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Advance in herpes simplex viruses for cancer therapy

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Oncolytic virotherapy is an attractive approach that uses live viruses to selectively kill cancer cells. Oncolytic viruses can be genetically engineered to induce cell lyses through virus replication and cytotoxic protein expression. Herpes simplex virus (HSV) has become one of the most widely clinically used oncolytic agent. Various types of HSV have been studied in basic or clinical research. Combining oncolytic virotherapy with chemotherapy or radiotherapy generally produces synergic action with unclear molecular mechanisms. Arming HSV with therapeutic transgenes is a promising strategy and can be used to complement conventional therapies. As an efficient gene delivery system, HSV has been successfully used to deliver various immunomodulatory molecules. Arming HSV with therapeutic genes merits further investigation for potential clinical application.

oncolytic viruses, herpesvirus, virotherapy, cancer, gene therapy

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Cancer cells are still difficult to destroy at present. Five-year survival rates for pancreatic, liver and lung cancer remain disappointing. It is reported that 15 million new cancer cases and 10 million new deaths are expected in 2020 [1]. Carcinogenesis is a multi-step process involving various mutation points in genome. A single treatment is insufficient and various therapeutic strategies are needed due to the cancer heterogeneity. Surgery, chemotherapy and radiotherapy are three major approaches to treat cancer at present. However, chemotherapy and radiotherapy often have limited effects with toxicities. Oncolytic virotherapy is based on the concept of using live viruses to selectively replicate in cancer cells, with minimal toxicity to normal tissue. It is a novel way to eradicate cancer cells. Oncolytic viruses can be genetically engineered to specially target cancer cells and induce cell lyses. The mechanisms of oncolytic viruses destroying cancer cells are still unclear. They may be associated with virus replication in cancer cells, virus cytotoxic protein expression and activation of host immune system [2]. As the mechanisms are different from chemotherapies and radiotherapies, oncolytic virotherapies often synergize with these traditional approaches [3]. Various different viruses are undergoing preclinical or clinical research, including vaccinia, adenovirus, herpes simplex virus, reovirus and Newcastle disease [4]. Herpes simplex virus (HSV) is one of the most widely clinically used oncolytic agent. As the first oncolytic viruses to be introduced in fighting against cancer, HSV are easily manipulated and can be inserted transgene. Moreover, previous work from our studies and others has shown that HSV combined with radiotherapies or chemotherapies produce synergistic action [5,6]. The HSV used as oncolytic agents offer some advantages: (i) HSV infects most tumor cells types and replicates rapidly in infected cells; (ii) HSV virions have the ability of viral penetration within tumor; (iii) HSV with a large genome is easily manipulated and can be inserted multiple transgenes; (iv) HSV replication can be shut off with anti-HSV drug if required [7–10]. In this review, we will focus on the anti-

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tumor capability of HSV in basic research and clinical application.

