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MINIREVIEW

Replicating poxviruses for human cancer therapy

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Naturally occurring oncolytic viruses are live, replication-proficient viruses that specifically infect human cancer cells while sparing normal cell counterparts. Since the eradication of smallpox in the 1970s with the aid of vaccinia viruses, the vaccinia viruses and other genera of poxviruses have shown various degrees of safety and efficacy in pre-clinical or clinical application for human anti-cancer therapeutics. Furthermore, we have recently discovered that cellular tumor suppressor genes are important in determining poxviral oncolytic tropism. Since carcinogenesis is a multi-step process involving accumulation of both oncogene and tumor suppressor gene abnormalities, it is interesting that poxvirus can exploit abnormal cellular tumor suppressor signaling for its oncolytic specificity and efficacy. Many tumor suppressor genes such as *p53*, *ATM*, and *RB* are known to play important roles in genomic fidelity/maintenance. Thus, tumor suppressor gene abnormality could affect host genomic integrity and likely disrupt intact antiviral networks due to accumulation of genetic defects, which would in turn result in oncolytic virus susceptibility. This review outlines the characteristics of oncolytic poxvirus strains, including vaccinia, myxoma, and squirrelpox virus, recent progress in elucidating the molecular connection between oncogene/tumor suppressor gene abnormalities and poxviral oncolytic tropism, and the associated preclinical/clinical implications. I would also like to propose future directions in the utility of poxviruses for oncolytic virotherapy.

Keywords: oncolytic virus, poxvirus, vaccinia virus, myxoma virus, squirrelpox virus, oncogenes, tumor suppressor genes

Introduction

Oncolytic viruses are live, replication-proficient viruses that preferentially infect human cancer cells while sparing normal cell counterparts. Such replication-proficient viruses provide a series of potentially viable anti-cancer therapeutic approaches. Oncolytic viruses have many advantages over the use of conventional chemotherapy/radiotherapy or replication-incompetent viral vectors. First, they generally target cancer cells specifically, because of their natural or engineered reduced ability to replicate in normal cells, while replicating vigorously in and killing transformed cells. Second, as compared to replication-incompetent viral vectors, they can propagate from initially infected cancer cells to surrounding or distant cancer cells, thereby achieving a wide distribution and exerting potent anti-cancer effects (Parato *et al*., 2005; Hartkopf *et al*., 2011; Russell *et al*., 2012).

Because of these unique features of the replicating nature of oncolytic viruses, they are highly dependent on the host cell physiology for optimal performance as viral cancer-targeting agents. Many naturally occurring viruses have shown great potential as cancer-targeting agents by exploiting various oncogene signaling pathways that are established by host cancer cells during tumorigenesis (Strong *et al*., 1998; Roberts *et al*., 2006; Wang *et al*., 2006). However, carcinogenesis is a multi-step process involving accumulation of not only oncogene abnormalities but also tumor suppressor gene abnormalities, and we recently discovered that cellular tumor suppressor genes such as *p53*, *ATM* (Ataxia telangiectasia mutated), and *RB* (Retinoblastoma-associated) are also important in determining oncolytic viral tropism, including in poxvirus (Kim *et al*., 2010). Thus, an important mechanism of viral oncolysis can be established by both cellular oncogene and tumor suppressor gene abnormalities.

Since the eradication of smallpox in the 1970s with the aid of vaccinia viruses, vaccinia viruses and other poxvirus genera have shown various degrees of safety and efficacy in preclinical or clinical application for human anti-cancer therapeutics. Here, I review recent progress in molecular studies and preclinical/clinical aspects of replication-proficient poxvirus oncolysis.

Origin of oncolytic poxviruses

Poxviruses, which belong to the *Poxviridae* family, are ubiquitous, enveloped viruses that replicate entirely in the cytoplasm of vertebrate or invertebrate cells. Poxvirus particles (virions) can be externally enveloped virion (EEV), although the intracellular mature virion (IMV) form of the virus, which contains a different envelope, is also infectious. They vary in shape depending upon the species, but are generally brick- or oval-shaped (similar to a rounded brick) wrapped by the endoplasmic reticulum. The virion is exceptionally large at

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around 200 nm in diameter and 300 nm in length, and carries its genome in a single, linear, double-stranded segment of a DNA molecule comprising 130 to 300 kb pairs (Moss, 2013).

