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The Anti-cancer Impact of Genetically Engineered Newcastle Disease Virus Expressing GFP Gene Against U87-MG Cell Line

Lubna Muhi Rasoul
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

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Abstract

A number of disorders characterized by aberrant cell proliferation are referred to as cancers. Cancer is a complicated group of mutagenic diseases that can move or infiltrate to other parts of the body. It develops through a multi-step process. The need for new therapeutic strategies is driven by malignancies resistance to conventional therapies. Use of the Newcastle disease virus as an oncolytic agent has advanced and expanded in immunocompetent carcinoma tumor models by utilizing reverse genetics techniques. Preclinical investigations have shown that recombinant NDV (rNDV-GFP), which expresses foreign genes, is proven to be effective in cancer treatment. Green fluorescent protein gene is usually used as an expression reporter for certain genetically encoded molecular biomarkers. To demonstrate that a gene may be expressed in many organs, interest cells or across an organism, it was utilized to make GFP-expressing biosensors. GFP has been detected in human cells as well as bacteria, yeasts, fungi, fish and other animals. The aim was to investigate the anti-tumor effects of rNDV expressing GFP gene on U78-MG glioblastoma cell line *in vitro*. This research examined *in vitro* the anticancer activity of genetically modified Newcastle disease virus strains that express GFP (rClone3-GFP) using the MTT test (a colorimetric assay for measuring cell metabolic activity) on the U87-MG glioblastoma cell line. Recombinant viruses were found to be able to trigger a time-dependent demise of tumor cells death starting 96 hours after inoculation. Using reverse genetics, we inserted GFP-coding regions between the F and HN genes in the lentogenic NDVClone30 strain's genome which was named as rNDV-GFP. The recombinant NDV-GFP strains that produce GFP showed promising results for inhibiting growth of tumor cells. Our study paved the way for the use of recombinant NDV as an anticancer viral vector. Our results suggest that NDV-GFP is a promising therapeutic for glioblastoma cancer treatment.

Keywords: rNDV, GFP, Reverse Genetics Technique, MTT, U87-MG cell line.

*Email: Iraqi_biologist@yahoo.com

التأثير المضاد للسرطان لفايرس مرض النيوكاسل المعدل وراثيا والذي يعبر عن جين (البروتين المشع الاخضر) ضد خط خلايا U87-MG.

لبنى محي رسول
قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة

يشار إلى مجموعة الاضطرابات التي تتميز بتكاثر الخلايا بشكل غير طبيعي على أنها سرطانات. السرطان عبارة عن مجموعة معقدة من امراض الطفرات الجينية التي يمكن أن تنتقل إلى أجزاء أخرى من الجسم أو تتسلل إليها، و يتطور المرض من خلال عملية متعددة الخطوات. إن الحاجة إلى استراتيجيات علاجية جديدة بسبب مقاومة الأورام الخبيثة للعلاجات التقليدية اصبح امرا ملحا. لقد تطور استخدام فيروس مرض نيوكاسل كعامل مُحلل للأورام في نماذج أورام السرطان ذات الكفاءة المناعية تطورا كبيرا من خلال استخدام تقنيات الوراثة العكسية. أظهرت التحقيقات قبل السريرية أن فايرس مرض نيوكاسل المؤتلف (rNDV-GFP) ، الذي يعبر عن الجينات الأجنبية ، يكون أكثر فعالية في علاج السرطان. يشيع استخدام جين GFP كمراسل تعبير. من أجل إظهار أنه يمكن التعبير عن الجين في جميع أنحاء الكائن الحي ، في أعضاء معينة ، أو في خلايا معينة ذات أهمية ، فقد تم استخدامه لصنع مستشعرات حيوية وحيوانات معبرة عن .GFP تم التعرف على. او البكتيريا والخمائر الفطريات والأسماك والحيوانات الأخرى. تهدف الدراسة الى التحري عن التأثير المضاد للسرطان لفايرس مرض نيوكاسل المحمل لجين البروتين المشع الاخضر على خطوط خلايا الورم الارومي الدبقي. يهدف هذا البحث التحري عن النشاط المضاد للسرطان في المختبر لسلالات فيروس مرض نيوكاسل المعدلة وراثيا والتي تعبر عن الجين المشع الاخضر على خط الخلايا الارومي الدبقي باستخدام اختبار قياس تقييم نشاط الخلية الاستقلابي للفيروسات المؤتلفة لتحديد قدرتها على التسبب في موت الخلايا السرطانية بطريقة تعتمد على الوقت بعد 96 ساعة من الاصابة. باستخدام الوراثة العكسية قمنا بادخال جين البروتين المشع الاخضر الى الجينوم الفايروسي واظهر الفايرس الناتج نتائج واعدة في تثبيط نمو الورم واستخدامه كناقل فايروسي مضاد للسرطان. اظهرت نتائجنا ان فايرس مرض نيوكاسل المحمل بجين(البروتين المشع الاخضر) اظهر نتائج واعدة لعلاج خلايا سرطان الدماغ خارج الجسم الحي.

