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Targeted Therapy for Gliomas: The Oncolytic Virus Applications

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1. Introduction

Cancer now is well described as a genetically defective disease that causes the overgrowth of particular cells. Cancer is phenotypically related to multiple sequential gene mutation and amplification, genetic translocalization, preservation of telomeres and loss of tumor suppressor gene that lead to immortal cell (Sinkovics & Horvath, 2000). In this regard, gliomas are particularly defined as pathological tumors that display histological, immunohistological and ultrastructural evidence of glial differentiation (Maher *et al*., 2001). Amongst gliomas, glioblastoma multiform (GBM) is one of the most killer cancer and maintains its prevalence despite current millennium intelligent treatment.

Taking advantage of the genetic defects that fuel cancer growth, targeted therapy using viruses to kill mutated or defective-gene cancer cells was investigated. Viruses with oncolytic properties and limited side effects to human were used as miniature biological machine to reach the targeted cancer cells. In this progressing treatment modality, the viruses were specifically studied to reach the targeted cancer without interfering the normal cells, directly inducing cancer cell death or activating the body immune system to infiltrate and mediate the destruction of tumor mass.

Basically, the targeted therapy using virus can be classified into two classes which are *replication-defective* virus that is mainly used as a vehicle for gene therapy by means as a vector for suicide gene delivery and also a *replication-competent* virus (Biederer *et al*., 2002). Some of the replication-competent viruses that have been studied, focused on its *natural-selective oncotropic* to invade cancerous cells and the others were armed with a genetically engineer technique to activate cell death promoter genes.

As the malignant gliomas are amongst the few rapidly proliferating ones in central nervous system, it is becoming an interesting subject for the study of selective-amplifications virus (Aghi *et al*., 2006).

In the beginning of this century, it was noted that a patient with cervical carcinoma experienced significant tumor regression after rabies vaccination. In addition, there were reports of remissions of Burkitt's and Hodgkin's lymphomas following natural infection with measles virus. In the 1950s, human trials with several potentially oncolytic viruses were initiated (Evert and Henk G., 2005).

Preclinical studies of oncolytic viruses in gliomas emerged in 1990's where the first attenuated Herpes Simplex Virus and Adenovirus were used followed by oncolytic

Reovirus. To date, four viruses have completed the clinical trials. The viruses are Herpes Simplex Virus (HSV-1, HSV-1716 & HSV-G207), Newcastle Disease Virus (MTH-68/H, NDV-Huj), Adenovirus (Onyx-015) and Reovirus. As the general outcomes of phase 1 trials, the viruses were declared as safe to be injected directly to the brain and no maximum tolerate dose (MTD) were reached. Some anti-glioma activities were also found. Amongst these, NDV showed the most promising benefits with 6 patients showed tumor regression and 3 patients have long term survival (Franz *et al*., 2010).

In this chapter, taking an example of Newcastle disease virus, we focus on the advantages of virotherapy, targeted pathway in oncolysis mechanism, methodology for virus with oncolytic properties study, and development of tumor model for glioma in a mouse model.

2. Oncolytic virus therapy

Oncolytic virus refer to the virus that kills tumor cells selectively without harming the normal surrounding tissue (Biederer *et al*., 2002; Franz *et al*., 2010). As a mode of therapy, oncolytic virus is used to "self-recognize" and infect the mutated cancerous cells which replicates within the infected cells followed by release of new virion that simultaneously amplifies the input dose. New virions later spreads and infect the adjacent cancerous cells. Consequently, infected cells often undergo pathological programmed cell death which also known as apoptosis.

This application modality reflects the application of viruses with *replication-competent* and inheriting the natural selective capability to the cancerous cells. Most studied natural oncotropic viruses are the RNA viruses such as paramyxovirus family including newcastle disease virus (NDV), reovirus, and vesicular stomatitis virus.

Moreover, some viruses were also enhanced with genetic modification by insertion or deletion of therapeutic transgenes respectively (Marianne *et al*., 2010). Virus can be made tumor selective by modification of the cellular tropism at the level of viral replication in a way that it becomes dependent on specific characteristics of tumor cells for viral replication. This can be achieved by deleting viral genes that are critical for viral replication in healthy cells but are dispensable upon infection of neoplastic cells. Modification of the cellular tropism at the level of cell recognition and binding by altering the viral coat for tumor-selective binding and uptake may also be performed (Evert and Henk G., 2005)

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

Research of the oncolytic virus also been studied along with current conventional treatments and the virotherapeutics have demonstrated synergy with the approved chemotherapeutics and radiotherapy (Liu *et al*, 2007).

