Development of a recombinant measles virus armed with BNiP3, a pro-apoptotic gene for oncolysis

Thesis submitted to Jawaharlal Nehru University in partial fulfilment for the award of the degree of

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By

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CERTIFICATE

This is to certify that the work entitled **"Development of a recombinant measles virus armed with BNiP3, a pro-apoptotic gene for oncolysis"** submitted to the School of Biotechnology, Jawaharlal Nehru University, New Delhi in partial fulfilment of the requirement for the award of the degree of Doctor of Philosophy, embodies faithful record of original research work carried out by **Geetanjali**. This work is original and has not been submitted so far in part or full for any other degree or diploma of any other university.

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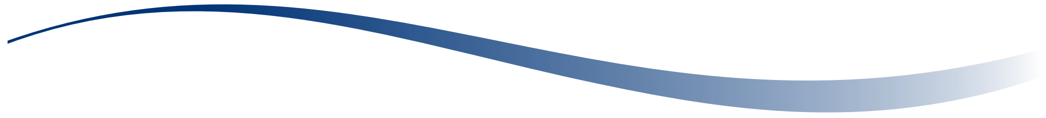
Dedicated
to
my Parents



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Geetanjali

LIST OF ABBREVIATIONS

Ad	Adenovirus
BLAST	Basic local alignment search tool
BNiP3	Bcl2/adenovirus E1b interacting protein 3
BRAF	Serine/threonine protein kinase B-raf
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CPE	Cytopathic effect
DAMPs	Damage associated molecular patterns
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco’s modified eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ER	Estrogen receptor
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FDA	Food and drug association
FITC	Fluorescein isothiocyanate
G418	Geneticin
H2	N’-(2-chlorobenzylidene)-4-(2-(dimethylamino)ethoxy)Benzohydrazide
Her2/neu	Human epidermal growth factor receptor 2
HSV	Herpes simplex virus
IC50	Inhibitory concentration
IFA	Immunofluorescence assay
kDa	Kilo Dalton
LB broth	Luria bertani broth
mA	Milli ampere
MAPK/MEK	Mitogen Activated Protein Kinase kinase
MEM	Modified eagle’s medium
$MgCl_2$	Magnesium chloride
MHC I	Major histocompatibility complex I

MOI	Multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MV	Measles virus
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NCBI	National Centre for biotechnology information
OV	Oncolytic virus
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pdn6	Random hexamer primer
PI	Propidium iodide
PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone receptor
RdRp	RNA dependent RNA polymerase
rMV	Recombinant measles virus
rMV-BNiP3	Oncolytic measles virus harboring bnip3 gene
rMV-Mcherry	Reporter measles virus harboring M-cherry gene
RNA	Ribonucleic acid
RT	Room temperature
SABR	Stereotactic ablative radiotherapy
SDM	Site directed mutagenesis
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SLAM	Signaling lymphocytic activation molecule
Taxol/Tax	Paclitaxel
TBE	Tris-borate EDTA
TCID50	Tissue culture infective dose
TE	Tris-EDTA buffer
vRNP	Viral ribonucleoprotein complex
VSV	Vesicular stomatitis virus
WHO	World health organization
XRT	X-ray therapy

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ABSTRACT

Conventional anti-cancer regimen of chemotherapy, radiotherapy and targeted therapies has limitations such as severe systemic side effects, narrow therapeutic index, non-specificity and non-availability of drugs for all types of cancer. The trials on developing a targeted therapy using virus have gained much interest in the recent past. Construction of a recombinant virus that selectively infects and kills cancer cells without exerting the same lytic effect on normal cells provides a promising platform for the development of a multimodal anticancer therapeutic. Oncolytic effect of a recombinant virus can be a part of natural profile of that virus or it can be achieved by reprogramming its genetic makeup. The lytic activity of a recombinant virus can further be enhanced by incorporating foreign genes of viral or non-viral origin with known functions hypothetically towards tumor suppression. Here, we aimed to construct the recombinant measles virus with BNiP3 gene, a pro-apoptotic gene of human origin inserted into the viral genome to enhance its anti-tumor activity. To ascertain the genomic stability and infectivity of virus upon insertional modifications in its genome, initially reporter virus encoding for M-cherry fluorophore was generated and then the virus with BNiP3. Gene encoding for M-cherry or BNiP3 was inserted in viral genome between the unique restriction sites *Pfl*2311 and *Aat*II upstream of M gene. Recombinant virus was generated by co-transfection of packaging cell line (HEK293 stably co-expressing viral nucleoprotein, phosphoprotein and T7 RNA polymerase) with p (+) MV-NSE-FlagP-M502-3p construct of full length viral genome with foreign gene element and L polymerase gene construct. Replication and infection profile of reporter virus (rMV-Mcherry) and virus with BNiP3 (rMV-BNiP3) was observed in breast carcinoma cell lines MCF-7 and MDA-MB-231. BNiP3 armed virus showed infective selectivity towards MDA-MB-231 cells as compared to MCF-7 cells. MDA-MB-231 cells were quantified for cell death and apoptosis where increased response was observed in cells infected with rMV-BNiP3. Synergistic potential of recombinant virus with established and novel chemotherapeutic agents was also explored and enhanced anti-cancer toxicity was observed. Our findings demonstrated the efficient rescue, internalization and replication of rMV-BNiP3. The infective bias of recombinant virus towards MDA-MB-231 cells and its oncolytic profile should be explored *in vivo* to establish the underlying mechanism and proclivity of anti-tumor response.

INTRODUCTION

According to the listings of WHO, cancer is one of the leading causes of death worldwide. This reflects in the current scenario of research amongst scientific community where cancer research, either to discern its causes and molecular approach for progression or its treatment, form a major part of the portfolio. Even with massive financial and manpower inputs towards development of an efficacious cancer therapy regimen, a comprehensive model with superior prognostic outcome has evaded us. So, there is a need to devise a therapeutic moiety which can be a template with provisions to make it effective towards any cancer type. Recently the focus of cancer research with respect to treatment has shifted towards the development of specifically targeted anti-cancer modalities. Examples can be found in case of immunotherapy, nanoparticle based therapy, gene therapy, virotherapy and many more.

In comparison, gene therapy projects a more sustainable therapeutic approach where the genetic defects associated with cancer can be substituted or anti-tumor genes can be introduced (Kay et al. 2001). This transfer is facilitated by various viral, bacterial and chemical vectors amongst which viral vectors have garnered interest for their targeted approach. Viral vector-mediated gene therapy had immense success in the treatment of various monogenetic diseases but is unable to replicate it in the majority of cancers where genetic variations amongst the individuals or within different tumor sites in a patient are substantial (Das et al. 2015). All these approaches are quite effective on their own but their reliance on conjecture for the progression of disease and the dynamic nature of cancer cells has caused lacunae to develop a therapy which addresses the obstacles of targeted delivery such as efficient internalization at the effector site and efficient expression of anti-tumor genes.

In contrast to non-replicating viruses used for gene therapy purpose, use of replication competent viruses has evolved into an interdisciplinary anti-cancer therapeutic approach. Viruses that are either selected or modified to specifically target and kill cancer cells without harming non-cancerous normal tissue are called oncolytic agents. Viruses such as newcastle disease virus, vaccinia virus, moloney leukemia virus etc. have natural tumor tropism and post-infection can lead to lysis of tumor cells. This natural tumor tropism is, to some extent, present in most of the viruses not due to biology of virus but due to immunocompromised, rapidly dividing nature of cancer cells presenting them as ideal hosts for viral propagation. To utilize this property of tumor bias, the infective potential of a candidate virus towards normal cells has to be removed. Undoubtedly, utilization of virus to treat tumor cell is a gruff tool,

but with careful construction of a recombinant virus to make it tumor selective, the risk of infecting normal cells can be avoided. Thus, the foundation of oncolytic virotherapy is either use of a naturally tumor tropic virus or manipulation of virus to be cancer specific for effective tumor destruction. The question arises about which virus to use, what targeting should be involved and if the naturally lytic ability of viruses can be enhanced for heightened anti-tumor response.

Currently there are many platforms of recombinant viruses which are being developed as oncolytic agents such as adenovirus, herpes simplex virus, poxvirus, measles virus amongst others. There are unique features of each platform which can be exploited for the generation of an oncolytic virus. Virus that is not naturally inclined for oncolysis can be developed into an oncolytic agent by manipulating its genome to enhance its specificity and toxic profile against cancer cells. Changes made are directed towards increasing the safety and specificity of a virus with respect to its efficient targeting to cancer cells either via selective internalization or selective replication. Selective internalization pertains to changes in the viral proteins engaged with host cell surface receptors for infection, utilizing which oncolytic viruses can be retargeted towards specific receptors or their mutants overexpressed in tumor cells. Tumor specificity can be introduced at many steps of viral infection including transcription and translation of viral genome. Targeting can also be done based on tumor microenvironment and the expression profile of cancer cells towards cell death. Viruses are also engineered to encode additional transcriptional units to modulate virus biology against cancer cells.

In addition to genetic modification for tumor-specific targeting, more robust oncolytic activity of recombinant virus can be achieved by inserting foreign genes into its genome. Replication of many viruses exploits host cellular survival pathways which are generally deregulated in cancer cells. Genetically modified viruses can be constructed by arming the virus with foreign genes of viral or non-viral origin to target the deregulated molecular events such as apoptosis, angiogenesis etc, in a cancer cell. Insertion of pro-drug activator genes and immunomodulators also contributes to anti-tumor activity of the recombinant virus. This ‘arming’ of oncolytic virus potentiates the therapeutic index of anti-cancer virus mediated gene therapy by efficient delivery and expression of transgene.

Oncolytic viruses armed with pro-viral, anti-tumor genetic elements can either be developed as a standalone anti-cancer regimen or as a synergistic component of an established anti-

cancer approach. Oncolytic viruses given in combination with other anti-cancer drugs improve the therapeutic efficiency and are also effective against drug resistant cancer cells. Therapies considered in combination with oncolytic viruses include chemotherapy, radiation, anti-angiogenic and immunotherapies. Other molecules considered for combinatorial therapies are histone deacetylase inhibitors, immune checkpoint inhibitors, monoclonal antibodies, cytokines and chemokines. The effect of oncolytic viruses combined with other anti-cancer agents has already been demonstrated in preclinical studies and some of the combinatorial therapies are FDA approved. The challenge in combinatorial therapy is to keep the combined toxicity limited, hence it is still imperative to observe and analyze the significance of anti-cancer potential of oncolytic viruses amidst all the established therapies and the role they can play in instituting a comprehensive anti-cancer regimen. Significant number of reports on construction of oncolytic viruses and their use as a synergistic component for anti-cancer effects has been documented in the literature (Ottolino-Perry et al. 2010). Also, a large number of studies reported the preclinical activity of various oncolytic viruses constructed, nevertheless their potential in clinical settings is yet to be established.

Till date no reports on construction of recombinant viruses for tumor lytic purpose from India is documented in the literature. This work was carried with an objective to construct a recombinant virus and arm the virus with foreign gene. Further, to ascertain the effects of an armed oncolytic measles virus against breast carcinoma cells and whether these effects can be enhanced by combination with chemotherapy. Here we used Edmonston strain of measles virus as backbone for the generation of a recombinant virus. Measles virus is an RNA virus having single-stranded negative sense RNA as genome. One major therapeutic advantage of measles virus as an oncolytic agent is its significant bystander effect mediated through spread of infection via cell-to-cell fusion and formation of large multinucleated syncytia (Iankov et al. 2010).

Recombinant measles virus was generated and rescued from the packaging cell line expressing viral structural proteins and T7 polymerase following co-transfection with full length measles viral genome and viral RNA dependent RNA polymerase constructs. The internalization and replication ability of the generated virus was checked in Vero cells. Further its ability to infect breast carcinoma cells; MCF-7 and MDA-MB-231 was evaluated. Most of the cancers have abrogated apoptotic machinery as one of the mechanism for continued growth. So, following the confirmation of acquired tumor tropic profile of

recombinant measles virus generated; a foreign gene BNiP3, a pro-apoptotic gene of human origin was inserted into the viral genome and virus with BNiP3 gene in its genome was generated. Prior to the insertion of pro-apoptotic gene, a red fluorescence reporter gene Mcherry was inserted into the viral genome to track the infection of the recombinant virus carrying additional gene in host cells. The apoptosis induced by recombinant virus with BNiP3 gene was measured in both the cancer cell lines. Our preliminary studies had shown better selectivity of the recombinant measles virus to MDA-MB-231, a triple negative (ER, PR, Her2/neu negative) breast cancer cell line.

Recent studies on oncolytic viruses demonstrated increased therapeutic efficacy when administered in combination with established anti-cancer therapeutic drugs. Thus we aimed to observe the combinatorial anti-tumor effects of armed oncolytic measles virus with well-established anti-cancer drug paclitaxel and a novel hydrazone derivative. Toxic and apoptotic effects exerted by recombinant measles virus armed with BNiP3 gene in combination with paclitaxel and hydrazone derivative independent of each other were also observed. Experimental evidences showed better selectivity of this virus in MDA-MB-231 cells as compared to MCF-7 cells. With this we successfully constructed and rescued the recombinant measles virus armed with a pro-apoptotic gene but whether this will lead to the development of another oncolytic virus based therapeutic approach to target breast cancer cells or not needs further investigation.

AIMS AND OBJECTIVES

1. **Generation of packaging cell line/complementing cell line expressing measles viral capsid Nucleoprotein and Phosphoprotein**

    1.1. Cloning and expression of measles virus gene encoding Nucleoprotein (structural protein).

    1.2. Cloning and expression of measles virus gene encoding Phosphoprotein (RNA dependent RNA polymerase co-factor).

    1.3. Cloning of T7 RNA Polymerase.

    1.4. Generation of Stable cell line stably expressing Nucleoprotein, Phosphoprotein and T7 RNA polymerase.

2. **Rescue of replication competent engineered measles virus from complementing cell line**

    2.1. Cloning and expression of measles virus gene encoding Large (RNA polymerase) protein.

    2.2. Co-transfection of packaging cell line (stably expressing ‘N’ and ‘P’ proteins) with recombinant ‘L’ gene (viral RNA dependent RNA polymerase) and genetically engineered viral construct.

    2.3. Recovery/rescue of engineered virus.

    2.4. Insertion of M-cherry into the full length viral genome to confirm viral internalization and replication.

3. **Evaluation of replication competency of recombinant measles virus**

    3.1. Confirmation of viral replication by transcript amplification and protein expression.

    3.2. Validation of cytopathic/cytotoxic effects induced by recombinant measles virus in breast carcinoma cell lines.

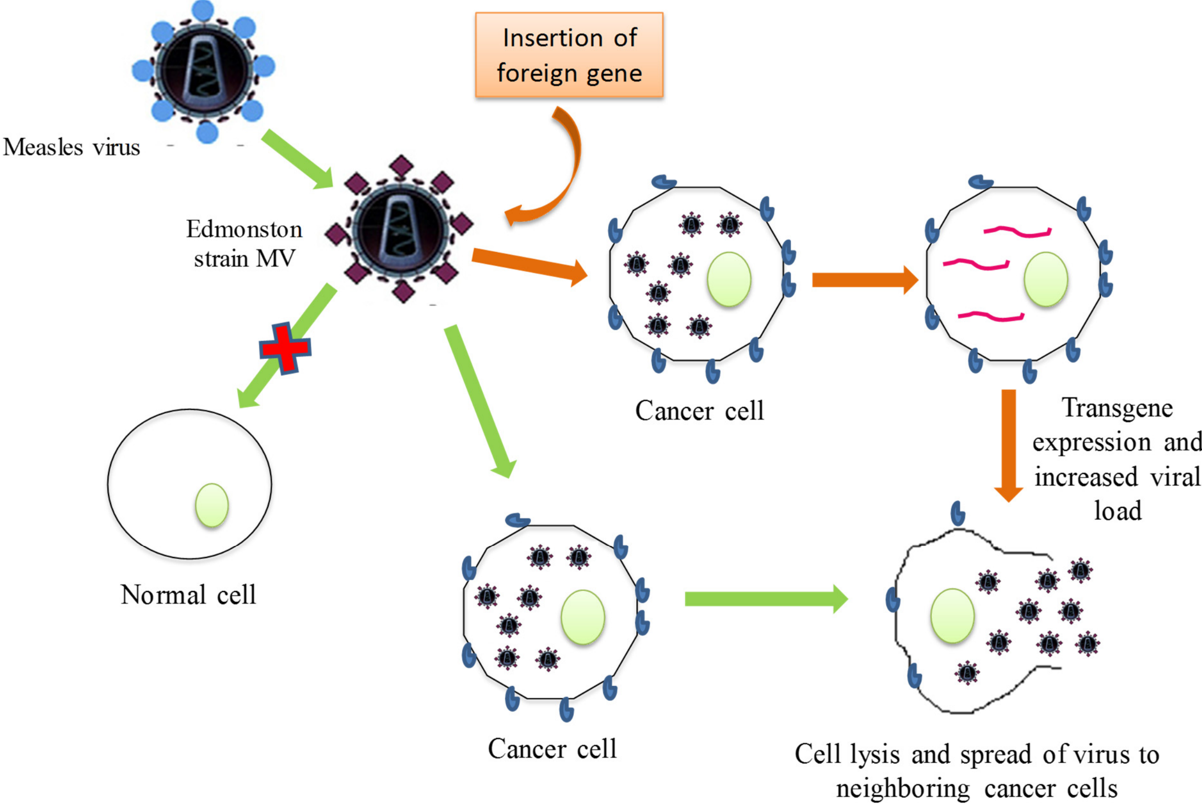
4. **Manipulation of measles viral genome by inserting pro-apoptotic genes or immunomodulators or cytotoxic gene to enhance anti-tumour activity**

    4.1. Insertion of desired gene/s in recombinant measles virus genome

    4.2. Confirmation of replication competency of engineered virus harboring BNiP3.

    4.3. To check the antitumor activity of genetically engineered virus in breast cancer cells.

RATIONALE OF THE STUDY



REVIEW OF LITERATURE

Cancer in itself is an uncontrolled pattern of growth. Even with various advancements in the field of cancer therapy, it remains one of the major causes of death worldwide. Recent advocacy of characterizing cancer as a lifestyle disease has been debunked due to recorded medical history of cancer patients and treatments throughout the years. But this underscores the fact, that cancer has always been one of the most studied diseases. Various studies have charted the routes and modulations of cancer establishment and progression. The detailed pathways and moieties involved in cancer and their complex interplay to thwart the host immune response is acutely characterized. But even with all these information, an effective therapy for control and destruction for majority of tumor types has evaded us.

This generalization of poor prognosis of many cancers is not due to lack of treatment modalities but due to dynamic nature of cancer cells where their responsiveness towards treatment varies not only in different cancer types but also in different patients. This non-uniform responsiveness is the major driving force behind continued development of strategies and treatment avenues against cancer. The fact remains, that the employment of sophisticated coping mechanisms by tumor cells against various anti-cancer therapies poses a major hurdle in cancer treatment. Effectiveness of conventional cancer therapies such as chemotherapy, radiation therapy and surgery is moderate at best and is mired with various side effects. Thus, it is imperative to design a therapeutic moiety which addresses these issues and would be able to effectively remain a step ahead with the evolving nature of cancers.

Conventional cancer therapies

Early references to cancer therapy have largely been associated with surgical resection of tumors. In case of cancers where surgery was not possible or generally inoperable malignancies, alternative cures were sought including various chemicals, plant extracts, tissue extracts of animals and other esoteric approaches. Even with advances in medicine with respect to knowledge, expertise, pharmacology, instrumentation and execution of procedures, surgery still remains one of the first opted strategies in cancer treatment. But these advances do not pre-empt the previous caveats of early diagnosis and accessibility, which still remain as a major criteria of the success of surgical resection. Conventional cancer therapy has been associated with a combination of surgery, radiation therapy and chemotherapy. In addition, promising results of gene therapy and targeted therapies as a part of anti-cancer regimen have also gained approval. In order to develop a successful cancer therapeutic or more optimistically a cure, it is essential to understand and address the lacunae of current anti-

cancer treatments and look for viable alternatives, not only to replace but to synergise with the available modalities.

a. Radiation therapy

Waves such as X-rays, gamma rays, LASER, near infrared, electron and proton beams and high-energy charged particles are some of the components of radiation therapy of cancer. Radiation therapy relies on DNA damage due to complex double-strand breaks, mitotic catastrophe and induction of apoptosis for cancer cell death (Eriksson & Stigbrand 2010). Localized radiation therapy, majorly at sub-lethal doses, results in upregulated expression of MHC I and tumor associated antigens at the afflicted site. Immunosuppressive nature of radiation therapy with respect to lymphocytic sensitivity has been challenged due to pro-inflammatory response elicited by the therapy priming an anti-cancer immune response. Thus with right dosage and combinations with other modules of conventional anti-cancer therapies, radiotherapy provides both palliative and curative responses (Wargo et al. 2015). These responses, while effective in halting the growth of cancer cells, are associated with severe side-effects. Aside from cosmetic damage, susceptibility of normal cells towards radiation-induced toxicity and limitations of lifetime quantity of radiation that can be given to a patient decreases the singular effectiveness of this therapeutic approach. Systemic toxicity in combination with heterogeneity of response with different patients and cancer type renders radiation therapy a supportive role, at best, within clinical anti-cancer approaches.

b. Chemotherapy

Chemical agents are the most widely used anti-cancer approaches. These are developed as both broad-range and targeted tumor effector molecules. Their nature and mode of action can broadly be classified as alkylating, cytostatic, DNA intercalators, cytoskeletal modifiers and nucleotide analogues. The variations in their mode of actions and targets allow them to have diverse range of effects with which growth and progression of cancer cells can be efficiently curbed and eventually destroyed. Chemotherapy has seen a metric rise in the number of candidate agents being studied at both pre-clinical and clinical settings but mere presence of a large number of agents does not necessarily translates into heightened degree of ensuing cancer cell death. Majority of chemotherapeutic agents affect rapidly dividing cells, thus compromising their specificity and show overlapping systemic toxicity towards normal cells.

In addition to precincts of efficacy, delivery and penetration of drugs to target site, chemotherapy is mired with various side effects as well as emergence of drug resistant tumor cells. In addition, drug inactivation, target alteration, DNA mutation and damage repair, cell death inhibition, epigenetics and epithelial-mesenchymal transition (Housman et al. 2014) leads to cancer relapse which is unresponsive to established chemotherapeutic drugs. Most widely used agents such as cyclophosphamide, cisplatin, paclitaxel, rapamycin, doxorubicin, 5-fluorouracil etc. suffer from similar inadequacies and promote the need for development of a moiety which has high therapeutic index with relatively manageable associated toxicities.

c. Immunotherapy and other Targeted therapies

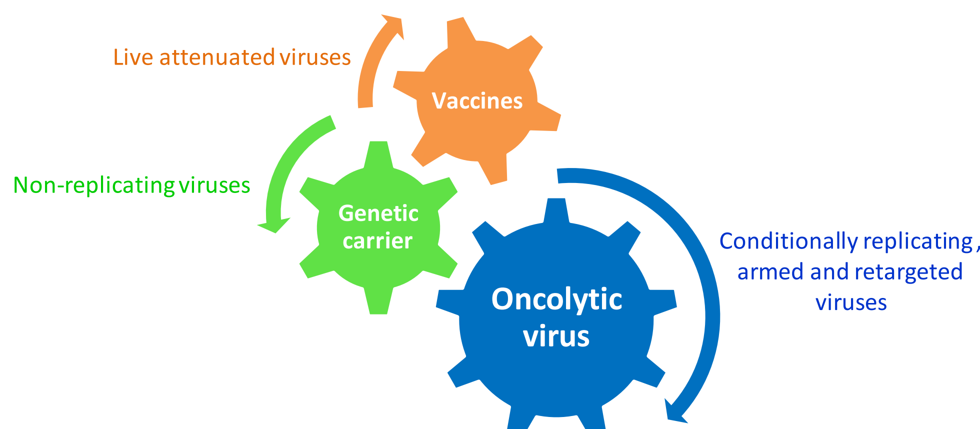
In addition to chemotherapeutic agents with cytotoxic profile many other molecules are also being pursued as anti-cancer effectors. The generalization of their activity can only be found in their anti-cancer toxicities but not with the mode of action. With increase in our understanding of the molecular landscape of tumor and its microenvironment, it has become possible to design agents targeting specific receptors or molecules involved in tumor mechanisms maintaining unchecked growth, immune suppression and evasion, metastasis as well as evolving resistance towards pre-existing therapies. These targeted therapies include antibodies against oncogenic receptors (e.g. Her2 and EGFR), immune checkpoint inhibitors (e.g. CTLA-4 and PD-1), inhibitors against kinases (e.g. BRAF kinase or MEK) and histone deacetylases (Xu et al. 2016). There have been reports of synergistic potentiation of anti-tumor effects and reversal of acquired resistance of either immunotherapy or targeted therapy when combined (Keller et al. 2015) but these therapies also exhibit problems pertaining to unforeseen effects of such immune-modulations. The effective delivery of these targeted molecules to the effector site is another major hurdle shared by other pre-existing therapies due to disrupted vasculature of tumor microenvironment as well as toxicities associated with IgG based antibody therapies due to their higher stability.

One of the major successes in the field of specificity and targeted delivery of therapeutic agents has been achieved with gene therapy especially in the case of monogenetic diseases where, gain or substitution of function due to delivery of functional copy of defective gene resulted in either reversal or stable maintenance of the progressive medical condition. The idea to replicate it with using targeting vectors to introduce anti-cancer elements within the tumor is promising but lacks effectiveness due to constraints of tumor breaching, sustenance

of vector and dynamic genetic nature of different cancers. But the idea holds merit of devolving into the repertoire of vectors for specific cancer targeting.

Despite the promising results and advances, an ideal therapeutic intervention which can be translated into a comprehensive treatment modality is yet to be found within the confines of conventional approach. At present, there is a need to devise a therapeutic moiety which can be a template with provisions to make it effective towards any cancer type. Expanding upon the idea of gene therapy, use of viral vectors to target cancer cells has gained momentum, giving rise to the field of oncolytic virotherapy. But unlike in the previous scenario, rather than favoring replication deficient virus there is retain of this propagative quality specifically within cancer cells, morphing this nature's nanoparticle into an oncolytic therapeutic which is self-replicating, cancer specific and can be manipulated for enhanced cancer toxicity.

Viruses are more than mere pathogens



Historically and even at present, viruses have been associated with pathogenesis of mild to severe diseases. Large scale deaths have been caused by outbreaks of viral pandemics such as Spanish flu of 1918, which caused death of millions, accounting for almost 5% of world population at the time. Recent examples can be seen in case of 2009 swine flu and 2014-2016 Ebola virus pandemic which caused death and widespread economic burdens. As a causative agent, virus displays one of most sophisticated systems of coup, where it hijacks and modulates the host's molecular machinery for its own progression and propagation simultaneously devising strategies to overcome or bypass defenses mounted by the host cell.

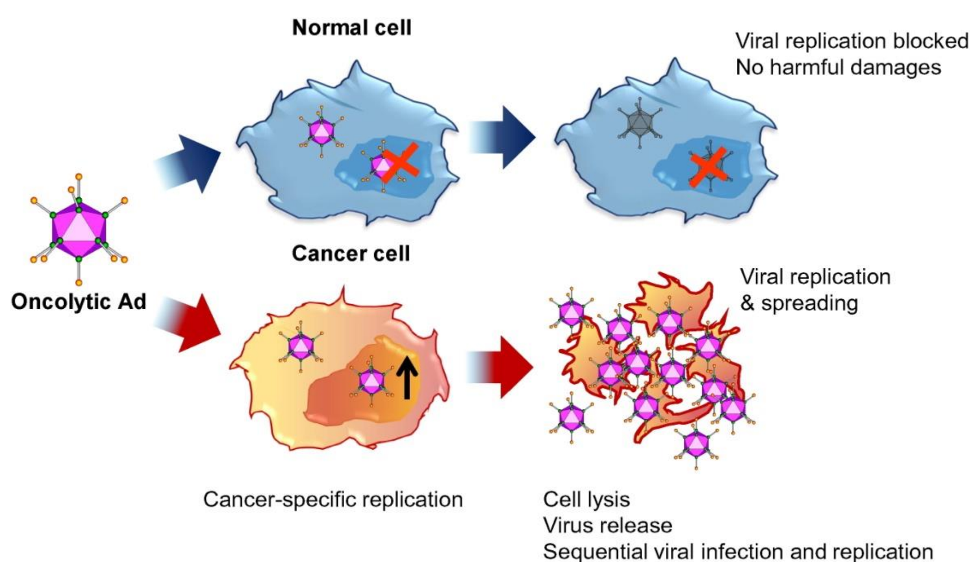
But the description of viruses is not limited to being a disease causing pathogen as they have been instrumental in development of vaccines against various diseases. In addition, with

advent of molecular biology techniques, they form a major biotechnological tool of gene transfer. They have found employment in not only gene therapy but also in cancer therapy in the way of oncolytic viruses which have moved from being crude viral isolates to specially designed viruses for cancer cell lysis.

What are oncolytic viruses?

Oncolytic viruses (OVs) are novel therapeutic moieties which selectively infects and lyse neoplastic cells sparing the normal cells. The idea behind using viruses as a tool of cancer therapy originated from documented accounts of spontaneous tumor regression upon virus infections such as burkitt's lymphoma (Bluming & Ziegler 1971) and hodgkin's disease (Zygiert 1971; Taqi et al. 1981) with natural measles infection as well as different forms of leukemia with natural influenza, chickenpox, measles infection and small pox vaccination (Dock & Company 1904; Bierman et al. 1953; Pasquinucci 1971; RM & JA 1978). This tumor tropism is in lieu of cellular tropism exhibited by viruses during their natural infective cycle dictating the type of cells infected by a virus and the kind of disease it causes, which can be exploited to generate OVs (Russell et al. 2012). Later studies have revealed how the cellular environment of cancer cells make them attractive viral targets for infection and opened up new avenues for development of viruses as anti-cancer agents.

Oncolytic strategy



Adapted from Choi et al, J of Controlled Release (2015) 219:189-191

Viruses may exhibit cancer specificity as their natural infection profile or they can be programmed to be selective towards cancer cells by genetic alterations. Earlier the use of viruses was limited to being non-replicative carriers of transgenes to the target cells for gene therapy but with generation of OVs, the role of a passive carrier has transformed into that of an active 'drug' agent (Fukuhara et al. 2016) whose replicative potential makes it a potent instigator of cancer cell death.

Oncolytic virotherapy relies on cancer specific replication of virus triggering tumor cell death by a number of mechanisms, including direct lysis, induction of apoptosis, expression of toxic proteins, autophagy, and induction of anti-tumoral immunity (Bilsland et al. 2016; Wong et al. 2010). In addition, oncolytic viruses can mediate the killing of uninfected cancer cells by indirect mechanisms such as induction of anti-angiogenic response (Toro Bejarano & Merchan 2015b), anti-cancer immune response (Loskog 2015) or through the specific activities of transgene-encoded proteins expressed from engineered viruses (rev by Russell & Peng 2007; Russell et al. 2012). There are a number of viruses with natural preference to infect cancer cells such as parvoviruses, reovirus (Reolysin), newcastle disease virus, mumps virus, moloneyleukemia virus whereas viruses such as measles, adenovirus, vesicular stomatitis virus, vaccinia and herpes simplex virus can be adapted by repeated laboratory cultures (Grote et al. 2001) or engineered for cancer specificity. But for a virus to be an oncolytic agent, stringent criteria related to safety of the population from pathogenic reversion or evolution of recombinant virus in the patient or of person-to-person transmission of the oncolytic virus or its quasi species have to be followed.

At present, studies are aimed towards generating an ideal OV platform which hosts the following features:

1. Oncoselectivity: replicative attenuation in normal tissues and safe for immunocompromised subjects
2. Oncotoxicity: exceedingly effective infectivity and killing of cancer cells
3. Low intrinsic pathogenicity
4. Ability to stimulate an anti-tumor adaptive immune response
5. Ability to resist premature clearance by host immune system
6. Easy to generate and manipulate
7. Highly stable with inability towards insertional mutations and chromosomal integrations

8. Safe for patient and population
9. Systemically deliverable (Bressy et al. 2017; Verheije & Rottier 2012)

Theoretically, these characteristics should be essential before employing a virus as an oncolytic agent, but in majority of cases no single virus embodies all these features thus it is imperative to programme a virus to generate an ideal OV with respect to the cancer type. Based on their preferential replication in tumor cells, viruses from nine families have progressed to clinical trials. DNA viruses include *Adenoviridae, Herpesviridae, Parvoviridae, and Poxviridae* and RNA viruses *Paramyxoviridae, Picornaviridae, Reoviridae, Retroviridae, and Rhabdoviridae* (Cattaneo & Russell 2017).

Naturally occurring Oncolytic Viruses

Viruses showing non-virulent infection profile in humans and tropism towards cancer cells are generally referred to as natural OVs which can be used as a therapeutic agent without any changes to their genome. This tropism being preferential by virus is generally not exclusive and in majority is due to molecular profile of tumor and its microenvironment and not due to any specific viral requisites. The activation of anti-viral immune response by the host cell is a major limiting factor in viral propagation, but malignant cells have evolved to avoid immune responses, apoptosis and translational suppression thus rendering them unable to elicit any anti-viral activity. In context there are many overlapping features between the hallmarks of cancer in a malignant cell and the hallmarks of infection in a virus-infected cell (Seymour & Fisher 2016). For example, there is downregulation of host immune responses such as IFN-β signal pathway (Stojdl et al. 2000), deregulation of cell cycle arrest, resistance towards apoptosis and translational suppression (Russell et al. 2012). Cancer cell lysis is achieved by a combination of various mechanisms. It includes direct cell lysis due to cytopathic effect and progression of infection (Yaacov et al. 2008) as well as modulation of host innate and adaptive immune response in form of natural killer cells and cytotoxic T lymphocytes to establish anti-cancer immunity in response to presence of viral antigens in infected cancer cells (Schirrmacher 2005; Schirrmacher et al. 2000).

These natural or adapted tumor-tropic viruses have emerged as potential oncolytic agents and are undergoing pre-clinical and clinical studies for their cytotoxic effect against various cancers. Amongst many candidate natural oncolytic viruses, the ones in advanced stages of clinical development are described below.

<u>Reovirus</u>: Reolysin, T3D strain of reovirus (RT3D) is currently being pursued as an oncolytic agent. It is a double stranded RNA virus which exhibits preferential replication in cancer cells with activated Ras signaling, with negligible virulence in normal human cells (Fukuhara et al. 2016). Wild type reovirus had shown enhanced infectivity and cytotoxicity against cells overexpressing EGFR and v-erbB which supports the observation of Ras activation (downstream signaling to EGFR) being a prerequisite for reovirus sensitivity (Strong & Lee 1996; Coffey et al. 1998; Strong et al. 1998). Mutational activation of Ras corresponds to hypophosphorylation of protein kinase R (PKR), a serine threonine protein kinase responsible for induction of antiviral response against reovirus and a contributor of interferon mediated anti-proliferative response towards viral infection, causing its inactive state and unchecked replication of reovirus (Meurs et al. 1990). This overexpression or constitutive mutational activation of EGFR or Ras gene, frequently associated with transformation, proliferation and chemotherapeutic resistance exhibited by cancer cells (Bos 1989), concurs with the infective bias of reovirus to cancer cells thereby making these tumors susceptible to viral lysis (Harrington et al. 2010).

Reolysin has shown promising oncolytic effects against various cancers in pre-clinical and clinical studies. It has shown endoplasmic reticular (ER) stress mediated induction of apoptosis in mouse xenograft model of pancreatic cancer, enhanced in combination with known inducers of ER stress such as bortezomib, tunicamycin and brefeldin A (Carew et al. 2013). Phase I clinical trials have shown safety of Reolysin upon intralesional introduction in patients with advanced solid tumors (Gollamudi et al. 2010). Its safety and oncolytic efficacy has also been observed in phase I and phase II trials with multiple myeloma, refractory or metastatic solid tumors, metastatic colorectal cancer, malignant glioma, prostate cancer and metastatic melanoma, head and neck cancer, lung squamous cell carcinoma respectively (rev by Fukuhara et al., 2016). The trials also included intravenous introduction of Reolysin as well as its combination with radiation therapy and established chemotherapeutic agents. This combinatorial approach has shown increased survival of patients of head and neck cancer in phase III clinical trials (Gong et al. 2016). FDA has granted Reolysin the designation of orphan drug for treatment of ovarian cancer, pancreatic cancer and malignant glioma.

<u>Newcastle disease virus (NDV)</u>: It is an avian virus with negative sense single-stranded non-segmented RNA genome belonging to the paramyxoviridae family. Its classification based on the pathogenicity, lentogenic (avirulent), mesogenic (intermediate) and velogenic (virulent),

corresponds to its oncolytic potential in cancer cells. Thus the more virulent mesogenic and velogenic strains present themselves with lytic properties whereas non-virulent lentogenic strains with non-lytic (Zamarin & Palese 2012). It causes no known disease in humans thus can be used in increasing doses in human subjects without any major morbidity or side effects as seen in phase I and II clinical trials (Sinkovics & Horvath 2000). It uses host sialic acid cell-surface receptors for internalization (Villar & Barroso 2006) thus exhibits broad cell tropism due to ubiquitous presence of these receptors. Thus rather than the internalization, it is replication and propagation of NDV which is tumor specific. Fusogenic strain of NDV has shown enhanced oncolytic potential due to its property of syncytia formation leading to lysis of uninfected neighboring cancer cells both in vitro and in vivo (Li et al. 2011). Due to NDV being an avian virus, its species-specific management of host anti-viral response for continued propagation is not very efficient in human cells. It elicits robust type I interferon response against infection leading to immune clearance. But this response is absent in cancer cells due to cancer mediated abrogation of IFN signaling to overcome growth inhibition and apoptosis. This property of cancer cells allows successful infection and lysis of cancer cells by NDV (Krishnamurthy et al. 2006).

Cancer cells also exhibit lower basal level expression of anti-viral genes such as RIG-I, IRF-3 and IFN-β, which increases their susceptibility towards NDV infection, making it an ideal candidate for oncolytic agent with natural tumor-tropism (Wilden et al. 2009; Fournier et al. 2012). But to assume that propensity of NDV towards cancer cells is solely due to disrupted native immune response in tumor cells would be oversimplifying the complex nature of infection and oncolysis achieved not only by NDV but other viruses with natural tumor-tropism. In case of NDV, abrogated apoptotic machinery also plays a major role in enhanced viral replication in cancer cells. The generally overexpressed apoptotic inhibitor proteins such as Livin in carcinoma cells (advanced melanomas) resulted in sensitization of melanoma towards NDV mediated lysis. In fact, the lysis is promoted though induction of previously inhibited or downregulated apoptotic pathways thus implying the greater role of NDV in modulation of host mechanisms to achieve lysis (Lazar et al. 2010).

Oncolytic effects of NDV have been observed in tumor xenografts of various carcinoma models including breast cancer, colon cancer, large cell lung cancer, prostate cancer and neuroblastoma, with various routes of administration. Most of the models showed tumor

growth inhibition with some showing complete regression with no recurrence of disease (Phuangsab et al. 2001).