Introduction

Cancer is a category of diseases that can invade and spread to different sections of the body [1, 2]. It is defined by abnormal cell proliferation. Both endogenous and exogenous factors can result in cancer. Examples of endogenous causes include genetics caused by the deletion or mutation of genes associated with cancer which results in uncontrolled cell growth. Exogenous impacts include factors like chemicals, physical effects and biological. The term "Cancer" describes a class of disorders characterized by abnormal cell development and the capacity to invade or spread to different body regions [1, 2].

An oncolytic virus is one that only affects and eradicates cancer cells. New infectious virus particles or virions are generated to help in the remaining tumor eradication as infected cancer cells are eliminated by oncolysis [3, 4]. In addition to directly destroying tumor cells, oncolytic viruses are hypothesized to also incite the host's immune system to fight the tumor [5, 6]. The tumor microenvironment may be impacted by a range of oncolytic viruses' potential actions [7].

The oncolytic potential of some naturally occurring viruses is unsurpassed, despite the fact that genetically edited viruses are currently being investigated for use in cancer therapy.

Newcastle disease virus (NDV) virulence can be explained on the basis of molecular biology, highly oncolytic strains of NDV with low toxicity were attenuated to reduce virulence[8,9]. With the discovery of NDV reverse genetics, point mutations, the insertion of foreign genes, and other genetic alterations of the NDV viral genome has become possible. This technology can be utilized to increase NDV's efficacy as a vaccination against chicken flu. Additionally, it can be applied to modify NDV in order to improve its oncolytic properties. Reverse genetics technology is currently being applied widely to boost NDV's anti-tumor effectiveness [10, 11].

GFP gene is an expression reporter that is widely employed. The concept that a gene can be expressed throughout an organism, in particular organs or in particular cells of interest, has been demonstrated through the use of customized biosensors and numerous animals that exhibit GFP. By using transgenic procedures, GFP can be injected into animals or other species and maintained in their genomes and offspring. In addition to human cells, GFP has also been found in fish, animals, yeasts, fungus and bacteria [12].

Adults are more likely to develop hepatocellular carcinoma than any other type of primary liver cancer which is currently the main cause of death among cirrhotic patients [13]. The third most frequent cancer-related cause of death worldwide is hepatocellular carcinoma [14].

The GFP gene is one that is frequently used as an expression reporter. Through the use of specialized biosensors and multiple animals that display GFP, it has been shown that a gene can be expressed throughout an organism, in certain organs or in specific cells of interest. GFP can be introduced into animals or other species and preserved in their genomes and offspring by using transgenic methods. GFP has been discovered in organisms other than human cells, including fish, mammals, yeasts, fungi and bacteria [12].

Glioblastoma is one of the most serious malignancies that develops in the brain. Initial signs and symptoms of glioblastoma are hazy. Headaches, behavioral changes, nausea, and symptoms resembling a stroke may be among them. Symptoms frequently worsen rapidly and may even put a person into a coma [13].

