Expert Opinion

Oncolytic vaccinia virus for the treatment of cancer

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Introduction: Gene therapy offers promising approaches for the development of anticancer agents with new modes of action. Among gene therapy vectors, vaccinia virus has emerged as an attractive agent especially when used as an oncolytic virus.

Areas covered: This review describes the use of vaccinia virus in cancer therapy as a gene therapy vector, as an oncolytic virus and in the generation of oncolysates. The main achievements of each field are summarized with a special emphasis on vaccinia as an oncolytic vector and its combination therapies. The virus that has advanced furthest in clinical trials, GM-CSF expressing JX-594, is described in detail and its preclinical and clinical data are reviewed.

Expert opinion: Vaccinia virus has great potential in cancer gene therapy, especially when used as an oncolytic virus. In particular, JX-594 has shown promising preclinical and clinical data, and a multi-continental randomized Phase III trial in hepatocellular carcinoma is expected to start soon.

Keywords: cancer, JX-594, oncolytic virus, vaccinia virus

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1. Introduction

New drugs with novel mechanisms of action are needed in the fight against cancer. Gene therapy offers great opportunities for developing new approaches for improved cancer treatment. In this regard, many different viral and non-viral vectors have been studied in the search for the ideal cancer gene therapy drug. One promising gene therapeutic agent is vaccinia virus (VV), which is a member of the poxvirus family and is characterized by a double-stranded DNA genome and enveloped, brick-shaped particles of about 300 × 240 × 120 nm. The majority of the VV particles are of the intracellular mature virion form with a single lipid bilayer envelope and mostly located inside the infected cell until lysis [1]. The other two infectious forms, the cell-associated enveloped virions and the extracellular enveloped virions (EEV), have an extra lipid bilayer and bud out from the host cell without lysing it. VV particles contain numerous virus encoded enzymes such as a DNA-dependent RNA polymerase, transcription factors, capping and methylating enzymes and a poly(A) polymerase [2]. These enzymes enable the virus to synthesize translatable mRNAs independently from the host cell in the so-called virus factories, which are located in the cytosol of the infected cell. VV became famous as an efficient vaccine in the worldwide smallpox eradication program [3] and it is due to this role that VV has the longest and most extensive history of use in humans of any virus [4]. This historical role has led to detailed studies of VV biology and pathogenesis and thus there is a wealth of knowledge available including basic, preclinical and clinical data on VV. From 1980s onwards, VV has been explored for its utility in other fields, for example,

Article highlights.

- Vaccinia virus (VV) has many attributes of a promising cancer gene therapy agent and is an efficient gene therapy vector for expression of therapeutic genes.
- Genetic deletions to increase cancer selectivity improve the safety profile of oncolytic VV.
- JX-594 is an armed, oncolytic VV that has been shown to be safe in patients and produced promising efficacy data in Phase I and II clinical trials.
- Combining VV with other therapy approaches enhances overall antitumor efficacy.
- VV generated oncolysates have shown promising preclinical antitumor efficacy.
- Oncolytic wild-type VVs have shown significant antitumor efficacy in early clinical trials.
- Arming oncolytic VVs with therapeutic transgenes significantly increases antitumor efficacy.
- Other recombinant oncolytic VVs have entered clinical trials but results have not been reported yet.
- VV oncolysates have shown encouraging results in Phase I – II trials but failed to show significant antitumor efficacy in a Phase III trial.
- Incidence and severity of adverse events as well as contagiousness as seen in clinical trials with smallpox vaccines will have to be addressed in large clinical trials with oncolytic VVs.
- The oncolytic, GM-CSF expressing JX-594 has emerged as the clinically most advanced VV with the potential to become the first oncolytic virus approved by Western regulatory agencies.

This box summarizes key points contained in the article.

as an expression vector in immunology [4] and as a vaccine platform against influenza [5], HIV [6] and other diseases. Furthermore, VV has been studied for its use in cancer therapy mainly in three ways: i) as a gene therapy vector for tumor specific delivery of therapeutic genes; ii) as a tumor selective replicating oncolytic virus and iii) as a cancer vaccine expressing tumor antigens and/or immunostimulatory molecules.

VV has several unique features that makes it attractive for biomedical research and in the development of biotherapeutics especially as a vaccine or cancer therapeutic. First, VV is a highly immunogenic virus, eliciting strong T-cell mediated [7] and antibody responses [8]. This feature is the basis for VV's efficiency as a vaccine and might be particularly useful in the treatment of cancer. Furthermore, VV has a wide host range and is able to infect and replicate in almost all human and many other species' cell types [9]. Therefore, it can be studied in many syngeneic animal models, which helps design meaningful preclinical experiments and facilitates translation into clinical trials. VV infection and subsequent gene expression occur with high efficiency and there are a number of viral promoters available that can be used to control timing and level of gene expression. Because the entire replication cycle occurs in the cytoplasm, the VV genome never enters the host cell nucleus [10]. Thus, there is no possibility of chromosomal integration in contrast to other vector systems. A further advantage of VV over its competitor viruses is that in the unlikely case of uncontrolled replication, there are a number of approved and experimental antiviral agents available to limit toxicity and spread of the virus, such as vaccinia immune globulin [11], cidofovir [12], ST-246 [13] and certain tyrosine kinase inhibitors [14]. Moreover, using current standard DNA manipulation techniques, recombinant VVs with large and/or several transgenes can be efficiently constructed as up to 25 kb are insertable into the VV genome [15]. Last, VV can be produced easily to relatively high titers and the particles are stable and can be stored frozen in solution or as dry powder for prolonged periods of time without significant loss of infectivity [4].

