IN VITRO AND PRECLINICAL EVALUATION OF NEWCASTLE DISEASE VIRUS STRAIN V4UPM AS AN ONCOLYTIC VIRUS CANDIDATE FOR NOVEL HUMAN MALIGNANT GLIOMA THERAPY

By

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**Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy**

September 2014

DEDICATION

This thesis is especially dedicated to my late father who inspired me to pursue my PhD. Dedication also goes to all National Cancer Council (MAKNA) patrons who worked hard to improve the quality of life of cancer patients in Malaysia.

ACKNOWLEDGEMENT

In the name of Allah, the most Gracious and the most Merciful. Thank you for granting me the strength to overcome the obstacles and the ability to successfully complete this study.

First and foremost, I would like to express my sincerest gratitude to my supervisor, Prof Jafri Malin Abdullah, who has supported me throughout my thesis with his ambitions, spirit and passion. The day when he hired me as PhD student was the major turning point of my life. I am thankful for all of the efforts to allow myself to the international trainings and conferences that changed my view in research.

I offer my utmost appreciation to Prof Aini Ideris, Prof Hasnan Jaafar and Prof Manaf Ali for their excellent guidance on virology, pathology aspect and cell culture techniques.

Furthermost, I would like to thank my wife Shazana Hilda Shamsuddin who became my best companion and kept me in harmony. I am forever grateful for your love. My grateful appreciation also goes to my mother, father and mother-in-law, as well as my siblings. Their encouragement and prayers have been crucial to my success and this PhD is meaningless without them.

Last but not least, my sincere thanks to all staffs in the Department of Neurosciences and my entire laboratory colleagues, as well as Dr Hidayah, Rohaya and Aisyah. I wish to thank all the people, although not individually named here, who have contributed for completion of my study.

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LIST OF ABBREVIATIONS

°C	Degree centigrade
µg	Microgram (10^{-6} g)
µl	Microliter (10^{-6} L)
aCSF	Artificial cerebro-spinal fluid
ALL	Acute lymphoblastic leukaemia
APMV	Avian paramyxovirus
AVMP-1	Avian paramyxovirus-1
BC	Beaudette C
BCIP	5-bromo-4-chloro-3-indoyle phosphate
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CEF	Chicken embryo fibroblast
CLL	Chronic lymphoblastic leukaemia
CML	Chronic myeloid leukaemia
CPE	Cytopathic effect
CSF	Cerebro-spinal fluid
DF1	Douglas Foster 1
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide DNA deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DsRNA	Double stranded ribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetate
EGFR	Epidermal growth factor receptor
eIF	Eukaryotic initiation factor
ELISA	Enzyme linked immunosorbent assay
EMEM	Essential modified Eagle's medium
F protein	Fusion protein
F-actin	Filamentous actin
FBS	Fetal bovine serum

FAK	Focal adhesion kinase
GBM	Glioblastoma Multiforme
GDP	Guanosine diphosphate
GEF	Guanine-nucleotide exchange factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GTP	Guanosine triphosphate
HA	Hemagglutination assay
HeV	Hendra Virus
HMPV	Human metapneumovirus
HN	Haemaglutinin
H-Ras	Harvey-Ras
HSV	Human simplex virus
IA	Intraartrial
IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
IM	Intramuscular
IRF	IFN-regulatory factor
ISG	IFN-stimulated genes
IT	Intratumoral
IV	Intravenous
kb	Kilobase
kDA	Kilodalton
KPS	Karnofsky performance status
K-Ras	Kirsten-Ras
L protein	Large polymerase
M protein	Matrix protein
MAPK	Mitogen activated protein kinase
MDT	Mean death time
MeV	Morbili Virus
mg	Miligram (10^{-3} g)
ml	Mililiter (10^{-3} L)
MOI	Multiplicity of infection
mRNA	message RNA
MTD	Maximum tolerate dose

MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium
MuV	Mumps virus
NA	Neuraminidase
NDV	Newcastle disease virus
NFκB	Nuclear factor kappa B
ng	Nanogram (10^{-9} g)
NiV	Nipah virus
nm	Nanometer (10^{-9} m)
NP	Nucleocapsid protein
OD	Optical density
P protein	Phosphoprotein
PAGE	Polyacrylamide gel electrophoresis
PAK	p21 activating kinase
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFU	Plaque forming unit
pH	*Puissance hydrogene*
pi	post infection
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PiV5	Parainfluenza Virus 5
PKR	Protein Kinase R
PRRs	Patern-recognition-receptors
PTEN	Phosphatase tensin homolog on chromosome ten
PVDF	Polyvinylidene fluoride
QOL	Quality of life
Rac1	Ras-related C3 botulinum toxin substrate 1
Ras	Rat sarcoma
Rb	Retinoblastoma
RBC	Red blood cell
RER	Rough endoplasmic reticulum
RhoA	Ras homolog gene family, member A
RNA	Ribonucleic acid

rNDV	recombinant Newcastle disease virus
RNP	ribonucleoprotein
RPM	Round per minute
RSV	Respiratory syncytial virus
RT	Radiation therapy
RTKs	Receptor tyrosine kinase
RT-PCR	Reverse transcription PCR
SDS-PAGE	Sodium dodecyl sulfate-poly acrylamide gel electrophoresis
SeV	Simian virus
siRNA	Small interfering RNA
SPF	Specific pathogen free
SsRNA	Single stranded ribonucleic acid
STAT	Signal transducer & activator of transcription
TAA	Tumours association antigen
TAE	Tris-acetate EDTA buffer
TBS	Tris-buffered saline
TCID	Tissue culture infective dose
TEMED	N,N,N’,N’-tetramethylethylenediame
TLR	Tol like receptor
UV	Ultraviolet
VLP	Virus like particle
VSV	Vesicular stomatitis virus

PENILAIAN IN VITRO DAN PRAKLINIKAL VIRUS SAMPAR AYAM STRAIN V4UPM SEBAGAI CALON VIRUS ANTIKANSER UNTUK TERAPI NOVEL BAGI GLIOMA MALIGNAN MANUSIA

