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Generation of Tanapoxvirus Ankyrin Repeat Gene Deletion Mutant for Evaluating its Role in Cell Tropism

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GENERATION OF TANAPOXVIRUS ANKYRIN REPEAT GENE DELETION MUTANT FOR EVALUATING ITS ROLE IN CELL TROPISM

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GENERATION OF TANAPOXVIRUS ANKYRIN REPEAT GENE DELETION MUTANT FOR EVALUATING ITS ROLE IN CELL TROPISM

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The host range of poxviruses is highly regulated and is limited to certain hosts. A number of genes in different poxviruses have been identified that contribute to cell tropism. The ankyrin (ANK) repeat genes encode for proteins characterized by the repetitive motifs in its amino acid sequence. These genes have been found to play a role in determining the ability of poxviruses to replicate in certain cell types. Tanapoxvirus (TPV) Kenya genome analysis indicated the presence of six ANK repeat genes of which T146R, T147R and T148R are present in the terminal part of the TPV-Kenya genome. To understand the possible role of T146R in determining the cell tropism, an insertional knockout TPV (TPV-ΔT146R) was constructed. The ability of TPV-ΔT146R to replicate in a variety of human colorectal cancer cell lines and human malignant melanoma cells was also tested. Cell monolayers were observed for viral cytopathic effect at 96 hours post infection (h.p.i.) indicated that both TPV-Kenya and TPV-ΔT146R replicated in most of the human colorectal cancer cell lines. Taken together, these results provide evidence that the open reading frame T146R in TPV is a non-essential gene.

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Yogesh Suryawanshi

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INTRODUCTION

Ankyrin repeat containing proteins were first reported in 1978, as a protein present on the membrane of erythrocytes which act as anchor proteins, hence named as ankyrin (ANK) proteins (Bennett, 1978). A repeat of ~33 amino acid residues was observed in the sequence of two cell-cycle regulators Swi6p and Cdc10, in yeast and a developmental regulator Notch in Drosophila melanogaster (Breeden and Nasmyth, 1987). Later on, multiple copies of a similar sequence were reported in the cytoskeletal protein ANK (Lux et al., 1990). Subsequently, ANK proteins were also reported to be present in a wide range of organisms from prokaryotes to eukaryotes. Based on further studies, the role of ANK proteins in mediating interaction between different proteins is well known and they have been found to be present in a variety of proteins like transcriptional mediators, developmental regulators, cytoskeletal organizers, cyclin-dependent kinase inhibitors and toxins. In addition, the role of ANK proteins in protein-protein interaction is not limited to a particular class of proteins but instead it has been found to be involved with a wide variety of proteins (Michaely and Bennett, 1992; Bork, 1993). ANK proteins have been shown to play an important role in expression, localization and regulation of ion channels and transporters in the cardiac cell membrane. Mutations or loss of these ANK genes have been linked to the development of cardiac arrhythmias (Cunha and Mohler, 2011). In addition, ANK proteins have also proven to be of great importance for multiple aspects of muscle function. They play a critical role at different stages of muscle development and in controlling the hypertonic responses in fully developed muscles, thus suggesting the importance of these proteins in the

developmental disorders of muscle (Tee and Peppelenbosch, 2010). Additionally, some ANK proteins have a role in the transduction of endogenous inflammatory factors, as well as exogenous irritating chemicals, such as the Transient Receptor Potential Ankyrin 1 (TRPA1) on non-myelinated pain receptors (Stucky et al., 2009).

Chordopoxviruses (poxviruses of vertebrates) encode a large number of ANK proteins and typically the terminal part of their large linear DNA is responsible for coding them. Amongst these viruses, Molluscum contagiosum virus is the only poxvirus to date, which does not encode for any ANK proteins. Certain ANK proteins from vaccinia virus (VACV), myxoma virus (MYXV) and cowpox virus (CPXV) have been studied and they have shown to play a role in determining the virulence and host tropism of these viruses. Still the role of many other ANK proteins encoded by Chordopoxviruses remains a mystery and need to be studied further (Sonnberg et al., 2011).

This study describes the potential role of ANK repeat genes, T146R in TPV as a host/cell range factor. The T146R knockout TPV (TPV-ΔT146R) was generated and tested for its ability to replicate in a bank of human cancer cell lines, owl monkey kidney cells (OMK) and human fetal lung fibroblasts (WI 38). The OMK and WI 38 cells served as a control.

REVIEW OF LITERATURE

Tanapoxvirus

Poxviruses are large DNA viruses which infect a wide range of hosts. They replicate in the cytoplasm of the host cells and carry a double stranded DNA genome. While the central part of genome is highly conserved, the terminal part of poxvirus genomic DNA is considerably divergent and bear the genes that encode proteins involved in host specificity and virulence. Poxvirus family is divided into two subfamilies, namely Entomopoxvirinae which infect insects and Chordopoxvirinae which infect vertebrates. Chordopoxviruses are further divided into eight genera, Orthopoxvirus, Parapoxvirus, Leporipoxvirus, Capripoxvirus, Avipoxvirus, Suipoxvirus, Molluscipoxvirus and Yatapoxvirus (Knipe and Howley, 2007). TPV belongs to the genus Yatapoxvirus which also includes two more members, namely Yaba-like disease virus (YLDV) and Yaba monkey tumor virus (YMTV). All the members of Yatapoxviruses replicate in closely related hosts. Monkeys are the host for YLDV and YMTV while TPV replicates in humans (De Harven and Yohn, 1966; Espana et al., 1971). TPV and YLDV genomes are very closely matched (98.6%), showing similarity levels on par with different strains of VACV; it has therefore been argued that TPV and YLDV are possibly different strains of the same virus. In TPV-Kenya, T8L, T11.1L, T11.2L, T146R, T147R and T148R are the ANK repeat genes that have been identified and may potentially play a role in determining the host range (Nazarian et al., 2007).

A disease caused by TPV was reported in 1957 in Nagu village, in the

flood plains of the Tana river in Kenya. Two widespread epidemics in 1957 and 1962 of TPV disease occurred in the region along Tana river. Analysis of biopsy specimens from the skin legions showed that the virus belongs to the poxvirus group but differ from other poxviruses and hence named as TPV. TPV has a very limited range of hosts and has been grown in human and monkey tissues successfully. Monkeys are the only animal found to be susceptible to TPV (Downie et al., 1971). Recently a case of TPV infection was reported in 2004 in a college student who was caring for chimpanzees and spent time in close proximity with them in Republic of Congo (Dhar et al., 2004). These instances suggest towards possibility of TPV being a zoonosis from monkeys to humans. In another instance, after spending 3 weeks in Tanzania, a patient reported being infected with TPV in Europe in 1999. Though the infection was suspected to be a zoonosis from mosquito or cat during his stay in Tanzania, the mechanism of transmission and the animal reservoir of the disease couldn't be confirmed (Stich et al., 2002). Another case of TPV infection was reported in 2002, in a traveler from Sierra Leone (Croitoru et al., 2002). After an outbreak of YLDV in some primate centers in the U.S. in 1966, some animal handlers were infected, possibly through the skin lesions (Downie and Espana, 1972). This supports the possibility of TPV infection being a zoonosis from animals to humans as both TPV and YLDV are the members of Yatapoxvirus genus. In humans, TPV causes a short febrile disease accompanied by few skin lesions, severe headache, backache and prostration. This was followed by development of macular or nodular skin lesions where some of the nodular skin legions may burst due to trauma or necrosis, developing into an ulcer. However the disease caused by TPV is

self-limiting (Jezek et al., 1985).

Ankyrin Repeat Proteins in Poxviruses

Ankyrin repeat proteins are the adaptor proteins which contain repetitive motifs of about 33 residues. Recently it has been shown that ANK proteins with F-box motif at C-terminal are capable of interacting with SCF-1 ubiquitin ligase complex through the adapter subunit SKP1 of the complex. This suggests that the ANK proteins are possibly manipulating the host cell's ubiquitin system by directing it towards specific proteins (Sonnberg et al., 2008). CPXV expresses an ANK protein CPXV-006 which has been shown to manipulate the host immune response by inhibiting NF-kB which plays a role in activation of immune response following infection and cell injury. This further supports the idea that just like many other viruses and bacteria, poxviruses have evolved these novel mechanisms to counter the host immune system (Mohamed et al., 2009). M-T5, an ANK protein encoded by MYXV has been demonstrated to interact with the host cell cullin-1 which is a cell cycle regulator. Host cell cullin-1 plays a role in the regulation of cell cycle through alteration of p27/kip-1 phosphorylation. This suggests a possible role of ANK protein M-T5 as a cell cycle regulator (Johnston et al., 2005). ANK proteins, M148R and M149R in MYXV have been demonstrated to be critical in determining the virulence. MYXV showed a drastic decrease in virulence when the genes encoding M148R and M149R were knocked out, although they didn't seem to affect the tissue tropism and host range (Blanie et al., 2009).

Cancer

Cancer is a group of diseases characterized by uncontrolled growth of cells which can spread from the site of origin to distant sites in the body. Cancer can be the result of point mutation or shift in the genome which can possibly be caused by physical, chemical and biological agents. Some of the known possible causative factors of cancer are UV light (physical agent), asbestos, dye, poly vinyl chloride (chemical agents), Viruses and bacterium (biological agents). It has been observed for most of the cancers that the risk of developing cancer increases with age, and middle aged and old aged population is mostly affected. Genetic makeup also plays a role by predisposing individuals with specific genes to certain types of cancer like BRCA1 and BRCA2 genes in breast cancer. Cancer has been a growing health concern due to the high incidence and mortality associated with it.

In the U.S. the age adjusted mortality rate of cancer is 178.7 per 100,000 men and woman per year. Most common cancers are lung cancer, colorectal cancer (CRC) and skin cancer, in addition, breast cancer (BC) in females and prostate cancer in males. CRC is the second highest cause of cancer related deaths while BC is one of the most frequently diagnosed cancers in women in US and is ranked second highest as a cause of cancer related deaths in women. According to the National Cancer Institute the age adjusted incidence rate of CRC is 46.3 per 100,000 men and woman per year and BC is 124.3 per 100,000 women per year in US. The age adjusted annual mortality rates for CRC and BC are 16.7 per 100,000 men and women and 23 per 100,000 women respectively (Howlader et al., 2012)

Diagnosis of cancer at an early stage is very important to improve the

survival rate as metastasis at advanced stage creates complications leading to death. Conventional diagnostic techniques that are currently used include; clinical examination, screening techniques like proctoscopy, mammography or CAT scans and histopathological examination of biopsy from the tissue. American Cancer Society has recommended some guidelines and runs awareness campaigns to increase the chances of detecting cancer at early treatable stages and improve survival rate. The treatment strategy for cancer is determined based on the stage at which cancer has been diagnosed and the conventional treatment options available for advanced cancers involve surgical treatment followed by radiotherapy and/or chemotherapy, but serious side effects associated with radiotherapy and chemotherapy limit their use. In addition adjuvant therapies like the use of Tamoxifen can be used in patients with breast cancer positive for Estrogen Receptors (ER). Oncolytic virotherapy is a relatively new option which seems to be promising but still under clinical trials. Effective measures to control the metastatic disease in advanced stages of cancer are the key to improve the survival rate and quality of life.

Oncogenes, Growth Factors and Secondary Signaling

Epidermal growth factor receptor (EGFR) is known to play a role in progression of tumor through activation of many signaling pathways that eventually result in cellular growth, angiogenesis, invasion and metastasis. The exact mechanism through which EGFR plays role in carcinogenesis is not completely understood, but amplification of genes is supposed to be

responsible for their overexpression. Altered expression of EGFR has been linked with BC, CRC, non-small cell lung carcinoma, pancreatic cancer, brain cancer and squamous cell carcinomas in the head and neck region (Brand et al., 2011).

Overexpression of HER-2/neu/erbB2 has been linked with cancer and the overexpression is possibly due to amplification of the gene. It has been observed that overexpression of HER-2 is associated with higher rate of lymph node metastasis, growth of tumor and decreased survival rate in cancer patients (Tsapralis, 2012; Lee et al., 2007). HER-2 overexpression has been noticed in the cancer tissue of both ductal carcinoma in situ and invasive ductal carcinoma, but interestingly HER-2 is very highly overexpressed in the ductal carcinoma in situ as compared to the invasive ductal carcinoma. This indicates towards a possible role of HER-2 in progression of ductal carcinoma in situ, a comparatively benign state to a highly malignant invasive ductal carcinoma. However, it has been found that development of vascular stroma in the adjacent tissue can be an important factor leading to invasion of ductal carcinoma in situ into adjacent tissue and HER-2 gene expression doesn't always play a significant role in progression of a tumor from a relatively benign to highly malignant state (Latta et al., 2002).

Another oncogene, c-Myc has been associated with the metastasis of the cancer cells. It promotes metastasis indirectly by favoring cancer cell growth, cell survival, genetic instability and angiogenesis. However, c-Myc has also been found to regulate metastasis through a more direct and indirect ways by manipulating downstream programs involving activation of SNAIL transcription factor through TGF-β, as reviewed in (Wolfer and Ramaswamy,

2011). The gene c-Myc has been reported to be amplified in ductal carcinoma in situ significantly and in few cases of invasive ductal carcinoma. The exact mechanism through which it is overexpressed is not yet clearly known. Co-amplification of HER-2 in most of the cases expressing altered c-Myc oncogene has been reported (Aulmann et al., 2002).