Vaccinia virus is a member of the *Orthopoxvirus* genus of the *Poxviridae* and is the most intensively studied poxvirus. It is most well known as the live vaccine virus that was used to eradicate smallpox caused by the variola virus, a feat completed in the 1970s and that remains the greatest triumph for the World Health Organization to date (Ellner, 1998). Yet, despite the effectiveness of vaccinia virus in eradicating smallpox, its origin and natural history are unknown and remain an enigma of virology (Baxby, 1977; Wilkinson, 1982). The live vaccinia Lister strain was developed at the Lister Institute in the United Kingdom. From 1968 to 1971, the Lister strain became the most widely used vaccine throughout the world (Rosenthal *et al*., 2001). More recently, the oncolytic nature of the Lister strain has been studied by several research groups (Timiryasova *et al*., 1999; Chen *et al*., 2001; Hung *et al*., 2007). Currently, a modified version of the Lister strain is in clinical trials for treating various human cancers as well as for feline/canine cancer therapy (Gentschev *et al*., 2014; Mell *et al*., 2014). The live vaccinia Wyeth strain was one of the smallpox vaccine viruses used mainly in the Americas and West Africa during the worldwide vaccination campaign (Jacobs *et al*., 2009; Nalca and Zumbrun, 2010). The oncolytic nature of the Wyeth strain has also been widely studied (Mastrangelo *et al*., 1999; Liu *et al*., 2014), and a modified version of the Wyeth strain is in clinical trials for treating various human cancers (Mastrangelo *et al*., 1999; Park *et al*., 2008; Heo *et al*., 2013). The live vaccinia Western Reserve (WR) strain was derived from serial passaging the New York City Board of Health (NYCBH) strain in the mouse brain, and has been shown to replicate to high titers in various mouse organs (Kaplan, 1989; Brandt and Jacob, 2001; Brandt *et al*., 2005). The oncolytic nature of the WR strain has been studied (Gnant *et al*., 1999; McCart *et al*., 2001; Thorne *et al*., 2007; Autio *et al*., 2014; Parviainen *et al*., 2015), and a modified version is in a clinical trial for various human cancers (Zeh *et al*., 2015). The modified vaccinia Ankara (MVA) strain was derived in the late 1950s by passaging the chorioallantois VACV Ankara (CVA) strain of vaccinia virus more than 570 times in chick embryo fibroblast cells, resulting in a host range-restricted virus that is replication-defective in most mammalian cells (McCurdy *et al*., 2004). This highly attenuated strain is unable to fully replicate in human cells and presented no adverse reactions in clinical trials (Sutter and Moss, 1992). MVA was safely used to vaccinate over 100,000 people in Germany (Mayr, 2003), yet its effectiveness against smallpox remains untested. Due to its viral replication potential being severely compromised, MVA has been used as a nonreplicating anti-cancer vector to deliver various transgenes rather than for replicating oncolytic virotherapy (Sutter and Moss, 1992; Carroll *et al*., 1997; Drexler *et al*., 1999). Currently, a modified version of the MVA strain is in clinical trials for treating various human cancers (Larocca Schlom, 2011; Amato *et al*., 2012; Gómez *et al*., 2013). Strain LC16m8 was developed in Japan in 1975, by passaging the Lister strain through primary rabbit kidney epithelial cells (PRK) at a low temperature (30°C) (Kenner *et al*., 2006). The Lister virus was initially passaged 36 times through PRK cells, and individual clones were then evaluated for growth on monkey kidney Vero cells, in order to evaluate their ability to replicate in primate tissues. Strain LC16, which grew to the lowest titer in Vero cells, was passaged 6 more times under identical conditions. Eventually, LC16mO, which formed medium-sized pocks on chick chorioallantoic membranes (CAM), was isolated from this stock and passaged 3 more times on PRK cells. LC16m8 was then isolated from this latter stock as a clone that both replicated poorly in Vero cells and formed small plaques on CAM, PRK, and continuous rabbit kidney epithelial RK13 cells. Currently, modified versions of LC16m series strains (LC16mO, LC16m8, etc.) are in preclinical studies for potential treatment of various human cancers (Hikichi *et al*., 2011). Raccoonpox virus (RCNV) is also a member of the *Orthopoxvirus* genus and is closely related to the vaccinia and cowpox viruses. RCNV was first reported in 1964 by Herman, and was isolated from a naturally occurring poxvirus from the respiratory tract of raccoons inhabiting an undeveloped forest and swamp area near Aberdeen, Maryland (Herman, 1964; Thomas *et al*., 1975). Recently, a modified version of the RCNV strain is in preclinical studies for its potential in treating various human cancers (Evgin *et al*., 2010).

Yaba-like disease virus (YLDV) is a member of the *Yatapoxvirus* genus of the *Poxviridae* family. YLDV is closely related to Yaba monkey tumor virus (YMTV) and tanapoxvirus, which also belong to the *Yatapoxvirus* genus. YLDV was first recognized in 1965 and 1966 in monkey caretakers who were working at primate centers in the United States, and was traced to a single source (Espana, 1971). YLDV infection in the caretakers produced a brief fever and the development of a few firm, elevated, round, necrotic maculopapular nodules, followed by complete resolution of the infection. Recently, a modified version of the YLDV strain is in a preclinical study for human ovarian cancer therapy (Hu *et al*., 2001).

