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Ectopic Expression of *DSCR1* in Conjunction with NDV Infection Reduces VEGF and Induces Apoptosis in Lung Cancer A549 Cell Line

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Abstract

Lung cancer is one the most cause of cancer related deaths in the world. Newcastle Disease Virus (NDV) is an oncolytic and targeted self-amplifying agent that is able to replicates and kills only cancer cells. The strongest limitation of NDV in cancer treatments is due to its angiogenesis effect for tumors formation. The NDV mechanism for angiogenesis has not been described. This study was to evaluate for the first time the anti-angiogenesis effect of *DSCR1* lonely and in conjunction with NDV in lung cancer cells. Ectopic expression of *DSCR1* was induced by lentiviral transfection to A549 cell line. Transfected A549 was treated with the effective dose of NDV. Total RNA was extracted and cDNA was synthesized to detect *DSCR1*, VEGF, PCNA, Bax, and *Bcl2* genes expressions compare to HPRT expression as a housekeeping gene using SYBR green Real-time PCR assay. Over expression of VEGF was detected in RNA level for the first time in NDV treated cells. Significant fold changes of PCNA, Bax, and *Bcl2* showed that NDV used mitochondrial pathway for induction of cell death. In LV*DSCR1*$^{+}$ treated cancer cells, *DSCR1* ectopic expression acts as an anti-angiogenesis factor, by reducing VEGF and inhibiting angiogenesis signaling pathway. Furthermore, the apoptotic effect for *DSCR1* gene was shown for the first time in this study in lung cancer cells. A non-significant change in Bax and *Bcl2* gene expression has suggested a lack of intracellular apoptosis pathway activity following *DSCR1* over expression in cancer cells. In LVDSCR^{+} + NDV treated cancer cells, over expression of *DSCR1* could modulate angiogenesis effect of NDV by VEGF reduction and accelerate apoptosis induction in cancer cells as well. This finding for the first time suggests the benefit and potential usefulness in simultaneous application of oncolytic viruses and gene therapy in cancer treatment.

Keywords: Newcastle disease virus; Lung cancer; Apoptosis; Anti-angiogenesis

Introduction

Cancer is a devastating life-threatening disease arises from both genetic and environmental factors and caused by defects in the signaling mechanisms that control cell proliferation and apoptosis. Molecular mechanism defects that regulate cellular growth and death, allowing tumor cells to have uncontrolled division and the ability to metastasis [1,2].

Lung cancer is the second newly diagnosed cancer that accounts for the most related cancer-deaths in both genders in the world [3,4]. The primary lung cancer can grow locally or cause metastasis in other tissues [4]. The most frequent lung cancers is Non-Small Cell Lung Cancer (NSCLC) that consist of 80% of all kinds of lung cancer [5-7]. Despite of conventional therapies and novel progressed techniques in diagnosis and therapy, breast cancer still a devastating disease worldwide, therefore it has provided an incentive for searching of new more effective and with less side effect therapeutics. The purpose of cancer therapy is to destroy malignant cells without causing destruction of normal cells. One of the novel therapeutic that gives much hopes is using of oncolytic viruses that can selectively replicate in cancer cells [8].

Newcastle Disease Virus (NDV) as a member of oncolytic viruses (OVs) is also known as a member of genus Avulavirus within the family *Paramyxoviridae* [9,10]. The NDV is a negative-sense single stranded RNA virus that has a genome with a length of 15 kb, including six genes encoding at least eight proteins by alternative splicing: structural proteins (NP, P, M, F, HN, L) and non-structural proteins (V and W) [11-14]. NDV is targeted self-amplifying agent that is able to replicates and kills only cancer cells [11,12,15,16] by fuses into and replicates within the infected cells' cytoplasm independent of cell proliferation [17]. NDV exert anti-tumor cytotoxic effect based on host's anti-tumor immunity stimulation and oncolytic activity [18]. NDV can replicates in cells with deficient interferon pathway [17,18]. Their selectivity for neoplastic cells are 10000 fold higher than normal cells and are relatively safe for normal cells. The significant feature of NDV is its ability to selectively replicate in cancer cells. Virus replication is limited to the cell cytoplasm without integration or recombination. Apoptosis naturally occurs in tissues in order to maintain the survival of the cell population. According to the previous researches, NDV induced cell death stems from apoptotic reaction and probably it leads to oncolysis natural feature of NDV. Possessing various unique properties considers NDV a great agent for cancer therapy; for instance: good cell binding properties, selectively replication in tumor cell cytoplasm, rather safe and also the property of acting as an adjuvant. It has been suggested that differences in innate immune responses between normal and tumor cells cause to the selectivity of NDV and other oncolytic viruses to cancer cells. The replication of NDV in cancer cells enhances due to the type I interferon (IFN) response defection of these cells and hence destruction has been increased. IFN production inhibits viral replication when normal cells have been infected. NDV infects the host cells and then replicates itself similar to other viruses but interesting point is that NDV can replicate itself more quickly (up to 10000 times) in human neoplastic cells than in normal cells and cause oncolytic effects. According to the primary researches NDV induced apoptosis through IFN-α, TNF-α and TRAIL

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signal molecules. TNF-α and TRAIL are apoptosis inducers and meanwhile they display an antitumor activity [19-22].