ABSTRAK

Virus pemusnah kanser merupakan virus aktif yang digunakan untuk menjangkiti sel kanser dan telah dikaji dengan meluas bagi tujuan rawatan kanser. Virus pemusnah kanser menjangkiti sel kanser secara spesifik kerana virus ini mampu mengeksploitasi mutasi yang merangsang pembiakan sel kanser tanpa menjejaskan sel yang normal. Kanser otak adalah malignan pembunuh didalam otak dan glioma merupakan kanser otak manusia yang bertumbuh dari sel glia dan ianya paling kerap dijumpai. Glioma tahap IV dikenali sebagai *glioblastoma multiform* (GBM) dimana pembiakan dan perebakan GBM ini dikaitkan dengan peningkatan ekspresi protin Rac1. Virus sampar ayam (NDV) adalah virus avian didalam keluarga paramyxovirus, dan merupakan salah satu virus pemusnah kanser yang mewarisi seleksi terpilih terhadap sel kanser. NDV dilaporkan menjadi pencetus kepada pembentukkan sel syncytia dan mengaruh bagi kematian sel didalam pelbagai jenis kanser, tetapi dilaporkan selamat untuk suntikan klinikal pada manusia. Didalam kajian ini, NDV strain V4UPM telah dikaji sebagai perangsang untuk mematikan sel glioma. Kesan ketoksikan V4UPM dan mekanisma molekular pada GBM terawat telah diuji pada model in vitro menggunakan ujikaji microtetrazolium (MTT), ujikaji kematian sel, teknik pengimejan langsung, mikroskopi *fluorescence* dan *western blot*. Ianya juga diuji pada model mencit bogel (in vivo) dan ex vivo. Penemuan kami telah membuktikan V4UPM pada dos 9 HAU telah mencetus kematian sel barah otak manusia dimana permulaan pemusnahan sel ini berlaku kurang dari 12 jam selepas rawatan, selain ianya tidak menjejaskan sel astrocyte normal (p>0.05). Analisis

melalui kaedah pengimejan 3 dimensi pada NDV dan tetulang sel menunjukkan kemungkinan kemasukan virus didalam sel barah ini adalah melalui mekanisma pembenaman *caveole*. Analisis seterusnya melalui pewarnaan immunofluorescence menunjukkan jangkitan V4UPM telah merangsang penyusunan semula struktur tetulang pada sel membentuk synsytia dan dikaitkan dengan peningkatan ekspresi protin Rac1 serta protin NFκB pada fasa awal jangkitan. Walaubagaimanapun, ekspresi protein ini menurun selepas 12 jam. Analisa praklinikal pada model glioma didalam mencit bogel dengan system imun yang gagal berfungsi sepenuhnya telah menunjukkan kemampuan V4UPM pada dos 520 HAU untuk mengaruhkan pengecutan ketulan glioma tersebut ($p<0.05$), selain tidak merangsang ketoksikan kepada hos ($p>0.05$)**.** Potensi V4UPM seterusnya diuji pada hirisan glioma liar yang diperolehi dari pesakit hospital Universiti Sains Malaysia dan penemuan menunjukkan rawatan ini mengaruhkan kematian sel secara signifikan ($p<0.05$) selepas 48 jam rawatan. Sebagai konklusi, NDV dilihat mengekploitasi aktin pada sel untuk pembenaman virus dan mengaruh penggabungan sel serta penyusunan semula tetulang sel yang seterusnya memanjangkan hayat sel yang dijangkiti, namun selepas seketika sel tersebut mengalami kemusnahan. Penemuan praklinikal pula menunjukkan potensi terapeutik V4UPM pada GBM dalam model haiwan dan model ex vivo serta ianya selamat digunakan. Keseluruhanya, V4UPM adalah calon yang bertepatan untuk kajian selanjutnya didalam model haiwan bukan tikus dan primat sebagai terapi baru bagi barah otak manusia.

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ABSTRACT

Oncolytic viruses are replicating viruses that have been used to infect neoplastic cells and are widely studied as a form of antitumor therapy. Oncolytic viruses specifically target tumorigenic cells because they are able to exploit the aberrations on the cellular level that promote tumor growth and the viruses preferentially infect cancer cells without interfering with normal cells. Brain cancer is a malignant growth within the skull and glioma is the most common human brain cancer arising from glial cells. Grade IV glioma is known as glioblastoma multiforme (GBM) where the proliferation and invasive behavior in GBM was associates with upregulation of Rac1 protein. Newcastle disease virus (NDV), an avian virus in the Paramyxovirus family, is one of the oncolytic viruses that inherit natural selectivity towards cancer. It is reported to robustly induce syncytium and apoptosis in multiple types of cancer cells but found to be safe for clinical injection into human. In this study, the NDV strain V4UPM has been evaluated as a potential agent for brain cancer therapy. The cytotoxicity and molecular mechanism of V4UPM effects on GBM was evaluated in in vitro model using microtetrazolium (MTT) assay, apoptosis assay, live cell imaging, fluorescence microscopy and western blot technique. The oncolytic NDV induce GBM regression were also evaluated in in vivo and ex vivo models. Findings have shown that V4UPM at 9 HAU induces the apoptosis of human brain cancer cells with the onset of cytolysis occurring less than 12 hours after infection. Besides, it is non-toxic to normal human astrocytes cell lines (p>0.05). The three-dimensional imaging analysis of NDV co-localization with the actin cytoskeleton revealed a

potential of caveolae-mediated endocytosis as a viral entry mechanism. V4UPM infection also led to the reorganization of the actin cytoskeleton in syncytium cells and was associated with the upregulation of Rac1 and NFκB proteins in early phase of infection, but subsequently downregulated after 12 hours. Preclinical evaluation in immune-compromised athymic nude mice revealed that V4UPM at 520 HAU could induce the subcutaneous regression ($p<0.05$) of homogenous glioma xenografts without inducing any acute toxicity ($p>0.05$) in the host. V4UPM was subsequently tested on ex vivo heterogeneous glioma slices obtained from Hospital Universiti Sains Malaysia patients and found to decrease ($p<0.05$) of tissue viability 48 hours after treatment. In conclusion, V4UPM seems to exploit cellular actin for viral entry and induces actin reorganization to sustain replication via the *Rac1* signaling pathway, subsequently inducing apoptosis. Preclinical study demonstrates the therapeutic potential of V4UPM against GBM in in vivo and ex vivo with a promising safety margin. Therefore, V4UPM is found to be a potential candidate for subsequent analyses in non-rodent models and non-human primate as novel therapies for human brain tumors.

CHAPTER ONE

INTRODUCTION

Glioma is a tumor of the central nervous system arising from glial cells, and grade IV glioma is known as glioblastoma multiforme (GBM). GBM is the most common adult primary brain tumor, with relatively low incidence compared to other types of tumors. Approximately 22,070 new cases of central nervous system tumors occurred in the United States in 2009, representing 1.5% of all tumor sites (Jemal *et al.*, 2009). In Malaysia, the National Cancer Registry report in 2006 showed that the incidence of all types of cancers are estimated as 1 in 4, whilst the incidence of brain and nervous system tumors is 3.3 per 100,000 people (CR) in 2006 (Omar *et al.*, 2006; Farooqui *et al.*, 2013; Mustafa *et al.*, 2013). Even though the brain and nervous system tumors does not even account for the top ten local cancers, the GBM shows rapid development, and its median survival of only 12-15 months has remained unchanged for 25 years with almost 100% mortality (Zemp *et al.*, 2010; Zhong *et al.*, 2010; Wollmann *et al.*, 2012). For this reason, GBM is also aptly called *The Terminator* (Holland, 2000).