Even after promising results of using NDV as an oncolytic agent against various carcinomas, its limitations regarding the extent of anti-cancer effects of native virus as well as significant amount of shedding found in studies with non-human primates (Buijs et al. 2014) supports the need of ongoing studies for genetic modifications for enhancing both its safety and efficacy.

Vesicular stomatitis virus (VSV): It is an enveloped, non-segmented negative sense RNA virus belonging to rhabdoviridae family. As with above mentioned naturally occurring oncolytic viruses, it does not present itself as a disease in humans and generally non-lethally affects horses, cattle, pigs and other mammals. Its transmission in humans is restricted to person directly in exposure to virus (Lyles & Rupprecht 2007). Absence of neutralizing antibodies and pre-existing immunity in humans allows it to be an efficient agent of oncolysis. It is able to infect majority of cell lines, independent of receptor or cell cycle restrictions and gives high virus yields. Cell-surface receptors for low density lipoproteins are recognized as the major receptors for VSV infection in human and mouse cells (Finkelshtein et al. 2013). Its cytoplasmic replication ablates the risk of host genome integration and host-cell transformation (Hastie & Grdzelishvili 2012).

As with other viruses, VSV exploits the abrogated or reduced type I IFN response to specifically replicate in tumor cells without affecting the normal cells(Stojdl et al. 2000). Atypical translational machinery and cellular protein levels including that of PKR, eIF2b, eIF4E, AKT and NFAR1/2(Barber 2005; Balachandran & Barber 2004) often found in cancer cells, also contribute towards the onco-selectivity of VSV(Oliere et al. 2008). Studies with non-human primates indicated no major shedding, marking its safety against environmental shedding (Jenks et al. 2010).

VSV in addition to selectively infect cancer cells can also infect endothelial cells in tumor vasculature and cause thrombosis in tumor vessels (Breitbach et al. 2011). As with oncolytic reovirus, VSV also induces apoptosis (Cary et al. 2011) and autophagy in infected cancer cells (Chakrabarti et al. 2012) promoting tumor immunogenicity by release of DAMPs (danger-associated molecular patterns) such as HMGB1(Thorburn et al. 2008), ATP and uric acid and antigen cross-presentation from cancer cells to DCs and T-cells (Bartlett et al. 2013;

Endo et al. 2007). Various pre-clinical studies have showed cytotoxic effects of oncolytic VSV against breast cancer (Shi et al. 2009), prostate cancer (NM et al. 2008; Moussavi et al. 2010), colorectal cancer (Shinozaki et al. 2005), melanoma (Fernandez et al. 2002), glioblastoma (Cary et al. 2011), liver cancer (Altomonte et al. 2009) and pancreatic ductal carcinoma (Murphy et al. 2012) amongst others. All these examples of promising oncolytic potential of VSV cannot be attributed to only wild type VSV. Currently most of the studies with VSV include recombinant VSV variants for enhanced oncolytic efficacy against cancer cells and reduced neurotoxicity generally encountered with WT VSV infections in mice and rats (van den Pol et al. 2002). These pre-clinical successes are undergoing development into a clinical model.

In addition to the above mentioned examples, there are other viruses with natural bias towards cancer cells, but there are associated limitations with use of wild type viruses. Even when the virus has appreciable safety profile, the oncolytic or anti-cancer immunogenic profile can be enhanced by genetically modifying the virus. Thus, the first generation of oncolytic virus includes naturally occurring viruses but subsequent generations harbor genetic modifications to enhance their overall safety and efficacy.

Genetic modifications to introduce or enhance oncolytic potential of viruses

Viruses in their natural profile may or may not be tumor tropic. Advent of molecular biology has allowed us to design viral vectors with desired characteristics of tumor specificity, establishment of host anti-tumor immunity, increased safety from genetic reversion, mutation or insertion as well as benign symptomatic presentation. It can be said that it is easier to target a viral vector for tumor specificity but harder to completely disengage its entry and replication in normal cells. Recombinant viruses designed on these tenets can be classified based on the purpose of modification within the viral genome, life cycle and effects on host cell.

Genetic modifications can be directed towards retargeting the virus towards cancer cells by modifying it receptors. Modifications can also include increasing the safety of virus by attenuating its pathogenicity and associated morbidity. Changes are also made to induce host immunity against cancer cells and induce cell death in infected and non-infected cancer cells by inserting cytotoxic transgenic elements of viral or non-viral origin in viral genome.

Majority of times these criteria overlap with the modifications involved in designing and generating the oncolytic virus.

a. Modifications for attenuation of pathogenicity of virus in normal cells

Wild type viruses generally encode for proteins essential to subjugate host defense mechanism to promote viral pathogenesis. This feature, being essential for effective viral propagation, is not desirable for the safety profile of oncolytic viruses. In order to restrict the replication of viruses in normal cells, the genes encoding for these proteins are either deleted or mutated. Some deletions are more substitutive in action, where the absence of deleted viral genes and corresponding proteins restricts the successful replication of viral genome in normal cells but its function is substituted in cancer cells. This kind of modification generates a virus which can successfully replicate in cancer cells due to their abrogated immune machinery but will be killed by the anti-viral response mounted by normal cells. In context, these modified viruses are generated to mimic the tumor-specific mechanism of naturally occurring oncolytic viruses described above. Many viruses also accumulate such mutations with selective passages in cancer cells or tissues. This targeted viral adaptation was the first manipulation towards generation of an oncolytic virus (Kelly & Russell 2007).

There are many examples of viruses being bereft of their virulence to enhance their overall safety. Some of the major examples include Herpes simplex virus and adenovirus which are the widely used viruses for oncolytic purpose following genetic modifications.

Herpes Simplex Virus: Thymidine kinase gene from HSV genome was deleted to make it conditionally replicative in cancer cells but these viruses were observed to have replication competency only in dividing cells. This deletion mutant was used for treatment of human glioma in mouse xenograft model (RL, Malick, JM, KL, & DM, 1991). In addition to using naturally attenuated HF10 (Takakuwa et al. 2003) strain of HSV as an oncolytic agent, $\gamma_1$34.5, gene responsible for neuro-virulence of HSV, is deleted in majority of oncolytic HSVs (McKie et al. 1996). It cripples the virus to counteract protein kinase R (PKR) response elicited by the normal cells thus allowing it to only replicate in cancer cells. Another gene deleted for conditional replication is U_L39, encoding for large ribonucleotide reductase subunit which made the high deoxyribonucleotide content of cancer cells as an ideal and selective environment for these deletion mutants (Campadelli-Fiume et al. 2011). G207, another HSV based vector has deletion of both copies of $\gamma_1$34.5 and LacZ gene instead of

U$_L$39 (Markert et al. 2000; Markert et al. 2009). G47Δ, a derivative of G207, has deletion of α47 coding region putting U$_S$11 under immediate early promoter for enhanced oncolysis. Product of α47 gene is responsible for inhibiting the transporter associated with antigen presentation (TAP) and loss of this inhibition causes increased MHC-I expression in infected cancer cells (Smith et al. 2014; Todo et al. 2001).

Adenovirus: Use of adenoviral vectors as agent of oncolysis started with replication incompetent viruses. It reached the pinnacle of success with Chinese FDA (CFDA) approval of Gendicine, an Ad5 vector with E1A gene deletion (Δ24) and human TP53 gene insertion, for the treatment of head and neck squamous cell carcinoma in 2004 (Peng 2005).Despite the phenomenal role it played as a vector of gene therapy, a major shortcoming was its one time effector molecule status. To overcome this flaw, replication competent adenoviral vectors were generated. The first conditionally replicating adenovirus (CRAds) were similar to Gendicine with deletion in Retinoblastoma (Rb) binding sites of E1A (Ad-Δ24).The rationale behind this deletion is the major role E1A gene plays in the progression of replication and pathogenesis of adenovirus. It is an immediate-early viral protein which binds to retinoblastoma (Rb) protein causing release of active transcription factor E2F. This leads to quiescent cells being pushed into G1 and S phase of cell cycle to aid the replication of virus. Since most cancer cells are in S phase, this deletion mutant can replicate in them without any anti-viral response but not in normal non-cycling cells with functional Rb (Alemany et al. 2000). Other CRAds followed suit with deletions in E1B 19k and E1B 55k genes.E1B 19K binds to inhibit Bax in normal cells to suppress induction of apoptosis as a defense mechanism by the infected normal cells. These deletion mutants exploit the defective apoptotic signaling in cancer cells for replication (Liu et al. 2004). Similarly E1B 55K deletion mutants exploited the defective p53 expression and functional profile of cancer cells as this protein forms an E3 ubiquitin ligase complex with E4orf6 targeting p53 degradation further inducing cyclin E expression and inhibiting export of cellular mRNA and promoting export of late viral mRNAs(Bischoff et al. 1996). One of the most widely studied E1B 55K mutant is ONYX-015. Studies with ONYX-015 revealed that the tumor-selectivity of E1B 55K deletion mutants was not due to p53 status of normal cells *vs* cancer cells but due to defective viral mRNA transport from nucleus (O'Shea et al. 2004).

In addition to the above mentioned viruses, other candidates such as measles virus, vaccinia virus etc. has also been attenuated for greater safety profile in normal cells.

Restrictive replication of virus owing to robust anti-viral response by normal cells as observed in above mentioned examples of deletion mutants is at best a palliative measure of tumor targeting. Because of these deletions and attenuations, oncolytic virus candidates lose their virulence which earlier was seen as a small price in exchange of continued safety of these agents but also limited the maximum potential of the viruses against cancer cells where the abrogation of immune responses, apoptosis and autophagy are not to a great extent or the cancer cells which can still elicit anti-viral response.

b. Modifications for retargeting of virus towards cancer cells: making the virus tumor-specific

Cancer-specificity of oncolytic viruses is not solely dependent on making them unable to counter the anti-viral response elicited by normal cells. Specificity can be introduced at the stage of viral attachment and internalization or at the transcription and translation of viral proteins. In fact this kind of targeting allows the virus to retain its robust replicative and infective potential without bearing any adverse effects on normal cells.

Tumor-selective infection or **transductional targeting** pertains to regulating the entry of oncolytic viruses into cancer cells thereby preventing them from entering normal cells. This is achieved by de-targeting them from their natural receptors and re-targeting them towards cancer-specific receptors or their mutants over-expressed in cancer cells. It includes mutational or insertional modification of the viral proteins engaged with host cell surface receptors for natural infection. Rationale behind transductional targeting is equipping an oncolytic virus with components to interact with tumor cell epitopes. There are various methods with which it can be accomplished. Chimeric viruses are also generated to serve the purpose of retargeting.

Viral surface protein modification: As the name suggests, the most widespread method of retargeting is to change or modify the viral surface proteins to preferentially or exclusively interact with cancer-cell surface receptors. This genetic modification includes either mutating or deleting the gene responsible for natural tropism of virus and replacing it with ligand of interest. The modification being genetic in nature is carried in all the progeny viral particles thus extending the efficacy and specificity of treatment to successive infection of neighboring cancer cells.

Modifications of surface glycoproteins depends on the viral vector to be used, thus comprehensive structural knowledge of proteins engaged in viral attachment and internalization as well as specific residues, domains and motifs of interaction is essential. In addition to incorporation of receptors of other viruses to generate a chimera, cancer cell specific antibody fragments are also incorporated as ligands. Such as single chain variable fragments (ScFvs) and antigen-binding fragments (Fabs).Majority of time the changes do not deter natural mode of infection of the virus, it just modifies the interacting partners. Strategies of viral surface glycoprotein modification, based on the viral system being used, are listed below.

Adenovirus: Majority of adenovirus based oncolytic platforms are human serotype 2 (Ad2) or serotype 5 species C (Ad5). These are well characterized, have easily manipulated genome with large capacity of foreign gene incorporation, can be purified to high titer and are associated with relatively mild form of disease (Yamamoto & Curiel 2010; Yamamoto 2004). Primary receptor of Ad infection, coxsackie adenovirus receptor (CAR), is not expressed by many cancers including ovarian cancer, pancreatic cancer, gastrointestinal cancer and hormone-refractory prostate cancer (Yamamoto 2004; Yamamoto & Curiel 2005). Thus modification of CAR dependence of adenovirus is essential for generation of a successful oncolytic agent against a wide spectrum of carcinomas. These modifications are as follows:

***Fibre modification*:** adenoviral interaction with CAR is mediated by the fibre protein present on the surface of adenovirus. It consists of a knob, shaft and tail domain. Binding of carboxy terminal knob domain to CAR facilitates the attachment and further internalization is mediated by RGD motifs in penton base with cellular $\alpha_v$integrins. Modifications have been done in various domains of fiber protein including H1 loop, C-terminus, L1 loop of hexon, RGD loop in penton base and in the minor capsid protein IX. Ad5-Δ24-RGD is the prime example of successful insertion of arginine-glycine-aspartic acid-4C (RGD-4C) motif in H1 loop of fiber-knob region. RGD-4C is a partial peptide sequence of fibronectin and its insertion resulted in CAR independent internalization of recombinant virus CAR-negative cancer cells *in vivo* and *in vitro* (Suzuki et al. 2001).

***Serotype switching*:** not all Ad serotypes use CAR as the primary receptor. For example, Ad35 uses CD46 receptor, generally overexpressed in majority of cancers (Gaggar et al. 2005; Sirena et al. 2004). It is easier to use the alternative serotype but in the process the robust cytolytic activity of Ad5/2 maybe lost. Thus it begets the notion of merging two

serotypes to create tumor-specificity and retain anti-cancer toxicity. Chimera containing Ad5 fiber and Ad3 knob or Ad5 fiber with Ad35 binding domain can infect CAR negative cell lines and retain the oncolytic activity to kill the cancer cells in liver metastasis mouse model (Liu et al. 2009; Hoffmann et al. 2007). Serotype switching also lends itself to increased systemic circulation and safety. Since Ad5 is known for sequestration and high toxicity in liver, a hexon chimeric virus with non-toxic hepatic profile can make the therapy safer for clinical settings (Youil et al. 2002). Similarly, a large percentage of population has neutralizing antibodies (Nab) against Ad5 (Bangari & Mittal 2006), so an oncolytic adenovirus with chimeric hexon or fiber from a non-reactive subtype can surpass the pre-existing immunity (Ranki & Hemminki 2010).

***Mosaic viruses*:** are adenoviruses with more than one binding molecule on the surface. These moieties can be derived from other parental viruses or can be targeted peptides. This allows the viral vectors to target a wide range of cancer cells. Mosaic viruses can be generated either by genetic insertion or by pseudotyping using cells expressing different binding motifs (Takayama et al. 2003; Murakami et al. 2008).For example, Ad5PTD has protein transduction domain (PTD) of HIV-1 Tat protein in the hypervariable region 5 of adenoviral hexon protein for CAR-independent attachment (Kurachi et al. 2007) to enhance the transduction capacity and oncolysis of neuroblastoma cells (Yu et al. 2011).

***Bridging molecule*:** earlier the concept of bridging molecule was limited to a one-time use targeting, not carried by the progeny viruses. It was done by incorporating the tumor-specific receptor ligand to anti-knob antibody and using it to outfit the virus for delivery to tumor site. Since, it was not genetic, thus the specificity only lasted with the parental particles. To overcome this, now ligand specific antibody fragments can be incorporated genetically in viral fibre or capsid protein IX to induce tumor-targeting (Belousova et al. 2008; Poulin et al. 2010).

Herpes Simplex Virus: attaches to heparin sulfate proteoglycans, present on the cellular surface, for infection. This attachment is mediated by HSV surface glycoproteins gC and gB. Post-attachment, gD interacts with HVEM (herpesvirus entry mediator), a receptor belonging to family of tumor necrosis factors, and nectin1 as well as nectin2. gH, gL and gB forms the conserved fusion apparatus of herpesviridae family (Campadelli-Fiume et al. 2016) where gB mediates virion to cell membrane fusion. Retargeting of HSV also involves the modification of either gD or gH as well as insertion of ligands or ScFvs in the gC or gD protein.

HSV with insertion of IL-13 at N-terminus of gD, with deleted gD ORF, retargeted the recombinant virus from HVEM based internalization to IL-13 receptor 2α, overexpressed in glioblastoma cells (Zhou et al. 2002). HSV was also retargeted towards uPAR by fusing it at different residues of gD to ascertain the specificity it renders to recombinant virus (Zhou & Roizman 2007). ScFv against Her 2 was also inserted at N terminus of gH between 23 and 24 amino acid residues. These recombinant viruses were also deletion mutants of gD with residues 6-38 being deleted for de-targeting purposes (Gatta et al. 2015).

Measles virus: A targeted approach utilizing the protease rich tumor micro-environment can also be employed where protease-cleavable linkers are inserted into the F protein to restrict proteolytic maturation of F at tumor sites (Springfeld et al. 2006). MicroRNA target sites (miRTSs) are also being incorporated at the 3′-untranslated region of both the fusion and hemagglutinin genes to inhibit translation of viral genome in the normal cells due to endogenous microRNA expression. For example, microRNA-122 (liver), microRNA-7 (brain), microRNA-148a (gastrointestinal) carrying recombinant measles virus have been generated with mentioned tissue specificity. These recombinant viruses carrying miRTSs can successfully replicate in cancer cells due to their differential and downregulated microRNA levels (Leber et al. 2011; Baertsch et al. 2014).This natural tumor tropism exhibited by vaccine strains of measles virus and ease of retargeting makes measles virus a promising oncolytic agent.

Scaffold insertion in viral surface protein: This modification is similar to the generation of mosaic viruses discussed in the retargeted adenovirus section. Here instead of insertion of a targeting ligand in the viral genome, incorporation of a scaffold moiety to the viral surface receptor is done. This scaffold acts as an attachment site for exogenously provided tumor-specific receptor ligand. The biggest drawback of this approach is the single usage of the targeting instead of regenerative potential of genetic approaches. But this restrictive single round-replication has ease of changing the scaffold-ligand partners without always resorting to genetic manipulation of viral genome. This allows a single viral parent vector to be used with various cancer cells by changing the externally provided ligand (Campadelli-Fiume et al. 2016). Most commonly used method is biotin-streptavidin coupling. When used with metabolically biotinylated adenovirus to bind with EGF-streptavidin complex and biotin-PEG-EGF coupled to an avidin-modified adenovirus, it retargeted the recombinant virus towards EGFR expressing cancer cells (Pereboeva et al. 2007; Park et al. 2008). Viruses such

as adeno-associated virus, Sindbis virus and Murine leukemia virus have been enhanced for tumor-tropism by incorporation of IgG binding domain of protein A as master scaffold for further interaction with wide varieties of antibody fragments against various cancer-specific receptors (Stachler et al. 2008; Ohno et al. 1997; Tai et al. 2003).

Bispecific adaptors: Bispecific adapters are proteins containing two domains, one of which binds to virion surface and other binds to cell surface receptor. These artificially manufactured proteins facilitate indirect interaction of recombinant virus to cancer cell. The virus binding domain generally is made up of pseudo-receptors, polymers, antibody fragments such as scFvs or Fabs. The cell-binding domain is generally a vitamin ligand, natural peptide and scFvs or Fabs. Both the arms are joined together either by chemical modifications or by combining them in a fusion protein. It generally has a flexible linker with targeting arm at C-terminus. These adapters are not dependent on the comprehensive knowledge of the receptor proteins of the virus and can incorporate larger sized targeting moiety as it will not be incorporated into viral protein. The biggest disadvantage of using such adapters is difficulty in its expression and folding due to its artificial nature.

These adapters can either be pre-complexed with virions before exposure to cells or their sequence can be incorporated in the viral genome with later option being conducive to efficient targeting through successive rounds of replication (Campadelli-Fiume et al. 2016). Targeting has been done using various cancer-specific receptors such as EGFR, EpCAM, integrins, FGF receptor, carbonic anhydrase IX protein G250 (Jongmans et al. 2003), CD40 (Tillman et al. 1999; Tillman et al. 2000), various organ- and tumor homing peptide receptors (Trepel et al. 2000), mesothelin (MSLN) (Breidenbach et al. 2004), prostate-specific membrane antigen (PSMA) (Kraaij et al. 2005), VEGFR2, Tie2 receptors (Haisma et al. 2010) and Ly-6D (van Zeeburg et al. 2010).

Tumor-selective replication involves both **transcriptional** and **translational** targeting. The main objective behind selective-transcription is to introduce regulatory elements of cancer-specificity in the viral genome to control the viral genes responsible for virulence and pathogenesis. These can either be the genes controlling the toxicity of virus or the immunomodulation induced by virus to subjugate host anti-viral response. Selective transcription involves developing a viral vector under the control of a tumor specific promoter such as hTERT (telomerase promoter), osteocalcin, hepatocellular carcinoma specific promoters (Foka et al. 2010), HIF-responsive promoter (Longo et al. 2011), prostate

cancer specific promoter (Lee et al. 2010)and alphafetoprotein amongst others. The promoter of osteocalcin, a protein induced by vitamin-D3 in bone forming cells incorporated into adenoviral vector makes it conditionally replicative in renal carcinoma cells (Hsiao et al. 2012). HSV with re-insertion of γ34.5 under E2F-responsive cellular B-myb promoter in an ICP6/γ34.5 deletion mutant replicate selectively in neuroblastoma cells and cycling fibroblasts (Chung et al. 1999). Similarly, Musashi1 promoter control over γ34.5 gene of HSV made the recombinant virus specific for malignant glioma cells (Kanai et al. 2005). Adenoviral vectors have also been generated with E2F-1 and CTP-1 promoters to induce targeted transcription (Dobbelstein 2004; Ko et al. 2005). Dual targeted oncolytic adenovirus has also been generated bearing both Oct-4 response elements (ORE) and Hypoxia response elements (HRE) to effectively lyse xenograft model of human bladder carcinoma (Lu et al. 2015).

Translational targeting aims to exploit the defects in signaling pathways for interferons and other anti-viral agents in cancer cells to facilitate the replication and multiplication of recombinant virus. This method can be seen as almost substitutive in action for an oncolytic virus with essential gene deleted or mutated leaving it susceptible to immune clearance by normal cells but thrive in its multiplication in cancer cells. For example, VSV with mutant M is unable to replicate in normal cells but can successfully infect and replicate in cancer cells (Barber 2005; Stojdl et al. 2003; Stojdl et al. 2000). Non-neurovirulent phenotype of poliovirus has been generated by replacing the cell-type specific IRES of poliovirus (neuronal cells) with IRES of human rhinovirus type 2 to target glioma cells (Gromeier et al. 2000).

In many cases oncolytic viruses are designed to exploit the pathways responsible for induction of apoptosis and cell death in cancer cells. Viruses are designed to multiply in cancer cells by exploiting the abrogated cell cycle machinery which may cause cell death to halt the virus progression. The general targets are cancer cells with defective or downregulated p53 tumor-suppressor pathway, RAS/PKR pathway, IFN/PKR pathway, p16/Rb pathway or other pro-apoptotic signals (Chiocca 2012).

c. Modifications for enhancing the toxicity of virus towards cancer cells: arming the virus

In addition to genetic modification for tumor-specific targeting, an oncolytic virus can also be enhanced for more robust anti-tumor activity by inserting cytotoxic elements into its genome. This ‘arming’ of oncolytic virus potentiates the therapeutic index of anti-cancer virus

mediated gene therapy by efficient delivery and expression of transgene of either viral or non-viral origin. Advent of replication competent oncolytic viruses has provided a platform where the viral genome can be outfitted with transgenes for tumor suppression, apoptosis, anti-angiogenesis, suicide and immunomodulation (Wong et al. 2010).It can act as partial compensation for the loss of virulence due to attenuation of viral vector for the safety of normal cells.

Inclusion of suicide genes presents an efficient mechanism of direct cell death but in majority of cases, after death of infected cell and immune clearance of virus, this effect is lost. To maintain a long term anti-tumor effect recombinant viruses can be used to incite downregulated host responses against cancer cells by encoding for secretable immunogenic factors. The induction of oncolytic viruses as tools for cancer therapy has opened up new avenues of establishing host immune response against tumor cells. Generally tumor cells produce immunosuppressive cytokines (e.g. TGFβ) and recruit cells to inhibit immune response (e.g. regulatory T-cells) to halt the host defense mechanism (Yang et al. 2010).With the alternative of oncolytic viruses, it is now possible to combine the debulking of tumor and attack on tumor vasculature due to viral infection and virus-induced cell lysis with effective activation of adaptive and innate immune response (Naik et al. 2012; Virotherapy 2014). In fact, 2015 FDA approval to T-Vec (Imlygic-talimogenelaherparepvec) for treatment of melanoma supports this possibility as in addition to double deletion of γ34.5 and α47genes of HSV-1, it has insertion of GM-CSF (granulocyte-macrophage colony-stimulating factor) at the deleted γ34.5 loci (Hu et al. 2006). Viruses are also being armed with secretory factors inducing apoptosis (Loya & Zhang 2015), functional p53 gene (Bressy et al. 2017), prodrug activation gene as well as immune checkpoints (Bauzon & Hermiston 2014) which are abrogated in tumor cells.

Prodrug activation gene: Systemic toxicity is one of the major side effects of chemotherapy. To overcome this shortcoming and to provide effective concentration of drugs at the tumor site, oncolytic viruses can be outfitted with prodrug activation gene. These are genes encoding for prodrug-converting enzymes such as thymidine kinase, cytosine deaminase, nitroreductase (Chen et al. 2004) etc. Gene directed enzyme prodrug therapy is where viral vectors are armed with suicide genes which can convert low cytotoxicity prodrugs into potent cytotoxic agents against cancer cells. This enables the therapy to be specific towards cancer with highly reduced systemic side effects as the prodrug is processed to become toxic only in

the infected cancer cells. This causes concentration of drug to become high in cancer cells and it can subsequently affect uninfected neighboring cancer cells. GLV-1h68, a strain of vaccinia virus carrying β-Galactosidase (*lacZ*) supplied with prodrug derived from a *seco*-analog of the natural antibiotic duocarmycin SA caused tumor regression and activation of intrinsic apoptotic pathway in breast cancer xenograft model. It circumvents the presence of anti-apoptotic viral genes in favor of toxicity of converted prodrug (Seubert et al. 2011).

Genes for enhancement of infective reach of oncolytic virus: Many enveloped viruses have fusion protein as one of their surface glycoproteins. It is responsible for cell to cell fusion spread of virus by syncytia formation. This property is very useful in an oncolytic agent as it engages uninfected neighboring cells to form syncytia ultimately leading to spread of virus and large scale cell death. Oncolytic viruses without this property can be armed with a fusogenic agent or protein to introduce fusion property. Generally, these proteins are derived from enveloped viruses such as measles virus and respiratory syncytial virus amongst others. There are some non-structural fusogenic proteins such as fusion associated small transmembrane proteins (FAST), expressed by several species of non-enveloped orthoreovirus (Chernomordik & Kozlov 2008).

Adenoviral vectors armed with a various fusogenic moieties have been developed and were found to have enhanced oncolysis such as Ad.RSV-F, Ad.VSV-G, Ad.MV-H/F etc. (Del Papa & Parks 2017). R849, a $\gamma_1$34.5 deletion mutant armed with LacZ gene at the deleted locus and co-infected with fusogenic HF strain of HSV-1, was able to generate a chimeric virus with deletion mutation of R849 and fusogenicity of HF which caused cancer cell-death of oral squamous cell carcinoma *in vitro* and *in vivo* (Takaoka et al. 2011).

Necrotic areas and fibrotic sarcomas present in tumor-microenvironment inhibit the lateral spread on oncolytic virus (Sauthoff et al. 2003). An Ad vector was designed harboring a peptide hormone relaxin to make tumor microenvironment more permeable. It increases the production of some matrix metalloproteases while decreasing the production of collagen and was found to have more effective intra-tumoral dissemination and anti-cancer activity than parental vector (Ganesh et al. 2007; Kim et al. 2006).

Immunomodulatory elements: Immunotherapy has been a success story in making with respect to cancer treatment. Idea of honing the host defense against transformed cells which thrive by subjugating various immune pathways has been equal parts achievable as it has

been out of reach. The complex interplay of roles by immune system as both anti and pro-tumorigenic activator has made anti-cancer immunotherapy an option which needs to maintain a delicate balance. Cancer immunotherapy relies on enhancing the potency of patient's immune system, ablating the tumor-induced immunosuppression and presenting the tumor as immunogenic target (Bauzon & Hermiston 2014). Immunosuppression and immune evasion is achieved by cancer cells by various evolving strategies such as: expression of immunosuppressive cytokines, down-regulation of MHC-I molecules, and expression of Fas ligand to kill reactive cytotoxic lymphocytes. This modified tumor microenvironment prevents the immune system from eradicating even tumor antigen expressing tumors (de Souza & Bonorino 2009). Oncolytic viruses by themselves illicit pro-inflammatory and immunogenic effects which leads to induction anti-tumor immune response (Tong et al. 2012). Insertion of cytokines and chemokines which can aid this immune response, into the viral genome, resulted in targeted delivery and activity of these molecules.

Non-replicating viral vectors have been employed for targeted gene therapy for a long time for various genetic disorders. Using similar approach replicating oncolytic viruses can be armed with these inhibitors for specific internalization and expression in tumor cells. The most studied and used molecule is GM-CSF (granulocyte macrophage-colony stimulating factor). T-Vec, an oncolytic HSV with GM-CSF insertion has been approved by FDA for cancer therapy and has given promising result (Andtbacka et al. 2015). Viruses encoding TAA have been generated to induce anti-tumor immune response. Attenuated VSV armed with dopachrometautomerase (DCT) caused upregulation of CD4 and CD8 antigen-specific T cell responses and showed enhanced oncolytic activity in the B16F10 murine melanoma model(Bridle et al. 2009). A recombinant VSV encoding interferon γ, VSVΔ51-IFNγ, showed enhanced anti-tumor immune response in murine tumor models (Bourgeois-Daigneault, Roy, et al. 2016). Vaccinia virus doubly expressing tumor antigen HY and GM-CSF showed enhanced anti-tumor immune response in MB49 bearing B6 mice, a murine transitional cell carcinoma tumor model. It was interesting to observe that MB49 cells normally express HY antigen but this expression is not able to elicit an immune response, whereas HY expression due to oncolytic vaccinia resulted in induction of HY specific CTLs (Yang et al. 2003). Recent study involving arming of vaccinia virus with DAI (DNA-dependent activator of IFN-regulatory factors) showed activation of adaptive immune cells in syngeneic melanoma mice models and demonstrated establishment of anti-tumor immunity by rejection of tumor establishment with the same cancer cells in treated mice (Hirvinen et al.

2016). Other molecules and viruses have also been tested such as Fas ligand, IL-27, IL-12, IFN α, β and RANTES reviewed by (Toth & Wold 2010) etc.

Recently, use of immune checkpoint inhibitors for reversal of immunosuppression of T-cells by cancer cells, has been a promising therapeutic approach. The bigger issue is targeted transport of these inhibitors in the cancer cells. Various checkpoint receptors have been identified as anti-cancer targets such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (Leach et al. 1996), programed cell death 1(PD1) (Keir et al. 2008) and its ligands PDL1 and PLD2, B and T-lymphocyte attenuator (BTLA), T-cell membrane protein 3 (TIM3), Lymphocyte activation gene 3 (LAG3), adenosine A2a receptor (A2aR), and the B7 family of inhibitory ligands (Bauzon & Hermiston 2014). Antibodies (IgGs), such as Ipilimumab (CTLA-4), Nivolumab (PD1) and Penbrolizumab (PD1) have been developed and in varying stages of clinical trials against various carcinomas. Ipilimumab has been approved by FDA for the treatment of metastatic melanoma (McDermott et al. 2013).

In many instances, use of immune checkpoint blocking antibodies lead to systemic immune-related adverse effects and many times cause restriction of viral replication (Marchini et al. 2016). To ensure the safety of this therapy and localization of antibodies to tumor site, it is beneficial to insert checkpoint inhibitors into the viral genome. Recent generation of Western Reserve (WR) oncolytic vaccinia virus harboring hamster monoclonal IgG (J43) recognizing murine PD-1 protein in three different constructs; the whole antibody (mAb), fragment antigen-binding (Fab) and single-chain variable fragment (scFv) tested on B16-F10 melanoma model and MCA 205 fibrosarcoma model showed significantly enhanced localization of J43 antibody at the tumor site with reduced tumor growth and increased survival in case of MCA 205 model (Kleinpeter et al. 2016). Earlier studies also support the feasibility of oncolytic viruses armed with antibodies against checkpoint inhibitors such as adenoviral vector Ad5/3-Δ24aCTLA4 coding and expressing complete human monoclonal antibody specific for CTLA-4 (Dias et al. 2012) and measles virus coding for anti CTLA-4 (MV-aCTLA-4) and PD-L1 (MV-aPD-L1) antibodies showed enhanced therapeutic benefits with antibody localization in B16-CD20 melanoma model with no immune-mediated toxicity (Engeland et al. 2014). With this approach alternative antibody formats such as Fabs and scFvs can also be used, preventing the IgG associated toxicities due to their large size causing increased serum half-life (Corsello et al. 2013).

Elements with direct anti-tumor effects: Oncolytic viruses can be generated to encode for molecules inducing cell death. These cytotoxic elements can enhance the inherent oncolytic properties of recombinant viruses by their targeted expression in cancer cells thus reducing the chances of systemic toxicity. It includes tumor-suppressors such as p16 and p53 (Bressy et al. 2017; Ma et al. 2009), pro-apoptotic proteins such as TNFα, TRAIL and IL-24 (Shashkova et al. 2008; Zhao et al. 2006), proliferation factors such as hTERT and surviving (Shen et al. 2009) as well as microRNAs or small hairpin RNAs targeting cell survival (Zheng et al. 2009). Adenovirus with wild type p53, Ad-Δ24-p53, showed enhanced cytotoxicity, reduced tumor mass, increased inflammatory response and increased survival of glioma and neuroblastoma xenograft models (Geoerger et al. 2004; Geoerger et al. 2005).

Anti-angiogenic agents: The sustained growth of tumor mass needs independent blood supply which is characterized by formation of new blood vessels (angiogenesis), vasculogenesis, vessel cooption and vasculogenic mimicry (Toro Bejarano & Merchan 2015a). This neovascularization is one of main factors responsible for inefficient therapeutic efficacy against tumors especially in case of systemically delivered therapies. Many agents have been approved by FDA to target this angiogenesis including bevacizumab, aflibercep, ramucirumab, sorafenib, sunitinib, pazopanib, axitinib, regorafenib against VEGF. The systemic toxicities as well as low bioavailability of the agents have proven to be a big hurdle in the complete success of this approach.

Insertion of anti-angiogenic factors in the genome of oncolytic virus can overcome this problem due to its targeted and conditional mode of infection and replication. Oncolytic viruses have been generated to specifically bind to tumor blood vessels, target tumor endothelial cell surface receptors and armed with anti-angiogenic elements. Most common target of this approach is VEGF wherein shRNA against VEGF (Yoo et al. 2007), soluble VEGF receptors (Guse et al. 2009; Jin et al. 2011) or artificial zinc finger proteins targeting VEGF promoter (Kang et al. 2008) have been generated. T-TSP1, a thrombospondin-1 expressing HSV-1 vector, showed enhanced anti-tumor effects (Tsuji et al. 2013). Soluble platelet factor-4 (PF-4) expressing HSV, bG47ΔPF4, showed anti-angiogenic effects in mouse models of glioblastoma and peripheral nerve sheet tumors (Liu et al. 2006). Theoretically this approach should lead to complete tumor cessation both due to damage to tumor vasculature leading to easy accessibility to the therapeutics and host immune effectors as well as inability of cancer cells to develop resistance.

In addition to above mentioned arming approaches, oncolytic viruses are also being armed against targets involved in transcriptional activation of tumorigenic genes as well as other factors of tumor progression. For example, adenoviral vector bearing p73 gene and a small hairpin RNA against HDAC1 (OV.shHDAC1.p73) was tested against malignant melanoma *in vitro* as well as *in vivo* and exhibited increased apoptosis due to caspase 3 cleavage, induction of autophagy, complete regression of tumor, extended survival and no resurgence within 16 weeks of observation (Schipper et al. 2014). Viruses are also armed for the purpose of imaging and visualization of tumor sequestration such as arming of measles virus with CEA and sodium iodide symporter (NIS) (Penheiter et al. 2012).

Oncolytic virus in combination therapies

Oncolytic viruses armed with pro-viral, anti-tumor genetic elements can either be developed as a standalone anti-cancer regimen or as a synergistic component of an established anti-cancer approach. These synergistic properties can be based on virus potentiating the anti-cancer toxicity of therapy or the therapy potentiating the replication and invasion of virus in the tumor cells.

The most logical option towards using oncolytic viruses for combination therapies would be to employ it with pre-existing therapy used for treatment of carcinomas. In doing so there is a possibility of either the chemotherapy or radiotherapy negatively affecting the viral replication (Mccart et al. 2000; Dingli et al. 2005). Thus it is imperative to observe and analyze the significance of anti-cancer potential of oncolytic viruses amidst all the established therapies and the role they can play in instituting a comprehensive model anti-cancer regimen.

Radiation therapy: Preclinical studies have indicated a synergistic role of radiotherapy and oncolytic Virotherapy (SJ et al. 2006). Variants of recombinant HSV have shown increased viral load (SJ et al. 1998) and appreciable toxicity against various carcinomas when combined with radiation therapy. For example G207, harboring ICP6/γ34.5 deletions combined with radiation therapy exhibited multi-fold increased toxicity and reduction of carcinoma in mice model of cervical cancer (Blank et al. 2002). Similar effects were seen in the case of colorectal cancer where the combination of G207 and low dose radiation resulted in upregulation of ribonucleotide reductase, causing increased anti-cancer toxicity (SF et al. 2002). Another HSV variant NV1066, harboring ICP0/ICP4/γ34.5 deletions, in combination

with radiation therapy resulted in reduction in non-small cell lung tumor mass (PS et al. 2005) with similar effects seen in mesothelioma (PS et al. 2007). Temporal sequestration of radiation with respect to viral gene expression has been reported to cause regression in high grade glioma mouse models as the irradiation is known to enhance the late promoter genes of HSV 1 (Advani et al. 2011; Markert et al. 2014; Bradley et al. 1999).

Combination of oncolytic adenovirus and radiation has also shown significantly greater toxicity as compared to both the therapies on their own (Idema et al. 2007; Rogulski et al. 2000). ONYX-015, mutant adenovirus with E1B-55k gene deletion has been reported to enhance radiation induced cytotoxicity in anaplastic thyroid cancer cells (Portella et al. 2003) as well as in human malignant glioma (Geoerger et al. 2003). Two prostate specific adenoviral vectors CV706 (Chen et al. 2001) and CV787 (Dilley et al. 2005) in combination with radiation resulted in inhibition of tumor growth, tumor mass reduction, and reduction of serum prostate-specific antigens in xenograft mouse models for prostate cancer. Gendicine (E1/E3 deletions expressing p53 under Rous sarcoma virus promoter) in combination with radiation (Ma et al. 2008) and chemotherapy is approved as intra-tumoral therapy against head and neck squamous cell carcinoma in China (Peng 2005; Xia et al. 2004). AdΔ24 (24-bp deletioninC2 domain of E1A region rendering the virus ineffective in cells with intact Rb pathways) and AdΔ24-p53 (p53 gene in deleted E3 region) have shown increased anti-tumor efficacy and apoptotic cell death in combination with radiotherapy in therapy resistant glioma models (Idema et al. 2007).