Cyclin D1 oncogene is overexpressed at all the stages during the development of ductal carcinoma of breast, beginning from hyperproliferative state of ductal epithelium of breast through ductal carcinoma in situ to invasive carcinoma of breast, cyclin D1 is overexpressed at all the stages. However, cyclin D1 expression is increased gradually from hyperproliferative state to invasive ductal carcinoma state, indicating that it plays an important role in development as well as progression of ductal carcinoma of breast (Alle et al., 1998). Cyclin D1 acts as a cell cycle regulator and overexpressed in many cancers. It regulates cell cycle by acting as a sensor for mitogenesis and activating factor for cyclin dependent kinase (CDK) 4/6. In neoplastic cells the nuclear trafficking of Cyclin D1 is impaired resulting in accumulation of Cyclin D1 inside the nucleus. High nuclear levels of Cyclin-D1 along with proteolysis are believed to be the reasons for the development of cancer (Kim and Diehl, 2009). This indicates that overexpression of cyclin D1 is not the sole factor for development of cancer, but its accumulation inside the nucleus plays a huge role.

Transforming growth factor- beta (TGF-β) is an important factor playing crucial roles in regulation of cell growth. Loss of functional TGF-β-receptor II has been linked with proliferation of tumor cells (Gobbi et al., 1999). In addition to EDGF, estrogen is another important hormone which

plays an important role in development of ductal carcinomas which are positive for estrogen receptors. Though the exact mechanism through which estrogen induces the growth in ductal carcinoma is not completely known, but it is believed that it acts through an indirect pathway (Palmieri et al., 2002). Different cytokines like IL-2, IL-6 and TNF- α has been reported to play an important role in carcinoma development by promoting growth of cells through different mechanisms (Garcia-Tunon et al., 2004; Garcia-Tunon et al., 2006).

The surrounding stromal tissue in ductal carcinoma of breast differs from that of normal breast tissue. Though the stromal tissue doesn't become malignant itself, it has been noted that different isoforms of extra-cellular glycoprotein called Tenascin are expressed at different stages during progression of cancer. These altered forms of Tenascin produced by stromal tissue adjacent to the cancerous tissue are believed to be responsible for the progression of cancer by promoting development of anchorage independence, invasion of cancer cells into the surrounding tissue and angiogenesis (Adams et al., 2002).

Tumor Suppressor Genes

The p53 is a commonly altered tumor suppressor gene by mutations in many cancers. Function of p53 gene is altered by missense mutation or nonsense or frameshift mutations result in loss of p53 gene functionality which makes it ineffective In terms of tumor suppression activity, resulting in development of oncogenesis (Vogelstein and Kinzler, 1992). Oncogene p53 is

known to regulate plasticity, synthesis, repair and transcription of DNA and cellular apoptosis as reviewed in (Greenblatt et al., 1994).

BRCA1 and BRCA2 are two major tumor suppressor genes which regulate the cell growth. BRCA1 gene is believed to be responsible for most of the familial or hereditary cases of breast cancer, while a smaller proportion of familial breast cancer cases are linked with BRCA2 gene (Easton et al., 1993; Miki et al., 1994; Wooster et al., 1994). It is believed that these tumor suppressor genes encode a regulatory protein that inhibits the cell growth and keeps the cell division under control. Loss of functionality is usually due to the mutations in these tumor suppressor genes which result in either non-production of the proteins or production of an altered form of protein with reduced or abolished function. Missense mutations are responsible for these alterations in these tumor suppressor genes most of the time (Miki et al., 1994). Another mechanism through which BRCA1 has been suggested to play a role is by regulating the activity of p27 and p21. Proteins p27 and p21 are believed to inhibit the cell growth and promote apoptosis by inhibiting the CDKs (Williamson et al., 2002). It has been observed that BRCA1 trans-activates the production of p21. Thus BRCA1 acts through regulation of p21 activity and causes arrest of cell cycle. This arrest of cell cycle caused by BRCA1 has been observed in the transformed cells (Somasundaram et al., 1997). In a normal breast tissue BRCA1 causes an up-regulation of p27 and keeps a check on cell division. In a cancerous tissue low levels of p27 have been observed as compared to normal tissue which shows that the mutations in BRCA1 gene might be rendering this mechanism inefficient to control the cell growth, leading to transformation of normal cells. Thus a decrease in the

level of p27 in breast tissue indicates towards a possible incompetent BRCA1 gene. Recently it has been suggested that methylation of BRCA1 gene can also be responsible for alteration of expression of BRCA1 gene. Methylation of BRCA1 gene has been linked to down-regulation of expression of p27. It is quite possible that methylation or mutations or both the mechanisms together render BRCA1 gene inefficient to control the cell growth (Chappuis et al., 2000; Niwa et al., 2000).

Researchers have been trying to understand the interaction between p53 and BRCA1 and its effect on regulation of cell cycle. BRCA1 is found to be involved in both p53 dependent as well as p53 independent transcription regulatory mechanisms. BRCA1 leads to transcriptional co-activation of p53 gene which causes increased expression of p21 and bax gene. Gene BRCA1 and p53 are the important factors in cell cycle regulation and apoptosis of cells. In normal breast tissue BRCA1 coded protein binds to the p53 and induces apoptosis in cancer cells. In patients with familial breast cancer the mutations in BRCA1 gene, truncated forms of protein are produced which competes for binding sites on p53 and interferes with the activation of the same. This results in inhibition of apoptosis in cancer cells by causing down-regulation of p21. It has been observed that BRCA1 binds with p53 in vivo as well as in vitro which supports the above hypothesis (Zhang et al., 1998).

In addition to BRCA1 and BRCA2, p16 and Rb tumor suppressor genes are found to be involved in development of carcinoma. Oncogenesis can be the result of an altered p16-Rb pathway in some of the cancers. The gene CDKN2 encodes for p16 and can be inactivated by deletion mutations and hypermethylation of genes (Gorgoulis et al., 1998). Transcription factor E2F

has been shown to interact with Rb, which suggests that alteration of p16-cyclin D1-Rb pathway can be linked to oncogenesis. Inability of altered Rb gene to interact with E2F can result in deregulation of E2F function and result in the development of cancer (Bernards, 1997).

Apoptosis

Apoptosis is a programmed cell death which can be triggered by different gene products. It is a highly regulated by fine balancing between the promoters and inhibitors of apoptosis. Apoptosis plays an important role as an important defense mechanism against infection and cancer development. The apoptotic process is triggered in a cell followed by infection with viruses as well as after transformation of normal cells into cancerous cells. Bcl-2 gene family has emerged as an important group of genes which control the apoptosis. The exact mechanism through which they act is a controversial issue, but it has been suggested that Bcl-2 prevents apoptosis by altering the damage caused by reactive oxygen radicals. It has also been found that the Bcl-2 genes avoid apoptosis in the cells lacking mitochondrial DNA. Cancer cells lack mitochondrial DNA and so have a deficient oxidative respiratory chain. The Bcl-2 gene is overexpressed in cancer cells, but less likely to act by avoiding the oxidative damage to cells (Jacobson et al., 1993). The bcl-2 family members interact with each other to regulate the apoptotic activity in the cells. The Bax is another member of the Bcl-2 family and is a homologous to Bcl-2 and has been known to bind with Bcl-2 and inhibit the Bcl-2 anti-apoptotic activity (Oltvai et al., 1993). The Bcl-2 family members consist of

promoters and inhibitors of apoptosis. Interaction among these promoters and inhibitors from Bcl-2 family makes apoptosis a highly regulated activity. The Bcl-2 genes also interact with the non-family members like IGF-II and cytokines to control the apoptosis (Christofori et al., 1994).

Another tumor suppressor which plays a key role in the regulation of apoptosis process is p53. It plays a role in multiple crucial events of cell life like transcription regulation, promoting apoptosis of cell, gene stabilization, inhibition of cell cycle progression and angiogenesis. Mutations in p53 lead to loss of its function to promote the apoptosis in cells. Cancer cells can bypass the apoptosis following transformation due to mutated p53 gene (Simpson and Page, 2000). It is believed that p53 has a nuclear binding site through which it can activate target elements. Another possible way through which p53 can act is by promoting oncogenesis through up-regulation of p21 which is a cyclin dependent kinase inhibitor (Bernards, 1997). The p53 is also found to repress the expression of Bcl-2 which inhibits apoptosis (Miyashita et al., 1994). The Rb gene is another tumor suppressor gene which is also found to be altered in few cases during development of malignancies. It has been found that mutations in the Rb gene cause abnormal DNA development which in turn triggers apoptosis of the cells (Morgenbesser et al., 1994). Recently the zinc finger transcription factors - snail and slug were found to be involved in the development of invasive ductal carcinoma. One possible way through which they can act is through repression of E-cadherin which is believed to be an important factor playing a role by altering the invasiveness of tumor cells. Slug is also found to play a key role in the maintenance of poorly differentiated cells and promote the growth of cancer tissue. Also

these gene expressions have been found to be regulated by different factors like estrogen, EGF/EGFR and c-kit pathways (Come, 2004).

Genomic Integrity

In a normal cell the rate at which mutations take place in the genome is significantly low. It is believed that the rate of mutation in a normal cell is as low as one mutation for every10^6 number of cell divisions (Strauss, 1992). Different protective mechanisms to counteract these mutations involve repair of the genome or induction of cell cycle arrest and apoptosis. This makes mutation a relatively rare event in a normal cell. The cells when transformed from normal to cancerous cells show an increased level of mutation events leading to an increasing number of defects in DNA due to possible failure of the DNA repair system. The cellular genome becomes unstable resulting in an uncontrolled growth of tumor cells (Jacinto and Esteller, 2007).

Passage of a cell from one phase to another phase during replication is a highly regulated procedure and many genes play crucial roles in it. Amongst various factors controlling the cell cycle progression, cyclins and cyclin dependent kinases exhibit vital roles in cell cycle progression. It is now clearly known that the CDK inhibitors play an important role in the regulation of CDK activity in response to different signals received from within the cell and from the surrounding environment of the cell. All these factors along with replication enzymes ensure the homogeneous distribution of the replicated genome between the two daughter cells. Mutations in any of these genes regulating the smooth and successful passage of cells through

different phases during the replication cycle leads to DNA damage and transformation of normal cells (van den Heuvel, 2005).

The checkpoint system plays an important role in the assessment of genomic integrity during the passage of cells from one phase to another during replication. The altered genome due to either mutations or replication errors are detected by the checkpoint system and the cell cycle progression is stopped allowing some time for a cell to repair the altered genome. Thus the checkpoint system ensures that all the necessary events are completed before the cell enters into the next phase (van den Heuvel, 2005). Many tumor suppressor genes like ATM, NBS1, BRCA1 and BRCA2 are found to play a crucial role in the maintenance of genomic integrity through the checkpoint system. At G1-S checkpoint the p53 plays an important role and causes a cell cycle arrest in case of any DNA damage (Dasika et al., 1999). ATM is found to act by up-regulating the expression of p53 which is another tumor suppressor in response to damage to the DNA (Meulmeester et al., 2005). This indicates that ATM basically acts as a caretaker gene and helps to maintain the genomic integrity.

Along with tumor suppressor genes p53 and ATM, the other two genes which have been found to play a central role in DNA repair are hMSH2 and hMLH1. Inactivation of these genes leads to a higher rate of mutations which are successfully carried further to the daughter cells. This mutated aberrant DNA leads to the uncontrolled growth of cancer cells (Murata et al., 2002). Thus the DNA repair mechanisms, cell cycle control mechanisms and checkpoint mechanisms at different phases of the cell cycle work together to avoid the mutations and maintain the integrity of genome.

Metastasis

Metastasis of tumor cells to distant locations involves mainly three steps: detachment, invasion and migration. The tumor cells are attached to surrounding cells through glycoproteins like laminin and firbonectin. Tumor cells finely regulate the activities of these attachment proteins as they need to increase or decrease their level of activity at different stages of the development of cancer since they have to attach and detach to the surrounding cells at different stages (Jones and DeClerck, 1980). Tumor cells invade the surrounding tissue by hydrolysis of the surrounding matrix by a group of proteolytic enzymes like metalloproteinases (MMPs). Tumor cells and the surrounding stromal cells secrete the MMPs (Jones and DeClerck, 1980; Liotta et al., 1977). These proteolytic enzymes together play an important role in extra-cellular matrix membrane remodeling. The proteolytic activity appears to be highly localized adjacent to the tumor cell which is invading the anatomical barriers to enter into the circulatory system (Liotta, 1984; Yee and Shiu, 1986). Recent study indicates that the activity of MMP's is finely regulated by tissue inhibitors of the matrix metalloproteinases (TIMP) (Vizoso et al., 2007).

Metastasis involves migration of the tumor cell through the anatomical barriers into the circulatory system. The cancer cells, after progressing to invasive stage initially metastasizes to axillary and internal mammary lymph nodes which are draining the tissue. The common distant sites where they metastasize are liver, lungs, skeleton and central nervous system (Aragon-Ching and Zujewski, 2007). Metastasis of ductal carcinoma of breast cells to distant sites is a sequential process as the cells have to cross multiple

anatomical barriers like basement membrane, stroma and the vessel wall to enter into the circulatory system. The tumor cells are further carried to distant sites where they form secondary tumors. Before crossing the anatomical barriers, the tumor cells have to free themselves from surrounding tumor cells. E-cadherin is a calcium dependent trans-membrane protein that plays an important role in the attachment of the cell surface. Tumor cells in invasive ductal carcinoma of the breast express an aberrant E-cadherin which allows the tumor cells to break their attachments with other tumor cells in the primary tumor mass. Aberrant expression of E-cadherin in the tumor cells possibly enables them to cross the vessel or lymphatic channel wall to enter into the circulatory system. E-cadherin is found to be re-expressed by the tumor cells when they reach the distant sites allowing them to attach with the other cells at the secondary site and colonize to form a secondary tumor. Highly regulated expression of E-cadherin plays an important role during metastasis (Kowalski et al., 2003). In addition, the chemokines promote the invasion of the tumor cells into surrounding tissue and into the circulatory system. The chemokines and chemokine receptors like CXCR4, CXCR1, CXCR6 and CXCR7 also regulate the migration of metastatic cells to the specific distant sites and their successful colonization at the secondary sites. These specific chemokine receptors show high density at the possible secondary sites of metastasis, making a favorable environment for the tumor cells to colonize. Chemokines have been found to be playing an essential role in terms of induction of angiogenesis which is a critical step in the formation of secondary colonies (Karnoub and Weinberg, 2006). It appears that chemokines play important roles at all the stages of metastasis, beginning

from the invasion of tumor cells from primary tumor mass into the circulatory system to their colonization at distant secondary sites.

