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Gene Expression Profiling in Apoptotic MCF-7 Cells Infected with Newcastle Disease Virus

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Abstract: Newcastle disease virus (NDV), an avian paramyxovirus, induces apoptosis in a variety of human malignant cells. A Multiplex Gene Expression Kit was used to measure the relative expression of 21 genes at the mRNA level in MCF-7 cells infected with a velogenic NDV strain AF2240. The results showed that virus changed the expression level of most of the genes involved in tumor progression, cell cycle regulation, cell growth, differentiation, apoptosis, cancer suppression, hormone response and DNA damage in the MCF-7 cells as measured by RT-PCR and capillary electrophoresis.

Key words: Newcastle disease virus · Oncolytic viruses · MCF-7 · PUMA · Bcl-2 · ESR1-α · MYBL2, TGFβ3 · GeXP · Gene Expression

INTRODUCTION

Oncolytic viruses (OVs) are able to selectively kill tumor cells. This property of OVs has allowed them to be used in an innovative approach to treat human cancers. With this view, different strains of non-engineered NDV have been used as experimental oncolytic agents for more than 30 years [1]. NDV has a single stranded negative-sense RNA comprising 15,186 nucleotides in length which encodes at least eight proteins. The envelope of NDV contains two glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion (F) proteins; the HN protein mediates attachment of the virus to the cell and the F protein fuses the viral envelope with cellular membranes [2]. Similar to other paramyxoviruses, through RNA editing NDV produces two additional proteins, V and W, from the *P* gene. The NDV transcriptive complex is made up of the nucleoprotein (NP), phosphoprotein (P) and the large protein as the viral RNA polymerase. The NDV P protein interacts with both assembled and unassembled NP to facilitate the viral replication and transcription, respectively [3].

NDV exerts its oncolytic property through direct induction of apoptosis; a multipathway programmed cell death, with two pathways: extrinsic and intrinsic. Both pathways are activated in NDV-induced apoptosis. The extrinsic pathway is triggered by death receptors (such as Fas ligand) engagement, which initiates the activation of caspase-8. The activated caspase-8 stimulates the activation of caspase-3 and the release of cytochrome c by the mitochondria leading to the degradation of cellular proteins. The intrinsic pathway occurs when various apoptotic stimuli trigger the release of pro-apoptotic proteins and cytochrome c from the mitochondria (independently of caspase-8 activation) [4]. NDV strains are known to evoke cellular apoptosis and the reported apoptosis signalling pathways induced by NDV in tumor cells are inconsistent and conflicting [5]. Although some aspects of such selective cytotoxicity have been elucidated, the gene regulation mechanisms are not well clarified which inspired the current study to investigate the gene expression pattern in NDV-infected MCF-7 cells.

MATERIALS AND METHODS

Virus and Cells: NDV strain AF2240 was propagated as described by [6]. Briefly, the virus was injected into 9-10 day-old embryonated chicken eggs. Prior to inoculation, the eggs were candled to sort out the dead embryos and a spot about 1 mm above the air sac was marked on each of them. The surface of the eggs were sterilised by swabbing with 70% ethanol before and after punching a hole on the egg shell at the chorioallantoic sac above the margin of the air sac membrane and away from embryo.

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The virus stock was diluted 1:100 in 1X Phosphate Buffer Saline (PBS), sterilized through 0.45 µm pore-size filters (Acrodise 32-Gelman Sciences, USA). By using a 1ml syringe with 26-gauge needle (Terumo, Belgium) the inoculum of 0.2 ml of the diluted virus was injected within the chorioallantoic sac (CAS) through the shell hole at 45° angle from the perpendicular in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) with antibiotic-antimycotic solution (Gibco, USA) at final concentration of 1% (v/v). The hole was sealed with melted wax. Non-inoculated eggs were used as control. Eggs were incubated in humidified incubator (Memmert, Germany) at 37% for 48 h with intermittent candling at 24 h. The infected allantoic fluid was harvested before the embryo's death.

Prior to harvesting, eggs were kept in 4°C overnight to ensure that the blood vessels were constricted, to reduce bleeding during harvesting. Under sterile condition in the laminar-flow cabinet (Micro-tech, USA) the egg shells were removed and the allantoic fluid was harvested using sterile 10 ml pipettes. The maximum yield was about 8-10 ml from single embryo of 13 day maturity. Following harvesting, the infected allantoic fluid was clarified by centrifugation at 5,000 rpm (J-251, Beckman, USA) for 30 min at 4°C.