The biggest limitation of using NDV for cancer treatments is the increasing level of angiogenesis in tumors. Cells that have been treated with oncolytic viruses, due to the destruction of tissue homeostasis conditions, disturb the balance between angiogenesis and anti-angiogenesis factors. These balance changes switch on the angiogenesis pathway and provides the conditions for the formation of new blood vessels [23,24]. In order to solve this problem has had many strategies were examined [23]. To date, some anti-angiogenesis genes were transfected to cancer cells by engineered oncolytic viruses [25-28].

Gene therapy is the leading way to treat cancer in many investigational models. This method is based upon the recognition of tumor suppressor or suicide proteins encoding genes and transferring them into tumor cells by means of a genetic vector. Viruses are mostly chosen as vehicles for gene therapy because they have evolved very effective mechanisms of gene transfer and expression. Angiogenesis has vital role to tumor progression therefore it can be a significant target for anticancer treatment. Vascular endothelial growth factor-A (VEGFA; also referred to as VEGF) is one of the critical factors for solid tumor growth through stimulating of angiogenesis. It is suggested that the reduction occurrence in many cancer types in Down syndrome patients can be due to the high expression of some genes on chromosome-21 such as Down syndrome critical region-1(*DSCR1*) encoding a protein which can suppress (VEGF)-mediated angiogenic signaling *via* the calcineurin pathway. *DSCR1* is a gene situated in the 21q22.1-q22.2 region of human chromosome 21 [29,30]. The *DSCR1* gene consists of seven exons [31,32]. Commonly, exons 1–4 can be alternatively spliced to produce four different mRNA isoforms [33] which have different N-terminal but identical at the C-terminal domains [29]. *DSCR1* (RCAN1) is a calcineurin binding protein [31,34] which is characterized as an inhibitor of the calcineurin phosphatase [35] by a negative feedback loop formation in cells regulating calcineurin-dependent dephosphorylation [36]. *DSCR1* overexpression blocks NFAT transcriptional activity and down regulates COX2 and VEGF genes [37]. *DSCR1*-4 is capable of inhibiting the formation of new blood vessels [38] thus it was used as an anti-angiogenic agent in this study and gene transfer to cells was performed *via* lentiviral vectors.

Lentiviruses are a large member of Complex Retroviridae diploid single-stranded positive sense RNA viruses [39,40]. Due to their ability to infect a wide variety of dividing and non-dividing cells, they have gained much attention as gene delivery tools over the past decade [39-41]. LVs can encompass large transgenes as large as about 10 kilobases (kb) [42]. These vectors, such as those based on human immunodeficiency virus 1 (HIV-1), transduce a variety of cell types, including embryonic and adult stem cells, and have been suggested as candidate vectors for both *in vivo* and *ex vivo* gene therapy applications. However, integration of viral genome into host cell genome may induce some adverse effects, such as insertional mutagenesis; this has been highlighted by the induction of malignancy in mouse models and development of leukemia in five patients in two clinical gene therapy trials. Improving safety and efficiency of LV has been achieved, for example, by modifications of packaging cassettes on the virus integrase gene or on other regions of virus genome. This virus, which is called the nonintegrated LV, cannot integrate into the host genome.

In this study, for the first time simultaneous using of NDV and DSCR 1 gene as a reducing agent of angiogenesis were examined. Both integrating and non-integrating LVs were used as viral vectors for gene transfer to investigate effects of NDV and ectopic *DSCR1* gene overexpression on apoptosis and expression of angiogenesis markers in A549 cells.

Materials and Methods

Chick embryo fibroblast cell culture

A 9–11 days-old embryonated chicken eggs were used to prepare Chicken Embryonic Fibroblast (CEF) monolayer [43]. Briefly the air sac of eggs was marked and embryos were aseptically removed from the eggs, washed in phosphate buffered saline (PBS) and head, legs, wings and viscera were cut and discarded. The rest of the body portions were washed by PBS and digested by collagenase solution (Sigma) at a final concentration of 0.075% for 30 minutes. Digested tissues were centrifuged at 400 g for 10 min. Pellets were resuspended and cultured at a density of 2×10^6 cells/mL in 25 cm^2 plastic culture flask in high glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) containing 50% fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin and incubated at 37°C in CO_2 incubator with 5% CO_2 for 24 h. Non adherent cells were washed away with PBS and CEFCs were cultured in DMEM containing 20% FBS and antibiotics. Upon 80% confluency, the cells were harvested using 0.25% Trypsin-0.02% EDTA for 1-2 min at 37°C and were kept frozen in liquid nitrogen for later use.

Newcastle Disease Virus (NDV)

A commercial vaccine (*LaSota* strain) was obtained from poultry vaccine production department, Razi vaccine and serum research institute, Tehran, Iran and kept at 4°C till used. A vial of lyophilized vaccine (2000 dose/vial) was reconstituted with 2 ml sterile phosphate buffered saline (PBS), and stored at -70°C in 0.2 ml aliquots.