Taken together, these attributes render recombinant VVs attractive agents for the treatment of many diseases, especially cancer. Consequently, a large number of recombinant VVs have been constructed and studied preclinically and some of them have entered clinical testing with exciting results. In this review, we discuss the utility of VV for cancer treatment with a special focus on oncolytic VV constructs, in particular JX-594, which is likely to be tested in a worldwide randomized Phase III trial for hepatocellular carcinoma. If this trial is successful, JX-594 could be among the first oncolytic virus based products ever approved.

2. Preclinical data on VV for cancer therapy

VV can be used for cancer therapy via three approaches: i) as a gene delivery vector to express therapeutic genes with certain mechanisms of action, ii) as a replication competent (oncolytic) vaccinia to directly lyse tumor cells and iii) as a cancer vaccine to induce anticancer immunity. In this review, we will not discuss the large field of VV based cancer vaccines, which has been reviewed elsewhere [4,16-19].

2.1 VV as a vector for therapeutic genes

VV has many attributes of an ideal gene therapy vector such as high infectivity of most tissues including tumors, highly efficient gene expression and the capacity to hold up to 25 kb of foreign DNA. Despite these features, VV has not been widely used as a gene therapy vector because of its high immunogenicity, which was thought to lead to a rapid clearance of the vector and prevent re-administration. However, studies in animals that had been vaccinated prior to VV administration showed that efficient infection was still possible [20].

The VV vectors that have been most widely used are highly attenuated strains, such as Modified Vaccinia Ankara (MVA) and New York Vaccinia virus (NYVAC), which are either replication incompetent or show markedly impaired replication (**Table 1**). Alternatively, inherently non-attenuated strains have been rendered non-replicative by physical–chemical methods.

Timiryasova *et al.* constructed and studied a Lister strain based VV that expresses p53 [21]. This vector was safe and

Table 1. Vaccinia virus strains.

Strain	Background	Characteristics	Ref.
Wyeth or New York City Board of Health	North American vaccine strain	Minimal inherent tumor selectivity Slow replication in mouse tissue Commonly used clinical strain	[42,114]
Western Reserve	Laboratory strain derived from Wyeth through passaging in mice	High tumor selectivity Strong oncolytic effect in mouse models Minimal clinical use in humans	[34-37,42]
Lister	European vaccine strain	Inherent tumor selectivity Extensive use in humans during smallpox eradication	[114,115]
Modified Vaccinia Ankara	Vaccinia strain derived from Ankara strain through passaging in avian cells	Does not replicate in mammalian cells Highly immunogenic, well suited for vaccination purposes	[114]
Copenhagen	Northern European vaccine strain	Inherent tumor selectivity Used as smallpox vaccine but withdrawn Relatively high incidence of adverse events	[116]
New York Vaccinia	Vaccine strain derived from Copenhagen through deletion of several genes	Does not replicate in mammalian cells Highly immunogenic, well suited for vaccination purposes	[114]
Tian Tan (temple of heaven)	Chinese vaccine strain	Unknown potential as oncolytic virus Extensive use in humans in China during smallpox eradication	[117]

Adapted from Kirn and Thorne [25].

effective in a murine glioma model [22] and when UV irradiated to make it replication incompetent, retained its antitumor activity while toxicity was reduced [23]. Although VV shows remarkable infectivity, penetration within the tumor and transduction of all cancer cells are difficult to achieve in complex and advanced human tumors featuring stromal components, necrotic, hyperbaric and hypoxic areas. Thus, vectors expressing therapeutic proteins with a bystander effect might be more useful. Erbs *et al.* studied an MVA vector expressing the suicide gene FCU1, demonstrated a bystander effect of the molecule and showed that the vector was more effective than a replication incompetent adenovirus vector expressing the same transgene in a colon cancer model [24].

2.2 VV as an oncolytic virus

VV's oncolytic potential is dependent on the strain the vectors are based on. Wyeth, Lister and Copenhagen strains have demonstrated oncolytic potency, while the Western Reserve strain seems to have the strongest oncolytic effect (**Table 1**). In contrast, strains such as MVA and NYVAC do not replicate in mammalian cells and, therefore, have no oncolytic potential at all.

The antitumor effect mediated by oncolytic VV is predominantly based on three different mechanisms of action. The first is direct infection of cancer cells and subsequent replication that leads to cell lysis. This mechanism seems to have features of both necrosis and apoptosis [25]. The second mechanism of action is immune-mediated cell death. VV infection results in cell destruction and release of cellular danger signals (danger associated molecular pattern molecules) [26,27] and viral danger signals (pathogen associated molecular pattern molecules) [28] as well as tumor associated antigens [25]. Third, oncolytic VV has been shown to induce vascular collapse within tumors in preclinical [29] and clinical settings [30].

VV's strong oncolytic effect is based on its high infectivity, fast replication cycle, efficient cell-to-cell spread and productive cell lysis [31]. The entire life cycle of VV takes place in the cytosol and is completed within 24 h releasing as many as 10,000 new virions [10]. The newly produced virions are of several distinct forms such as the EEV form of the virus, which buds out from the host cells covering itself with a cell membrane derived second lipid bilayer [10]. The EEV envelope contains, therefore, several host complement control proteins and only few exposed viral antigens making it efficient in spreading throughout the system without being recognized by the immune system [32,33].

2.2.1 Cancer selective oncolytic VVs

VV has a natural tropism to tumors. After intravenous administration of VV into tumor bearing animals, the highest amounts of virus have been recovered from tumors followed by ovaries with little virus detected in other organs [34-36]. It has been suggested that leaky vasculature found in tumors and ovaries is one of the major determinants of VV tropism [31,37].