In past decade, the oncolytic viruses have been tested on various human cancer cells in-vitro and animal model with very promising benefits (Schirrmacher and Fournier, 2009). Some of the oncolytic virus been studied and reported in phase 1 against glioma are the herpes simplex virus (HSV), adenovirus, reovirus, and NDV, while the measles virus, vaccinia virus, myxoma virus, polio virus, and vesicular stomatitis virus were at the preclinical level. (Parker *et al*., 2009)

For NDV, the strain been studied on glioma are the MTH/68H, NDV-HUJ,OV-001, 73-T and V4UPM. The V4UPM is the avirulent strain of ND virus that has been used as a thermostable feed pellet vaccine for poultry. It is been tested to poses excellent oncolytic activity against glioma cell lines. (Zulkifli *et al*., 2009)

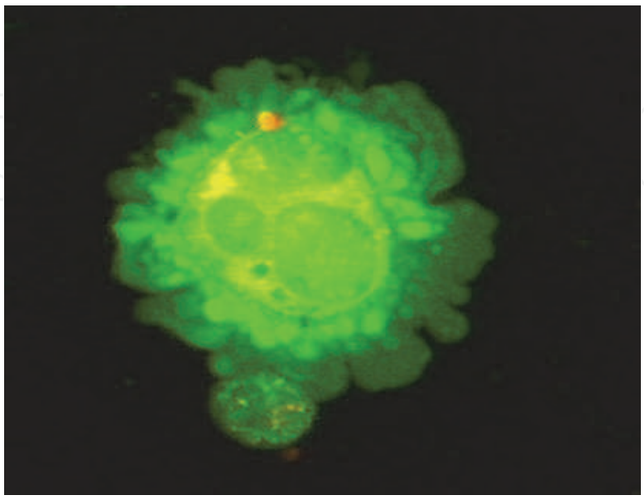

Fig. 1. An example of apoptotic glioma cells infected with newcastle disase virus (40x)

To date, there are 119 patients recruited for clinical trials. HSV (strain G207 & HS-1716), adenovirus (strain ONYX-015), reovirus and NDV (strain NDV-HUJ) have completed the phase 1 clinical trials. NDV strain MTH-68/H reported in several case reports against gliomas. (Franz *et al*., 2010) .

In the clinical practice, China is reported to be the first country that has approved the oncolytic virus application in 2006. The virus is the genetically modified adenovirus strain H101 that has been reported to enhance anticancer rate response compared to chemotherapy alone.

3. Advantages and disadvantages of viral therapy

In current conventional treatments, gliomas are managed by chemotherapy, resection surgery and also radiation therapy. However, the disease remains incurable. One of the exclusive factors of glioma is the diffuse infiltrative nature of tumor cells into adjacent brain parenchymal. This has led to incomplete resection and regrowth of the cancer. Therefore, oncolytic virus offered promising technique of eradicating the gliomas as it well known to selectively infect only the cancerous cell without harming the normal brain tissue.

As reviewed by Biederer *et al*., 2002, some of the major advantages of gene therapy and oncolytic virus therapy includes: oncolytic viruses can be engineered by recombinant genetic technology to meet specific targets, pose unique pharmacokinetics properties as its ability to amplify its own input dose and limited side effects to normal tissue. This limited side effect is extended with no adverse effect concerning allergy and asthmatic. (Schirrmacher and Fournier, 2009)

Oncolytic viruses are the foreign body that have innate capacity to stimulate host cytokines for potential anticancer activity. In this regards, some oncolytic viruses infect the target cells of different species and produce non-infectious virion but infected cells will express viral

antigens that later attract host antibody against the cancerous cells. (Sinkovics & Horvath, 2000). Tumor cell is immunogenically poor that leads them to escape from being killed by the body immune system. For example, NDV can also infect freshly isolated patient-derived melanoma cells that further lead to increase of viral antigens at the cell surface (Schirrmacher *et al*., 1999). On the other hand, the different host origin or animal viral are capable of infecting human cells where the pre-existing antibody is low.

Particularly to the reovirus, NDV virus and other simple single stranded RNA virus, their replications are incapable of recombination. In another words, the virus replication does not involve the intermediate DNA steps during their replication and thus no possibility of mutation insertion of viral RNA to host genome. Besides, the virus did not carry the oncogenes.

However, gliomas are always reported with single cell infiltration. Surrounding normal tissue therefore, may inhibit the virus spread that requires cell to cell contact and limits the local treatment implantation.

4. Viral genomic and infection

On the genomic basis, every oncolytic virus is characterized with several proteins that help them to establish the infection to the host cell as well as the cancerous cells.

For the ND virus, it is an avian virus with the genome consisting of 15 kilo base pairs of non-segmented, single-stranded RNA, coded for 6 main structural proteins. These genes namely nucleocapsid (NP), phosphorylation (P), matrix (M), fusion (F), hemaglutinin-neuraminidase (HN), and RNA-dependent RNA polymerase (L) proteins are found to be in 3′ NP-P-M-F-HN-L 5′ arrangement.

The NP protein is the most abundant protein found in the virion. Electron microscopic study reveals that the protein exists as flexible helical structure with a diameter of about 18 nm in length and 1 μm height. Its structure resembles classical morphology with spikes protruding from the central channel (Yusoff and Tan, 2001). Each NP subunits consists of 489 amino acids with molecular mass of about 53 kDa. In viral replication process, NP subunits in association of P and L proteins encapsidate the genomic RNA into RNase-resistant nucleocapsid. This complex, instead of naked viral RNA, becomes the RNA template for transcription and replication processes of the viral RNA genome.