1 An overview of HSV

HSV is a double-stranded DNA virus encoding more than 84 different polypeptides. HSV DNA consists of two unique sequences: Unique Long (U_L) and Unique Short (U_S), which are separated by terminal inverted repeats [11]. One or more α sequences with 400 base pair (bp) join the L and S segments. Gene-modified HSV includes inactivation of *ICP*6 gene and deletion of γ-34.5 [12]. *ICP*6 encodes the large subunit of ribonucleotide reductase, which is essential for viral DNA replication. *ICP*6-muated HSV can only replicate in rapidly dividing cells but not in quiescent cells. Virus gene γ34.5 is the major neuropathogenicity gene in HSV [13]. Phosphorylates the α-subunit of eIF-2α will block the host proteins synthesis upon viral infection. However, the γ-34.5 that encodes protein *ICP*34.5 can overcome the response. *ICP*34.5 expression results in dephosphorylation of eIF-2α and disinhibition of protein synthesis. Ras/Mitogen activated protein kinase kinase (MEK) pathway is often activated in cancer cells, which enable γ34.5-deficient HSV to replicate selectively in cancer cells. As a consequence of genetic modification, ICP6/γ34.5-deleted HSV imposes low pathogenicity to normal tissue and high oncolytic capacity to tumor cells. Multi-genes deletions confer great selectivity and safety on HSV; however, deletions of mutations lead to a reduction in neurovirulence and clearness of the virus by host immune response [14,15]. HSV oncolytic nature is determined by lystic genes. The viral genes are divided into three categories: immediate early or α genes, early or β genes, and late or γ genes. These proteins encoded by lystic genes promote replication of virus and react against host immune response [16]. A lystic replication cycle includes that α genes enhance β genes to transcript and synthesize proteins, which are required for DNA replication. The main function of α gene is to regulate the transcription of β and γ genes. After DNA replication, γ genes encode the structural components of virion. The viral DNA replication and protein synthesis lead to cancer cells lysis, and subsequently progeny viruses are released to infect adjacent cancer cells, amplifying the cancer-killing ability. A HSV can be delivered via regional delivery (peritoneal or pleural perfusion and intratumor injection) and systemic delivery (vascular administration). Intratumor injection limits delivery to tumor site. Physical barrier such as extracellular matrix and host immune response against HSV inhibit virus replication and distribution [17]. Clinical experiment has demonstrated that intravascular delivery of HSV is a safe and effective way to produce oncolytic effects [18]. Types of HSV used as oncolytic viruses are summarized in Table 1. *Dlsptk* is the first engineered HSV-1 derivate with deletion of thymidine kinase (TK) gene mutation. *Dlsptk* showed potent antitumor property and attenuated neurovirulence in the treatment of malignant human gliomas cells [19,20]. However, intracranial inoculation of dlsptk was limited by fatal encephalitis at higher doses [21]. Therefore, other engineered herpes simplex mutants with reduced neurovirulence are studied.

R3616 is a recombinant virus derived from HSV-1(F) strain with deletion of two copies of γ-34.5 genes. Compared with chemotherapy, R3616 can elicit more effective host anti-tumor immune responses. R3616 induces a greater number of infiltrating T cells, macrophages and dendritic cells in injected murine colon cancer model [22]. There is a complicated interaction among viruses, cells and chemotherapy agents. Combination of R3616 with gemcitabine could produce synergic action against advanced pancreatic cancer [23]. Recently, Kanzaki et al. [24] reported a new delivery of R3616 by absorption of the virus onto tumor antigen-specific lymphocytes. R3616 has an oncolytic effects on not only primary tumor lesions, but also multiple metastatic lesions. This method inhibits virus neutralization and will become one of the most effective ways of systemic virus delivery.

DM33 is a recombinant HSV-1 with deletions of γ-34.5 and *LAT* gene. Being different from *dlsptk*, mutations in DM33 are based on the McKrae strain, which enhance the virus to better grow and kill cancer cells. DM33 was tested on nude mice bearing intracranial U-87 MG human gliomas. Prolonged survival times were achieved in mice receiving DM33 compared with control group. Histological analyses showed increased tumor cells lyses and decreased tumor size in DM33-treated group. DM33 spreading was only limited to the tumor without infecting normal tissue [25]. In another study, DM33 demonstrated dose-dependent antitumor activity in glioma cell lines. The safety of DM33 was supported by its attenuated toxicity against neuronal cells, astrocytes, and endothelial cells [26]. Based on its efficacy and safety, DM33 shows considerable promise as an oncolytic virus for cancer treatment.

G207 is the first oncolytic HSV-1 used in clinical trial in the United States. It contains deletions of two copies of the γ-34.5 and insertion of an *Escherichia coli LacZ*. Moreover, thymidine kinase gene derived from HSV is intact in G207, enabling amplifying its anti-tumor effect by treatment with prodrugs [27]. G207 induces systemic anti-tumor immunity in the course of oncolytic activity. The anti-tumor immunity is associated with an increasing cytotoxic T lymphocyte activity [10]. G47 delta is a HSV-1 vector derived from G207 with an additional deletion of nonessential α47 gene. This deletion places the late U_S11 gene under control of the immediate-early α47 promoter, which promotes replication of G47delta. Moreover, mutation of α47 increases MCH-I expression in infected cells, thus enhancing tumor antigen presentation and anti-tumor immunity [28]. Both G207 and