Decades of studies revealed that tumorigenesis is a multistep process in which mutations in tumor suppressors and proto-oncogenes accumulate (Biederer *et al.*, 2002; Nakada *et al.*, 2011), thus deregulating normal cellular signaling and the cell cycle. These mutations lead to the self-sufficiency of growth signals, insensitivity to anti-growth signals, deregulated proliferation, escape from the apoptosis pathway, enhanced angiogenesis and the acquisition of invasive properties

(Conti *et al.*, 2010). All of these defects are associated with specific gene or pathway alterations, which occur in gliomagenesis and sustain malignant progression (Krakstad and Chekenya, 2010). In particular, several oncogene aberrations have been reported in GBM, such as the amplification of epidermal growth factor receptors (EGFR) and the activation of the receptor tyrosine kinase (RTK) family, PI3K, and NFκB (Nakada *et al.*, 2011).

In addition, a pathway that is less emphasized but has exhibited remarkable and important aberrations in GBM is overexpression of *Rac1* protein. This protein signalling controls proliferation and regulates autonomous behavior in GBM (Gjoerup *et al.*, 1998; Chan *et al.*, 2005; Michaelson *et al.*, 2008; Bosco *et al.*, 2009). *Rac1*, known as *Ras*-related C3 botulism toxin substrate 1, is a member of the monomeric G-protein Rho GTPases. This protein is involved in the regulation of the cell cytoskeleton, migration, gene transcription, and G1 cell-cycle progression (Senger *et al.*, 2002; Villalonga *et al.*, 2004; Sun *et al.*, 2006). Former studies have reported that the suppression of *Rac1* leads to glioma inhibition (Senger *et al.*, 2002). Thus, novel treatments have focused on exploiting this aberration in GBM (Kanu *et al.*, 2009), notably in dealing with the obstacles encountered in temozolamide resistant GBM (Bredel *et al.*, 2011).

Oncolytic viruses have been extensively evaluated, due to their potential to infect cancer cells preferentially without interfering with normal cells (Parato *et al.*, 2005; Liu *et al.*, 2007; Parker *et al.*, 2009; Wollmann *et al.*, 2012). Taking advantage of the genetic defects or aberrations that fuel cancer growth, targeted therapy using oncolytic viruses to kill cancer cells with genetic defects or mutations

was investigated. Viruses with oncolytic properties and limited side effects were used as miniature biological machines to reach the targeted cancer cells.

Newcastle disease virus (NDV) is an avian pathogen that exhibits selective oncolytic properties and is one of the most intensively studied oncolytic viruses, affecting many types of human cancer. NDV is a single-stranded negative-sense avian RNA virus in the family of Paramyxovirus that inherits selective oncolytic properties (Sinkovics and Horvath, 2000) . The virus encodes six viral proteins in the order 3'-NP-P-M-F-HN-L-5' and has been divided into three pathotypes according to pathogenicity: velogenic, mesogenic and lentogenic (Schirrmacher and Fournier, 2009). The cells that are infected by NDV undergo cell-cell fusion, which is called syncytium formation (Zamarin and Palese, 2012).

To date, several replication-competent strains of NDV have been tested for their oncolytic capacities in phase II clinical trials, including the MTH-68 and HuJ strains (Freeman *et al.*, 2006; Yaacov *et al.*, 2008). NDV is reported to be safe for injection into human (Zemp et al., 2010). Other strains, such as 73-T (Phuangsab et al., 2001), PV-701 (Pecora et al., 2002), Ulster (Fiola et al., 2006), Beaudette C (Krishnamurthy et al., 2006), AF2240 (Meyyappan *et al.*, 2003) and V4UPM (Zulkifli et al., 2009), are currently being investigated for their oncolytic potential at a preclinical level.

In particular, the V4UPM strain is a modified avirulent V4 strain that has been cloned as a thermostable virus and used as feed pellet vaccine for poultry in Malaysia and Nigeria (Ideris *et al.*, 1990; Nwogu and Olaji, 2012). Study has also indicates that V4UPM induces apoptosis in a glioma cell line (Zulkifli et al., 2009). Nevertheless, the fundamental mechanism that drives NDV infection in tumorigenic

cells remains to be elucidated. Recently, a study showed that *Rac1* was required for NDV replication in human cancer cells, and this finding established a link between tumorigenesis and sensitivity to the oncolytic virus (Puhlmann *et al.*, 2010). In Puhlmann *et al.*,(2010) the dynamic siRNA approach using the skin carcinoma HaCaT cell line and *Rac1* knockdown with two different siRNAs led to the significant inhibition of viral replication, thereby demonstrating that Rac1 protein is an essential component of efficient NDV replication in tumorigenic cells.

The primary aim of this thesis is to evaluate the potential of NDV V4UPM as an oncotherapeutic virus against human brain cancer in in vitro, in vivo and ex vivo experimental settings. In in vitro studies, the cytotoxic dose of V4UPM on GBM cells was determined and observed the temporal morphological changes of infected GBM cells by live cell imaging. The Annexin V apoptosis assay was performed to determine the mode of infected cell death, and the expression of Rac1 and NFκB proteins was monitored in GBM following the V4UPM treatment. As the Rac1 protein primarily regulates the actin cytoskeleton of the cells, actin was also visualized at an early phase of cell infection.

For in vivo study, the preclinical antitumor potential of V4UPM was tested on the subcutaneous xenograft glioma model in nude mice, and the acute toxicity was evaluated as a safety measurement. Finally, an ex vivo study was performed to evaluate the effects of V4UPM infection on authentic GBM obtained from patients in Universiti Sains Malaysia hospital, and V4UPM infectivity was evaluated in human cerebral spinal fluid (CSF) media (Mustafa *et al.*, 2013). All of these analyses were designed to address the general objective to determine the potential of V4UPM as a safe and potent oncotherapy for human brain cancer.

CHAPTER TWO

LITERATURE REVIEW

2.1 Oncolytic viral therapy

2.1.1 Definition

Oncolytic viruses are viruses that selectively eradicate tumor cells without harming the normal surrounding tissues (Biederer *et al.*, 2002; Russell, 2002; Zemp *et al.*, 2010). Oncolytic viruses are used to recognise and infect mutated cancerous cells, where they replicate and then release new virions that amplify the input dose. Newly produced virions can also spread and infect the adjacent cancerous cells. Consequently, infected cells often undergo pathological programmed cell death known as apoptosis. Infected cells are also targeted by the host immune system. Selective oncolysis can be caused by naturally occurring viruses that inherit oncolytic properties or by genetically modified viruses that only target cancer cells by design.