Most glioblastoma cases have unidentified origins. Prior radiation therapy and genetic disorders including neurofibromatosis and Li-Fraumeni syndromes are infrequent risk factors. Glioblastomas account for 15% of all cases of brain tumors. They might develop from healthy brain cells or an existing low-grade astrocytoma [14].

Numerous people are dying from cancer and the incidence is increasing globally at a rate of 3% each year [15]. Statistics showed that cancer deaths have become the leading cause of mortality for many Chinese metropolitan inhabitants. As a result, more focus is being paid to expanding cancer therapy research in an effort to find an innovative way to treat malignancies. Reverse genetics technology enables individuals to request genetic alterations to the Adenovirus genome in order to increase the virus's oncolytic activity and reduce its potentially harmful side effects.

After introducing sequences encoding the GFP gene into the genome of the Newcastle disease virus strain, we examined and assessed the recombinant virus's anticancer activity *in vitro*. This virus was given the name (NDV-GFP). Our results suggest that NDV-GFP is a promising therapeutic for the treatment of glioblastoma cancer.

Materials and Methods

Cell Line and Viruses

Helper plasmids, rClone30 and transcription of the entire length of cDNA plasmids were ready to use. The NDV lentogenic strain rClone30 was used to clone the plasmids of prClone30 (pBrClone30). Sequence analysis was used to determine the nucleotide sequence of the GPF gene and compare it to the previously reported GPF gene. The U87-MG human glioblastoma cell line was grown at 37°C with 5% CO_2. To sustain the cells, EMEM (EBSS) was utilized, which contains 2 mM glutamine, 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate (NaP), and 10% fetal bovine serum (FBS).

The digestion system was as follows:

rNDV-GPF	20.0μL (20μg)
Mlu I	10.0μL
Sfi I	10.0μL
K buffer	20.0μL
ddH2O	140.0uL

The mixture was incubated for 2.5 hours, the enzyme reaction system at 37°C. Later, gel digestion products for plastic recycling were collected, yielding roughly 730 bP fragment. Enzyme products (200 µl, 20g) were placed on a 1% agarose and electrophoresed. The target was detected in a UV light irradiation and cut roughly 730 bP of the target gene with rubber cutting knives. According to Axygen Company Gel Extraction Kit description, 300 ml of DE-A solution was added to each 100mg agarose, and then the water bath was set at 70°C until the agarose had entirely melted and shaken 2-3 times for melting the colloidal melt as quickly as possible during the solubilization.

Cell Recovery

U87-MG cells were frozen in liquid nitrogen (obtained from the Chinese Academy of Sciences (Shanghai, China) using normal methods, and then swiftly removed and placed in a 37°C water bath, quickly shaken to room temperature before transferring it to a sterile centrifuge tube. The cell bank used a short tandem repeat polymerase chain reaction to verify the cell lines. The cells were grown in DMEM (Gibco, Carlsbad, CA, USA) containing 4.5 g/L glucose, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco). The cells were kept at 37°C in an incubator that was humidified and contained 5% CO_2.

$TCID_{50}$ Assay for rNDV

When cell density reached around 2-3 $\times 10^5$/ml, the supernatant containing virus was diluted 10 times, and was then made accessible to the holes and incubated with the cells. Each dilution was seeded in 12 holes, each hole was injected with 100 µl. 200 µl of complete Medium was added 1 hour after infection, with normal cells as a reference. Normal cells were two rows (100 µl + 100 µl cell suspension growth medium). Following Reed-method Muench's to compute the two-half cell culture infective dose, we examined and recorded the number of infected wells in each dilution 24, 48, 72, 96 hours later ($TCID_{50}$). Formula for the Reed-Muench method (1938), step two:

Distance Scale = (more than 50% disease rate percentage - 50% lower than the percentage rate of the lesions) / (50% - 50% higher than the percentage rate of the lesions) LgTCID50 is the distance multiplied by a dilution ratio that is higher than the number of lesions at a 50% dilution rate.

