Review

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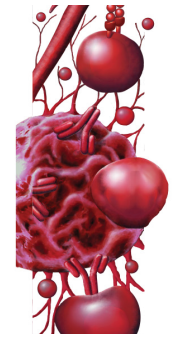
Immunotherapy



Vaccinia virus, a promising new therapeutic agent for pancreatic cancer

The poor prognosis of pancreatic cancer patients signifies a need for radically new therapeutic strategies. Tumor-targeted oncolytic viruses have emerged as attractive therapeutic candidates for cancer treatment due to their inherent ability to specifically target and lyse tumor cells as well as induce antitumor effects by multiple action mechanisms. Vaccinia virus has several inherent features that make it particularly suitable for use as an oncolytic agent. In this review, we will discuss the potential of vaccinia virus in the management of pancreatic cancer in light of our increased understanding of cellular and immunological mechanisms involved in the disease process as well as our extending knowledge in the biology of vaccinia virus.

Keywords: immunotherapy • oncolytic virus • pancreatic cancer • vaccinia virus

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Pancreatic cancer remains one of the most difficult cancers to diagnose and treat. It is the fifth most common cause of cancer death in the UK with 1 and 5 years survival of 20.8 and 3.3%, respectively. These figures have hardly improved since the early 1970s [1]. Complete surgical resection remains the only curative treatment. Unfortunately, less than 20% of pancreatic tumors are amenable to surgical excision at the time of diagnosis. However, even with complete surgical resection prognoses remains poor with 5 years survival around 20% [2,3]. Gemcitabine is the main chemotherapeutic agent approved for advanced pancreatic cancer. Despite being shown to improve life expectancy compared with 5-flurouracil, effect remains modest with median survival around 6 months [4]. Combining gemcitabin therapy with erlotinib led to minimal increase in life expectancy from 5.9 to 6.2 months [5]. Therefore, new treatment strategies are clearly imperative.

Vaccinia virus (VV) has played a prominent role in one of the greatest achievements in medical history: the eradication of smallpox (caused by Variola virus). Since then, VV has been developed as a vector for vaccines against infectious diseases such HIV, influenza, malaria and tuberculosis as well as in immunotherapies [6] and oncolytic therapies for cancer [7,8]. With regards to the latter, the earliest studies, which mainly used replication attenuated VV recombinants for fear of toxicity, were relatively disappointing in the clinic. Replication competent VVs retain their ability to lyse tumor cells and spread through tumor tissue. Recent advances in DNA recombinant technology enabling the rational manipulation of the viral backbone, coupled with the ever increasing knowledge gains in the fields of molecular virology and cancer cell biology have aided the development of safe and efficacious tumor-targeted oncolytic VVs. These are currently at the forefront of the most promising novel anticancer agents.

In this review, we will explore the potential of tumor-targeted oncolytic VV in the management of pancreatic cancer in light of our increased understanding of cellular and immunological mechanisms involved in the disease process as well as our extending knowledge in the biology of VV.

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Tumor-targeted oncolytic viruses as a new class of cancer therapeutics

Targeted therapy of cancer using oncolytic viruses (OV) has generated much interest over the past decades in the light of the limited efficacy and the significant side effects of standard cancer therapeutics for advanced disease [9]. OVs have become an increasingly popular anticancer therapy platform due to their ability to selectively infect and lyse tumor cells (Figure 1). Cancer selectivity of OVs could be a result of natural tropism [10,11] or via genetic modification [9]. OVs can target multiple cellular pathways [12–14] minimizing the risk of tumor resistance and induce different modes of cell death [15–18]. In addition, OVs can break down the immunosuppressive tumor microenvironment and induce a long-lasting tumor-specific immunity [19,20] (Figure 2). OVs can specifically deliver therapeutic proteins into tumors at increasing levels following viral replication within the malignant cells. Furthermore, OVs can function in synergy with conventional cancer treatments of chemoradiotherapy [21–24]. Finally, OVs as a treatment platform are amenable to adjustment and development following our ever-increasing understanding of cancer cells, the virus and host immune responses to both tumor and virus.