Viruses have also been used as a vehicle for nucleic acid transfer (Parker *et al.*, 2009). These viral vectors are intended to re-establish the wild-type copies of mutated tumor suppressor genes in cancer, affect the metabolism of tumor cells, activate the host immune response, or sensitize the neoplastic tissue to standard therapies (Cervantes-Garcia *et al.*, 2008). These methods are all within the field of gene therapy. In summary, viruses with oncolytic properties and limited side effects were used as miniature biological machines to reach the targeted cancer cells and lead to cancer cell death (Russell, 2002; Zamarin and Palese, 2012).

2.1.2 History of oncolytic virus therapy

Oncolytic viruses were noted as early as 1912, when a female patient suffering from cervical carcinoma showed significant tumor shrinkage after being vaccinated against rabies. The attenuated rabies vaccine was administered as a prophylactic treatment following a dog bite (Nemunaitis, 1999).

Further reports of the regression of Burkitt's and Hodgkin's lymphomas were documented after natural infection by the measles virus. Later, several potential oncolytic viruses were proposed for clinical trials in the 1950s (Everts and van der Poel, 2005). Summarized by Kelly *et al* (Table 2.1), five significant virotherapy clinical trials were reported: Hodgkin's disease was treated with Hepatitis B virus in 1949, "unresponsive neoplastic disease" was treated with West Nile virus in 1952, cervical carcinoma was treated with adenovirus in 1956 and terminal cancers were treated with the wild type non-attenuated mumps virus in 1974 (Kelly and Russell, 2007). In a revised timeline, the first virus-induced tumor recovery was reported in chronic myeloid leukemia as early as 1904 (Liu *et al.*, 2007).

Even though oncolytic potential was present in some cases, official analyses demonstrated a lack of the desired anticancer activities. Therefore, this therapy was abandoned for almost two decades. Modern knowledge of the basic principles of molecular biology, including cell cycle control and cell death, tumorigenesis, viral biology and the discovery of recombinant DNA technology in the intervening years have resulted in the current revival of oncolytic viruses as a form of cancer therapy (Everts and van der Poel, 2005; Todo, 2008). These advances led to the first trials with engineered oncolytic viruses in the 1990s.

With respect to human brain cancer, the preclinical studies of oncolytic viruses in glioma emerged in the 1990s, when the first attenuated herpes simplex viruses (HSVs) and adenoviruses were used, followed by oncolytic reoviruses. To date, four viruses have completed the clinical trials: herpes simplex virus (strains HSV-1, HSV-1716 & HSV-G207), Newcastle disease virus (strains MTH-68/H, NDV-Huj), adenovirus (Onyx-015) and reovirus. From the phase 1 trials, the viruses were declared as safe to be injected directly to the brain, and no maximum tolerated dose (MTD) was reached. Some anti-glioma activities were also observed. Among these, NDV had the most promising benefits, as six patients exhibited tumor regression and 3 patients exhibited long-term survival (Zemp *et al.*, 2010).

Table 2.1 Five significant historical virotherapy clinical trials against human cancer. Modified from (Kelly and Russell, 2007)

Year	Disease	Virus	No of patients	Route	Outcome	Side effects
1949	Hodgkin's disease	Hepatitis B virus	22	Parenteral injection of unpurified human serum	7/22 improve in clinical aspect of disease and 4/22 reduction in tumor size	Fever, malaise, death
1952	Advance unresponsive neoplastic disease	Egypt 101 virus (early passage West Nile)	34	IV, IM injection of bacteriologically sterile mouse brain, chick embryo, human tissue	27/34 infected, 14/34 oncotropism and 4/34 transient regression	Fever, malaise, mild encephalitis
1956	Cervical carcinoma	Adenovirus adenoidal-pharyngeal-conjuctival virus (APC)	30	IT, IA, IV injection of TC supernatant	26/40 inoculation resulted in localized necrosis	Vaginal haemorrhage, infrequent fever (3/30), malaise
1964	Myelogenous Leukaemia	NDV	1	IV	"responded to treatment"	No description
1974	Terminal cancers; gastric, pulmonary, uterine	Mumps virus (wild-type, non-attenuated)	90	IT, IV, oral, rectal; inhalation of purified human saliva or TC supernatant	37/90 complete regression or decrease >50%, 42/90 decrease <50%, and 11/90 unresponsive	7/90 adverse reactions; bleeding fever

2.1.3 Classification of oncolytic viruses

The oncolytic viruses are divided in two groups according to their nature: naturally occurring viruses with innate oncolytic activity and engineered viruses that alter specific genes by design to achieve selective oncolysis (Cervantes-Garcia *et al.*, 2008). The viral modifications in engineered viruses were also intended to attenuate their pathogenicity.

Summarized in Figure 2.1, viruses in cancer therapy are also divided into replication-defective viruses and replication-competent viruses. Most of the naturally occurring viruses are replication competent. In contrast, replication-defective viruses may also be used in oncotherapy as carriers of gene therapy through suicide gene delivery to targeted cells (Biederer *et al.*, 2002; Prestwich *et al.*, 2008). The suicide genes can be prodrug activating enzymes, death genes, tumor suppressor genes or antisense against oncogenes (Biederer *et al.*, 2002; Liu and Kirn, 2008).

NDV, reovirus, bovine herpes virus 4, coxsackie virus, vesicular stomatitis virus (VSV) and parvovirus are examples of natural viruses with innate oncolytic activity against human tumors (Cervantes-Garcia *et al.*, 2008).

Viruses were also enhanced with genetic modification to improve their selectivity by the insertion or deletion of therapeutic transgenes (Stanford *et al.*, 2010). Viruses can be made tumor selective by the modification of cellular tropism at the level of viral replication such that they become dependent on the specific characteristics of tumor cells. This specificity can be achieved by deleting viral genes that are critical for viral replication in healthy cells but are dispensable upon the infection of neoplastic cells. The modification of cellular tropism at the level of cell recognition and binding can be achieved by altering the viral coat for tumor-selective

binding and uptake (Everts and van der Poel, 2005; Russell *et al.*, 2012). Adenovirus, HSV and vaccinia virus are DNA viruses that have been modified using recombinant technology to attenuate their pathogenicity (Prestwich *et al.*, 2008).

Despite the direct cytolysis of cancerous cells, oncolytic viruses also stimulate the inflammatory response (Fournier *et al.*, 2012). The virally induced lysis of carcinogenic cells will release a wide range of tumor-associated antigens (TAAs). These TAAs will be processed by infiltrating dendritic cells and migrating to the lymphatic system, cross-presenting antigens to T cells, and potentially generating an adaptive anticancer immune response (Prestwich *et al.*, 2008).

For example, the most common immune-modulatory protein inserted into the oncolytic viruses is the granulocyte-macrophage colony-stimulating factor (GM-CSF), which has been inserted into adenovirus, herpes simplex virus and vaccinia virus to stimulate an inflammatory response within the tumor microenvironment (Stanford *et al.*, 2010) and lead to cancer cell death.