Myxoma virus (MYXV) is a member of the *Leporipoxvirus* genus of the *Poxviridae* family. Two distinct types of MYXV have been identified: South American MYXV (Lausanne strain; Brazil/Campinas/1949/1), which circulates in the tapeti (*Sylvilagus brasiliensis*), and Californian MYXV, which circulates in the brush rabbit (*Sylvilagus bachmani*). Each virus is highly adapted to its host, causing a benign cutaneous fibroma at the site of inoculation. Both types of MYXV infect the European rabbit (*Oryctolagus cuniculus*), causing myxomatosis with mortality rates reaching up to virtually 100% (Fenner, 1983; Kerr *et al*., 2012). The Lausanne strain of MYXV originated from a Brazilian rabbit, but is so-named because it was obtained from a laboratory in Lausanne, Switzerland (Regnery, 1971). Currently, a modified version of the MYXV Lausanne strain is in preclinical studies for treating various human cancers (Lun *et al*., 2005; Kim *et al*., 2009, 2010; Kim and Johnston, 2014; Zemp *et al*., 2014).

Squirrelpoxvirus (SQPV) is a member of an as-yet-unassigned genus of the *Poxviridae* family. SQPV was isolated from a grey squirrel (*Sciurus carolinensis*) in Maryland in 1953 and initially placed into the genus *Leporipoxvirus* by Kilham *et al.* (1953); it was later thought to be a member of the genus *Parapoxvirus* (Housawi *et al*., 1998), but a sub-

Table 1. Oncolytic strains of replicating poxviruses

Poxviruses				Applications for cancer therapy		Engineered strains
Genus	Species	Backbone strain	Genomic modification	Pre-clinical	Clinical	
Orthopoxvirus	Vaccinia (VACV)	Lister	Attenuated, transgenes inserted (Chen *et al.*, 2001; Zhang *et al.*, 2007; Kochneva *et al.*, 2012; Chan and McFadden, 2014)	Breast tumor model, Ovarian tumor model, etc (Hung *et al.*, 2007; Zhang *et al.*, 2007; Hiley *et al.*, 2010; Gholami *et al.*, 2014)	Various solid tumors, Breast cancer (Gentschev *et al.*, 2014; Mell *et al.*, 2014)	GLV-1h68*, GLV-1h99, GLV-1h108, VV-mIL2, VVhEA, VV-hup53, etc
		Wyeth	Attenuated, transgenes inserted (Mastrangelo *et al.*, 1999; Liu *et al.*, 2014)	Melanoma model, Bladder cancer model, etc (Mastrangelo *et al.*, 1999; Gomella *et al.*, 2001)	Melanoma cancer, Liver cancer, etc (Mastrangelo *et al.*, 1999; Park *et al.*, 2008; Heo *et al.*, 2013)	JX-594*
		WR	Attenuated, transgenes inserted (Gnant *et al.*, 1999; McCart *et al.*, 2001; Thorne *et al.*, 2007; Parviainen *et al.*, 2015)	Colon cancer model. etc (Gnant *et al.*, 1999; McCart *et al.*, 2001; Autio *et al.*, 2014)	Various tumors (Zeh *et al.*, 2015)	JX-795, JX-963*, vvDD*, VV-TRAIL, etc
		MVA	Severely attenuated, transgenes inserted (Sutter and Moss, 1992; Kochneva *et al.*, 2012)	Various cancer model (Drexler *et al.*, 1999; Carroll *et al.*, 1997)	Various tumors (Larocca and Schlom, 2011; Amato *et al.*, 2012; Gómez *et al.*, 2013)	MVA-5T4*, MVAhup53
		LC16m series	Severely attenuated, transgenes inserted (Hikichi *et al.*, 2011)	Various cancer model (Hikichi *et al.*, 2011)	ND	MRVV (miRNA-regulated vaccinia virus)
	Raccoonpox (RCNV)	Derived from a cat	Wild type, Attenuated, transgenes inserted (Evgin *et al.*, 2010)	Various cancer model (Evgin *et al.*, 2010)	ND	
Yatapox	Yaba-like disease (YLDV)	Derived from a monkey caretaker	Wild type, transgenes inserted (Hu *et al.*, 2001)	Ovarian cancer model (Hu *et al.*, 2001)	ND	
Leporipoxvirus	Myxoma (MYXV)	Lausanne	Wild type, transgenes inserted (Opgenorth *et al.*, 1992; Johnston *et al.*, 2003; Sypula *et al.*, 2004; Chan and McFadden, 2014)	Glioma tumor model, Hematologic cancer model, etc (Lun *et al.*, 2005; Kim *et al.*, 2009; Kim *et al.*, 2010; Zemp *et al.*, 2014)	ND	vMyx-gfp(tdTr), MYXV-F11L, vMyx-M135KO-gfp
Unassigned	Squirrelpox (SQPV)	Kilham	Wild type (Kim *et al.*, 2014b)	Various cancer model (Kim *et al.*, 2014b)	ND	

Abbreviations: WR, Western Reserve; YLDV, Yaba-like disease virus; MVA, Modified Vaccinia Virus Ankara; Wyeth, A strain produced by Wyeth Laboratories and derived from the New York City Board of Health strain. ND, not done yet. *Strains undergoing clinical trials. Representative literatures are listed in parenthesis.

sequent study revealed that SQPV does not belong to any known poxviral genus, and was hence reassigned to an unknown genus (McInnes *et al.*, 2006). Currently, the wild type SQPV Kilham strain is in preclinical studies for its potential in treating various human cancers (Kim *et al.*, 2014a, 2014b). A summary of the oncolytic strains of replicating poxviruses is shown in Table 1.