VSV expressing tumor associated antigens (TAA) has shown significant reduction in locally established and metastasized mouse model of oligo-metastatic melanoma in combination with stereotactic ablative radiation therapy (SABR). The tumor regression was associated with priming of substantial tumor-infiltrative CD8+ T-cell response (Blanchard et al. 2015).

Reovirus in combination with radiation therapy in murine-human colorectal carcinoma model has shown synergistic oncolytic effect as compared to standalone therapy even at low input MOI of reovirus. This combination showed statistically significant death in cell lines relatively resistant to reovirus mediated oncolysis, hinting towards the fact that this synergism is not simply additive but is causative due to increased apoptosis and bystander effect (Twigger et al. 2008). Combination of T3D, a non-pathogenic reovirus with radiation therapy showed increased viral replication due to CUG2 upregulation causing downregulation of pPKR and peIF2-α activating mitochondrial apoptotic signaling in both

BRAF- Ras mutant , BRAF- Ras wild type cell lines and BRAF mutant xenograft mouse model of malignant melanoma which is generally resistant towards radiation and chemotherapy (McEntee et al. 2016).

GLV-1h68, construct of oncolytic vaccinia virus has shown induction of intrinsic apoptotic pathway by downregulation of anti-apoptotic Bcl-2 proteins when combined with external beam radiation therapy as compared to both standalone therapies leading to decreased tumor mass and increased survival in a human xenograft model (Wilkinson et al. 2016).

Chemotherapy: The road to develop OVs as an efficient standalone therapy is still not completely paved thus all major studies and trials focus towards using these viruses with ongoing chemotherapeutic modalities. In many instances the dosage of both chemotherapy and OV dictates the synergism and outcome of the combination. Case in point is the CFDA approval of Gendicine for treatment of not only head and neck squamous cell carcinoma in combination of chemotherapy but of clinical studies involving both the agents against various cancers such as hepatocellular carcinoma. The use of Gendicinein combination with doxorubicin, camptothecin or 5-flurouracil resulted in increased patient survival and the quality of life (Guan YS, Sun L, Zhou XP 2005). Oncorine (H101), a derivative of ONYX-015 showed promising anti-cancer effects against many tumor cells having either mutated or normal p53 gene. It also showed enhanced anti-tumor effects in naso-pharyngeal and squamous cell carcinoma patients especially in combination with cisplatin and 5-fluorouracil (Xia et al. 2004; Ma et al. 2008) and was approved as therapy for head and neck squamous cell carcinoma in China. Similarly, Advexin (E1/E3 deletions expressing p53 under CMV promoter) in combination with methotrexate showed enhanced toxicity as compared to both the therapies as standalone in phase III clinical trials against advanced recurrent head and neck squamous cell carcinoma (Nemunaitis et al. 2009). Silica implants bearing Ad5-Δ24-RGD and Ad-Δ24-RGD-GMCSF in combination with gemcitabine have shown marked increase in survival of mouse and hamster xenograft model of peritoneal disseminated pancreatic cancer (Kangasniemi et al. 2012). Also both the viral constructs showed decrease in tumor markers and conversion of progressive state of different cancers to stable disease in almost 50% of patient population tested (Pesonen et al. 2012).

Oncolytic WT reovirus has shown significant synergistic anti-tumor toxicity with a low dose of docetaxel in a murine flank model of hormone refractory metastatic prostate cancer as compared to modest or negligible affects respectively as a single therapeutic agent. This effect was partially due to microtubular stabilization of cells by docetaxel promoting mitotic arrest resulting in apoptotic induction (Heinemann et al. 2011). Combination of chemotherapy and virotherapy has also been exploited to overcome the constraints of neutralizing antibodies in the patients by using chemotherapeutic agent as an immuno-modulator and for efficient delivery of virus to the tumor site. For example, administration of cyclophosphamide prior to using reovirus in phase I clinical trials of refractory or metastatic solid tumors resulted in no rise in neutralizing antibody baseline level (Roulstone et al. 2015). Similar effects were seen during co-administration of gemcitabine and Reolysin (Lolkema et al. 2011). Combination of cisplatin, paclitaxel and Reolysin also showed significant rise in overall survival of refractory or metastatic head and neck cancer patients (Karapanagiotou et al. 2012). Paclitaxel in combination with oncolytic rhabdovirus, Maraba-MG1 showed prolonged survival in various murine breast cancer models (Bourgeois-Daigneault, St-Germain, et al. 2016). Doxorubicin and rituximab in combination with Newcastle disease virus has shown enhanced cytotoxicity against haematological malignancies such as plasmacytoma and non-hodgkin lymphoma *in vitro* (Al-Shammari et al. 2016).

Recombinant vaccinia virus, GLV-1h68 used with cyclophosphamide in mice model of human lung adenocarcinoma has shown complete loss of characteristic haemorrhagic phenotype of the disease in addition to reported decrease in tumor growth, angiogenesis, further leading to EGF downregulation as well as an increase in viral distribution within tumor and elevation of pro-inflammatory cytokines such as M-CSF-1, MCP-1, MCP-5 and chemokine, eotaxin (Hofmann et al. 2014). In a larger context the oncolytic potential of vaccinia virus has been observed in pre-clinical studies with various cancers such as breast cancer including breast cancer stem-like cells (Wang et al. 2012), squamous cell carcinoma (Yu et al. 2009), salivary gland carcinoma (Chernichenko et al. 2013), human sarcomas (He et al. 2012) etc. Cyclophosphamide has been used as a chemotherapeutic agent for treatment of various carcinomas thus it can be arguably said that this combination if successful in clinical settings can generate a viable therapeutic model.

Histone deacetylase inhibitors (HDACi): These are a class of chemotherapeutic agents which have already found approval for lymphoma therapy (Garber 2007). In many instances

carcinogenesis and tumor progression have been attributed to deregulation of HDACs. There has been a rising interest in use of HDACis with oncolytic viruses to enhance the oncolysis as these have been shown to not only hyperacetylate nucleosome core proteins to drive expression of anti-tumor genes but also acetylate non-histone proteins such as chaperones, regulators of DNA damage repair, transcription factors including p53 (Bressy et al. 2017). Many molecules are being investigated at clinical levels for treatment of various malignancies out of which vorinostat (cutaneous T-cell lymphoma), romidepsin (cutaneous and peripheral T cell lymphoma) and belinostat (refractory peripheral T cell lymphoma) have been approved by FDA (Marchini et al. 2016).

VSV variant VSVΔ51 in combination with vorinostat has been found to increase viral replication, apoptosis, decrease IFN expression and IFN-mediated response in xenograft models of refractory prostate, melanoma, colon, breast and ovarian tumors(Nguyen et al. 2008). Replication-deficient adenoviral vector Ad.CMV-GFP in combination with romidepsin caused increase in viral entry CAR receptors in xenograft model of melanoma causing increased viral infectivity with respect to internalization of virus into the tumor cells (Goldsmith et al. 2007). Patient derived xenograft model of glioblastoma showed differential activation of multiple cell death pathways upon synergistic use of LBH589 and Scriptaid with Ad-Δ24-RGD (Pont et al. 2015). HSV-1 variant G47Δ and trichostatin A decreased VEGF secretion and angiogenesis in xenograft model of glioma and colorectal cancer (Liu et al. 2008). Alternatively, this combination of oncolytic virus and HDACi is not limited to a two-component therapy as described earlier under arming of oncolytic viruses.

Immune checkpoint inhibitors: Emerging studies suggest that immunogenic cell death is a major component of oncolytic virus induced cell death. It causes establishment of an anti-tumor immunity by either secretion/release or exposure of DAMPs (danger-associated molecular patterns) and PAMPs (pathogen-associated molecular patterns) causing maturation of antigen-presenting cells leading to activation of antigen-specific CD4+ and CD8+ T-cells (Bartlett et al. 2013; Guo et al. 2014). Antibodies such as Ipilimumab (CTLA-4), Nivolumab (PD1) and Penbrolizumab (PD1) have been approved by FDA for treatment of advanced metastatic melanoma (Bauzon & Hermiston 2014) due to observed reversal of tumor cell mediated repression of T-cell response by blocking immune checkpoint proteins (Marchini et al. 2016). Examples can be found in pre-clinical studies with VSV and CTLA-4 inhibitor in Her2/neu positive D2F2/E2murine mammary tumor model showing complete remission and

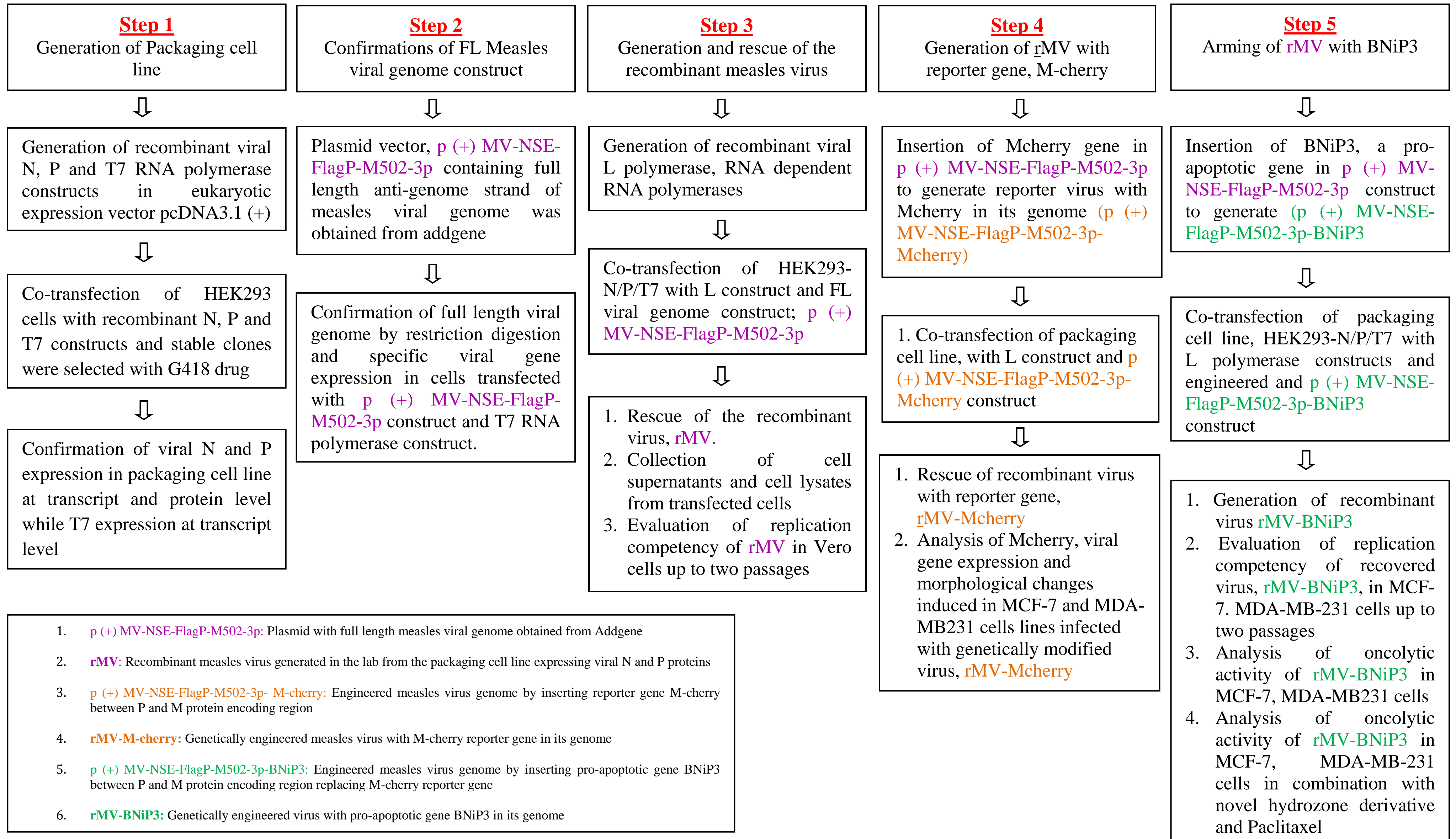
immunity towards tumor antigens (Gao et al. 2008). Intra-tumoral newcastle disease virus and anti-CTLA-4 antibody therapy caused tumor regression with increased survival rate in bilateral B16-F10 melanoma mouse model and TRAMP C2, a prostate adenocarcinoma transgenic mouse model (Zamarin et al. 2014). Phase I clinical trials with T-Vec and Ipilimumab or Penbrolizumab for metastatic melanoma therapy have shown encouraging results (Puzanov et al. 2013).

In many instances, use of immune checkpoint blocking antibodies lead to systemic immune-related adverse effects and restriction of viral replication (Marchini et al. 2016). To ensure the safety of this therapy and localization of antibodies to tumor site, it is beneficial to insert checkpoint inhibitors into the viral genome. Recent generation of Western Reserve (WR) oncolytic vaccinia virus harboring hamster monoclonal IgG (J43) recognizing murine PD-1 protein in three different constructs; the whole antibody (mAb), fragment antigen-binding (Fab) and single-chain variable fragment (scFv) tested on B16-F10 melanoma model and MCA 205 fibrosarcoma model showed significantly enhanced localization of J43 antibody at the tumor site with reduced tumor growth and increased survival in case of MCA 205 model (Kleinpeter et al. 2016). Earlier studies also support the feasibility of oncolytic viruses armed with antibodies against checkpoint inhibitors such as adenoviral vector Ad5/3-Δ24aCTLA4 coding and expressing complete human monoclonal antibody specific for CTLA-4 (Dias et al. 2012) and measles virus coding for anti CTLA-4 (MV-aCTLA-4) and PD-L1 (MV-aPD-L1) antibodies showed enhanced therapeutic benefits with antibody localization in B16-CD20 melanoma model with no immune-mediated toxicity(Engeland et al. 2014).

There are other modalities which can also be combined with oncolytic viruses such as radionucleotides, nucleotide analogues (Ottolino-Perry et al. 2010) and another oncolytic virus as observed in the study involving intra-tumoral administration of reovirus and systemic delivery of VSV encoding cDNA library of melanoma antigens (VSV-ASMEL) in a B16-melanoma model showed significant increase in survival (Ilett et al. 2017).

Genetically modified viruses for oncolytic purpose therefore have much to offer as a therapeutic to target a wide variety of cancers. They can either directly be used following desired manipulations or in combination with pre-existing cancer therapeutics to improve their anti-cancer effects. Viruses, disease causing pathogens might soon become accessible to oncologists as effective drugs to treat cancer; not just one disease but group of diseases.

MATERIALS & METHODS


WORK FLOW
Step 1
Generation of Packaging cell line
Generation of recombinant viral N, P and T7 RNA polymerase constructs in eukaryotic expression vector pcDNA3.1 (+)
Co-transfection of HEK293 cells with recombinant N, P and T7 constructs and stable clones were selected with G418 drug
Confirmation of viral N and P expression in packaging cell line at transcript and protein level while T7 expression at transcript level
Step 2
Confirmations of FL Measles viral genome construct
Plasmid vector, p (+) MV-NSE-FlagP-M502-3p containing full length anti-genome strand of measles viral genome was obtained from addgene
Confirmation of full length viral genome by restriction digestion and specific viral gene expression in cells transfected with p (+) MV-NSE-FlagP-M502-3p construct and T7 RNA polymerase construct.
Step 3
Generation and rescue of the recombinant measles virus
Generation of recombinant viral L polymerase, RNA dependent RNA polymerases
Co-transfection of HEK293-N/P/T7 with L construct and FL viral genome construct; p (+) MV-NSE-FlagP-M502-3p
1. Rescue of the recombinant virus, rMV.
2. Collection of cell supernatants and cell lysates from transfected cells
3. Evaluation of replication competency of rMV in Vero cells up to two passages
Step 4
Generation of rMV with reporter gene, M-cherry
Insertion of Mcherry gene in p (+) MV-NSE-FlagP-M502-3p to generate reporter virus with Mcherry in its genome (p (+) MV-NSE-FlagP-M502-3p-Mcherry)
1. Co-transfection of packaging cell line, with L construct and p (+) MV-NSE-FlagP-M502-3p-Mcherry construct
1. Rescue of recombinant virus with reporter gene, rMV-Mcherry
2. Analysis of Mcherry, viral gene expression and morphological changes induced in MCF-7 and MDA-MB231 cells lines infected with genetically modified virus, rMV-Mcherry
Step 5
Arming of rMV with BNiP3
Insertion of BNiP3, a pro-apoptotic gene in p (+) MV-NSE-FlagP-M502-3p construct to generate (p (+) MV-NSE-FlagP-M502-3p-BNiP3
Co-transfection of packaging cell line, HEK293-N/P/T7 with L polymerase constructs and engineered and p (+) MV-NSE-FlagP-M502-3p-BNiP3 construct
1. Generation of recombinant virus rMV-BNiP3
2. Evaluation of replication competency of recovered virus, rMV-BNiP3, in MCF-7. MDA-MB-231 cells up to two passages
3. Analysis of oncolytic activity of rMV-BNiP3 in MCF-7, MDA-MB231 cells
4. Analysis of oncolytic activity of rMV-BNiP3 in MCF-7, MDA-MB-231 cells in combination with novel hydrozone derivative and Paclitaxel
1. p (+) MV-NSE-FlagP-M502-3p: Plasmid with full length measles viral genome obtained from Addgene
2. rMV: Recombinant measles virus generated in the lab from the packaging cell line expressing viral N and P proteins
3. p (+) MV-NSE-FlagP-M502-3p- M-cherry: Engineered measles virus genome by inserting reporter gene M-cherry between P and M protein encoding region
4. rMV-M-cherry: Genetically engineered measles virus with M-cherry reporter gene in its genome
5. p (+) MV-NSE-FlagP-M502-3p-BNiP3: Engineered measles virus genome by inserting pro-apoptotic gene BNiP3 between P and M protein encoding region replacing M-cherry reporter gene
6. rMV-BNiP3: Genetically engineered virus with pro-apoptotic gene BNiP3 in its genome

Cell lines

African green monkey kidney epithelium cell line Vero, human embryonic kidney epithelium cell line HEK293 and human breast carcinoma cell lines MCF-7 and MDA-MB-231 were procured from NCCS, Pune. Vero cells were propagated in Modified Eagle Medium (MEM) whereas HEK293, MCF-7 and MDA-MB-231 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% 100X antibiotic solution.

Cell cultures

Vero cells were used for the propagation of wild type measles virus. Packaging cell line was generated with HEK293 cells to support the formation of engineered virus particles from plasmid encoding measles virus full length genome. MCF-7 and MDA-MB-231 cells were used as representative breast carcinoma to ascertain the oncolytic activity of recombinant virus generated. Cells were maintained as monolayers in humidified atmosphere of 5% CO_2 at 37ºC. Experiments were performed with cells of early passages. Cells were cryopreserved at regular intervals at initial passages by preserving the exponentially growing cells in media supplemented with 20% FBS and 10% DMSO which acts as a cryoprotectant. Cells were kept at -80°C for long term storage.

Reagents

Reagents for cell culture were procured from Hi-media. All conventional and fast digest restriction enzymes, dNTPs mix, random hexamer primer, RibolockRNAse inhibitor, M-MuLV reverse transcriptase, Dreamtaq DNA polymerase, T4 DNA ligase and DNA markers were purchased from Fermentas, USA. Kapa Hifi polymerase with 25mM dNTP mix was obtained from Kapa Biosystems, USA and Herculase polymerase from Agilent technologies, USA. Trizol, RNA extraction reagent, was obtained from Biobasic Inc. Agarose Gel Elution Kit was obtained from MDI. GeneJET Plasmid Miniprep kit was obtained from Thermo Fisher Scientific., UK. Primary antibodies against measles virus nucleoprotein, phosphoprotein and hemagglutinin protein as well as BNiP3 protein were procured from Santacruz, USA. GenElute™ HP Plasmid Midiprep Kit, Anti-mouse FITC conjugated secondary antibody, Hoechst stain and Paclitaxel was purchased from Sigma-Aldrich, USA. H2 compound was a kind gift from Prof. Amir Azam, JMI, New Delhi. Lipofectamine 2000,

G418 (geneticin), EnzCheck Caspase 3 apoptosis kit and Alexa fluor 488 Annexin V/PI Dead cell apoptosis kit were procured from Life technologies, Invitrogen, USA.

Virus and its propagation

Clinical isolate of wild type measles virus was obtained from AIIMS, New Delhi. Virus was propagated in Vero cells as they serve as a natural host for laboratory propagated measles virus. Initial seeding density of 0.1×10^6cells/ml was used for a T25 cm^2 flask. For infection, 100µl of viral lysate was diluted in 2ml of DMEM and inoculated in T25 cm^2 flask for viral adsorption at 37ºC; 5% CO_2 with the same volume of DMEM, without virus, used as inoculum for mock infected flask to serve as control. Two hours post-adsorption inoculum was removed and flasks were fed with DMEM supplemented with 2% FBS. Cells were observed for the development of cytopathic effect of syncytia formation after which virus was harvested by three freeze-thaw cycles, collected in cryovials and stored at -80°C.

Centrifugation enhanced culture (CEC) method of viral infection was used to increase the adsorption and subsequently the titer of virus. In this, the cells with viral inoculum were centrifuged at 700×g at room temperature (RT) for one hour to enhance the adsorption of virus onto the host cell surface. Once the viral titers were increased, routine cultures were done as per the protocol described above.

Generation of packaging cell line/complementing cell line expressing measles virus structural proteins and T7 RNA polymerase

1. Cloning of measles virus genes encoding nucleoprotein and phosphoprotein in eukaryotic expression vector pcDNA3.1+

Nucleoprotein (N) is a capsid protein and phosphoprotein (P) is a co-factor for viral L Protein, an RNA dependent RNA polymerase. Both of them are integral components of viral ribonucleoprotein complex which is essential for viral replication, transcription and packaging.

a. Viral RNA isolation

Total RNA was isolated from cells infected with measles virus using Trizol method. Micropipette tips and micro centrifuge tubes used for RNA isolation were treated with 0.1% DEPC and autoclaved. Frozen stocks of virus-infected cells were thawed; 1ml Trizol reagent

was added to 200µl of suspension and incubated for 5min at RT to allow complete lysis followed by addition of 200µl of chloroform. Sample was gently shaken for 15sec, incubated at RT for 2-3min and centrifuged at 12,000×g for 15min at 4ºC. Upper aqueous phase was transferred to a fresh 1.5ml tube and chloroform extraction was repeated to enhance the purity. RNA was precipitated by adding 500µl of isopropanol with 10ng glycogen to the aqueous phase followed by incubation for 10min at RT or alternatively for 1hour at -20 ºC for enhanced precipitation. Sample was centrifuged at 12,000×g for 10min at 4ºC and the pelleted RNA was washed with 75% ethanol (prepared in DEPC-treated water) at 7,500×g for 5min at 4ºC. RNA pellet was air-dried, re-suspended in 10µl of DEPC-treated water, incubated at 55ºC for 10min and kept on ice. Ribolock RNAse inhibitor, 0.5µl, was added to the sample and RNA was stored at -80ºC until further use.

b. cDNA synthesis

Isolated viral RNA was reverse transcribed to generate positive strand cDNA following reverse genetics paradigm. For a reaction volume of 20µl, 5µl of total RNA, 1µl of random hexamer primer and 7µl of DEPC-treated water was added in a nuclease free PCR tube, incubated at 65ºC for 5min and kept on ice. Reaction mix was completed by adding 4µl of 5X reverse transcriptase buffer, 1 µl (20U) of M-MuLV reverse transcriptase enzyme and 2µl of 10mM dNTPs. It was further incubated at 25ºC for 10min, 37ºC for 60min followed by enzyme inactivation at 70ºC for 10min. The cDNA was stored at -20ºC until further use.

c. Amplification and cloning of Nucleoprotein and Phosphoprotein gene

Using cDNA as the template, genes encoding measles virus Nucleoprotein (N) and Phosphoprotein (P) were amplified using specific primers with restriction enzymes *Eco*RI and *Xho*I recognition sites.

Gene	Primer	Sequence (5’→ 3’)
Nucleoprotein	Forward primer	ATATGAATTCACCATGGCCACACTTTTGAGGAG
	Reverse primer	ATATCTCGAGCTAGTCTAGAAGATCTCTGTC
Phosphoprotein	Forward primer	ATATGAATTCACCATGGCAGAAGAGCAGGCAC
	Reverse primer	ATATCTCGAGCTACTTCATTATTATTATCTTCATC

For a 25µl reaction volume, 5µl of Kapa HiFi buffer, 1µl of 10mM dNTPs, 1µl of forward and reverse primer each, 0.5U of Kapa HiFi Polymerase, 0.5µl of $MgCl_2$, and 5µl of cDNA template was added with remaining volume made up with nuclease free water. Initial denaturation was done at 95ºC for 3 min followed by 30 cycles of denaturation at 95ºC for 45sec, annealing at 55ºC for 1min and extension at 72ºC for 1min. Final extension was done at 72ºC for 10min. The amplified product was resolved on 1% agarose gel, visualized under UV and image was captured on GelDoc System (Bio-Rad).

Amplified products of N and P were excised from the gel and purified using the Agarose Gel Elution Kit (MDI) as per the manufacturer's instructions. Purified amplicons were cloned in vector pcDNA3.1+ between *Eco*RI and *Xho*I restriction sites.

2. Cloning of T7 RNA Polymerase gene in eukaryotic expression vector pcDNA3.1+

As the full length measles virus genome was cloned under bacteriophage T7 promoter, packaging cell line stably expressing T7 RNA polymerase and measles virus proteins, N and P was generated. For the same, T7 RNA polymerase was cloned into eukaryotic expression vector, pcDNA3.1+.

a. Genomic DNA isolation from BL21pLysS cell culture

Genomic DNA was isolated from overnight BL21pLysS cells (contains T7 RNA polymerase) grown in LB medium, cells were pelleted down at 12000×g for 1min in a micro centrifuge tube; re-suspended in 600µl of lysis buffer (10mM Tris-Cl pH 8.0; 1mM EDTA pH 8.0 (TE), 10% SDS, proteinase K) and solution was incubated at 37ºC for 1hour. Phenol: chloroform extraction was done twice to isolate DNA and remove protein contamination. Phenol was removed by adding equal volume of chloroform to the upper aqueous phase and solution was centrifuged at 12000×g for 5min and aqueous layer was transferred to a new tube. DNA was precipitated by adding 2.5 volumes of cold ethanol to the sample and incubating at -20ºC for 1hour. Precipitated DNA was pelleted by centrifuging the tube at 12000×g; 4ºC for 15min. DNA pellet was rinsed with 1ml of 70% ethanol (RT); centrifuged at 12000×g for 2min; supernatant was discarded and pellet was air-dried. DNA was re-suspended in 100µl TE buffer and stored at -80ºC.

b. *Amplification of T7 RNA Polymerase gene*

Using genomic DNA as the template, T7 RNA Polymerase encoding gene was amplified using specific primers with restriction enzymes *Eco*RI and *Xho*I recognition sites.

Gene	Primer	Sequence (5'→ 3')
T7 RNA polymerase	Forward primer	ATATGAATTCACCATGAACACGATTAACATCGC
	Reverse primer	ATATCTCGAGTTACGCAACGCGAAGTCCG

For a 25µl reaction volume, 5µl of Kapa HiFi buffer, 1µl of 10mM dNTPs, 1µl of forward and reverse primer each, 0.5U of Kapa HiFi Polymerase, 0.5µl of $MgCl_2$, and 0.5µl of genomic DNA template was added with remaining volume made up with nuclease free water. Initial denaturation was done at 95ºC for 3min followed by 30 cycles of denaturation at 95ºC for 45sec, annealing at 51ºC for 1min and extension at 72ºC for 2min. Final extension was done at 72ºC for 10min. The amplified product was visualized and excised from the gel and purified using the Agarose Gel Elution Kit as per the manufacturer's instructions. Purified T7 RNA polymerase amplicon was cloned into pcDNA3.1+ between *Eco*RI and *Xho*I restriction sites.

c. *Confirmation of clones*

Colony PCR: Clones were screened for the presence of recombinant plasmid by colony PCR directly from bacterial cells. Single colonies were picked and inoculated in 3ml of LB broth. The primary culture was incubated overnight at 37°C; 200rpm. Using 2µl of the primary culture PCR was set up using N, P and T7 RNA polymerase gene specific primers respectively under the same reaction conditions as used for gene amplifications.

Fallout of restriction digestion: Plasmid DNA was isolated from overnight primary culture of positive clones, ascertained by colony PCR, using Gene JET Plasmid Miniprep kit (Thermo Fisher Scientific) following the manufacturer's instructions. Insertion of N, P and T7 RNA polymerase genes was screened by digesting 1µg of isolated plasmid DNA with 1U each of *Eco*RI and *Xho*I restriction enzymes. Digestion mixture was incubated for 2hours at 37ºC and analysed on 1% agarose gel.

Confirmation of clones by sequencing: Recombinant clones generated were further confirmed by sequencing of the inserted N, P and T7 RNA polymerase genes using the gene specific forward and reverse primers. DNA sequences obtained were aligned with the reference sequences available for measles virus nucleoprotein and phosphoprotein genes and T7 RNA polymerase gene of bacterial origin, within the NCBI nucleotide database using the BLAST tool.

3. Co-transfection of HEK293 cells for the generation of packaging cell line stably expressing Nucleoprotein, Phosphoprotein and T7 RNA Polymerase

a. Linearization of plasmid constructs of Nucleoprotein, Phosphoprotein and T7 RNA Polymerase

Plasmid constructs of measles virus nucleoprotein, phosphoprotein and T7 RNA polymerase were linearized using internal *Sca*I restriction site of pcDNA3.1+ vector backbone. It was done to avoid genomic integration or recombination in HEK293 cells. A digestion reaction of 20µl volume was setup, each consisting 1X fast digest buffer, 1µg plasmid vector and 1U of fast digest *Sca*I restriction enzyme. Reactions were incubated at 37ºC for 30min in a water bath. Digested products were resolved on agarose gel, excised out and purified using the Agarose Gel Elution Kit. Eluted plasmids were quantified and further used for transfections in HEK293 cells for the generation of cell line stably expressing nucleoprotein, phosphoprotein and T7 RNA polymerase.

b. Transfection

HEK293 cells, at a density of 0.1×10^6cells/ml, were seeded in a 35mm culture dish and transfection experiments were carried out at 90% confluency (in duplicate). The cells were transfected with linearized plasmids for recombinant N, P and T7 RNA polymerase and mock transfected with empty vector pcDNA3.1+ alone, using Lipofectamine 2000 reagent. In a microcentrifuge tube, 1.5µg each of plasmid DNA of N and P and 1µg of T7 RNA polymerase were added to 250µl of Opti-MEM reduced serum medium (Invitrogen). In another tube, 10µl of Lipofectamine 2000 reagent was diluted in 250µl of Opti-MEM. Both tubes were incubated for 5min at RT. The diluted transfection reagent was added to the diluted plasmid DNA and the mixture was further incubated at RT for 20min. HEK293 cells were washed with 1X PBS (pH7.2) followed by serum free DMEM; the DNA-Lipofectamine solution was added to the cells and medium was made up to 2ml using DMEM supplemented

with 10% FBS and cells were kept at 37ºC; 5% CO_2. Expression of N, P and T7 RNA polymerase was checked 24-48hours post transfection by transcript amplification as well as immunoblotting and immunofluorescence assays using specific antibodies.

c. Generation of clones

Twenty four hours post-transfection, cells (2nd set) were fed with DMEM supplemented with 10% FBS and 600µg/ml G418 as a selection marker. Approximately 72hours after introduction of G418 selective medium, massive cell death was observed. Media with dead cells was removed and the remaining cells were fed with fresh G418 selective medium. Regular media changes were done till the cells formed visible clones which were picked by adding 10µl of warm trypsin-EDTA on the clone with an enzyme tip to disassociate the cells and seeding them in a single well of a 24-well culture dish. This process was followed with all the observed clones. The expanded clones were maintained in G418 selective medium till they formed a confluent monolayer where after they were dissociated and sub-cultured in two 35mm dishes each. Expression of measles virus nucleoprotein and phosphoprotein was confirmed by western blot analysis and immunofluorescence assay, transcripts were also checked for N, P and T7 RNA polymerase genes.

d. ***Confirmation of transfection and stable expression of nucleoprotein, phosphoprotein and T7 RNA polymerase in HEK 293 cells.***

Reverse transcription-polymerase reaction: RNA was isolated from co-transfected HEK 293 cells by Trizol method and cDNA was generated as described earlier. The cDNA was used as a template for amplification of N, P and T7 RNA polymerase genes using specific primers. Amplified products were analyzed in agarose gel. Similarly, presence of transcripts was also confirmed in clones after G418 selection.

Immunofluorescence staining: Presence of measles virus nucleoprotein and phosphoprotein was confirmed by immunofluorescence assay and immunoblot analysis. Transiently transfected HEK293 cells (24hours post-transfection) and clones obtained post G418 selection were grown onto the coverslips, washed twice with cold 1X PBS and fixed with chilled acetone for 20min at -20ºC. Cells were again washed with 1X PBS and incubated with anti-measles nucleoprotein and anti-measles phosphoprotein antibodies (1:100 dilution prepared in 1X PBS) overnight at 4ºC in a moist chamber. Unbound antibody was removed by repeated washes with 1X PBST (0.1% tween-20). Cells were further probed with anti-

mouse FITC conjugated secondary antibody (1:200 dilution prepared in 1X PBS) for 1hour at 37ºC in the moist chamber followed by three washes with 1X PBST. Nuclei were stained using 2.5µg/ml of Hoechst stain (Sigma) for 10min and excess stain was removed by two washes with 1X PBST. Coverslips were mounted on clean glass slides in 90% glycerol in PBS (pH8.0). Slides were visualized to detect the fluorescence signals under a Confocal Laser Scanning Microscope (Olympus FluoviewTM - FV1000) equipped with HeNe laser (488 nm) and pulsed diode laser (408nm). Images were acquired with PLAPON 60X O NA: 1.42 oil immersion objective using FV10SW1.7 software.

Immunoblot analysis: Total protein was isolated from transfected cells at 24hours post-transfection as well as from clones for selection. Cells were washed twice with cold 1X PBS; lysed with 100µl of lysis buffer (50mM Tris-CL pH 7.4, 150mM NaCl, 1% Triton X-100, 1mM EDTA pH8.0, 1mM Na_3VO_4, 1mM PMSF, 1X protease inhibitor cocktail) and incubated on ice for 30min to allow complete lysis. The cells were then scraped off, collected in pre-chilled 1.5ml micro centrifuge tubes and centrifuged at 14,000rpm for 20min at 4ºC. Supernatant containing the total cellular protein was collected in a fresh tube and protein concentration was determined by bradford assay.

Protein lysates were resolved on 10% SDS-PAGE and transferred to PVDF membrane at 150-170mA for 3hours. The membrane was blocked overnight with 3% BSA (in 1X TBST) at 4ºC and thereafter probed with mouse monoclonal anti-nucleoprotein (1:1000) and anti-phosphoprotein (1:1000) antibody in blocking buffer overnight. Unbound antibody was removed by repeated washes with 1XTBST (0.1% Tween-20). Anti-mouse HRPO (1:10,000 in 1XTBST) was used as the secondary antibody.

Clones with high expression of nucleoprotein and moderate expression of phosphoprotein were selected and further propagated for experiments. Clones were also cryopreserved at initial passages for future work.

Rescue of replication competent engineered Measles virus from complementing cell line

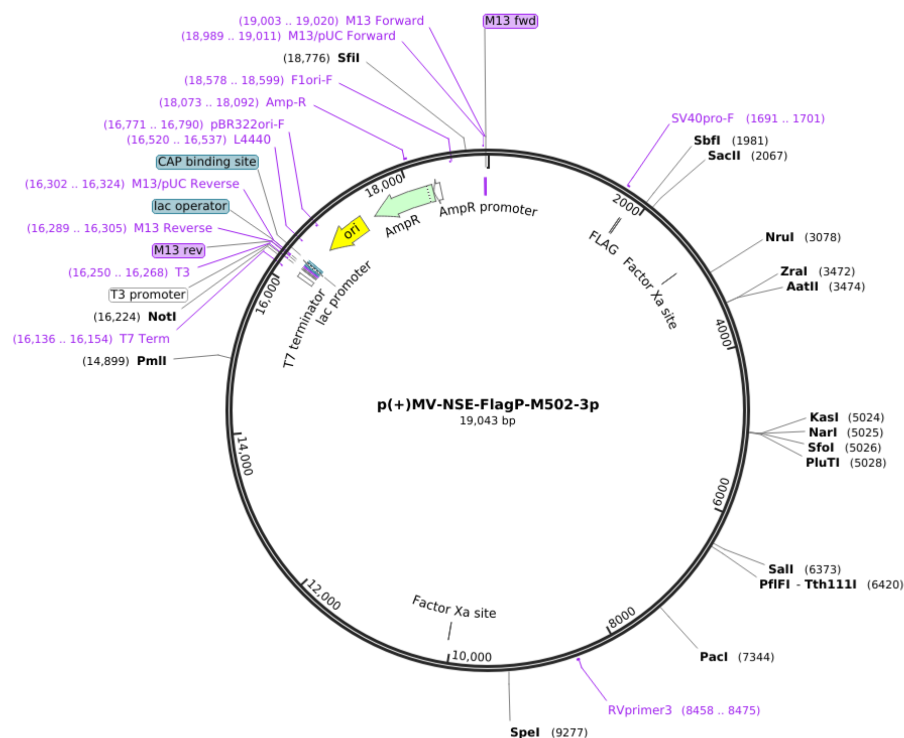
1. Measles virus full length genome plasmid construct

Plasmid vector, p (+) MV-NSE-FlagP-M502-3p, containing complete anti-genome sequence (positive sense) of measles virus Edmonston strain was generated in Branka Horvat's lab and obtained from Addgene, a non-profitable plasmid repository (plasmid # 58748). It is derived from p(+) MVNSe made in Roberto Cattaneo's lab which was further derived from the original p(+) MV15894 plasmid described by Martin Billeter's group and contains additional characteristic features (*refer appendix*).

Vector backbone: Measles virus anti-genome Species, insert Size: 15996bp (*Complete measles virus genome sequence: refer to appendix)*

Promoter: T7 Promoter

a. p(+) MV-NSE-FlagP-M502-3p plasmid map with measles virus anti-genome insert



b. Confirmation and Validation of measles virus genome plasmid construct

Cells transformed with p (+) MV-NSE-FlagP-M502-3p were supplied as stab culture; streaked onto LB agar plate with ampicillin and kept at 30ºC overnight. A single colony was picked and inoculated in 3ml LB broth. The primary culture was incubated overnight at 30°C; 200rpm.