In contrast, the replication cycles of RNA viruses are not subject to the mechanism of nuclear transcription factors and must rely on a different pathway for their replication selectivity in cancerous cells. RNA virus life cycles involve the formation of dsRNA, which activates a spectrum of infected cellular defense mechanisms, including the activation of PKR and the release of interferons. The most promising oncolytic RNA viruses currently documented are attenuated strains of reovirus, measles virus, VSV, NDV, mumps virus, influenza virus and polio virus (Russell, 2002; Liu and Kirn, 2008; Russell *et al.*, 2012).

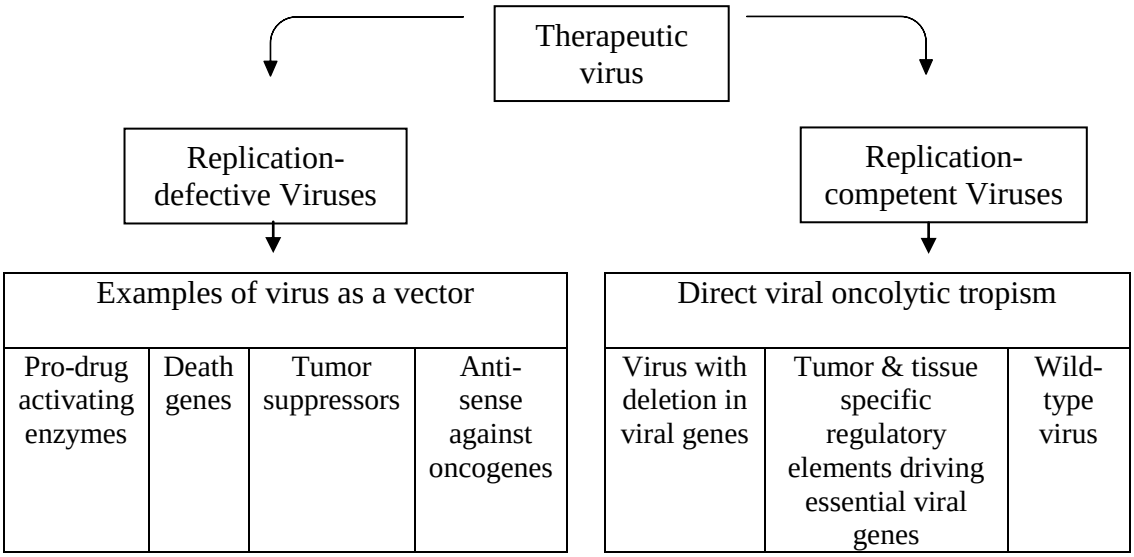


Figure 2.1 Applications of viruses for oncotherapy. Viruses can be used as vectors for therapeutic molecules (Group I) or used for the direct selective oncolysis of cancerous cells (Group II). Modified from Biederer *et al.*, (2002)

2.1.4 Characteristic of oncolytic viruses

Taking advantage of the genetic defects that fuel cancer growth, targeted therapies using oncolytic viruses to kill cancer cells with genetic defects or aberrations offer tremendous therapeutic potential. The modern era of cancer therapy research is now moving toward “biological weapons” that have evolved in parallel with cancer cells.

Several features are required for oncolytic viruses to be used as an effective cancer treatment (Biederer *et al.*, 2002; Parato *et al.*, 2005; Liu and Kirn, 2008; Parker *et al.*, 2009; Zemp *et al.*, 2010; Wollmann *et al.*, 2012) which include:

I. The virus is not a human pathogen but is capable of infecting human cells. This property will reduce the likelihood of pre-existing immunities against the vectors that would limit their therapeutic effectiveness (Zamarin and Palese, 2012).
II. Limited side effects to human (Zamarin and Palese, 2012).
III. Applicable for recombinant technology, such as the introduction of suicide genes into transformed cells and the insertion of transgenes for monitoring of viral shedding (Wollmann *et al.*, 2012).
IV. The viral life cycle of replication-competent viruses should include rapid replication and spread to induce cytolysis. This accelerated life cycle will facilitate the amplification of the viral therapeutic dose (Russell *et al.*, 2012).
V. The oncolytic viruses should be able to tolerate systemic administration (Russell *et al.*, 2012).

VI. The virus should not only eradicate the transformed cells but should also be able to establish anti-tumor immunity and should allow the virus to act as a potent anticancer vaccine adjuvant (Zamarin and Palese, 2012).

VII. The virus should not enter the nucleus or recombine with the host cell genome. This property will minimize the risk of virus-host genetic recombination events (Zamarin and Palese, 2012).

VIII. Selective replication should occur in transformed cells, sparing the non-transformed normal cells (Zamarin and Palese, 2012).

IX. Potential for high-titer virus production, simple and safe manufacturing.

X. The availability of specific antiviral agents is necessary to help regulate the viral distribution (Russell *et al.*, 2012).

XI. Viruses that demonstrate efficacy in the treatment of tumors can be used clinically in combination with standard treatment modalities (Parker *et al.*, 2009).

2.1.5 Advantages of oncolytic viruses

An ideal oncolytic virus for cancer therapy offers numerous advantages. The therapeutic benefits of this emerging therapy include natural selectivity during virus infection, which allows the viruses to selectively infect mutated cancer cells and helps to spare the normal tissue. Therefore, the viruses do not adhere to conventional toxicity and dose-response relationships (Biederer *et al.*, 2002; Wollmann *et al.*, 2012).

Oncolytic viruses are also “engineerable”. If they are not already naturally discriminatory, oncolytic viruses can be genetically engineered to be selective for mitotically active neoplastic cells. This property is especially appealing for glioma

therapy, as the tumor-adjacent, quiescent neurons often remain resistant and intact after treatment. Additionally, oncolytic viruses enable the introduction of therapeutic genes, allowing them to act as vectors for the augmentation of specific antitumor effects (Aghi and Chiocca, 2006; Zemp *et al.*, 2010; Wollmann *et al.*, 2012).

Moreover, oncolytic viruses theoretically offer unique pharmacokinetics, as the input dose can be amplified following viral replication. Current findings also show that along with current conventional treatments, the virotherapeutics have demonstrated synergy with approved chemotherapeutics and radiotherapy (Zemp *et al.*, 2010). For example, oncolytic adenovirus has been intratumorly injected along with docetaxel for treatment of prostate cancer (Russell *et al.*, 2012)

Furthermore, several oncolytic viruses have passed phase I clinical trials, and the results indicate that most of the oncolytic viruses are safe for direct administration into humans (Biederer *et al.*, 2002; Parato *et al.*, 2005; Cervantes-Garcia *et al.*, 2008; Liu and Kirn, 2008; Russell *et al.*, 2012).