Adaptation and propagation of NDV to *in vitro* conditions

For virus adaptation, medium of the confluent CEFCs monolayer plate was discarded and cells were gently washed with PBS. 100 µl of virus from stocks stored at -70°C was added to the CEFCs monolayer plate. PBS was added to other confluent CEFCs monolayer as negative control. The plate was incubated at 37°C in an atmosphere of 5% CO_2 and 80% relative humidity for 1 hr. 4 ml DMEM growth medium supplemented with 3% FBS, 100 U/mL penicillin/streptomycin was added to the confluent monolayer cells and kept in CO_2 incubator at 37°C with 5% CO_2 for 3 days. After 3 days plate was incubated in -20°C until freeze-thaw cycles rapidly for cell explosion and virus releasing into medium. The medium was filtered with 0.22 µm filter. The process repeated with 100 µl of this adapted virus and Subsequent passage was done as well for virus propagation. Medium containing virus stored at -70 °C. Infected monolayers were checked twice a day under an inverted microscope to observe any changes compare to non-infected cells.

Cancer and normal cell lines

The human NSCLC A549 lung cancer cell line and human fibroblasts HU02 were purchased from National Center of Genetic and Biological Reserves (Tehran, Iran). Cell line A549 was cultured using Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F-12, GIBCO) and cell line HU02 was cultured in DMEM (GIBCO) both supplemented with 10% fetal bovine serum (FBS, GIBCO).

Construction of integrated and non-integrated lentiviruses *DSCR1*$^+$ particles

The LV *DSCR1*$^+$ virus particles were constructed in transfected HEK

293T cells. HEK 293T cells were transfected with plox-MD2 (pmd2G) plasmid which contained G protein of the vesicular stomatitis virus (InvivoGen), pLV-HELP expression vector encoding the non-integrate packaging proteins Gag- Pol- Rev- Tat (InvivoGen), psPAX, integrated packaging plasmid (InvivoGen) and finally transfer vector EX-S0552-LV-105-B harboring *DSCR1* gene(GeneCopia). The culture medium was discarded 14 hour after transfection and fresh culture medium was added to the culture flask. 24 hours after changing the medium, lenti virus particles were released into culture medium. Lenti viruses were collected and concentrated by MILLIPORE falcons to be kept in -70°C.

Ectopic expression of *DSCR1* gene in HEK293T

HEK 293T cells were infected with LV *DSCR1*$^+$. Total RNA was extracted 24 hour after infection (RNX plus, Fermentas, EU) and cDNA synthesis was performed using cDNA synthesis kit and Oligo-(dT) primers (Fermentas, EU). Real time-PCR was performed in a 15 µl reaction volume by designed primers for *DSCR1* gene (Table 1).

MTT Assay to determine effective concentrations of NDV and LV-*DSCR1*$^+$

MTT assay was performed to determine the effective concentrations for NDV and LV-*DSCR1*$^+$ concentrations in A549 and HU02. These cell lines were seeded in three separate 96-well microtiter plates (7000 cell/well) in three repeat wells and incubated for 24 hours. NDV was added to A549 (0- 0.01- 0.1- 1- 10 µl) and HU02 (0- 0.1- 0.5- 1- 2- 4- 8- 16- 32 µl). They were infected with different volumes of Lenti viruses (LV Int. DSCR$^+$ (0- 1- 2- 3- 4- 5- 6-7 µl for A549 and 0- 1- 3- 6- 12- 24- 48 µl for HU02 cell lines) and LV Non Int. DSCR$^+$ (0- 0.1- 0.2- 0.4- 0.8- 1.6- 3.2 µl for A549 and 0- 0.8- 1.6- 3.2- 6.4- 12.8- 25.6 µl for HU02 cell lines) in two separate 96-well microtiter plates. MTT assay was done on Sigma Protocol. The plates were shaken gently and optical density (OD) was measured at 580 nm to determine the relative cell viability.

Apoptotic and angiogenesis effects of NDV and LV-*DSCR1*$^+$ in A549 cell line

A549 cell was cultured in two 24 wells plates 3.5×10^4 A549 cells/well for 24 hours to become 70% confluence in three repeat wells and one well non-treated control for each treatment in five different treatment conditions. First one treated with NDV (0.5 µl) alone, and second and third treatments with integrated (30 µl) and non-integrated LV-*DSCR1*$^+$ (4 µl) and forth and five conditions with simultaneous treatment NDV+ integrated LV-*DSCR1*$^+$ (0.5 µl NDV/ 30 µl LV-*DSCR1*$^+$) and NDV + Non-integrated LV-*DSCR1*$^+$ (0.5 µl NDV/ 4 µl LV-*DSCR1*+). First pale was harvested after 24 hours and second plate was harvested after 72 hours of treatment. Cells from all wells were harvested after treatment, total RNA was extracted (RNX plus, Fermentas, EU) and cDNA synthesis was performed using cDNA synthesis kit and Oligo-(dT) primers (Fermentas, EU).

Real Time PCR

To determine expressions of genes involve in apoptosis and angiogenesis after five different treatments with NDV and LV-*DSCR1*$^+$, all samples were tested for *DSCR1*, VEGF, PCNA, Bax, *Bcl2*, expressions of compare to HPRT expression as a housekeeping gene with SYBR green Real-time PCR assay. The SYBR green reaction was obtained from BIONEER, Korea, 2X Greenstar qPCR Master Mix kit. The Real-time PCR was performed in 42 cycles each cycle 95°C for30 sec, and 62°C for 60 sec with initial 95°C for 5 min.