Plasmid Isolation: A fraction of primary culture was used to isolate the plasmid DNA using Gene JET Plasmid Miniprep kit (Thermo Fisher Scientific) following the manufacturer's instructions. The isolated plasmid DNA was resolved on 1% agarose gel in TBE buffer to observe the approximate size of 19kb against 1kb DNA marker. To prepare the plasmid DNA for transfection, 50ml of LB broth was inoculated with 100µl of primary culture (1:500) and the culture was grown overnight at 30°C; 200rpm. Plasmid DNA was isolated using GenElute™ HP Plasmid Midiprep Kit (Sigma) as per the manufacturer's instructions.

Restriction digestion: Confirmation of plasmid was also done by digesting the isolated plasmid at unique restriction site listed by depositor and internal restriction sites obtained after analyzing the sequence available at Addgene. Depositors have listed presence of unique *Aat*II restriction site in p (+) MV-NSE-FlagP-M502-3p plasmid. To confirm this, 1µg of isolated plasmid DNA was digested in a 20µl reaction with 1U of fast digest *Aat*II enzyme for 30min at 37ºC. Linear DNA, following digestion, was incubated and analysed on 1% agarose gel. p (+) MV-NSE-FlagP-M502-3p has internal restriction sites for *Sca*I and *Bam*HI. To confirm the corresponding fragments sizes which would be generated upon digestion, 1µg of plasmid DNA was digested in a 20µl reaction with 1U each of fast digest *Sca*I and *Bam*HI and 1X fast digest buffer at 37ºC for 15min and analyzed on 1% agarose gel.

Amplification of viral transcripts: To check the transcription potential of p (+) MV-NSE-FlagP-M502-3p in HEK293, cells were co-transfected with varying ratios of p (+) MV-NSE-FlagP-M502-3p and T7 RNA polymerase constructs (following protocol described earlier). Twenty four hours post-transfection RNA was isolated from cells using Trizol and cDNA was synthesized using random hexamer (pdn6) primers. This cDNA was subjected to PCR amplification using measles viral nucleoprotein and phosphoprotein gene specific primers.

2. Cloning of measles viral Large Polymerase gene (viral RNA dependant RNA polymerase) in eukaryotic expression vector pcDNA3.1+

Large polymerase gene is a viral RNA dependent RNA polymerase (RdRp) responsible for transcription and replication of negative sense measles viral genome. Lack of machinery for RNA based transcription in host cells makes its external delivery to the packaging cell line essential.

a. Amplification of Large Polymerase gene

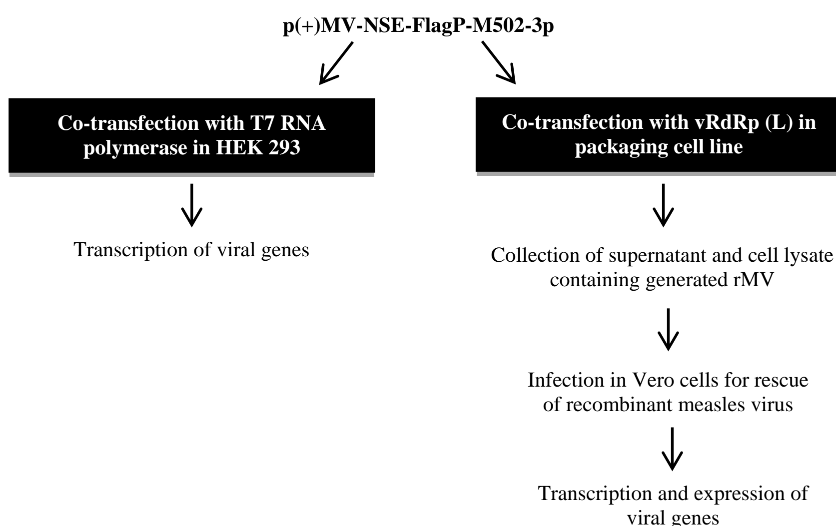
Using p(+) MV-NSE-FlagP-M502-3p as the template, viral Large Polymerase (L) gene was amplified using specific primers with restriction enzymes *Bam*HI and *Not*I recognition sites:

Gene	Primer	Sequence (5’→ 3’)
Large polymerase	Forward primer	ATATGGATCCACCATGGACTCGCTATCTGTCAAC
	Reverse primer	TATAGCGGCCGCTTAGTCCTTAATCAGGGCACTG

For a 25µl reaction volume, 5µl of 5X Herculase buffer (Agilent technologies, USA), 1µl of 10mM dNTPs, 1µl of forward and reverse primer each, 0.5µl of Herculase Polymerase, 0.5µl of $MgCl_2$, and 0.2µl of plasmid DNA template was added. Initial denaturation was done at 95ºC for 2min followed by 30 cycles of denaturation at 95ºC for 20sec, annealing at 57ºC for 30sec and extension at 72ºC for 3min 30sec. Final extension was done at 72ºC for 3min. The amplified product was excised from the gel, purified and cloned into eukaryotic expression vector pcDNA3.1+ between *Bam*HI and *Not*I restriction sites.

Recombinant L clone was confirmed by colony PCR and restriction enzyme digestion. Recombinant clone giving expected fallout was further confirmed by sequencing of the inserted L gene using 4 sets of gene specific internal nested primers to cover the complete gene sequence of approximately 6.5kb. DNA sequences obtained were aligned with the reference sequence available for measles virus L gene within the NCBI nucleotide database using the BLAST tool.

3. Generation and rescue of recombinant measles virus from packaging cell line



Replication-competent measles virus was generated from plasmid construct containing the genome of measles virus Edmonston strain by using reverse genetics paradigm. It has been reported in literature that in presence of necessary proteins, a complete virion can be generated from viral genetic material in absence of a parent virus in the host cell. This method was followed for this study, where packaging cell line provided the necessary proteins for the formation of viral ribonucleoprotein (vRNP) complex to propagate the replication and packaging of viral genome (Radecke et al. 1995).

a. Co-transfection of packaging cell line

Packaging cell line was co-transfected with p (+) MV-NSE-FlagP-M502-3p (plasmid carrying full length measles viral anti-genome) and viral RNA dependent RNA polymerase (L gene) as described earlier. Forty eight hours post-transfection medium was collected from co-transfected cells and cells were replenished with new medium. Following 24hours incubation after replenishing the medium, cells were harvested by repeated freeze-thaw cycles; cell lysates were transferred to cryovials and stored at -80ºC for further use.

b. Infection and replication competency of rescued recombinant measles virus

The supernatant and lysates collected from L plasmid and p (+) MV-NSE-FlagP-M502-3p co-transfected packaging cell line, were subjected to repeated freeze thaw cycles and filtered through a 0.22 micron filter to remove cell debris. It was further used as inoculum for

infection of Vero cells using centrifugation enhanced culture as described earlier. Infected cells were harvested 4 to 5 days post-infection by repeated freeze thaw cycles to recover the generated viral particles. Virus generated in Vero cells was considered the master stock of recombinant measles virus (rMV) and stored at -80ºC for further use.

c. Confirmation of rescue of recombinant virus in Vero cells

Transcripts of measles virus genes: Total RNA was isolated from Vero cells infected with rMV for two successive passages; cDNA was generated and subjected to amplification of measles viral nucleoprotein and phosphoprotein genes using specific primers. Late viral matrix gene was also amplified using specific primers.

Gene	Primer	Sequence (5’→ 3’)
Matrix gene	Forward primer	ATATGTTTAAACACCATGACAGAGATCTACGATTTC
	Reverse primer	ATATGCGGCCGCCTACAGAACTTTGAATAGTCC

For a 25µl reaction volume, 2.5µl of 10X Dreamtaq buffer, 1µl of 10mM dNTPs, 1µl of forward and reverse primer each, 0.3µl of Dreamtaq Polymerase, and 5µl of cDNA was added. Initial denaturation was done at 94ºC for 5min followed by 30 cycles of denaturation at 94ºC for 30sec, annealing at 53ºC for 1min and extension at 72ºC for 1min. The amplified product was resolved on agarose gel to observe amplicon corresponding to approximately 1kb.

Immunofluorescence staining: Presence of viral proteins was further confirmed by IFA staining. Cells were grown on coverslips in a 35mm culture dish in duplicates and infections were done at 70-80% confluency; 48hours post-infection, cells were washed twice with chilled 1X PBS and then fixed in ice cold acetone for 20min at -20ºC. Cells were again washed with 1X PBS and incubated with anti-measles N and anti-measles P antibody and visualized for fluorescence as described earlier.

4. Generation reporter measles virus having M-cherry gene in its genome

To establish the genetic stability and successful rescue of an engineered rMV with a foreign genetic element inserted in its genome, a reporter rMV was generated harboring M-cherry gene inserted between measles phosphoprotein and matrix protein genes between unique restriction sites for *Pfl*2311 and *Aat*II.

a. Restoration of Pfl2311 restriction site by site directed mutagenesis

Measles virus full length genome construct p (+)MV-NSE-FlagP-M502-3p, obtained from Addgene, contained additional nucleotides 'CGTACGATGACGTCCTAG' inserted just after nucleotide 3368 to introduce unique restriction sites including *Aat*II and *Pfl*2311 which were to be used for insertion of M-cherry reporter gene. Site for *Pfl*2311 was disrupted due to a single nucleotide substitution at position 3373 (g3373c) and was restored by site directed mutagenesis (SDM) where specific primers were used to introduce the single point mutation. For this, primers were synthesized with a single base substitution:

Primer	Sequence (5'→ 3')
MV-c467g-sdm-FP	TTGTACTAGGACGTCATCGTACGCTAGTTGGGTAT
MV-c467g-sdm-RP	ATACCCAACTAGCGTACGATGACGTCCTAGTACAA

For a 25µl reaction volume, 5µl of 10X Herculase buffer, 1µl of 10mM dNTPs, 1µl of forward and reverse primer each, 0.5µl of Herculase Polymerase, and 10ng of plasmid DNA template was added. Initial denaturation was setup at 95ºC for 2min followed by 18 cycles of denaturation at 95ºC for 30sec, annealing at 60ºC for 30sec and extension at 68ºC for 9min. Final extension was done at 68ºC for 10min. The amplified product was visualized under UV to confirm the presence and amplification of plasmid DNA.

To remove the non-mutated parent plasmid, the amplified product was digested with 1U of *Dpn*I enzyme at 37ºC for 1hour. The digested product was concentrated by ethanol precipitation and re-suspended in 10µl TE buffer, 5µl of which was transformed and colonies were screened for incorporation of mutation in the plasmid by restriction digestion with 1U of *Pfl*2311 and analyzed on agarose gel for linearization.

b. Amplification of M-cherry gene

Plasmid construct pmcherry-C1 (Clontech) was taken as template for amplification of M-cherry gene encoding for a red fluorescent protein. Gene specific primers were used for amplification with *Pfl*2311 site in forward primer and *Aat*II site in reverse primer:

Gene	Primer	Sequence (5'→ 3')
M-cherry	Forward primer	ATATCGTACGACCATGGTGAGCAAGGGCGAGG
	Reverse primer	TATAGACGTCTTACTTGTACAGCTCGTCCATG

A fraction of purified plasmid construct of pmcherry-C1 was amplified with specific M-cherry primers using Dreamtaq polymerase enzyme. Amplification was setup for 30 cycles of denaturation at 94ºC for 30sec, annealing at 57ºC for 30sec and extension at 72ºC for 30sec. Amplified product was purified and inserted into p (+) MV-NSE-FlagP-M502-3p construct with restored *Pfl*2311 site between *Pfl*2311 and *Aat*II restriction sites. Insertion of M-cherry in recombinant measles virus genome plasmid construct was confirmed by colony PCR using specific primers, restriction digestion with *Aat*II and *Pfl*2311 and sequencing.

c. Generation and rescue of reporter virus

Packaging cells line (HEK293-N-P-T7) stably expressing nucleoprotein, phosphoprotein and T7 polymerase was co-transfected with plasmid constructs of recombinant measles virus harboring M-cherry reporter gene (p (+) MV-NSE-FlagP-M502-3p-Mcherry) and L polymerase in duplicates. Forty eight hours post-transfection, cell lysate was collected and reporter virus, rMV-Mcherry, was rescued in Vero cells as described earlier. Vero cells, 24hours post-infection with rMV-Mcherrywere visualized under fluorescent microscope for the expression of M-cherry.

Evaluation of replication competency of recombinant measles virus with M-cherry reporter gene (rMV-Mcherry) in breast carcinoma cells

Vero cells serve as preferential host platform for laboratory culture of measles virus. Despite Edmonston strain of measles virus being directed towards CD46 instead of SLAM receptors, rescued virus may not propagate efficiently in cancer cells. Additionally, insertion of a foreign gene may hinder or disrupt the replication of virus thus to ascertain the generation, retained replication competency, transcription efficiency and infectivity of rescued rMV and rMV-Mcherry, subsequent infections were done in MCF-7 and MDA-MB-231 breast cancer cells and the expression of reporter gene was visualized under fluorescent microscope.

1. Confirmation of infection and replication competency of rMV-Mcherry

Further, total RNA was isolated and viral gene expression was confirmed by reverse transcription-polymerase chain reaction using measles virus gene specific primers. Total protein was recovered from successive infections in cancer cells and protein lysates were subjected to SDS-PAGE followed by western blot analysis using specific antibodies against measles virus nucleoprotein and phosphoprotein.

Immunofluorescence staining: Cells were grown on coverslips in a 35mm culture dish in duplicates and infected with rMV and rMV-Mcherry. Twenty four hours post-infection, cells were washed twice with chilled 1X PBS and then fixed in ice cold methanol for 5min at RT. Cells were further washed with 1X PBST and subjected to IFA staining with anti-measles N and anti-measles P antibodies as described earlier and visualized for co-expression of M-cherry and measles virus proteins.

2. Validation of cytopathic/cytotoxic effects induced by rMV in breast carcinoma cell line

To ascertain the integrity of viral infectiousness, recombinant virus-induced cytopathic effect and morphological changes need to be evaluated. For this, MCF-7 cells were seeded in a 6-well plate. At 80% confluency cells were infected with rMV, keeping mock infected cells and wild type measles virus infected cells as control. Infected cells were kept at 37ºC; 5% CO_2. Cells were observed daily for development of CPE (syncytia) and upon CPE development images were recorded.

Manipulation of rMV to enhance its oncolytic activity

To enhance the oncolytic ability of rMV its genome was further manipulated. For this, BNiP3, pro-apoptotic gene of human origin was selected. Prior to insertion of the foreign gene into rMV genome, recombinant BNiP3 gene construct was generated and its activity in MCF-7 cells was measured by transfecting BNiP3 construct followed by infection with previously generated rMV.

1. Generation of BNiP3 construct

For isolation of BNiP3 gene, MDA-MB-231 cells were treated with 100µM hydrazone derivative to induce apoptotic gene activation. Total RNA was isolated from drug treated cells, reverse transcribed and cDNA was amplified using BNiP3 gene specific primers with restriction enzymes *BamHI* and *Xho*I recognition sites. Reaction setup of, initial denaturation at 94ºC for 5min followed by 30 cycles of denaturation at 94ºC for 30sec, annealing at 47ºC for 30sec and extension at 72ºC for 30sec was done. Amplicons were purified and cloned into eukaryotic expression vector pcDNA3.1+ between specific restriction sites. Recombinant clones were confirmed by colony PCR, restriction digestion and sequencing.

Gene	Primer	Sequence (5'→ 3')
BNiP3	Forward primer	ATATGGATCCACCATGTCGCAGAACGGAGC
	Reverse primer	TATACTCGAGTCAAAAGGTGCTGGTGG

2. Oncolytic activity of BNiP3 construct in combination with rMV

Vero and MCF-7 cells were grown in a 24-well plate (corning). At ~80% confluency, cells were transfected with recombinant construct of BNiP3 keeping using Lipofectamine 2000 as described earlier. Cells transfected with empty vector pcDNA3.1+ was taken as control. Twenty four hours post-transfection, cells were infected with rMV and caspase activity was measured 48hours post-infection.

a. Caspase activity assay

Effect of recombinant plasmid encoding for BNiP3 gene in combination with recombinant virus towards induction of apoptosis was measured using EnzCheck caspase-3 apoptosis kit (Life technologies) as per instruction. For this, above treated cells were harvested and lysed in 1X lysis buffer. 50µl of lysate was incubated with specific substrate for 30min at RT and fluorescence was measured at 342/441nm excitation-emission spectra with Varioskan Flash microplate reader (4.00.53) using SkanIt software 2.4.5 RE. Fluorescence detected was a direct measure of caspase 3 activity.

3. Generation of recombinant measles virus with BNiP3 gene inserted in its genome

Plasmid construct of BNiP3 generated in pcDNA3.1+was taken as template for amplification of BNiP3 with *Pfl*2311 site in forward primer and *Aat*II site in reverse primer following same parameters as described earlier.

Gene	Primer	Sequence (5'→ 3')
BNiP3	Forward primer	ATATCGTACGACCATGTCGCAGAACGGAGC
	Reverse primer	TATAGACGTCTCAAAAGGTGCTGGTGG

For insertion of desired foreign gene, BNiP3, p (+) MV-NSE-FlagP-M502-3p with M-Cherry reporter gene between *Aat*II and restored *Pfl*2311 restriction site was used where M-cherry reporter gene was replaced with BNiP3. The amplified product was resolved on agarose gel, visualized under UV and the image captured on Gel Doc System (Bio-Rad). Purified BNiP3

amplicon was cloned in p (+) MV-NSE-FlagP-M502-3p-Mcherry. Recombinant construct was confirmed by colony PCR using gene specific primers, restriction enzymes digestion for fall out and sequencing.

Recombinant measles virus plasmid construct harboring BNiP3, p (+) MV-NSE-FlagP-M502-3p-BNiP3, was co-transfected in packaging cell line with L polymerase construct and recombinant armed virus (rMV-BNiP3) was rescued from packaging cells as described earlier.

4. Evaluation of antitumor activity of genetically engineered virus in breast cancer cells

Oncolytic ability of genetically engineered virus harboring BNiP3 gene (rMV-BNiP3) and its replication competency was evaluated in breast cancer cells MCF-7 and MDA-MB-231. Prior to evaluation of its oncolytic ability, BNiP3 expression was confirmed in cells infected with rMV-BNiP3 24hours post-infection by western blot analysis and IFA staining using anti-BNiP3 antibody. Expression of viral proteins was also confirmed in both the cell lines with anti-measles nucleoprotein and anti-measles hemagglutinin antibodies. For anti-cancer activity, breast carcinoma cells were seeded at a density of 1×10^4 cells in 96-well plate (MTT assay) and 1×10^5 cells in 24-well plates (caspase activity assay). At ~80% confluency, cells were infected with rMV-BNiP3 and incubated at 37°C; 5% CO_2 incubator for 48 to 72hours for MTT assay and 24hours for caspase activity assay as described earlier. Apoptosis was also measured by Annexin V assay using FACS.

a. MTT assay

Cell viability was measured in transfected-infected cells keeping un-transfected, vector transfected, only infected and wild-type measles infected cells as control. The assay was performed in triplicates. Forty eight hours post-infection, cells in each well were incubated with MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide) reagent at a working concentration of 0.5mg/ml from stock solution of 5mg/ml MTT in 5% methanol for 3hours. MTT reagent was subsequently removed and the formazan crystals were solubilized in 200µl of DMSO. The absorbance of solution was recorded at 560nm using Spectra max plus plate reader (Molecular devices). The value of absorbance is a measure of number of viable cells and was calculated keeping untreated cells as control.

$$\%\ Cell\ viability = \frac{OD\ of\ test}{OD\ of\ control} \times 100$$

b. Annexin V assay/FACS

Apoptosis and cell death was also measured by Annexin V/PI assay using FACS analysis in rMV and rMV-BNiP3 infected cells keeping un-infected cells as control. The assay was performed in triplicates. MDA-MB-231 cells were seeded in 12-well plates at 0.1×10^6 cells/well. At ~80% confluency cells were infected with rMV and rMV-BNiP3 and 24hours post-infection, cells in each well were harvested, washed with ice cold 1X PBS and processed for FACS analysis using Alexa fluor 488 Annexin V/Dead cell apoptosis kit. In brief, harvested cells were re-suspended in 100µl of 1X annexin binding buffer; incubated with propidium iodide (PI) and alexa-fluor 488 conjugated annexin V for 15min at RT; volume was made upto 400µl with 1X annexin binding buffer and cells were analysed by FACS using BD AriaFusion with DiVa ver. 8.0.1 (excitation with 488nm laser and emission at 530 and 575nm).

5. Combinatorial effect of chemical compounds and genetically engineered virus

Effect of chemotherapeutic agents and novel hydrazone compound, in combination with oncolytic virus was also observed with breast carcinoma cells. Paclitaxel, a chemotherapeutic drug was used for comparison of the effects induced by hydrazone derivative.

a. Determination of IC_{50} value of drugs

Synthesized hydrazone derivative H2 was obtained from Department of Chemistry, Jamia Milia Islamia, New Delhi. Paclitaxel was procured from Sigma. Compounds were reconstituted in 100% DMSO and further dilutions were made in serum free medium DMEM. MDA-MB-231 and MCF-7 cells were treated with increasing concentration of H2 (0.5-200µM) and Paclitaxel (0.05-1µM) in triplicates. Medium without respective drugs was added in control wells. At 56hours post drug treatment, MTT assay was performed and the values obtained were plotted in graph against the concentration of drug. The concentration at which cells viability was unaffected was chosen for further experiments.

b. Oncolytic effect of rMV and rMV-BNiP3 in the presence of hydrazone derivative and Paclitaxel

MCF-7 and MDA-MB-231 cell were grown in 24 well plates. At 80% confluency, cells were treated with desired concentration of H2 or Paclitaxel for 4hours. Post drug treatment, cells were washed with serum free medium and infected with rMV and rMV-BNiP3. At 2hours

post viral adsorption, cells were washed once with serum free medium and replenished with medium supplemented with 2% serum and drug with desired concentration. Cells were incubated and observed for CPE and morphological changes. At 24 to 72hours post infection, cell death and apoptosis was measured by MTT assay, Caspase assay, and Annexin V assay as described earlier.

Every experiment including MTT assay was done in biological triplicates. Data are represented as means and standard deviations. In MTT assay, means of percentage of cell viability and in caspase assay means of fluorescence in each group was compared with its corresponding control by student's t test. A p value <0.05 was considered significant.

RESULTS

1. Generation of packaging cell line/complementing cell line

The components required for the construction and rescue of recombinant measles virus are (1) packaging cell line (2) plasmid carrying full length measles virus genome and (3) viral RNA dependent RNA polymerase construct. HEK293 cell line was used for the generation of packaging cell line stably expressing measles virus capsid nucleoprotein (N), phosphoprotein (P) and T7 RNA polymerase. As measles viral full length genome (obtained from addgene) was cloned under bacteriophage T7 promoter, T7 RNA polymerase was supplied in the packaging cell line.

Generation of recombinant Nucleoprotein (N) construct

Nucleoprotein is a capsid protein of measles virus forming an integral part of viral ribonucleoprotein complex (vRNPs), which is essential for replication and packaging of progeny virus particles. Nucleoprotein gene was amplified from total RNA isolated from Vero cells infected with wild type measles virus using gene specific primers and cloned into eukaryotic expression vector pcDNA3.1+. Single colony was observed post-transformation and was screened for recombinant nucleoprotein by colony PCR. Plasmid isolated from the colony was subjected to restriction digestion and expected fallout of ~1.57kb was observed. Further confirmation was done by sequencing (*refer appendix*). Recombinant clone for measles virus nucleoprotein gene was successfully generated.

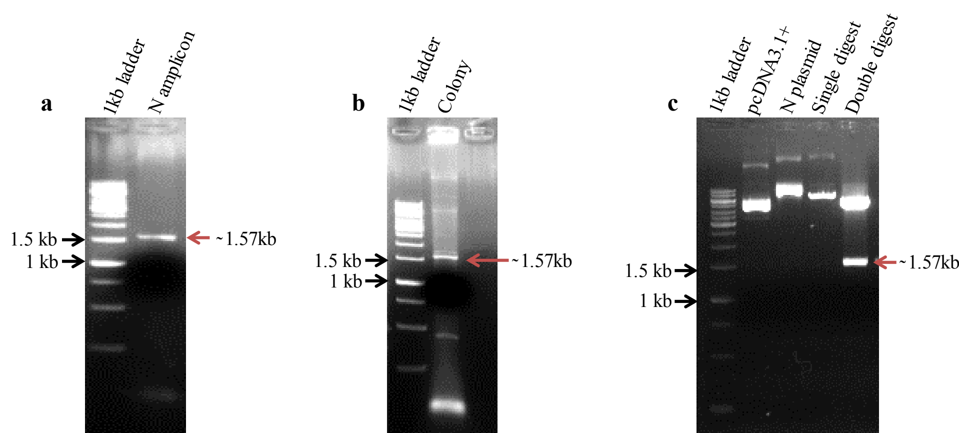


Figure 1: Agarose gels showing (a) amplification of N gene (b) amplified product of recombinant N gene by colony PCR and (c) fallout of ~1.57kb following digestion of recombinant N construct with *Eco*RI and *Xho*I restriction enzymes.

Generation of recombinant phosphoprotein (P) construct

Phosphoprotein, co-factor of viral RNA dependent RNA polymerase (RdRp) is also a component of vRNP complex and is required for the activity of RdRp (Large polymerase). Phosphoprotein gene was amplified from total RNA isolated from wild type measles virus infected cells. The amplified product was cloned into pcDNA3.1+ vector. Transformed colonies were screened for recombinant phosphoprotein by colony PCR. Plasmid isolated from positive colony was subjected to restriction digestion and expected fallout of ~1.52kb was observed. Recombinant clone was confirmed by sequencing (*refer appendix*).

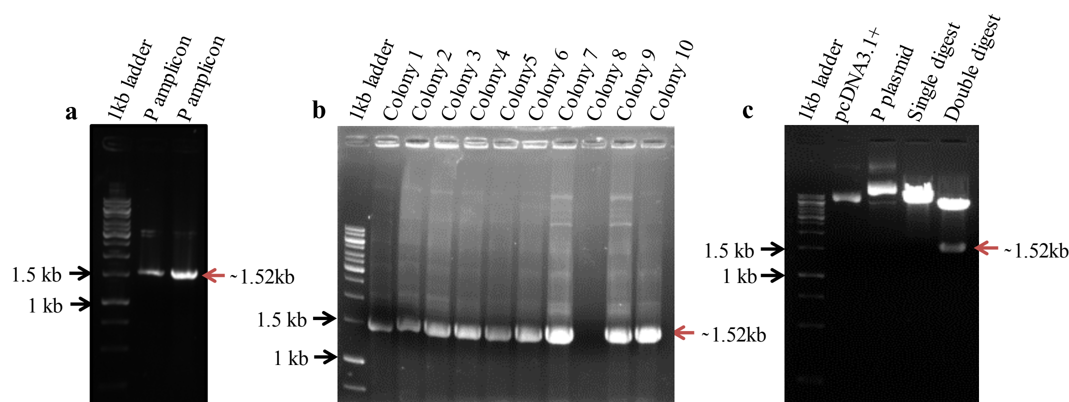


Figure 2: Agarose gels showing (a) amplification of P gene (b) amplified product of recombinant P gene by colony PCR and (c) fallout of ~1.52kb following digestion of recombinant viral phosphoprotein construct with *Eco*RI and *Xho*I restriction enzymes.

Generation of recombinant T7 RNA polymerase construct

T7 RNA polymerase/promoter system provides robust transcription specificity and efficiency. The agility of this combination allows circumnavigation of plasmid constraints and generation of larger RNA fragments. T7 promoter controlled measles virus full length genome necessitates the inclusion of T7 RNA polymerase in the packaging cell line. T7 RNA polymerase gene was amplified from genomic DNA of BL21pLysS cells using gene specific primers and amplified product was cloned into pcDNA3.1+vector. Transformed colonies were screened for recombinant T7 RNA polymerase by colony PCR. Plasmid isolated from positive colony was subjected to restriction digestion and expected fallout of ~2.6kb was observed. Recombinant clone for T7 RNA polymerase gene was confirmed by sequencing (*refer appendix*).

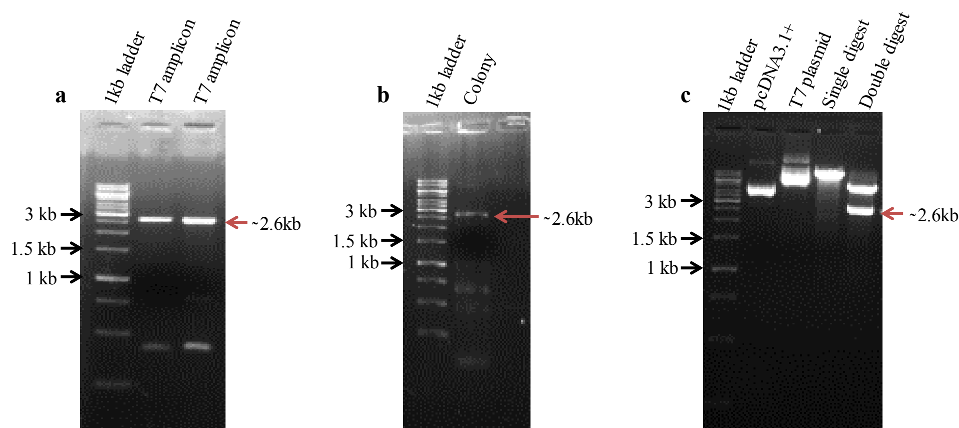


Figure 3: Agarose gels showing (a) amplification of T7 RNA polymerase gene (b) amplified product of recombinant T7 by colony PCR and (c) fallout of ~2.6kb following digestion of recombinant viral T7 RNA polymerase construct with restriction enzymes.

Generation of packaging cell line stably expressing Nucleoprotein, Phosphoprotein and T7 RNA polymerase

HEK293 cells were used to generate the rescue platform of recombinant measles virus using reverse genetics for generation of a complete virion with the use of cloned genome of virus. Plasmid constructs for recombinant nucleoprotein, phosphoprotein and T7 RNA polymerase were co-transfected in HEK293 cells and their expression was confirmed by amplification of respective transcripts from total RNA isolated from transiently transfected cells. Transcripts corresponding to nucleoprotein, phosphoprotein and T7 RNA polymerase were observed (Fig4a). Expression was also confirmed by immunofluorescence staining for measles virus nucleoprotein and phosphoprotein where cytoplasmic expression of both the proteins was observed (Fig4b).

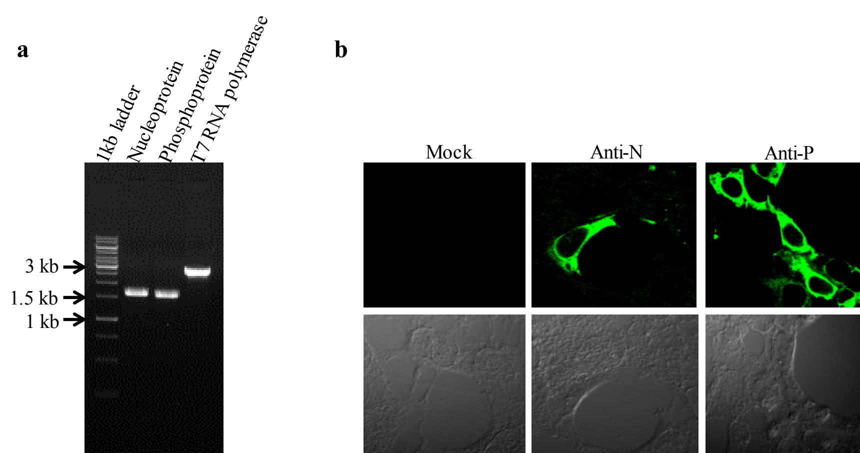


Figure 4: Transient transfection: (a) Expression of N, P and T7 polymerase at transcript level (b) expression of N and P at protein level in packaging HEK293cells.

Selection of stable clones

These transfected cells generated clones upon G418 selection and the clones were screened for the presence of recombinant nucleoprotein and phosphoprotein by immunoblotting and by immunofluorescence staining. All the selected clones were observed to be positive for N and P expression (Fig 5a, 5b). However, the seventh clone from N, P and T7 RNA polymerase expressing cells showed relatively higher expression of N and P than other clones, Thus it was selected and propagated as the rescue/complementing cell line. Expression levels of N and P of this clone was further confirmed by western blot analysis (Fig5c).

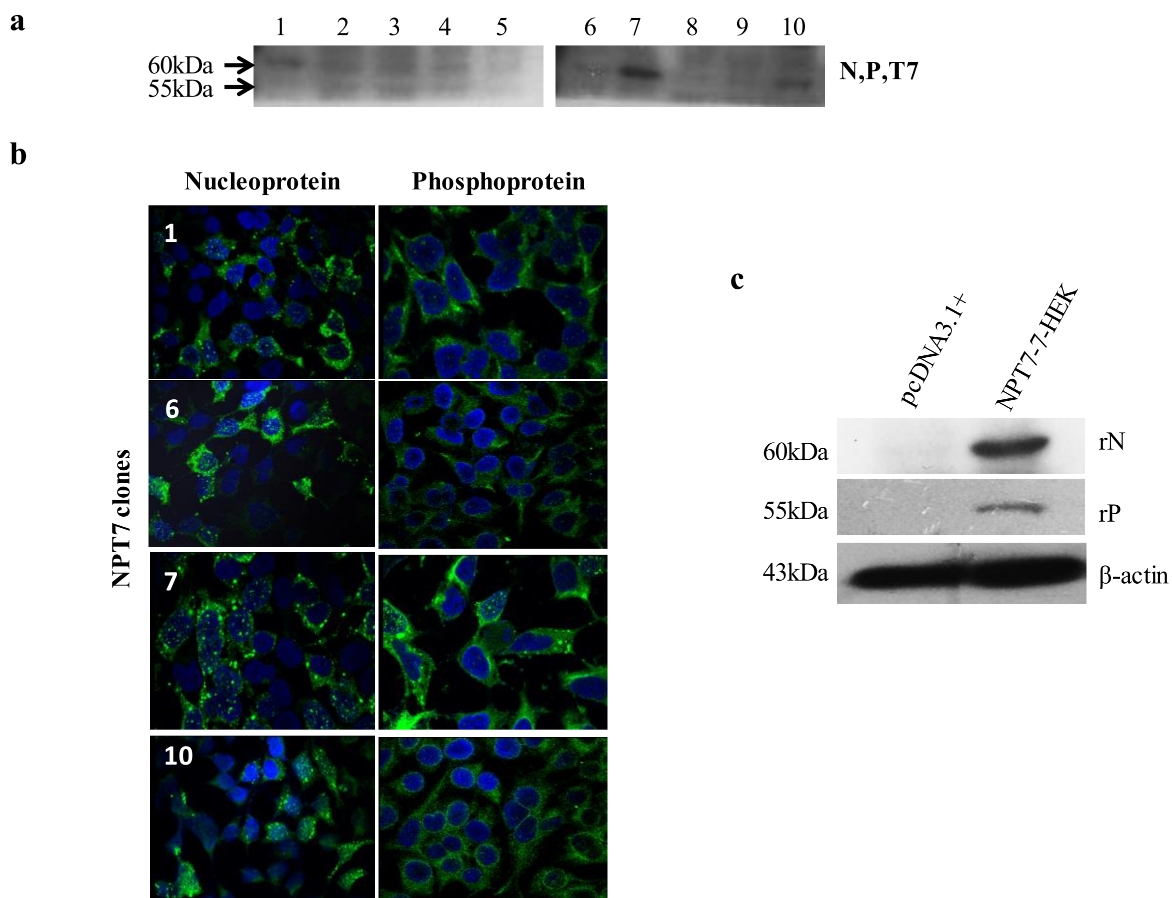


Figure 5: Stable clones: Screening of clones generated for rescue cell line by (a) western blot analysis and (b) IFA staining of nucleoprotein and phosphoprotein in N, P and T7 RNA polymerase co-transfected HEK 293 cells. (c) Confirmation of selected clone (#7) by western blot analysis using anti-N and anti-P antibodies.

2. Rescue of replication competent engineered Measles virus from complementing cell line stably expressing viral N, P and T7 RNA Polymerase

Rescue of virus refers to successful isolation of viral population from infected host. In the context of this study, rescue of recombinant virus refers to generation of viable virion with replicative potential from a laboratory based system in the absence of a parental virus population. It is possible through introduction of viral genome with all the accessory viral proteins, necessary for viral replication and packaging, into a cell line supportive of virus generation and subsequent isolation of recombinant virus without the loss of its infective or propagating potential.

First component, packaging cell line stably expressing viral N, P and T7 was successfully generated. The second component is the measles viral genome construct. Plasmid encoding for Edmonston strain of measles virus genome was obtained from Addgene. The length of non-segmented negative sense RNA genome of measles virus is approximately 15.8kb encodes for 8 proteins.

Plasmid contained the full length anti-genome strand (positive sense) of measles virus was confirmed by linearization using the unique restriction site listed by depositor. Measles virus genome plasmid was also confirmed by restriction digestion using internal sites ascertained from the deposited sequence (Fig 6).

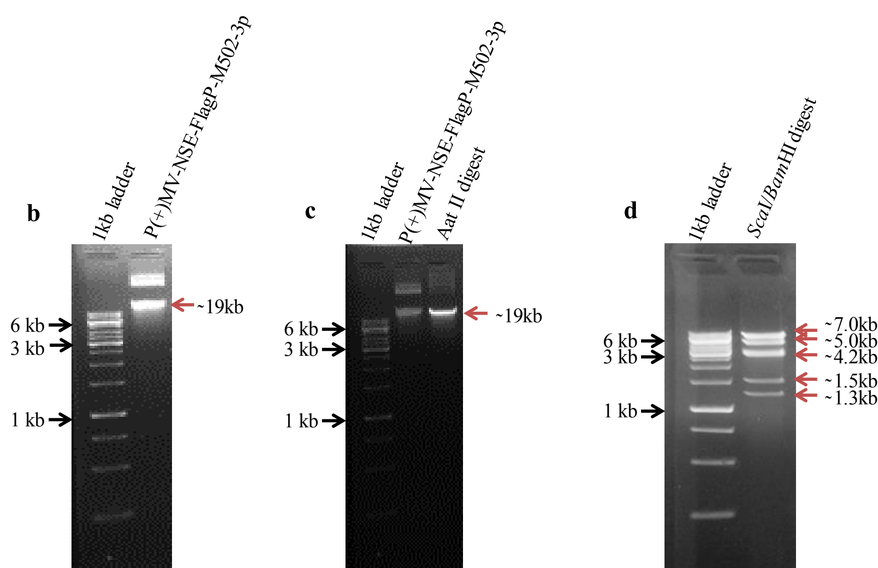


Figure 6: (a) Measles virus complete anti-genome insert (b) confirmation of unique *Aat*II restriction site by linearization of the plasmid and (c) fragments observed following digestion of plasmid with internal *Sca*I and *Bam*HI sites.