Safety of the oncolytic poxvirus strains

Although vaccination with vaccinia viruses, which belong to the *Orthopoxvirus* genus, led to the complete worldwide eradication of smallpox, the viruses have a relatively poor safety record compared to other viruses used as live vaccines. For instance, in the USA in the late 1960s, vaccination with the vaccinia Wyeth strain induced approximately one death per million vaccinees and other types of very rare but serious complications occurred, particularly in immunocompromised individuals (Nalca and Zumbrun, 2010). The frequency of viral complications varied with the particular strain of vaccinia virus used, and the more pathogenic strains were replaced by those with a better safety profile (Jacobs *et al.*, 2009). The frequency of all complications was greater during primary vaccination than during revaccination. The major types of complications noted were skin disorders, neurological conditions, systemic infection, and progressive vaccinia (Halsell *et al.*, 2003; Lane and Goldstein, 2003). Because cancer patients occasionally undergo periods of severe immunosuppression due to heavy chemo/radiation treatments or advanced tumor progression, these potential complications could be a hurdle in applications of replicating poxviruses for human anticancer therapeutics.

In an effort to search for attenuated vaccinia virus variants, several approaches have been followed, including the continuous passage of the virus in tissue culture cells and removal of particular genes in the viral genome. Mayr and colleagues used primary chicken embryo fibroblast cells to grow CVA, a Turkish smallpox vaccine, and, after more than 570 passages, isolated attenuated variant mutants to eventually obtain what we now know as the modified vaccinia Ankara (MVA) (Mayr *et al.*, 1975). This virus was sequenced and shown to have lost about 30 kb, particularly at both ends of the viral genome (Antoine *et al.*, 1998). An important characteristic of MVA is that it is host range-restricted, is unable to replicate in most mammalian cells, and is a highly

attenuated phenotype, as shown in several animal models and in humans. In fact, MVA was used during the eradication campaign in Germany in over 120,000 individuals without adverse effects (Mayr *et al*., 1978; Stickl *et al*., 1974; Hochstein-Mintzel *et al*., 1975). However, it remains questionable as to whether MVA actually effectively induced protective immunity against smallpox, because these vaccinees could not be exposed to the real variola viruses. As mentioned above, MVA-based anticancer therapeutics are currently in clinical trials, but because viral replication competency is severely compromised, transgenes expressing MVA viruses are always used instead of the virus itself (Larocca and Schlom, 2011; Amato *et al*., 2012; Gómez *et al*., 2013).

LC16m8 was produced by repeated passage of the Lister strain in cell culture and has a small plaque phenotype (Hashizume, 2004). It was used in Japan for smallpox vaccination between 1974 and 1975, although, as with MVA, it is unknown whether it is effectively protective in humans because smallpox was no longer endemic in Japan at this time. LC16m8 produced milder reactions in children and was less virulent (including neurovirulence) in animals compared to the Lister strain; there were no serious complications reported from its use. The genomes of LC16m8, its parent LC16m0, and the original Lister strain have been sequenced. An important genetic difference between LC16m8 and other VACV strains, including the parental Lister and LC16m0 strains, is the disruption of the B5R gene by a frameshift mutation (Morikawa *et al*., 2005). The B5R protein is present on the surface of EEV and is essential for spread of the virus (Mathew *et al*., 1998). Although loss of B5R gene function may be important for the safety of LC16m8, its oncolytic viral potential could be significantly compromised due to the lack of EEV-mediated oncolysis. Because its viral replication competency and EEV function are severely compromised, a genetically modified version of LC16m series viruses are used for preclinical studies (Hikichi *et al*., 2011).

YLDV belongs to the *Yatapox* genus, and was derived from a monkey caretaker (Hu *et al*., 2001). As mentioned above, YLDV infection in caretakers produced a brief fever and the development of a few firm, elevated, round, necrotic maculopapular nodules, followed by complete resolution of the infection. The *Yatapoxvirus* genus consists of Yaba monkey tumor virus (YMTV), tanapoxvirus, and YLDV. YMTV was first isolated in 1958 from rhesus monkeys in Yaba, near Lagos, Nigeria (Bearcroft and Jamieson, 1958). Tanapox virus was isolated from human skin biopsy specimens during outbreaks of illness in 1957 and 1962 among natives living along the Tana River Valley in Kenya (Downie *et al*., 1971). The clinical manifestations included fever, headache, backache, and prostration. A pock lesion (generally solitary) appeared during the fever, from which tanapox virus could be isolated. YLDV symptoms were almost identical to tanapox disease, and the viruses were originally believed to be the same. Knight *et al.* (1989) differentiated tanapox virus and YLDV by restrictive mapping analysis. Yatapoxviruses are not orthopoxviruses, and thus their human pathogenic nature could be entirely different from that of orthopoxiruses such as vaccinia viruses. Taken together, the human pathogenicity of the YLDV oncolytic strain seems to be very limited and solitary, as shown by inoculation of a closely related tanapox virus in a human volunteer (Downie *et al*., 1971), although confirmation awaits further human studies including immunocompromised individuals.