MTT Assay for Detecting rNDV-induced Tumor Cell Growth Inhibition

After trypsin digestion, cells were collected at the logarithmic growth phase of human U87-MG cell lines to prepare 1×10^4 cells / ml cell suspension, to seed in 96 well. Added 200 µl cancer cell suspension in each well, incubated at 37°C, 5 percent CO_2 incubator and cultured overnight. Then the medium was removed. Wells were infected for 24 hours, 48 hours, 72 hours, and 96 hours. Later on 20 µl of MTT solution (5 mg/ml) was added to each well and incubated for 4 hours. The medium was then discarded and 150 µl of DMSO was added to each well, and then waited 10 minutes [16]. The OD value of 490 nm wavelength was read by an enzyme-linked immunosorbent assay instrument.The cancer cell growth inhibition ratio was calculated as follows:
(OD control group mean - OD value for the treatment group) / mean OD control 100% inhibition is equal to (mean OD control group - OD value for treatment group) / mean OD control 100 percent.

Amplification of GPF Gene by PCR

According to the GPF gene sequences available on GenBank (accession number KJ668651.1), the following primers and PCR amplification condition were used for GPF gene cloning.
PCR steps are 95℃ for 5min, 94℃ for 1min, 54℃ for 30s, 72℃ for 1min, cycle for 30 times, system are as follows:
10X PCR buffer 2.5uL
dNTPs 2.5uL
Template 2.0uL (10ng)
Prime1 1.0uL(10p mol)
Prime2 1.0uL(10p mol)
Taq enzyme 1.0uL
Deionized water 15uL
Primer sequence for the target gene is as follows
Forward primer: 5'- GCTTATCGATGACTTTATTAG-3'
Reverse primer: 5'- GGATCGCACTCTACCGATT-3'

Cytotoxic Assay

Human brain cancer cell line (U87-MG) was used. Cells were collected at the logarithmic growth phase, after trypsin digestion, to prepare a 1×10^4 cells/ml cell suspension, to be seeded in 96 wells. Then added 200 l of cancer cell suspension in each well, and incubated at 37°C, 5% CO_2 incubator and cultured overnight. The medium was then removed, washed once with PBS, and then divided into 4 groups of experimental wells. 1MOI Wells were infected for 24, 48, 72, and 96 hours before adding 20 l of MTT solution (5 mg/ml), and incubated for 4 hours before discarding the medium, and adding 150µ l of DMSO to each well. After waiting 10 minutes, the enzyme-linked immunosorbent assay apparatus read the results in OD.
% Inhibition is equal to (mean OD control group - OD value of treatment group) / mean OD control 100%.

Statistical Analysis

To identify the impact of various factors on study parameters, the Statistical Analysis System- SAS (2012) application was employed. In this study, a meaningful comparison between percentage (0.05 and 0.01 likelihood) was made using the Chi-square test.

Results

PCR amplification of GPF gene

GFP gene was amplified using condition and primers mentioned earlier as shown in (Figure. 1).