2.1.6 Disadvantage of oncolytic viruses

Conversely, some oncolytic viruses operate optimally using cell-cell contact to assist viral spread. An oncolytic virus entering a normal cell triggers the cellular antiviral response but cannot counterattack, so the infection is quickly eliminated (Russell *et al.*, 2012).

In the case of GBM, for example, single-cell infiltration into the normal cell population is thought to be a major obstacle because viral proliferation could be inhibited by the surrounding normal tissue. Therefore, the objective of targeting multifocal tumors in the brain might be difficult (Zemp *et al.*, 2010).

In addition, circulating antibodies potentially neutralize free virus, and the rapid loss of oncolytic cells may entirely negate the therapeutic potential (Russell, 2002; Russell *et al.*, 2012). Therefore, the therapeutic index and ultimately the clinical outcome will depend on a complex balance between the host and viral factors (Biederer *et al.*, 2002; Liu and Kirn, 2008).

Moreover, DNA viruses can potentially undergo genetic shift or drift and thus can evolve over time. Thus, working with these viruses could become a major hurdle, specifically in terms of inducing the genotoxic effects (Liu and Kirn, 2008).

2.1.7 Current oncolytic virus candidates in trials on various cancers

Oncolytic viruses have several features that are unique from other therapeutics. Specific properties or features of the viruses determine different targets on the cancerous cells. The molecular aberrations of many cancerous cells are widely distinct. Therefore, several viruses have been screened for their oncolytic capabilities. In the past decade, the oncolytic viruses have been tested on various human cancer cells in vitro and in animal models with very promising benefits (Schirrmacher and Fournier, 2009; Zamarin and Palese, 2012).

Nevertheless, the studies conducted are not limited to replication-mediated oncolysis only but have also been evaluated for anti-tumor immune induction, anti-angiogenesis, and the induction of apoptosis and autophagy (Liu and Kirn, 2008). Some of the viruses that have been evaluated against multiple types of cancers include adenovirus, reovirus, HSV1, NDV, vaccinia virus, coxsackie virus, measles virus, VSV, retrovirus and myxoma virus (summarized in Table 2.2) (Parato *et al.*, 2005; Wollmann *et al.*, 2012).

Table 2.2 Oncolytic viruses, their mechanisms and the phase of studies in all targeted tumor cells that underwent trials. Modified from Parato *et al.*, (2005).

Virus	Mechanism of tumor targeting	Phase of development	Types of Cancer
Adenovirus	Targets to tumor antigens; conditionally replicating	Phase III conducted	Squamous head and neck carcinoma
Reovirus	Selectively infect Ras-transformed cells	Phase I conducted	Melanoma and malignant glioma
Herpes Simplex virus 1 (HSV-1)	Only replicates in tumor cells	Phase I conducted	Glioma
Newcastle Disease virus	Selectively replicates in interferon defective cells	Phase I conducted	Advance Solid cancers
Vaccinia virus	Gain access to tumor by vascular leakiness	Phase I conducted	Melanomas
Coxsackie-virus	Selectively infects tumor cells that over express DAF	Phase I conducted	Melanoma
Measles virus	Virus re-targeting to tumor antigens; overexpression of virus receptor (CD46) on some tumor cells	Phase I ongoing	Ovarian cancer
Vesicular Stomatitis virus	Selectively replicates in interferon defective cells	Preclinical mouse model	Metastatic tumor
Influenza virus	Non-structural protein 1-deleted virus specifically replicate in interferon defective cells	Preclinical mouse model	Tumorous cell
Retro virus	Tumor specific promoter allows expression only in cancer cells	Preclinical mouse model	Tumorous cell
Myxoma virus	Replicates selectively in signal transducer and activator of transcription 1 (STAT-1) or interferon defective cells	In vitro study	Tumorous cell

2.1.8 Oncolytic viruses on brain cancer

Brain cancer is the malignancy found in the brain parenchyma, where grade IV brain cancer is characterized as GBM. As malignant GBM is among the few rapidly proliferating tumors of the central nervous system, it is becoming an interesting subject for the study of selective-amplification viruses (Aghi and Chiocca, 2006; Mustafa *et al.*, 2011).

A review by Parker *et al*. in 2009 explained that the modern oncolytic targeted therapy has been initiated by engineered replication-attenuated viruses in non-dividing cells, such as neurons. The HSV with thymidine kinase deleted, *dlsptk*, has been designed for this purpose. The deletion of the *tk* gene controls the virus by making it dependent on actively dividing cells for its supply of both thymidine kinase and nucleotide pools for DNA replication. In animal studies, the *dlsptk* mutant virus exhibits a favorable therapeutic benefit in the treatment of glioma (Parker *et al.*, 2009). Unfortunately, the inactivation of the *tk* gene also renders this mutant resistant to acyclovir, an antiviral agent that targets the viral thymidine kinase. This antiviral resistance prevented further evaluation of this modified virus in clinical trials (Parker *et al.*, 2009).

To date, members of various virus families with distinctly different biologies have been tested on human GBM. Several modern clinical trials have been initiated for different oncolytic viruses (Parker *et al.*, 2009; Russell *et al.*, 2012). Wollmann *et al.* (2012) has reviewed all the clinical trials that had been initiated using attenuated strains and results from the trials have clearly established the proof of concept and have confirmed the general safety of oncolytic virus application in the brain (Wollmann *et al.*, 2012).

A total of fourteen virus families that target human glioma have been tested (summarized in Table 2.3). The viruses are HSV, adenovirus, reovirus, NDV, measles virus, vaccinia virus, myxoma virus, poliovirus, VSV, parvovirus, sindbis virus and SVV. Among these, HSV, adenovirus, reovirus and NDV have completed the early phase clinical trials in brain cancer patients (Table 2.4) (Zemp *et al.*, 2010).

The findings of all phase I clinical trials, which were conducted with the primary goal of evaluating the safety of oncolytic viruses, were largely successful. It was reported that these viruses were nontoxic and safe, with no maximum tolerate dose MTD reached. Although efficacy is not a major objective of this phase, some anti-glioma activity was observed in a handful of the subjects. These preliminary studies have demonstrated that the live replication-competent viruses can be safely administered into the brains of GBM patients (Zemp *et al.*, 2010).

Table 2.3 List of oncolytic virus candidates that have been evaluated in the context of human brain cancer in preclinical and clinical trials. Extracted from Wollmann *et al.*, (2012).