Prior to use of the viral construct for generation of recombinant virus, transcription potential of full length viral genome was confirmed by co-transfecting regular HEK293 cells with p (+) MV-NSE-FlagP-M502-3p construct and recombinant plasmid of T7 RNA polymerase as the viral genome construct is cloned under T7 promoter. Viral transcripts for nucleoprotein and phosphoprotein were amplified from total RNA isolated from co-transfected cells. Varying ratios of both the plasmids were used for co-transfections. Different ratios did not affect transcription and showed almost similar expression pattern.

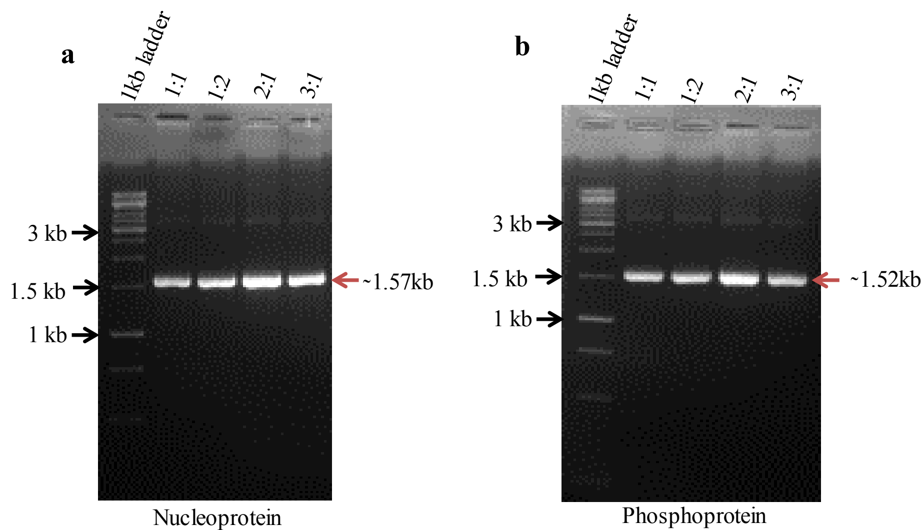


Figure 7: Amplification of (a) nucleoprotein and (b) phosphoprotein transcripts from p (+) MV-NSE-FlagP-M502-3p and T7 RNA polymerase co-transfected HEK 293. Numbered lanes represent different ratio of p (+) MV-NSE-FlagP-M502-3p and T7 RNA polymerase used for transfection.

Generation of recombinant measles virus L polymerase (RNA dependent RNA polymerase) construct

The third component for the generation of recombinant virus is L polymerase, an RNA dependent RNA polymerase. It is essential for replication and transcription of negative sense RNA genome of measles virus in the host cell as it does not support the RNA directed RNA synthesis. L-protein in the presence of phosphoprotein, which acts as a co-factor, transcribes the viral genomic RNA. Transcription of viral anti-genomic strand (positive strand), encoded by the plasmid, is essential for generation of individual viral mRNAs encoding for viral proteins and to generate genomic negative sense strand thus it necessitates the presence of L-protein in rescue cell line (Fig 8a). Gene encoding for L-protein was amplified using p (+) MV-NSE-FlagP-3p as template and amplified product was confirmed by digestion with internal *Sca*I site (Fig8b). Purified amplicon was cloned into pcDNA3.1+ vector, transformed

colonies were screened by colony PCR, restriction digestion and expected fallout of ~6.55kb was observed. Owing to the large size of gene, nested primers were generated for sequencing (*refer appendix*). Recombinant L-protein plasmid was successfully generated.

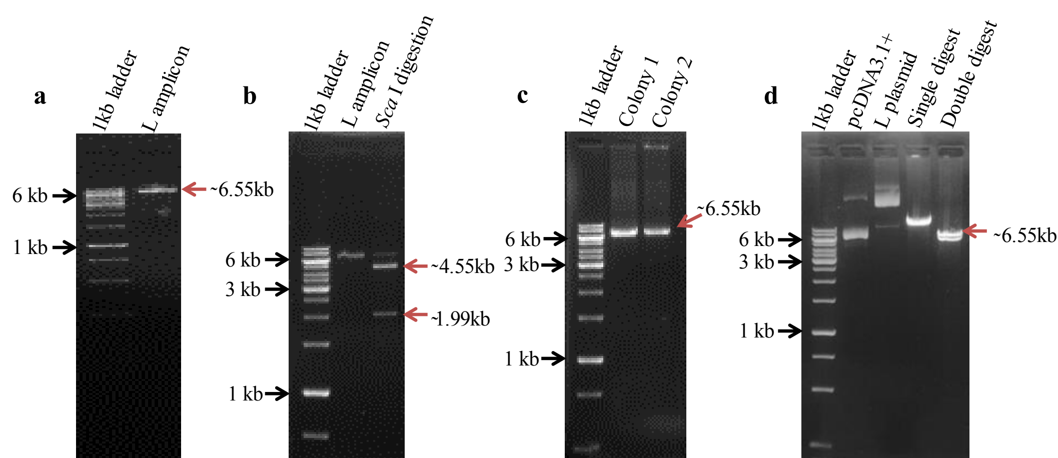


Figure 8: Agarose gel images showing (a) amplification of Large polymerase gene (b) confirmation of L-gene by digestion at internal *Sca*I site (c) colony PCR and (d) fallout of ~6.55kb above plasmid backbone of 5.4kb resulted after digestion with restriction enzymes.

Rescue of the recombinant measles virus

Packaging cell line (HEK293-N/P/T7) was co-transfected with the plasmids encoding for measles virus anti-genome strand (p (+) MV-NSE-FlagP-M502-3p) and large polymerase protein. At 24-48hours post tranfection, supernatants were collected to recover cell free virus as measles virus is an enveloped virus and released into the extracellular environment through budding. Cells were harvested to rescue the cell associated virus as well. Virus recovered here will be referred as rMV.

To ascertain the generation of a viable, replication competent recombinant measles virion (rMV), the supernatant of packaging cell line and cell lysate recovered after transfection were used for infection of Vero cells. Presence of viral transcripts, with increasing levels in successive passages, was indicative of rescue of complete virus particles from transfected packaging cells (Fig9). It also confirmed the internalization of rescued virus through successive passages into the host cells indicative of structural and genetic integrity of rMV. Further, one of the viral genes, N expression at protein level by IFA staining (Fig10) indicated unhindered replication and infectivity of rMV upon rescue.

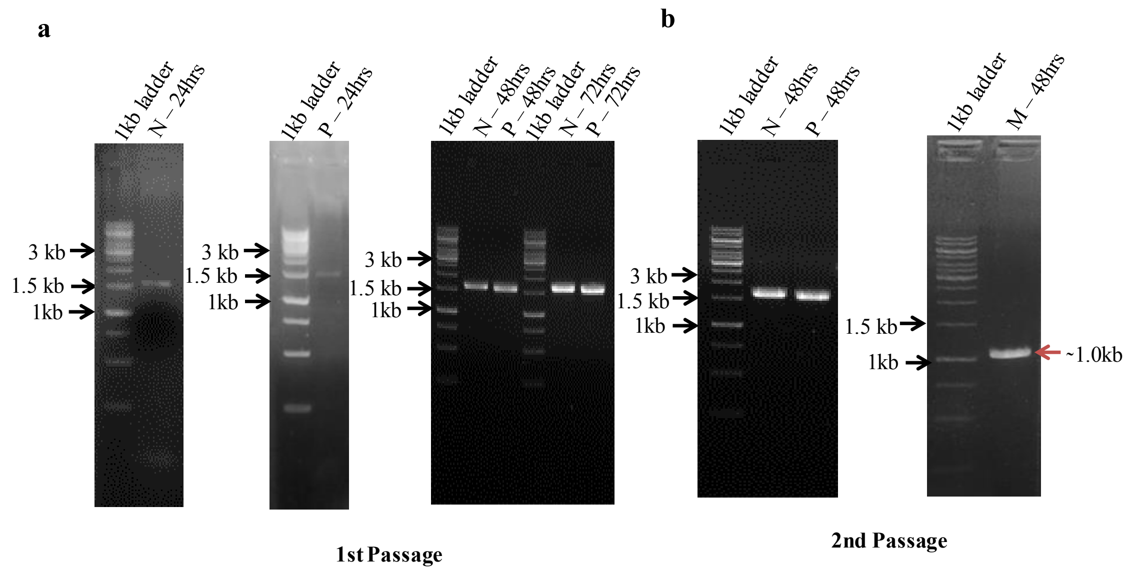


Figure 9: Agarose gel images showing (a and b) amplification of viral N, P and M (protein encoding matrix gene) transcripts from infected Vero cells for two successive passages.

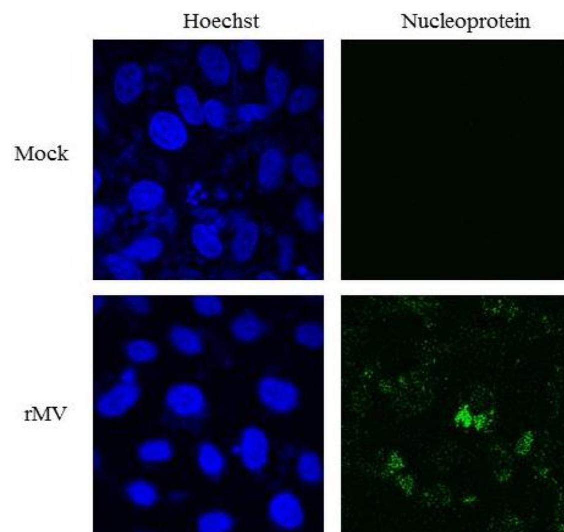


Figure 10: IFA staining of Vero cells infected with rMV and probed with anti-N antibody.

Insertion of M-cherry into the full length viral genome to confirm viral internalization and replication

To establish the genetic stability and successful rescue of an engineered rMV with a foreign genetic element inserted in its genome, a reporter rMV was generated harboring M-cherry gene inserted between the region encoding measles virus phosphoprotein and matrix protein genes. p (+) MV-NSE-FlagP-M502-3p contained additional nucleotides inserted just after nt3368 to introduce unique restriction sites. Restriction sites for *Aat*II and *Pfl*2311 were used

for insertion of M-cherry reporter gene. Site for *Pfl*2311 was disrupted and was restored by site directed mutagenesis (SDM) where specific primers were used to introduce the single nucleotide mutation.

Figure 11: (a) Point mutation at 3373nt (c3373g) to restore *Pfl*2311 restriction site and (b) confirmation by linearization of plasmid by *Pfl*2311 digestion.

M-cherry gene was amplified from pMcherry-C1 plasmid and cloned into p (+) MV-NSE-FlagP-M502-3p between *Aat*II and regenerated *Pfl*2311 sites. Recombinant clones were screened by colony PCR, restriction digestion and sequencing. Recombinant clone positive for M-cherry gene referred as p (+) MV-NSE-FlagP-M502-3p-M-cherry was further confirmed by sequencing (*refer appendix*).

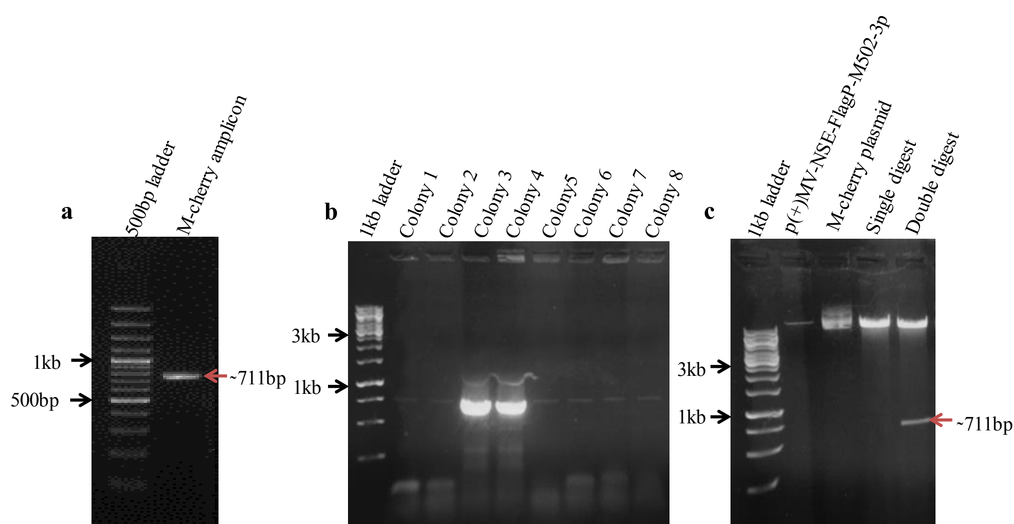
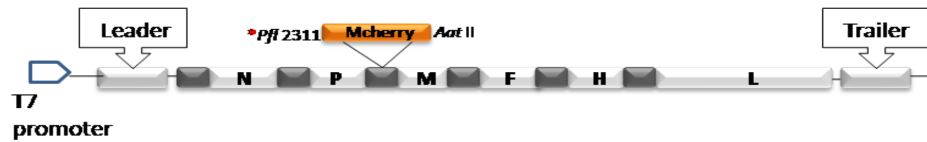


Figure 12: Agarose gel images showing (a) amplification of M-cherry gene (b) confirmation by colony PCR and (c) fallout of ~ 711bp was observed after restriction digestion.

Location at which Mcherry, a red fluorescence reporter gene was inserted into full length viral genome construct.



Recombinant measles virus expressing M-cherry was generated and rescued by co-transfecting the packaging cell line with p (+) MV-NSE-FlagP-M502-3p-M-cherry plasmid construct and L polymerase plasmid as described earlier. Rescued virus was referred as rMV-Mcherry. Vero cells were infected with rMV-Mcherry had shown M-cherry expression, red fluorescence indicative of insertion of foreign gene not hampering internalization and replication of recombinant viral genome or its protein expression thus reconfirming the lack of any loss of replicative or infective potential of engineered Measles virus. Successful generation of rMV-Mcherry also paved the way for arming of recombinant measles virus for oncolysis.

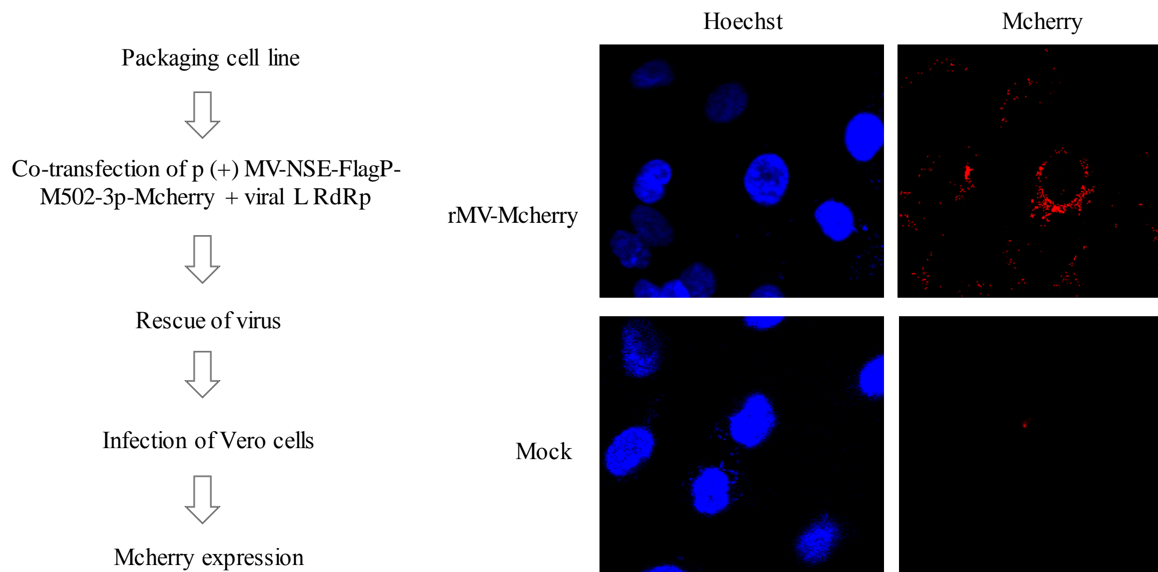


Figure 13: (a) Schematic of rescue of measles virus harboring M-cherry and confirmation by (b) fluorescence.

3. Evaluation of replication competency of rMV and rMV-Mcherry

Expression of M-cherry by reporter virus suggested generation of a viable replication competent measles virion. Vero cells were used for these confirmations as they serve as model cell line for propagation of measles virus. To ascertain the infective potential of recombinant virus, rMV-Mcherry in cancer cells, breast cancer cell line MCF-7 was used. Infections were done similar to that in Vero cells and presence of viral transcripts and protein was observed. Virus propagated to second passage in cancer cells was used as inoculum. Results demonstrated the ability of virus to infect cancer cells which can be attributed to expression of CD46 and nectin-4 receptors in cancer cells.

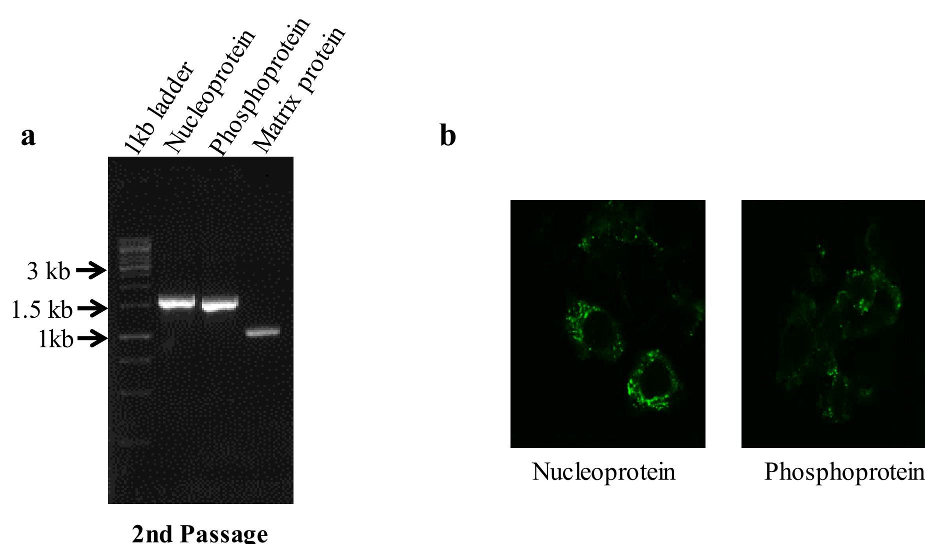


Figure 14: Agarose gel images showing (a) amplification of N and P transcripts from infected MCF-7 cells and (b) confirmation by IFA staining.

Cytopathic/cytotoxic effects induced by recombinant measles virus in MCF-7 cell line

Infected cells also showed morphological changes including formation of cell aggregates and rounding off cells leading to death. This effect was absent in HEK 293 cells infected with recombinant virus or MCF-7 cells infected with wild type virus, indicative of cancer specificity of the rescued virus as supported by literature. Classical measles CPE of syncytia formation was not clearly observed attributable to low amount of virus particles.

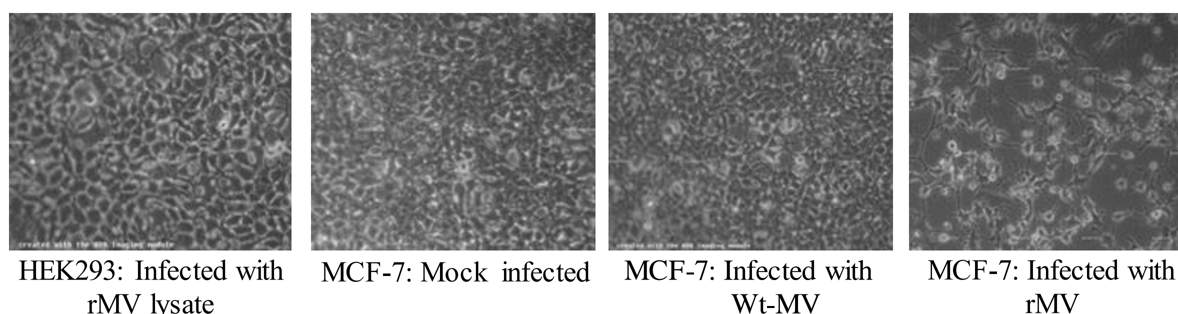


Figure 15: Morphological changes in breast carcinoma cells infected with recombinant measles virus.

Mcherry and viral phosphoprotein expression was confirmed in triple-negative (ER, PR, Her2/neu negative) breast carcinoma cell line, MDA-MB-231 infected with rMv-Mcherry as well. All the results confirmed the formation of a virus particle which is successfully packaged and rescued from the packaging cell line, retaining its replicative and infective potential.

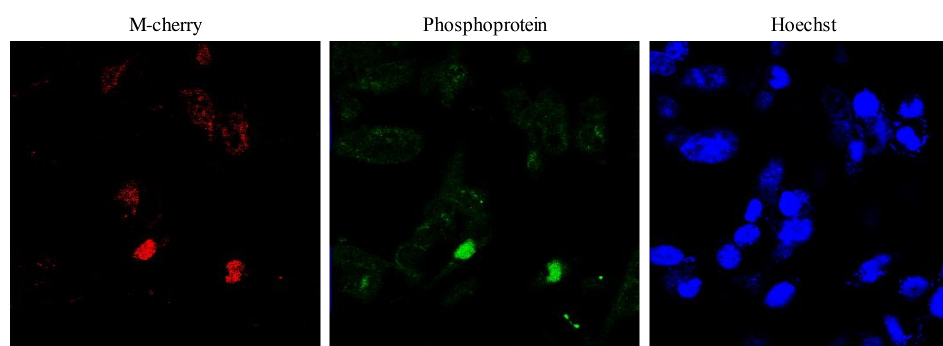


Figure 16: Mcherry and viral phosphoprotein expression in MDA-MB-231 cells infected with rMV-Mcherry.

4. Arming of recombinant measles virus (rMV) to enhance its anti-tumour activity

Oncolytic potential of recombinant measles virus was aimed to be enhanced by insertion of BNiP3 protein encoding gene of human origin, a pro-apoptotic protein belonging to Bcl2 interacting protein 3 family. It is a mitochondrial protein containing a BH3 domain and is silenced in tumors by DNA methylation. Loss of its function has been indicted in diseases such as leiomyomatosis, renal cell cancer and colorectal cancer. In addition, its downregulation in tumors have been associated with metastasis especially in breast carcinomas.

Prior to insertion of BNiP3 into measles viral genome, recombinant clone of BNiP3 was generated and its activity was assayed in conjunction with rMV. BNiP3 gene was amplified from total RNA isolated from MDA-MB-231 cells treated with a novel hydrazone derivative. Amplified product was cloned into pcDNA3.1+vector, transformed colonies were screened by colony PCR followed by restriction digestion of positive clone whereby expected fallout of ~585bp was observed. Recombinant clone for BNiP3 was confirmed by sequencing (*refer appendix*).

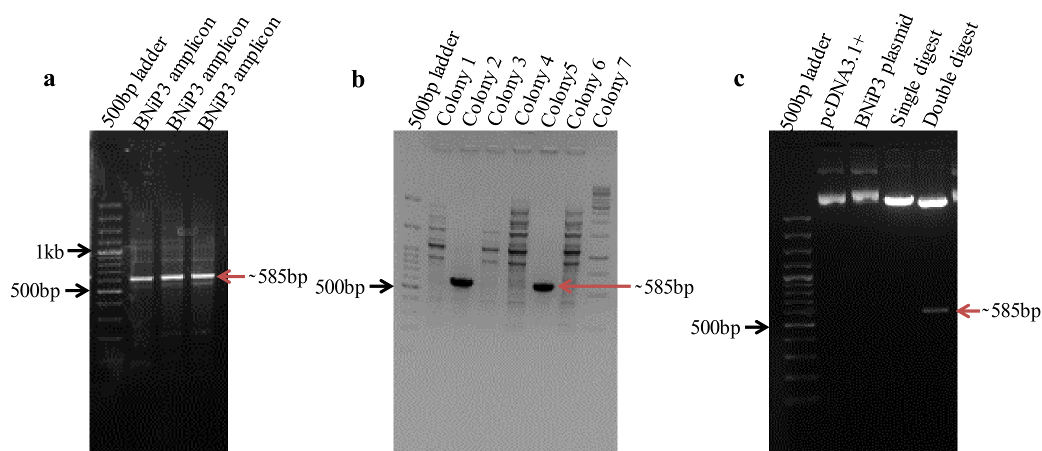


Figure 17: Agarose gel images showing (a) amplification of BNiP3 (b) amplified product by colony PCR and (c) fallout of ~585bp by restriction digestion of positive clone.

Anti-tumor potential of BNiP3 in combination with rMV was established in Vero and MCF-7 cells with BNiP3 plasmid construct and subsequent infection with rMV. Caspase activity was measured in infected and transfected cells. Measurement was done by using EnzCheck Caspase 3 apoptosis detection kit and fluorescence was a direct measure of apoptosis and caspase 3 activity. As compared to vector transfected cell, BNiP3 transfected cells showed slightly higher caspase activity corresponding to apoptotic activity. This effect was compounded when BNiP3 was overexpressed in combination with rMV infection. Caspase activity was observed to be significantly high in both the cell lines transfected with BNiP3 construct followed by rMV infection as compared to cells transfected withBNiP3 (Vero, p = 0.0032; MCF-7, p = 0.0024) infected with rMV (Vero, p = 0.002; MCF-7, p = 0.014) or alone.

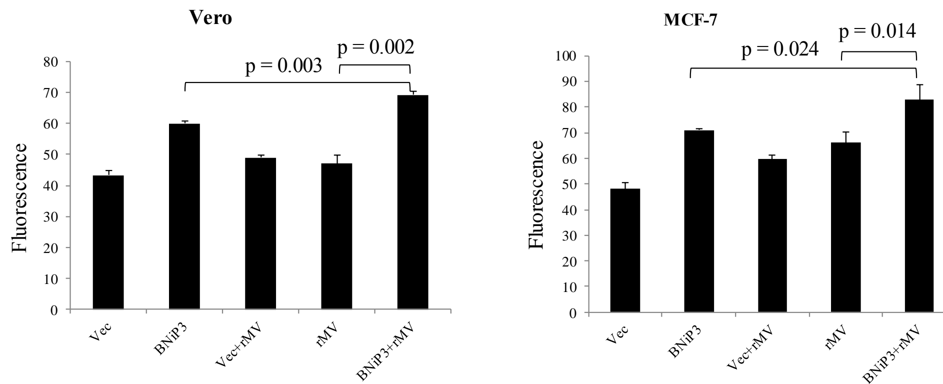
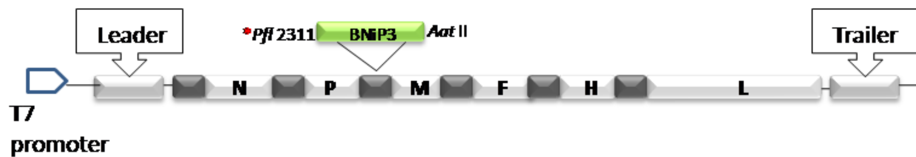


Figure 18: Caspase 3 activity of MCF-7 and Vero cells transfected with BNiP3 followed by infection with rMV. Activity was measured 48hours post-infection using EnzCheck Caspase3 apoptosis detection kit.

Insertion of BNiP3 into full length measles virus genome

Induction of increased caspase activity in breast carcinoma cells in response to BNiP3 combined with rMV infection proposes its potential to increase the anti-tumor effects of rMV. Therefore, recombinant virus harboring BNiP3 gene was generated. Amplified product of BNiP3 gene was inserted into p (+) MV-NSE-FlagP-M502-3p plasmid between *Aat*II and regenerated *Pfl*2311 sites.



Recombinant clones were screened by colony PCR and restriction digestion. Recombinant clone positive for BNiP3 gene (p (+) MV-NSE-FlagP-M502-3p-BNiP3) was further confirmed by sequencing (*refer appendix*).

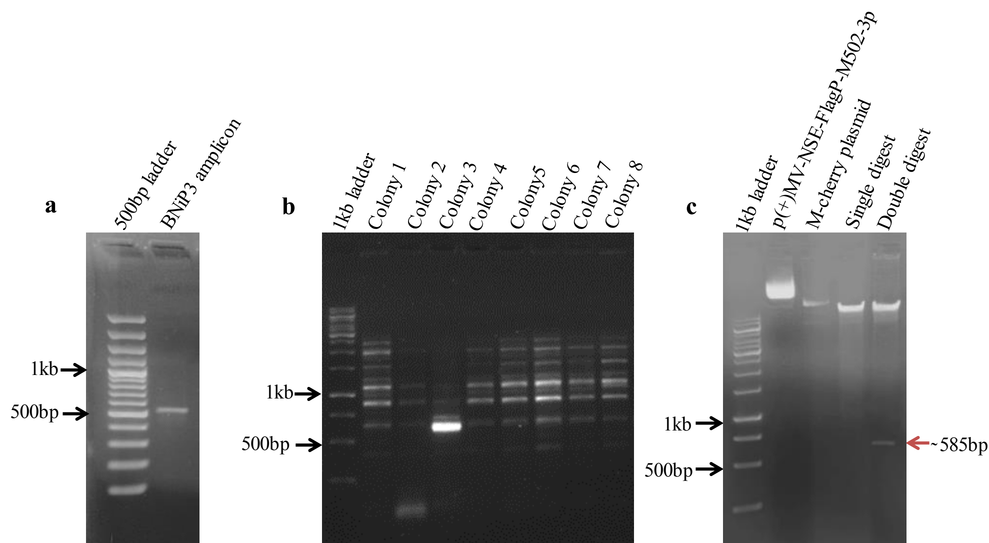


Figure 19: (a) amplified product of BNiP3 gene (b) screening of transformants by colony PCR and (c) restriction digestion of positive clone for fallout.

Recombinant measles virus harboring BNiP3 (rMV-BNiP3) was successfully generated and rescued from packaging cell line transfected with p(+)MV-NSE-FlagP-M502-3p-BNiP3 construct and L plasmid as described earlier. BNiP3 and viral gene expression was confirmed in cells 48hours post transfection.

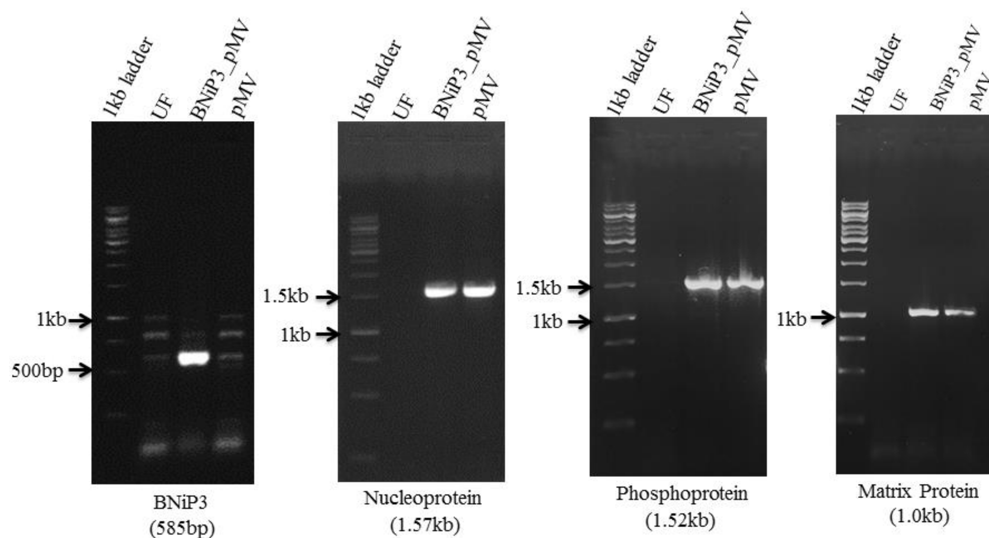


Figure 20: Agarose gels showing expression of (a) BNiP3 (b) nucleoprotein (c) phosphoprotein (d) matrix protein.

Supernatants and cell lysates collected were used to infect MCF-7 and MDA-MB-231 cells. Increased length of the viral genome, more than its unit length, due to insertion of foreign

gene (BNiP3) did not affect the replication competency in subsequent infections. However, in comparison to MCF-7 cells, MDA-MB-231 showed higher expression of BNiP3 by IFA staining and western blot analysis.

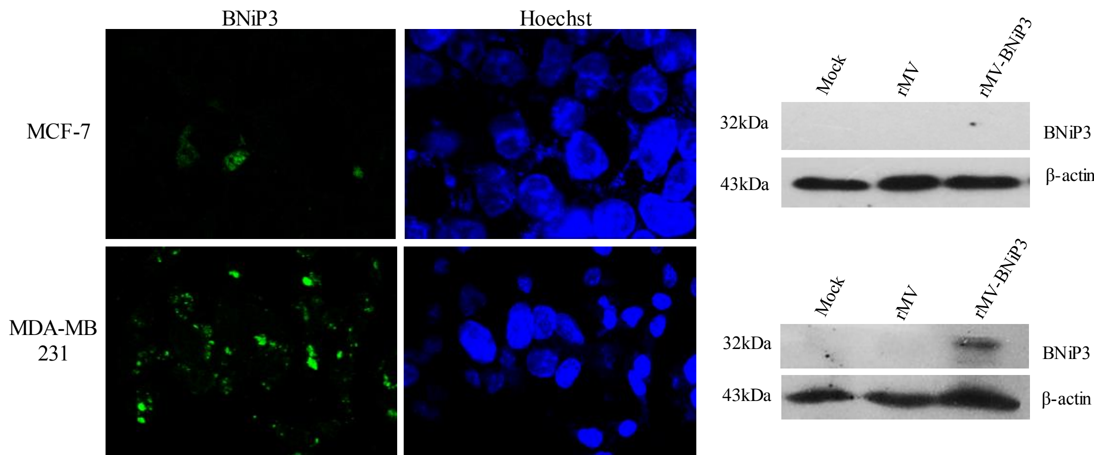


Figure 21: BNiP3 expression in MCF-7 and MDA-MB-231 cells infected with rMV-BNiP3 by IFA staining and Western blot analysis using anti BNiP3 antibody.

Viral gene expression at transcript and protein level was confirmed in MCF-7 and MDA-MB-231 cells. Higher expression levels of BNiP3 and viral genes in MDA-MB-231 cells suggest the higher affinity of rMV-BNiP3 towards triple negative breast carcinoma cells.

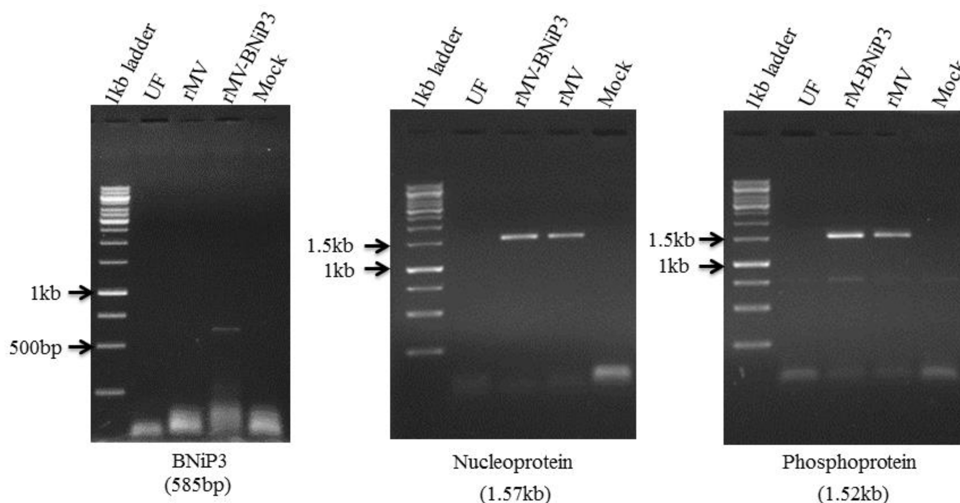


Figure 22: Agarose gels showing expression of (a) viral nucleoprotein (b) phosphoprotein and (c) BNiP3 in MCF-7 cells.

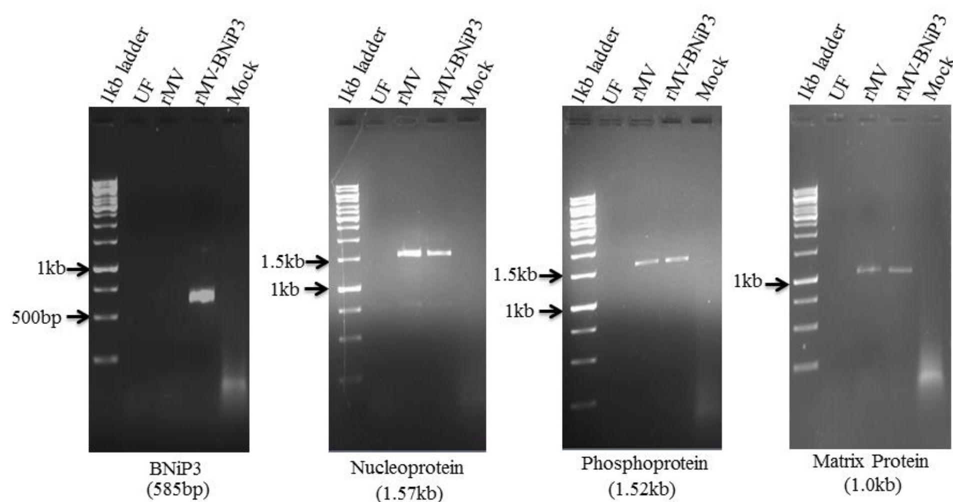


Figure 23: Agarose gels showing expression of BNiP3, viral nucleoprotein, phosphoprotein and matrix protein genes in MDA-MB-231 cells.

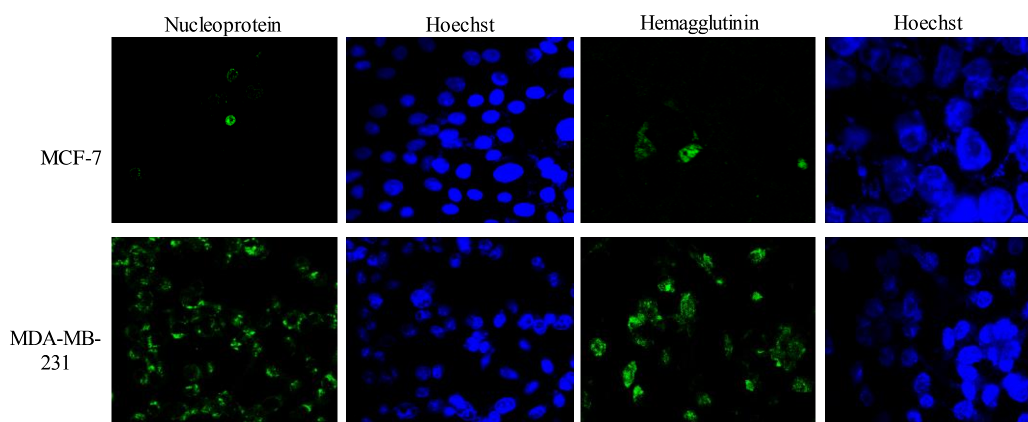


Figure 24: Expression of nucleoprotein and hemagglutinin protein in cells infected with rMV-BNiP3 in MCF-7 and MDA-MB-231 by IFA staining.