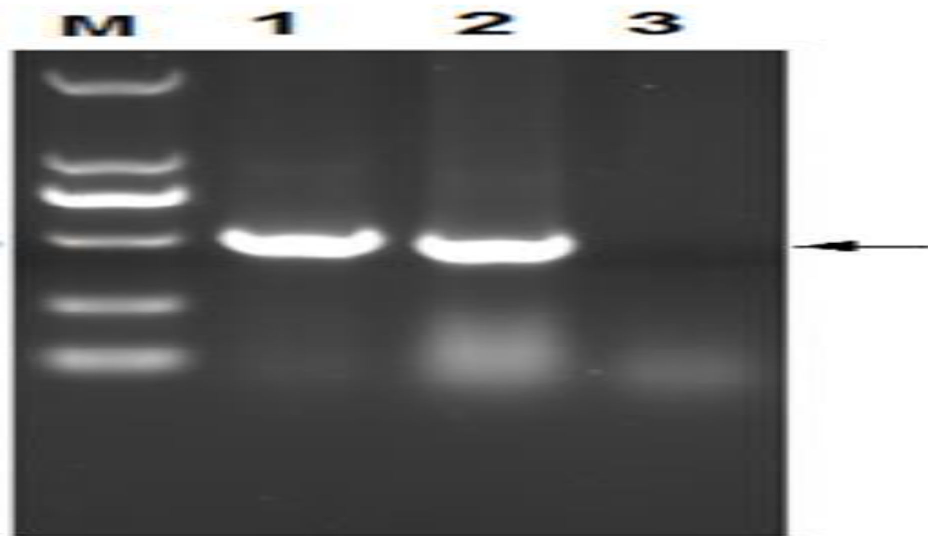

Figure 1: Amplification of GFP gene from plasmid

M：DL2000； 1： GPF ’PCR product; 2： GPF PCR product (molecular weight 730 bp； 3 ： control (each of the two primer pairs amplified with GFP, GFP encoding box and GFP (730 bp) gene. (Agarose concentration 1%, voltage 4–10 V/cm, Typically, 6X concentration (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol) gel loading dye is created, for 90 minutes)

Preparation of PBrClone30 Vector

After being doubly digested with HpaI and MluI to obtain 18000bp fragments, the full-length cDNA plasmid from the NDVClone30 strain was purified and stored at -20°C until usage (Figure 2).

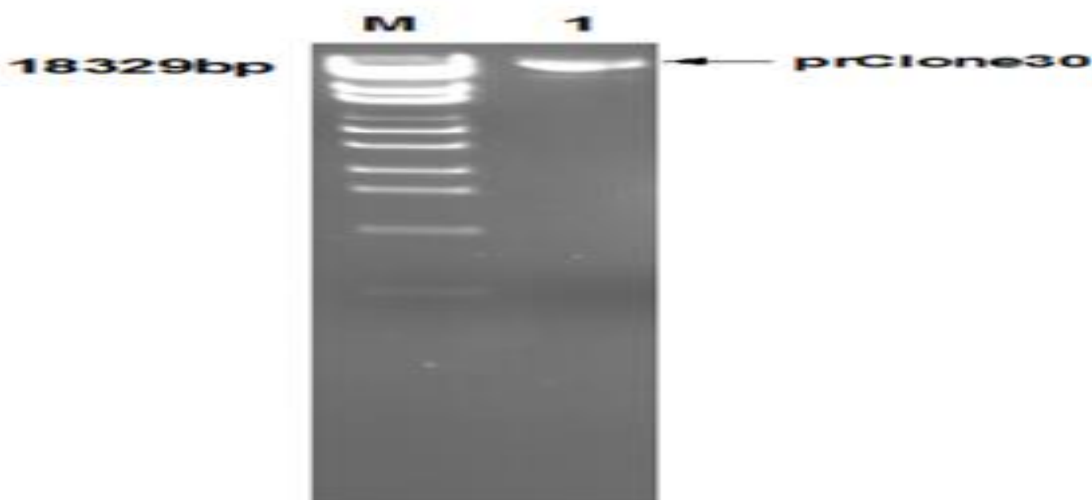

Figure 2: Gel extraction of prClone30 M：λ-EcoT14 1prClone30 vector cycling product

Cellular (*in vitro*) Experiments

MTT test for rNDV-GPF suppression of U87-MG tumor cells

Results revealed that recombinant virus rNDV-GFP in U87-MG cell inhibition rate in a time-dependent relationship between virus infection. The infection time of 24h, 48h, 72h, 96h, rNDV-GFP on U87-MG tumor cell line was 24.97%, 27.97%, 76.2%, 82.03% respectively. Compared with parental rNDV, the tumor inhibitory effect significantly enhanced. Whereas, rNDV showed 14.81%, 15.33%, 22.7% and 22.9% at the same exposure times (Figure 3).