Host Tropism of Poxviruses

Poxviruses are able to infect relatively wide range of eukaryote hosts except Molluscum contagiosum virus which is highly host restricted. However each family within the poxvirus group is restricted to limited hosts (McFadden, 2005). Poxviruses encode certain host range proteins which are believed to play a role in determining their tissue tropism. Host cells that are permissive to certain poxviruses support the successful replication of viruses inside the cell by providing the required factors. It has been suggested that the host range proteins work by manipulating with certain intracellular pathways of the host cells and compromising the anti-viral defense mechanisms of the host, creating a favorable environment for the viruses to replicate successfully. This suggests that in non-permissive cells, the virus fails to replicate successfully due to either lack of the host factors or its inability to manipulate anti-viral pathway mechanisms of the host cell. (Werden et al., 2008) Creating specific gene-knockout virus and testing their ability to replicate in the cells permissive to wild type virus has been a commonly used strategy for identification of the host range genes.

Vaccinia Virus Ankyrin Repeat Gene K1L

In a study to determine the genes responsible for the replication of VACV in human cells, a knock-out K1L VACV which lost its ability to

replicate in human cells was able to regain it after inserting 855bp long DNA fragment from the wild-type VACV. This DNA insert from wild-type VACV contained only K1L open reading frame (ORF) (Gillard, 1986). Though the exact mechanism of action is not completely clear, K1L gene appears to play a direct or indirect role during the production of early delayed viral gene products. (Gillard et al., 1989) Modified vaccinia virus (MVA) lacking K1L gene was able to successfully replicate, when K1L was ectopically expressed in transfected RK13 cells, which otherwise was unable to replicate in RK13 cells. This supports the role of K1L as host range gene. (Sutter et al., 1994)

K1L interacts with host factors like ACAP2, which is a GTPase activating protein (Bradley and Terajima, 2005; Meng and Xiang, 2006). K1L has been shown to inactivate NF-kB by degradation of IkBα which can be a possible mechanism of action for K1L to regulate the host tropism (Shisler and jin, 2004). However, later studies have indicated that this is a less likely mechanism of action for K1L as a double-knockout VACV lacking K1L and C7L, another host range gene in VACV, could not be rescued even after knockdown of NF-kB in Hela cells (Chang et al., 2009). Recently K1L has been shown to inhibit anti-viral response induced by type I interferon (INF) in the host cells. Replication of VACV double-knockout which lacked both K1L and C7L was blocked at the intermediate stage of viral gene translation in the cells pre-treated with type I INF. However, the virus regained its INF- resistant phenotype with restoration of K1-L or C7L (Meng et al., 2009). K1L is an ANK repeat gene in VACV and most of the Orthopoxviruses encode ortholog genes, indicating that it has been conserved fairly well amongst Orthopoxviruses (Werden et al., 2008).

Vaccinia Virus Ankyrin Repeat Gene C7L

C7L is another host range protein encoding gene in VACV which is functionally equivalent to the K1L gene. Ability of VACV strain Copenhagen to replicate in human cells after deletion of K1L gene indicated towards a possibility of presence of another functionally equivalent gene. Further investigation led to the discovery of C7L gene. Although C7L was able to retain the potential of VACV strain Copenhagen to replicate in human cells, it failed to do the same in rabbit cells RK13 (Perkus et al., 1990). This indicates that though K1L and C7L are functionally equivalent genes present in the VACV, their presence helps the virus to widen its host range. Recent study has shown that C7L inhibits the induction of apoptosis of the infected host cell. NYVAC is a knockout virus obtained from VACV strain Copenhagen which lacks both K1L and C7L. NYVAC failed to prevent an increase in the rate of phosphorylation of eIF-2α, a eukaryotic protein synthesis initiation factor which coincided with the shutdown of translation process. Insertion of C7L gene resulted in re-establishment of the ability of the virus to prevent apoptosis of host cells (Najera et al., 2006). C7L also plays a role in inhibiting anti-viral responses by host cells that are induced by type I INF (Meng et al., 2009).

Vaccinia Virus Ankyrin Repeat Gene B5R

B5R was first identified in VACV as a gene affecting host range and viral plaque size in a temperature sensitive mutant of VACV, LC16m8.

Nucleic acid sequence analysis of LC16m8 revealed that a single amino acid was deleted from its sequence in comparison with the parental VACV strain which resulted in early termination of the gene, resulting in loss of the gene function (Takahashi-Nishimaki et al., 1991). VACV strain WR showed a significant reduction in the production of extracellular enveloped virus (EEV) particles when B5R gene was deleted. It also affected the viral plaque size and virulence as compared to the parental strain (Wolffe et al., 1993). B5R, present in VACV strain WR as a membrane protein is essential for trans-Golgi/endosomal membrane wrapping of intracellular mature virus (IMV) (Wolffe et al., 1993). Short consensus repeats (SCR) which are associated with the complement control proteins superfamily are also found to be present in the protein encoded by B5R. B5R encoded protein contains four short consensus repeats (SCR) and deletion or mutagenesis of one or more of B5R protein domains resulted in alteration of viral plaque size in vitro studies (Mathew et al., 1998). B5R protein is well conserved among Orthopoxviruses and orthologs are present in other poxvirus family members. Deletion of B5R gene from RPXV resulted in the failure of plaque formation in otherwise permissive cells like Vero, Pk-15, chicken embryo fibroblasts and QT-6. (Martinez-Pomares et al., 1993) In a recent study, it has been indicated that SCR4 domain of B5R protein binds and activates Src which is important for the actin polymerization induced by VACV. (Newsome et al., 2004) VACV strain LC16m8 is highly attenuated due to the mutation of B5R gene and B5R is highly immunogenic which makes it an ideal vaccination agent against VACV. Monkeys immunized with VACV strain LC16m8 showed no symptoms when infected with MPXV strain Liberia or Zr-599 (Saijo et al.,

2006).

Vaccinia Virus Ankyrin Repeat Gene E3L

E3L is another gene which regulates host range and pathogenesis of VACV. It is known to play a role in anti-IFN mechanism used by VACV in host immune system evasion along with another host range gene, K3L. E3L encodes for a 25-kDa product which contains a double stranded RNA (dsRNA) binding domain at the C-terminus and Z-DNA binding motif at the N-terminus (Langland et al., 2006). As a response to viral infection, cellular enzymes dsRNA binding protein kinase (PKR) and 2'-5' oligoadenyl synthase inhibit protein synthesis in the host cell. PKR bound to dsRNA is autophosphorylated and leads to phosphorylation of eIF-2 on the small α subunit which results in the shutdown of protein synthesis (Williams, 1999). A 2'-5' synthase shuts down the protein synthesis by activating a latent endoribonuclease, which cleaves cellular mRNA and rRNA (Iordanov et al., 2000). E3L has also been shown to phosphorylate transcription factor IFN regulatory factor 3 (IRF-3) and IRF-7 which blocks induction of IFN-α/β. (Smith et al., 2001; Xiang et al., 2002).

As mentioned earlier, N-terminus of E3L protein binds with Z-double stranded DNA which is essential for inhibiting PKR in an effective manner (Kim et al., 2003; Kim et al., 2004; Langland and Jacobs, 2004). Deletion of 83 amino acids from the N-terminus of VACV strain WR resulted in significant reduction in virulence in the mouse model (Brandt, and Jacobs, 2001). This indicates that there is a strong correlation between the N-terminus of E3L

protein and virulence. However, for full pathogenesis of VACV strain WR in mice, both the C-terminus and N-terminus are necessary (Brandt, and Jacobs, 2001). The N-terminus of E3L does not appear to play any role in IFN resistance mechanism and host range of VACV (Shors et al., 1997). VACV demonstrated loss of ability to replicate in Vero, Hela and a murine epidermis-derived cell line XS52 after deletion of E3L gene (Beattie et al., 1995; Langland and Jacobs, 2002; Deng et al., 2006) which indicates the role of E3L as a host range gene. Host range function of E3L has also been demonstrated in other poxviruses. MVA virus showed loss of replication ability in CEF cells following E3L gene deletion due to the inhibition of viral protein synthesis (Hornemann et al., 2003).

Mutant VACV with deletion of E3L gene showed accumulation of cytoplasmic granules containing stress granule associated proteins G3BP, TIA-1, USP10 and poly(A)-containing RNA around the viral factories at an early stage of infection. These anti-viral granule (AVG) structures are characterized by the absence of ribosomal subunits and are likely incapable of translation. It is assumed that they are associated with restricted replication of virus as they are seen in more than 80% of cells infected with mutant VACV with E3L gene deleted. PKR and phosphorylated eIF2α are correlated with formation of AVGs. Lack of PKR in mouse embryonic fibroblasts (MEFs) resulted in a decrease of AVG formation, while a lack of eIF2α in MEFs resulted in an absence of AVG formation (Simpson-Holley et al., 2011). NS1 gene in influenza virus has shown a functional similarity with E3L gene in VACV. NS1 and E3L promote viral pathogenesis by interfering with IFN mediated innate immune response. Mutant VACV with E3L gene deleted and

NS1 gene inserted was able to promote viral replication in vitro by preventing shutdown of protein synthesis, breakdown of RNA and preventing apoptosis but failed to conserve the virulence in vivo. This indicates that E3L and NS1 share some functional similarities as well as differences (Guerra, 2011).

Vaccinia Virus Ankyrin Repeat Gene K3L

K3L is another host range gene which works through the same mechanism as E3L gene. It prevents the phosphorylation of eIF2α by PKR which has been demonstrated in vitro as well as in vivo studies (Davies et al., 1992; Davies et al., 1993) and inhibits the protein synthesis shutdown. Deletion of K3L gene in VACV strain Copenhagen demonstrated a wider range of host as compared to the E3L knockout VACV, but was sensitive to IFN in mouse L929 cells (Beattie et al., 1995). In a recent study it has been demonstrated that K3L may not be playing a role in modulating disease caused by VACV but possibly plays a key role in dissemination of VACV from lungs to distal organs, but the exact mechanisms involved are not completely known (Rice et al., 2011).

Cowpox Virus Ankyrin Repeat Gene CHOhr

CHOhr or CP77 is an ANK repeat protein encoded by CPXV025 gene in CPXV. It is responsible for the ability of CPXV to replicate successfully in Chinese hamster ovary (CHO) cells (Spehner et al., 1988). CHO cells are non-permissive for VACV as early gene expression is prevented at an early stage

of infection and host as well as viral protein synthesis is shutdown in an extremely rapid manner (Drillien et al., 1978; Hruby et al., 1980). Interestingly, CHOhr has shown functional equivalency with K1L and C7L genes in VACV. Replication ability of VACV strain Copenhagen with both K1L and C7L genes knocked-out, was successfully re-established in human and porcine kidney cells when complemented with CHOhr (Perkus et al., 1990). In another study, CHOhr/CP77 has been shown to bind with a host cellular protein, HMG20A. In the absence of CP77, HMG20A protein remained bound to the viral DNA of mutant VACV which lacked both K1L and C7L genes but when CP77 gene was expressed in the same virus, CP77 protein bound to the HMG20A and removed away from viral clusters in CHO-K1 cells. This suggests that CP77 binding with HMG20A plays an important role in deciding the host range of virus in CHO-K1 cells (Hsiao et al., 2006). Similarly, when CHOhr gene was inserted in Ectromelia virus (ECTV), it extended the host tropism of ECTV to CHO cells (Chen et al., 1992). CHOhr possibly acts by preventing early and sudden stoppage of protein synthesis which otherwise occurs when CHO cells are infected with VACV. CHOhr gene expression in VACV resulted in prevention of apoptosis of the host cell and provided the virus longer duration for replication (Bair et al., 1996; Ink et al., 1995). CP77 also plays a role in preventing the innate immune response in host cells by suppressing NF-kB activation by TNF-α. TNF-α is known to induce an innate immune response through activation of NF-kB. CP77 modulates the innate immune response by interactions with p65 subunit of NF-kB through its N-terminal and SCF complex through C-terminal (Chang et al., 2009).