The clarified supernatant was then subjected to another centrifugation at 19,500 rpm for 3 h at 4°C (rotor JA-20, Beckman, USA). The supernatant was discarded and the pellet was dissolved in NTE buffer (10 mM Tris, 0.1 M NaCl, 1 mM EDTA, pH 8.0). The virus was purified by centrifugation in 30 to 60% (w/v) sucrose gradient at 38,000 rpm (SW 41 Ti-rotor, XL-1-K ultracentrifuge Beckman, USA) for 4 h at 4°C. The visible band of the virus was collected at the interphase of 40% to 50% sucrose concentration. This portion which contained the virus was removed and suspended in NTE buffer and centrifuged again at 38,000 rpm for 2 h at 4°C to pellet down the virus. The pellet of the virus obtained was suspended in 500 ml NTE buffer and stored at 20°C until further use [7]. Haemaglutanin (HA) assay was performed for the virus titration. Human breast cancer cell line MCF-7 (ATCC) was grown in RPMI medium 1640 supplemented with 10% FBS, 1% antibacterial and antimycotic. At 90% confluence, the cells were infected with NDV at a MOI of 0.7.

Gene Expression: Three and six h post-infection (PI) of 1 x 10^7 MCF-7 cells with NDV, total RNA was extracted using Trizol (Invitrogen, USA). The resulting RNA had a 260 nm/280 nm absorbance ratio of 2. GenomeLab GeXP Genetic analysis system (Beckman Coulter, USA) was used to evaluate the mRNA levels. The GeXP system is a capillary gene expression analysis system by which the expression level of 21 genes related to tumor progression, cell cycle regulation, cell growth, differentiation, apoptosis, cancer suppression, hormone response and DNA damage are assayed. In addition, the Multiplex kit contains an internal control and three reference genes. RT-PCR was carried out according to the manufacturer's instructions (GeXP, Beckman Coulter). Treatment of the cells with NDV and the mock-infected control were performed in duplicates; all samples were run in triplicates. For the analysis of GeXP data, all genes were normalized against the geometric mean of the three normalisation genes: actin (ACTB), peptidylprolyl isomerise A (Cyclophilin A) and GAPDH. Fragment analysis, the eXpress Profiler and the eXpress Analysis software modules were used for the analysis of data. Fold change was measured relative to the expression of treated samples/un-treated samples. In Table 1, negative fold changes in gene expression was divided by -1, e.g -1/0.233 [8].

Cell Viability: Following tyrpsinization, the single cells were taken with micropipette and diluted with trypan blue (100 µl). The cells were counted using hemocytometer. The cells were mixed thoroughly and 10 µl of cells suspension were placed in counting chamber with coverslip using micropipette. The viability of the cells were counted and the number of cells calculated.

Flow Cytometry: Cell cycle analysis was performed using the following procedures. First both the control and treatment were washed twice with PBS to remove any traces of serum, then the number of cells was adjusted at $2x10^6$ cells/100 µl in PBS and fixed with 75% ethanol dropwise while vortexing gently. Prior to analysis, the cells were washed by spinning twice with PBS and one ml of staining medium (Merck, USA) (900 µl of 1x PBS 50 µl of propidium iodide (1mg/ml) and 50 µl of RNase A (1mg/ml) was added. Analyses were carried out using Flow cytometry (DakoCytomation, USA).

RESULTS AND DISCUSSION

The gene expression pattern was compared at three and six h post-infection (PI) between NDV-infected and non-infected (control) MCF-7 cells using Multiplex gene expression kit. Cell cycle analysis using propidium iodide staining was also performed to confirm the induction of apoptosis in the infected cells.

Table 1: Genes whose mRNA expressions are significantly regulated by NDV

Number	Target Gene	[a, b]Fold *change 3 h post infection	[a,b] Fold *Change 6 h post infection
1.	Estrogen Receptor, (ESR1)	-1.9	-2.3
2	Keratin 18 (KR18), Transcript variant 1	1.7	2.4
3	3-oxoacid CoA transferase 1 (OXCT1), nuclear gene encoding mitochondrial protein	-2.5	-5
4	Deoxycytidine kinase (dCK), mRNA	-3	-4.7
5	V-myb myeloblastosis viral oncogene homolog (avian)-like 2 (MYBL2)	1.8	2.3
6	Transforming growth factor, beta 3 (TGFβ3)	1.2	1.3
7	Protein regulator of cytokines 1 (PRC1), Transcript variant 1	-6.25	-5.5
8	CDC42 binding protein kinase alpha (DMP-like) (CDC42BPA), Transcript variant 1	-1.3	-1.4
9	Kinetochore associated 2 (KNTC2)	-3	-3
10	RAB6B, member RAS oncogene family (RAB6B)	-1.1	-1.2
11	Bcl-2 binding component 3 (BBC3)	3.2	3.9
12	Adaptor-related protein complex 2, beta 1 subunit (AP2B1)	9	9
13	WNT1 Inducible signalling pathway protein 1(WISP1), Transcript variant 1	-2.8	-1.8
14	HSC4A2 Type IV collagen alpha(2) chain	No Detection in Treatment	No Detection in Treatment
15	Replication factor C (Activator 1) 4, 37kDa (RFC4), transcript variant 1	-3.5	-3.6