The polycistronic phosphoprotein (P) gene codes for protein of 395 amino acids with a calculated molecular weight of 42 kDa (McGinnes *et al*., 1988). In the viral transcriptase complex, P protein acts as a cofactor with dual functions; stabilizing the L protein as well as placing the polymerase complex (P:L) on the formed NP:RNA template for mRNA synthesis. Apart from 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. Insertion of one G residue at the conserved editing site (UUUUUCCC, genome sense) will produce the V protein, while insertion of two G residues at the same site will give W protein. The real functions of these two non-structural proteins are yet to be identified but some studies shows that V protein significantly contributes to the virus virulence (Huang Z *et al*., 2003).

M gene codes for the matrix protein. It can be found between the nucleocapsid and viral envelope proteins. The protein which consists of 371 to 375 amino acids, is considered to be the central organizer of viral morphogenesis such as in making interactions with the cytoplasmic tails of the integral membrane proteins, the lipid bilayer, and the nucleocapsids.

The F gene, an important determinant of NDV pathogenicity, consists of 540 to 580 amino acids which codes for fusion protein or known as fusion glycoprotein Type 1. Virulent and

avirulent NDV are characterized by the presence of multibasic and single basic residues in the F_o cleavage site respectively as there is a difference in the amino acid sequences surrounding the precursor F_o for both the virulent and avirulent strain. The amino acid sequences in a virulent strain renders the F protein to be susceptible to cleavage by the host protease and leads to a fatal systemic infection. It was reported that combination of F and HN proteins initiates the NDV infection (McGinnes and Morrison, 2006).

The HN gene codes for hemaglutinin-neuraminidase protein which is the major antigenic determinant of all paramyxoviruses. This multifunctional protein is responsible for attachment to receptors containing sialic acid and neuraminidase (NA) activity. It has been considered that the role of the neuraminidase activity is to prevent self-aggregation of viral particles during budding at the plasma membrane. In addition, HN also has a fusion-promoting activity meaning that coexpression of HN and F is required for cell-cell fusion to be observed.

RNA-dependent RNA polymerase (L) protein is the largest protein in the NDV genome. It consists of 2200 amino acid residues. This protein forms a complex with P protein, and both of these components are required for polymerase activity with NP:RNA templates. The P:L complex can make mRNA *in* vitro that is both capped at its 5′ end and contains a polyA tail at the 3′ end.

Newcastle disease virus initiates infection through attachment of viral membrane to host cell surface receptor, the sialic acid-containing molecule which fused with the viral HN protein. Activated HN protein causes conformational changes to the viral F protein which brings the virion and the host membranes into close proximity (Morrison, 2003). This process allows for the viral nucleocapsid to enter the host cytoplasm. After this step, the nucleocapsid complex (RNA:NP:P:L) directs primary transcription of mRNAs which is complementary to the viral negative strand genome. These mRNA will later serve as templates for further negative strand RNA resulting in genome amplification. Secondary transcription then occurs in the same manner as the primary transcription but using the progeny nucleocapsids instead of the earlier parental's. After transcription is the translation process which produce the viral proteins followed by nucleocapsid assembly, association of P and L proteins, and further encapsidation. All these processes occur in the host cytoplasm. The almost completed virion then moves to the plasma membrane and is released by budding and rendered itself with an envelope coat from the host plasma membrane.

In the oncolytic study, the new NDV's virion was detected as early as 3 hours post infection and apoptotic cell death of glioma was detected via life cell imaging in our study as early as 7 hours post infection. The specific protein of NDV that interacts in the oncotropic mechanism however remains unclear but Elankumaran *et al*, (2007) reported that it was associated with the HN protein.

Schirrmacher and Fournier, 2009 summarised that following the intravenous injection of NDV, the virus was mainly detected in the lung, blood, liver and spleen at 0.5 hour. The amount of virus decreased rapidly over time and reached the detection limit at less than 1 day (blood and thymus), 2 days (kidney), and around 14 days (in lung, liver and spleen).

5. Glioblastoma and oncolytic selective mechanism

The molecular biology of gliomas has provided new insights in the development of brain tumors. These dysregulated cell signalling pathways that have been identified are now becoming the focus of a specific molecular targeted therapy (Chamberline et al., 2006). The

overexpression of these defective genes gives the opportunity to oncolytic virus to infect the gliomas.

In the study of reovirus, the virus infection leads to activation of dsRNA-activated protein kinase (PKR), which phosporylates the a-subunit of eIF-2, resulting in termination in the initiation of translation of viral transcript in normal cells. However, PKR kinase activity is impaired, allowing the virus replication to proceed. Ras-mediated signal transduction is activated in most human cancers due to either mutated Ras or mutated epidermal growth factor receptor (EGFR) (Kirn *et al*., 2001)). In GBM, 50% was found with EGFR overexpression and high Ras expression especially in the primary GBM. (Aghi M. & Chiocca E.A., 2006)

Oncolytic viruses are believed to replicate and and lyse different malignant cells in vitro and in vivo as a result of an impaired type I interferon response in cancer cells. In Miyakoshi et al study, oncogenes activation in human glioblastoma multiform had increased the activation of protein kinase. This leads to interferon synsthesis and the inhibition of tumoregenesis. (Miyakoshi *et al*, 1990). In gliomas however, the anti-tumor response is impaired by glioma-derivative immunosuppressing factors such TGF-b, IL-10, prostaglandin E2 and gangliosides. TGF-b is the most prominent immunosuppresor that plays a major role in glioma biology whose overexpress and become the hallmark of the gliomas.