Serin Protease Inhibitor

Poxviruses encode several serin protease inhibitors (SPIs) which are members of the serpin superfamily and are known to be involved in inflammation and apoptosis. SPI-1, SPI-2 and SPI-3 are the three highly conserved SIPs encoded by the Variolavirus (VV), VACV, rabbitpoxvirus (RPXV) and MYXV (Silverman, et al., 2001). SPI-1 affects the host range which was evident by inability of SPI-1 knockout RPXV to replicate in pig kidney cells and human lung carcinoma cells which otherwise are permissive for RPXV (Ali,A.N., 1994). SPI-1 appeared to inhibit proteinases of host cell origin during RPXV infection that they act by inhibiting caspase-independent apoptotic pathway (Brooks et al., 1995). SPI-1 in RPXV seems to inhibit human neutrophil cathespin G and along with SPI-2, inhibits granule-mediated cell killing pathways (Macen et al., 1996a; Moon et al., 1999). SPI-2 appears to inhibit proteolytic activity of interleukin 1β converting enzyme, granzyme B and block apoptosis induced through Fas receptor or type 1 TNF receptor (Dobbelstein and Shenk, 1996; Macen,et al., 1996b; Kettle et al., 1997). Role of SPI-3 in inhibiting apoptotic pathways has not been yet established. VACV with both the SPI-1 and SPI-2 deletions has shown better growth in transformed cells or p53-null cells as compared to normal cells. This selectivity of replication can be attributed to the loss of the anti-apoptotic property of VACV due to the absence of SPI-1 and SPI-2 (Guo et al., 2005).

Ankyrin Repeat Gene p28/N1R

Protein p28/N1R is a well conserved zinc-finger protein in

Orthopoxviruses and most of the Chordopoxviruses encode orthologous proteins. It is expressed in cytoplasm during early stages of viral infection (Senkevich. et al., 1994). Protein p28/N1R from many Orthopoxviruses has exhibited ubiquitin ligase activity (Huang et al., 2004; Nerenberg et al., 2005). Ubiquitin and p28/N1R co-localize in cytoplasm of the host cell and the anti-apoptotic activities by p28/N1R are possibly executed through the ubiquitination of host proteins (Nerenberg et al., 2005). In ECTV p28/N1R has demonstrated anti-apoptotic activity in host cells induced by UV light (Brick et al., 2000). ECTV virulence was affected to a great extent in murine models by non-expression of p28/N1R, however its replication ability was least affected in most of the cell lines tested (Senkevich et al., 1994). Murine macrophages proved as an exception, where p28/N1R knockout ECTV was able to express early genes but failed to replicate later, resulting in failure of successful viral infection (Senkevich et al., 1995). Though the exact mechanism of action through which p28/N1R acts is not clear, the experimental studies suggest a possibility of p28/N1R playing an essential role in viral DNA replication. Infection of poxviruses is significantly affected by proteasome inhibitors. Late gene expression and viral DNA replications seems to be mainly affected as the cytoplasmic viral factories fail to appear, but early gene expression is unaffected. Ubiquitin-activating enzyme inhibitors affect the early and late protein expression during viral infection. Though the exact molecular targets involved in the process are not known, it is quite clear that a functional proteasome system in the host cell is very much required for poxvirus replication (Zhang et al., 2009).

Myxoma Virus Ankyrin Repeat Gene M-T2

M-T2 is a host range gene encoded by MYXV which is believed to promote viral replication by inhibiting apoptosis of host cells. M-T2 knockout MYXV was able to replicate in RK13 cells but failed to establish successful infection in rabbit T-lymphocytes in vitro, as the virus failed to prevent the rapid apoptotic response by the host cell triggered by the infection. Infection of RL-5 cells by M-T2 knockout MYXV resulted in an abortive infection even after supplementing exogenous M-T2 indicating towards the possibility of intracellular form of M-T2 blocking TNF mediated apoptosis (Macen et al., 1996b). It has been demonstrated that intracellular M-T2 inhibits cellular TNFR signaling by the formation of a complex with cellular TNFR through preligand assembly domain (PLAD) which is distinct from TNF binding domain site. This phenomenon has also been demonstrated in human cells indicating that it is not restricted to a single species (Sedger et al., 2006).

M-T2 has also been correlated to the virulence of MYXV as M-T2 knockout MYXV showed a significant attenuation as compared to the parental virus when used to infect European rabbits. Most of the rabbits launched an effective immune response against M-T2 knockout MYXV and recovered completely. M-T2 also encodes for a secretory form of protein which shares the sequence similarity with the N-terminus of cellular TNFR and acts as a viroceptor (Upton et al., 1991). M-T2 inhibits the cytolytic activity of rabbit TNF by binding with it. It has been demonstrated that M-T2 has high affinity for TNF (Schreiber and McFadden, 1994; Schreiber et al., 1996).

Myxoma Virus Ankyrin Repeat Gene M-T5

MYXV encodes an ANK repeat host range protein named as M-T5. It is a 49-kDa protein which is expressed throughout the course of viral infection (Mercer et al., 2005; Johnston, 2005; Mossman et al., 1996). M-T5 has been shown to affect the ability of MYXV to replicate in different cell lines in vitro. M-T5 knockout MYXV has been able to infect RK-13 cells just like parental virus, but it showed an inability to establish a successful infection in RL-5 cells. Failure of infection in RL-5 cells by M-T5 knockout MYXV resulted in the shutdown of host and viral gene synthesis and the induction of apoptosis (Mossman et al., 1996). This Indicates that M-T5 possibly inhibits the shutdown of protein synthesis and induction of apoptosis and helps MYXV to establish infection in T-lymphocytes and spread to the distant sites (Mossman et al., 1996).

M-T5 also works as a virulence factor as demonstrated by in vivo studies. M-T5 knockout MYXV failed to establish myxomatosis when used to infect rabbits. Rabbits were able to launch an immune response against M-T5 knockout MYXV and recovered completely. M-T5 forms a complex with a cellular component which increases the ubiquitination of p27/Kip1, which is a cell cycle inhibitory factor. Infection with M-T5 knockout MYXV results in the arrest of cell cycle at G0/G1 stage as a response by host cells, but wild-type MYXV prevents the cell cycle arrest and promotes cell cycle progression beyond G0/G1 (Johnston et al., 2005). M-T5 has been shown to bind and promote phosphorylation of Akt protein which is a serine-threonine kinase, essential for many critical cellular signaling pathways. Many human cancer cell lines express low levels of phosphorylated Akt and M-T5 helps MYXV to

expand its ability to infect a wide range of human cancer cell lines by increasing phosphorylation of Akt (Wang et al., 2006).

Many other known genes playing role in determination of host range have been identified in the poxviruses, but the exact mechanisms utilized by these genes are largely unknown and need to be studied further.

Oncolytic Viruses

In spite of significant advances in surgical treatment and chemotherapy over the last few decades, cancer remains one of the leading causes of death all over the world. Advances in surgical treatment and chemotherapy have improved the survival rate in patients with advanced cancer, but controlling the disease progression and the recurrence of cancer is still a major issue. Novel options of cancer treatment are an essential need for our time and oncolytic virotherapy has shown some promising results.

Oncolytic effect of viruses was a coincidental finding in 19th century when viral infections appeared to help in regression of some hematological cancers. A woman who was a known case of cervical carcinoma showed regression of the disease when inoculated with a rabies virus. It has been noted that viruses like VACV, Reovirus and New Castle Disease virus (NCDV) preferentially infect cancer cells over the normal cells (Kelly and Russell, 2007).

In the process of the development of an efficient oncolytic virus, a key step is to uncover the functions of different viral genes. A better understanding of functionality of viral genes will be helpful to decide on which viral genes can be manipulated or used as exogenous genes to

construct an effective oncolytic virus. Some of the mechanisms through which oncolytic viruses work include cell death by either by direct lysis following viral replication or due to cytotoxicity caused by the viral proteins. Adenovirus proteins E3 and E4ORF4 are known to cause cell death by inducing toxicity (Tollefson et al., 1996). Another mechanism of action used by oncolytic viruses is induction an immune response against tumor cells, leading to the destruction of cells. Following the viral infection, the cancer cells secrete cytokines and growth factors which induce an initial trigger immune response, which in turn establish a full-fledged immune response involving cytotoxic T lymphocytes against the cancer cells (Toda et al., 1999). Oncolytic viruses can increase the sensitivity of the tumor cells to chemotherapy or radiotherapy. E1A gene in adenovirus has been shown to be responsible for the upregulation of the p53 function in the tumor cells with intact p53 gene, following chemotherapy and radiotherapy (Martin-Dugue et al., 1999).

Though many oncolytic viruses have shown promising results and can be used as a treatment option for cancer, there many obstacles that needed to be cleared. Use of oncolytic virotherapy has been limited by number of factors like host immune response against virus, lack of specificity against cancer cells and inefficient viral delivery at the target site and toxicity. Using the modern genetic engineering technology, researchers are trying to overcome the hurdles in the path of use of oncolytic viruses as an efficient treatment option for cancer by using different strategies. One of the strategies used to increase the specificity of oncolytic viruses and enable them to infect the cancer cells is deletion of the genes from the virus genome whose

expression is usually upregulated specifically in the cancer cells. Deletion of thymidine kinase (TK) gene is a strategy used on the similar line. Absence of TK gene in the viral genome makes the virus dependent on host cell TK for its growth. This strategy has been used to generate an oncolytic virus HPSV1 genome to enable herpes simplex virus type 1 (HPSV1) to selectively grow in the cancer cells (Martuza et al., 1991). Deletion of genes responsible for the virulence is another strategy used to modulate the oncolytic viruses, in an effort to minimize the damage to the normal cells. HPSV1 mutant NV1020 is an oncolytic virus which has been modulated by deletion of one copy of each of the diploid genes $\alpha 0$, $\alpha 4$, $\gamma_1 34.5$ and $U_L 56$from the viral genome has shown promising results, when used in the treatment of hepatic cancer (Kelly et al., 2008). In a similar pattern to increase the target specificity, E1B gene was deleted from the adenovirus to interfere the late viral RNA export, restricting adenovirus growth in normal cells (O'shea et al., 2004). Insertion of transgenes into the viral genome to express the exogenous gene products favoring viral growth in cancer cells and modulating the host immune response against the virus are relatively new strategies which appear promising, opening new doors to improve the efficiency of oncolytic virotherapy (Cheever, 2008). Insertion of promoter sequences of the specific genes that are upregulated in the cancer cells into the viral genome can enhance the ability of virus to selectively replicate in cancer cells. Promoter sequences of carcinoembryonic antigen in HPSV-1 genome and alphafeto protein, prostate-specific antigen and MUC1 genes in adenovirus genome have been inserted to increase their target specificity and have shown encouraging results (Reinblatt et al., 2004; Hallenbeck et al., 1999; Yu et al.,

1999; Kurihara et al., 2000). Following the insertion of these promoter sequences in the viral genome, HPSV-1 showed a better replication ability in colorectal cancer cells and adenovirus showed selectively enhanced replication in hepatic, prostate and breast cancer cells. Two major routes of administering the oncolytic viruses are intratumoural and intravenous (IV). The intratumoural route has been tried with limited success to treat easily reachable solid tumors. Virus injected intratumourally needs to replicate in the tumor tissue and then disseminate through circulatory system to the distant metastatic sites. This provides the time and opportunity to the host to develop an immune response against the virus. This limits the chances of intratumoral virus targeting metastatic sites which is a major concern. On the other hand, IV administration of viruses provides the opportunity to the virus to target primary as well as distant metastatic tumors at the same time. However, IV route also has some obstacles like host immune response (Shashkova et al., 2008), nonspecific uptake by the lung, liver and spleen, and poor viral delivery into the extravascular compartment.

Viruses that are present ubiquitously in nature or have been used for vaccination cannot be used as oncolytic viruses, as most of the population has immunity against them. Similarly use of the same oncolytic virus for the second time will be ineffective in the same patient due to the development of an immune response against the virus during the first use. This makes it necessary to develop more oncolytic viruses. Viral genes regulating the host or tissue tropism can possibly be manipulated to expand the horizon of available options to develop oncolytic viruses.

MATERIALS AND METHODS

Cells and Virus

Owl monkey kidney (OMK) cells, colorectal cancer cell lines (WiDr, LS513, SW1463) and human fetal lung fibroblasts (WI-38) were obtained from American Type Culture Collection (ATCC). Colorectal cancer (HCT-116) and malignant melanoma (SK-MEL-5) cell line were received from Dr. McFadden as a gift. OMK cells were cultured in growth medium, Eagle's minimum essential medium (EMEM) (Sigma-Aldrich) supplemented with 10% (v/v) newborn calf serum (Atlanta Biologicals, Lawrenceville, GA), 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, and antibiotics (Penicillin G sodium 100 units/ml, streptomycin sulfate 100 μg/ml) (Invitrogen). OMK cells after infection were maintained in maintenance medium, EMEM supplemented with 2% (v/v) newborn calf serum, 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, and antibiotics (Penicillin G sodium 100 units/ml, streptomycin sulfate 100 μg/ml). All the cancer cell lines (HCT 116, SK-MEL-5, WiDr, SW1463, LS513) and WI-38 were grown in growth medium, Dulbecco's modified eagle medium (DMEM) (Gibco) supplemented with 10% (v/v) fetal bovine serum, 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, and antibiotics (Penicillin G sodium 100 units/ml, streptomycin sulfate 100 μg/ml). All the cancer cell lines (HCT-116, SK-MEL-5, WiDr, SW1463, LS513) and WI-38 after infection were maintained in maintenance medium, DMEM supplemented with 2% (v/v) fetal bovine serum, 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, and antibiotics (Penicillin G sodium 100 units/ml, streptomycin sulfate 100 μg/ml). All the cell lines were incubated at 37°C in

humidified incubators with 5% CO_2. TPV-Kenya was obtained from Dr. Joseph Esposito.