Despite its remarkable natural tumor tropism, researchers have tried to make VV even more tumor specific. Different strategies have been pursued based on genetic engineering of

the VV genome to increase cancer tissue specific replication (**Table 2**). Thymidine kinase (TK) is involved in the synthesis of deoxyribonucleotides in dividing cells in which there is a suboptimal nucleotide pool for DNA replication [4]. In normal cells, TK is necessary for replication as these cells have generally low nucleotide concentrations. However, in cancer cells, high concentrations of nucleotides are found and TK is, therefore, dispensable for cell proliferation [38]. Consequently, a TK deleted VV preferentially replicates in cancer cells as it needs to rely on sufficient nucleotides provided by the host cell, which is not present in normal cells. Tumor selective replication of TK deleted VVs has been shown in many animal models including colon cancer, melanoma, sarcoma and liver metastasis [35,39].

In another approach, vaccinia growth factor (VGF) can be deleted in the VV genome to achieve tumor specific replication. VGF is a secreted protein, which is expressed early during VV infection. It is an EGF homologue and binds the EGF receptor on infected and surrounding non-infected cells, thereby, stimulating cell proliferation [40,41]. This function is important for virus spread in normal tissue as VV relies on proliferating cells for efficient virus production but it is dispensable in tumor tissue because cancer cells are naturally proliferating. VGF deletion has been combined with TK deletion in the so-called double deleted VVs (also known as vvdd). These viruses demonstrated enhanced tumor specificity compared with VV that have a single deletion in either TK or VGF [37,42].

Another strategy is to disrupt the A56R gene coding for hemagglutinin, which has shown reduced virulence in normal tissue in combination with TK deletion [43]. Other approaches to generate tumor specific VVs include deletion of the host range genes SP-1 and SP-2 which are antiapoptotic serpins [31] as well as the antiapoptotic F1L protein, an inhibitor of cytochrome c release [44].

2.2.2 *Armed oncolytic VVs*

Oncolytic VVs that amplify in tumor tissue can provide high local concentrations of transgenes in a large number of cancer cells, which is ideal for gene transfer of therapeutic proteins [39]. Thus, engineering oncolytic VVs to express therapeutic proteins is a good way to potentiate antitumor efficacy and consequently many constructs have been made and tested preclinically (**Table 2**). Secreted transgene products that have a cytotoxic effect on surrounding non-infected cells (the so-called bystander effect) are particularly attractive. The cytosine deaminase/5-fluorocytosine (CD/5-FC) system is probably the most widely used suicide gene system with a strong bystander effect. When cloned into a TK deleted VV, enhanced cytopathic effect was observed *in vitro* at low doses when the prodrug 5-FC was added [45,46]. The *in vivo* antitumor effect was also augmented with VV-CD when 5-FC was administered although 5-FC inhibited virus replication both *in vitro* and *in vivo* [46]. Foloppe *et al.* fused the genes for CD with the uracil phosphoribosyltransferase for improved conversion of 5-FC into toxic metabolites and incorporated this gene into a TK deleted VV [47]. This virus demonstrated efficient antitumor activity after administration of 5-FC in murine colon cancer and liver metastatic models after local and systemic virus injection. Another group of promising therapeutic transgenes are those that inhibit tumor angiogenesis. Improved antitumor efficacy in syngeneic kidney cancer models as well as xenograft prostate and lung cancer models was demonstrated with anti-angiogenically armed oncolytic VVs compared with their unarmed control viruses [48,49].

Arming VV with immunostimulatory molecules to increase antitumor efficacy is another strategy that has been pursued. VV has evolved mechanisms to suppress early development of T_H1 responses [10]. Thus, wild-type VV is not efficient in cross-priming the immune system to tumor antigens [31]. On the other hand, one of the major limitations to effective *in vivo* replication, efficient intratumoral spread and high transduction is premature immune clearance of the virus. Thus, arming VV with immunostimulatory molecules would be expected to exacerbate this problem. Indeed, incorporation of FAS-L [31], IL-2 [50], IL-15 [50], CD40 ligand [51] or TNF [52] into oncolytic VVs has been shown to reduce viral replication *in vivo*. However, diminished *in vivo* replication due to stimulation of the immune system does not necessarily lead to reduced overall antitumor efficacy. For example, a VV expressing the immunostimulatory cytokine IL-12 and the antigen HIV-env demonstrated attenuated virus replication but augmented cellular immune response against HIV-env [53]. Thorne *et al.* constructed a TK-VGF double deleted Western Reserve VV armed with GM-CSF [42]. This virus (termed JX-963) showed significant cancer selectivity in tumor bearing mice, rabbits and primary surgical samples *ex vivo*. Moreover, intravenous administration led to systemic efficacy against primary carcinomas and widespread metastases in immunocompetent mice and rabbits. Increased neutrophil, monocyte and basophil concentrations in peripheral blood of treated rabbits were measured and enhanced cytotoxic T-lymphocyte induction was noted [54].

To create a simultaneously diagnostic and therapeutic agent, Zhang *et al.* incorporated three reporter genes into an oncolytic VV [43]. A renilla luciferase–green fluorescent protein fusion gene, β-galactosidase and β-glucuronidase genes were inserted in the F14.5L (which encodes a 49 amino-acid peptide with a not clearly identified function [55]), TK and hemagglutinin loci for tumor specific replication. GLV-1h68 has been reported effective in breast cancer [43], thyroid cancer [56], mesothelioma [57], pancreatic cancer [58] and prostate cancer models [59] and tumor regression could be monitored real time. This virus has recently been fully sequenced and the genomic features were compared to those of the parental wild-type lister strain and wild-type viruses of other strains [55]. The analysis indicated that GLV-1h68 has lost several open reading frames including genes for virulence such as the cytokine response modifier E and a viral Golgi anti-apoptotic protein. These genomic changes seem

Table 2. Examples of oncolytic VVs used in preclinical cancer studies.