The MYXV Lausanne strain, which belongs to the *Leporipoxvirus* genus, was isolated from a Brazilian rabbit in a laboratory of Lausanne, Switzerland (Regnery, 1971). Poxviruses are known to have either a heterogeneous host range or a very specific host range, depending on the genus and species (McFadden, 2005). For instance, variola viruses belong to the genus *Orthopoxvirus* and exert their viral pathogenic nature only in humans, whereas cowpoxviruses can have a very broad host range, including rodents, dogs, cat, horses, cows, primates, and humans. Leporipoxviruses are known to have a very narrow and specific host range and exert their viral pathogenic nature only in a certain rabbit species, and the MYXV Lausanne strain has been extensively used as a public biological control agent to diminish the European rabbits species (*Oryctolagus cuniculus*) without affecting other non-rabbit species, including humans (Hayes and Richardson, 2001; Fenner, 2010). Taken together, the human pathogenicity of the MYXV Lausanne oncolytic strain seems to be highly unlikely due to its poxviral host range restriction, although further human studies are warranted that include immunocompromised individuals.

The SQPV Kilham strain, which belongs to an unassigned genus, was isolated from a grey squirrel in Maryland, USA (Kilham *et al*., 1953). In grey squirrels, SQPV exerts a subclinical infection that rarely manifests into disease (Sainsbury *et al*., 2000; Tompkins *et al*., 2002). However, red squirrel (*Sciurus vulgaris*) infection by SQPV causes ulceration with crusted lesions and scabs around the eyes, lips, feet, and genitalia, and an exudative dermatitis, which may be similar to the myxomatosis pathogenesis caused by MYXV infection in the European rabbit species that is almost always fatal (Duff *et al*., 1996; Carroll *et al*., 2009). Red squirrels have been in decline in Great Britain for the last century due to a combination of habitat loss and the introduction of the North American Eastern grey squirrel (*Sciurus carolinensis*) (Gurnell 1996). The dramatic decline of the native red squirrel in the United Kingdom has been attributed to both direct and disease-mediated competition with the grey squirrel, where the competitor acts as a reservoir host of SQPV (Collins *et al*., 2014). SQPV natural infection in humans has not been reported since the decline of the red squirrel population in Great Britain. SQPV seems to have a very narrow and specific host range and exerts its viral pathogenic nature only in a certain squirrel species without affecting other non-squirrel species (Collins *et al*., 2014). Taken together, the human pathogenicity of the SQPV Kilham oncolytic strain seems to be highly unlikely due to the poxviral host range restriction, although further human studies are warranted that include immunocompromised individuals.

Attenuation through deletions and insertions in the poxviral genome

Since thymidine kinase (*TK*)-deleted vaccinia viruses are less pathogenic to mice than wild-type viruses, nonessential poxviral genomic loci such as the TK-encoding locus (J2R) are

frequently modified by insertion/deletion approaches (Buller *et al.*, 1985). The vaccinia Lister strain was genetically modified by inserting *IL-2* and *lacZ*, *IL-12*, and *lacZ*, or two reporter genes (*luc* and *lacZ*) into the *J2R* locus of the Lister viral genome (Chen *et al.*, 2001). The vaccinia Lister strain was also genetically modified by inserting three expression cassettes (*Renilla* luciferase-*Aequorea* green fluorescent protein fusion, beta-galactosidase, and beta-glucuronidase) into the *F14.5L*, *J2R*, and *A56R* loci of the Lister viral genome, respectively (Zhang *et al.*, 2007). Zhang *et al.* (2007) reported that whereas the triple insertions greatly reduced replication of the modified virus in a normal mouse cell line (e.g., murine embryonic fibroblasts), replication of the modified virus in a tumor cell was not detrimentally affected.