[a]Fold change in mRNA abundance for 3 and 6 hours NDV treatment relative to control. Fold change was calculated by Exp/Cont. Below two-fold decrease in gene expression have values less than 0.5 in the table, thus converting them into negative fold change, fraction fold change was divided into -1, e.g -1/0.233= -4.29.

[b]A positive fold change indicates increased mRNA abundance in 3 and 6 hours treatment relative to control, a negative fold change indicates decreased mRNA abundance in 3 and 6 hours relative to control

*All experiments were run in triplicates.

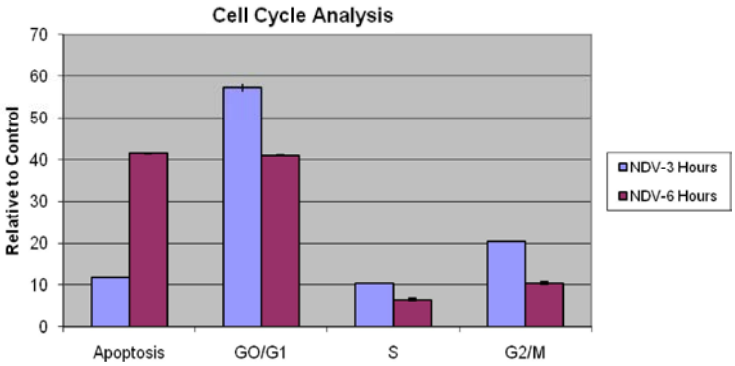

Fig. 1: Cell cycle analysis of MCF-7 cells at three and six hours PI with Newcastle disease virus relative to control.

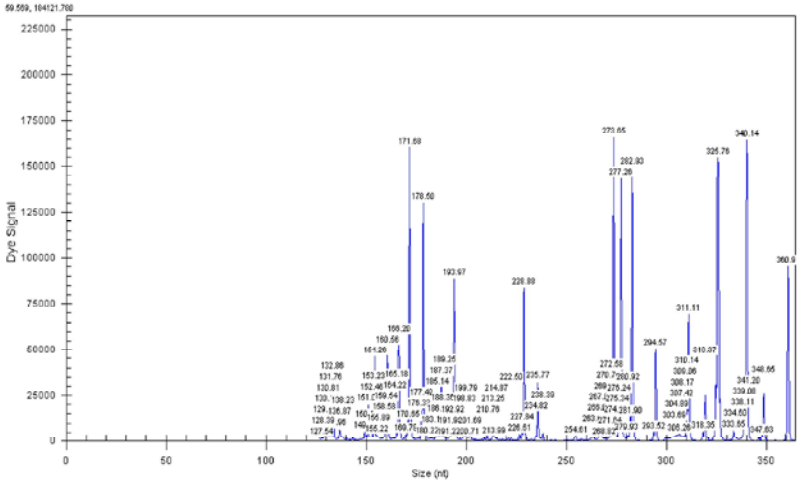

Fig. 2: Example of gene expression analysis in MCF-7 cells using GeXP. Each peak represents the expression of a gene relative to the dye signal.

As shown in (Figure 1) at 3 and 6 h PI, 11 and 41% of NDV-infected MCF7 cells underwent apoptosis, respectively. The cell viability was assay by Trypan blue method as mentioned in Materials and Methods. The results from the gene expression analysis showed that out of 21 genes, the mRNA expression level of 15 genes changed. The fold-change differences in both infected and the control groups were summarised in Table 1. Results for each gene will be discussed according to numbering sequence given in Table 1 and an example for the gene expression measurements in Figure 2.

Estrogen receptor, (ESR1): Estrogen receptor 1 (ESR1) α is a member of the superfamily of nuclear receptors, which are ligand-dependent transcription factors [9]. Estrogen modulates gene expression through interaction with estrogen responsive genes that are important for cell growth, proliferation and malignant transformation [10]. ESR1 receptors are over expressed in almost 70% of breast cancer cases known as ER positive [11]. There are several studies that support the notion that ERs signalling and expression are related to breast tumorigenesis and progression [12]. As shown in the Table 1, NDV down regulated the expression level of ESR1 α at three and six h post-infection 1.9 and 2.3-fold, respectively. This down-regulation may significantly contribute to the induction of apoptosis.