After normalizing the data, the variation rates of the expression of the genes were calculated using the comparative C_t ($2^{-\Delta\Delta CT}$) method and statistical analysis was then performed by independent sample T-Test utilizing SPSS version 16.0. Statistical significance was determined as p *value<0.05*.

Investigation of apoptosis induction in cultured cells after viral infection using flowcytometry

Induction of apoptosis in A549 cell line was detected using annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Biolegend, San Diego, CA). According to the manufacturer's instruction, Cancer cells were plated in 6-well tissue culture plate at a concentration of 100000 cells per well in 2 ml of culture media. Cells were infected by NDV+ integrated LV-*DSCR1*$^+$ (the best treatment for induction of apoptosis on cancer cells). After 72 hrs, apoptosis were investigated in the infected and non-infected cells were harvested and washed with cold PBS buffer, pelted and resuspended in kit binding buffer. They were treated with annexin V-FITC conjugate and incubated in dark place at RT for 15 mints. After antibody conjugation and performing PI staining (5 µg/ml) cells were analyzed for induction of apoptosis by flow cytometry equipment (Accuri® C6 Flow Cytometer, AnnArbor, MI) within 1 hrs. The flow cytometry data obtained from a minimum of 10000 cells per sample was analysed using BD Accuri C6 Flow software.

Results

Expression analysis of *DSCR1* in HEK 293

Virus particles LV *DSCR1* was added medium of HEK293 culture. After 24 hours, total RNA was extracted and cDNA was synthesized. *DSCR1* expression was quantified by real-time PCR in transformed and untransformed cultures compared to HPRT housekeeping gene expressions. Four times ectopic expression of *DSCR1* was detected in transformed HEK293 compared to untransformed culture (Figure 1).

MTT assay for NDV and LV *DSCR1*$^+$ effective dose

The effective and lethal doses for NDV and LV *DSCR1* were determined on A549 and fibroblast cells using MTT assay. The optimum volume required for killing 50% of A549 cells was 0.1 µl per 7000 A549 cells. The IC_{50} value for fibroblast cells was 0.5 µl per7000 fibroblasts (Figure 2). Therapeutic index of NDV was calculated 5. For integrated LV *DSCR1*, ED50 and LD50 values were 6 µl/7000 A549 and 48 µl/7000 fibroblast cells lines respectively (Figure 3), whereas they were 0.8 µl/7000 A549 cells and 6.4 µl/7000 fibroblast cells for LV *DSCR1* Non-Integrated respectively (Figure 3). Therapeutic index for Int. and Non Int. LV *DSCR1*+ was calculated as both 8 which is safer than 5 obtained for NDV.

Expressions of *DSCR1*, in LV *DSCR1*$^+$ and NDV

DSCR1 expression is significantly increased in A549 cells transfected with integrated and non integrated LV-*DSCR1*$^+$, 7.3 (p=0.02) and 6.8 folds (p=0.04) compared to non-transfected cells. For NDV+ LV *DSCR1*$^{+\ in}$ integrated and non integrated LV-*DSCR1*$^+$, 9.8 (p=0.039) and 12.6 folds (p=0.036) compared to non-transfected cells after 24 hours (Figure 4).

Expression of VEGF angiogenesis marker

Expression of VEGF is significantly decreased in A549 cells transfected with integrated and non integrated LV-*DSCR1*$^+$, 5.6 (p=0.016) and 2.6 folds (p=0.021) compared to non-transfected cells. For NDV+ LV *DSCR1*$^+$ in integrated and non integrated LV-*DSCR1*$^+$,

3.3 (p=0.04) and 1.5 folds (p=0.041) compared to non-transfected cells after 24 hours. Expression of VEGF in NDV transfected A549 cells has increased significantly equal to 2.3 fold (p=0.023) (Figure 5).

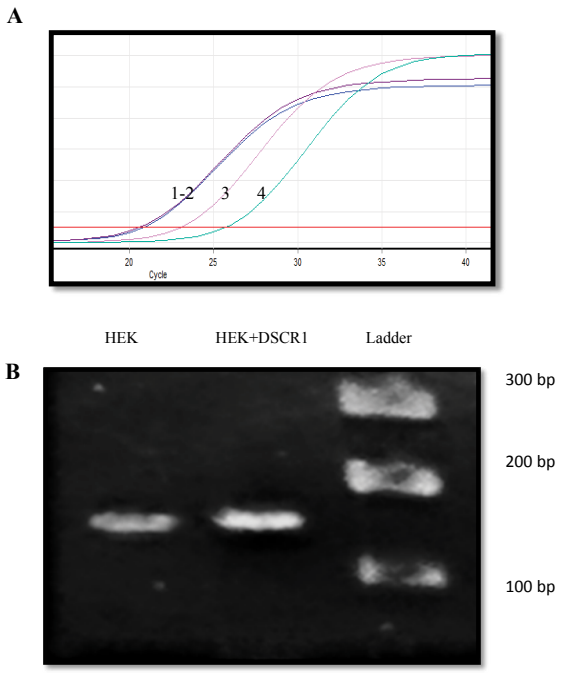

Figure 1: Real-time PCR SYBR of DSCR1 and HPRT genes from infected and non-infected HEK 293 cells. **A**: Standard curves of both samples and their Cts. (1: HEK+DSCR1 HPRT/ 2: HEK HPRT/ 3: HEK+DSCR1 DSCR1/ 4: HEK DSCR1) **B**: PCR products bands on 2% agarose gel (140 bp for DSCR1 PCR products).