Virus name	VV strain	Genetic deletion for tumor specificity	Transgene expressed	Ref.
vCB025	Western Reserve	TK	Luciferase	[35]
vvdd-GFP	Western Reserve	TK, VGF	GFP	[37]
GLV-1h68	Lister	TK, F14.5L, A65R (hemagglutinin)	Renilla luciferase-GFP fusion protein, β-galactosidase, β-glucuronidase	[43,56-59]
GLV-1h99	Lister	TK, F14.5L, A65R (hemagglutinin)	Human norepinephrine transporter, β-galactosidase, β-glucuronidase	[60]
vvCD	Western Reserve	TK	CD	[45,46]
VV-FCU1	Copenhagen	TK	CD/uracil phosphoribosyltransferase fusion gene (FCU1)	[47]
vvdd-VEGFR-1-Ig	Western Reserve	TK, VGF	Soluble VEGFR receptor 1 construct	[48]
GLV-1h107, GLV-1h108, GLV-1h109	Lister	TK, F14.5L, A65R (hemagglutinin)	VEGF single chain antibody GLAF-1, Renilla luciferase-GFP fusion protein, β-glucuronidase	[49]
JX-594	Wyeth	TK	GM-CSF	[69]
JX-963	Western Reserve	TK, VGF	GM-CSF	[42,54]

CD: Cytosine deaminase; GFP: Green fluorescent protein; TK: Thymidine kinase; VGF: Vaccinia growth factor; VV: Vaccinia virus; vvdd: Double deleted VV.

to contribute to the tumor selectivity in addition to the engineered deletions of F14.5L, TK and hemagglutinin.

Chen *et al.* constructed GLV-1h99, which is a variant of GLV-1h68 that expresses the norepinephrine transporter instead of the luciferase–green fluorescent protein fusion protein [60]. Norepinephrine expression in infected cells resulted in specific uptake of meta-iodobenzylguanidine isotopes, which could be imaged using single photon emission computed tomography and positron emission tomography. Moreover, compared with GLV-1h68, GLV-1h99 retained its systemic antitumor efficacy in a murine subcutaneous pancreatic cancer model.

2.2.3 *Preclinical data on JX-594*

JX-594 is a Wyeth strain oncolytic VV that has the TK gene deleted and is armed with human GM-CSF. GM-CSF is among the most potent activators of antitumor immunity [61] and acts through several mechanisms including direct recruitment of NK cells and antigen presenting cells [62]. It has been incorporated into many different oncolytic viruses including adenovirus [63-65], herpesvirus [66] and vaccinia [67]. However, evaluation of human GM-CSF in preclinical cancer models is difficult because the human version is not active in mice [68]. Thus, although combining an oncolytic virus with GM-CSF expression holds great theoretical promise, it is difficult to evaluate the contribution of human GM-CSF to antitumor efficacy in most preclinical models.

Given these limitations, JX-594 was studied in a rabbit liver cancer model as it has been shown that human GM-CSF has significant biologic activity in this species [68]. Intravenous administration of JX-594 was well tolerated and had significant efficacy, including complete responses against intrahepatic primary tumors [69]. Moreover, JX-594 treated rabbits did not develop lung metastases while all controls did. In addition, tumor specific replication was demonstrated as well as systemic levels of human GM-CSF and tumor infiltrating cytotoxic T lymphocytes.

2.2.4 *Oncolytic VVs in combination with other therapies*

Despite promising preclinical results, oncolytic VVs, when used as single agents, may not be able to eradicate advanced solid tumors due to their high complexity and capacity for developing resistance. Combination treatments with chemo-, radio- or immunotherapy or other oncolytic viruses can exploit additional mechanisms of action to augment antitumor efficacy with often synergistic results. Because tumors consist of heterogeneous populations of cancer cells and some populations might be resistant to certain single therapies, combination treatments could result in an improved overall outcome. Moreover, it is likely that initial regulatory approval for an oncolytic VV will only be granted for use in combination with well-established therapies.

2.2.4.1 *VV in combination with chemotherapy*

VVs have been evaluated in combination with various standard chemotherapeutics such as alkylating agents, nucleoside analogs, cytoskeleton modifiers and cytostatic agents. Cisplatin is a commonly used chemotherapeutic that binds and crosslinks cellular DNA leading to apoptosis. In a pancreatic cancer model, the oncolytic VV GLV-1h68 combined with cisplatin resulted in enhanced therapeutic efficacy with a significantly increased number of complete responses compared with virus treatment alone [58]. The nucleoside analog gemcitabine, which leads to apoptosis when it is incorporated into the replicating cellular DNA strand, has also been studied in combination with GLV-1h68. Combination therapy demonstrated significantly improved antitumor efficacy in a pancreatic cancer model [58]. Taxanes stabilize microtubules thereby interfering with the cytoskeleton function to prevent mitosis.

Paclitaxel is one of the most widely used taxanes and has shown synergistic antitumor efficacy in combination with an oncolytic double deleted Western Reserve VV in a subcutaneous colorectal tumor model [70].