H101, an adenovirus with *E1B 55K* gene deletion (Oncorine; Shanghai Sunway Biotech, Shanghai, China) was licensed in China in 2005 as the world's first OV for treatment of head and neck cancer when combined with chemotherapy [25]. The similar virus, *dl1520* (also known as, ONYX-015) has been administered by intratumoral injection under CT guidance into locally advanced primary tumors of pancreatic cancer patients in Phase I/II trials. The treatments were well tolerated, but no objective responses were seen in any of the patients with virus alone, and only 10% (2/21) patients showed objective response when gemcitabine was used in combination [26–28]. Another virus that entered clinical trials is HF10, a Herpes Simplex virus armed with granulocyte-macrophage colony-stimulating factor (GM-CSF). Phase I trial of intratumoral injection into nonresectable pancreatic tumors proved to be safe with some encouraging clinical results [29]. These early results warrant further investigation to seek more powerful agents for this cancer.

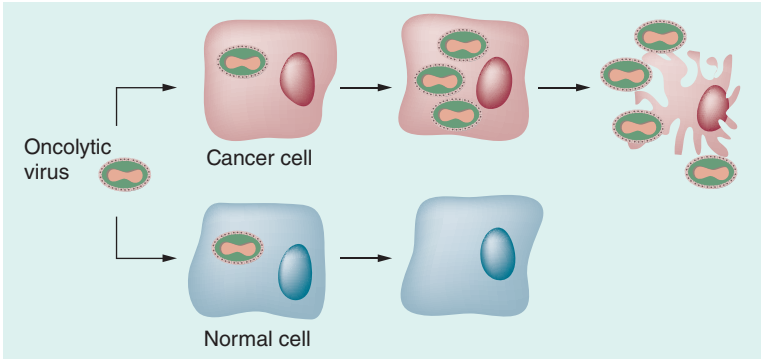

Figure 1. Tumor selectivity of oncolytic viruses. Tumor-targeted oncolytic viruses can exploit defective cellular pathways in cancer cells (top). oncolytic viruses can infect and replicate in cancer cells leading to cell lysis and release of viral particles. These in turn infect neighbor tumor cells and so forth. In normal cells (bottom) cellular defense mechanisms prevents viral replications.

Favorable features of vaccinia virus for cancer treatment

VV is a member the poxvirus family. It is a double-stranded DNA virus ~192 kbp in size. It can be stably accommodate up to 25 kbp of cloned exogenous DNA [30]. Structurally, it consists of a core region composed of viral DNA and a various viral enzymes including RNA polymerase and polyA polymerase encased in a lipoprotein core membrane. The outer layer of the virus consists of double lipid membrane envelope [31,32]. VV has two major forms of infectious virions; the intracellular mature virions, as described above, which is released upon cell lysis and the extracellular enveloped virion released from the cells via cell membrane fusion. The latter has an additional lipid bilayer membrane wrapped around the intracellular mature virion particle.

VV has many inherent characteristics that make it an ideal choice for oncolytic virotherapy. VV has a short life cycle of 8 h that takes place in its entirety in the cytoplasm eliminating the risk of genome integration. Replication usually starts 2 h after infection, at which time the host cell nucleic acid synthesis shuts down as all cellular resources are directed toward viral replication [33,34]. Cell lyses takes place between 12 and 48 h releasing packaged viral particles. Furthermore, the virus does not depend on host mechanisms for mRNA transcription making it less susceptible to biological changes of the host cell [33,35].

Unlike other OVs, VV does not have a specific surface receptor for cell entry allowing it to infect a wide range of cells unhindered by the lack of expression of said receptor. They depend on a number of membrane fusion pathways for cell entry [36,37].

The existence of various antigenically distinct forms of the mature virus allows it to evade host immune system. extracellular enveloped virion form of the virus is encapsulated in a host-derived envelope, with incorporated viral proteins, that contains several host complement control proteins [38–40]. In addition, VV infected cells secret Vaccinia complement control protein which binds an inactivate C4b and C3B inhibiting the classic and alternative complement activation pathways [41–43]. VV therefore can be disseminated relatively unharmed in the blood stream to reach distant tumors allowing

systemic delivery of the virus [44], which is more suitable for the treatment of the advanced pancreatic cancer.

The hypoxic nature of pancreatic cancer contributes to its aggressive and treatment-resistant phenotype. In contrast to adenovirus [45], we have found that hypoxic conditions did not affect replication, viral proteins production, cytotoxicity and transgene expression of the Lister strain of VV [46]. These results suggest that VV could be suitable for management of pancreatic cancers and potentially other hypoxic tumors.