Oncolytic Virus Candidates for Glioma Therapy With Summary of Tumor Selectivity Factors				
Virus	**Genome and Structure**	**Host and Virus Family**	**Determinants for Tumor-Selective Targeting or Replication**	**Progress Relating to Glioma**
HSV-1	ds DNA Enveloped	Human Herpes-viridae	(1) HSV-TK and RR mutations compensated by activated cell cycle in tumors. (2) PKR defects in tumors allow F134.5 deleted HSV to replicate. (3) ICP47 gene deletion acts immune-stimulatory	Clinical phase I/II
NDV	ss (-) RNA Enveloped	Avian Paramyxo-viridae	(1) Mainly induction of anti-tumor cytokines and immune response (2) Possibly exploiting IFN defects	Clinical phase I/II
Adeno-virus	ds DNA Naked	Human Adeno-viridae	(1) Defects in p53 or RB pathway targeted by E1B and E1A mutants (2) RGD modification targets tumor integrins (3) E2F1 responsive elements control viral replication	Clinical phase I
Reovirus	ds RNA Naked	Mammalian Reoviridae	(1) Tropism for Ras-activated, transformed tumor cells	Clinical phase I
Vaccinia	ds DNA Enveloped	Cow/horse Poxviridae	(1) TK deletion compensated by nucleotide abundance in transformed tumors (2) VGF deletions compensated by aberrant EGFR receptor activation (3) Large size requires leaky tumor vessels for viral extravasation	Pre-clinical in vivo
Polio	ss (+) RNA Naked	Human Picorna-viridae	(1) Polio receptor CD155 expressed on glioma (2) Pathogenic polio IRES replaced with rhinovirus IRES	Clinical phase I
VSV	ss (-) RNA Enveloped	Livestock /mosquito Rhabdoviridae	(1) Selective replication depends on defective IFN pathway in tumor cells	Pre-clinical in vivo
MVM	ss DNA Naked	Mouse Parvoviridae	(1) Actively dividing cells required for replication (2) Defects in PKR augment viral replication	Pre-clinical in vivo
Sindbis	ss (+) RNA Enveloped	Mammalian/ mosquito Togaviridae	(1) Sindbis binds to 67-kd laminin receptor, which is overexpressed on some tumors	Pre clinical in vivo
PRV	ds DNA Enveloped	Pig Herpes-viridae	(1) HSV-TK and RR mutations compensated by activated cell cycle in tumors	Pre-clinical in vivo
Measles	ss (-) RNA Enveloped	Human Paramyxo-viridae	(1) Binding to CD46 receptor, overexpressed on tumors	Clinical phase I
Myxoma	ds DNA Enveloped	Rabbit Poxviridae	(1) Replication in IFN-deficient tumor cells (2) High affinity to cells with activated Akt	Pre-clinical in vivo
H1PV	ss DNA Naked	Rat Parvoviridae	(1) Virus requires actively dividing cells in S phase for replication (2) Defects in PKR augment viral replication	Clinical phase I
SVV	ss (+) RNA Naked	Pig Picorna-viridae	(1) Strong tropism to neuroendocrine and solid pediatric tumors, possibly mediated through integrin >4 receptor binding	Pre-clinical in vitro

Table 2.4 Summary of the oncolytic viruses strain and types of studies that completed clinical trials in human brain cancer. For oncolytic NDV, 20 patients have been recruited in total (Zemp *et al.*, 2010).

Oncolytic viruses for gliomas in completed clinical trials—overview.				
Virus (strain)	**Genetics**	**Study Type**	**Patient number**	**Dose/schedule/route of administration**
HSV-1 (G207)	g1-34.5 gene deletion lacZ insertion in UL39	Phase I	21	$1x10^6$ to $3x10^9$ pfu/single injection /intratumoral to enhancing area
HSV-1 (G207)	g1-34.5 gene deletion lacZ insertion in UL39	Phase I	9	$1.5x10^8$ to $1x10^9$ pfu/intratumoral injections pre- and post-resection
HSV-1 (HS-1716)	g1-34.5 gene deletion	Phase I	9	$1x10^3$ to $1x10^5$ pfu/single injection/ intratumoral
HSV-1 (HS-1716)	g1-34.5 gene deletion	Phase I	12	$1x10^5$ pfu/single injection/ intratumoral
HSV-1 (HS-1716)	g1-34.5 gene deletion	Phase I	12	$1x10^5$ pfu/single injection/ intratumoral
AdV (ONYX-015)	E1B-55 kDa gene deletion	Phase I	24	$1x10^7$ to $1x10^{10}$ pfu/single injection/ tumor bed post-resection
Reovirus	Wildtype virus	Phase I	12	$1x10^7$ to $1x10^{10}$ pfu/single injection/ intratumoral
NDV (MTH-68/H)	Attenuated NDV (mesogenic)	Case report 1	1	$2x10^7$ to $2.5x10^8$ pfu/daily for years/ intravenous
NDV (MTH-68/H)	Attenuated NDV (mesogenic)	Case report 1	4	$2x10^7$ to $2.5x10^8$ pfu/daily for years/ intravenous
NDV (MTH-68/H)	Attenuated NDV (mesogenic)	Case report 1	1	$4x10^8$ pfu/daily for months/ alternating intravenous inhalational
NDV (NDV-HUJ)	Selected NDV (lentogenic)	Phase I/II	14	$0.1x10^9$–11x109 IU/qd5; q1-2weeks, 3 cycles of 55 11_109 IU/ intravenous

2.2 Newcastle Disease Virus (NDV)

NDV is a highly contagious pathogen that affects avian species and causes severe economic losses to the poultry industry worldwide. NDV outbreaks were first reported in poultry from Java, Indonesia, followed by Newcastle-upon-Tyne in 1926 (Seal *et al.*, 2000; Zamarin and Palese, 2012). Eighteen NDV strains from four lineages were later identified and classified as either avirulent or virulent strains (Dortmans *et al.*, 2011; Nidzworski *et al.*, 2011).

2.2.1 NDV virus taxonomy

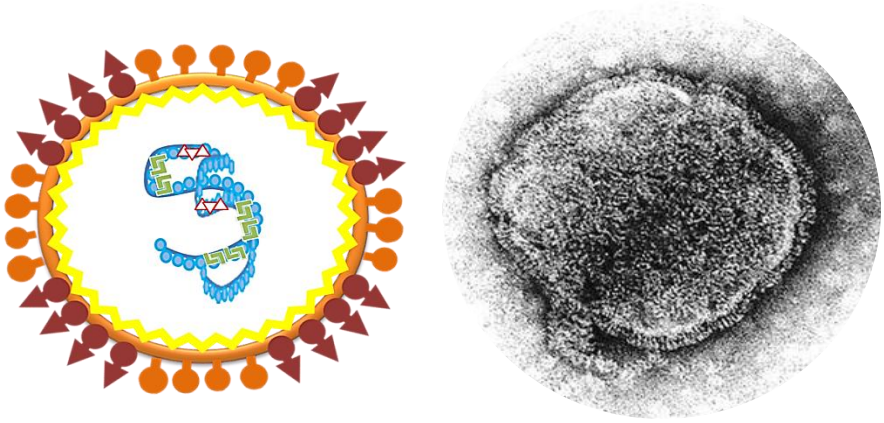
NDV is classified as a member of the Paramyxoviridae family of the Mononegavirales superfamily. This family is further divided into two subfamilies: the Paramyxovirinae and the Pneumovirinae (Seal *et al.*, 2000; Dortmans *et al.*, 2011). Other members of the Paramyxovirinae are Rubulavirus, Morbilivirus, Respirovirus, and Henipavirus. The genus of Avulavirus contains nine serotypes of avian paramyxoviruses (APMV-1-9); NDV represents type 1 (APMV-1). The disease potential of APMV-2 to -9 is not well known. APMV-2, -3, -6, and -7 have been associated with disease in turkeys, chickens and caged birds (Kumar *et al.*, 2011).