Table 1 Types of HSV used as oncolystic viruses

Name	Mutation	Tumor type	Therapeutic traits	Ref.
Dlsptk	Deletion of viral thymidine kinase (TK) gene	Malignant human gliomas cells	Dlsptk virus has significant safety concerns. Its neurotoxicity is seen at high doses.	[19–21]
R3616	Deletion of two copies of γ-34.5 genes	Pancreatic cancer; colon carcinoma cell	R3616 induced a greater number of infiltrating T cells, macrophages and dendritic cells than chemotherapy.	[22–24]
DM33	Deletions of γ-34.5 and *LAT* gene	Human gliomas and glioma cell lines	Mutations in DM33 are based on the McKrae strain, enhancing the virus to better kill cancer cells with attenuated virulence.	[25,26]
G207	Deletions of two copies of the γ-34.5; insertion of an *Escherichia coli LacZ*	Prostate adenocarcinoma; glioblastoma; hepatocellular carcinoma; colorectal cancer	G207 induces systemic anti-tumor immunity with an increasing cytotoxic T lymphocyte activity specific to tumor cells.	[10,27, 29,30]
G47 delta	Derived from G207 with additional deletion of α47 gene	Prostate adenocarcinoma; glioblastoma; rectal cancer; nasopharyngeal carcinoma; breast cancer	G47 delta has better efficacy than that of G207.	[28–30]
NV1020	Deletion of a 15-kb region at the UL/S junction and 700 bp deletion in thymidine kinase (tk) locus	Pancreatic cancer; colon carcinoma; bladder cancer; pleural cancer	Its efficacy and safety have been tested in clinical trials.	[31–37]
HF10	Deletion of 3.9 kbp in the right end of the UL and UL/IRL junction	Breast cancer; malignant melanoma; pancreatic cancer	HF10 enhances angiogenesis and induced a cytotoxic T lymphocyte response directed against tumor cells.	[38–42]
HSV1716	Derived from HSV-1(17+) strain with the deletion of γ-34.5 genes	Glioblastoma multiforme; anaplastic astrocytoma; oral squamous cell carcinoma	HSV1716 is safe and well tolerated at high dose up to 1×10^5 PFU.	[43–46]
Oncovex$^{GM\text{-}CSF}$	Deletion of two copies of ICP34.5 gene and the viral ICP47 genes; insertion of (GM-CSF)	Breast cancer; head and neck cancer; gastrointestinal cancers; malignant melanoma	Oncovex$^{GM\text{-}CSF}$ induces antitumor effects through both direct tumor lysis and secondary initiation of tumor-specific immune response.	[47–50]
FusOn-H2	Replacing the serine/threonine protein kinase (PK) domain of the ICP10 gene with the DNA sequence expressing enhanced green fluorescent protein (EGFP)	Renal cell carcinoma; metastatic ovarian cancer; pancreatic cancer; breast cancer; lung carcinoma	It selectively targets the activated Ras signaling pathway in tumor cells and induces cancer cell apoptosis.	[51–56]

G47delta target the human cancer specimens specifically. However, G47delta has better efficacy of replication than that of G207 and exhibits greater cytopathic effects in xenogenic models and majority of cell lines [29,30].

NV1020 is an attenuated, recombinant virus derived from HSV-1. A 15-kb region at the UL/S junction encompassing one copy of the diploid genes (α0, α4 and γ134.5) and one copy of U_L56 is deleted. NV1020 is further attenuated by deletion of thymidine kinase (TK) gene and the promoter for the gene U_L24. In addition, an exogenous copy of *tk* gene is inserted under control of the *ICP*4 promoter [31]. These modifications enable NV1020 to be highly attenuated and only propagate in tumor cells. Safety and efficacy of NV1020 have been tested in pancreatic cancer, colon carcinoma, bladder cancer and pleural cancer [32–35]. Given such promising preclinical data, a Phase I clinical study of NV1020 was taken by enrolling 12 patients with colorectal adenocarcinoma metastatic to the liver [36]. NV1020 virus was delivered into hepatic artery in four cohorts of three patients each. The administered doses were 3×10^6, 1×10^7, 3×10^7 and 1×10^8 plaque-forming units. All the 12 patients exhibited reduction in tumor size in response to subsequent chemotherapy. No serious adverse events associated with NV1020 were observed, supporting that NV1020 could be safely administered into the hepatic artery. Based on data from the Phase I clinical study, an additional Phase I/II trial of NV1020 for colorectal cancer hepatic metastases has been completed. The study evaluated the safety, pharmacokinetics, and anti-tumor effects of NV1020. The results showed that the NV1020 stabilized liver metastases in patients, and extended survival by re-sensitizing to chemotherapy [37]. A larger, randomized phase II/III trial study combining NV1020 with cytotoxic and targeted agents is underway.