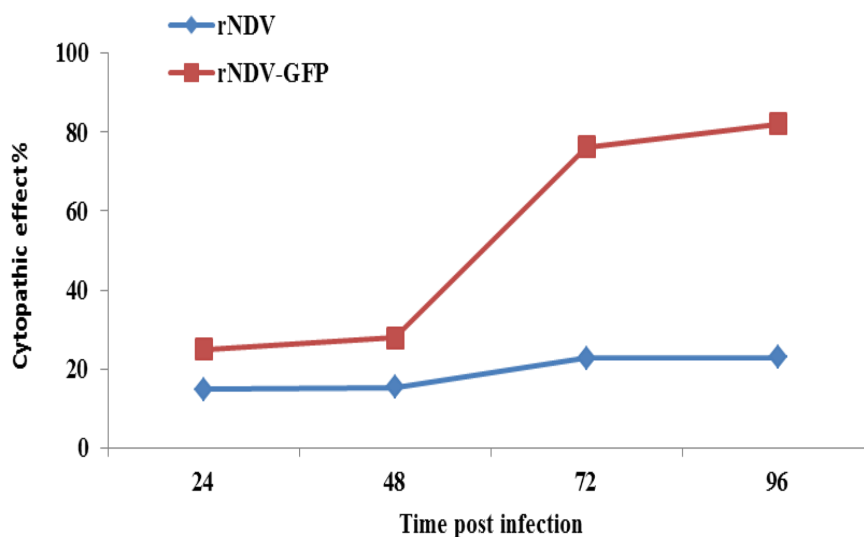

Figure 3: Tumor inhibitory effect of rNDV-GFP assessed by MTT assay on U87-MG cells at 24, 48, 72 and 96 hours after inoculation.

At the logarithmic growth phase of the human glioblastoma cell line UG87-MG were seeded in 6-well plates for overnight to access the culture. At a concentration of 1 MOI. recombinant NDV virus was used in viral infections, 24h, 48h, 72h, 96h later. In addition to the empty viral vector, added to each well 20ul of MTT solution (5mg/ml, ie 0.5% MTT). Later incubated for 4h, and then added 150 µl DMSO solution, and used enzyme-linked immunosorbent assay reader 490 nm OD values. The results showed that recombinant virus rNDV-GFP in UG87-MG cell inhibition rate in a time-dependent relationship between virus infection. When the infection time of 96h, rNDV-GFP on tumor cell line was 78.3 % compared with parental rNDV (22.17%), the tumor inhibitory effect significantly enhanced.

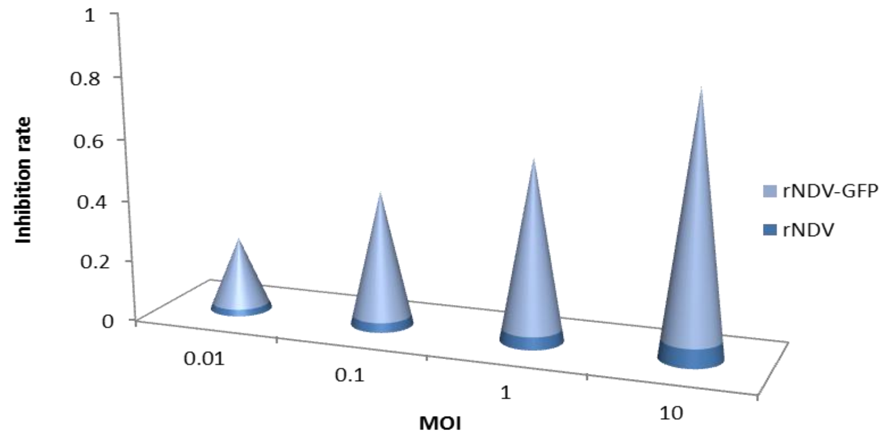

Figure 4: Inhibition rate induced by various MOI of rNDV-GFP compared to empty viral vector strain

