	OncoVEX$^{GALV/CD}$	(85)
Light-activated cytotoxicity	KR	G47Δ-KR	(97)
Anti-inflammatory	IL-4	R8306	(98)
Weaken the replication and re-transmission	UL55	HF10/C-REV	(99)
	UL56		
	US3	R7041, MG18L	(100, 101)
	UL23	Dlsptk	(102)
Reduce immune escape	UL43	HF10/C-REV	(99)
	UL49.5		
	LAT		

HCMV, human cytomegalovirus; GADD34, growth arrest and DNA damage gene 34; MyD116, mouse myeloid differentiation protein 116; GALV-GP R−, gibbon ape leukemia virus membrane R− glycoprotein; EphA2, ephrin type-A receptor 2; Flt3L, Fms-related tyrosine kinase 3 ligand; IL-15, interleukin-15; anti-CTLA4, anti-cytotoxic T lymphocyte-associated protein 4; CCL2, chemokine (C–C motif) ligand 2; CCL4, chemokine (C–C motif) ligand 4; CCL5, chemokine (C–C motif) ligand 5; Fcy::Fur, yeast cytosine deaminase/uracil phospho-ribosyltransferase fusion; KR, KillerRed; IL-4, interleukin-4; LAT, linker for activation of T cells; PD-L1B, programmed death-ligand 1 B; hPD-1scFv, humanized single-chain variable fragment against human PD-1; UL55, UL56, UL23, UL43, UL49.5, unique long region 55, 56, 23, etc.; US3, unique short region 3.
Gray indicates the distinct objectives, pink denotes the inserted genes, and blue signifies the representative strains harboring different types of inserted genes. Green marks the knockout genes, while yellow highlights the representative strains with various knockout genes.

systemic symptoms, such as fatigue and elevated body temperature. More attention is paid to the inflammatory status of patients during treatment.

3.2 IL-12

To enhance the tumor resistance of NK cells and cytotoxic T lymphocytes, interleukin-12 (IL-12) was selected as a candidate gene for the insertion of oncolytic HSV-1. Its ability to reshape the tumor microenvironment while augmenting responses to checkpoint inhibitors underscores its therapeutic potential particularly when combined with other cancer immunotherapies establishing it as a formidable agent in anti-tumor immunity.

IL-12 facilitates CD4+ T-cell differentiation into Th1 cells that secrete elevated levels of interferon-gamma (IFN-γ), which subsequently activates NK cells and cytotoxic T lymphocytes (CTLs), thereby enhancing their anti-tumoral functions (74). Moreover IL-12 amplifies both growth rates and cytotoxic activities among NK cells alongside CD4+ and CD8+ T lymphocytes, resulting in increased production of perforin and granzyme B, which are key molecules essential for CTLs' capacity to eradicate tumor cells (74, 75). Additionally, IL-12 promotes differentiation toward memory or effector T-cell phenotypes, thus improving precision persistence within targeting residual or metastatic malignant populations (76, 77).

Furthermore, IL-12 diminishes regulatory T-cell (Treg) and myeloid-derived suppressor cell (MDSC) populations within tumoral environments alleviating suppression mechanisms detrimental toward effective antitumoral responses (5, 78). It also drives macrophage polarization toward an M1 phenotype, a state characterized by pro-inflammatory properties conducive for inducing tumoricidal activity. By downregulating vascular endothelial growth factor (VEGF), IL-12 effectively reduces angiogenesis associated with tumors (25, 79).

Moreover, IL-12 sensitizes neoplasms toward checkpoint inhibitors like PD-1/PD-L1 blockade, thereby amplifying therapeutic efficacy (24, 80). With regard to promotion of tumor antigen presentation, death induced through IL-12-stimulated effectors releases TAAs further stimulating adaptive immunity assisting remaining malignant targets recognized by activated T cells (76, 77).

4 Others

In addition to the aforementioned wide-ranging applications in genetic modification techniques, several strategies for genetic modification demonstrate significant potential for clinical application (Table 1). A category of genetically modified viruses has been developed to enhance viral replication and tumor-specific cytotoxicity. The IRS1 and TRS1 genes from human cytomegalovirus (HCMV) have been inserted into HSV-2 to improve protein synthesis and replication by inhibiting PKR kinase activity and autophagy, thereby facilitating robust viral protein production and survival within tumor cells (81).