On the other hand, normal brain cells response to the viral infections leads to stimulation of the patern-recognition-receptors (PRRs) and later activates the Type 1 interferon.(Franz *et al*., 2010). The type 1 interferon futher binds and activates the Janus kinases JAK1 and TYK2 which inturns phophorylates the activators for transcription of STAT1 and STAT2. The STAT proteins later heterodimers and forms a complex with IRF9. The complex is known as ISGF3 that futher provides DNA recognition and simultaneously produces the interferon-stimulated genes (IGS) that creates the antiviral state in the target cells and blocks viral replication. In this regards, interferon-beta is the principle antiviral factor secreted by NDV-infected cells. Consequently, the interferon defective tumor cells gives more opportunity for the NDV to effectively replicate compared to normal cell and it is concluded that this replication-competent virus selective mechanism is associated with the defect of the host interferon (Krishnamurthy *et al*., 2006).This is summarised in the Figure 2 below.

Recently, Puhlmann *et al* has established Rac 1 as a protein which activity is critical for both oncolysis virus sensitivity and autonomous growth behaviour of cancer (Puhlmann *et al*., 2010). Rac1 plays a role as a pleiotropic regulator of multiple cellular functions including actin skeleton reorganization, gene transcription and cell migration. Rac1 is a key contributor to glioma cell survival, probably via multiple signaling pathways including JNK (Halatsch *et al*, 2009) but is found to be critical for the replication of oncolytic NDV to the highly tumorigenic ras-transformed skin carcinoma cells

However, despite several entry-line association, there are several physicals barriers that is present in the gliomas microenvironment that blocks the virus distribution. The extracellular matrix (ECM), hypoxic region, and high interstitial pressure are amongst the major challenge in achieving lasting oncolytic virus infection (Franz *et al*., 2010)

Besides that, GBM is currently described with multiple interactive dysregulated cell signalings pathway and this could lead to the inconsistency of outcome (Chamberline et al., 2006) in bigger population target.

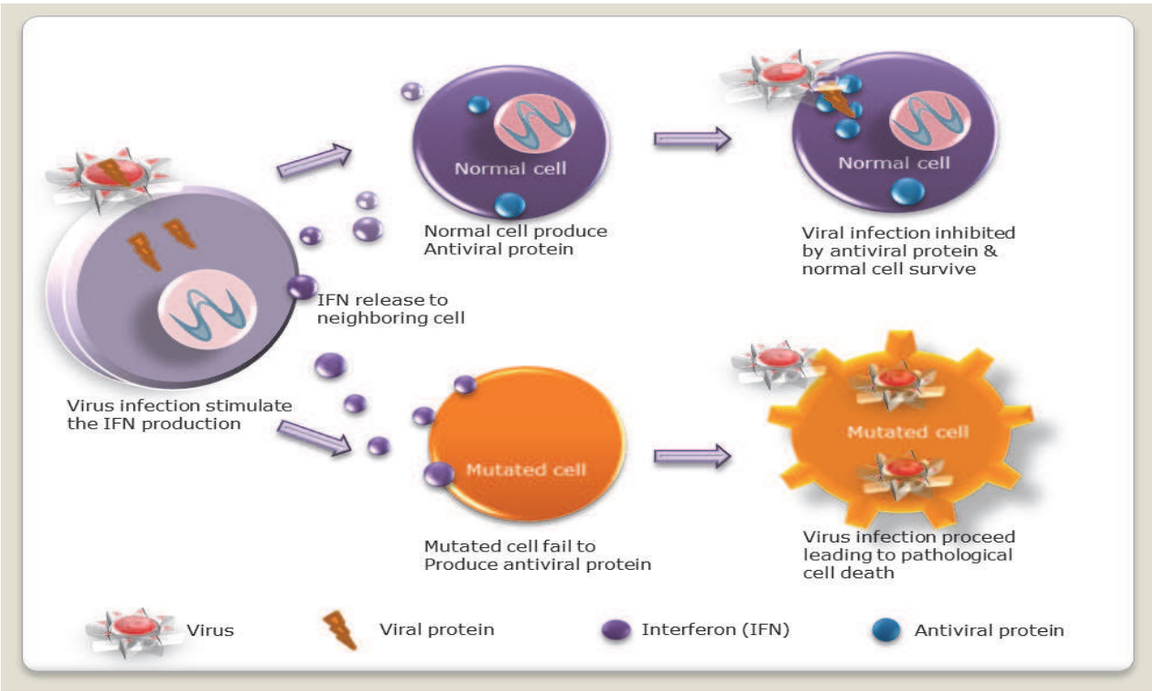

Fig. 2.