Generation of pPoxCherry-ΔT146R Plasmid

Vector p-mCherry was purchased from Clontech which contains mCheery reporter gene. The mCherry cassette was cloned into pBlueScript II (KS+) plasmid followed by insertion of a single early late synthetic poxvirus promoter into the plasmid to create pPoxCherry plasmid. The DNA sequences flanking the left and right sides of T146R ORFs were amplified by PCR using TPV-Kenya genomic DNA as a template. Primers used for the upstream and downstream flanking regions of T146R ORF are listed in Table 1. Following amplification by PCR, the left flanking DNA segment was treated with restriction endonucleases *Xho*I and *Cla*I, while the right flanking DNA segment was treated with restriction endonucleases *Not*I-HF and *Sac*I-HF for 3 hours at 37°C. Vector, Plasmid pPoxCherry was treated with restriction endonucleases *Xho*I and *Cla*I in the same manner as insert T146R left flank and both the vector and insert were gel purified. T146R left flank and pPoxCherry plasmid were mixed in approximately 3:1 ratio, along with T4 DNA Ligase (Invitrogen) enzyme and the ligation reaction mixture was incubated at room temperature for one hour to clone the insert into the vector. T146R left and right flanking regions were cloned into pPoxCherry plasmid (Fig. 1) between *Xho*I-*Cla*I and *Not*I-*Sac*I restriction sites respectively to obtain pPoxCherry- ΔT146R plasmid. Confirmation of the cloning of T146R left and right flanking regions into the plasmid was done by colony PCR,

using the respective primers. Plasmid pPoxCherry-ΔT146R was treated with the restriction enzymes at 37°C for 3 hours to cut the left flank with *Xho*I and *Cla*I, and the right flank with *Not*I-HF and *Sac*I-HF. Presence of the left and right flanking regions was confirmed by agarose (1%w/v) gel electrophoresis, stained with ethidium bromide (0.5ng/ml).

Table 1

List of primers used to amplify flanking regions of T146R by PCR. Underlined sequences indicate the restriction sites for the endonucleases *Xho*I. *Cla*I, *Not*I and *Sac*I.

146R-left/for-*Xho*I	GGATATCTCGAGAGTTTATGTGTAAGT
146R-left/rev-*Cla*I	GTAAAATATCGATGTCATTTTTAGTATATTG
146R-right/for-*Not*I	CGGAATAAATAGCGGCCGCATTTAATACC
146R-right/rev-*Sac*I	CATCTTGAGCTCTTGATCCTCTTGC

Generation of Recombinant TPV-ΔT146R

OMK cell monolayers were grown in EMEM growth medium in a 35 mm Petri-dish. Approximately 80% confluent OMK cell monolayer was infected with TPV-Kenya by adsorbing the virus at room temperature for one hour at the multiplicity of infection (pfu/cell) of 1. The virus was removed following the adsorption and 2 ml of EMEM maintenance medium was added. After incubating the Petri-dishes at 37°C for one hour, cells were transfected with 1 μg of pmCherry-ΔT146R plasmid DNA using jetPRIME transfection reagent (Polypus-transfection) as per the manufacturer's

instructions. Following Incubation of the transfected cells for 4 hours at 37°C, the medium was completely removed and overlay EMEM maintenance medium with 0.5% methylcellulose was added gently along the wall of the Petri-dish to prevent any damage to the monolayer. At 96 hours post-transfection, isolated viral plaques expressing red colored m-Cherry protein were identified, picked and plaque purified by passing through four cycles of plaque purification. Deletion of T146R ORF from the recombinant virus TPV-ΔT146R obtained through the process summarized in figure 2 was confirmed using the internal primers for T146R ORF. Internal primers for T146R are listed in Table 2. TPV-Kenya genomic DNA was used as a template to design internal primers for T146R ORF.

Table 2

List of T146R internal forward (for) and reverse (rev) primers

T146R-internal/for	GATAAAAAAAATAACGTTAATGAAATTTATAGGGG
T146R-internal/rev	CGTTAATTTTTTTCCCATATATAATAGAGTTTC

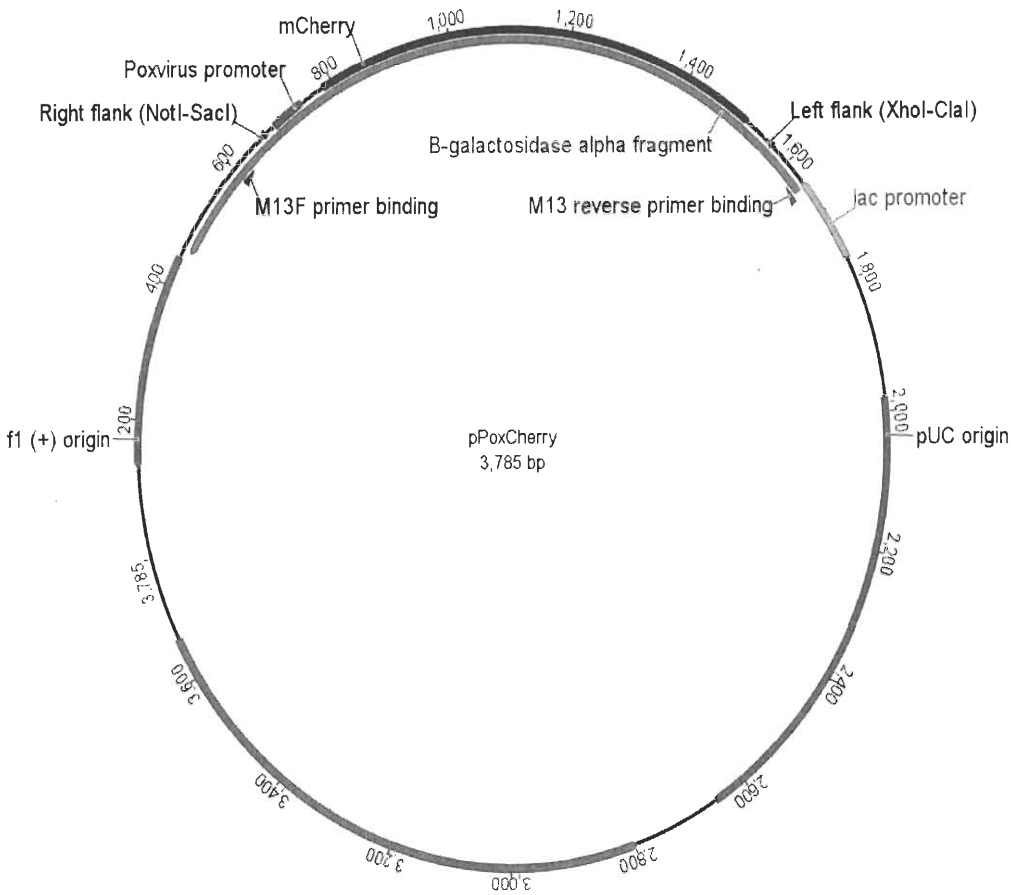

Figure 1

Map of pPoxCherry. Plasmid constitutes a synthetic poxvirus promoter and mCherry reporter gene. Sites of insertion for T146R left and right flanking sequences are indicated on either side of mCherry reporter gene.

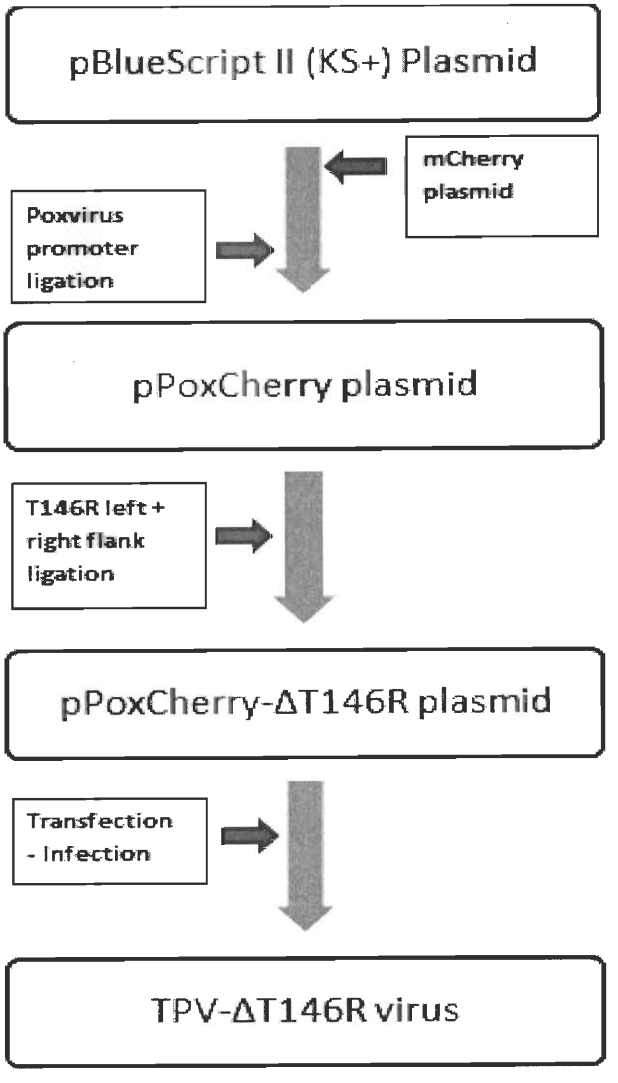

Figure 2

Overview of construction of TPV-ΔT146R virus. Synthetic poxvirus promoter and mCherry cassette were cloned into pBSII(KS+) plasmid to create pPoxCherry plasmid. Left and right flanks of T146R ORF were ligated into the pPoxCherry plasmid to obtain pPoxCherry- ΔT146R plasmid which was transfected into TPV-Kenya to create the recombinant TPV- Δ146R.

100X Virus Concentration

Following infection of OMK cells with TPV-ΔT146R virus, the monolayer showed cytopathic effect (CPE) at around 5 days post infection, and mCherry protein was expressed which could be observed under UV light as shown in Figure 3. Multiple roller bottle 490cm2 flasks were used to grow TPV-ΔT146R in larger quantities. OMK cells were grown in roller bottle 490 cm2 flasks in minimum essential medium eagle with Hank's salt (HMEM) supplemented with 10% (v/v) newborn calf serum, 1.5g/L sodium bicarbonate, 2mM L-glutamine, and antibiotics (Penicillin G sodium 100 units/ml, streptomycin sulfate 100 μg/ml). OMK monolayer was infected with the TPV-ΔT146R when they were about 80% confluent and maintained in HMEM with 2% (v/v) newborn calf serum, 1.5g/L sodium bicarbonate, 2mM L-glutamine, and antibiotics (Penicillin G sodium 100 units/ml, streptomycin sulfate 100 μg/ml). All roller bottle flasks were kept at 37°C on a roller apparatus. The OMK cell monolayer was completely destroyed in 7-10 days after infection. The infected culture was collected and centrifuged at 1500 x g for 20 minutes at 4°C to separate cells. The cell pellet was re-suspended in sterile deionized water and left at 4°C overnight. The cell pellet was freeze thawed 3X and centrifuged at 1500 x g for 20 minutes at 4°C. After collecting the supernatant, the cell pellet was re-suspended in sterile deionized water and subjected to sheer force with a homogenizer (20 strokes) and centrifuged at 1500 x g for 20 minutes at 4°C. All the supernatant collected following every centrifugation was pooled and ultracentrifuged using Beckman Ti45 rotor at 125,000 x g (40,00rpm) for 90 minutes at 4°C. The virus pellet was soaked in EMEM maintenance medium overnight at 4°C and

re-suspended later to store at 4°C or -20°C.

Plaque Assay

Viral titers were determined by plaque assay using serial dilutions of virus. OMK cell were plated in 6-well plates at a density of $1x10^6$ cells per well and the monolayer was infected with 300 µl of dilutions from 10^{-4} to 10^{-7} of the virus. The virus was adsorbed over the OMK monolayer for 1 hour at room temperature (RT) and removed from the well. Two milliliter of EMEM maintenance medium with 0.5% methyl cellulose was added to each well of the 6-well plate. After 9 days of incubation at 37°C, medium was removed from the wells carefully and monolayer was stained for 1 hour with 0.1% (w/v) crystal violet in 10% formaldehyde (plaque staining solution). After removing the staining solution, the wells were rinsed with water and air-dried and plaques were counted in each dilution to determine the plaque forming units per milliliter.

Infection of Cell Lines with TPV-ΔT146R

OMK, WI-38, HCT-116, SW1463, WiDr, LS513 and SK-MEL-5 cells were planted in a 12 well plate at a density of $1x10^5$ cells per well. The monolayers were infected with TPV-ΔT146R at 1 pfu/cell in 100 µl medium by adsorbing the virus over the monolayer for 1 hour at RT. The virus was removed and 1.0 ml of DMEM maintenance medium was added to all the wells except the well with OMK cells, which received 1.0 ml of EMEM maintenance medium. The plates were incubated at 37°C for 96 hours in a

humidified incubator with 5% CO2. Microscopic examination of infected cells was carried out for CPE.

RESULTS

Conservation of Ankyrin Repeat Protein T146R in Poxviruses

ANK repeat protein T146R was compared with other poxvirus ANK repeat proteins. BLAST result of the ANK repeat protein T146R indicated that the YLDV (146R), YMTV(146R), DPXV W848-83 (DpV83gp164), Swinepox virus (SPV142), MYXV (M149R), Lumpy skin disease virus (LSDV) NI-2490 (LSDV142) encoded ANK repeat proteins share 98%, 75%, 42%, 38%, 34% and 38% similarity respectively. Multiple sequence alignment data of all the above mentioned proteins showed conservation of many amino acids as shown in Figure 3.