HF10 has a deletion of 3.9 kbp in the right end of the UL and UL/IRL junction, resulting in the loss of U_L56 expression. HF10 replicates more efficiently with low virulence than wild-type HSV-1. Its safety and effectiveness are tested in many animal models, and it has been used for treatment of breast cancer, malignant melanoma and pancreatic cancer in clinical trial [38–41]. Recent report showed that HF10 also had effects on the tumor microenvironment in patients with recurrent carcinoma tumor [42]. HF10 enhances the angiogenesis and induces a cytotoxic T lymphocyte response against the tumor. These characteristics confer HF10 as a promising oncolytic virus for the treatment of advanced cancer.

HSV1716 is derived from HSV-1(17+) strain with the

deletion of both copies of γ-34.5 genes. Safety and toxicity of HSV1716 administration was first addressed in patients with glioblastoma multiform and anaplastic astrocytoma [43]. This study showed that HSV1716 was well tolerated and no adverse events were observed at high dose up to 1×10^5 PFU. HSV1716 has been applied to treat oral squamous cell carcinoma and gloma [44–46].

Oncovex$^{GM\text{-}CSF}$ is a genetically engineered strain of HSV-1 with deletion of two copies of *ICP34.5* genes and *ICP47* genes, and insertion of human granulocyte macrophage colony-stimulating factor (GM-CSF). Its anti-tumor response is enhanced through the deletion of *ICP47* gene and the delivery of GM-CSF. Oncovex$^{GM\text{-}CSF}$ induces anti-tumor effects through both direct tumor lysesand secondary initiation of tumor-specific immune response. It has been evaluated in clinical trial under name of OncoVex$^{GM\text{-}CSF}$ (BioVex Ltd, Abingdon, UK). The phase I study established the safety of Oncovex$^{GM\text{-}CSF}$ in patients with various metastatic tumor types, such as breast, head and neck cancer, gastrointestinal cancers, and malignant melanoma [47]. A multi-institutional Phase II clinical trial of Oncovex$^{GM\text{-}CSF}$ enrolling 50 patients with metastatic melanoma showed that direct injection of Oncovex$^{GM\text{-}CSF}$ into melanoma lesion resulted in a 28% objective response. Oncovex$^{GM\text{-}CSF}$ exhibited therapeutic response via increasing antigen-specific T cells response and decreasing the level of suppressor $CD4^+$ Tregs, $CD8^+$ Ts and myeloid-derived suppressive cells [48]. A prospective, randomized Phase III clinical trial in patients with unresectable melanoma has been initiated (OPTIM clinical trial) [49]. Current information about the clinical trial can be found on the Biovex, Inc. (MA, USA) sponsored website [50].

FusOn-H2 is constructed from HSV-2 by replacing the serine/threonine protein kinase (PK) domain of the *ICP*10 gene with the DNA sequence expressing enhanced green fluorescent protein (EGFP). It selectively targets the activated Ras signaling pathway in tumor cells and induces cell apoptosis. Moreover, the promoter of *ICP*10 is also replaced with the cytomegalovirus immediate early promoter, which can induce syncytia formation in cancer cells. Therefore, FusOn-H2 owns an additional oncolytic mechanism that enhances its anti-tumor effects [51]. FusOn-H2 showed potent oncolytic activity against renal cell carcinoma, metastatic ovarian cancer, pancreatic cancer and human breast cancer [51–54]. These studies reported that administration of FusOn-H2 at large dose (up to 1×10^7 pfu) was less toxic than parental wt virus and was well-tolerated by the animals. Li H and colleagues reported that FusOn-H2 induced strong T cells response against primary and metastatic mammary tumor in animal model [55]. Another study showed that co-administration of FusOn-H2 and cyclophosphamide produced a synergistic anti-tumor effect against lung carcinoma growing in mice [56].