Mumps, human parainfluenza, sendai, simian virus-5 and the recently emerged nipah and hendra viruses are important human viruses in this subfamily (Ravindra *et al.*, 2009). These serotypes are summarized in Figure 2.2A. The paramyxoviruses comprise a large group of enveloped RNA viruses, some of which cause significant human and animal diseases. Virus particles are capsules built within infected cells that transmit infection from cell to cell and from host to host (Harrison *et al.*, 2010).

A

Family	Subfamily	Genus	Species
Paramyxoviridae	Paramyxovirinae	Rubulavirus	Mump virus, PIV5
		Avulavirus	NDV
		Respirovirus	Se Virus, Human PI Virus 3
		Henipavirus	He Virus, Ni Virus
		Morbilivirus	Me Virus
	Pneumovirinae	Pneumovirus	Respiratory Syncytial Virus
		Metapneumovirus	HMPV, AMPV

B



C

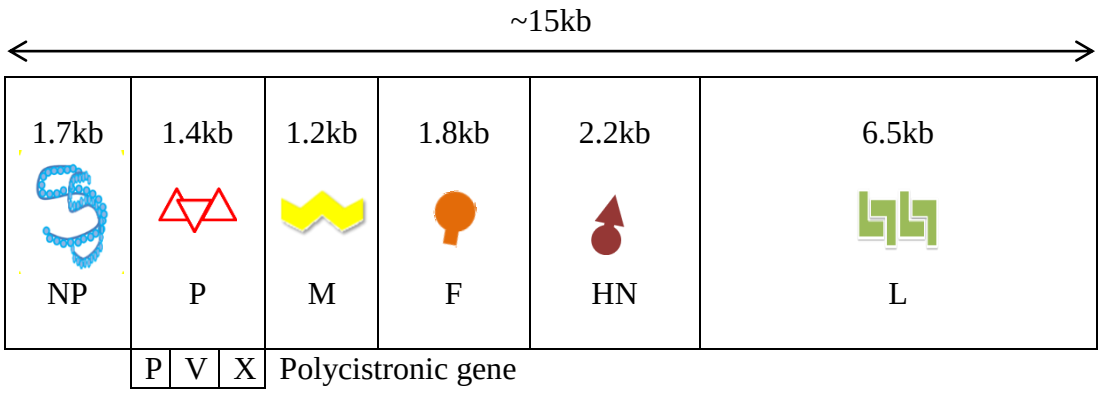


Figure 2.2 Serotypes and molecular structure of paramyxovirus. A, the members of the paramyxoviridae family. B, schematic diagram and an electron microscopy picture of a paramyxovirus particle. C, a schematic diagram of NDV genome organization and viral transcript. The F and HN proteins project from the virion envelope to form spike-like instruments. The M protein is beneath the envelope. NP protein is tightly bound to the viral genome forming the nucleocapsid complex, and the L and P proteins are loosely bound to the nucleocapsid complex. (Veits *et al.*, 2006; Harrison *et al.*, 2010).

2.2.2 NDV virus properties

On the genomic basis, every oncolytic virus is characterized by several proteins that help to establish the infection of the host cells. The NDV is morphologically pleomorphic, often filamentous and may be polyploid (containing more than one genomic equivalent) with an envelope particle diameter between 100 and 300 nm. The NDV genome consists of 15 kilobase pairs of non-segmented, single-stranded RNA, which code for 6 main structural proteins (Figure 2.2B and C page 22). These genes, nucleocapsid (NP), phosphorylation (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and RNA-dependent RNA polymerase (L) proteins, are found in a 3' NP-P-M-F-HN-L 5' arrangement (Sinkovics and Horvath, 2000; Schirrmacher and Fournier, 2009).

The most abundant protein in NDV is the NP protein. This protein has a flexible helical structure with a diameter of approximately 18 nm and a height of 1 μm, as observed by electron microscopy. The NP protein structure resembles classical morphology with spikes protruding from the central channel (Tan *et al.*, 2009). Each NP subunit consists of 489 amino acids with a molecular mass of approximately 53 kDa. Upon replication, NP subunits are associated with P and L proteins to encapsulate the viral genomic RNA into RNase-resistant nucleocapsid. This complex, instead of naked viral RNA, becomes the RNA template for the transcription and replication of the viral RNA genome (Harrison *et al.*, 2010).

The polycistronic phosphoprotein (P) gene codes for a protein of 395 amino acids with a calculated molecular weight of 42 kDa (Kumar *et al.*, 2013). In the viral transcriptase complex, the P protein acts as a cofactor with dual functions: stabilizing the L protein and orienting the polymerase complex (P:L) on the formed NP:RNA

template for mRNA synthesis. In addition to the P protein, which is encoded by an unedited transcript of the P gene, NDV was also shown to edit its P gene mRNA to produce V and W proteins. The insertion of one G residue at the conserved editing site (UUUUUCCC, sense strand) will produce the V protein, while the insertion of two G residues at the same site will produce W protein. The real functions of these two non-structural proteins are yet to be identified, but some studies show that V protein significantly contributes to the virulence in avian cells (Huang *et al.*, 2003; Jang *et al.*, 2010). The V protein is known to have an inhibitory effect on the alpha/beta interferon response of the avian host (Farkas *et al.*, 2009).

The M gene codes for the matrix protein. It can be found between the nucleocapsid and the viral envelope proteins. The M protein, which consists of 371-375 amino acids, is considered to be the central organizer of viral morphogenesis, due to its interactions with the cytoplasmic tails of the integral membrane proteins, the lipid bilayer, and the nucleocapsids. The self-association of M protein and its affinity for the nucleocapsid are the driving forces behind the formation of a budding virus particle (Andres Chang, 2012). For NDV, membrane deformation and vesicle budding have even been reconstituted in vitro using purified M protein and unilamellar vesicles, demonstrating that all of the activities necessary for inducing the curvature and fission of a membrane are contained within the M protein itself (Harrison *et al.*, 2010).

The F gene, an important determinant of NDV pathogenicity, consists of 540 to 580 amino acids and is known as the fusion protein or fusion glycoprotein Type 1. Virulent and avirulent NDVs are characterized by the presence of multibasic or single basic residues, respectively, in the F_o cleavage site, as there is a difference in