Construction of Mutant Virus TPV- ΔT146R

T146R left and right flanks were ligated in the pPoxCherry plasmid which carried mCherry reporter cassette. T146R left and right flanks and the mCherry cassette were successfully cut from the plasmid pPoxCherry-T146R using appropriate restriction enzymes and analyzed by DNA gel electrophoresis, which confirmed the ligation of the T146R left (868bp) and right (564bp) flanks in the plasmid as shown in Figure 4. T146R left flank was cut with restriction enzymes *Xho*I and *Cla*I , while T146R right flank was cut with restriction enzymes *Not*I-HF and *Sac*I-HF.

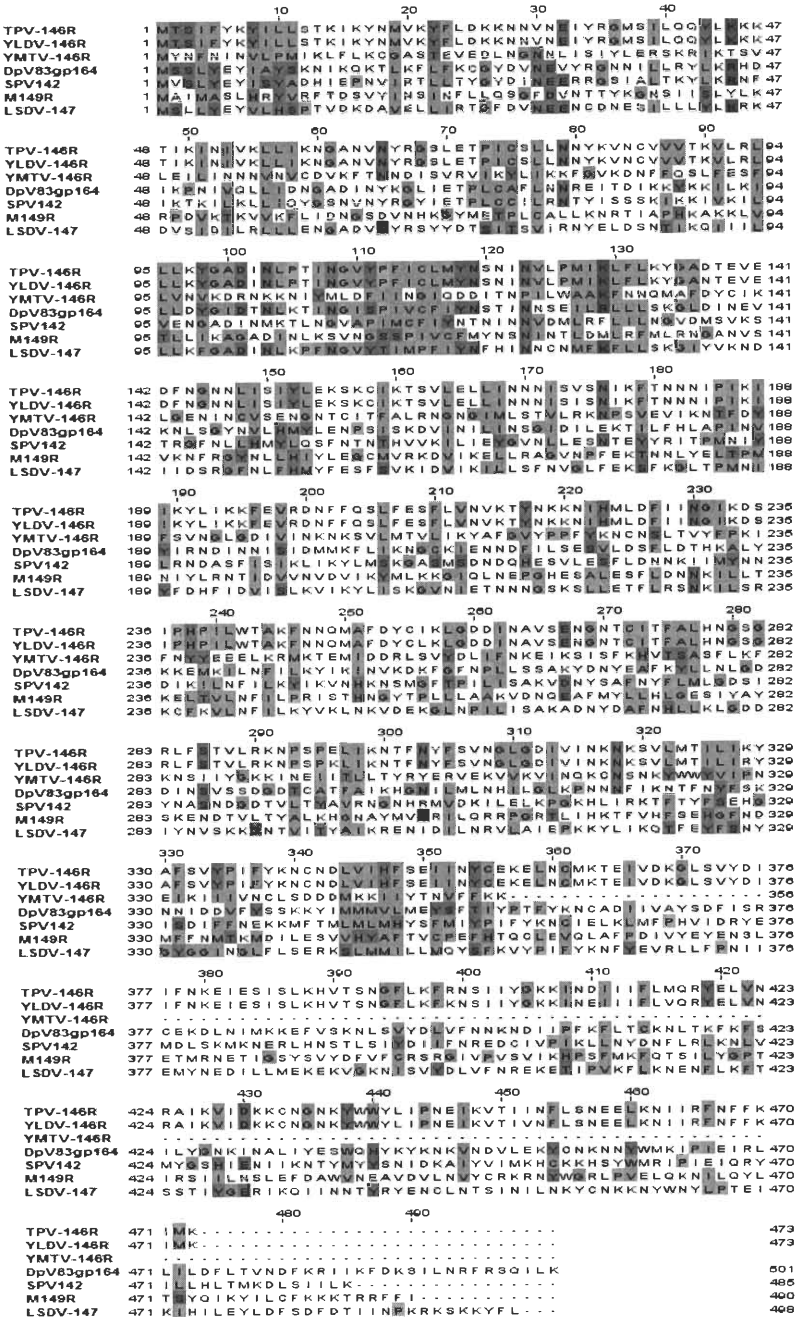

Figure 3

Multiple sequence alignment of TPV (146R), YLDV (146R), YMTV (146R), DPXV W848-83, SPV (SPV142), MYXV (M149R), LSDV NI-2490 (LSDV142). Conserved amino acids are color coded based on their nature. Green - Polar/Hydrophilic: N T S Q, Blue - Hydrophobic: L V I M F W A C, Violet - Acidic: D E, Red - Basic: K R, Magenta - Large aromatic polar: H Y

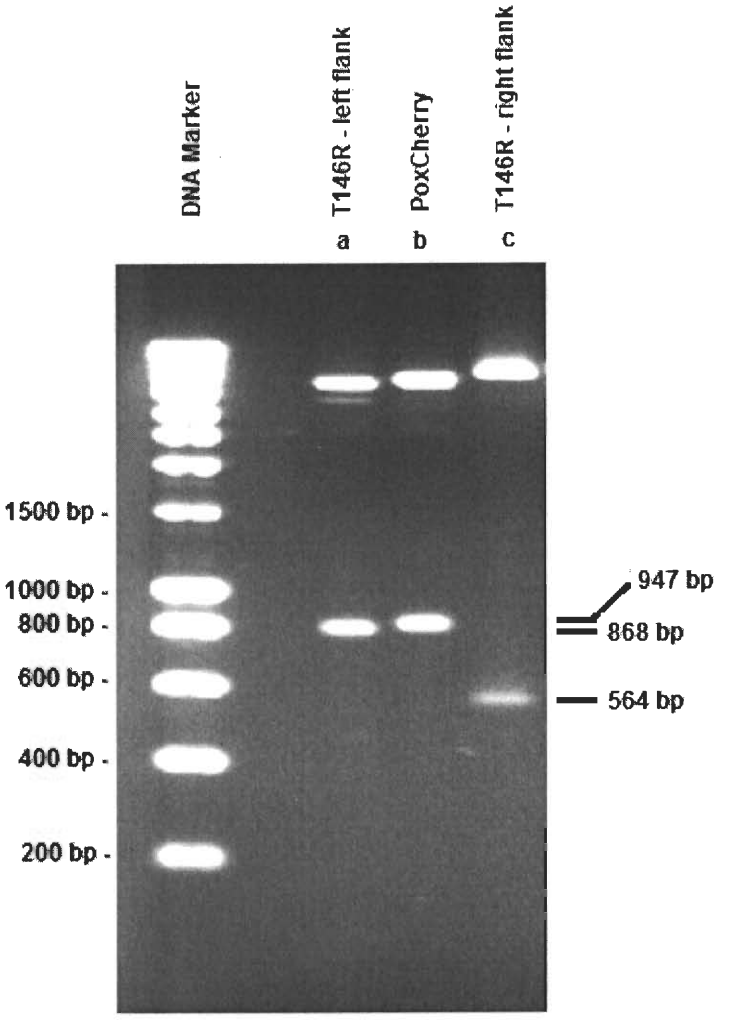

Figure 4

Agarose gel DNA electrophoresis of T146R left and right flanks cut from the pPoxCherry-ΔT146R plasmid. Plasmid pPoxCherry-ΔT146R was treated with restriction enzyme pairs *Xho*I-*Cla*I, *Not*I HF-SacI HF and *Cla*I-*Not*I HF to cut the T146R left flank (lane a), mCherry cassette+synthetic poxvirus promoter (lane b) and T146R right flank (lane c) respectively. Plasmid pPoxCherry-ΔT146R was treated with respective restriction enzymes for 3 hours at 37°C and run on agarose (1%w/v) gel stained with ethidium bromide (0.5ng/ml).

The T146R gene from TPV-Kenya genome was replaced with T146R left and right flanks along with mCherry cassette by homologous recombination. Mutant TPV-ΔT146R was constructed by infection-transfection with TPV-Kenya and pPoxCherry-ΔT146R plasmid on OMK cell monolayer. Expression of mCherry reporter gene facilitated the isolation of mutant TPV-ΔT146R plaques which were clearly visible under ultraviolet (UV) light as red colored plaques as shown in Figure 5. TPV-ΔT146R was purified through three cycles of plaque purification.

To confirm the knockout of T146R gene from TPV- ΔT146R genome, genomic DNA of TPV-ΔT146R was extracted by phenol-chloroform extraction technique. Internal primers for the T146R (Table 2) were used to amplify T146R ORF, using TPV- ΔT146R DNA as a template. No amplification of T146R ORF was noted as shown in Figure 6, which confirmed the knockout of T146R gene from the mutant TPV- ΔT146R genome.

During infection-transfection process, the segment of pPoxCherry-ΔT146R plasmid which only includes T146R left and right flanking sequences along with the poxvirus promoter and mCherry cassette is supposedly incorporated into the TPV-Kenya genome during a double crossover homologous recombination event as shown in Figure 7(a). In the event of the possible single crossover recombination, the entire pPoxCherry-ΔT146R plasmid including ampicillin resistance (ampR) gene may get incorporated into the viral genome as shown in Figure 7(b). To rule out the possibility of single crossover recombination event, PCR amplification of ARG was attempted using the recombinant TPV-ΔT146R genomic DNA as a template. No amplification of the ARG fragment from TPV- ΔT146R genome was

noticed as shown in Figure 8. This confirmed the absence of ARG in the TPV-ΔT146R genome which in turn assured that the T146R gene was knocked out by a double crossover homologous recombination event as shown in Figure 7(a).

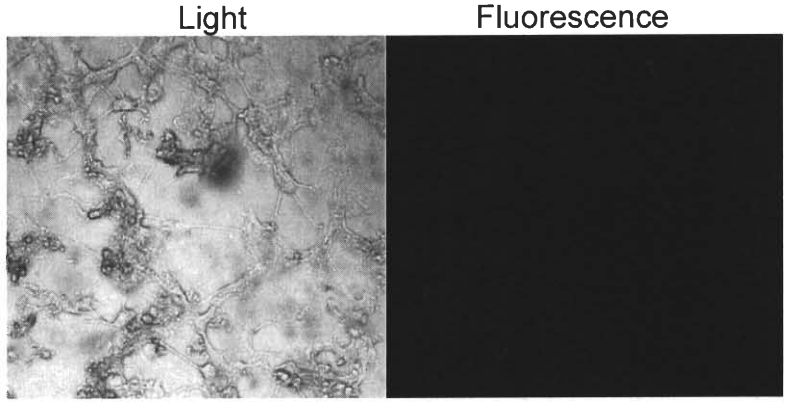

Figure 5

UV fluorescence microscopy of TPV- ΔT146R infected OMK cells at day 4 post infection- transfection.

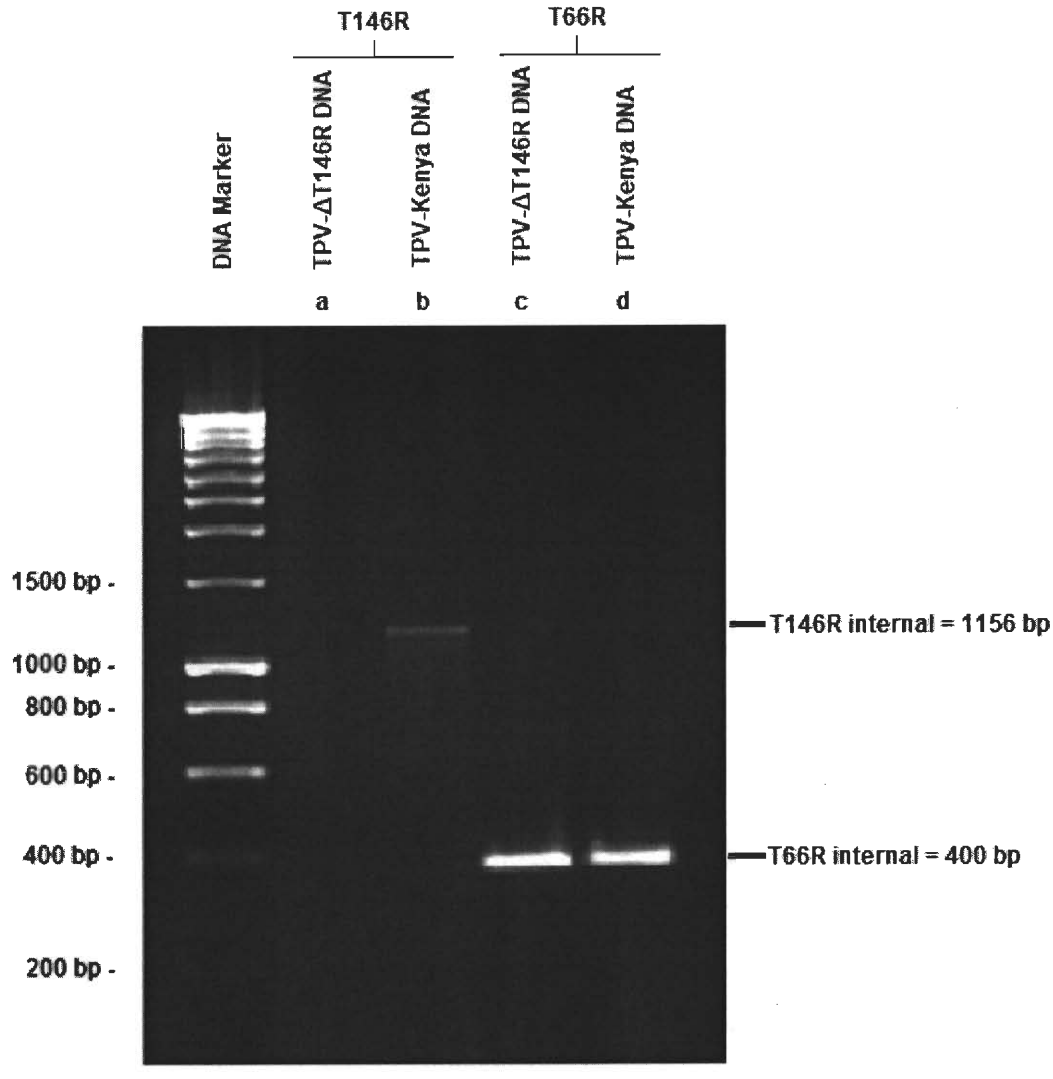

Figure 6

PCR analysis of T146R ORF amplification from TPV- ΔT146R genomic DNA. T146R internal primers were used to PCR amplify T146R ORF fragment from TPV- ΔT146R genomic DNA and TPV- Kenya genomic DNA. T146R ORF amplification was not seen from TPV- ΔT146R genomic DNA (lane a), but T146R ORF was successfully amplified from TPV-Kenya genomic DNA (lane b). T66R ORF fragment was successfully amplified from both TPV-ΔT146R (lane c) and TPV- Kenya (lane d) genomic DNAs.