Anti-tumor activity of genetically engineered virus, rMV-BNiP3

Oncolytic activity and its enhancement in BNiP3 harboring rMV was ascertained by both cytotoxicity assays as well as assays measuring apoptosis. MDA-MB-231 and MCF-7 cell lines were infected with rMV and rMV-BNiP3 respectively. Cells were also treated with a novel hydrazine derivative; H2 (N’-(2-chlorobenzylidene)-4-(2-(dimethylamino) ethoxy)

Benzohydrazide) to check the combinatorial effect of drugs on the anti-tumor activity of oncolytic virus generated. H2 drug was used in this study, as it showed tumor cell selectivity in our previous study with different cancer cell lines. Validation of this synergism was done by combining Paclitaxel, a known cancer therapeutic agent with rMV and rMV-BNiP3. Both the drugs in combination with rMV-BNiP3 viruses induced morphological changes in both

the cell lines as compared to their respective controls. But the effects were observed to be more prominent in MDA-MB-231 cells (Fig26).

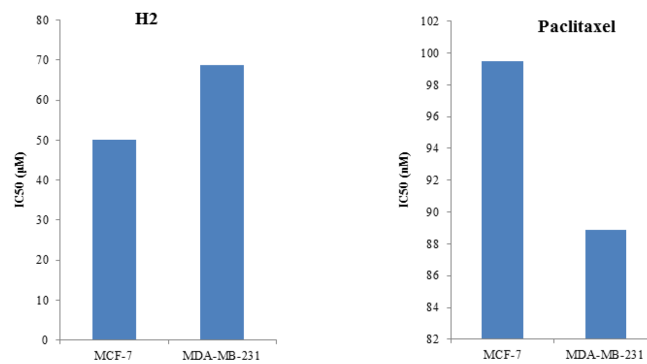


Figure 25: IC_{50} value of H2 and Paclitaxel in MCF7 and MDA-MB-231 cells

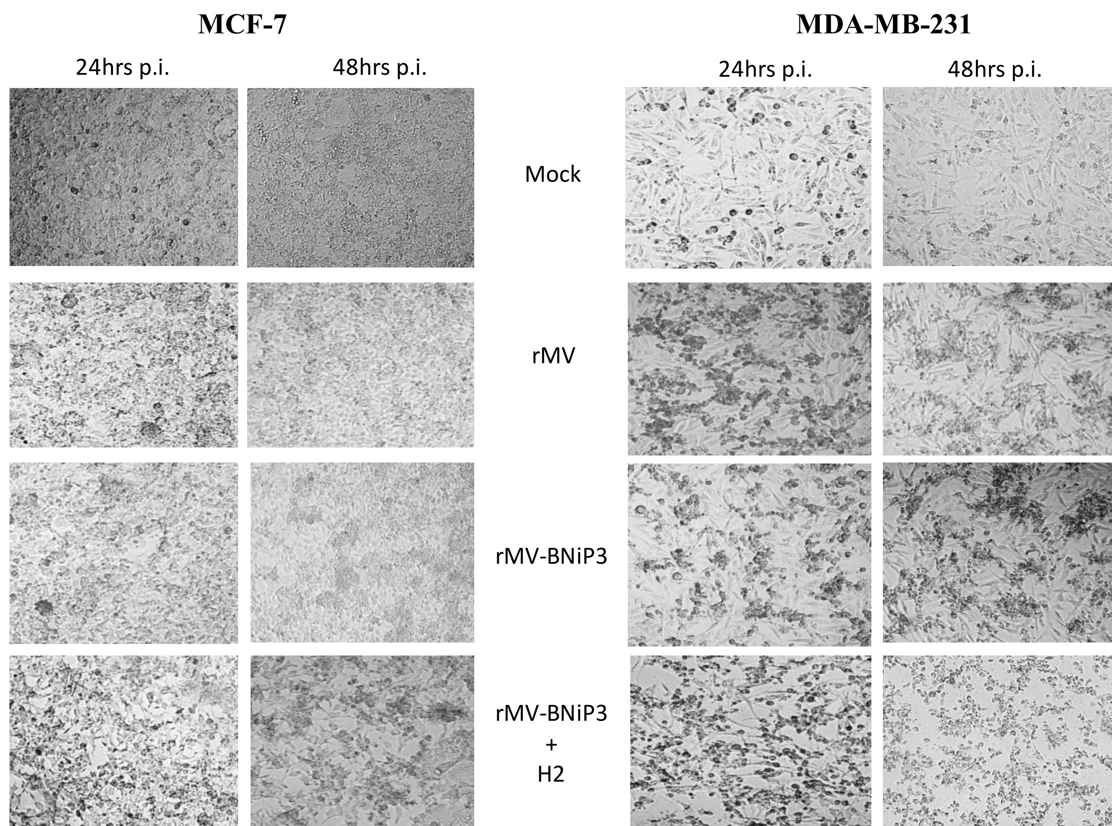


Figure 26: Photomicrographs showing morphological changes induced in cells following with treatment with drugs and rMV, rMV-BNiP3, rMV-BNiP3+H2, 24 and 48hours post infection.

Morphological changes were validated by measuring the percentage of viability in drug treated rMV-BNiP3 infected cells 56hours post-infection keeping mock infected cells as control. H2 drug treatment in combination with rMV-BNiP3 induced significant cell death

(p=<0.001) than cell treated with drug or infected with virus alone in MDA-MB-231 cells. Results were correlated with viral gene expression in both the cell lines and confirmed the better selectivity of the recombinant virus to triple negative breast cancer cells. Increased caspase activity was observed in MCF-7 cells corresponding to cell death when transfected with BNiP3 construct followed by rMV infection, but no such result was noted when directly infected with recombinant measles virus armed with BNiP3 gene (rMV-BNiP3).

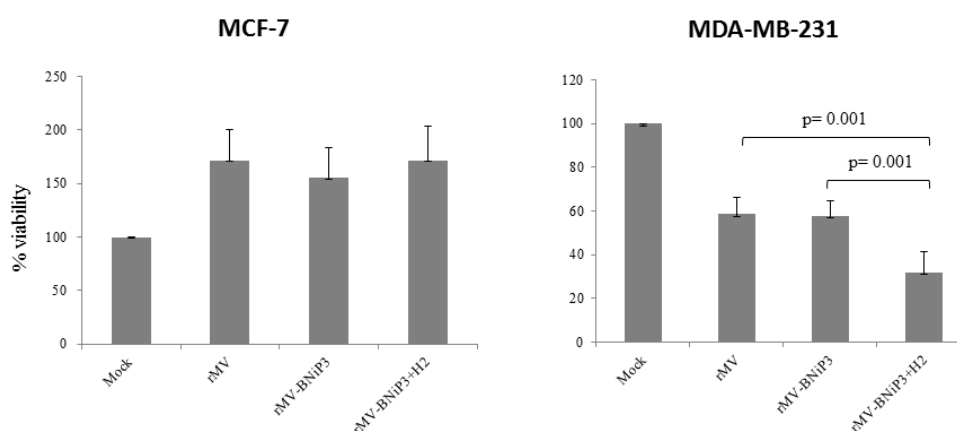


Figure 27: MTT assay of MCF-7 and MDA-MB-231 cells infected with rMV and rMV-BNiP3 in combination with H2 compound.

Owing to the limited observed effect of recombinant virus on MCF-7 cells, further assays were carried out in MDA-MB-231 cells which were shown to be more susceptible to infection. To re-confirm the toxic effects recorded in the above experiment, MTT assay was repeated with MDA-MB-231 cells and similar results were obtained with significantly induced toxicity with H2 drug treatment.

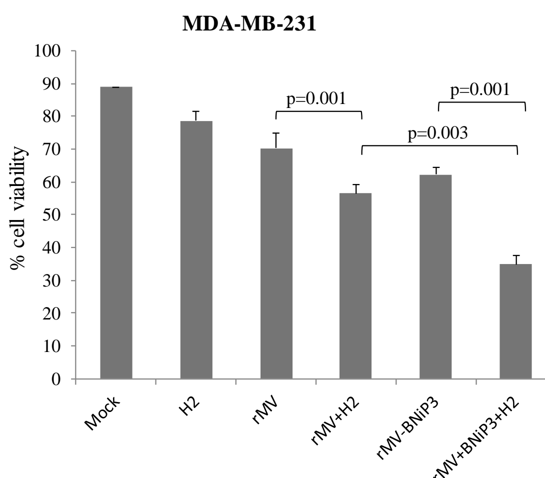


Figure 28: MTT assay of MDA-MB-231 cells infected with rMV and rMV-BNiP3 in combination with H2 compound.

Caspase 3 activity assay was measured as a representation of apoptotic induction in MDA-MB-231 cells infected with recombinant virus and treated with paclitaxel keeping mock infected cells as control. It was observed that cells infected with rMV-BNiP3 showed higher caspase 3 activity than cells infected with rMV (p=0.0038). Presence of 30nM of paclitaxel resulted in marginal increase in apoptotic activity but no clear or enhanced synergism was observed.

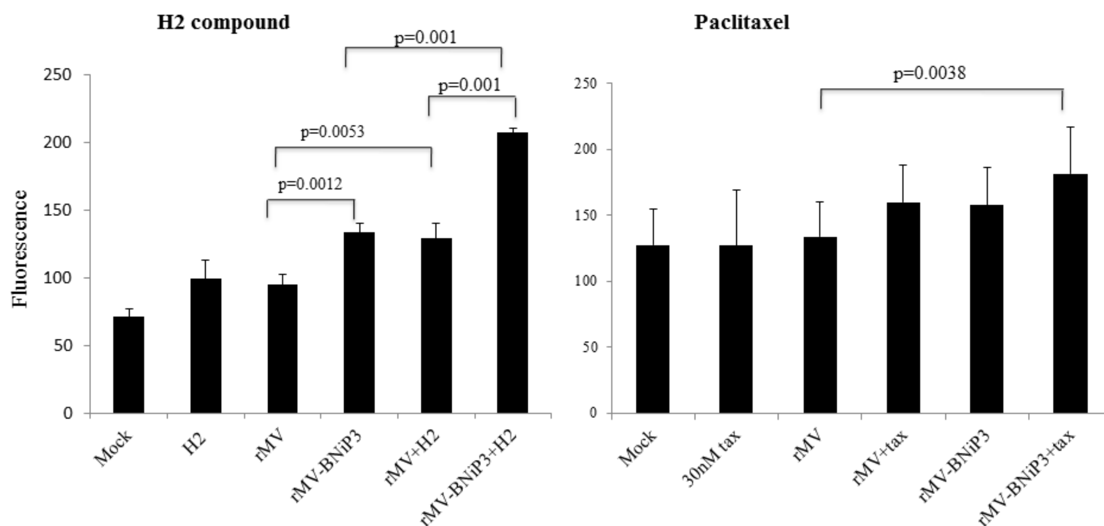


Figure 29: Caspase activity assay in MDA-MB-231 cells infected with rMV and rMV-BNiP3 in combination with H2 compound and Paclitaxel.

It is evident from the given photomicrographs; paclitaxel treatment changed the morphology of rMV and rMV-BNiP3 infected cells extensively but no correlation with apoptosis was recorded. Paclitaxel is a chemotherapeutic drug known to induce necrosis at higher concentration but apoptosis at lower concentrations. Despite using lower concentration; 30nM drug did not induce apoptosis; the observed toxicity in cells might be due to necrosis.

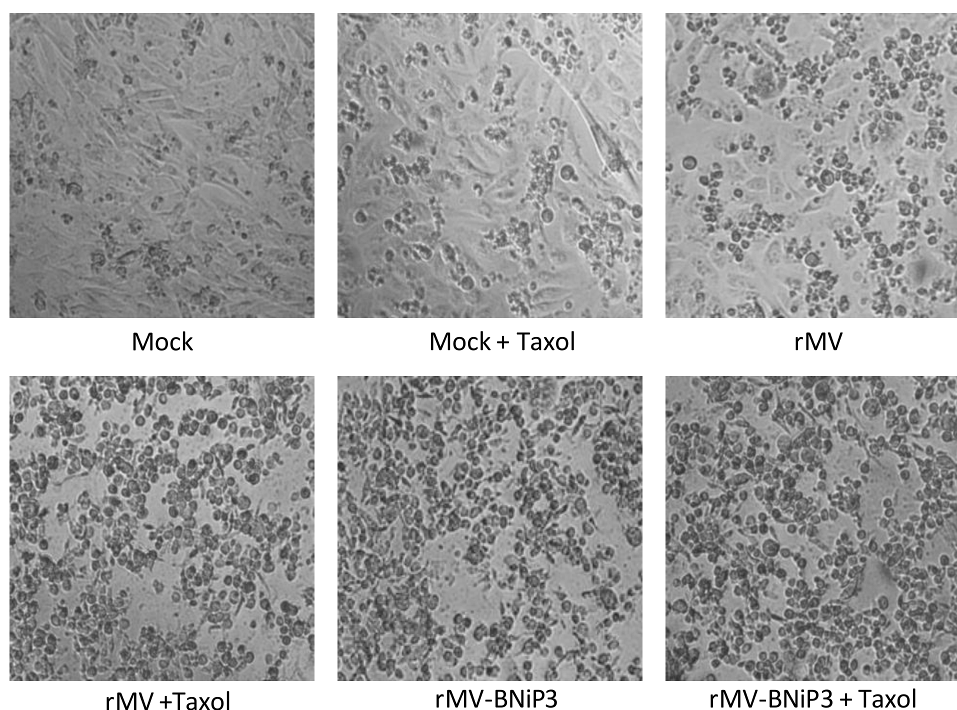
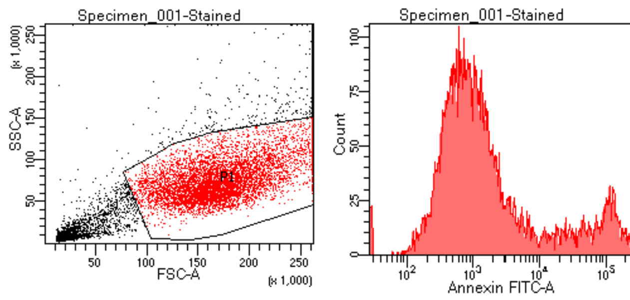


Figure 30: Photomicrographs showing changes induced in MDA-MB-231 cells infected with rMV and rMV-BNiP3 in combination with Paclitaxel (Taxol).

Oncolytic activity of armed recombinant measles virus was also ascertained by FACS analysis of MDa-MB-231 cells infected with rMV-BNiP3 and supplemented with paclitaxel (30nM) prior to infection. Annexin V and PI were used as markers for apoptosis and cell death respectively. The means of the ratio of Annexin V/PI stained population and Annexin V stained population was plotted. The data represented as column graph indicates the level of apoptosis in rMV-BNiP3 infected cell as compared to rMV infected cells but the difference was not statistically significant seemingly due to higher standard error. Significant difference (p=0.03) between rMV- BNiP3 *vs*. rMV-BNiP3+taxol treated cells could be due to paclitaxel induced necrotic death. Increased apoptotic activity in rMV-BNiP3 infected cells suggests that the expression of BNiP3 in infected cells could contribute to anti-tumor activity in association with recombinant measles virus. Correlating with caspase3 activity results, paclitaxel did not induce any significant apoptotic effect. However, due to technical problems we could not measure apoptosis in H2 treated cells by FACS.

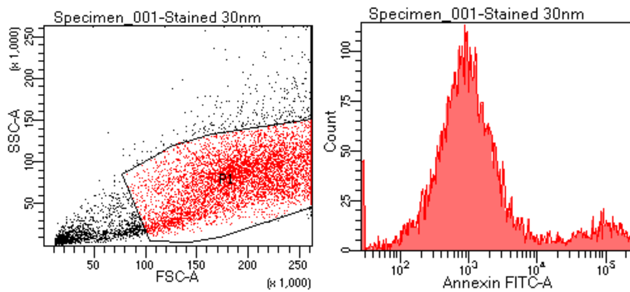
Mock infected



Tube: Stained

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	6,614	66.1	66.1
Q1	1	0.0	0.0
Q2	897	13.6	9.0
Q3	1,937	29.3	19.4
Q4	3,779	57.1	37.8

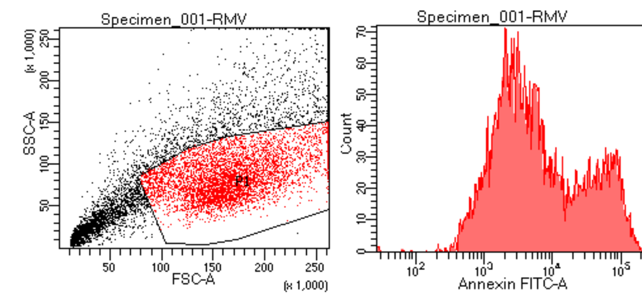
Mock infected + Taxol



Tube: Stained 30nm

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	6,684	66.8	66.8
Q1	1	0.0	0.0
Q2	640	9.6	6.4
Q3	1,877	28.1	18.8
Q4	4,166	62.3	41.7

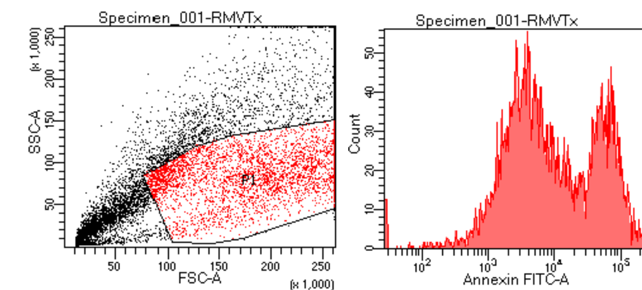
rMV infected



Tube: RMV

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	5,037	50.4	50.4
Q1	0	0.0	0.0
Q2	418	8.3	4.2
Q3	134	2.7	1.3
Q4	4,485	89.0	44.8

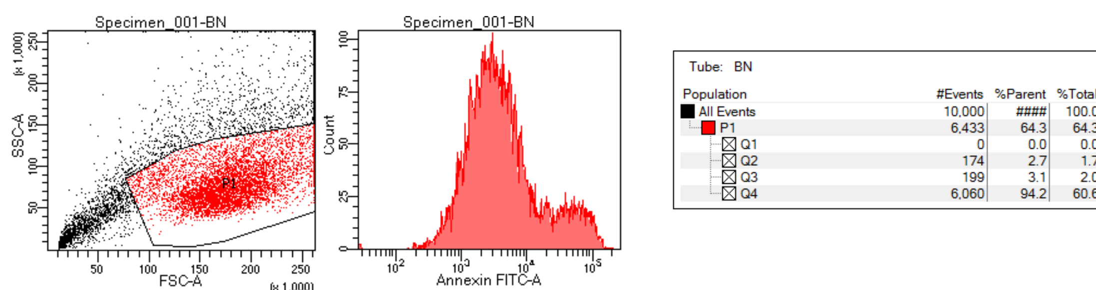
rMV infected + Taxol



Tube: RMVTx

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	4,103	41.0	41.0
Q1	0	0.0	0.0
Q2	458	11.2	4.6
Q3	123	3.0	1.2
Q4	3,522	85.8	35.2

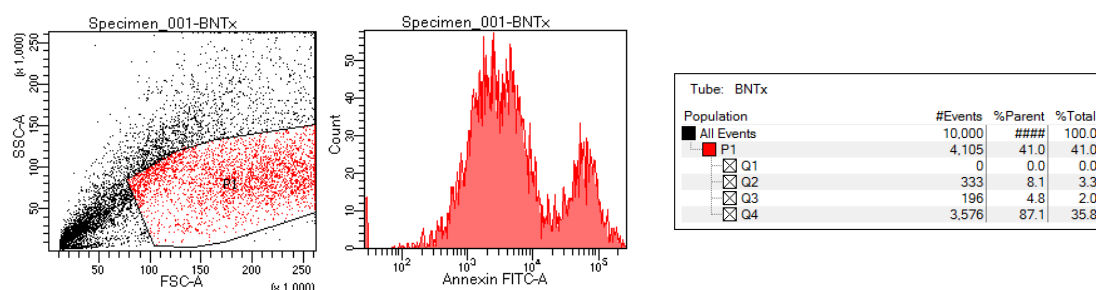
rMV-BNiP3 infected



Tube: BN

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	6,433	64.3	64.3
Q1	0	0.0	0.0
Q2	174	2.7	1.7
Q3	199	3.1	2.0
Q4	6,060	94.2	60.6

rMV-BNiP3 infected + Taxol



Tube: BNTx

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	4,105	41.0	41.0
Q1	0	0.0	0.0
Q2	333	8.1	3.3
Q3	196	4.8	2.0
Q4	3,576	87.1	35.8

Figure 31: Scatter plot and histogram of each representative sample and the corresponding table shows the percentage of cells in all quadrants. Upper right quadrant (Q2) shows the percentage of cells stained for AnnexinV/ PI; lower right quadrant (Q4) shows the percentage of Annexin V stained population.

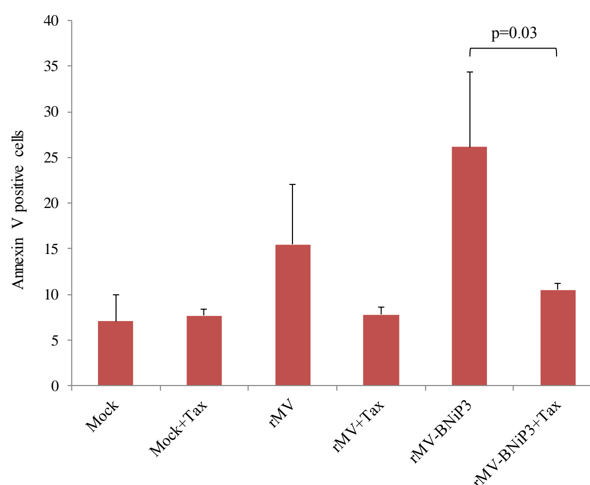


Figure 32: Annexin V staining in MDA-MB-231 cells infected with rMV and rMV-BNiP3 in combination with Paclitaxel.

The mechanism of rMV-BNiP3 mediated cell death in triple negative cell line remains elusive, so our future studies will be focused on this aspect. But the scope of the current study was to construct a stable and replication competent recombinant virus armed with a foreign genetic element to enhance its oncolytic potential.

DISCUSSION

Viruses have long been associated with devastating epidemics, pandemics and diseases. Death rates associated with outbreaks as seen in case of SARS, swine flu and Ebola makes them a dreaded pathogen of nature. Viruses employ the most sophisticated evasion maneuvers to counteract host defense and subjugate its cellular machinery for their own propagation. This property, while making them a strong adversary, can be exploited for therapeutic purposes. The strong survival instincts of virus can be honed against cancer cells to generate a cancer specific pathogen for oncolysis.

Viral vectors used for gene therapy are not ideal for treatment of cancer due to its complexity and difficult to achieve tumor specificity. Replication competent viruses with intrinsic tumor tropism or recombinant viruses can be used specifically to infect and kill cancer cells without affecting the normal cells. They can be the missing link in the evolutionary pattern of ever changing cancer therapy scenario. Conventional cancer therapeutics such as surgery, chemotherapy and radiotherapy are mired with severe side-effects and limited efficacy. Also the conventional cancer therapies impart their toxicity to neighboring cells. Their lack of specificity towards cancer cells and adaptability towards drug resistant cancer cells has led to a lacuna in development of a comprehensive anti-cancer approach. Poor prognostic outcome in metastatic form of disease has made cancer the dreaded foe it is today. Thus the option of rewired and reprogrammed viruses to direct their pathogenicity towards cancer cells provides a promising tool in the arsenal of anti-cancer regimens. Viruses can be manipulated to target the deregulated pathways in cancer cells. Several such strategies have already been under investigation to develop effective oncolytic viruses.

Virotherapy is a new anti-cancer treatment modality with improved therapeutic efficacy. Until now more than ten different viruses have been exploited and manipulated for the development of oncolytic agents to treat a wide variety of cancers. Among them measles, mumps, Newcastle disease and Sendai viruses; members of paramyxoviridae family have great potential for inducing tumor cell death efficiently. Some of them are already under clinical trials (Matveeva et al. 2015). In the current study we aimed to develop measles virus based oncolytic agent to target the deregulated apoptotic pathway in cancer cells. It was done by inserting a foreign gene encoding for pro-apoptotic protein in measles viral genome. It is hypothesized that the infection of recombinant virus in cancer cell will lead to expression of pro-apoptotic gene and inability of cancer cells to suppress this expression will induce apoptosis.

Measles virus is an enveloped virus with a non-segmented negative sense RNA genome spanning a length of approximately 16kb leading to translation of eight viral proteins; N, P, M, F, H and L. N is nucleocapsid protein, P, co-factor for L protein, M, F and H are structural proteins; matrix, fusion and hemagglutinin respectively and L is RNA dependent RNA polymerase. Two small proteins C and V, involved in pathogenesis, are generated through RNA editing of P transcripts. Live attenuated measles virus has been used as a vaccine for more than half a decade and has well documented population safety due to the widespread vaccination programs. This safety can be attributable to the stable nature of its genome, with no recorded case of reversion of vaccine or attenuated strain to its pathogenic state. Its stable genome, with rigid reluctance for natural insertional mutations make it a safe and viable option as a platform for generation of current and future oncolytic agents. As reported earlier, Edmonston strain of measles virus is biased towards cancer cells owing to its utilization of CD46 and nectin-4 receptors which are overexpressed in cancer cells as opposed to wild type measles virus using signaling lymphocyte activation molecule (SLAM) receptor for its internalization. The altered tropism of measles virus-Edmonston strain was achieved by a single amino acid substitution N481Y in its hemagglutinin, host cell receptor binding protein (Naniche et al. 1993; Dörig et al. 2017; Nielsen et al. 2001).

Here, we developed genetically modified measles virus with a pro-apoptotic gene inserted in its genome to increase its oncolytic ability. For which, we generated a packaging cell line stably expressing measles virus nucleocapsid protein, phosphoprotein (co-factor for viral RNA polymerase) and T7 RNA polymerase. Recombinant measles virus was generated and rescued following co-transfection of packaging cell line with full length recombinant measles virus genome that was cloned under T7 promoter and viral RNA polymerase (L polymerase) construct. The replication competency of the recombinant virus generated was confirmed in Vero cells, an *in vitro* model for virus propagation. Replication competency and oncolytic ability of this recombinant virus was evaluated in breast carcinoma cell lines; MCF-7 and MDA-MB-231. Expression of recombinant viral genes confirmed the replication ability of rescued virus in MCF-7 and MDA-MB-231 cells and showed better cytopathic effect (CPE), as compared to normal epithelial HEK293 cells. Measles virus is an enveloped virus and manifests syncytia due to cell to cell spread, but more of rounding of cells than syncytia was noticed in cells infected with recombinant virus.

Even amongst the breast cancer cells, recombinant virus with BNiP3 gene (rMV-BNiP3) showed higher toxicity against MDA-MB-231 cells as compared to MCF-7 cells. This observation was made more interesting with the fact that MDA-MB-231 cells are the triple negative population of breast cancer cells which neither expresses estrogen and progesterone receptors nor it is her2/neu positive. Both the cell lines do express CD46 receptor but the infection ability and the relatively higher expression of viral genes at transcript and protein level including BNiP3 could be due to better targeting of this virus to triple negative cells. Tumor targeting is the major feature of the oncolytic virotherapy but viral tropism can be modified for efficient targeting of the oncolytic virus to tumor cells. Recent studies with mutated hemagglutinin (H) gene of wild type measles virus has demonstrated the preferential use of nectin-4 receptors by SLAM blind viruses and have shown oncolytic activity against breast cancer, lung cancer and colorectal carcinoma (Sugiyama et al. 2012; Fujiyuki et al. 2015; Amagai et al. 2016). Insertion of cancer specific ligands including peptides and ScFvs as C-terminus extensions of mutated H gene has also contributed towards tumor-specificity of the recombinant measles virus. Measles virus with insertion of specific DARPins (designed ankyrin repeat proteins), in its H protein, for Her2/neu , EGFR or EpCAM was retargeted towards tumors positive for these surface receptors (Friedrich et al. 2013; Hanauer et al. 2016). No modification with respect to targeting was done to the recombinant virus generated in this study, yet the higher affininty of this virus towards MDA-MB-231 cells is an important feature to be explored. Apart from tropism, other factors might have contributed to the better selectivity of the virus, rMV-BNiP3 to MDA-MB-231 cells.

In addition to retargeting the virus for tumor specificity, measles virus also exhibit ease of genetic manipulation as evident by various studies involving arming of virus for enhanced anti-tumor toxicity as well as immunomodulation to incite host anti-tumor immune response. The advent of incorporation of genetic changes can be traced back to the generation of plasmid encoding complete measles cDNA giving way for the establishment of a reverse genetics system for generation of recombinant virus within a cell line based recovery system (Radecke et al. 1995). Since then various studies have used this system to generate measles virus with unique genetic features contributing towards its oncolytic ability. Examples can be sought from studies involving measles virus expressing NIS (sodium iodide symporter) which in combination with radioisotope of iodine (^{131}I), sequesters it in the tumor and leads to lysis of radiosensitive cancer cells as seen in case of preclinical models of multiple myeloma (Dispenzieri et al. 2017), prostate cancer, glioblastoma multiforme and medulloblastoma

(Kadia 2016). Measles virus has also been armed with genes encoding for pro-drug activation such as purine nucleoside phosphorylase (PNP) from *E.coli* which showed increased survival in murine model of colon adenocarcinoma in combination with pro-drugs 6-methylpurine-2′-deoxyriboside (MeP-dR) and fludarabine (Ungerechts et al. 2007). Insertion with GMCSF and IFN β in measles genome has resulted in heightened anti-cancer immune response (Cattaneo & Russell 2017). Measles virus was also armed with IL-12 which acts as an immunomodulaor and mediates anti-tumor effects through T-cell activation (Veinalde et al, 2017). Measles virus armed with neutrophil activating protein of *Helicobacter pylori* also acts as immunomodulator and enhances the oncolytic activity (Iankov et al. 2012) of the virus. It facilitates the efficient killing of metastatic breast cancer cells through cell to cell virus spread. All the above reports collectively suggest that the oncolytic potential of measles virus can be enhanced by insertional modifications.

Present study was also aimed to generate an ‘armed’ oncolytic virus with a foreign genetic element inserted within the measles viral genome to increase its tumor toxicity. This was achieved by stable incorporation of BNiP3 gene, a pro-apoptotic gene of human origin into the viral genome in between the coding region for measles virus phosphoprotein and matrix protein. Expression of BNiP3 at transcript and protein level in cells infected with rMV-BNiP3 confirmed the successful generation of a recombinant virus with extra genome than its unit length. Measles virus capsid exhibits helical morphology. Helical capsid is characterized by length of the viral genome and the open symmetry of helix facilitates the incorporation of extra genome into the capsid. But, increased genome length neither affected its assembly and packaging nor replication efficiency. Besides, measles virus, as many other viruses, follows the rule of six to maintain genetic integrity and dissuade insertional mutations. This rule allows efficient transcription, translation and packaging of viral genome only when the number of bases is in multiples of six. It is due to the fact that nucleoprotein, an essential protein for the formation of viral ribonucleoprotein complex, always binds to six bases of viral genome thus rendering anything not within the sextuplet to be lost during transcription leading to loss of function and infectivity. While inserting the foreign gene in viral genome, the rule of six was followed. As described earlier the level of expression of BNiP3 and viral genes was more in MDA-MB-231 cells as compared to MCF-7 cells. Due to more robust viral response in MDA-MB-231 cells as compared to MCF-7 cells, majority of assays for apoptosis were done in MDA-MB-231 cells.

Measles being a virus with its infective life cycle carried out specifically in cellular cytoplasm, showed BNiP3 expression in cytoplasm of infected MDA-MB-231 cells. This cytoplasmic expression has clinical efficacy due to the fact that there have been clinical reports linking downregulation and nuclear expression of BNiP3 to increased potential of metastasis in breast cancer patients (Koop et al. 2009). Since Measles virus replicates exclusively in cytoplasm of host cells, thus rMV-BNiP3 can prove to be an efficient tool against breast carcinoma. BNiP3 is a Bcl-2/adenovirus E1B 19kDa interacting protein with a BH3 domain. It is a pro-apoptotic protein localized on the outer mitochondrial membrane, induced under hypoxia and is absent or downregulated in many cancers. BNiP3 functionality has been associated with caspase independent cell death without induction of cytochrome c release (Vande Velde et al. 2000; Imazu et al. 1999) and induction of autophagy and mitophagy (Zhang & Ney 2009; Azad et al. 2008).

The idea behind inclusion of BNiP3 in the viral genome was to induce apoptosis upon infection of cancer cells, as evasion of programmed cell death is the major hallmark of cancers including breast cancer. Preliminary experiments performed in measuring apoptosis following transfection of BNiP3 construct and rMV infection had demonstrated increased caspase activity in both the cell lines. But assays done to observe the induction of apoptosis in cells infected with the recombinant virus with BNiP3 had shown higher toxicity and caspase activity in MDA-MB-231 cells than rMV without BNiP3. The lack of significance of the comparative activity can be attributed to various facts. One of the major reasons could be the double pronged consequences of inclusion of a suicide or pro-apoptotic gene in the virus. As it stands, a toxic gene will lead to the death of cancer cells, but it also deprives the virus of the associated host machinery for its propagation. This may result in less number of viral progenies as compared to that of virus without any cytotoxic element. It has been reported in literature that Edmonston strain of measles virus downregulates BNiP3 to ablate the apoptotic machinery for its continued propagation. By outfitting the virus with BNiP3 we may be able to induce apoptosis in breast cancer cells but it may also compromise the production of viral progeny particles thereby allowing the rMV to compensate for lack of apoptosis with sheer debulking effect. The incorporation of additional coding sequence in the viral genome may also affect the replication potential of rMV-BNiP3 and slow it down as compared to that of rMV. In addition to the replication and propagation, the mechanism of action of an oncolytic virus is a complex process. It does not solely depend on virus-induced lysis after host machinery exhaustion to induce tumor-cell death but also incorporates the host immune

response for effective oncolysis. Studies have demonstrated the major role immunogenic cell death plays in oncolytic virus induced cancer cell death (Iankov et al. 2010). In case of rMV-BNiP3, lack of immune component and responsiveness of an *in vitro* cellular platform may have played a role in the comparable anti-cancer effect observed with rMV and rMV-BNiP3. Thus, it remains to be seen that whether it has an effective oncolytic role in case of *in vivo* tumor models or whether it potentiates the apoptotic response towards invasion.

The induction of apoptosis or cell death in cancer cells either virus-induced or by some other mechanism, is not a case of singular gene expression. It is a complex multi-focal pathway, thus it is difficult to predict the ultimate outcome of a single incorporation in an *in vitro* system. This unpredictability of host and tumor response towards the oncolytic viral infection as well as presence of neutralizing antibodies is a shortcoming of majority of viral platforms. Thus to increase their efficacy against cancer, they are combined with an arm of conventional cancer therapeutics. The vulnerability of oncolytic therapy, rather than being reduced in virus, can be overcome by the elicited effect of synergistic conventional therapy. There are recent examples of combination of oncolytic viruses with either chemotherapeutic agents or radiotherapy. Even FDA approval of T-Vec and CFDA approval of H101 and Gendicine is in combination with known therapeutic agents. Thus we aimed to observe the combinatorial anti-tumor effects of armed oncolytic measles virus with Paclitaxel and a novel hydrazone derivative (H2).

Oncolytic virus, as a standalone monotherapy, still has ways to go before achieving greater success. With keeping this in mind we combined rMV and rMV-BNiP3 with a known and a novel anti-tumor compound from our previous study, H2 compound). Paclitaxel was taken as a drug control. Generally paclitaxel is given as chemotherapy in various cancers, but as with all chemotherapeutic agents, it also has systemic side-effects. By treating the breast carcinoma cells with paclitaxel at a sub-lethal dose followed by infection with rMV or rMV-BniP3 or vice-versa we observed marked increase in the caspase 3 activity of cells treated with rMV and cells treated with the combination of rMV-BNiP3 and 30nM paclitaxel and H2 compound. FACS analysis of cells treated with paclitaxel followed by infection with rMV or rMV-BNiP3 had shown no difference and increase in apoptosis as compared to untreated cells. Annexin V stained population, representing apoptotic cells was relatively high in cells infected with rMV-BNiP3 than rMV in the absence of paclitaxel.

This observation of synergism was made even more interesting when in combination to a novel hydrazone derivative N'-(2-chlorobenzylidene)-4-(2-(dimethylalamino)ethoxy) benzohydrazide) H2, the cytotoxic effect against MDA-MB-231 increased to a significant level as compared to only rMV or rMV-BNiP3 infected cells. It may be noted that the working concentration of H2 used (20μM) may seem quite improbable for actual use but its relatively low toxicity at even higher concentrations of approximately 240μM (IC_{50} value) in HEK293 cells makes the utilized concentration of drug sub-lethal. Annexin V staining corresponding to apoptosis had shown high Annexin V stained population in cells infected with rMV-BNiP3 in MDA-MB-231 as compared to rMV infected cells in absence of paclitaxel. The possible explanation is that paclitaxel induce necrotic death, thus the addition of paclitaxel to rMV and rMV-BNiP3 infected cells did not contribute to apoptosis. The reason for inconsistency of apoptosis results demonstrated by caspase activity and FACS analysis needs further investigation.

The approval of T-Vec by FDA for the treatment of carcinoma has been seen as an unprecedented success of an alternative therapeutic approach. But this approval comes with the caveat of loco-regional application of virus to lesions and there is not much significant improve of overall survival. As discussed earlier, combination of oncolytic virotherapy with conventional approaches may result in increase of the overall tumor death and survival but this response is still not achieved even in the case of FDA approved T-Vec (Bommareddy et al. 2017). Thus it is imperative to evolve the viruses according to the needs and requirements of therapy rather than trying to fit a single representative in every role.

Measles virus is one of the most widely investigated viruses for oncolyic purpose and showing promising results at pre-clinical and clinical settings. Its therapeutic efficacy was demonstrated at various levels. Considering the safety record of vaccine strain and its ability to be manipulated, its effectiveness as an oncolytic agent will soon be established. Significant number of studies has reported the use and manipulation of measles virus genome to target various cancers, also in combination with pre-existing cancer therapeutics. The main goal of our study was to construct the recombinant measles virus and we successfully developed genetically stable recombinant virus armed with foreign gene element. The results we achieved in our study further points to the fact that in addition to developing viruses as oncolytic agents, it is also imperative to thoroughly establish the mode of cell death induced by oncolytic viruses as even now it is poorly understood as in the case of increased toxicity of

rMV-BNiP3 against MDA-MB-231 cells as compared to MCF-7 cells. As triple negative breast tumors are more metastatic and resistant with limited responsiveness towards available therapeutics this infective bias can be explored against breast carcinomas showing poor reactivity towards conventional therapeutics.