GADD34 is homologous to γ134.5; like MyD116, it can substitute for γ134.5 to restore viral replication in glioblastoma and breast cancer cells, enhancing selective cytotoxicity (82, 83). The Gibbon leukemia virus fusion glycoprotein (GALV-GP) increases the efficiency of viral vector entry while inducing cell fusion, significantly boosting tumor cell death *in vitro* and promoting tumor shrinkage *in vivo* (84, 85). The Nestin promoter drives selective replication in glioma cells, enhancing glioma suppression when combined with cyclophosphamide (86, 87).

To induce and facilitate host immune responses against tumors, numerous attempts have been made to insert various genes into oncolytic viruses (OVs). EphA2 induces anti-tumor immunity by generating EphA2-specific CD8+ T cells that are effective against resistant tumors (89). Flt3L promotes dendritic cell development, thereby enhancing both local and systemic anti-tumor immune responses (90). IL-15 amplifies NK cell and CD8+ T-cell responses while enhancing tumor-specific immune cycles as demonstrated in pancreatic cancer models (91). Anti-CTLA4 antibody ONCR-177 increases the CD8+ T-cell response specific to tumor antigens, effectively inhibiting metastatic tumors while bolstering memory responses (103).

Some studies focus on immunorecruitment and chemotactic infiltration of immune cells into tumors to improve the efficacy of oncolytic viruses against malignancies. Chemokine genes such as CCL2, CCL4, and CCL5 are incorporated into OVs to enhance immune cell infiltration within tumors. For instance, a γ134.5-deficient HSV-1 expressing CCL2 along with IL-12 enhances glioma killing capabilities (92), whereas OV-CIMab-CCL5 improves outcomes in glioblastoma patients (93).

Certain genetic engineering studies target synergy with immune checkpoint inhibitors for enhanced anti-tumor effects. PD-1, associated synergistic genes inserted into the HSV genome, include single, stranded variable fragment PD-1 (ScFvPD-1), variable region components of antibodies targeting programmed death receptor one (anti-PD-1 Fab),and portions acting as PD -1 blockers (PD-L1B). Incorporating ScFvPD-1 sequences into NG34 virus augments anti-tumoral responses prolonging survival rates observed across ovarian carcinoma models alongside those exhibiting glioblastomas, demonstrating synergistic benefits when paired with PI3K inhibitors (94, 104). The ScFvPD-1 gene is also integrated within YST-OVH aiming at promoting systemic antitumoral reactions through CTLA–4 or TIM–3 blockade (95).

Another broad category concerning genetic modifications applied toward OV focuses upon prodrug activation mechanisms. Infected tumoral environments allow the synthesis of prodrug invertase produced intracellularly via virally encoded proteins, converting non-toxic precursors and directly transforming them into therapeutic agents. As early as 1998, cytochrome P450 was introduced within HSV-I, enabling conversion processes whereby cyclophosphamide becomes activated specifically inside malignant tissues, leading toward notable anticancer effects evidenced across medulloblastoma atypical teratoid/rhabdomyosarcoma brain neoplasms among others (105, 106). This approach yielded substantial advantages during treatment regimens involving

TABLE 2 Published clinical trials with oHSV.