6. Cell cycle arrest and pathway

Glioma has been classified according to their hypothesized line of differentiation that is whether they display features of astrocytic, oligodendroglial or ependymal cells. These are graded on the scale of grade one to grade four according to the degree of malignancy judged by histological features. At the molecular level, the mutation that leads to different glioma grades presented with several relevant pathways. In the high grade astrocytoma for example, retinoblastoma mutation is found at approximately 25 percent. In this regard, retinoblastoma is a major regulator of cell cycle progression where mutational inactivation of retinoblastoma leads to unschedule cell cycle entry (Maher *et al*., 2001).

Cell cycle is a series of cellular events which leads to cell division and replication. Generally, cell cycle mechanism involves 4 different stages; G_1 (gap 1), S (synthesis), G_2 (gap 2) and M (mitosis). G_1 is an interphase between the end of M (mitosis) phase and the beginning of a new cell cycle. At this phase, the cell either prepares to enter the S (synthesis) phase or stops dividing (quiescence). If the cell receives growth signal to replicate, it will move to S phase where it starts synthesizing nucleic acid. Once DNA replication completes, the cell will enter G_2 phase. Here, synthesis of crucial proteins involved in cell division like microtubules occurs and once complete, these cells will move to the M phase and divide itself into 2 daughter cells. All these 4 processes should occur without any disturbance from inside or outside of the cell. Each of the cycle phases is very critical and if anything goes wrong at any stage especially during G_1 and M, they can cause mutations and may lead to cancer. Normal cell usually has several systems to check for errors at each phase and this is known as phase checkpoints.

Transition from G_1 to S phase is controlled mostly by cyclin-dependent kinases (Cdk2, Cdk4, and Cdk6) and their substrates. These Cdks regulate the retinoblastoma family proteins

(p107, p130, and pRb) by phosphorylation. Initial partial inactivation of pRb by Cdk4 and Cdk6 induce transcription of E-type cyclins. These cyclins activate Cdk2 which further phosphorylates the pRb and other substrates. The Cdks are regulated by different levels involving interaction with positive and negative partners. Amongst the inhibitors are the Cip/Kip family proteins and one of them is $p27^{Kip1}$. This protein can binds to cyclin D either alone or when complexed to its catalytic subunit CDK4. By doing so, it inhibits catalytic activities (phosphorylation) of CDK4 towards pRB protein. It was reported that phosphorylation at the Thr187 of $p27^{KIP1}$ by Cyclin E/CDK2 complex promotes its degradation by recognition of this phosphorylated $p27^{KIP1}$ by SCF(Skp2) which has the E3 ubiquitin ligase activity (Ungermannova *et al.*, 2005**)**. Degradation of $p27^{KIP1}$ starts the generation of cyclin A-dependent kinase activity which will push the cells from G phase to S phase in the cell cycle. In several studies done in viral infections which induces cell cycle arrest, it was found that the level of this inhibitor protein increases significantly after infection, which leads to arrest of the cell replication process.

A successful introduction of viral genome into a host cell usually causes chaos in the host system and ends-up with the production of viral genome instead of host. Studies done on certain viruses revealed that these viruses are able to stop the host cellular replication mechanism namely cell cycle arrest which may render the virus with advantages in taking over the whole machinery system. This cell arrest is usually associated with elevation or suppression of players in the cell cycle from members of cyclins, CDKs, and inhibitors. For instance, infection of influenza A virus A/WSN/33 (H1N1) causes changes in the host protein expression level of cyclin E and cyclin D1, as well as p21 which are amongst the key molecules of cell cycle (He *et. al.,* 2010). Besides that, p27 is one of the protein involve in the retinoblastoma pathway where glioma cell lines exhibit an inverse corelation between the level of p27 protein and proliferation index (Maher *et al.*, 2001) that could be investigated as a rational component target in cell cycle arrest.

Many other viruses were also reported to cause cell cycle arrest upon infection such as measles virus, human immunodeficiency virus-type 1 (HIV-1), and herpesvirus. Our recent findings on NDV infection also reveal the potential of this virus to block cell cycle in tumor cells.

7. Methods

7.1 Virus propagation

Avirulent NDV propagation is carried out in the Class II laboratory (Laboratory Biosafety Level). Generally, the egg shell is cleaned with 70% ethanol and then candling is done to ensure that the embryo is still alive. Then a mark is made slightly above the air-sac where the mark is pricked with a 21G needle. Through this tiny hole dilution of NDV in PBS is introduced into the allantoic fluid cavity. The hole is then sealed with melting wax and the egg is incubated at 37^{o}C for 48 to 72 hours (subjected to viral virulence) to allow for the viral propagation as well as the embryo growth.

Prior to allantoic fluid collection, the embryo is killed by placing it at 4^{o}C for about 5 hours. Besides killing the embryo, this cold temperature also shrinks the blood capillary inside the egg, thus, extracting the allantoic fluid would be easier. Allantoic fluid is then collected and clarified at 8,000 x *g* for 30 minutes to remove the debris such as red blood cells and yolk if any. Further centrifugation at 20,000 x *g* for 2 and half hours will precipitate the virus at the

bottom of the centrifuge tube. The pellet is then re-suspended in NTE or PBS buffer according to further work specifications.