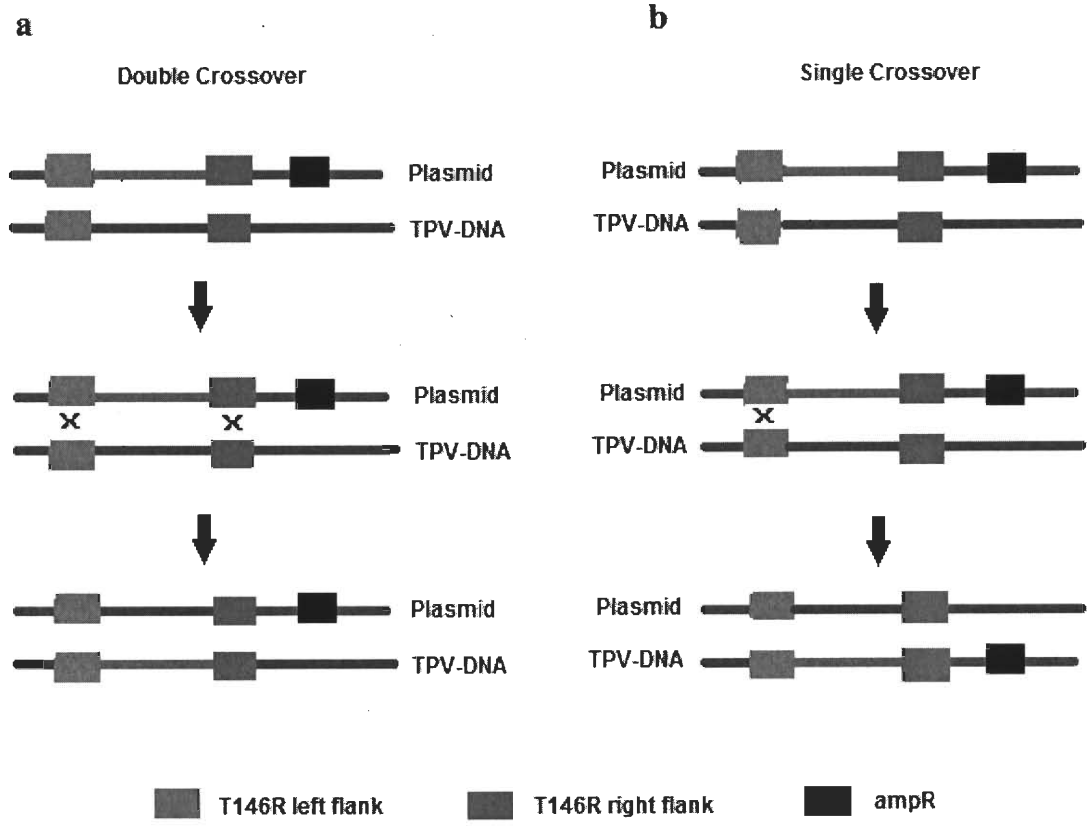

Figure 7

T146R gene knockout from TPV-Kenya genomic DNA. (a) Schematic map of TPV-Kenya genome and the plasmid pPoxCherry-ΔT146R designed for deletion of T146R gene. TPV-Kenya DNA sequences adjacent to T146R (T146R left and right flanks) were cloned along with poxvirus promoter and mCherry cassette into the TPV-Kenya DNA by double crossover homologous recombination. (b) Schematic map of TPV-Kenya genome and the plasmid pPoxCherry-ΔT146R showing the possible single crossover homologous recombination event in which the entire plasmid pPoxCherry-ΔT146R including ampR gene can get cloned into TPV-Kenya DNA while knocking out T146R gene.

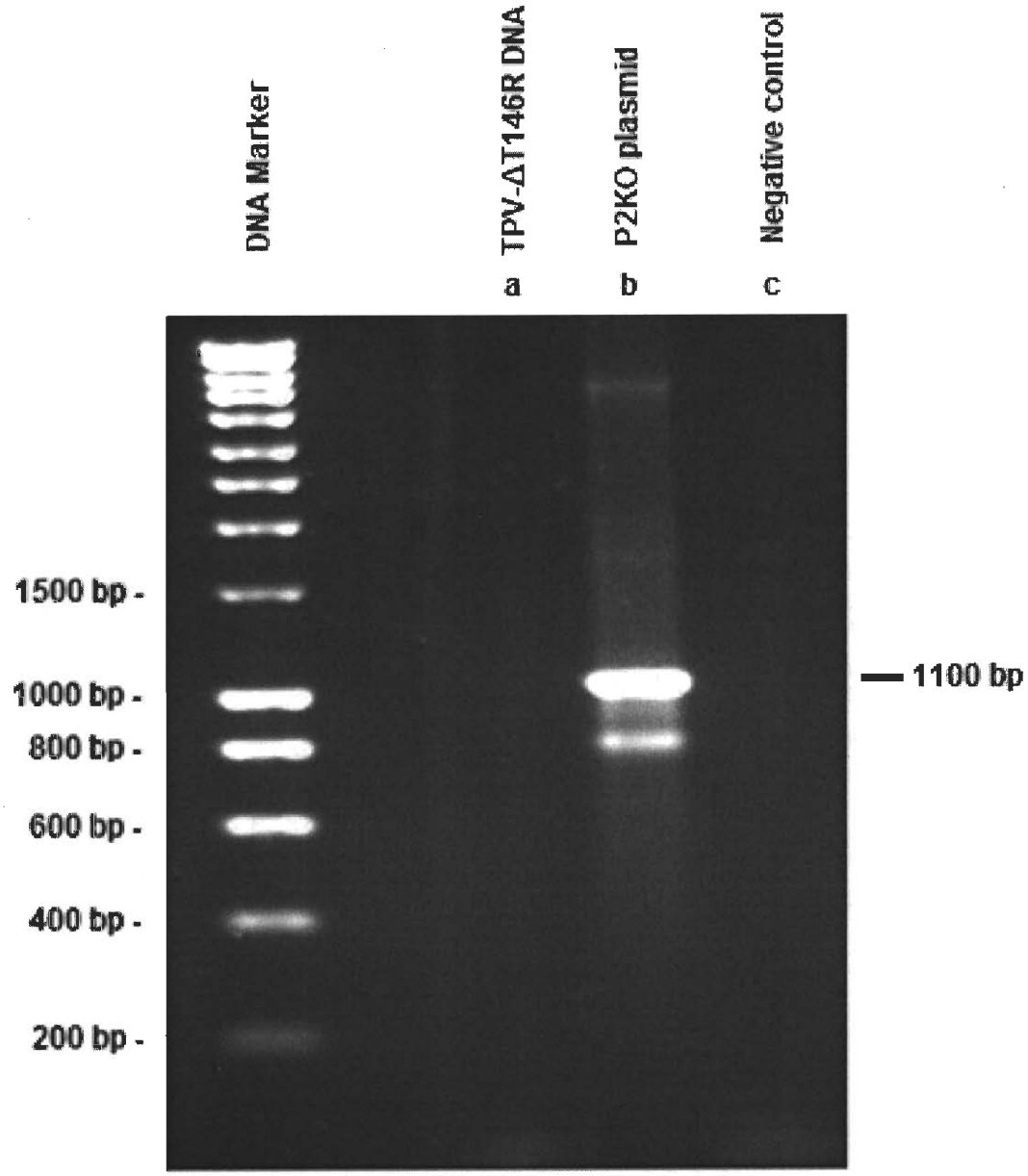

Figure 8

PCR analysis of absence of ampicillin resistance (ampR) gene from TPV-ΔT146R genome. Internal primers for ampR gene were used to PCR amplify the ampR gene fragment from TPV-ΔT146R DNA (lane a) and pBSII (+KS) plasmid (lane b). Negative control sample PCR was run without any vector (lane c).

Replication of TPV- ΔT146R in Human Cancer Cell Lines

TPV- ΔT146R replication in different human colorectal cancer cell lines (WiDr, LS513, SW1463, and HCT 116) and human malignant melanoma (SK-MEL-5) was studied. Human lung fibroblasts (WI-38) and OMK cells were also used in the study as control. All cells used in the study were planted in 12-well plates at an approximate density of 1 x 10^5 cells per well and the cell monolayers were infected with TPV- ΔT146R and TPV-Kenya at 1 pfu/cell. Cell monolayers were observed for the expression of mCherry under fluorescent microscopy and CPE, at 96 h.p.i. Although mCherry expressing cells were observed in all the TPV-ΔT146R infected cell lines (Figure 9A, Figure 9B and Figure 9C), marked CPE was only noted in OMK, WI-38 and HCT116 cells, but not in LS513, SW1463 and WiDr cells (Table 3). These results suggest that virus replication did occur in all cell lines.

OMK cells were permissive to both TPV-ΔT146R and TPV-Kenya as shown from the marked CPE. Similarly, CPE was also observed in WI-38 cells infected with TPV-ΔT146R and TPV-Kenya.

Amongst colorectal cancer cell lines, only HCT 116 showed pronounced CPE, at 96 h.p.i. following the infection with TPV-ΔT146R and TPV-Kenya. No marked CPE was observed after infection of LS513, SW1463 and WiDr cells with TPV-ΔT146R and TPV-Kenya as compared to HCT 116. Human malignant melanoma (SK-MEL-5) cells also supported TPV-ΔT146R replication. SK-MEL-5 demonstrated CPE at 96 h.p.i. following infection with TPV-ΔT146R.

Since all cell lines used in the experiment demonstrated red colored virus plaques at 96 h.p.i, following infection with TPV- ΔT146R, it indicates

that the ankyrin repeat gene T146R in the tanapoxvirus genome is not an essential gene for the replication of virus in cell cultures.

Table 3

Microscopic observation of TPV- ΔT146R infected cells at 96 h.p.i. OMK cells, human fetal lung fibroblasts (WI-38), human colorectal cancer cell lines (LS513, HCT 116, SW1463, WiDr) and human malignant melanoma cells (SK-MEL-5) were infected with TPV- ΔT146R and the cell monolayers were observed at 96 h.p.i. under Nikon Diaphot 300 epifluorescence microscope at 100X magnification.

	Cytopathic effect
OMK	++
WI-38	++
HCT 116	++
LS 513	+
SW 1463	+
WiDr	+
SK-MEL5	+

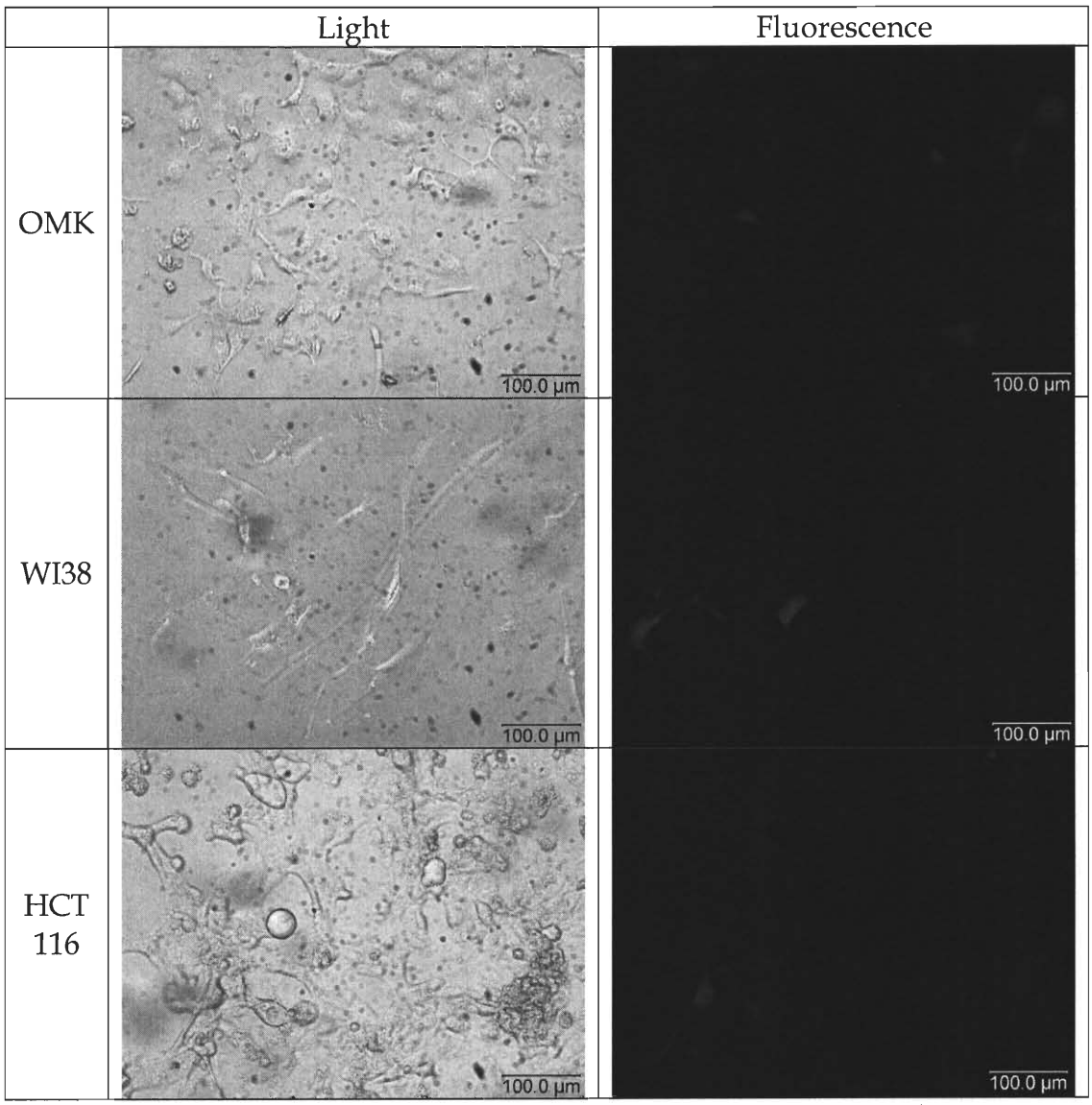

Figure 9A

Replication of TPV-ΔT146R in OMK cells, human fetal lung fibroblasts (WI-38) and human colorectal cancer cell line (HCT 116). Cells were infected with TPV- ΔT146R at 1 pfu/cell, fluorescence and phase contrast images of the infected monolayers were captured at 96 h.p.i. Images were taken using a 35 mm camera on a Nikon Diaphot 300 epifluorescence microscope at 100X magnification.