Year (published)	Phase	oHSV applied	Method	Tumor	References
2024	Phase IB	orienx010	orienx010+anti-PD-1	Toripalimab melanoma	(110)
2024	Phase II	T-VEC	T-VEC+radiotherapy	cutaneous metastases from solid tumors	(111)
2024	Phase II	T-VEC	T-VEC+pembrolizumab	melanoma	(112)
2023	Phase I	CAN-3110	CAN-3110	glioblastoma	(113)
2023	Phase II	T-VEC	T-VEC+surgery	melanoma	(114)
2023	Phase II	T-VEC	T-VEC+ipilimumab	melanoma	(115)
2022	Phase I	T-VEC	T-VEC+CD1c (BDCA-1)+ +/− CD141 (BDCA-3) + myDCs	melanoma	(116)
2022	Phase III	T-VEC	T-VEC+pembrolizumab	melanoma	(117)
2022	Phase II	G47Δ	G47Δ	glioblastoma	(118)
2022	Phase I/II	G47Δ	G47Δ	glioblastoma	(119)
2022	Phase I	T-VEC	T-VEC	melanoma	(120)
2022	Phase IB	orienx010	orienx010	melanoma	(73)
2021	Phase II	T-VEC	T-VEC	breast cancer	(121)
2021	Phase II	T-VEC	T-VEC+surgery	melanoma	(122)
2021	Phase IB/II	T-VEC	T-VEC+external beam radiation therapy	sarcoma	(123)
2021	Phase II	T-VEC	T-VEC	melanoma	(124)
2021	Phase I	T-VEC	T-VEC+neoadjuvant chemotherapy	breast cancer	(125)
2020	Phase IB	T-VEC	T-VEC+pembrolizumab	head and neck squamous cell carcinoma	(126)
2020	Phase II	T-VEC	T-VEC+pembrolizumab	sarcoma	(127)
2019	Phase I	HSV1716	HSV1716	relapsed or refractory extra-cranial solid cancers	(128)
2019	Phase II	T-VEC	T-VEC	melanoma	(129)
2019	Phase III	T-VEC	T-VEC	melanoma	(130)
2018	Phase I	HF10	HF10+erlotinib and gemcitabine	pancreatic cancer	(131)
2018	Phase II	T-VEC	T-VEC+ipilimumab	melanoma	(132)
2017	Phase I	G207	G207	malignant brain tumors	(133)
2016	Phase III	T-VEC	T-VEC	melanoma	(134)
2016	Phase I	M032	M032	malignant brain tumors	(135)
2015	Phase III	T-VEC	T-VEC	melanoma	(136)
2014	Phase I	HF10	HF10	refractory superficial solid tumors	(137)
2014	Phase I	G207	G207+radiation	malignant brain tumors	(138)
2010	Phase I/II	T-VEC	T-VEC+chemoradiotherapy	head and neck squamous cell carcinoma	(139)
2010	Phase III	T-VEC	T-VEC	melanoma	(140)
2009	Phase II	T-VEC	T-VEC	melanoma	(141)
2006	Phase I	NV1020	NV1020	hepatic colorectal metastases	(142)

diverse oncological conditions including but not limited to those previously mentioned (96, 107, 108).

An additional strategy involves inserting a gene-encoding yeast cytosine deaminase/uracil phospho-ribosyltransferase fusion(Fcy::Fur) into HSV-I, prompting infected neoplastic entities capable of synthesizing said construct. Fcy::Fur fusion catalyzes transformation processes wherein five-fluorocytosine (5-FC) is converted selectively, yielding toxic derivatives known as five-fluorouracil (5-FU), effectively targeting only malignant cellular populations without adversely affecting surrounding healthy tissue structures (85).

Recently, Kazuhide's team successfully integrated killer red (KR) gene allowing light-induced singlet oxygen generation, which markedly enhanced overall effectiveness regarding treatments administered under laser irradiation particularly noted among cases involving both gliobastomatosis multiple myelomas (97).

To enhance safety profiles related specifically toward employing HSV-1-based therapeutics aimed at combating cancers, certain critical genomic deletions occur preventing uncontrolled propagation/infection events. Two primary methodologies exist focusing upon limiting risks tied closely together utilizing these engineered strains. One method entails restricting replicative capacity particle assembly through deletion, such as UL55, UL56, US3, and UL23, thus confining resultant virulence strictly localized around affected sites (99, 102, 109). Another tactic employs removing particular loci inclusive of UL43, UL49.5, and LAT, mitigating escape routes available and henceforth increasing the likelihood of successful elimination efforts directed toward residual pathogenic threats encountered post-treatment interventions (99).

5 Clinical trials

Preclinical studies have identified a substantial number of oHSVs with diverse antitumor properties. To gain a deeper understanding of the clinical application of oHSVs, we conducted a review of 34 published oHSV clinical trials spanning the past two decades (Table 2). Over half of these clinical trials were concentrated in Phases I and II, comprising 67% of the total. The three most common treatment methods were the injection of T-VEC, the combination of T-VEC injection and pembrolizumab, and the injection of G47Δ, which accounted for 26%, 11%, and 5.9% of the total, respectively. Among the tumors targeted by oHSV clinical trials, the top 2 were melanoma and brain tumors, representing 50% and 17.6% of the total, respectively. The oHSV type most frequently reported in clinical trials was T-VEC (n=22), accounting for 64% of all clinical trials. Notably, 22 out of the 34 clinical trials were conducted in the past 5 years, indicating a significant increase in research interest in this field.