Virus obtained at this stage can be used in work like HA, HI and other several tests but not for infection and other works that need pure virus application. Virus purification is achieved by separating the virus from other tiny contaminants in the viral suspension through glucose gradient. Special ultracentrifuge with vacuum function is needed for this process since the virus will be spinning at 38,000 x *g* for 4 hours to get better separation. Band containing virus is then identified and extracted for further centrifugation at the same speed in 2 hours. This will pellet the virus at the bottom of the tube. Pure virus pellet is now re-suspended in NTE or PBS buffer and kept at -20^{o}C or -80^{o}C for longer storage.

7.2 In vitro cytotoxic study

Cell lines were cultured in the media and supplemented according to supplier recommendations. For the cytotoxicity study, adapted from the method by Zulkifli *et al.*, 2009, the normal and glioma cell lines were seeded at 1 x 10^5 in 96-well plate and incubated overnight in 37°celcius incubator supplemented with 5% CO_2 gas. Following the attachment of the cells next day, the media was changed. The cells later treated with virus at MOI of log_{10} serial concentrations and incubated for 24, 48 and 72 hours. Relative cell viability later tested with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent. The absorbance values were expressed as a percentage and the sigmoidal dose response curve were plotted versus virus dose. The EC50 achieved by the curve represents the dose of virus that reduces the maximal light absorbance capacity of an exposed cell culture by 50% and is proportional to the percentage of cells killed by the virus.