The alkylating agent cyclophosphamide is used as a chemotherapeutic for the treatment of cancers and as an immune suppressive agent for autoimmune diseases. Use of high doses in combination with oncolytic viruses results in enhanced infection, spread and persistence of the virus due to suppression of innate and adaptive immune responses [71]. An oncolytic Western Reserve VV combined with cyclophosphamide demonstrated increased viral replication and prolonged survival in a rat glioma model [72]. In the same study, the oncolytic VV was also combined with rapamycin, an immune suppressant used in transplant patients. Also, this combination exhibited improved antitumor efficacy due to enhanced viral replication.

2.2.4.2 *VV in combination with radiotherapy*

It has been shown in several preclinical cancer models that radiotherapy can enhance viral oncolysis and oncolytic viruses can sensitize cancer cells to radiation therapy, which can both result in synergistic antitumor efficacy [71]. Most of these radiotherapy combination studies have been performed with oncolytic herpes and adenoviruses, whereas oncolytic VV has not yet been extensively studied.

A Lister strain VV deleted in TK and coding for p53 has been evaluated in combination with radiotherapy glioma models [73,74]. *In vitro*, p53-negative glioma cells were significantly more susceptible to treatment with UV attenuated virus in combination with radiotherapy compared to either treatment alone. The observed enhanced cell killing was shown to be due to increased apoptosis. Moreover, mice bearing rat glioma tumors responded significantly better to the combination treatment with non-attenuated VV-p53 and radiotherapy.

Another approach is to arm VV with receptors, which can be utilized for radionuclide therapy. McCart *et al.* generated an oncolytic vvdd expressing somatostatin receptor and showed that the somatostatin analog ^{111}In-pentetreotide was specifically taken up by infected cells *in vitro* [75]. Moreover, in a murine subcutaneous colon cancer model also, systemically administered ^{111}In-pentetreotide localized specifically to virus injected tumors in contrast to tumors injected with control viruses.

2.2.4.3 *VV in combination with other oncolytic viruses*

Another approach which has recently been studied is combining VV with other oncolytic viruses for improved antitumor efficacy. This strategy reduces the virus specific toxicity because lower doses of the single viruses are used. At the same time, each of the viruses is an adjuvant for the other, which enhances the overall immune responses. If rationally combined to utilize complementing features of different oncolytic viruses, the overall antitumor efficacy of the treatment might be higher than with either of the viruses alone. Recent work by Le Boeuf *et al.* demonstrated that the combination of VV with oncolytic vesicular stomatitis virus resulted in a synergistic interaction with enhanced tumor cell killing in murine cancer models [76].

In a similar approach, Zhang *et al.* combined VV with Semliki Forest Virus to increase tumor immunity in an ovarian cancer model [77]. This virus combination exhibited improved antitumor efficacy against murine ovarian surface epithelial carcinomas due to an augmented T-cell immune-mediated response.

2.2.4.4 *VV in combination with hyperthermia*

Hyperthermia has been shown to increase the vascular permeability of tumor vessels, which results in enhanced delivery of various macromolecules, such as antibodies [78] and liposomes [79]. VV's *in vivo* tropism to tissues with leaky vasculature such as tumors and ovaries [31,37] is, among other factors, determined by its large particle size (300 × 240 × 120 nm). Chang *et al.* demonstrated that hyperthermic treatment (41.5°C for 30 min) of subcutaneous murine tumors enhances virus uptake after systemic delivery > 100-fold [80]. Moreover, the authors showed that hyperthermia in combination with systemic oncolytic VV treatment resulted in significantly improved antitumor response compared with normothermic virus treated controls. Another mechanism which might contribute to the utility of the approach is hyperthermia mediated enhancement of antitumor immune response [81].

2.2.4.5 *Cell carriers for oncolytic VV*

Most viruses that enter the systemic circulation are rapidly complexed by complement, antibodies, phagocytosing immune scavenger cells or blood factors [82]. For example, viruses such as vesicular stomatitis virus and adenovirus are difficult to detect in the blood 30 min after intravenous injection [82,83]. Consequently, it has been attempted to protect viruses from inhibitory factors and direct them to tumors by loading them in or onto carrier cells, such as T cells, cytokine-induced killer (CIK) cells, macrophages, dendritic cells, mesenchymal stem cells and tumor cells. This approach has proven successful with oncolytic reovirus, vesicular stomatitis virus and herpes simplex virus [82].

Thorne *et al.* used CIK cells to carry an oncolytic VV [84]. Cells infected with VV retained their ability to traffic to the tumor and infiltrate it. Virus replication in CIK cell, compared to cancer cells, was initially slowed down followed by a rapid virus burst 2 – 3 days after infection. Thus, the infected CIK cells had sufficient time to home to the tumor before virus was released. Tumor bearing mice treated with these VV loaded CIKs showed significantly better tumor response and survived significantly longer than mice treated with virus or cells alone. Cell carriers for oncolytic viruses are covered more in depth in a recent review [82].

2.2.5 Oncolysates

Oncolysates are tumor cells that have been infected with an oncolytic virus and subsequently lysed by the virus or physical methods. They are, therefore, a mixture of cancer cell proteins, other cell components and live or attenuated virus. The major goal of oncolysate therapy is to increase the immunogenicity of tumor associated antigens because they often appear to be too weak to induce active immune responses in patients.

Different oncolytic viruses have been used to generate oncolysates but VV has emerged as one of the most useful ones because of its high immunogenicity and relatively low pathogenicity [85]. VV oncolysates have been shown to induce protective immunity in syngeneic cancer models [85,86] and result in tumor growth reduction and prevention of metastatic spread in animals with established tumor [87].