SUMMARY
AND
OVERVIEW OF THE STUDY

SUMMARY

- ✓ Recombinant clones encoding measles virus structural nucleoprotein and polymerase co-factor phosphoprotein were successfully generated in eukaryotic expression vector pcDNA3.1+

- ✓ Recombinant clone encoding bacteriophage T7 RNA polymerase gene was successfully generated in eukaryotic expression vector pcDNA3.1+

- ✓ Packaging cell line stably co-expressing measles viral nucleoprotein, phosphoprotein and T7 RNA polymerase was successfully generated using HEK293 as platform to rescue the generated recombinant virus. Expression of recombinant genes was confirmed by amplification of corresponding transcripts and protein expression was confirmed by immunofluorescence assay and immunoblotting.

- ✓ Plasmid encoding full length anti genome of measles virus, p (+) MV-NSE-FlagP-M502-3p, was procured from addgene plasmid repository.

- ✓ Presence of viral genome was confirmed by amplification of measles virus nucleoprotein and phosphoprotein genes using specific primers. Plasmid was also confirmed by restriction digestion of listed unique site as well as of internal observed sites.

- ✓ Recombinant clone encoding measles virus large polymerase gene, an RNA dependent RNA polymerase, was successfully generated in eukaryotic expression vector pcDNA3.1+.

- ✓ Co-transfection of packaging cell line with p (+) MV-NSE-FlagP-M502-3p and recombinant large polymerase plasmid construct resulted in formation of virus particles and demonstrated the success of reverse genetics paradigm of generation of complete virion in cells supplied with viral genome with associated proteins without the presence of parental virus.

- ✓ Generated recombinant virus particles (rMV) were rescued from the packaging cell line and confirmed by amplification of transcripts and viral protein expression in infected Vero cells for two consecutive passages. Presence of viral transcripts and

proteins in successive passages suggest generation of complete virion with intact structural and replicative integrity.

- ✓ Disrupted site for *Pfl*2311 restriction enzyme was restored in the viral genome (p (+) MV-NSE-FlagP-M502-3p) between phosphoprotein and matrix protein encoding region by site directed mutagenesis. Linearization of mutated p (+) MV-NSE-FlagP-M502-3p with *Pfl*2311 restriction enzyme confirmed the introduction of point mutation and restoration of restriction site.

- ✓ Reporter gene encoding for Mcherry fluorophore was successfully inserted in the viral genome between phosphoprotein and matrix protein encoding region using unique restriction sites for *Pfl*2311 and *Aat*II. Its cytoplasmic expression in MCF-7 and MDA-MB-231 cells confirmed the functional restoration of *Pfl*2311 restriction site, stable integration of reporter gene in viral genome and formation of modified virus (rMV-Mcherry) expressing Mcherry in the host cells.

- ✓ Expression of measles viral early proteins, nucleoprotein and phosphoprotein, and late hemagglutinin protein in addition to Mcherry protein in rMV-Mcherry infected breast cancer cells further confirmed the successful generation of reporter virus and its ability to infect and form replication competent progeny virions in cancer cells.

- ✓ It was curious to observe that rMV and rMV-Mcherry, both showed infective bias towards triple negative MDA-MB-231 cells as compared to ER+ve MCF-7 cells. This observation was corroborated with low expression levels of early and late viral proteins in MCF-7 cells as compared to MDA-MB-231 cells.

- ✓ Recombinant measles virus encoding a human origin pro-apoptotic protein, BNiP3, was successfully generated (rMV-BNiP3) and showed similar infective bias towards MDA-MB-231 cells as compared to MCF-7 cells. High expression of BNiP3 was observed in MDA-MB-231 cells infected with rMV-BNiP3 as compared to rMV infected cells.

- ✓ Cytotoxicity measured by MTT assay of rMV and rMV-BNiP3 infected MDA-MB-231 and MCF-7 cells also validated the proclivity of recombinant viruses towards MDA-MB-231 cells with rMV-BNiP3 infected cells showing higher cell death.

- ✓ Combinatorial anti-tumor effect of recombinant virus was also observed by addition of Paclitaxel, a known chemotherapeutic agent and H2, a novel hydrazone compound at sub-lethal dosage.

- ✓ MDA-MB-231 cells treated with combination of rMV-BNiP3 and either paclitaxel or H2 showed significantly higher cytotoxicity as compared to cells treated with similar combination of rMV and drug or only viruses.

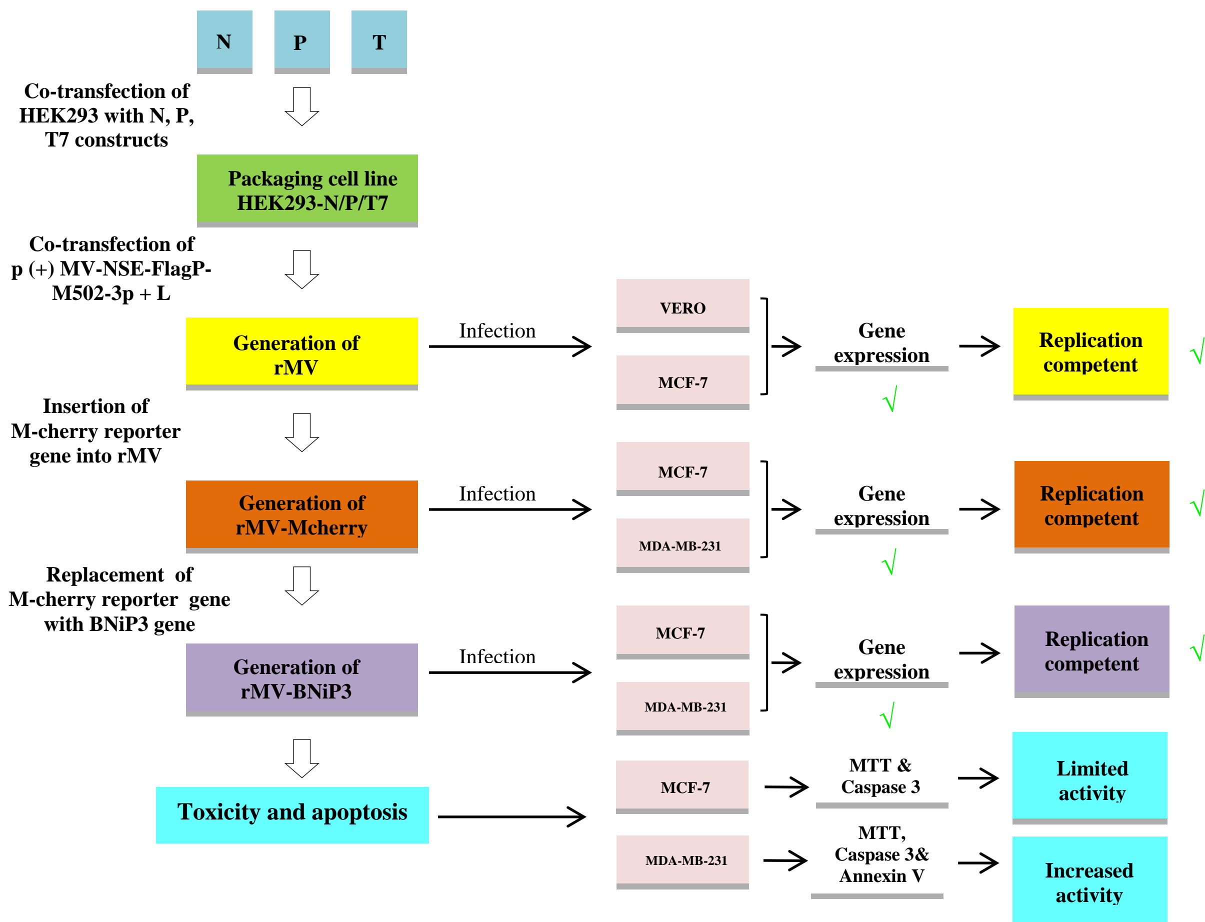
- ✓ In addition to cytotoxicity, apoptosis was also measured by caspase 3 activity and annexin V/PI staining by FACS.

- ✓ MDA-MB-231 cells treated with either paclitaxel or H2 and infected with rMV-BNiP3 showed significantly higher caspase 3 activity as compared to rMV treated cells. Annexin V/PI staining showed higher number of apoptotic cell population in cells treated with rMV-BNiP3 as compared to rMV. This observation was significant with respect to the fact that BNiP3 is a pro-apoptotic gene which is downregulated in majority of cancers including breast carcinoma and its incorporation in the viral genome corroborates the heightened apoptotic response of infected cells.

- ✓ The underlying mechanism of selectivity exhibited by recombinant virus towards MDA-MB-231 cells is yet to be explored and if characterized, can open new avenues for the treatment of resistant or refractory form of disease.

- ✓ It remains to be seen whether the infective bias exhibited by rMV-BNiP3 towards triple negative breast carcinoma cells and its contribution towards inducing apoptosis in infected cells can translate into *in vivo* and clinical settings and whether it emerges as a viable oncolytic agent for cancer therapy.

Overview of the study



Legend of figure:

- **N, P, T7:** Recombinant constructs of viral N, P and T7 RNA polymerase generated in eukaryotic expression vector, pcDNA3.1+
- **HEK293-N/P/T7 (Packaging cell line)**: HEK293 cells stably expressing MV nucleoprotein-N (capsid protein), Phosphoprotein-P(cofactor for L protein, RNA dependent RNA polymerase) and T7 RNA polymerase
- **p(+)MV-NSE-FlagP-M502-3p:** Plasmid vector containing full length MV genome (19.4kb) cloned under T7 Promoter
- **rMV**: Recombinant MV rescued from packaging cell line following transfection with p(+)MV-NSE-FlagP-M502-3p construct and viral recombinant L plasmid, RNA dependent RNA polymerase generated in expression vector pcDNA 3.1+
- **rMV-MCherry:** Genetically engineered recombinant MV harboring reporter red fluorescent gene rescued from packaging cell line transfected with p (+) MV-NSE-FlagP-M502-3p-Mcherry plasmid and viral recombinant L plasmid, RNA dependent RNA polymerase generated in expression vector pcDNA 3.1+
- **rMV-BNiP3:** Genetically engineered recombinant MV harboring pro-apoptotic gene BNiP3 of human origin rescued from packaging cell line transfected with p (+) MV-NSE-FlagP-M502-3p-BNiP3plasmid and viral recombinant L plasmid, RNA dependent RNA polymerase generated in expression vector pcDNA 3.1+
- √: Cells had shown viral gene expression at transcript and protein level 24-48 hours post-infection with the respective genetically modified recombinant virus indicating its replication efficiency in Vero and MCF-7 cells up to 2-3 passages.

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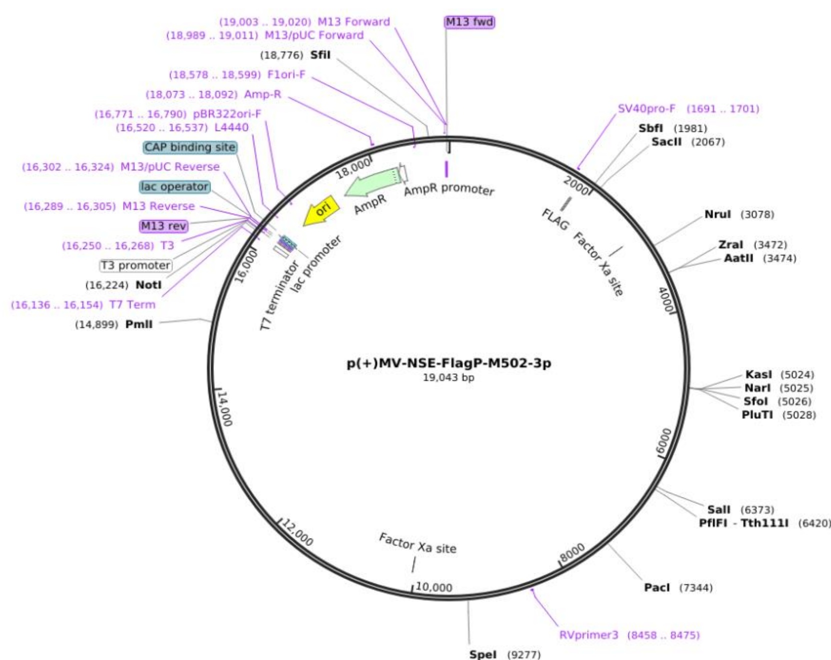
APPENDIX