Finally, VV has a good safety track record following its use as a vaccine for over a century. Minor and less severe side effects include fever, rash and inadvertent inoculation. Moderate-to-severe side effects include eeczema vaccinatum, generalized vaccinia, progressive vaccinia and postvaccinial encephalitis [47]. Sides effects are rare with an incident of less than 1:10,000 and severe side effects in particular are extremely rare [48]. Genetically modified recombinant VV could be potentially safer due to their tumor selectivity. Recent clinical trial of JX-594 virus in hepatocellular carcinoma showed the treatment to be well tolerated with mainly flu-like symptoms in all patients and a single severe side effect [8].

How Vaccinia virus selectively kills cancer cells by multiple action mechanisms

VV has a natural tropism to cancer cells [49,50]. The virus can utilize activated molecular pathways in tumor cells to aid its replication [51–53]. In fact, many of the hallmarks of cancer [54] make tumor cells susceptible to viral replication including immune escape, sustained cell proliferation and resisting cell death. In the case of VV, the EGFR family [55], potentially plays an important role in tumor selectivity. The viral SPGF, an EGF-like growth factor carried by VV, can activate host cellular pathways leading to increased viral replication [56]. In addition, Ras–GTP-activating protein S3H domain-binding protein, overexpressed in most human cancers [57], plays a role in VV replication by complementing the activity of the VITF-2 [58].

Various approaches can be utilized to enhance tumor selectivity of OVs. The virus depends for its replication in normal cells on a set of genes that prepare the cell resources for viral replication and block apoptotic pathways. Deleting these genes will limit the virus ability to replicate in normal cells. However, these pathways are often disrupted in cancer cells allowing the mutant virus to replicate despite the defective genes. One such example is the disruption of the vaccinia thymidine kinase gene (*TK* gene) affecting the virus ability to synthesize deoxyribonucleotides [59,60]. Normal cells have a much smaller reserve of deoxyribonucleotides, compared with tumor cells, limiting the ability of VV to replicate. Another example is the deletion of the *B18R* gene encoding the secreted IFN-binding protein that blocks IFNα signaling [61]. In normal cells, this gene deletion attenuates viral replication due to IFN antiviral effect while cancer cells remain permissive to VV replication as IFN signaling is often disrupted [62,63]. In addition, altering the expression of crucial vaccinia viral gene by microRNA also enables tumor-specific viral replication, which is a potentially novel and versatile platform for engineering VVs for cancer virotherapy [64].

GLV-1h68 is a replication-competent VV targeted at tumor cells by mutation of *J2R* (encoding thymidine kinase) and *A56R* (encoding hemagglutinin) loci. This virus was shown to be effective against human pancreatic cancer cell line *in vitro* and in nude mice xenografts. Importantly this efficacy was enhanced