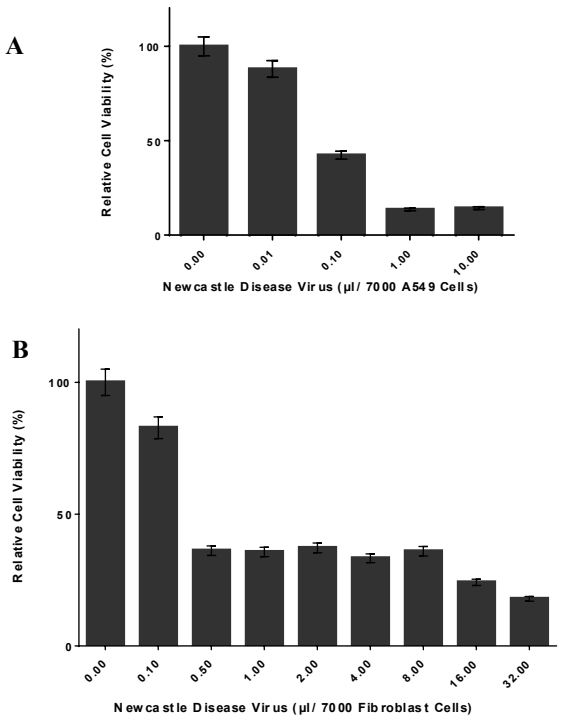

Figure 2: Cytolytic activity of NDV on A549 lung cancer cell line **(A)** and fibroblast cells **(B)**.

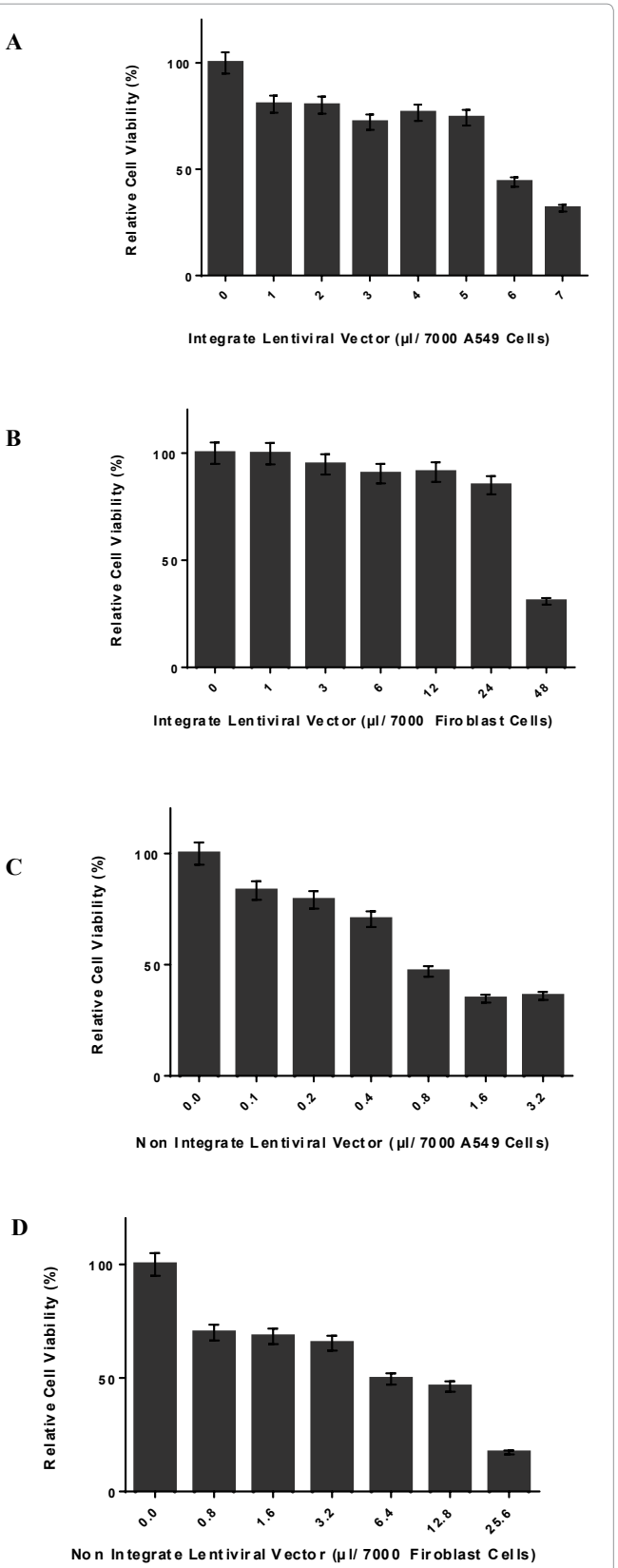

Figure 3: DSCR1 overexpression mediated by lentiviral vectors inhibited cancer cell growth. Cell viability of A549 cells (A) and fibroblasts (B) 3 days after LV Int. infection measured by MTT assay. The effects of LV Non-Int. on A549 cells (C) and fibroblasts (D).