The vaccinia Wyeth strain was genetically modified by inserting granulocyte-macrophage colony-stimulating factor (GM-CSF) and beta-galactosidase reporter gene into the *J2R* locus of the Wyeth viral genome (Mastrangelo *et al.*, 1998). Similarly, the Wyeth strain was also genetically modified by incorporating the human thyroidal sodium iodide symporter gene (hNIS) as a reporter gene (Liu *et al.*, 2014). The vaccinia WR strain was genetically modified by inserting the lacZ gene and the enhanced green fluorescent protein (EGFP) gene into vaccinia growth factor (*VGF*) and *TK* sites of the WR viral genome, respectively (McCart *et al.*, 2001). In addition to the double insertion/deletion, the GM-CSF gene was also incorporated into the *TK* site of the WR viral genome, generating a triple insertion/deletion WR strain (Thorne *et al.*, 2007). Similarly, GM-CSF and tdTomato fluorophore genes were incorporated into the TK sites of the WR viral genome (Parviainen *et al.*, 2015). For a therapeutic suicidal purpose, the cytosine deaminase gene was incorporated into the TK site of the WR viral genome (Gnant *et al.*, 1999). As mentioned above, through long-term passaging/attenuation, MVA has significant genomic deletions and its replicative potential is nearly compromised (Antoine *et al.*, 1998). Similarly, in LC16m8, the *B5R* gene is disrupted and its replication potential is also severely compromised (Mathew *et al.*, 1998; Morikawa *et al.*, 2005).

An RCNV strain derived from a cat was genetically modified by inserting *EGFP* into the *TK* site of the RCNV viral genome (Evgin *et al.*, 2010). A YLDV strain, derived from a monkey caretaker, was also genetically modified by inserting *EGFP* into the *TK* site of the YLDV viral genome (Hu *et al.*, 2001). The MYXV Lausanne strain was genetically modified by inserting the *lacZ* gene into an innocuous site between open reading frames M010L and M011L in the MYXV viral genome (Opgenorth *et al.*, 1992). Furthermore, the MYXV Lausanne strain was genetically modified by intergenic insertion of *GFP* between open reading frames *M135R* and *M136R* of the MYXV viral genome (Johnston *et al.*, 2003). The SQPV Kilham strain has not been genetically modified for oncolytic poxviral studies (Kim *et al.*, 2014b).

Mechanisms of the oncolytic nature of poxviruses

In 1985, Buller *et al.* reported that a *TK*-deleted vaccinia virus showed decreased pathogenicity compared with wild-type vaccinia virus, with preserved replication potential in tumor cells. A *TK*-deleted vaccinia virus may obtain thymidine triphosphate for DNA synthesis from the nucleotide pool that is only present in actively dividing cells such as cancer cells. Thus, this could lead to preferential viral replication in dividing cells, and hence partially explain the observed oncolytic nature of *TK*-deleted vaccinia virus, since some actively dividing normal cells could be targeted by the virus as well.

In 2000, McCart *et al.* reported that a modified vaccinia WR strain (vvDD), which has TK and VGF deletions, was able to preferentially replicate in tumor tissues in an animal model. Nude mice bearing s.c. murine colon cancer xenografted tumors were injected systemically (intraperitoneally [i.p]) with 10^8 plaque-forming units (pfu) of vvDD. A significant antitumor effect was observed in the mice treated with vvDD-GFP (McCart *et al.*, 2000). McCart *et al.* (2001) further showed that the vvDD-GFP-modified strain, which has *TK* and *VGF* deletion/ insertions, was able to preferentially replicate in tumor tissues *in vivo*. Nude mice bearing s.c. murine colon cancer xenografted tumors were injected systemically (i.p.) with 10^9 pfu of vvDD-GFP, and a significant antitumor effect was observed in the mice treated with vvDD-GFP (McCart *et al.*, 2001). Remarkably, these antitumor effects are attributable to the systemically replicating

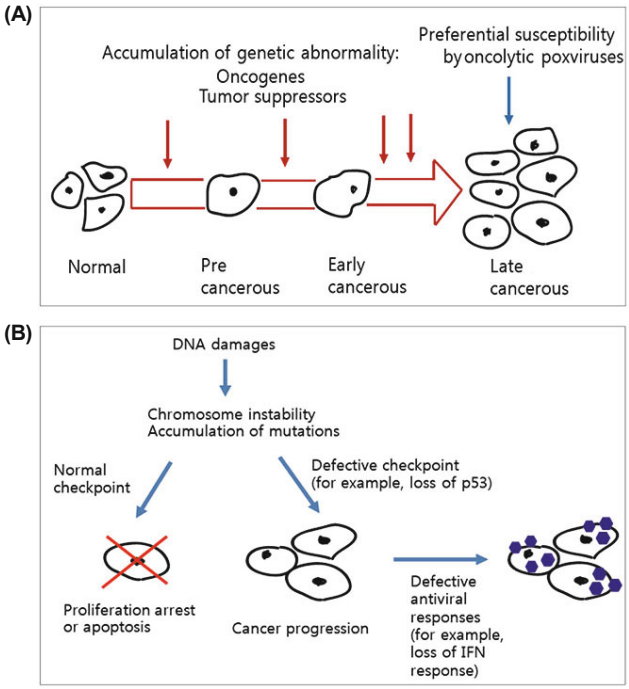

Fig. 1. Mechanistic basis of poxvirus oncolysis. (A) Carcinogenesis is a multi-step process involving accumulation of cellular oncogenes and tumor suppressor gene abnormalities both genetically and/or functionally. (B) In the case of normal cells, DNA damages (externally or internally) could be repaired by normal checkpoint activities such as cell cycle arrest or apoptosis restricting viral propagation due to preservation of anti-viral integrity. However, tumor suppressor defective cancers couldn't handle genotoxic challenges, resulting in genomic instability and defective checkpoints/antiviral responses. Taken together, abnormal cells can be preferentially infected by poxviruses due to loss of intact anti-viral functionality.