2 HSV combined with conventional therapies

Like other therapeutic strategies, oncolytic viruses can not eradicate the cancer cells completely. Combining oncolytic virotherapy with chemotherapy or radiotherapy generally produces synergic action in preclinical models [57–59]. The interaction between HSV and conventional therapies has been studied by many groups (summarized in [60]). However, the molecular mechanism underlying this interaction is unclear. A widely-accepted hypothesis is that radiotherapy or chemotherapy increases the GADD34 expression, resulting in greater viral replication and increased anti-tumor efficacy (Figure 1). The expressions of GADD family (GADD34, GADD45 and GADD153) can be promoted by various stresses, such as radiation or chemotherapeutic agents. The function of GADD family protein is mainly to stop cell cycle progression at G1 and G2 checkpoint for viral DNA repair. A region of GADD34 protein is homologous to the carboxyl terminal domain of the protein encoded by γ34.5. Deletion of γ34.5 significantly reduces viral virulence; however, it also inhibits viral replication and decrease cytotoxicity. Therefore, upregulation of GADD34 expression complements and substitutes for γ34.5 gene, leading to increased viral protein synthesis and viral replication [61]. The upregulation of GADD34 expression by radiation in tumor cell infected with HSV was seen in head-and-neck cancer, cholangiocarcinoma and lung cancer [62–64]. It is also reported that enhanced expression of host cellular ribonucleotide reductase (RR) following radiation treatment also increased viral replication and tumor cell killing [65,66]. We found that radiotherapy and HSV produced the synergistic oncolytic activity against pancreatic cancer cell lines. However, the radiotherapy at different doses had no impact on HSV viral replication in our study [5]. Other studies also showed that synergistic cytotoxicity between HSV-1 and chemotherapy was not related to alteration in viral replication [67,68]. It is clear that multiple mechanisms are involved in the interaction between HSV and conventional therapies. First, radiotherapy or chemotherapy may induce a number of alterations in gene expression, and these molecular changes can impact the viral life cycle. Second, impact of radiotherapy or chemotherapy on viral entry into tumor cells could also influence antitumor effects [63]. Moreover, the synergistic interaction is not universal, depending on cell type, chemotherapeutic agents and viral strain [6]. Combined therapeutic regimens are promising strategies in order to maximize efficacy and reduce treatment-associated toxicity.

3 Arming HSV with therapeutic genes

The discovery of genetic mutation in cancer cells has promoted the gene therapy, which is to restore, enhance or in-

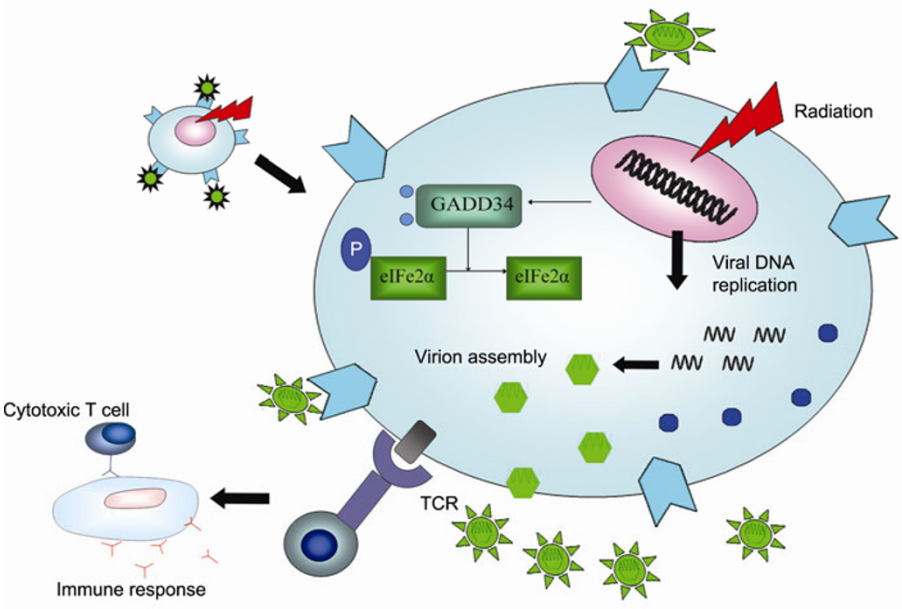

Figure 1 Radiation-mediated enhanced viral replication and inducing tumor cells lysis. As the specific cell-surface receptors are over-expressed by tumor cells, the viruses will infect the tumor cells. Simultaneously, radiation or chemotherapy induces DNA damage, resulting in upregulation of GADD34 transcription. Increase of GADD34 protein expression blocks the phosphorylation of eIF-2α, leading to increased viral protein synthesis and viral replication. Viral gene expression and replication activate the immune response. Excessive amount of viral budding and immune attack by $CD8^+$ T cells result in infected tumor cells lysis.