6 Discussion

In this review, we observed that the majority of oHSV clinical trials have employed various forms of viral modifications, such as deletions of genes γ134.5, US11, US12, and UL39, or the expression of transgenes like GM-CSF and IL-12. We also explored various gene modifications, which, despite not having been evaluated in clinical trials, represent a promising direction for future oncolytic virus research. Although oncolytic virotherapy is a promising anti-tumor technique, it is still facing several challenges.

The effectiveness of oncolytic viruses (OVs) is modest despite good safety. Viral genetic engineering improvements may enhance efficacy, but there are still obstacles in clinical trials, like balancing viral replication and immune responses, optimizing delivery routes, and achieving tumor-specific targeting.

During oncolytic virotherapy, it is imperative to achieve equilibrium between viral proliferation and the host's anti-viral immune response. The ideal immune response is to allow viral replication early in oncolytic virotherapy and to initiate humoral immunity and clear the virus quickly at the end of treatment. The host immune system is crucial for tumor elimination but can clear OVs prematurely, limiting their therapeutic potential. Optimizing virus delivery and suppressing early immune responses give the virus more time for anti-tumor action. One of the strategies currently ongoing is to optimize delivery methods so that the virus moves silently into tumor cells before the host generates an immune response to clear the virus. Another strategy is to suppress the host immune response early on treatment, thereby improving the infection efficiency of the oncolytic virus. Upon completion of therapy, the introduction of antiviral medications expedites the virus' elimination (143).

The current delivery methods include intratumoral injection and intravenous delivery. Intratumoral injection has the limitation of accessible tumors and is practically difficult in deep-seated or metastatic cases. For inaccessible tumors, imaging-guided or surgical approaches are required, which further complicate intratumoral injection. Intravenous delivery is more convenient than intratumoral injection. However, it requires high specificity to target tumors effectively, not to mention that it has risks of systemic toxicity and immune clearance.

Moreover, OVs as monotherapy may not achieve best therapeutic results. OVs are usually combined with other therapies, including immune checkpoint blockade or traditional anti-tumor therapies, to increase efficacy. Recently, integrating OVs with chimeric antigen receptor (CAR)-T cell therapy has emerged as an option. It could facilitate targeted delivery while improving bioavailability and enhancing tumor specificity. Furthermore, optimizing timing and dosing remains crucial for maximizing synergy between OVs and CAR-T cells (144, 145). A comprehensive regimen combining stereotactic body radiotherapy, oncolytic virotherapy, and pembrolizumab was used in clinical studies of metastatic non-small-cell lung cancer. The results demonstrate the superior prognosis of the comprehensive treatment regimen over conventional chemotherapy and pembrolizumab alone (146).

Potential safety issues of oncolytic virus therapy have also been suggested in clinical trials. For example, tumor cells died in large numbers after virus injection, resulting in the release of large amounts of antigenic material and cytokines. If the above-

mentioned process occurs in a short time, it can lead to the life-threatening cytokine release syndrome. In addition, after the death of tumor cells, intracellular substances enter the circulation system and affect the coagulation system, which can lead to thrombosis or bleeding events. In addition, viruses may also cause insertional mutagenesis in host cells; for instance, the oncolytic adenovirus-based studies have found out the integration of viral genes into the host genome. As a kind of DNA virus, the possibility of insertional mutagenesis of HSV-1 virus is relatively small in theory, while long-term observation and studies are also needed toward this issue (147). Oncolytic HSV-1 has the potential to move through blood–brain barrier and infect the central nervous system, which, on the one hand, makes this type of oncolytic virus a candidate for the treatment of neurogenic malignancies, and on the other hand, increase the risk of central nervous system virus infection during the treatment of other tumors. Genetic modification is commonly used as one of the preventive strategies to reduce the pathogenicity of oncolytic viruses and improve their specificity for tumor cells. For example, G47Δ silenced γ134.5, UL39, US12, and US11 genes simultaneously (118). Clinical trials have shown that this kind of virus can barely replicate *in vivo*; therefore, treatment with the right dose of injected virus can safely treat tumors. Another preventive strategy is to combine oncolytic virus therapy with tumor immune checkpoint therapy or chemotherapy to kill the tumor while reducing the amount of oncolytic virus injection during the treatment. This strategy is currently widely used in clinical trial, such as the use of T-VEC virus strain combined with anti-PD-1 treatment (112, 126, 127).

As an increasing number of clinical trials explore newly engineered oncolytic virotherapies, these advancements are poised to yield significant breakthroughs in related research and promote the widespread adoption of oncolytic virotherapy for cancer treatment.

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