Keratin 18 (KR18), Transcript variant 1: Human keratin 18 is a type 1 intermediate filament which is structurally related to cellular proteins involved with cytoskeleton. Keratin 18 has two transcript variants. Type 1 keratin is the most divergent type with the N-terminal and C-terminal domains [13]. It has been shown that high Keratin 18 expression level in tumor cells is associated with reduced invasiveness *in vitro*, lack of tumorigenicity in nude mice and good prognosis [14]. Interestingly, NDV infection increased the expression of Keratin 18 (KR18), Transcript variant 1 to 2.4 fold compared to non-treated breast cancer MCF-7 cells.

Oxoacid CoA transferase 1 (OXCT1), Nuclear Gene Encoding Mitochondrial Protein: OXCT1 encoded by 3-oxoacid CoA-transferase gene family, playing central role in ketone body utilization and loss of this gene causes ketoacidosis. Besides, it has been shown that OXCT1 has a role in cell proliferation and differentiation [15]. Feng *et al.* [16] showed that OXCT1 is abundantly expressed in hepatocarcinoma cell. There is no data available for the expression level of this gene in breast cancer cell line. However, in this study we showed that the mRNA expression level of OXCT1 is down-regulated after NDV treatment.

Deoxycytidine Kinase (DCK), mRNA: Deoxycytidine kinase mRNA is essential for the phosphorylation of several deoxyribonucleosides and their nucleoside analogs. Cells need deoxynucleotides to repair the damaged DNA and an increased dCK activity could also help supply of all dNTPs thus helping DNA repair. Deficiency of dCK is suspected to be related with the resistance to antiviral and anticancer chemotherapeutic agents [17]. In this study, NDV induced down-regulation of *dCK* gene at three and six h PI compared to the control MCF-7 cells.

V-myb Myeloblastosis Viral Oncogene Homolog (avian)-like 2 (MYBL2) and Transforming Growth Factor β (TGF β-1): *V-myb* and its cellular progenitor *c-myb* are retroviral oncogenes encoding nuclear DNA binding proteins. MYBL2 gene has been suggested to have a dual role in cancer, both cell cycle progressions [18] as well as proapoptotic activity [19]. The gene has been reported to be highly expressed in human breast cancer [20]. Transforming growth factor β-1 has also both tumor suppressive and progression properties. Reduced expression of TGF β-1 or loss of its inhibitory effects has been linked to cell hyperproliferation and tumor progression [21]. Perry *et al.* [22] studied the effect of Tamoxifen, a chemotherapeutic drug, on TGF-beta 1 mRNA expression and observed a 2-fold up-regulation at six h post-treatment. In infection with influenza virus, cytomegalovirus and HIV, TGF-β was over-expressed which lead to apoptosis [23-25]. Bies *et al.* [26] proved that there was a correlation between the expression levels of MYBL2 and TGF-β. They showed that the over-expression of MYBL2, accelerated apoptosis in TGF β-1 treated cells. Interestingly, we demonstrated that NDV up-regulated both MYBL2 and TGFB1 in favour of the induction of apoptosis.

Protein Regulator of Cytokines 1 (PRC1), Transcript Variant 1: This gene encodes the protein involved in cytokinesis [27]. Elevated levels of PRC1 was previously shown to be important in the development of breast cancer and its down-regulation resulted in growth suppression of breast cancer cells [28]. In the present report, it was shown that NDV down-regulated PRC1 at three and six h by 5-6 fold in infected MCF-7 cells compared to non-treated cells.

CDC42 Binding Protein Kinase Alpha (DMP-like) (CDC42BPA), Transcript Variant 1: Similar to Rac and Rho, CDC42BPA is a member of Ras superfamily of GTPase which share a significant homology at the amino acid level [29]. Increased levels of these subgroups have been implicated in breast cancer [30]. The T47D breast cancer cells exhibit over-expression of CDC42 [31]. We observed that NDV down-regulated the expression level of CDC42BPA 1.4-fold at six h post-infection (PI).

Kinetochore Associated 2 (KNTC2): Kinetochore associated 2 is a member of the evolutionarily conserved centromere protein complex [32] and it is believed to be up-regulated in many cancers including breast cancer [33]. This gene is also known as highly expressed in cancer (HEC1) [34]. In the current study a 3-fold down-regulation of this gene at three and six h PI with NDV was observed.