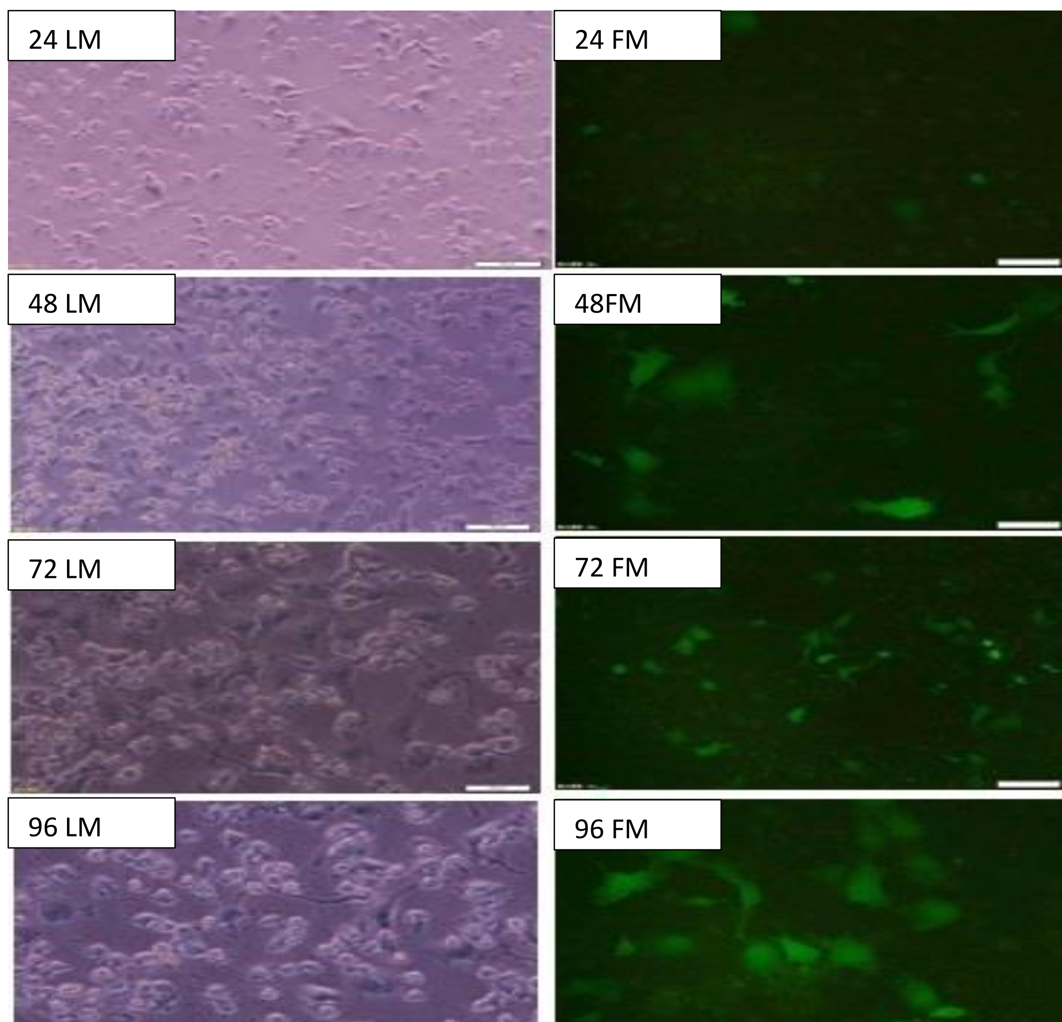

Figure 5: After 24, 48, 72, and 96-hours infections, rNDV-GPF recombinant virus-treated U87-MG cells were examined under a microscope. (LM) Light microscope (FM) Fluorescent magnifying glass (magnification power 10x)

rNDV-GPF recombinant virus-treated U87-MG cells were examined under a microscope at 24, 48, 72, and 96 hours after infection Light microscope (left column) and fluorescent microscope (right column) (Figure 5). After infection for 24 hours, a minor cytopathic effect with some inclusion bodies was noted under a light microscope. Whereas, nothing was noted under a fluorescent microscope. After infection for 48 hours, a minor cytopathic effect with more inclusion bodies was noted under a light microscope. However, very few fluorescent points were noted under a fluorescent microscope. After infection for 72 hours, more cells death was recorded with the fusion (swelling) of some cells. Lastly, after infection for 96 hours, under a light microscope, the total death of the monolayer cells indicated subtotal viral infection annihilation.