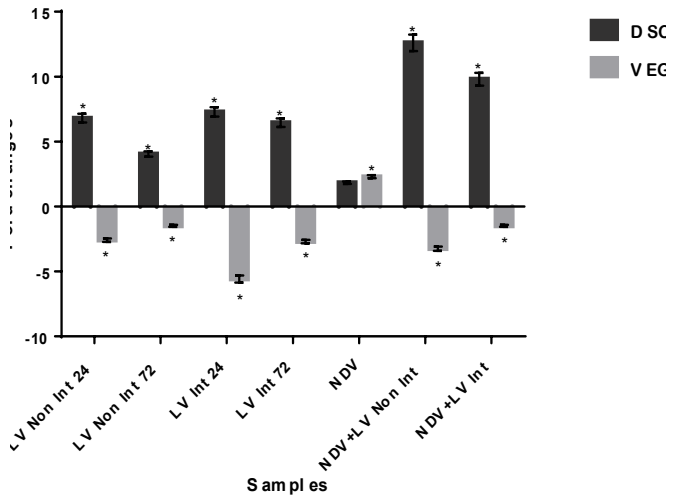

Figure 4: Changes in the expression of DSCR1 and VEGF genes within all samples. Statistical analyses were done by data normalization using HPRT housekeeping gene (*p=0.05*).

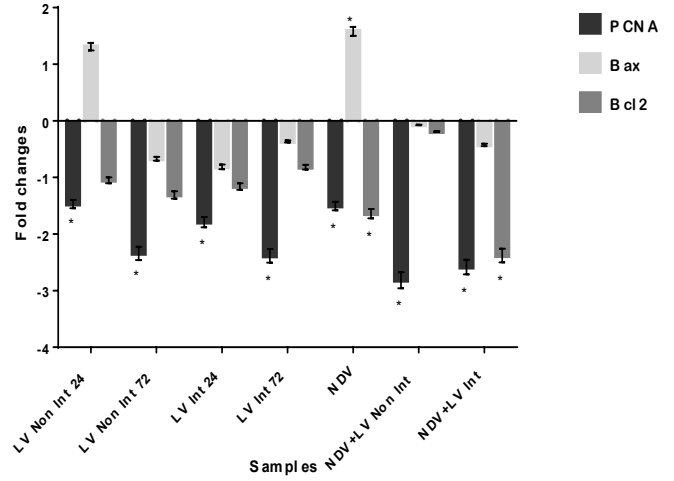

Figure 5: Expression level of PCNA, Bax and Bcl_2 genes expression in all treated samples. Relative gene expressions of PCNA, Bax and Bcl2 were investigated in all treated samples. All PCR products were verified by electrophoresis and sequencing and relative gene expression data were analyzed using $2^{-\Delta\Delta Ct}$ method. Increased and decreased expression of these genes in treated cells is meaningful (*p<0.05*).

Genes	Sequence (5'-3')	Size (bp)
HPRT	CCCTGGCGTCGTGATTAGTG	163
	GCCTCCCATCTCCTTCATCA	
DSCR1	AACAAGTGGAAGATGCGAC	140
	AACAAGTGGAAGATGCGAC	
VEGF	AACTTTCTGCTGTCTTGGGTG	179
	AACTTTCTGCTGTCTTGGGTG	
PCNA	AGCACCAAACCAGGAGAAAGT	191
	TCACTCCGTCTTTTGCACAG	
Bax	CTGACATGTTTTCTGACGGCAA	140
	GAAGTCCAATGTCCAGCCCA	
Bcl_2	ATTGTGGCCTTCTTTGAGTTCG	150
	ATCCCAGCCTCCGTTATCCT	

Table 1: Sequences of primers used in real-time PCR.

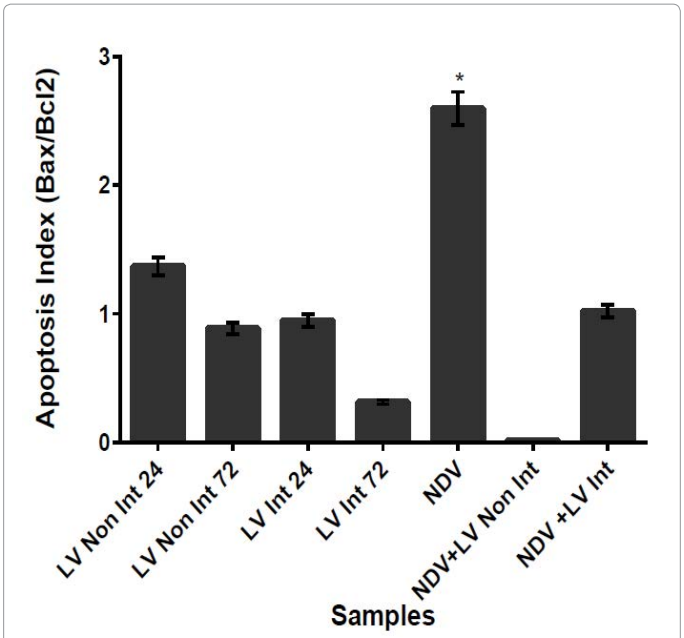

Figure 6: Apoptosis indexes for all treated samples (*p<0.05*)