Sequence features of p (+) MV-NSE-FlagP-M502-3p

```
Name                type                location
mut agc --> tcg     misc_feature        3076..3078
C cds NB maybe elongated from two ATG upstream & inmisc_feature        1853..2067
N cds               misc_feature        108..807,809..1685
F cds               misc_feature        5551..7212
H cds               misc_feature        7373..9226
L cds               misc_feature        9336..15887
Trailer (as)        misc_feature        15960..15996
Leader              misc_feature        1..41
Flag                misc_feature        1810..1833
C cds               misc_feature        2068..2413
M cds               misc_feature        3540..4547
mut XD Schwarz GTG --> aTGmisc_feature  3334..3334
mut site BsiWI cgtacg --> cgtacCmisc_feature  3467..3467
P cds               misc_feature        1807..2067
P cds               misc_feature        2068..3354
Amp pro             misc_feature        18352..18380
T3                  primer_bind         rev:16248..16267
M13-fwd             primer_bind         19003..19020
M13-rev             primer_bind         rev:16285..16305
ColE1 origin        rep_origin          rev:16673..17355
LacZ alpha          CDS                 rev:18864..18932
LacO                misc_binding        rev:16311..16333
AmpR                CDS                 rev:17453..18112
AmpR promoter       misc_feature        rev:18352..18380
Lac promoter        misc_feature        rev:16338..16367
M13rev(-29) MWG seqmisc_feature         rev:16288..16305
M13rev(-49) MWG seqmisc_feature         rev:16302..16325
M13 uni (-21)       misc_feature        19003..19020
M13uni(-43) MWG seq misc_feature        18981..19003
si2(siP) target     misc_feature        2923..2942
si2(siP) target     misc_feature        3380..3399
si2(siP) target     misc_feature        3406..3425
si2(siP) target     misc_feature        3432..3451
```



```
LOCUS       Exported               19043 bp ds-DNA     circular SYN 20-JUN-2017
DEFINITION  Expresses recombinant measles virus with Flag-P gene tagged si2
            (siP) target sequence.
ACCESSION   .
VERSION     .
KEYWORDS    p(+)MV-NSE-FlagP-M502-3p
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 19043)
  TITLE     biG-biS plasmids
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 19043)
  AUTHORS   .
  TITLE     Direct Submission
  JOURNAL   Exported Tuesday, Jun 20, 2017 from SnapGene Console 4.0.0a8
            http://www.snapgene.com
FEATURES             Location/Qualifiers
     source          1..19043
                     /organism="synthetic DNA construct"
                     /mol_type="other DNA"
     primer_bind     1691..1701
                     /label=SV40pro-F
                     /note="SV40 promoter/origin, forward primer"
     CDS             1810..1833
                     /codon_start=1
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                     cleavage site"
                     /label=FLAG
                     /translation="DYKDDDDK"
     CDS             2853..2864
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                     /product="Factor Xa recognition and cleavage site"
                     /label=Factor Xa site
                     /translation="IEGR"
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                     /label=RVprimer3
                     /note="pGL3 vector, forward primer"
     CDS             10038..10049
                     /codon_start=1
                     /product="Factor Xa recognition and cleavage site"
                     /label=Factor Xa site
                     /translation="IEGR"
     primer_bind     complement(16136..16154)
                     /label=T7 Term
                     /note="T7 terminator, reverse primer"
     terminator      16150..16197
                     /label=T7 terminator
                     /note="transcription terminator for bacteriophage T7 RNA
                     polymerase"
     promoter        complement(16250..16268)
                     /label=T3 promoter
                     /note="promoter for bacteriophage T3 RNA polymerase"
     primer_bind     complement(16250..16268)
                     /label=T3
                     /note="T3 promoter, forward primer"
     primer_bind     complement(16289..16305)
                     /label=M13 rev
                     /note="common sequencing primer, one of multiple similar
                     variants"
     primer_bind     complement(16289..16305)
                     /label=M13 Reverse
                     /note="In lacZ gene. Also called M13-rev"
     primer_bind     complement(16302..16324)
                     /label=M13/pUC Reverse
                     /note="In lacZ gene"
     protein_bind    16313..16329
                     /label=lac operator
                     /bound_moiety="lac repressor encoded by lacI"
```

```
                     /note="The lac repressor binds to the lac operator to
                     inhibit transcription in E. coli. This inhibition can be
                     relieved by adding lactose or
                     isopropyl-beta-D-thiogalactopyranoside (IPTG)."
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12421 tgttaaaagg attattccat gatgacagta aagaagagga cgagggactg gcggcattcc
12481 tcatggacag gcatattata gtacctaggg cagctcatga aatcctggat catagtgtca
12541 caggggcaag agagtctatt gcaggcatgc tggataccac aaaaggcttg attcgagcca
12601 gcatgaggaa gggggggttta acctctcgag tgataaccag attgtccaat tatgactatg
12661 aacaattcag agcagggatg gtgctattga caggaagaaa gagaaatgtc ctcattgaca
12721 aagagtcatg ttcagtgcag ctggcgagag ctctaagaag ccatatgtgg gcgaggctag
12781 ctcgaggacg gcctatttac ggccttgagg tccctgatgt actagaatct atgcgaggcc
12841 accttattcg gcgtcatgag acatgtgtca tctgcgagtg tggatcagtc aactacggat
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12901 ggttttttgt cccctcgggt tgccaactgg atgatattga caaggaaaca tcatccttga
12961 gagtcccata tattggttct accactgatg agagaacaga catgaagctt gccttcgtaa
13021 gagccccaag tcgatccttg cgatctgctg ttagaatagc aacagtgtac tcatgggctt
13081 acggtgatga tgatagctct tggaacgaag cctggttgtt ggctaggcaa agggccaatg
13141 tgagcctgga ggagctaagg gtgatcactc ccatctcaac ttcgactaat ttagcgcata
13201 ggttgaggga tcgtagcact caagtgaaat actcaggtac atcccttgtc cgagtggcga
13261 ggtataccac aatctccaac gacaatctct catttgtcat atcagataag aaggttgata
13321 ctaactttat ataccaacaa ggaatgcttc tagggttggg tgttttagaa acattgtttc
13381 gactcgagaa agataccgga tcatctaaca cggtattaca tcttcacgtc gaaacagatt
13441 gttgcgtgat cccgatgata gatcatccca ggatacccag ctcccgcaag ctagagctga
13501 gggcagagct atgtaccaac ccattgatat atgataatgc acctttaatt gacagagatg
13561 caacaaggct atacacccag agccatagga ggcacccttgt ggaatttgtt acatggtcca
13621 caccccaact atatcacatt ttagctaagt ccacagcact atctatgatt gacctggtaa
13681 caaaatttga gaaggaccat atgaatgaaa tttcagctct catagggggat gacgatatca
13741 atagtttcat aactgagttt ctgctcatag agccaagatt attcactatc tacttgggcc
13801 agtgtgcggc catcaattgg gcatttgatg tacattatca tagaccatca gggaaatatc
13861 agatgggtga gctgttgtca tcgttccttt ctagaatgag caaaggagtg tttaaggtgc
13921 ttgtcaatgc tctaagccac ccaaagatct acaagaaatt ctggcattgt ggtattatag
13981 agcctatcca tggtccttca cttgatgctc aaaacttgca cacaactgtg tgcaacatgg
14041 tttacacatg ctatatgacc tacctcgacc tgttgttgaa tgaagagtta gaagagttca
14101 catttctctt gtgtgaaagc gacgaggatg tagtaccgga cagattcgac aacatccagg
14161 caaaacactt atgtgttctg gcagatttgt actgtcaacc agggacctgc ccaccaattc
14221 aaggtctaag accggtagag aaatgtgcag ttctaaccga ccatatcaag gcagaggcta
14281 tgttatctcc agcaggatct tcgtggaaca taaatccaat tattgtagac cattactcat
14341 gctccctgac ttatctccgg cgaggatcga tcaaacagat aagattgaga gttgatccag
14401 gattcatttt cgacgccctc gctgaggtaa atgtcagtca gccaaagatc ggcagcaaca
14461 acatctcaaa tatgagcatc aaggctttca gaccccccaca cgatgatgtt gcaaaattgc
14521 tcaaagatat caacacaagc aagcacaatc ttcccatttc agggggcaat ctcgccaatt
14581 atgaaatcca tgctttccgc agaatcgggt tgaactcatc tgcttgctac aaagctgttg
14641 agatatcaac attaattagg agatgccttg agccagggga ggacggcttg ttcttgggtg
14701 agggatcggg ttctatgttg atcacttata aggagatact taaactaagc aagtgcttct
14761 ataatagtgg ggtttccgcc aattctagat ctggtcaaag ggaattagca ccctatccct
14821 ccgaagttgg ccttgtcgaa cacagaatgg gagtaggtaa tattgtcaaa gtgctcttta
14881 acgggaggcc cgaagtcacg tgggtaggca gtgtagattg cttcaatttc atagttagta
14941 atatccctac ctctagtgtg gggtttatcc attcagatat agagaccttg cctgacaaag
15001 atactataga gaagctagag gaattggcag ccatcttatc gatggctctg ctcctgggca
15061 aaataggatc aatactggtg attaagctta tgcctttcag cggggatttt gttcagggat
15121 ttataagtta tgtagggtct cattatagag aagtgaacct tgtataccct agatacagca
15181 acttcatatc tactgaatct tatttggtta tgacagatct caaggctaac cggctaatga
15241 atcctgaaaa gattaagcag cagataattg aatcatctgt gaggacttca cctggactta
15301 taggtcacat cctatccatt aagcaactaa gctgcataca agcaattgtg ggagacgcag
15361 ttagtagagg tgatatcaat cctactctga aaaaacttac acctatagag caggtgctga
15421 tcaattgcgg gttggcaatt aacggaccta agctgtgcaa agaattgatc caccatgatg
15481 ttgcctcagg gcaagatgga ttgcttaatt ctatactcat cctctacagg gagttggcaa
15541 gattcaaaga caaccaaaga agtcaacaag ggatgttcca cgcttacccc gtattggtaa
15601 gtagcaggca acgagaactt atatctagga tcacccgcaa attttggggg cacattcttc
15661 tttactccgg gaacagaaag ttgataaata agtttatcca gaatctcaag tccggctatc
15721 tgatactaga cttacaccag aatatcttcg ttaagaatct atccaagtca gagaaacaga
15781 ttattatgac gggggggtttg aaacgtgagt gggtttttaa ggtaacagtc aaggagacca
15841 aagaatggta taagttagtc ggatacagtg ccctgattaa ggactaattg gttgaactcc
15901 ggaaccctaa tcctgcccta ggtggttagg cattatttgc aatatattaa agaaaacttt
15961 gaaaatacga agtttctatt cccagctttg tctggtggcc ggcatggtcc cagcctcctc
16021 gctggcgctg gctgggcaac attccgaggg gaccgtcccc tcggtaatgg cgaatgggac
16081 gcggccgatc cggctgctaa caaagcccga aaggaagctg agttggctgc tgccaccgct
16141 gagcaataac tagcataacc ccttggggcc tctaaacggg tcttgagggg ttttttgctg
16201 aaaggaggaa ctatatccgg atgcggccgc aggtacccag cttttgttcc ctttagtgag
16261 ggttaatttc gagcttggcg taatcatggt catagctgtt tcctgtgtga aattgttatc
16321 cgctcacaat tccacacaac atacgagccg gaagcataaa gtgtaaagcc tggggtgcct
16381 aatgagtgag ctaactcaca ttaattgcgt tgcgctcact gcccgctttc cagtcgggaa
16441 acctgtcgtg ccagctgcat taatgaatcg gccaacgcgc ggggagaggc ggtttgcgta
16501 ttgggcgctc ttccgcttcc tcgctcactg actcgctgcg ctcggtcgtt cggctgcggc
16561 gagcggtatc agctcactca aaggcggtaa tacggttatc cacagaatca ggggataacg
16621 caggaaagaa catgtgagca aaaggccagc aaaaggccag gaaccgtaaa aaggccgcgt
16681 tgctggcgtt tttccatagg ctccgccccc ctgacgagca tcacaaaaat cgacgctcaa
16741 gtcagaggtg gcgaaacccg acaggactat aaagatacca ggcgtttccc cctggaagct
16801 ccctcgtgcg ctctcctgtt ccgaccctgc cgcttaccgg atacctgtcc gcctttctcc
16861 cttcgggaag cgtggcgctt tctcatagct cacgctgtag gtatctcagt tcggtgtagg
16921 tcgttcgctc caagctgggc tgtgtgcacg aacccccccgt tcagcccgac cgctgcgcct
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16981 tatccggtaa ctatcgtctt gagtccaacc cggtaagaca cgacttatcg ccactggcag
17041 cagccactgg taacaggatt agcagagcga ggtatgtagg cggtgctaca gagttcttga
17101 agtggtggcc taactacggc tacactagaa ggacagtatt tggtatctgc gctctgctga
17161 agccagttac cttcggaaaa agagttggta gctcttgatc cggcaaacaa accaccgctg
17221 gtagcggtgg tttttttgtt tgcaagcagc agattacgcg cagaaaaaaa ggatctcaag
17281 aagatccttt gatcttttct acggggtctg acgctcagtg gaacgaaaac tcacgttaag
17341 ggattttggt catgagatta tcaaaaagga tcttcaccta gatcctttta aattaaaaat
17401 gaagttttaa atcaatctaa agtatatatg agtaaacttg gtctgacagt taccaatgct
17461 taatcagtga ggcacctatc tcagcgatct gtctatttcg ttcatccata gttgcctgac
17521 tccccgtcgt gtagataact acgatacggg agggcttacc atctggcccc agtgctgcaa
17581 tgataccgcg agacccacgc tcaccggctc cagatttatc agcaataaac cagccagccg
17641 gaagggccga gcgcagaagt ggtcctgcaa ctttatccgc ctccatccag tctattaatt
17701 gttgccggga agctagagta agtagttcgc cagttaatag tttgcgcaac gttgttgcca
17761 ttgctacagg catcgtggtg tcacgctcgt cgtttggtat ggcttcattc agctccggtt
17821 cccaacgatc aaggcgagtt acatgatccc ccatgttgtg caaaaaagcg gttagctcct
17881 tcggtcctcc gatcgttgtc agaagtaagt tggccgcagt gttatcactc atggttatgg
17941 cagcactgca taattctctt actgtcatgc catccgtaag atgcttttct gtgactggtg
18001 agtactcaac caagtcattc tgagaatagt gtatgcggcg accgagttgc tcttgcccgg
18061 cgtcaatacg ggataatacc gcgccacata gcagaacttt aaaagtgctc atcattggaa
18121 aacgttcttc ggggcgaaaa ctctcaagga tcttaccgct gttgagatcc agttcgatgt
18181 aacccactcg tgcacccaac tgatcttcag catcttttac tttcaccagc gtttctgggt
18241 gagcaaaaac aggaaggcaa aatgccgcaa aaaagggaat aagggcgaca cggaaatgtt
18301 gaatactcat actcttcctt tttcaatatt attgaagcat ttatcagggt tattgtctca
18361 tgagcggata catatttgaa tgtatttaga aaaataaaca aataggggtt ccgcgcacat
18421 ttccccgaaa agtgccacct aaattgtaag cgttaatatt ttgttaaaat tcgcgttaaa
18481 tttttgttaa atcagctcat tttttaacca ataggccgaa atcggcaaaa tcccttataa
18541 atcaaaagaa tagaccgaga tagggttgag tgttgttcca gtttggaaca agagtccact
18601 attaaagaac gtggactcca acgtcaaagg gcgaaaaacc gtctatcagg gcgatggccc
18661 actacgtgaa ccatcaccct aatcaagttt tttggggtcg aggtgccgta aagcactaaa
18721 tcggaaccct aaagggagcc cccgatttag agcttgacgg ggaaagccgg ccatttaggc
18781 catagggcgc tggcaagtgt agcggtcacg ctgcgcgtaa ccaccacacc cgccgcgctt
18841 aatgcgccgc tacagggcgc gtcccattcg ccattcaggc tgcgcaactg ttgggaaggg
18901 cgatcggtgc gggcctcttc gctattacgc cagctggcga aagggggatg tgctgcaagg
18961 cgattaagtt gggtaacgcc agggttttcc cagtcacgac gttgtaaaac gacggccagt
19021 gaattgtaat acgactcact ata
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Sequence alignment of measles virus nucleoprotein recombinant plasmid construct

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CLUSTAL 2.0.12 multiple sequence alignment


Nucleoprotein-MV        ATGGCCACACTTTTGAGGAGCTTAGCA--TTGTTCAAAAGAAACAAGGACAAACCACCCA
Nucleoprotein-FP        ------------------CCTTTAGCCATTTGTTTCAAAAGAACAAGGACAAACCACCCA
                                             *****   *****  ***  *******************

Nucleoprotein-MV        TTACATCAGGATCCGGTGGAGCCATCAGAGGAATCAAACACATTATTATAGTACCAATTC
Nucleoprotein-FP        TTACATCAGGATCCGGTGGAGCCATCAGAGGAATCAAACACATTATTATAGTACCAATCC
                        ********************************************************** *

Nucleoprotein-MV        CTGGAGATTCCTCAATTACCACTCGATCCAGACTACTGGACCGGTTGGTCAGGTTAATTG
Nucleoprotein-FP        CTGGAGATTCCTCAATTACCACTCGATCCAGACTTCTGGACCGGTTGGTCAGGTTAATTG
                        ********************************** *************************

Nucleoprotein-MV        GAAACCCGGATGTGAGCGGGCCCAAACTAACAGGGGCACTAATAGGTATATTATCCTTAT
Nucleoprotein-FP        GAAACCCGGATGTGAGCGGGCCCAAACTAACAGGGGCACTAATAGGTATATTATCCTTAT
                        ************************************************************

Nucleoprotein-MV        TTGTGGAGTCTCCAGGTCAATTGATTCAGAGGATCACCGATGACCCTGACGTTAGCATCA
Nucleoprotein-FP        TTGTGGAGTCTCCAGGTCAATTGATTCAGAGGATCACCGATGACCCTGACGTTAGCATAA
                        ********************************************************** *

Nucleoprotein-MV        GGCTGTTAGAGGTTGTTCAGAGTGACCAGTCACAATCTGGCCTTACCTTCGCATCAAGAG
Nucleoprotein-FP        GGCTGTTAGAGGTTGTCCAGAGTGACCAGTCACAATCTGGCCTTACCTTCGCATCAAGAG
                        **************** *******************************************

Nucleoprotein-MV        GTACCAACATGGAGGATGAGGCGGACCAATACTTTTCACATGATGATCCAAGCAGTAGTG
Nucleoprotein-FP        GTACCAACATGGAGGATGAGGCGGACCAATACTTTTCACATGATGATCCAATTAGTAGTG
                        ***************************************************  *******

Nucleoprotein-MV        ATCAATCCAGGTCCGGATGGTTCGAGAACAAGGAAATCTCAGATATTGAAGTGCAAGACC
Nucleoprotein-FP        ATCAATCCAGGTTCGGATGGTTCGAGAACAAGGAAATCTCAGATATTGAAGTGCAAGACC
                        ************ ***********************************************

Nucleoprotein-MV        CTGAGGGATTCAACATGATTCTGGGTACCATTCTAGCCCAGATCTGGGTCTTGCTCGCAA
Nucleoprotein-FP        CTGAGGGATTCAACATGATTCTGGGTACCATCCTAGCCCAAATTTGGGTCTTGCTCGCAA
                        ******************************* ******** ** ****************

Nucleoprotein-MV        AGGCGGTTACGGCCCCAGACACGGCAGCTGATTCGGAGCTAAGAAGGTGGATAAAGTACA
Nucleoprotein-FP        AGGCGGTTACGGCCCCAGACACGGCAGCTGATTCGGAGCTAAGAAGGTGGATAAAGTACA
                        ************************************************************

Nucleoprotein-MV        CCCAACAAAGAAGGGTAGTTGGTGAATTTAGATT-GGAGAGAAAATGGTTGGATGTGGTG
Nucleoprotein-FP        CCCAACAAAGAAGGGTAGTTGGTGAATTTAGATTTGGAGAGAAAATGGTTGGATGTGGTG
                        ********************************** *************************

Nucleoprotein-MV        AGGAACAGGATTGCCGAGGACCTCTCTTTACGCCGATTCATGGTGGCTCTAATCCTGGAT
Nucleoprotein-FP        AGGAACAGGATTGCCGAGGACCTCTCCTTACGCCGATTCATGGTCGCTCTAATCCTGGAT
                        ************************** ***************** ***************

Nucleoprotein-MV        ATCAAGAGGACACCCGGGAACAAACCTAGGATTGCTGAAATGATATGTGACATTGATACA
Nucleoprotein-FP        ATCAAGAGAACACCCGGAAACAAACCCAGGATTGCTGAAATGATATGTGACATTGATACA
                        ******** ******** ******** *********************************

Nucleoprotein-MV        TATATC-GTAGAGGCAGGA-TTAGCCAGTTTTATCCTGACTATTAAGTTTGGATAGAAA
Nucleoprotein-FP        TATATCCGTATAGGCTGGAATTAGCCAGTTT-ATCCTGACTATTAT--------------
                        ****** *** **** *** *********** *************
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Sequence alignment of measles virus phosphoprotein recombinant plasmid construct

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CLUSTAL 2.0.12 multiple sequence alignment


Phosphoprotein-MV      ATGGCAGAAGAGCAGGCACGCCATGTCAAAAACGG--ACTGGAATGCATCCGGGCTCTCA
Phosphoprotein-FP      --------------------TTGGTTCAAAAACGGGAATTGGAATGCATCCGGGCTCTCA
                                                **********  * *********************

Phosphoprotein-MV      AGGCCGAGCCCATCGGCTCACTGGCCGTCGAGGAAGCCATGGCAGCATGGTCAGAAATAT
Phosphoprotein-FP      AGGCCGAGCCCATCGGCTCACTGGCCATCGAGGAAGCTATGGCAGCATGGTCAGAAATAT
                       ************************** ********** **********************

Phosphoprotein-MV      CAGACAACCCAGGACAGGACCGAGCCACCTGCAAGGAAGAGGAGGCAGGCAGTTCGGGTC
Phosphoprotein-FP      CAGACAACCCAGGACAGGAGCGAGCCACCTGCAGGGAAGAGAAGGCAGGCAGTTCGGGTC
                       ******************* ************* ******* ******************

Phosphoprotein-MV      TCAGCAAACCATGCCTCTCAGCAATTGGATCAACTGAAGGCGGTGCACCTCGCATCCGCG
Phosphoprotein-FP      TCAGCAAACCATGCCTCTCAGCAATTGGATCAACTGAAGGCGGTGCACCTCGCATCCGCG
                       ************************************************************

Phosphoprotein-MV      GTCAGGGATCTGGAGAAAGCGATGACGACGCTGAAACTTTGGGAATCCCCTCAAGAAATC
Phosphoprotein-FP      GTCAGGGACCTGGAGAGAGCGATGACGACGCTGAAACTTTGGGAATCCCCCCAAGAAATC
                       ******** ******* ********************************* *********

Phosphoprotein-MV      TCCAGGCATCAAGCACTGGGTTACAGTGTTATCATGTTTATGATCACAGCGGTGAAGCGG
Phosphoprotein-FP      TCCAGGCATCAAGCACTGGGTTACAGTGTTATTATGTTTATGATCACAGCGGTGAAGCGG
                       ******************************** ***************************

Phosphoprotein-MV      TTAAGGGAATCCAAGATGCTGACTCTATCATGGTTCAATCAGGCCTTGATGGTGATAGCA
Phosphoprotein-FP      TTAAGGGAATCCAAGATGCTGACTCTATCATGGTTCAATCAGGCCTTGATGGTGATAGCA
                       ************************************************************

Phosphoprotein-MV      CCCTCTCAGGAGGAGACGATGAATCTGAAAACAGCGATGTGGATATT-GGCGAACCTGAT
Phosphoprotein-FP      CCCTCTCAGGAGGAGACAATGAATCTGAAAACAGCGATGTGGATATTTGGCGAACCTGAT
                       ***************** ***************************** ************

Phosphoprotein-MV      ACCGAGGGATATGCTATCACTGACCGGGGATCTGCTCCCATCTCTATGGGGTTCAGGGCT
Phosphoprotein-FP      ACCGAGGGATATGCTA--------------------------------------------
                       ****************
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Sequence alignment of T7 RNA polymerase recombinant plasmid construct

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CLUSTAL 2.0.12 multiple sequence alignment


T7_RNA_polymerase         ATGAACACGATTAACATCGCTAAGAACGACTTCTCTGACATCGAACTGGCTGCTATCCCG
T7_RNA_polymerase-FP      -TTGGTCCTTCAAAAAACAAAAATAACCAATTCT---AGAATCAACTGGCTGCTATCCCG
                           *     *    ** * *   ** *** * ****   * *   *****************

T7_RNA_polymerase         TTCAACACTCTGGCTGACCATTACGGTGAGCGTTTAGCTCGCGAACAGTTGGCCCTTGAG
T7_RNA_polymerase-FP      TTCA-CACTCTGGCTGACCATTACGGTGAGCGTTTAACTCGCGAACAGTTGGCCCTTGAG
                          **** ****************************** ************************

T7_RNA_polymerase         CATGAGTCTTACGAGATGGGTGAAGCACGCTTCCGCAAGATGTTTGAGCGTCAACTTAAA
T7_RNA_polymerase-FP      CATGAGTCTTACGACATGGGTGAAGCACGCTTCCGCAAGATGTTTGAGCGTCAACTTAAA
                          ************** *********************************************

T7_RNA_polymerase         GCTGGTGAGGTTGCGGATAACGCTGCCGCCAAGCCTCTCATCACTACCCTACTCCCTAAG
T7_RNA_polymerase-FP      GCTGGTGAGGTTGCGGATAACGCTGCCGCCAAGCCTCTCATCACTACCCTACTCCCTAAG
                          ************************************************************

T7_RNA_polymerase         ATGATTGCACGCATCAACGACTGGTTTGAGGAAGTGAAAGCTAAGCGCGGCAAGCGCCCG
T7_RNA_polymerase-FP      ATGATTGCACGCATCAACGACTGGTTTGAGGAAGTGAAAGCTAAGCGCGGCAAGCGCCCG
                          ************************************************************

T7_RNA_polymerase         ACAGCCTTCCAGTTCCTGCAAGAAATCAAGCCGGAAGCCGTAGCGTACATCACCATTAAG
T7_RNA_polymerase-FP      ACAGCCTTCCAGTTCCTGCA-GAAATCAAGCCGGAAGCCGTAGCGTACATCACCATTAAA
                          ******************** ************************************** 

T7_RNA_polymerase         ACCACTCTGGCTTGCCTAACCAGTGCTGACAATACAACCGTTCAGGCTGTAGCAAGCGCA
T7_RNA_polymerase-FP      ACCACTCTGGCTTGCCTAACCAGTGCCGAAAATACAACCGTTCAGGCTGTAACAAGCGCA
                          ************************** ** ********************* ********

T7_RNA_polymerase         ATCGGTCGGGCCATTGAGGACGAGGCTCGCTTCGGTCGTATCCGTGACCTTGAAGCTAAG
T7_RNA_polymerase-FP      AACTGTCGGGCCATTGAGGACAAGGCCCGCTTCCGTCATATCCCTAACCTTAAAACTAA-
                          * * ***************** **** ****** *** ***** * ***** ** **** 

T7_RNA_polymerase         CACTTCAAGAAAAACGTTGAGGAACAACTCAACAAGCGCGTAGGGCACGTCTACAAGAAA
T7_RNA_polymerase-FP      CACTTCAAGAAAAACGTTGAATAAAACCTCAAAATATTATAAGGG---------------
                          ********************  ** * ***** *       ****               
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Sequence alignment of measles virus large polymerase recombinant plasmid construct

For sequencing the complete 6.5kb gene, four sets of nested primers were used and are represented below as FP1-4

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CLUSTAL 2.0.12 multiple sequence alignment


L polymerase-MV      ATGGACTCGCTATCTGTCAACCAGATCTTATACCCTGAAGTTCACCTAGATAGCCC-GAT
L polymerase-FP1     -------------------------------------------------ATAGCCCCGAT
                                                                      ******* ***

L polymerase-MV      AGTTACCAATAAGATAGTAGCTATCCTGGAGTATGCTCGAGTCCCTCACGCTTACAGCCT
L polymerase-FP1     AGTTACCAATAAGATAGTAGCCATCCTGGAGTATGCTCGAGTCCCTCACGCTTACAGCCT
                     ********************* **************************************

L polymerase-MV      GGAGGACCCTACACTGTGTCAGAACATCAAGCACCGCCTAAAAAACGGATTCTCCAACCA
L polymerase-FP1     GGAGGACCCTACACTGTGTCAGAACATCAAGCACCGCCTAAAAAACGGATTTTCCAACCA
                     *************************************************** ********

L polymerase-MV      AATGATTATAAACAATGTGGAAGTTGGGAATGTCATCAAGTCCAAGCTTAGGAGTTATCC
L polymerase-FP1     AATGATTATAAACAATGTGGAAGTTGGGAATGTCATCAAGTCCAAGCTTAGGAGTTATCC
                     ************************************************************

L polymerase-MV      GGCCCACTCTCATATTCCATATCCAAATTGTAATCAGGATTTATTTAACATAGAAGACAA
L polymerase-FP1     GGCCCACTCTCATATTCCATATCCAAATTGTAATCAGGATTTATTTAACATAGAAGACAA
                     ************************************************************

L polymerase-MV      AGAGTCAACAAGGAAGATCCGTGAGCTCCTAAAAAAGGGAAATTCGCTGTACTCCAAAGT
L polymerase-FP1     AGAGTCAACGAGGAAGATCCGTGAACTCCTCAAAAAGGGGAATTCGCTGTACTCCAAAGT
                     ********* ************** ***** ******** ********************

L polymerase-MV      CAGTGATAAGGTTTTCCAATGCCTGAGGGACACTAACTCACGGCTTGGCCTAGGCTCCGA
L polymerase-FP1     CAGTGATAAGGTTTTCCAATGCTTAAGGGACACTAACTCACGGCTTGGCCTAGGCTCCGA
                     ********************** * ***********************************

L polymerase-MV      ATTGAGGGAGGACATCAAGGAGAAAATTATTAACTTGGGAGTTTACATGCACAGCTCCCA
L polymerase-FP1     ATTGAGGGAGGACATCAAGGAGAAAGTTATTAACTTGGGAGTTTACATGCACAGCTCCCA
                     ************************* **********************************

L polymerase-MV      ATGGTTTGAGCCCTTTCTGTTTTGGTTTACAGTCAAGACTGAGATGAGGTCAGTGATTAA
L polymerase-FP1     GTGGTTTGAGCCCTTTCTGTTTTGGTTTACAGTCAAGACTGAGATGAGGTCAGTGATTAA
                      ***********************************************************

L polymerase-MV      ATCACAAACCCATACTTGCCATAGGAGGAGACACACACCTGTATTCTTCACTGGTAGTTC
L polymerase-FP1     ATCACAAACCCATACTTGCCATAGGAGGAGACACACACCTGTATTCTTCACTGGTAGTTC
                     ************************************************************

L polymerase-MV      AGTTGAGCTGTTAATCTCTCGTGACCTTGTTGCTATAATCAGTAAGGAGTCTCAACATGT
L polymerase-FP1     AGTTGAGTTGCTAATCTCTCGTGACCTTGTTGCTATAATCAGTAAAGAGTCTCAACATGT
                     ******* ** ********************************** **************

L polymerase-MV      ATATTACCTGACGTTTGAACTGGTTTTGATGTATTGTGATGTCATAGAGGGGAGGTTAAT
L polymerase-FP1     ATATTACCTGACATTTGAACTGGTTTTGATGTATTGTGATGTCATAGAGGGGAGGTTAAT
                     ************ ***********************************************

L polymerase-MV      GACAGAGACCGCTATGACCATTGATGC-TAGGTATGCAGAACTTCTAGGAAGAGTCAGAT
L polymerase-FP1     GACAGAGACCGCTATGACTATTGATGCCTAGGTATACAGAGCTTCT--------------
                     ****************** ******** ******* **** *****
L polymerase-MV      GCCTACCTCCATGACCCTGAGTTCAACCTGTCTTACAGCCTGAAAGAAAAGGAGATCAAG
L polymerase-FP2     ------------GACCCTGAGTTCAACCTGTCTTACAGCCTGAAAGAAAAGGAGATCAAG
                                 ************************************************

L polymerase-MV      G-AAACAGGTAGACTTTTCGCTAAAATGACTTACAAAATGAGGGCATGCCAAGTGATCGC
L polymerase-FP2     GCMAACAGGTAGACTTTTTGCTAAAATGACTTACAAAATGAGGGCATGCCAAGTGATTGC
                     *  *************** ************************************** **
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L polymerase-MV     TGAAAATCTAATCTCAAACGGGATTGGCAAGTATTTTAAGGACAATGGGATGGCCAAGGA
L polymerase-FP2    TGAAAATCTAATCTCAAACGGGATTGGCAAATATTTTAAGGACAATGGGATGGCCAAGGA
                    ****************************** *****************************

L polymerase-MV     TGAGCACGATTTGACTAAGGCACTCCACACTCTGGCTGTCTCAGGAGTCCCCAAAGATCT
L polymerase-FP2    TGAGCACGATTTGACTAAGGCACTCCACACTCTAGCTGTCTCAGGAGTCCCCAAAGATCT
                    ********************************* **************************

L polymerase-MV     CAAAGAAAGTCACAGGGGGGGGCCAGTCTTAAAAACCTACTCCCGAAGCCCAGTCCACAC
L polymerase-FP2    CAAAGAAAGTCACAGGGGGGGGCCAGTCTTAAAAACCTACTCCCGAAGCCCAGTCCACAC
                    ************************************************************


L polymerase-MV     AAGTACCAGGAACGTTAAAGCAGAAAAAGGGTTTGTAGGATTCCCTCATGTAATTCGGCA
L polymerase-FP2    AAGTACCAGGAACGTGAGAGCAGCAAAAGGGTTTATAGGGTTCCCTCAAGTAATTCGGCA
                    *************** * ***** ********** **** ******** ***********

L polymerase-MV     GAATCAAGACACTGATCATCCGGAGAATATAGAAACCTACGAGACAGTCAGCGCATTTAT
L polymerase-FP2    GGACCAAGACACTGATCATCCGGAGAATATGGAAGCTTACGAGACAGTCAGTGCATTTAT
                    * * ************************** *** * ************** ********

L polymerase-MV     CACGACTGATCTCAAGAAGTACTGCCTTAATTGGAGATATGAGACCATCAGCTTATTTGC
L polymerase-FP2    CACGACTGATCTCAAGAAGTACTGCCTTAATTGGAGATATGAGACCATCAGCTTGTTTGC
                    ****************************************************** *****

L polymerase-MV     ACAGAGGCTAAATGAGATTTACGGATTACCCTCATTTTTTCAGTGGCTGCATAAGAGGCT
L polymerase-FP2    ACAGAGGCTAAATGAGATTTACGGATTGCCCTCATTTTTCCAGTGGCTGCATAAGAGGCT
                    *************************** *********** ********************

L polymerase-MV     TGAAACCTCTGTCCTCTATGTAAGTGACCCTCATTGCCCCCCCGACCTTGACGCCCATGT
L polymerase-FP2    TGAGACCTCTGTCCTGTATGTAAGTGACCCTCATTGCCCCCCCGACCTTGACGCCCATAT
                    *** *********** ****************************************** *

L polymerase-MV     CCCGTTATGCAAAGTCCCCAATGACCAAATCTTCATCAAGTACCCTATGGGAGGTATAGA
L polymerase-FP2    CCCGTTATATAAAGTCCCCAATGATCAAATCTTCATTAAGTACCCTATGGGAGGTATAGA
                    ********  ************** *********** ***********************

L polymerase-MV     AGGGTATTGTCAGAAGCTGTGGACCATCAGCACCATTCCCTACTTATACCTGGCTGCTTA
L polymerase-FP2    AGGGTATTGTCAGAARCTGTGGACCATCAGCACCATTCCCTATCTATACCTGGCTGCTTA
                    *************** **************************  ****************

L polymerase-MV     TGAGAGCGGGGTAAGGATTGCTTCGTTAGTGCAAGGGGACAATCAGACCATAGCCGTAAC
L polymerase-FP2    TGA---------------------------------------------------------
                    ***
L polymerase-MV     GAGTGTGGATCAGTCAACTACGGATGGTTTTTTGTCCCCTCGGGTTGCCAACTGGATGAT
L polymerase-FP3    --------------------CGGATGGTTTTTTGTCCCCTCGGGTTGCCAACTGGATGAT
                                        ****************************************

L polymerase-MV     ATTGACAAGGAAACATCATCCTTGAGAGTCCCATATATTGGTTCTACCACTGATGAGAGA
L polymerase-FP3    ATTGACAAGGAAACATCATCCTTGAGAGTCCCATATATTGGTTCTACCACTGATGAGAGA
                    ************************************************************

L polymerase-MV     ACAGACATGAAGCTCGCCTTCGTAAGAGCCCCAAGTAGATCCTTGCGATCTGCCGTTAGA
L polymerase-FP3    ACAGACATGAAGCTTGCCTTCGTAAGAGCCCCAAGTCGATCCTTGCGATCTGCTGTTAGA
                    ************** ********************* **************** ******

L polymerase-MV     ATAGCAACAGTGTACTCATGGGCTTACGGTGATGATGATAGCTCTTGGAACGAAGCCTGG
L polymerase-FP3    ATAGCAACAGTGTACTCATGGGCTTACGGTGATGATGATAGCTCTTGGAACGAAGCCTGG
                    ************************************************************

L polymerase-MV     TTGTTGGCAAGGCAAAGGGCCAATGTGAGCCTGGAGGAGCTAAGGGTGATCACTCCCATC
L polymerase-FP3    TTGTTGGCTAGGCAAAGGGCCAATGTGAGCCTGGAGGAGCTAAGGGTGATCACTCCCATC
                    ******** ***************************************************

L polymerase-MV     TCGACTTCGACTAATTTAGCGCATAGGTTGAGGGATCGTAGCACTCAAGTGAAATACTCA
L polymerase-FP3    TCAACTTCGACTAATTTAGCGCATAGGTTGAGGGATCGTAGCACTCAAGTGAAATACTCA
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L polymerase-MV      GGTACATCCCTTGTCCGAGTGGCAAGGTATACCACAATCTCCAACGACAATCTCTCATTT
L polymerase-FP3     GGTACATCCCTTGTCCGAGTGGCGAGGTATACCACAATCTCCAACGACAATCTCTCATTT
                     *********************** ************************************

L polymerase-MV      GTCATATCAGATAAGAAGGTTGATACTAACTTTATATACCAACAAGGAATGCTTCTAGGG
L polymerase-FP3     GTCATATCAGATAAGAAGGTTGATACTAACTTTATATACCAACAAGGAATGCTTCTAGGG
                     ************************************************************

L polymerase-MV      TTGGGTGTTTTAGAAACATTGTTTCGACTCGAGAAAGATACTGGATCATCTAACACGGTA
L polymerase-FP3     TTGGGTGTTTTAGAAACATTGTTTCGACTCGAGAAAGATACCGGATCATCTAACACGGTA
                     ***************************************** ******************

L polymerase-MV      TTACATCTTCACGTCGAAACAGATTGTTGCGTGATCCCGATGATAGATCATCCCAGGATA
L polymerase-FP3     TTACATCTTCACGTCGAAACAGATTGTTGCGTGATCCCGATGATAGATCATCCCAGGATA
                     ************************************************************

L polymerase-MV      CCCAGCTCCCGCAAGCTAGAGCTGAGGGCAGAGCTATGTACCAACCCATTGATATATGAT
L polymerase-FP3     CCCAGCTCCCGCAAGCTAGAGCTGAGGGCAGAGCTATGTACCAACCCATTGATATATGAT
                     ************************************************************

L polymerase-MV      AATGCACCTTTAATTGACAGAGATGCAACAAGGCTATACACCCAGAGCCATAGGAGGCAC
L polymerase-FP3     AATGCACCTTTAATTGACAGAGATGCAACAAGGCTATACACCCAGAGCCATAGGAGGCAC
                     ************************************************************

L polymerase-MV      CTTGTGGAATTTGTTACATGGTCCACACCCCAACTATATCACATTCTAGCTAAGTCCACA
L polymerase-FP3     CTTGTGGAATTTGTTACATGGTCCACACCCCAACTATATCACATTTTAGCTAAGTCCACA
                     ********************************************* **************

L polymerase-MV      GCACTATCTATGATTGACCTGGTAACAAAATTTGAGAAGGACCATATGAATGAAATTTCA
L polymerase-FP3     GCACTATCTATGATTGACCTGGTAACAAAATTTGAGAA----------------------
                     **************************************
L polymerase-MV      TTATCGATGGCTCTACTCCTTGGCAAAATAGGATCAATACTGGTGATTAAGCTTATGCCT
L polymerase_FP4     -----------------------CAAAATAGGATCAATACTGGTGATTAAGCTTATGCCT
                                            *************************************

L polymerase-MV      TTCAGCGGGGATTTTGTTCAGGGATTTATAAGCTATGTAGGGTCTCATTATAGAGAAGTG
L polymerase_FP4     TTCAGCGGGGATTTTGTTCAGGGATTTATAAGTTATGTAGGGTCTCATTATAGAGAAGTG
                     ******************************** ***************************

L polymerase-MV      AACCTTGTCTACCCTAGGTACAGCAACTTCATATCTACTGAATCTTATTTAGTTATGACA
L polymerase_FP4     AACCTTGTATACCCTAGATACAGCAACTTCATATCTACTGAATCTTATTTGGTTATGACA
                     ******** ******** ******************************** *********

L polymerase-MV      GATCTCAAAGCTAACCGGCTAATGAATCCTGAAAAGATCAAGCAGCAGATAATTGAATCA
L polymerase_FP4     GATCTCAAGGCTAACCGGCTAATGAATCCTGAAAAGATTAAGCAGCAGATAATTGAATCA
                     ******** ***************************** *********************

L polymerase-MV      TCTGTGCGGACTTCACCTGGACTTATAGGTCACATCCTATCCATTAAGCAACTAAGCTGC
L polymerase_FP4     TCTGTGAGGACTTCACCTGGACTTATAGGTCACATCCTATCCATTAAGCAACTAAGCTGC
                     ****** *****************************************************

L polymerase-MV      ATACAAGCAATTGTGGGAGGCGCAGTTAGTAGAGGTGATATCAACCCTATTCTGAAAAAA
L polymerase_FP4     ATACAAGCAATTGTGGGAGACGCAGTTAGTAGAGGTGATATCAATCCTACTCTGAAAAAA
                     ******************* ************************ **** **********

L polymerase-MV      CTTACACCTATAGAGCAGGTGCTGATCAGTTGCGGGTTGGCAATTAACGGACCTAAACTG
L polymerase_FP4     CTTACACCTATAGAGCAGGTGCTGATCAATTGCGGGTTGGCAATTAACGGACCTAAGCTG
                     **************************** *************************** ***

L polymerase-MV      TGCAAAGAATTAATCCACCATGATGTTGCCTCAGGGCAAGATGGATTGCTTAACTCTATA
L polymerase_FP4     TGCAAAGAATTGATCCACCATGATGTTGCCTCAGGGCAAGATGGATTGCTTAATTCTATA
                     *********** ***************************************** ******

L polymerase-MV      CTCATCCTCTACAGGGAGTTGGCAAGATTCAAAGACAACCAAAGAAGTCAACAAGGGATG
L polymerase_FP4     CTCATCCTCTACAGGGAGTTGGCAAGATTCAAAGACAACCAAAGAAGTCAACAAGGGATG
                     ************************************************************

L polymerase-MV      TTCCACGCTTACCCCGTATTGGTAAGTAGTAGGCAACGAGAACTTGTATCTAGGATCACT
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L polymerase_FP4      TTCCACGCTTACCCCGTATTGGTAAGTAGCAGGCAACGAGAACTTATATCTAGGATCACC
                      ***************************** *************** ************* 

L polymerase-MV       CGCAAATTTTGGGGGCATATTCTTCTTTACTCCGGGAACAGAAAGTTGATAAATCGGTTT
L polymerase_FP4      CGCAAATTTTGGGGGCACATTCTTCTTTACTCCGGGAACAGAAAGTTGATAAATAAGTTT
                      ***************** ************************************  ****

L polymerase-MV       ATCCAGAATCTCAAGTCCGGTTATCTAGTACTAGACTTACACCAGAATATCTTCGTTAAG
L polymerase_FP4      ATCCAGAATCTCAAGTCCGGCTATCTGATACTAGACTTACACCAGAATATCTTCGTTAAG
                      ******************** *****  ********************************

L polymerase-MV       AATCTATCCAAGTCAGAGAAACAGATTATTATGACGGGGGGTTTAAAACGTGAGTGGGTT
L polymerase_FP4      AATCTATCCAAGTCAGAGAAACAGATTATTATGACGGGGGGTTTGAAACGTGAGTGGGTT
                      ******************************************** ***************

L polymerase-MV       TTTAAGGTAACAGTCAAGGAGACCAAAGAATGGTACAAGTTAGTCGGATACAGCGCTCTG
L polymerase_FP4      TTTAAGGTAACAGTCAAGGAGACCAAA---------------------------------
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Sequence alignment of M-cherry gene inserted in p (+) MV-NSE-FlagP-M502-3p

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CLUSTAL 2.0.12 multiple sequence alignment


M-cherry gene      ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAG
rMV-Mcherry        -----------------TAGAGGTAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAG
                                        * *************************************

M-cherry gene      GTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGC
rMV-Mcherry        GTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGC
                   ************************************************************

M-cherry gene      CGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC
rMV-Mcherry        CGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC
                   ************************************************************

M-cherry gene      TTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCAC
rMV-Mcherry        TTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCAC
                   ************************************************************

M-cherry gene      CCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGC
rMV-Mcherry        CCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGC
                   ************************************************************

M-cherry gene      GTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCTTGCAGGAC
rMV-Mcherry        GTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGAC
                   *************************************************** ********

M-cherry gene      GGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTA
rMV-Mcherry        GGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTA
                   ************************************************************

M-cherry gene      ATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGC
rMV-Mcherry        ATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGC
                   ************************************************************

M-cherry gene      GCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCT
rMV-Mcherry        GCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCT
                   ************************************************************

M-cherry gene      GAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTC
rMV-Mcherry        GAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTC
                   ************************************************************

M-cherry gene      AACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAA
rMV-Mcherry        AACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAA
                   ************************************************************

M-cherry gene      CGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAA---------
rMV-Mcherry        CGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAAGACGTCTTG
                   ***************************************************
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Sequence alignment of BNiP3 gene inserted in p (+) MV-NSE-FlagP-M502-3p

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CLUSTAL 2.0.12 multiple sequence alignment


BNiP3-human      ATGTCGCAGAACGGAGCGCCCGGGAT-GCAGGAGGAGAGCCTGCAGGGCTCCTGGGTAGA
rMV-BNiP3        --------------TGGGCCAGGGGCAGCAGGAGGA-AGCCTGCAGGGCTCCTGGGTAAA
                                * *** ***   ********* ********************* *

BNiP3-human      ACTGCACTTCAGCAATAATGGGAACGGGGGCAGCGTTCCAGCCTCGGTTTCTATTTATAA
rMV-BNiP3        ACTGCACTTCACCAATAAGGGGAACGGGGGCACCGTTCCACCCTCGGTTTCTATTTATAA
                 *********** ****** ************* ******* *******************

BNiP3-human      TGGAGACATGGAAAAAATACTGCTGGACGCACAGCATGAGTCTGGACGGAGTAGCTCCAA
rMV-BNiP3        GGGAAACATGGAAAAAATACGGCTGGACCCACAGCATGATTCGGGACGGATTACCTCCAA
                  *** *************** ******* ********** ** ******* ** ******

BNiP3-human      GAGCTCTCACTGTGACAGCCCACCTCGCTCGCAGACACCACAAGATACCAACAGGGCTTC
rMV-BNiP3        AACCTCTCACTGTGACACCCCACCTCGCTCGCAAACACCACAAAATACCAACAAAGCTTC
                  * ************** *************** ********* *********  *****

BNiP3-human      TGAAACAGATACCCATAGCATTGGAGAGAAAAACAGCTCACAGTCTGAGGAAGATGATAT
rMV-BNiP3        TGAAACAAATACCCATAGCATTGGAAAAAAAAACAGCTCACAGTCTGAGGAAAATGATAT
                 ******* ***************** * ************************ *******

BNiP3-human      TGAAAGAAGGAAAGAAGTTGAAAGCATCTTGAAGAAAAACTCAGATTGGATATGGGATTG
rMV-BNiP3        TGAAAAAAGGAAAAAATTTGAAACCATCTTGAAAAAAAAACTCAAATTGAATATGGGATTG
                 ***** ******* ** ****** ********* ********* **** ***********

BNiP3-human      GTCAAGTCGGCCGGAAAATATTCCCCCCAAGGAGTTCCTCTTTAAACACCCGAAGCGCAC
rMV-BNiP3        GTCAAGTCGGCCGGAAAATATTCCCCCCAAGGATTTCCTCTTTAAACACCCGAAGCGCAC
                 ********************************* **************************

BNiP3-human      GGCCACCCTCAGCATGAGGAACACGAGCGTCATGAAGAAAGGGGGCATATTCTCTGCAGA
rMV-BNiP3        GGCCACCCTCAGCATGAGGAACACGAGCGTCATGAAAAAGGGGGGCATATTCTCTGCAAA
                 ************************************ ** ****************** *

BNiP3-human      ATTTCTGAAAGTTTTCCTTCCATCTCTGCTGCTCTCTCATTTGCTGGCCATCGGATTGGG
rMV-BNiP3        ATTTCTGAAATTTTTCCTTCCATCTCTGCTGCTCTCTCATTTGCGGGCCATCGGATTGGG
                 ********** ********************************* ***************

BNiP3-human      GATCTATATTGGAAGGCGTCTGACAACCTCCACCAGCACCTTTTGA--------------
rMV-BNiP3        GATCTATATTGGAAGGCGTCTGACAACCCCCACCACCACCTTTTGAGACGTCTAGGCAAA
                 **************************** ****** **********
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General reagents

10× Phosphate Buffered Saline

Chemical	Concentration	Amount
NaCl	1.36M	79.47g
KCl	26.8mM	1.99g
$Na_2HPO_4.2H_2O$	80mM	11.35g
KH_2PO4	17.6mM	3.3g

Dissolve in 800ml of sterile ddH_2O and make up the volume to 1 litre. Autocalve. Store at RT.

To prepare 1×PBS, add 100 ml of 10×PBS stock solution to 800ml of ddH_2O. Adjust pH to 7.2. Finally make up the volume to 1000ml. Autoclave and store at RT.

To preare 1×PBST, add 100µl of Tween-20 to 100ml of 1×PBS.

0.5M EDTA (pH 8.0)

Add 18.6g EDTA to 80ml of ddH_2O. Adjust pH to 8.0 with NaOH. Make volume upto 100ml. Autoclave and store at RT.

Tris EDTA buffer (TE)

Chemical	Concentration	Amount
1M Tris-Cl pH7.4	10mM	12.114g
0.5M EDTA pH8.0	1mM	200µl

Mix and make up the volume to 100ml. Store at RT

10× Tris Borate EDTA Buffer

Chemical	Concentration	Amount
Tris base	891.53mM	107.99g
Boric acid	889.53mM	54.99g
EDTA pH8.0	20mM	20ml

Dissolve in 800ml of sterile ddH_2O and make up the volume to 1 litre. Autocalve. Store at RT.
To prepare 1×TBE, add 100 ml of 10×TBE stock solution to 900ml of ddH_2O.

To prepare 0.5×TBE, add 50 ml of 10×TBE stock solution to 950ml of ddH_2O.

1% Agarose gel

To make a 1% agarose minigel, add 0.3g of Agarose to 30 ml of 1×TBE. Heat till it dissolves. Cool and add 2µl EtBR. Pour in the cassette. Let it solidify at RT.

200mM PMSF

To prepare a 200 mM stock solution, dissolve 34.8 mg of PMSF in 1ml of isopropanol. Make aliquots of 200μl each. Store at −20°C.

200mM Sodium orthovanadate (Na_3VO_4)

To prepare a 200mM stock solution, dissolve 36.78mg of Sodium orthovanadate in 1ml of autoclaved milliQ water. Adjust pH to 10. Boil the solution until translucent. Re-adjust the pH to 10. Make aliquots of 200μl each. Store at −20°C.

Protease inhibitor cocktail

To make a 50× stock solution, dissolve 1 vial of lyophilised protesae inhibitor cocktail powder in 5ml of molecular biology grade water. Make aliquots of 200μl each. Store at −20°C.

100mg/ml Ampicillin

Dissolve 100mg of ampicillin in 1ml of autoclaved ddH_2O. Store at −20°C.

LB broth

Add 1.25g of Luria Bertani medium in 50ml of ddH_2O. Autoclave.

LB Agar

Add 1.25g of LB and 0.75g of Agar to 50ml of ddH_2O. Autoclave.

5mg/ml MTT

Add 5mg MTT powder in 1ml of 5% methanol. Use freshly prepared stock. Remaining reagent can be stored at 4°C protected from light.

Paclitaxel

Dissolve 1mg Paclitaxel in 118μl of DMSO to make 0.01M stock solution. Protect from direct light, aliquot and store at −20ºC.

H2 compound

Dissolve 20mg H2 compound in 1ml of DMSO to make 20mM stock solution. Store at −20ºC.

For SDS PAGE and Western blotting

1.5M Tris HCl

Dissolve 18.71g of Tris base in 80 ml of sterile ddH_2O. Adjust pH to 8.8. Make up final volume to 100ml. Autoclave and store at RT.

0.5M Tris HCl

Dissolve 6.057g of Tris base in 80 ml of sterile ddH_2O. Adjust pH to 6.8. Make up final volume to 100ml. Autoclave and store at RT.

30% Acrylamide

Dissolve 29.2g of Acrylamide and 0.8g of Bis Acrylamide in 80 ml of sterile ddH_2O in an amber bottle. Stir overnight. Filter through Whatman No. 1. Make up final volume to 100ml. Store at 4°C.

10% SDS

Dissolve 10g of SDS in 100ml of sterile ddH_2O. Store at RT.

10% APS

Dissolve 10g of APS in 100ml of sterile ddH_2O. Store at 4°C.

Resolving gel composition

For 10ml

Reagents	**8%**	**10%**	**12 %**
ddH_2O	4.6ml	4ml	3.3ml
1.5M Tris HCl pH8.8	2.5ml	2.5ml	2.5ml
30% Acrylamide	2.7ml	3.3ml	4.0ml
10 % SDS	0.1ml	0.1ml	0.1ml
10% APS	0.1ml	0.1ml	0.1ml
TEMED	0.006ml	0.004ml	0.004ml

Stacking Gel Composition

For 5ml

Reagents	**5%**
ddH_2O	3.4ml
0.5MTris HCl pH6.8	0.63ml
30% Acrylamide	0.83ml
10 % SDS	0.05ml
10% APS	0.05ml
TEMED	0.005ml

10× SDS Running Buffer

Chemical	**Concentration**	**Amount**
Tris base	250mM	30.3g
Glycine	1.92mM	144.10g
SDS (10%)	1%	10ml

Weigh and dissolve in 800ml of sterile ddH_2O. Stir overnight to set pH to 8.3. Make up final volume to 1000ml. Store at RT.

3×SDS Sample loading buffer (Laemmli Buffer)

Chemical	Concentration	Amount
1MTris-Cl pH6.8	187.5mM	2.4ml
20% SDS	6%	3ml
Glycerol	30%	3ml
Bromophenol blue	0.03%	6mg
β mercaptoethanol		1.6ml

Make up the volume to 10ml and store at RT

10× Western Blotting Buffer

Chemical	Concentration	Amount
Tris base	480mM	30.3g
Glycine	390mM	144.10g

Weigh and dissolve in 800ml of sterile ddH_2O. Make up the volume to 1000ml and store at RT

To make 1 litre of 1× buffer, dilute 100 ml of 10× stock solution with 700ml of water. Add 200ml methanol.

Stripping Buffer

Chemical	Concentration	Amount
0.5M Tris-Cl pH6.8	625mM	6.25ml
10%SDS	2%	10ml
ddH_2O		33.75ml

Just before use, add 400ul of β-mercapto-ethanol per 50ml of buffer.
Prepare the buffer in dark bottle and preheat at 55°C before use.

10× Tris buffer Saline (TBS)

Chemical	Concentration	Amount
Tris base	200mM	24.23g
NaCl	137mM	80.06g

Weigh and dissolve in 800ml of sterile ddH_2O. Adjust pH to 7.6. Make up the volume to 1000ml. Autoclave. Store at RT.

To prepare 1×TBS, add 100 ml of 10× stock solution to 900ml of ddH_2O. Check pH. Autoclave and store at RT.

To preare 1×TBST, add 100µl of Tween-20 to 100ml of 1×TBS.

For genomic DNA extraction

Lysis buffer

Chemical	Concentration	Amount
1M Tris-Cl pH8.0	10mM	0.010ml
0.5M EDTA (TE)	1mM	0.002ml
10%SDS		
Proteinase K	1×	0.020ml
Molecular biology grade water		0.827ml

Dissolve and make up the volume to 10ml with ddH_2O.

For protein isolation

Mammalian cell lysis buffer

Chemical	Concentration	Amount
1M Tris-Cl pH7.5	10mM	0.010ml
5M NaCl	150mM	0.030ml
0.5M EDTA	5mM	0.010ml
Sodium deoxycholate	1%	0.01g
10% SDS	0.1%	0.001ml
Triton X-100	1%	0.010ml
200mM Na_3VO_4	1mM	0.005ml
200mM PMSF	1mM	0.005ml
50×Protease Inhibitors	1×	0.020ml
Molecular biology grade water		0.909ml

Makes 1ml cell lysis buffer

LIST OF CONSUMABLES

Serial No.	Item	Catalogue No.	Company
Regular chemical reagents			
1.	Absolute Ethanol	K43342083215	Merck, Germany
2.	Acetic acid	012885	SRL, India
3.	Acrylamide	A3553	Sigma Aldrich/China
4.	Agar	FB0010	Biobasic, Canada
5.	Ampicillin	AB0028	Biobasic, Canada
6.	APS	AB0072	Biobasic, Canada
7.	Boric acid	21765	Fisher Scientific, India
8.	Bradford reagent	B6916	Sigma Life Sciences, USA
9.	Calcium chloride	CD0050	Biobasic, Canada
10.	Chloroform	12305	Merck, India
11.	DEPC	D5758	Sigma Life Sciences, USA
12.	DMSO	20323L05	SDFCL, India
13.	DNA ladder,100bp	#SM0323	Thermo Scientific, USA
14.	DNA ladder,1Kb	#SM0311	Thermo Scientific, USA
15.	DTT(DL-Dithiothreitol)	43815	Sigma Life Sciences, USA
16.	ECL	WBRLSO100	Millipore, USA
17.	EDTA	18455	Fisher Scientific, India
18.	Ethidium Bromide	EB0195	Biobasic, Canada
19.	Glycerol	G5516	Sigma Aldrich, USA
20.	Glycine	G8898	Sigma Aldrich, Japan
21.	Glycogen	G0885	Sigma Life Sciences, USA
22.	Isoamyl Alcohol	092945	SRL, India
23.	Isopropanol	092956	SRL, India

24.	KCl	19255	Fisher Scientific, India
25.	KH_2PO_4	PRB0445	Biobasic, Canada
26.	LB broth	29817	SRL, India
27.	Magnesium chloride	15535	Qualigens, India
28.	Methanol	82911	SRL, India
29.	Molecular biology grade water	TCL018	Himedia, India
30.	MTT	33611	SRL, India
31.	N,N,N′,N′-Tetramethylethylenediamine	T9281	Sigma Aldrich, USA
32.	N,N′-Methylenebis(acrylamide)	146072	Sigma Aldrich, USA
33.	$Na_2HPO_4 .2H_2O$	S0404	Biobasic, Canada
34.	NaCl	15915	Fisher Scientific, India
35.	Phenol	P4682	Sigma Life Sciences, USA
36.	PMSF	PB0425	Biobasic, Canada
37.	Protease inhibitor	P2714	Sigma Aldrich, USA
38.	Protein ladder	#26619	Thermo Scientific, USA
39.	PVDF membrane	IPVH00010	Millipore, USA
40.	SDS	L3771	Sigma Aldrich, USA
41.	Sodium acetate	20125	Fisher Scientific, India
42.	Sodium bicarbonate	Q|1Q611435	Merck,India
43.	Sodium orthovandate	SB0869	Biobasic, Canada
44.	Sodium thiosulphate	72049	Sigma Aldrich, USA
45.	Triton X 100	T8787	Sigma Life Sciences, USA
46.	Tris base	15965	Fisher Scientific, India
47.	Trizol reagent	UN1671	Biobasic, Canada
48.	Tween 20	1628154	SRL, India
49.	β-mercaptoethanol	MB0338	Biobasic, Canada

	Enzymes/PCR reagents		
50.	DNases	EN0521	Thermo Scientific, USA
51.	dNTPs	R0192	Thermo Scientific, USA
52.	*Eco*RI	ER0271	Thermo Scientific, USA
53.	HiFi DNA Polymerase	KK2101	KAPA Biosytems, USA
54.	Gene specific Primers	NA	Sigma Aldrich, India
55.	M-MuLV RT enzyme	EP0352	Thermo Scientific, USA
56.	Proteinase K	RM2957	Himedia, India
57.	Oligo dT primers	S0132	Thermo Scientific, USA
58.	Random Primer	S0142	Thermo Scientific, USA
59.	Ribolock RNase inhibitor	EO0381	Thermo Scientific, USA
60.	RNases	R4875	Sigma Aldrich, USA
61.	T4 DNA ligase	EL0014	Thermo Scientific, USA
62.	*Xho* I	FD0694	Thermo Scientific, USA
63.	*Bam*HI	FD0054	Thermo Scientific, USA
64.	*Not*I	FD0593	Thermo Scientific, USA
65.	*Pfl*2311	FD0894	Thermo Scientific, USA
66.	*Aat*II	FD0994	Thermo Scientific, USA
67.	*Dpn*I	ER1701	Thermo Scientific, USA
68.	Herculase	600675	Agilent technologies, USA
69.	DreamTaq polymerase	EP1701	Thermo Scientific, USA
Kits			
70.	Agarose gel elution kit	K0691	Thermo Scientific, USA
71.	Plasmid DNA miniprep kit	400761	Thermo Scientific, USA
72.	Plasmid DNA midiprep kit	NA0200	Sigma Aldrich, USA
73.	Caspase 3 activity kit	KK4601	Thermofischer scientific, USA

74.	Alexa fluor-488 annexin V /dead cell apoptosis kit.	V13241	Thermofischer scientific, USA
Mammalian cell culture /Transfection reagents			
75.	100X Antibiotic Solution	A001	Himedia, India
76.	MEM	AT154	Himedia, India
77.	DMEM	AT007	Himedia, India
78.	FBS Brazil, EU approved, gamma irradiated.	RM1112	Himedia, India
79.	HEPES	H3375	Sigma Aldrich, USA
80.	Lipofectamine^{TM}-2000	15338100	Life Technologies, USA
81.	Molecular biology grade water	TCL018	Himedia, India
82.	Nuclease free water	B-003000-WB-100	Dharmacon, USA
83.	Opti MEM	31985062	Life Technologies, USA
84.	Trypsin EDTA Solution	TCL007	Himedia, India
Antibodies			
85.	Anti-β-actin(C-2) antibody	sc-47778	SantaCruz Biotechnology, USA
86.	Anti-FITC antibody	F0257	Sigma Aldrich, USA
87.	Anti-nucleoprotein antibody	H1029	Sigma Aldrich, USA
88.	Anti-phosphoprotein antibody	sc-69824	SantaCruz Biotechnology, USA
89.	Anti-Mouse IgG (whole molecule)–Peroxidase antibody	A4416	Sigma Aldrich, USA
90.	Anti-hemagglutinin antibody	M4439	Sigma Aldrich, USA
91.	Anti-BNiP3 antibody	sc-13057	SantaCruz Biotechnology, USA
Instruments			
92.	DNA gel electrophoresis unit	NA	Local Make
93.	FACS	BD FACS Aria Fusion	BD biosciences, USA
94.	Fluorescence plate reader	Varioskan Flash	Thermoscientific, USA
95.	SDS PAGE unit	Mini-PROTEAN® Tetra Cell	Biorad, USA
96.	Spectrophotometer/ELISA Reader	Spectra max plus	Molecular devices
97.	Thermal Cycler	2720Thermal Cycler	Applied Biosystems, USA

POSTERS
AND
PUBLICATIONS

List of Posters Presented

- **Lal G** and Rajala MS. Engineering of measles virus to target cancer cells, an attempt. 17th international congress on infectious diseases. March 2-5, 2016, Hyderabad, India.

- **Geetanjali** and Rajala MS. Developing recombinant measles virus as an oncolytic agent. Poster presented in 5th Molecular Virology Meeting, THSTI, Faridabad. 11-12th Feb, 2017.

- **Geetanjali** and Rajala MS. Replication competent recombinant measles virus as an oncolytic agent. Poster presentation, National Science Day, 28th Feb, 2017

List of Publications

- **Lal G** and Rajala MS. Engineering of measles virus to target cancer cells, an attempt *International J of Infectious Diseases* 2016, Vol 45 Suppl1(ICIID abstract suppl): 333-334. Poster was published in F1000Research 2016, 5: 717 (doi:10.7490/f1000 research.1111756.1)

- **Geetanjali** and Rajala MS. Recombinant viruses and Oncolysis: a step towards comprehensive cancer therapy. Communicated to Gene therapy.
  Current status: under review