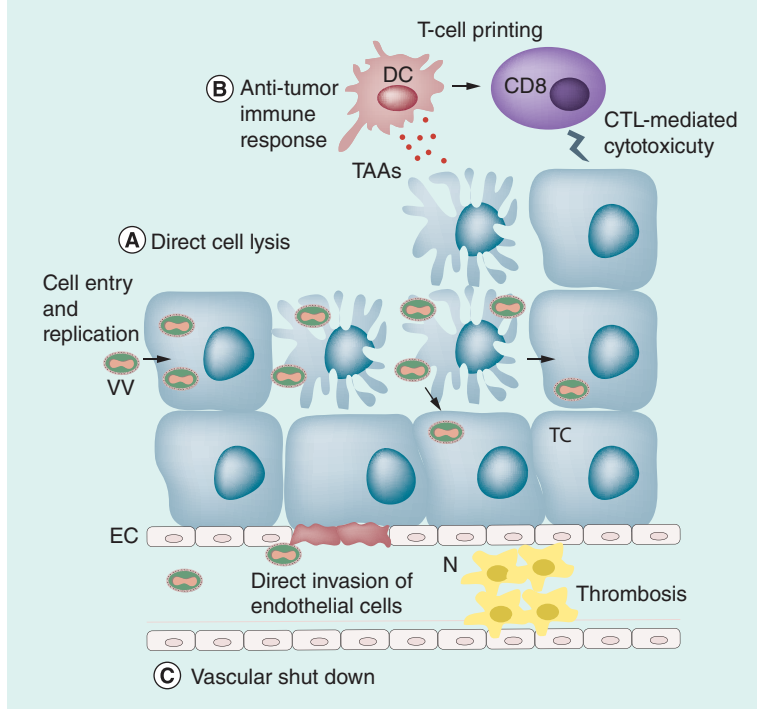

Figure 2. Multiple modes of actions of tumor-targeted oncolytic viruses.
Oncolytic viruses (OV) can kill cancer cells via a variety of mechanisms. First, they directly infect, replicate and lyse tumor cells sparing normal cells. Released virions can infect neighbor tumor cells and so forth. Second, OVs can induce immunogenic cell death associated with the release of pathogen-associated molecular patterns and damage-associated molecular patterns. In addition viral infection results in the release of cytokine and chemokines deviating the immune response toward a cytotoxic profile. Dendritic cells can pick tumor-associated antigens released from lysed tumor cells and prime $CD8^+$ T cells to induce a tumor-specific immune response. Third, OV infection can result in vascular shutdown caused by direct viral invasion of endothelial cells and thrombosis caused by cytokine-mediated neutrophils accumulation.
CD8: Cytotoxic T cell; DC: Dendritic cell; EC: Endothelial cell; N: Neutrophil; TAA: Tumor-associated antigen; TC: Tumor cell; VV: Vaccinia virus.

when virus therapy was combined with gemcitabine and cisplatin [65]. GLV-1h151, a virus with similar gene deletions but different marker proteins transgenes [66], was found to be effective *in vivo* and *in vitro* against human pancreatic cancer cell lines. Combining the virus with radiotherapy resulted in a synergistic antitumor effect [67].

In addition to direct cell lysis, VV can utilize vascular shut down to kill noninfected tumor cells [44,68–69]. This is believed to be caused by accumulation of neutrophils in blood vessels, mediated by cytokines and chemokines, leading to intravascular thrombosis [69]. In addition, VV can infect and destroy tumor-associated endothelial cells further contributing to vascular collapse [62]. Although this process has not been specifically shown in pancreatic tumors, we believe it to play an important role in the multimechanistic antitumor effect of VV, as pancreatic cancers are often well-vascularised and high microvascular density correlates with poor outcome after surgical excision [70]. To further capitalize on this process we have rationally armed Lister strain VV with endostatin–angiostaten fusion gene, a well-documented angiogenesis inhibitor [71]. The resultant VVhAE virus proved to be tumor selective *in vitro* and *in vivo*. It resulted in suppression of angiogenesis and prolonged survival of mice bearing human pancreatic cancer xenografts [50].

Vaccinia virus as immunomodulatory agent

The ability of OVs to alter the immune composition of the, ordinarily, immune-suppressive tumor microenvironment led to a new line of thinking of their mechanism of action. Large body of evidence suggests that antitumor immunity, where the virus is acting as an oncotropic immunomodulator, is the key determinant of a successful onclytic virotherapy [72–74].

VV kills cancer cells via a combination of necrosis and immunogenic apoptosis resulting in the release of damage associated molecular patterns [75–78] and pathogen associated molecular patterns [79–81] as well as the release of viral antigens into the tumor. This process leads to a strong inflammatory response that can overcome the immune suppression within the tumor microenvironment. In addition, tumor cell lysis releases tumor-associated antigens (TAA) into this inflammatory environment. Dendritic cells recruited by the virus can in turn pick up these exposed TAAs and cross-prime $CD8^+$ T cells resulting in a potent antitumor adaptive immune response. It has been demonstrated that an oncolytic VV (JX549) could induce tumor-specific immunity in human cancer patients [82] and preclinical study [20]. Therefore, oncolytic virotherapy may be considered as a method of vaccination *in situ*, enabling the adaptive immune response to clear residual disease as well remote metastatic cancer cells and provide long-term surveillance against relapse.

In the context of vaccination, heterologous prime-boost immunization regimen using recombinant adenovirus prime and VV boost has been shown to enhance $CD8^+$ T-cell immunogenicity with protective efficacy against malaria in a mouse model [83,84]. So, it seems logical that combining two different OVs for cancer treatment may induce a stronger tumor-specific immunity. We have, for the first time, combined the use of oncolytic adenovirus and VV, in a prime-boost strategy, for treatment of established tumors in the hope to harness the host immune response to the infected tumor cells. We found that sequential treatment via intratumoral injection with oncolytic adenovirus followed by oncolytic VV resulted in complete eradication of subcutaneous pancreatic cancer grafts in Syrian hamsters. More importantly, the surviving animals developed a long-lasting tumor-specific immune response that protected them against tumor rechallenge. This process was shown to be T-cell dependent [20].