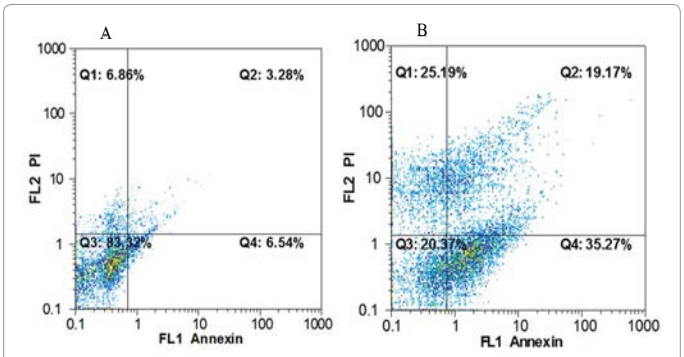

Figure 7: Investigation of apoptosis by flowcytometry assay using Annexin V-FITC/PI. NDV+ integrated LV. DSCR1+ induce apoptosis in infected cancer cells (B) in comparison to A) non-infected cancer cells.

Apoptotic effects of *DSCR1* gene and NDV viruses

After 24 hours expression of PCNA gene an indirect apoptosis marker was significantly decrease 1.8 folds in LV Int. (p=0.007), 1.75 folds in LV Non-Int. (p=0.004), 1.5 folds in NDV alone . (p=0.012), 2.6 folds in NDV+LV Int. . (p=0.008) and 2.8 fold in NDV+LV Non Int. (p=0.001).

Expressions of a pro-apoptotic marker Bax and an anti-apoptotic marker *Bcl2* involved in mitochondrial apoptosis pathway were compared after treatments to investigate the effects of *DSCR1* ectopic expression and NDV infection in A549 cells. Results showed a significant 1.58 folds (p=0.04) increased expression of Bax and 1.6 folds (p=0.04) significant decreased the expression of *Bcl2* gene only in NDV infected cells. Non significant expression changes were observed in other treatment conditions (Figure 6). Flowcytometry assay using Annexin V-FITC/PI has also confirmed the results of real time PCR (Figure 7).

Discussion

Cancer is a major cause of mortality in humans. In spite of current

immense progress cancer remains a destructive disease worldwide. Considering of prevalent and high amount of lethality of this disease and also the limited efficacy and toxicities of common treatments such as chemo- and radiotherapies there will need to use effective combination therapy to suppress such a complex disease. One of this combined novel anti-cancer approaches is applying oncolytic viruses such as HSV vectors and NDV viruses in a combined cancer treatment approach as well as gene therapy for the destruction of tumor cells. In this investigation NDV were used as an oncolytic agent with apoptotic property in addition to *DSCR1* gene as an anti-angiogenic factor.

Accordingly, it is important to find a cancer therapy with high efficacy selectivity killing malignant cells with fewer obstacles. Based on many studies, NDV replicates selectively in human cancerous cells while it spars normal cells and cause activate cell death program in tumor cells. The ability of NDV to replicate selectivity in cancer cells was previously known [10,16,21,44]. NDV could induce apoptosis in cancer cells by both intracellular and extracellular pathways [16,21,45]. One limitation for using NDV virus as oncolytic agent is related to its angiogenesis properties in tumor tissues [23]. Subsequently several different NDV strains including 73-T, Ulster, MTH-68, Italien, Hickman, PV701, HUJ and LaSota were used against a variety of human neoplasms [46]. The first report of the application of NDV to treat human cancers was in the early 1950s, in that research adenovirus and NDV were injected directly into uterine carcinoma and partial necrosis and sloughing was observed, but in follow regrowth was happened [47]. Othman et al. showed that NDV strain AF2240 has the ability to infect MCF7 cell line resulting in apoptosis [48]. NDV-LaSota strain was shown to be more susceptible to apoptosis resistant cells [49]. Another study was showed that MCF7-CR cells are susceptible to NDV infection which highlight the importance of surviving in the oncolytic effects of NDV in chemo resistant cancer cells [50]. Mansour et al. demonstrated that NDV-HUJ-induced oncolysis in chemoresistant malignant melanoma specimens [49]. Several solid tumors including prostate, epidermoid, colon, large cell lung, breast and low passage colon carcinoma xenografts treated with the strain 73-T regressed effectively [51]. Other study suggested that use of NDV strains AF2240 and V4-UPM as an oncolytic agent of WEHI-3B myelomoncytic leukemia cells and its cytotoxicity increased with increasing titers of the virus [52]. NDV AF2240 strain induced apoptosis in MCF-7 cells was most probably mediated by HN protein expression alone [53].