hibit a particular gene of interest by introducing exogenous genes [69]. The therapeutic genes include suicide genes, cytokines, chemokines, immuno-stimulatory molecules or tumor suppressor genes. Viruses are the most efficient gene delivery systems. The armed oncolytic viruses produce potent anti-tumor effects [70]. HSV-1 vector can be incorporated large therapeutic genes within the viral genome and it has been successfully used to deliver various immuno-modulatory molecules (includingIL-12, IL-24, IL-4, CD80, IL-18, and IFN-α). These transgenes inhibit tumor growth by stimulating local inflammatory and/or host immune response. Arming HSV with therapeutic genes merits further investigation for potential clinical application.

Interleukin-12 (IL-12), mediated by Th-1 response, shows strong anti-tumor activity. IL-12 has stimulatory effects on CTLs, helper T lymphocytes, and natural killer cells. IL-12 is a central regulator of the immune response, which is involved in recruiting and activating the NK and T cells. In addition to its immunomodulatory effects, IL-12 also possesses the property of antiangiogentic capability via stimulating secretion of IFN-γ from helper T lymphocytes. HSV armed with IL-12 showed enhanced oncolytic effects on squamous cell carcinoma. In a murine model of squamous cell carcinoma or micro-metastatic liver disease, NV1042 expressing IL-12 displayed significantly stronger anti-tumor in comparison with its non-IL-12-expressing analog, NV1023 [71–73]. Moreover, NV1042 also exhibited more efficacious effects than GM-CSF-expressing NV1034 [74]. Immunohistochemistry revealed an infiltration of $CD4^+$, $CD8^+$ T cells and macrophages in tumors treated with NV1042 [10]. The influx of lymphocytes seemed to account for the potent antitumor effects. However, the infiltration of immune cells results in not only rapid clearness of the virus but also damaging normal tissues.

Granulocyte macrophage colony-stimulating factor (GM-CSF) is secreted by activated lymphocytes and macrophages. It produces multiple immuno-stimulatory effects, including recruiting antigen-presenting cells such as macrophages and dendritic cells, and promoting their activation and differentiation [75]. GM-CSF is a cytokine involved in initiation phases of the immune response. $Oncovex^{GM\text{-}CSF}$ has been described as “oncolytic vaccine” or “oncolytic immunotherapy”. It has been tested in various tumor cell lines and mouse tumor models, both showing potent antitumor effects. GM-CSF may exert cytotoxic effects on tumor cells by activating T cells. Depletion of T cells inhibited the efficacy of NV1034, and eliminated the statistical difference in antitumor between animals treated with NV1034 and NV1023 [72]. It is reported that high dose of GM-CSF was toxic and brought immuno-suppressive effects [76].

Interleukin-24 (IL-24), Interleukin 4 (IL-4) and Interferon (IFN) also have been shown to selectively induce tumor cells apoptosis via inhibiting tumor angiogenesis, regulating maturation of T helper cells or modulating the tumor microenvironment (summarized in [77]). All the above transgenes approaches are likely to produce antitumor effects.

However, they can only produce one particular mediator and thus their oncolytic effects are limited. Moreover, it is difficult to control the amount of cytokine and there is a risk of excessive cytokine expression [61].

4 Future perspectives

The use of oncolytic viruses as an adjuvant therapy after tumor resection seems an attractive approach. As various types of oncolytic viruses have been tested for their anti-tumor properties, it is also important to elucidate the interaction between oncolytic viruses, the tumor microenvironment, and the immune system. It is still unclear what decreases or enhances their oncolysis *in vivo*, and what factors influence oncolytic viruses spread within the tumor microenvironment. In addition, the efficacy of combining oncolytic viruses and conventional therapies needs to be confirmed. Combination therapy regimens that produce synergic action against tumor cells without overlapping side effects are expected. Arming HSV with therapeutic transgenes is a promising strategy in cancer therapy and can be used to complement conventional therapies. However, issues such as oncolytic viruses' specificity and keeping virus replication under control should be addressed.

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