Arming VV with various cytokines and chemokines can further enhance its antitumor activity. IL-10, a cytokine produced by Th2 T cells, is a potent inhibitor of antiviral immune response [85]. We have found that arming VV with IL-10 dampened antiviral immune response resulting in prolonged viral persistence in pancreatic tumors. This led to stronger antitumor immunity and improved survival in both subcutaneous and transgenic pancreatic cancer mouse models [86].

Vaccinia virus as vaccine vector

The first use of a recombinant virus armed with an antigen from a different organism as a vaccine vector was reported over 30 years ago. VV armed with hepatitis B surface antigen gene was able to induce a protective immunity against hepatitis in chimpanzees [87,88]. Since then there has been a great progress in recombinant VV vaccines in the veterinary field [89,90]. Unfortunately this success did not extend to human infectious diseases vaccines, mainly due to the lengthy and more stringent process for human licensing, with only a handful of recombinant VV vectors in current clinical trials [91–94].

One of the significant challenges for cancer vaccination lies in developing strategies to improve the delivery of antigens to antigen-presenting cells *in vivo*, allowing effective antigen processing and presentation and activation of a potent immune response against a unique background of immune tolerance toward 'self' TAAs. Viral vectors have become attractive antigen delivery systems as they mimic a natural viral infection, resulting in induction of cytokines and co-stimulatory molecules

that provide a powerful adjuvant effect and elicit potent cellular immunity [74,95].

Survivin is a member of the inhibitor of apoptosis family expressed in a variety of cancers. It plays a crucial role in tumor survival and drug resistance [96]. It is expressed during embryonic development but absent from differentiated cells [97]. Survivin is overexpressed in 70–80% of pancreatic cancers and is associated with resistance to chemoradiotherapy [98,99]. Vaccination with Vaccinia Ankara virus, a nonreplicating attenuated VV strain, armed with survivin induced survivin-specific CD8+ immune response resulting in a modest antitumor effect. When combined gemcitabine antitumor immunity and efficacy improved significantly. This is likely to be related to gemcitabine suppression of myeloid-derived suppressor cells [100].

The only VV-based cancer vaccine to enter clinical trials is PANVAC-V, a VV expressing carcinoembryonic antigen and mucin-1, both highly expressed in pancreatic cancers. The two antigens were packaged with three costimulatory molecules: B7.1 (cluster of differentiation 80), ICAM-1 (intracellular adhesion molecule one) and LFA-3 (leukocyte function-associated antigen-3) known collectively as TRICOM. To further enhance the immune response, the vaccination was delivered as a heterologous prime/boost regimen using a nonreplicating fowlpox vector expressing the same antigens and costimulatory molecules (PANVAC-F) [101]. GM-CSF was administered at the injection site as an adjuvant to enhance local antigen processing and presentation. In a Phase I clinical trial, the vaccine was found to be safe and well tolerable. It generated an antigen-specific immune response toward carcinoembryonic antigen and mucin-1 which correlated with increased survival [102]. However, Phase III trial (NCT00088660) targeting patients with metastatic pancreatic cancer who failed gemcitabine treatment failed to meet its therapeutic targets and was terminated [103]. The vaccine is currently under investigation for direct intratumoral injection under endoscopic ultrasound guidance with encouraging results of Phase I trial [104].

Future perspective

There has been a great interest in VV in recent years. Its safety, cancer tropism, amenability to genetic modification and ability to target solid tumors via a variety of mechanism of actions have made it a near-perfect onclytic virus to target pancreatic cancers. Nevertheless, as with any new therapeutic agents VV therapy need to overcome many hurdles and challenges before it enters routine clinical practice.

The first challenge is the selection of the right VV strain. The nonvaccine strain Western Reserve (WR) VV is widely used in the lab. JX-963, a GM-CSF armed mutant of WR VV with deletion of both the Thymidine Kinase and the Viral Growth Factor gene, has been reported as the most potent tumor-targeted oncolytic VV [52]. Other strains, such as the European vaccine Lister strain, are largely untested. We recently evaluated the antitumor potency and biodistribution of different VV strains using *in vitro* and *in vivo* models of cancer, including pancreatic cancer models. The Lister strain virus with Thymidine Kinase gene deletion (VVΔTK) demonstrated superior antitumor potency and cancer-selective replication *in vitro* and *in vivo*, compared with WRDD, especially in human cancer cell lines and immune-competent hosts. Further investigation of functional mechanisms revealed that Lister VVΔTK presented favorable viral biodistribution within the tumors, with lower levels of proinflammatory cytokines compared with WRDD, suggesting that Lister strain may induce a diminished host inflammatory response [105]. Our comprehensive study indicates that the Lister strain VV with TK deletion is a particularly promising VV strain for the development of the next generation of tumor-targeted oncolytic therapeutics. We anticipate that more and more people will use the Lister stain of VV as a backbone to develop new OVs for cancer treatment in the future.