Discussion

Immuno stimulation can contribute to the development of post-oncolytic adaptive immunity. NDV has been shown to have both oncolytic and immunostimulatory effects in humans [17]. By selectively reproducing in tumor cells and ignoring healthy cells, it encourages oncolysis [18]. Delivering foreign genes to cancer patients, especially those with solid tumors, is a great use of NDV. Preclinical studies, after utilizing various NDV strains, have produced positive outcomes in a range of murine and human cancer models [19]. The safest strain of avian species (the NDV lentogenic strain rClone30) was used to produce recombinant viruses and to convey the foreign EGFP gene.

According to MTT results, both experimental and control virus groups were able to inhibit U87-MG tumor cells, and as the inhibitory MOI value was raised, this ability grew stronger. Research has shown that 1MOI dosages of the recombinant Newcastle disease virus, rNDV-EGFP, can prevent the growth of NCI-H727 cells for 24, 48, 72, and 96 hours, with the effects of the treatment being time-dependent. The recombinant Newcastle disease virus's capacity to suppress tumor cells differed statistically significantly from the rNDV parent virus, thus indicating that the introduction of a foreign gene expression enhances NDV tumor cell suppression.

Recombinant viruses have the ability to kill cells *in vitro*, however not in the same quantity or at the same MOI. According to these findings, cell death *in vitro* was not a result of foreign gene function but rather viral killing activities.

Fluorescent proteins have quantum yields that can exceed 0.8. Due of these characteristics, fluorescent proteins are exceedingly bright. The ability of GFP to absorb two photons is essential for *in vivo* deep tissue imaging. Another interesting characteristic of fluorescent proteins is that various family members have distinct spectrum characteristics which enable the simultaneous use of a range of multicolored fluorescent proteins for multifunctional *in vivo* imaging. Spectral separation imaging can be used to discriminate between various tones, including auto fluorescence [20].

In other studies, macrophages that expressed GFP absorbed cancer cells that expressed RFP (A useful biological marker known as red fluorescent protein (RFP) can be used to monitor physiological processes, see where proteins are located, and identify transgenic expression in living things). RFP tumor cells eventually retreated and GFP lymphocytes were discovered surrounding them [21, 22].

A stable reporter cell line that uses GFP-tagged cytochrome C to measure its release in response to anti-cancer therapy has been successfully created through prior research. The reporter cell line was examined prior to drug stimulation to validate the distribution and existence of cytochrome CGFP fusion proteins inside mitochondria. The release of cytochrome C GFP was monitored using time-lapse imaging and fluorescence microscopy.
These results also demonstrated that the extrinsic or intrinsic apoptosis pathway is activated by the release of cytochrome C-GFP, following the administration of apoptosis-inducing drugs [23, 24].

In another study, Amoh *et al*. [25] developed a somewhat simple conceivable *in vivo* angiogenesis assay after Gelfoam transplanting in ND-GFP mice. They demonstrated that the Gelfoam is quickly vascularized with GFP-expressing vessels in the presence of an angiogenesis activator. Anti-angiogenesis medications prevent this from occurring. As a result, this new simple assay might find angiogenesis stimulators and inhibitors very rapidly.

Conclusion

Reverse genetics technology was used to successfully insert the GFP gene into the NDV genome. NDVrClone30s smoothly infected tumor cells and expressed foreign gene. Our findings of *in vitro* test of NDVrClone30s demonstrated good suppressive capacity on glioblastoma U78-MG tumor cells.

Conflict of Interest

No conflict of interest declared by the authors.

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