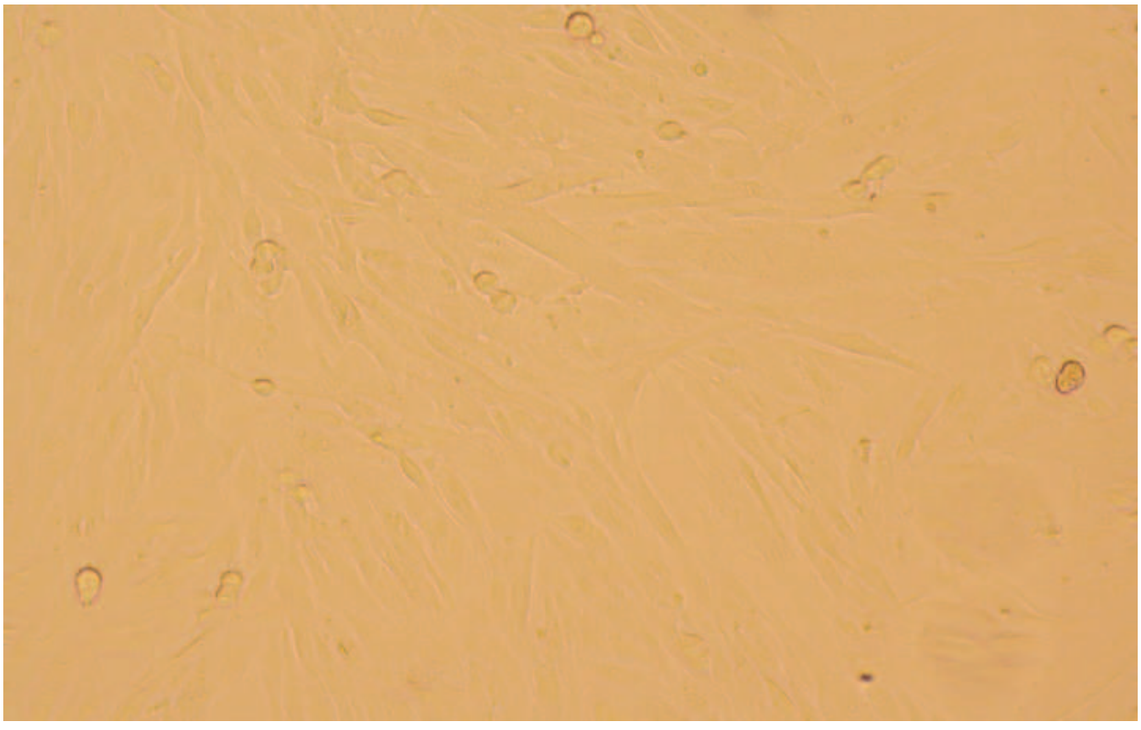

Fig. 3. Confluence glioma cell line in cell culture

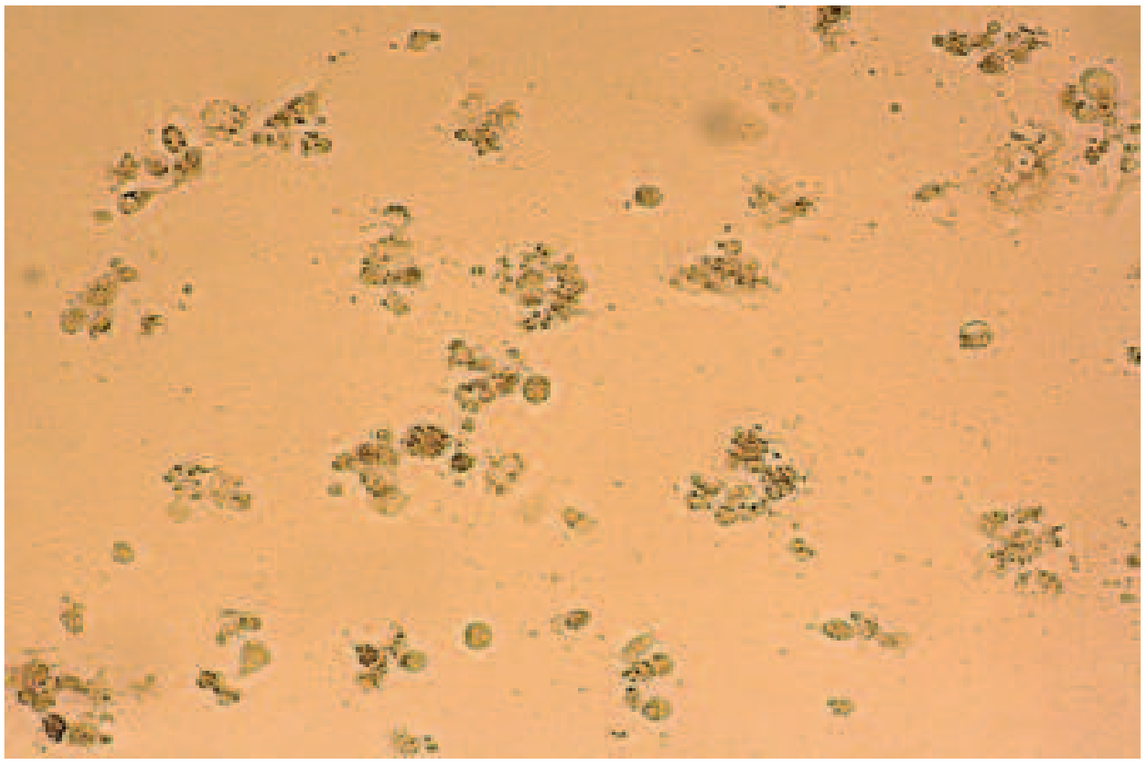

Fig. 4. Apoptotic glioma cells after 48 hours infection with newcastle disease virus

7.3 Protein analysis by SDS-PAGE

Cell cycle process occurs by interactions of many protein players. These proteins are produced at specific rate and amount to meet the purpose; overexpression or reduction in the amount suggests for disturbance or changes in the cycle process, perhaps causing cell cycle arrest. There are several options that can be carried out in a laboratory in order to find out on this changes of protein expression level, but the established method are the SDS-PAGE and Western blot. These two methods allow researchers to find the difference of specific protein level between different samples qualitatively and even quantitatively.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique to separate different proteins based on their size by running proteins through a gel forced by electrical voltage. SDS-PAGE gel consists of two parts; the bottom part namely the resolving gel and the upper part which is the stacking gel. Different percentage of resolving and stacking gels can be prepared depending on the size of protein of interest but the standard percentage in most practice is 12% resolving gel and 5% stacking gel. A 12 % resolving gel was prepared first by mixing 30% acrylamide solution, 1.5M Tris (pH 8.8), 10% (w/v) SDS in distilled water, 10% (w/v) ammonium persulfate (APS) in distilled water, deionized water (dH_2O) and N,N,N′,N′-Tetramethylethylenediamine (TEMED) in a clean beaker and immediately poured into a set of SDS-PAGE gel casting apparatus (Bio-Rad, USA) to an appropriate level. Immediately after this step, appropriate amount of n-butanol was layered onto the gel and it was left for 45 minutes at room temperature to solidify. After butanol layer was removed and rinsed with dH_2O, stacking gel was prepared by mixing 30% Acrylamide solution, 0.5M Tris (pH 6.8), 10% (w/v) SDS in distilled water, 10% (w/v) APS

in distilled water, dH_2O, and TEMED in a clean beaker. This mixture was then mixed properly and immediately layered on top of the resolving gel. A comb was inserted into the stacking gel and everything was allowed to set for about 45 minutes. Then the comb was removed carefully and the glass plates holding the gel was transferred into an inner chamber of Mini Protean-3 Cell electrophoresis tank (BioRad, USA). About 400 ml SDS-PAGE running buffer consisting of 0.025 M Tris, 0.192 M glycine, and 0.1% (w/v) SDS in distilled water (pH 8.3) was prepared and poured into the inner chamber until full while the rest was emptied into the electrophoresis tank.
Samples were then prepared by mixing the cell lysate with 2X sample buffer [125 mMTris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.0.2% Bromophenol blue, 10% 2-mercaptoethanol (added fresh before use)] at the ratio 1:1 and the mixture was boiled for 5 minutes. After that, the mixture was loaded into SDS-PAGE gel and electrophoresed at 180V for 50 minutes.