Sivanandham *et al.* evaluated a murine colon cancer oncolysate prepared with a VV which encodes IL-2 in a syngeneic murine colon adenocarcinoma hepatic metastases model in comparison to an oncolysate prepared with a control VV [88]. VV-IL-2 oncolysate treatment was significantly more potent in reducing tumor burden and improving survival than treatment with VV-IL-2 alone or with control VV oncolysate. It was suggested that the superior effect was due to higher levels of cytotoxic T lymphocytes that were observed in the blood of VV-IL-2 oncolysate treated mice.

In another study, vaccinia melanoma oncolysate prepared with VV encoding GM-CSF was evaluated in a melanoma pulmonary metastasis model [89]. The number of metastasis was significantly reduced and survival was prolonged in the VV-GM-CSF oncolysate group compared to the group treated with oncolysate prepared with a control VV. Lymphocytes isolated from VV-GM-CSF oncolysate treated mice showed higher cytolytic activity against melanoma cells than lymphocytes isolated from other treatment groups. The cytotoxic activity of macrophages was also significantly enhanced in the VV-GM-CSF oncolysate group.

3. Clinical data on oncolytic VV

3.1 Clinical data on wild-type VV

Several clinical trials with non-engineered VV were performed in 1960s – 1990s. Burdick and Hawk [90,91] and Belisario and Milton [92] treated several melanoma patients repeatedly with wild-type VV injections directly into the lesions. Regression of most of the injected and also uninjected lesions was noted as well as transient adverse events such as skin inflammation, fever, chills and malaise. In a study involving 19 melanoma patients who had previously been vaccinated against smallpox, Hunter-Craig *et al.* inoculated wild-type VV into the lesion by scarification or direct injection [93]. In 6 out of 10 evaluable cases, injected nodules completely disappeared whereas no response was seen in uninjected tumor sites. In another clinical trial, Roenigk *et al.* treated 20 melanoma patients with wild-type VV directly injected into the lesions [94]. Immunological responses against the tumors were seen and eight patients showed major regression of their lesions. Also, Mastrangelo *et al.* treated melanoma patients by local injection of wild-type VV in a Phase I trial [95]. Infection of tumor cells was demonstrated despite presence of anti-VV antibodies and intratumoral replication lasting at least 4 days was shown. Altogether, 44 patients were treated in these early trials and the overall objective tumor response rate of injected tumors was estimated to be ~ 50% with complete regression in 25% of the cases [96]. In many cases, durable tumor responses were seen (> 2 years) and in some cases, regression was also seen in uninjected nodules. Furthermore, these studies demonstrated that repeated injections upon tumor recurrence are feasible and lead to further responses.

Gomella *et al.* performed a Phase I study to evaluate whether wild-type VV can infect bladder cancer cells after intravesical administration [97]. One day after infection, radical cystectomy was performed on the four treated patients and the tissue was analyzed microscopically. Three patients showed significant infiltration of inflammatory cells and evidence of viral infection in normal and tumor urothelial cells. No clinical or laboratory manifestation of vaccinia related toxicity was observed except mild dysuria. Three of the four patients survived and were disease free at 4-year follow-up.

Arakawa *et al.* [98] used an attenuated vaccinia strain to treat patients with metastatic lung and kidney cancer. The patients were repeatedly injected intravenously and the primary as well as the metastatic lesions responded well to the treatment. Kawa and Arakawa [99] treated a multiple myeloma patient with the same attenuated VV strain and noted a significant drop in immunoglobulin levels as well as increased NK cell activity. These case reports suggest that repeated injections of this attenuated VV strain had significant antitumor efficacy in various types of cancer, while causing only mild adverse events.

3.2 Clinical data on recombinant oncolytic VVs

3.2.1 Clinical data on JX-594

JX-594 is the most clinically advanced oncolytic VV to date. Two Phase I studies have been published, another one has been completed but only been presented at scientific meetings so far and one Phase II study is ongoing with interim results presented at a meeting. In the first Phase I clinical study, Mastrangelo *et al.* treated seven patients with surgically incurable cutaneous melanoma by direct intratumoral injection [67]. Multiple injections with JX-594 at doses up to 2×10^7 pfu/lesion up to twice weekly were given over 6 weeks. Treatment was well tolerated, and only transient flu-like symptoms and local inflammation, at times with pustule formation, at high doses were reported. Five of seven patients responded to the treatment with one patient having complete remission. Interestingly, antitumor responses were seen in injected and non-injected lesions suggesting systemic efficacy despite local administration, perhaps due to the immune response and/or virus dissemination through the circulation.

In the second Phase I trial, Park *et al.* utilized CT guided injection of JX-594 to treat primary liver cancers and tumors metastatic to the liver [100]. In this dose escalation study, 14 heavily pretreated patients were injected with up to 3×10^9 pfu every 3 weeks with a mean of 3.4 treatments. Ten patients were available for response assessment, revealing three partial responses, six stable diseases and one progression. All patients experienced flu-like symptoms and four patients had thrombocytopenia. Significant hyperbilirubinemia was seen in both patients injected with the highest dose, setting the maximum tolerated dose at 1×10^9 pfu. Virus dissemination in blood and infection of non-injected distant tumor sites was observed as well as increases in neutrophil counts suggesting that biologically relevant GM-CSF levels were produced by JX-594. Three of the patients had advanced refractory HBV associated hepatocellular carcinoma and it was shown that JX-594 treatment suppressed underlying HBV replication in these subjects [30].