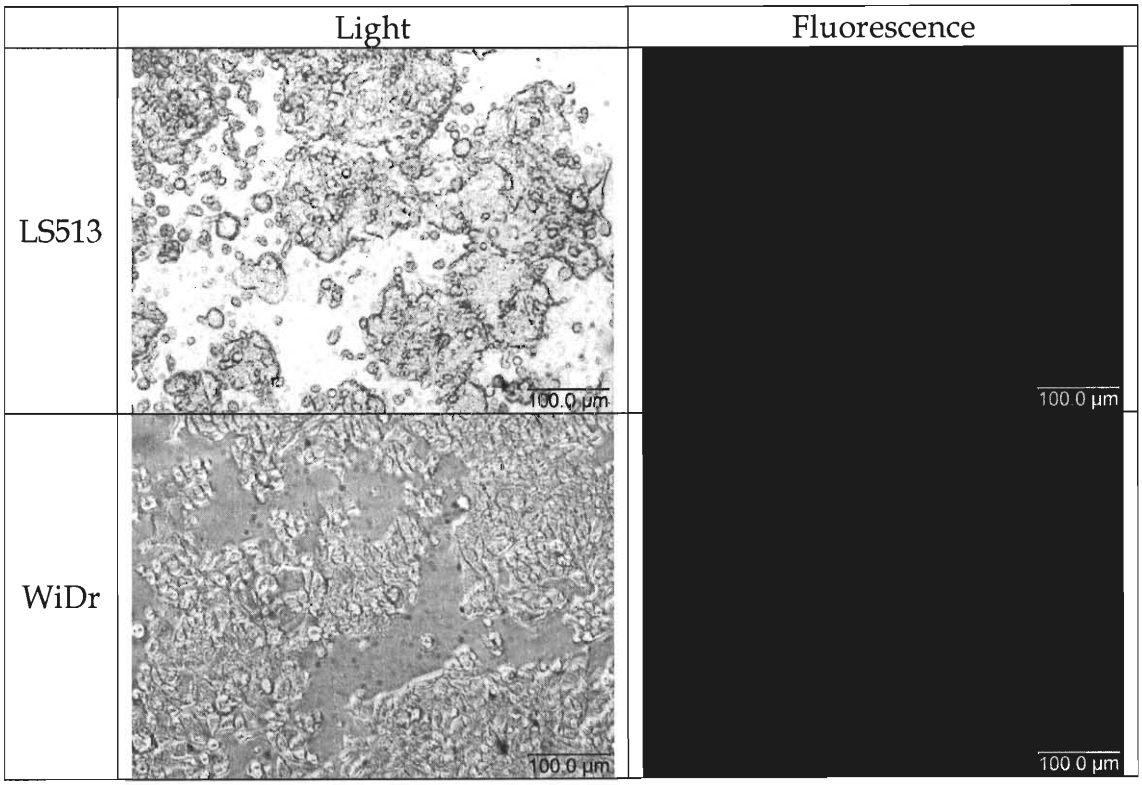

Figure 9B

Replication of TPV- ΔT146R in human cancer cell lines (LS513, WiDr). Cells were infected with TPV- ΔT146R at 1 pfu/cell, fluorescence and phase contrast images of the infected monolayers were captured at 96 h.p.i. Images were taken using a 35 mm camera on a Nikon Diaphot 300 epifluorescence microscope at 100X magnification.

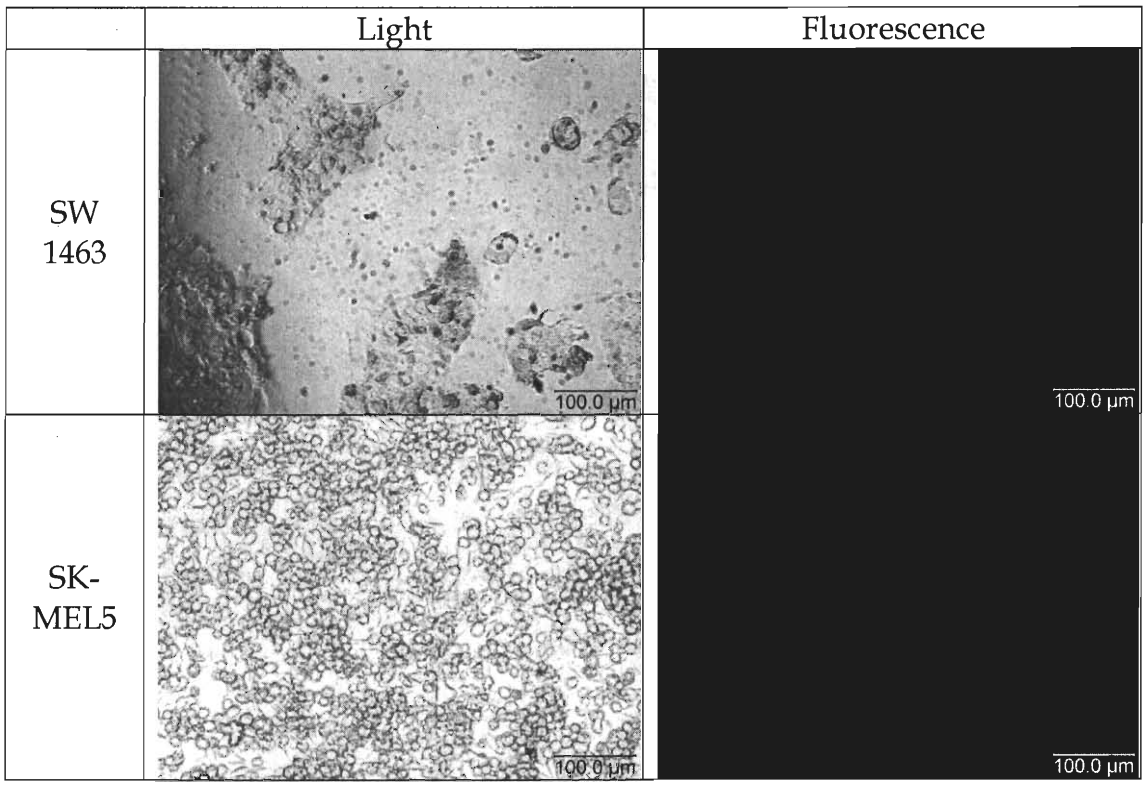

Figure 9C

Replication of TPV- ΔT146R in human cancer cell lines (SW1463, SK-MEL-5). Cells were infected with TPV- ΔT146R at 1 pfu/cell, fluorescence and phase contrast images of the infected monolayers were captured at 96 h.p.i. Images were taken using a 35 mm camera on a Nikon Diaphot 300 epifluorescence microscope at 100X magnification.

DISCUSSION

Cancer remains a major health concern, being one of the leading causes of death all over the world. Certain oncogenes like HER-2/neu/erbB2 (Tsapralis et al., 2012), c-Myc (Wolfer, 2011), Cyclin D1 (Kim, and Diehl, 2009), EGFR (Brand et al., 2011) are known to be altered leading to the development of different types of cancer. Mutational inactivation of the tumor suppressor genes p53 (Greenblatt et al., 1994), BRCA1 (Somasundaram et al., 1997), p16 and Rb (Gorgoulis et al., 1998) leads to a loss of control over the cell growth and has been linked with the different types of cancer. These genes exhibit the oncogenic activity through complex processes, some of which has been understood up to certain extent. However the exact mechanisms involved in the oncogenesis are largely unknown.

Novel effective treatment options for cancer need to be developed due to the high mortality associated with cancer and limitations of conventional treatment options like surgery, radiotherapy and chemotherapy. Oncolytic virotherapy is a relatively new treatment option for cancer and is still in its primitive stages. Rapid clearance from the system, target specificity, efficiency and host immune response are some of the limitations which restrict the use of oncolytic virotherapy in an effective manner. Number of viruses like HPSV1, VACV, adenovirus, NCDV, reovirus and many more are being used to develop oncolytic viruses (Kelly and Russell, 2007). TPV can be developed into an efficient oncolytic virus as some of the advantages associated with TPV are that humans are one of its known natural hosts (Espana et al., 1971). In addition, TPV causes only mild febrile disease in humans which is self-limiting (Downie et al., 1971), making it comparatively

safer to use in humans. Our lab has been involved in performing growth studies of TPV and its temperature sensitive mutant variants in different cell lines including human cancer cell lines. Previous studies in our lab have established the growth characteristics of TPV in various primate cell lines, like African green monkey kidney cell lines, OMK cells, human lung fibroblasts, human laryngeal epidermoid cancer cells and human cervical cancer cells (Mediratta and Essani,, 1999). TPV demonstrated the best replication ability in OMK cells.

In order to develop an oncolytic virus it is necessary to study its growth characteristics. Role of the ANK repeat genes in determination of the cell tropism and host range of viruses is well documented. In some cases, ANK repeat genes play role in determining the permissiveness of viruses into certain cell types through manipulation of the host immune response to avoid cell death and provide the necessary time for viral replication (Mohamed at al., 2009). Some ANK proteins can manipulate the host cell's ubiquitin system by directing it towards specific proteins and thus providing the virus the opportunity to grow (Sonnberg et al., 2008). MYXV ANK repeat gene M-T5 has been demonstrated to interact with cellular pathways to regulate the cell cycle (Johnston et al., 2005). Other ANK repeat proteins act by inhibiting cellular apoptotic pathways to provide the opportunity to the virus to grow (Brooks et al., 1995).

In order to develop TPV as an oncolytic virus, it is essential to study the role played by the ANK repeat genes in determination of the cell tropism. TPV genome has been completely sequenced and carries six ANK repeat genes, namely T8L, T11.1L, T11.2L, T146R, T147R and T148R. This study was

aimed at understanding the role of T146R gene in virus replication in various human cancer cell lines. The recombinant TPV-ΔT146R which lacks the T146R ANK repeat gene replicated in monkey cells (OMK) and human fetal lung fibroblasts (WI-38) as CPE was visible following infection with TPV-ΔT146R. Additionally, human colorectal cancer cell lines as well as human malignant melanoma cells also demonstrated red colored plaques after infection with TPV-ΔT146R. Marked CPE was observed in HCT 116 cells, but LS513, SW1463 and WiDr cells demonstrated no pronounced CPE. It indicates that absence of the ankyrin repeat gene T146R from TPV genome has a potential to affect the replication ability of the virus in LS513, SW1463 and WiDr. Though it appears that T146R is not an essential gene for the replication of virus and didn't seem to affect the permissiveness in the cell lines used in the experiment, a possibility of presence of other functionally equivalent gene in TPV genome needs to be investigated. The ANK repeat genes C7L and K1L in VACV strain Copenhagen demonstrated functional equivalency in terms of anti-interferon activity of the virus (Meng et al., 2009).

However, to investigate a possible role played by T146R gene in TPV, in determination of cell tropism, the replication cycle of TPV-ΔT146R in different cells including a panel of human colorectal cancer cells need to be studied. The human colorectal cancer cell lines to be used in the experiment include the cells derived from different sites of the colorectal tract. Cell line LS513 is derived from a Duke's type C, mucin secreting cecal tumor (Suardet et al., 1992). Cell line SW1463 is obtained from Duke's type C, rectal adenocarcinoma (Leibovitz et al., 1976). Cell lines HCT116 and WiDr are derived from colon (Brattain et al., 1981; Noguchi et al., 1979). In addition,

future studies include the investigation of the role of other ANK repeat genes T8L, T11.1L, T11.2L, T147R and T148R in TPV in determining cell tropism. Difference in the ability of any virus to replicate in different cell lines can be attributed to a number of factors within the cell. These factors vary from cell surface receptors to number of different biochemical pathways. Additional studies are needed to understand the quantitative variability of virus replication in different cell lines.

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