7.4 Western blotting and chemiluminescence

Separated proteins in SDS-PAGE gel was then transferred using Western blotting. Briefly, the gel was removed carefully and layered onto a polyvinylidene fluoride (PVDF) placed at the middle of 4 filtered paper. Protein in the gel was transferred by electrical charges attraction at 100V for 1h in a protein wet transfer tank filled with Towbin's transfer buffer [25 mMTris, 190 mM glycine (pH 8.0), 20% (v/v) methanol]. After that, blocking step was carried out by soaking the PVDF in 5% milk diluents for 1h at room temperature or overnight at 4^{o}C. The membrane was washed in TBST buffer [50 mMTris-HCl, 150 mMNaCl, 0.01 Tween 20] 3 times (5 minutes each time) to remove the blocking reagent. Specific protein detection was achieved by incubating the membrane in primary antibody for 1h at room temperature. The membrane was then washed with TBST buffer 3 times (5 minutes each) before it was incubated in secondary antibody conjugated with horseradish peroxidase (HRP) for another 1 h at room temperature. After that, the membrane was washed again with TBST for 3 times (5 minutes each wash).
Protein band of interest was obtained by chemiluminescence method. Reagents from Supersignal West Pico Chemiluminescent Substrate or Supersignal West Dura Chemiluminescent Substrate (Pierce, Thermo Scientific, USA) kit were added onto the membrane for 5 minutes incubation period, allowing for the HRP from the secondary antibody to bind to the substrate and fluoresce. This signal was then captured onto a film in an autoradiography cassette (Fisher Scientific, PA) and developed by a film developer machine (AFP Imaging Corp, USA). These bands can be compared quantitatively using software like Image J. Data used for the purpose of comparison helps in identifying the changes in protein expression level and further other changes or disturbance in the pathway involved.

7.5 Development of glioma model in mouse

Growing the glioma model is one of the critical issue in brain tumor therapy study. One of the common model is the xenograft implant of glioma cell line into the immunosuppresive mouse. Some of the advantages of the implant model are the predictable growth rate of the tumor and it is reproducible in term of location besides the precise histology features. The implant model however fails to give the characteristic of single cell infiltration.
The glioma model in the nude mouse were done according to Zulkifli *et al.* In brief, actively growing glioma cell lines such as DBTRG.05MG and U-87MG were harvested from culture

flask, counted at 1 x 10^7 in the PBS and subcutaneously injected to a flank of female 6-weeks old homozygous nu/nu Balb/c mice. Tumors grows until they were clearly growing and palpable measuring (by digital caliper) at 20mm^3 were obtained. Tumor size is measured using the ellipsoid formula (Length x Width x Height x 0.5) (Tanaka *et al.*) twice weekly .All animal were kill when they lost 25% of their body weight or had difficulty in ambulating, feeding or grooming. The experiments must be conducted according to guidelines and approval by respective animal ethic committee.

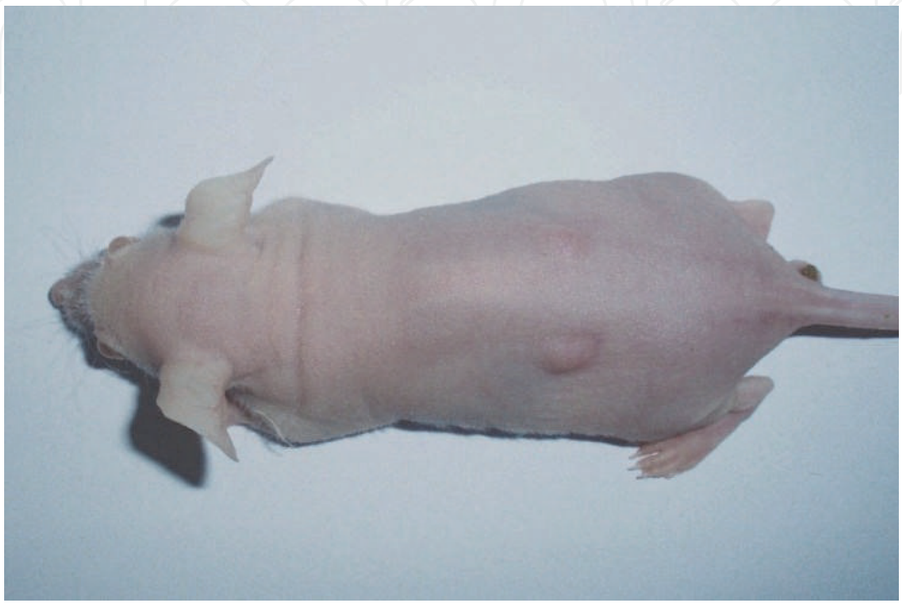

Fig. 5. The glioma growth on the nude mouse flank.

8. Conclusion

The oncolytic virus therapy is now amongst the fastest growing study compare to other salvage therapy as the current progress giving great potential in treating various cancer.
Specific relationship of virus with tumors however shows wide variable thus multiple dosages and optimum temperature have to be specifically studied. Besides that, current outcomes of oncolytic viruses studies show the direction of specific virus to be used for the specific cancers.

9. Acknowledgment

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10. References

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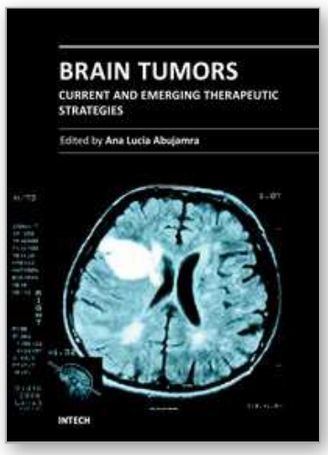

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