The third Phase I study is an open-label, dose escalation study in patients with treatment refractory cancers who are injected with a single intravenous infusion of JX-594 [101]. The 21 enrolled patients had different tumor types including colorectal carcinoma, melanoma, ovarian cancer and lung cancer. Patients generally experienced flu-like symptoms, which were dose related. Otherwise, the treatment was well tolerated without significant toxicity up to the maximum feasible dose of 3×10^7 pfu/kg. JX-594 delivery to tumors could be detected in three of six patients treated with the highest dose whereas virus could not be detected in any of the patients injected at lower doses. In the lower dose cohorts, 33.3% of the patients exhibited disease control while in the higher dose cohorts 75% of the patients demonstrated disease control.

Interim results of an ongoing randomized Phase II trial investigating low- versus high-dose intratumoral JX-594 in patients with hepatocellular carcinoma have recently been reported at a scientific meeting [102]. The treatment was typically associated with flu-like symptoms lasting < 24 h. Based on Kaplan-Meier analysis, the 6-month survival of patients treated at low- and full-dose were 48 and 75% and 12 months survival was 18 and 75%, respectively. Of 17 treated patients evaluated 8 weeks after treatment, 15 exhibited objective radiographic response or stable disease and 50% achieved a 'Choi' necrotic response on dynamic contrast enhanced MRI scan. The study has so far enrolled 24 patients and is supposed to be completed later during the year 2010.

A multi-continental Phase III trial with JX-594 in combination with sorafenib as first-line therapy for advanced hepatocellular carcinoma is anticipated to open later in 2010 (JC Bell, pers. commun.).

3.2.2 *Clinical data on other oncolytic VV*

To our knowledge, there are only two other oncolytic VVs besides JX-594 being tested clinically at the moment. A Phase I study with GL-ONC1 (also known as GLV-1h68, described in section 2.2.2) aims to determine safety and tolerability after intravenous injection in patients with solid tumors [103]. Results have not yet been published or presented at scientific meetings.

Another Phase I trial is in progress evaluating vvdd-CDSR (also known as JX-929), which is a TK-VGF double deleted Western Reserve oncolytic VV expressing CD and somatostatin receptor. This virus is tested in a dose escalation study to determine the maximum tolerated dose after intratumoral injection [103] but no results have been reported to date.

3.3 Clinical data on oncolysates

Oncolysates prepared with VV have been tested in several Phase I – III clinical trials predominantly in the 1980s. In most of these trials, VV oncolysates were evaluated in melanoma patients because melanoma is thought to be particularly immunologically sensitive [104].

A study with melanoma patients at high risk for recurrence after surgery demonstrated antimelanoma serological activity after VV oncolysate application in all patients [105]. Moreover, a positive correlation between serological activity and increased survival was observed. A Phase I–II study was subsequently conducted to assess the effective dose capable of inducing antimelanoma serological responses [106]. In this trial, 24 of the 48 enrolled patients showed disease-free intervals up to 28 months. Another trial enrolled 39 melanoma patients, who were treated with VV melanoma oncolysates as an adjuvant to surgery [107]. Pretreatment analysis showed that all patients were negative for antibodies to tumor associated antigens whereas 25 of these patients turned positive after treatment. Statistical comparison with matched control patients demonstrated a significant increase in disease-free survival for patients treated with VV melanoma oncolysates.

Wallack *et al.* subsequently performed a randomized, double-blinded, multi-institutional Phase III trial using VV melanoma oncolysates for 217 patients with resected melanoma [108]. The final analysis of the data showed no significant improvement of disease-free interval or overall survival of the VV oncolysate group compared with the control group, which was treated with VV alone. Only a small subset of male patients aged 44 – 57 years with one to five positive nodes showed a survival advantage with VV oncolysates as determined in a retrospective analysis. Several potential problems of this study that might have led to the disappointing outcome were later highlighted such as the absence of a true no treatment control arm, the use of a biologically active agent (VV) in the control arm and the insufficient number of patients in each group [104]. Therefore, Kim *et al.* compared the results of the VV oncolysates arm with the non-treated control arms of other prominent randomized anti-melanoma biologic trials [104]. The statistical analysis concluded that VV melanoma oncolysates may be statistically superior to observation arms from other trials. Moreover, patients of the control group treated with VV alone survived longer

compared with observation groups of other trials, which is in line with current thinking that oncolytic viruses may be useful for *in situ* vaccination without the need for tumor removal [64,65].

4. Safety concerns

Wild-type and engineered VVs have generally shown only mild toxicity in clinical cancer trials, mostly consisting of transient fever, malaise, skin reactions and pain at the injection site. However, oncolytic VVs have not yet been tested in large populations to reliably determine the occurrence of adverse events. A review of VV smallpox vaccination reports from 1924 to the 1960s indicated an incidence of eczema vaccinatum between 8 and 80/million vaccinations, progressive vaccinia at 1/million vaccinations, generalized vaccinia at an incidence of 1 – 70/million vaccinations, encephalitis between 2 and 1200/million vaccinations and an incidence of encephalopathy between 3 and 50/million vaccinations [109]. The type and severity of adverse events greatly depend on the VV strain used, as tropism and virulence varies significantly among different strains. Strains with high oncolytic potential such as the Western Reserve strain are likely to have more serious side effects than others. With regard to Western Reserve VV, it should also be kept in mind that this strain was derived from the New York City Board of Health (NYCBOH) by serial passaging in mouse brains and is, therefore, highly neurovirulent in mice [110]. The occurrence of encephalitis should thus be closely monitored in future clinical trials with large patient populations.