RAB6B, Member RAS Oncogene family (RAB6B): *RAB6B* gene is a subgroup of RAS superfamily, subgroup of RAB family of small GTPases. This family consists of nearly 60 subgroups [35]. The function of RAB proteins is the regulation of intracellular vesicle trafficking. RNA microarray analyses in ovarian cancer revealed that ~45% to 50% of RAB genes display increase in mRNA expression. Other studies have also shown that RAB25 gene was over-expressed in ovarian and breast cancers [35]. The exact expression level of RAB6B is not known in breast cancer. However, the gene expression analysis in the present study showed that NDV infection imposed an over-expression of this gene by 1-1.2 at three and six h post-infection (PI), respectively.

Bcl-2 Binding Component 3 (BBC3 or PUMA): bbc3 or p53-upregulated modulator of apoptosis (PUMA) is a member of bcl-2 pro-apoptotic family. It is transcriptionally induced upon diverse apoptotic stimuli leading to apoptosis via the mitochondrial pathway. BBC3 is activated by p53; it is also up-regulated after endoplasmic reticulum stress, independently to p53 status. It has been shown that bbc3 induces apoptosis in various cancer cells including breast cancer by promoting mitochondrial Bax conformational change [36]. In agreement with these findings, we recently confirmed that infection of Hela cells with NDV leads to the conformational change of Bax protein which is associated with the translocation of Bax from the cytoplasm to mitochondria and the release of cytochrome c into the cytoplasm [37]. In this study, the up-regulation of PUMA in MCF-7 cells after treatment with NDV suggested that NDV certainly exerts its oncolytic property through this pathway.

Adaptor-related Protein Complex 2, Beta 1 Subunit (AP2β1): The protein encoded by *AP2β1* gene is one of the two large chain components of the assembly protein complex 2, which serves to link clathrin to receptors in coated vesicles. As it is known the mode of entry of viruses may be different. Earlier, it has been shown that NDV enters the host cells through caveolea endocytosis [38]. In the current analysis, NDV up-regulated the expression of *AP2B1* gene in MCF-7 cells at three and six h by almost 9-fold compared to non-treated cells. This result may be explained by the possibility that NDV had used other routes of entry; there may be strain specific entry mode of this virus or it may use more than one entry mode to selectively replicate in cancer cells.

WNT1 Inducible Signalling Pathway Protein 1(WISP1): Wnt-1 signal is a secreted glycoprotein that plays an important role in embryonic development and carcinogenesis. The Wnt-1 gene expression increases in a variety of human cancers [39]. Studies have shown that Wnt-1 signalling blocked by either Wnt-1 small interference RNA or anti Wnt-1 antibody leading to apoptosis in human cancer cells [39]. In the current study we demonstrated that NDV down-regulated the expression of *Wnt-1* gene in MCF-7 cells by 1.8-2.8 fold at three and six h post infection.

HSC4A2 Type IV Collagen Alpha (2) Chain: Collagen type IV acts as basement membrane that structurally defines the architecture on epithelial and endothelial cells. However, during malignancy, normal architecture of the tissue is disrupted by factors secreted or produced by cancer cells [40]. Studies by Tanaka *et al.* [41] demonstrated the loss of collagen IV during malignancy. In agreement with these findings, this study did not detect any HSC4A2 expression in both treated and non-treated MCF-7 cells.

Replication factor C (Activator 1) 4, 37kDa (RFC4), Transcript Variant 1: The elongation of primed DNA templates by DNA polymerase delta and DNA polymerase epsilon requires the action of two accessory proteins: Replication factor C, which is an enzyme that contains five distinct subunits (145, 40, 38, 37, 36.5 kDa) and proliferating cell nuclear antigen PCNA having a role in DNA metabolic pathways. When bound to DNA, PCNA organizes various proteins involved in DNA replication, DNA repair and DNA modification. Replication factor C promotes the loading of PCNA onto DNA at template-primer junctions by an ATP-dependent process [42].

In this work, the decreased level of expression of *RFC4* gene after induction of apoptosis by NDV was observed up to 3-fold at three and six h post-treatment.

In conclusion, to unravel the molecular mechanism of oncolytic property of NDV we used GeXP Multiplex Kit by which the regulation of several breast cancer cells' genes involved in apoptosis, cell cycle, proliferation and cell growth were evaluated at their mRNA expression levels. The data obtained in this study would help identify target molecules for the development of more effective tools for breast cancer prevention and treatment.

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