Further genetic modifications of VV might enhance its oncolytic ability. Disruption of the *N1L* gene reduces virulence and inhibits VV replication in the brain reducing the risk postvaccinial encephalitis, a rare but significant complication of VV vaccination [106,107]. Our unpublished work on *N1L*-deleted VV suggests that *N1L*-deleted VV resulted in a superior antitumor efficacy compared with N1L-intact VV [Ahmed J *et al.*, Unpublished Data]. In addition, arming the new generation of VV with immune-modulatory genes or other therapeutic genes that enhance the antitumor immunity is a future for cancer treatment using tumor-targeted OVs.

Achieving the right immune response is of a paramount importance. Increasingly safer viruses permits the use of higher doses to maximize therapeutic effect [8], however higher viral load might deviate the immune response toward antiviral immunity resulting in rapid viral clearance and reduced antitumor immunity. Manipulating the immune system with cytokine-armed viruses is not without its risks including serious autoimmune side effects [108].

Systemic delivery of VV is particularly relevant in pancreatic cancer as most pancreatic tumors present with distant metastasis at the time of diagnosis. One such virus (JX594) has recently been shown to effectively target tumors after intravenous infusion, making it an ideal OV for treatment of inaccessible tumors such

as pancreatic cancer [7]. To date, the systemic delivery of OVs has been shown to be safe but not efficacious mainly due to the rapid clearance of these agents by the immune system [109]. When designing new strategy to enhance the systemic delivery of VV lessons can be learnt from other OVs. Serotype exchange [110,111], engineering new serotypes [112] and the use of chemical shielding [113] have been successfully used with other OVs. In fact the latter strategy have been used to modify the nonreplicating Vaccinia Ankara vaccine vector to circumvent pre-existing anti-VV immunity [114]. Other approaches include pharmacologically modifying the immune response to reduce the neutralization of the systemically delivered OVs [115–117].

Combining oncolytic virotherapy with traditional cancer treatments is an area of great promise. Gemcitabine can suppress myloid-derived suppressor cells in the tumor microenvironment resulting in a stronger antitumor immune response [118]. On the other hand, gemcitabine is a nucleoside analogue that inhibits DNA synthesis including that of double-stranded DNA viruses [119]. Using these agents in a sequential rather than combination manner might be the key to effective therapy [120]. Similarly, combining OVs with immune checkpoints inhibitors is an area that requires more investigation and optimization. PD-1 and CTLA-4 inhibitors might enhance the OV-induced antitumor immunity by creating a favorable immune profile in the tumor microenvironment [121,122].

Despite the challenges, the field of oncolytic virotherapy is generating a great interest of both researchers and pharmaceutical companies alike. The recent US FDA approval of talimogene laherparapvec (T-VEC, an engineered herpes simplex virus-1 expressing GM-CSF), for the treatment of melanoma has given the field a much needed boost. As safety and efficacy data start to accumulate the process of licensing new OVs will get easier. We anticipate other cytokine- and chemokine-armed viruses to enter clinical practice within the next few years. In addition, combining OVs with immune checkpoint therapies, monoclonal antibodies and CAR-T therapies will be an area of major research interest in the near future. Combining immune checkpoint antibodies with other immune-stimulating agents such as conventional drugs, targeted agents and most of all OVs, may increase the tumor types and individual patient profiles in which a durable clinical benefit can be achieved. OVs are finally being recognized for their ability to stimulate antitumor immunity, and with anti-CTLA-4 and anti-PD-1 agents on the market, OVs may finally have met their perfect match. It has never been a more promising era for cancer immunotherapy and personalized medicine.

We believe at the current rate of development it will not be long before OVs are part of routine clinical practice.

Financial & competing interests disclosure

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Executive summary

- Pancreatic cancer is one of the most aggressive human cancers, without effective therapies.
- Tumor-targeted oncolytic viruses is a new class of cancer therapeutic agents.
- Oncolytic Vaccinia virus (VV) has distinctive features that make it ideal for treatment of pancreatic cancer.
- The antitumor efficacy of oncolytic VV can be further improved by modification of viral genes and arming the virus with therapeutic genes.
- Combination of oncolytic VV with other cancer therapies could be the future for treatment of pancreatic cancer.

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