Table 2. Summary of clinical trials with completed and ongoing using replicating poxviruses alone or in combination with other therapies

Tumor types	Trial location	Phase	Highest doses (PFU)/ Backbone strain	Safety/Efficacy	ClinicalTrials. gov identifier	Current status*	Ref
Head and neck cancer	US	1	3×10^9/Lister		NCT01584284	Recruiting	Mell *et al.* (2014)
Peritoneal carcinomatosis	Germany	1/2	NR/Lister		NCT01443260	Recruiting	
	Canada	2	NR/Wyeth		NCT02017678	Withdrawn prior to enrollment	
Malignant pleural effusion	US	1	NR/Lister		NCT01766739	Recruiting	
Advanced solid tumors	UK	1	NR/Lister		NCT00794131	Recruiting	
	US	1	3×10^9/WR	Well tolerated/ limited efficacy	NCT00574977	Active, not recruiting	Zeh *et al.* (2015)
Skin cancer	US	1/2	NR/Wyeth		NCT00429312	Completed in 2009	
		Pilot	8×10^7/Wyeth	Well tolerated/ limited efficacy		Completed in 1999	Mastrangelo *et al.* (1999)
Liver cancer	South Korea	1	3×10^9/Wyeth	Well tolerated/ limited efficacy	NCT00629759	Completed in 2007	Park *et al.* (2008)
	South Korea, US, Canada	2	1×10^9/Wyeth	Well tolerated/ limited efficacy	NCT00554372	Completed in 2013	Heo *et al.* (2013)
	South Korea, US, Canada, Germany, France, Hong Kong, Taiwan	2	1×10^9/Wyeth		NCT01387555	Unknown	
Colorectal cancer	South Korea	1	3×10^7/Wyeth		NCT01380600	Active, not recruiting	
	US, Canada, France	1/2	NR/Wyeth		NCT01394939	Active, not recruiting	
	Canada	2	1×10^9/Wyeth		NCT01329809	Terminated	
Pediatric solid cancers	US	1	3×10^7/Wyeth		NCT01169584	Completed in 2014	

* As of Jan 2015. Abbreviations: NR, Not reported; WR, Western Researve

vaccinia viruses alone, because, except for viral modifications made for reporter or attenuation purposes by the TK-deletion/insertion, no therapeutic gene had been inserted in the poxviral genome. However, the underlining mechanism of the innate preference of vaccinia replication in tumor tissues was not well understood at that time.

Several molecular rationales of innately oncolytic poxvirus tropism have been proposed. In 2004, Katsafanas *et al.* reported that the cellular *Ras* oncogene signaling pathway may play an important role in vaccinia virus oncolytic tropism. In a human cervical cancer cell line, Ras-GTPase-activating protein SH3 domain-binding protein (G3BP) was copurified during screening for a host cell factor required for transcription of the vaccinia viral genome. The involvement of this cellular protein in transcription of intermediate-stage viral genes may indicate that it regulates the transition between the early and late phases of vaccinia virus replication (Katsafanas and Moss, 2004). In 2006, Wang *et al.* (2006) reported that that hyperactive *AKT* oncogene signaling pathway, either via constitutive phosphorylation or induced by MYXV infection, dictates MYXV oncolytic tropism in a variety of human cancer cell lines.

Recent studies have indicated that chromosome instability caused by the combined dysfunctional effect of oncogenes and tumor suppressor genes may be more central to tumorigenesis than previously thought (Coschi and Dick, 2012). Thus, not only oncogenes but also tumor suppressor genes may play an important role in determining the oncolytic nature of poxviruses, since genomic instability could compromise the integrity of normal cellular anti-viral networks. In 2010, we were able to show that the p53, ATM, and RB genes and their abnormal functions are important in determining MYXV oncolytic tropism. Abnormal functions of these tumor suppressors render cells to become susceptible upon MYXV challenge (Kim *et al.*, 2010). We also showed that highly dividing normal human hematopoietic stem cells were preserved upon MYXV challenge, whereas highly dividing hematopoietic human cancer stem cells were significantly infected upon MXYV challenge (Kim *et al.*, 2009), strongly suggesting that genetic abnormality of cancer cells plays an important role in the oncolytic nature of poxviruses in general. We proposed that abnormalities of tumor suppressor genes should increase genomic instability, which would in turn increase the occurrence of new mutations, including those affecting antiviral-related host genes. Furthermore, genomic instability appears to be the engine of both tumor progression and stagnation of the normal function of antiviral-related cellular genes. Thus, mechanistically, poxvirus oncolysis can be established by both oncogenic hyper-activation and/or tumor suppressor abnormalities. Fig. 1 shows the mechanistic basis of poxvirus oncolysis in regards to cellular oncogenes and tumor suppressor genes.