A recently published review on the newly developed smallpox vaccine ACAM2000 raises concerns that might also apply to oncolytic VVs [111]. ACAM2000 is a NYCBOH strain VV, which was derived from the previously widely used Dryvax vaccine by selecting a clone with the same ability to form skin lesions after scarification but with less neurovirulence after intracerebral injection into mice. Thousands of individuals were vaccinated with ACAM2000 in Phase I – III clinical trials with up to 2.2×10^8 pfu/ml. Besides common mild adverse events such as flu-like symptoms and pain at injection site, an unexpectedly high incidence of cardiac complications with severity ranging from mild to fatal was observed. In particular, myocarditis, pericarditis, arrhythmias and dilated cardiomyopathy were seen. In vaccinia naive subjects, myo- or pericarditis occurred in 5730/million cases whereas none of the 1819 vaccinia-experienced subjects showed signs of cardiac complications. The authors estimate that in case of a global vaccination with ACAM2000 as many as 1 in 145 vaccinees could experience myo- or pericarditis with varying severity.

Another potential safety concern is the contagiousness of VV. Several case reports exist on VV infections that were transmitted by recently vaccinated subjects [112]. Most of these infections occurred in hospitals and in almost all cases the individual were naive to VV. Moreover, mostly children or immunosuppressed patients were affected and the routes of transmission appeared to be through skin lesion, contaminated catheters and possibly by aerosol. Probably because of the nature of the affected population, these nosocomial VV infections may be fatal in up to 11% of the cases.

These reports highlight possible safety concerns that have to be addressed in clinical trials with oncolytic VVs involving large populations. However, it has to be noted that the described adverse effects and contagiousness refer to strains that were/are used for smallpox vaccination purposes. These vaccines typically contain non-engineered VVs derived from strains such as NYCBOH. This is not directly comparable to oncolytic VVs with genetic modifications for enhanced tumor selectivity such as the double deleted Western Reserve, which was shown to be 10,000-fold more tumor selective than the parental unmodified virus [37].

5. Conclusion

Gene therapy holds great promise for the development of novel and effective therapies for cancer. Among gene therapy agents that have been studied for their use in cancer therapy, oncolytic VV has emerged as a promising candidate. Many different recombinant VVs have been constructed and showed convincing preclinical and early clinical results; however, the ultimate proofs of antitumor efficacy and safety still need to be provided by randomized Phase III clinical trials. VV seems to be an ideal agent for these therapy approaches due to many of its attributes such as high immunogenicity. Importantly, unlike other oncolytic viruses, VV infects and replicates in many mammalian species and can thus be studied in immunocompetent, syngeneic cancer models. This enables researchers to study the effect VV has on the immune system and the effect the activated immune system has on tumors. In fact, preclinical and clinical results highlight the importance and effectiveness of the immune activating properties of VV.

VV as a vector for expression of therapeutic transgenes has had limited efficacy in cancer models. One way to improve VV mediated cancer therapy is to utilize the remarkable oncolytic ability of the virus, which adds to the transgene's antitumor efficacy and the immunostimulatory potential of the virus. Several oncolytic VVs armed with different therapeutic transgenes have been studied. For the choice of the right therapeutic protein, it is key to select molecules that exhibit a bystander effect so that uninfected tumor cells are also targeted. Besides suicide enzymes such as CD, immunostimulatory molecules are most attractive. One of the most promising immunostimulatory agents is GM-CSF, which is known to induce potent antitumor immunity [61].

JX-594 is a cancer selective oncolytic VV that expresses GM-CSF. It harnesses many of the unique properties that make VV so attractive for cancer therapy, most notably the oncolytic potential and the immunogenicity, which were further enhanced by arming the virus with GM-CSF. Moreover, VV in general and JX-594 in particular have exhibited good

safety in several clinical trials [100-102,113]. In an ongoing, randomized Phase II trial for hepatocellular carcinoma, JX-594 has shown promising interim results with a 12 month survival of 75% in the high-dose group versus 18% in the low-dose group. Based on these promising data, a Phase III study is planned to commence soon. This makes JX-594 the clinically most advanced oncolytic virus product in development at the moment. Although JX-594 has so far shown only mild toxicity in patients, adverse events will have to be carefully monitored in the planned Phase III clinical trial.

6. Expert opinion

Many promising cancer gene therapy agents are being developed and some of them have shown convincing results in clinical trials. However, the great breakthrough is still to come and no products have been approved yet outside of China. In our opinion, VV has great potential in this field. Among the oncolytic VVs that are being developed, JX-594 is the furthest along with three Phase I and one Phase II trial completed or ongoing. Safety and efficacy results of these studies are highly encouraging and warrant further trials. JX-594 combines strong oncolytic potential and enhanced immunostimulatory properties. Studies with JX-594 have so far been mostly focused on hepatocellular carcinoma but it will be interesting to see how this virus performs in other tumor types. In fact, as VV is able to infect and replicate in most tissues, it is likely that JX-594 will be similarly successful in other cancer types. Another attribute that makes VV stand out from many other viruses used in gene therapy is that VV can be administered systemically and is able to reach tumors, as shown in several animal studies and a human trial [101]. Moreover, the EEV form of VV, which is produced during virus replication, may be able to travel throughout the circulation and reach distant tumor sites and metastases.

In conclusion, we think that the striking features of VV in general and the wealth of positive preclinical and clinical data on JX-594 make this virus one of the most promising novel anticancer agents currently under development. Thus, it is possible that JX-594 will be among the first oncolytic viruses approved by Western regulatory agencies.

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Declaration of interest

A Hemminki is a shareholder in Oncos Therapeutics, Ltd. The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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