In this study to improve the oncolytic effect of NDV virus, its angiogenesis and apoptotic effects were studied in conjunction with ectopic expression of *DSCR1* gene by lentiviral vector in lung cancer cells A549. *DSCR1* play its anti-angiogenesis role due to interruption in angiogenesis signaling pathways by down regulating VEGF gene expression [31,54]. Our result has indicated for the first time significant increase in VEGF gene expression (p=0.023) up to 2.3 fold in A549 cancer cells after NDV infection alone. However, simultaneously infections with NDV and integrated and Nonintegrated LV-*DSCR1*+ reduce VEGF expressions significantly by 1.5 (p=0.04) and 3.25 (p=0.04) times respectively in comparison to non-treated A549 cancer cells. Increasing level of *DSCR1* gene expression could block the VEGF-Calcineurin-NFAT pathway in endothelial cells *in vitro* [55]. Shine et al. observed that a single extra copy of *DSCR1* gene could suppress lung tumor angiogenesis in mouse model developing human adenocarcinoma [56]. Our data on integrated and non-integrated LV-*DSCR1*+ indicated its 7 folds after 24 his however after 72 hrs its expression remained 6.4 in integrated and decreases to 4 folds in nonintegrated. It means a significant decrease in the expression of *DSCR1*in on integrated LV after 72 hrs. *DSCR1* expression simultaneous infection of NDV + LV-*DSCR1* increased significantly to a higher level 10 and 12 fold for integrated and non-integrated LV respectively.

PCNA decreased 1.5 fold (p=0.0126) and Bax pro apoptotic gene increased 1.6 fold (p=0.04) and *Bcl2* anti-apoptotic gene decreased 1.6 fold (p=0.04) significantly. Moreover, apoptosis index for NDV sample was about 2.6 and significant. This result showed that NDV used mitochondrial pathway for induction of cell death (Figure 6).

When *DSCR1* increase in neural and primary cells, total ROS accumulate and similarly decrease cell viability in response to high level of H_2O_2 [30]. This is the first study to investigate apoptotic effect of *DSCR1* ectopic over expression on lung cancer cells. Over expression of *DSCR1* has been suggested to induce apoptotic activation through caspase 9 and 3 pathway in neurons [57]. Activation of caspase 3 reduces PCNA gene expression an indirect apoptosis marker to induce cell death. After *DSCR1* ectopic expression, PCNA was decreased significantly but variations in Bax and *Bcl2*, two mitochondrial apoptosis markers, were not to be significant. This means that *DSCR1* does not use apoptotic intracellular pathway (Figure 6).

Baek et al. showed that a transgenic copy of *DSCR1* is sufficient to confer significant suppression of tumor growth in mice by dampening VEGF- calcineurin signaling [58]. VEGF-mediated angiogenesis is central to tumor progression and has become a therapeutic target for anticancer treatment. Minami et al. demonstrated that overexpression of *DSCR1* inhibited lung metastases in mouse models. *DSCR1* is directly blocking calcineurin phosphatase function and deletion of *Dscr1* in a transgenic mouse model leads to hyperactivation of calcineurin-NFAT signaling. In this study for the first time *DSCR1* gene was transferred to lung cancer cells using non-integrated lentiviral vectors. After cell infection the increasing in the *DSCR1* expression and the VEGF reduction were observed by real time RT-PCR. Decreasing of VEGF expression caused to the reduction in angiogenesis that is effective in tumor growth inhibition and cancer treatment. Lentiviral vectors can deliver transgenes to a wide variety of dividing and nondividing cells and maintain stable long-term transgene expression. This is the first study to investigate the application of non-integrated lentiviruses harboring *DSCR1* in inhibition of angiogenesis in lung cancer cells in the culture.

Su et al. shown that Anti-angiogenesis gene therapy combining with viral oncolytic therapy provides new slights for human cancer therapy. Antiangiogenesis effect due to gene expression and the selective viral replication and oncolysis of cancer cells, suggesting that the combination of gene therapy and virotherapy can provide a new therapeutic strategy to increase the probable of cancer treatment. In this approach for the first time we investigated the apoptotic effects on NDV virus in combination with the anti angiogenic effect of *DSCR1* gene *via* lenitviruse in lung cancer cells. In current study LV-*DSCR1*$^+$ and NDV-Lasota were used simultaneously to infection of A549 cancer cells for intensify their anti-cancer properties. Based on our results, PCNA decreased 1.5 fold (p=0.0126) and Bax pro apoptotic gene increased 1.6 fold (p=0.04) and *Bcl2* anti-apoptotic gene decreased 1.6 fold (p=0.04) significantly. Moreover, apoptosis index for NDV sample was about 2.6 and significant. This result showed that NDV used mitochondrial pathway for induction of cell death.

Conclusion

Based on these results, we can conclude that the higher levels of apoptosis occurred in cancer cells in co-infection by NDV and lentiviruses. The higher levels of apoptosis in cancer cells have occurred is result of high proliferation and cytotoxicity in cancer cells compared

to normal cells infected by NDV resulting in elevated levels of apoptosis in both virus inoculum to inoculate NDV virus alone caused more damage to cell membranes caused by the simultaneous arrival of two NDV virus and lentiviral the simultaneous inoculation. In summary, results demonstrated that combination of NDV virus with LV-*DSCR1*$^{+}$ can be efficient for lung cancer treatment.

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