Clinical trials for human cancer therapy

Various modified versions of vaccinia strains, such as Lister, Wyeth, and WR, are currently in human clinical evaluations. Various tumor types are challenged by these replicating poxviruses, which may or may not include a therapeutic transgene in the poxviral genome as described above and shown in Table 2; since MVA and the LC16m series can be regarded as nearly replication-defective poxviruses, their relevant trials are not included in the table. Most clinical trials are being

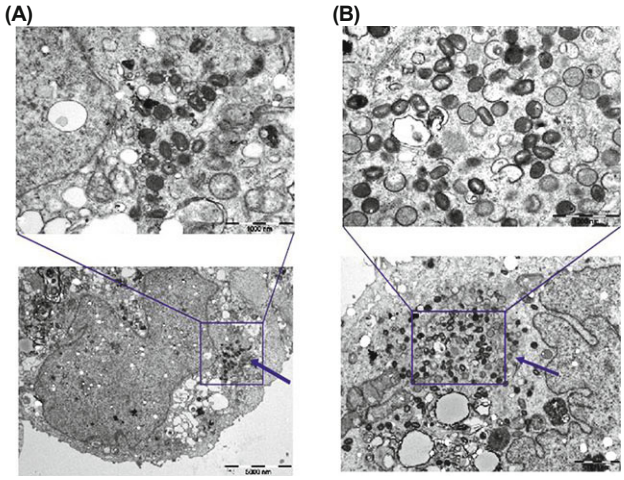

Fig. 2. SQPV tropsim on human gastric and liver cancer cell. SQPV viral particles are detected in cytoplasmic area of AGS human gastric cancer cell (A) or Hep3B human liver cancer cell (B) upon SQPV challenges at 3-5 days post-infection (10 MOI). Arrow indicates poxviral factory (lower) and mature/immature poxviral particles are shown in higher power EM (electron microscope) (upper panel). EM magnification: 6K or 8K (lower panels), 30K (upper panels).

conducted in North America and Europe. Since the Chinese and Korean governments recently approved the use of MYXV and SQPV for human cancer therapy (Kim *et al.*, 2014b; Kim and Johnston, 2014), our group is preparing an application for clinical trials of oncolytic poxviruses in Asian countries.

Future directions

Since the eradication of smallpox in the 1970s, the oncolytic nature of replicating vaccinia virus and other poxvirus strains have been identified. During the past 20 years of molecular research, the involvement of cellular oncogenes and tumor suppressor genes in determining poxviral oncolytic tropism is now fairly well established. Currently, clinical trials are being conducted in North America, Europe, and a few Asian countries, using modified versions of vaccinia virus strains. The safety aspects of attenuated versions of vaccinia viruses are now fairly established through the many trials that have already been conducted, although rare vaccinia viral complications could occur at a large population scale including immunocompromised individuals (Jacobs *et al.*, 2009; Nalca and Zumbrun, 2010). MVA and LC16m8 could be safer oncolytic vaccinia virus candidates; however, because their innate replication potency is severely compromised due to extensive genetic attenuation, the inherent oncolytic potency of these strains is questionable. As mentioned previously, the use of non-vaccinia poxviruses such as YLDV, RCNV, MYXV, SQPV, and other as-yet-unidentified non-vaccinia poxviruses, could be an alternative strategy to reduce these potential risks. Non-vaccinia poxviruses could also be used for pregnant or childhood cancer patients due to the apparent nature of their non-pathogenicity in human species and the lack of viral pathogenicity in immunocompromised animal studies (McFadden, 2005; Kim *et al.*, 2009, 2010, 2014b).

As shown in Table 2, all of the clinical trials conducted to date or that are on-going use modified versions of vaccinia viruses for human cancer therapy. Our study showed that gastric and liver cancers, which are prevalent in Asian countries, are highly susceptible to SQPV challenge, as shown in Fig. 2. Thus, clinical application of non-vaccinia poxviruses for gastric and liver cancers could be promising in the future. Furthermore, since a combinatorial oncolytic viral regimen using different families of viruses could synergistically enhance the oncolytic potency (Kim *et al.*, 2007; Le Boeuf *et al.*, 2010; Alkassar *et al.*, 2011), replicating poxviruses could be used as a DNA oncolytic virus candidate in a DNA-plus-RNA oncolytic viral combination strategy (Le Boeuf *et al.*, 2010).

Disclosure

M KIM is a scientific founder and shareholder of ViroCure which has replicating poxvirus patents under clinical development.

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