Oncolytic effect of Newcastle Disease Virus is associated with interferon pathway expression in canine mammary cancer cell lines.

Mariana R. Santos¹, Pedro L.P. Xavier¹, Pedro R.L. Pires¹, Arina L. Rochetti¹, Daniele Rosim¹, Muhammad Munir ², Helena L. Ferreira³, Heidge Fukumasu¹*.

¹ Laboratory of Comparative and Translational Oncology, Department of Veterinary Medicine, School of Animal Science and Food Engineering, University of Sao Paulo, Av. Duque de Caxias Norte n°225, Pirassununga 13635-900, Sao Paulo, Brazil.
² Division of Biomedical and Life Sciences, Lancaster University, Lancaster, LA1 4YG, United Kingdom.
³ Department of Veterinary Medicine, School of Animal Science and Food Engineering, University of Sao Paulo, Av. Duque de Caxias Norte n°225, Pirassununga 13635-900, Sao Paulo, Brazil.

*Correspondence to: Heidge Fukumasu, email: fukumasu@usp.br
Abstract

Canine Mammary Carcinomas (CMC) are one of the major health threats in dogs, globally accounting for about 40% of all tumors in intact females and being malignant in half of the cases. The oncolytic virotherapy is a promising strategy to treat canine as well as human cancer patients with non-pathogenic replicating viruses. Here we evaluated the antitumor activity of one lentogenic, non-lytic NDV LaSota strain expressing GFP (NDV-GFP) on 5 different CMC (2 epithelial-like and 3 mesenchymal-like) and one non-tumorigenic cell line, regarding cell viability, cell death, selectivity index, morphology and transcriptome analysis. As evidenced by the selectivity index, all CMC cell lines were more susceptible to NDV-GFP than the normal cells ranging from ~3.1x to ~78.7x. The oncolytic effect of NDV-GFP was more evident in cell lines with mesenchymal-like instead of epithelial-like phenotype. Also, there was an inverse association of IFN pathway expression and selective oncolysis of NDV, demonstrating this mechanism as the most prominent for oncolysis by NDV. To our knowledge, this is the first description of oncolysis by an NDV strain in canine mammary cancer cells. We also demonstrated specific molecular pathways related to NDV susceptibility in these cancer cells, opening the possibility of using NDV as therapeutic-targeted option for more malignant CMCs. Therefore, these results greatly urge for more studies using oncolytic NDVs, especially considering genetic editing to improve efficacy in dogs.

Keywords: epithelial-mesenchymal transition; comparative oncology; selectivity index; RNA-seq; Paramyxovirus
Canine Mammary Carcinomas (CMC) are one of the major health threats in dogs, globally accounting for about 40% of all tumors in intact females, and being malignant in half of the cases (SLEECKX et al., 2011). The standard treatment for small animals with CMTs is surgery and adjuvant therapies (e.g. post-operative chemotherapy) can be advocated for dogs with advanced disease. But adjuvant therapies have demonstrated limited effects on prognosis and a surplus at treatment-related side effects in patients with CMTs (SLEECKX et al., 2011). Since current adjuvant therapies in veterinary medicine are insufficient to treat advanced stages of CMC, the development of new and improved therapeutic options is in a high demand to reduce cancer patient’s mortality.

The Oncolytic Virotherapy is a promising strategy to treat canine as well as human cancer patients with non-pathogenic replicating viruses (SÁNCHEZ et al., 2018). Oncolytic Viruses (OVs) are natural or engineered infectious agents that show three fundamental mechanisms of action: (i) tumor selective infection, replication and spread after direct oncolysis; (ii) tumor microenvironment reshape, and (iii) tumor-associated antigens release and trigger of adaptive anti-tumor immune responses (ENGELAND; BELL, 2020). These principles end up in tumor vaccination effects, prompting therapeutic and protective antitumor immunity with minimal toxicity to normal cells, hence fewer side effects than conventional cancer treatments (RUSSELL; BARBER, 2018; ENGELAND; BELL, 2020).

A recent review on OVs considered the Newcastle Disease Virus (NDV) as a breakthrough for improvements in cancer therapy (SCHIRRMACHER; VAN GOOL; STUECKER, 2019). This virus features a natural preference for replication in many tumor cells comparing to normal cells and the observed antitumor effect of NDV appears to be a result of both selective killing of tumor cells and induction of immune responses (ZAKAY-RONES; TAYEB; PANET, 2015). Although cells of various human tumors were sensitive for NDV, only one study demonstrate the potential of NDV as oncolytic for canine lymphoma cells (SÁNCHEZ et al., 2015). Therefore, here we evaluated the antitumor activity of one lentogenic recombinant NDV LaSota strain expressing the GFP protein (NDV-GFP) on 5 different canine mammary cancer cell lines and one non-
tumorigenic cell line and demonstrated that the loss of Interferon pathway response was the main pathway associated with the oncolytic effects of NDV.

Material and Methods

Cell lines

Five canine mammary cancer cell lines (CMC) were used in this experiment: E20, E37, M5 and M25 cell lines were isolated and established in our laboratory as previously described (CORDEIRO et al., 2018; XAVIER et al., 2018). The CF41.Mg cell line was kindly provided by Dr. Debora A. P. C. Zuccari (Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, São Paulo, Brazil). The E20 and E37 cell lines presented an epithelial-like morphology whereas the M5, M25 and CF41.Mg presented a mesenchymal-like morphology (XAVIER et al., 2018). The canine embryonic fibroblasts were kindly provided by Dr. Carlos Eduardo Ambrósio (Laboratório de Cultivo de Células Tronco e Terapia Gênica, Faculdade de Zootecnia e Engenharia de Alimentos, Pirassununga, Sao Paulo) (GONÇALVES et al., 2017). All CMC cells were maintained in 75 cm² flasks at 37°C and 5% CO₂ with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. The canine embryonic fibroblasts were cultured with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) supplemented with 15% fetal bovine serum, 1% of glutamine and 1% antibiotic/antimycotic. Passaging was performed when cells were 70-80% confluent. Culture evolution was evaluated daily by optical microscopy (Axio Vert A1, Zeiss, Germany). All reagents used for cell culture were purchased from Thermo Fisher Scientific, USA.

Virus titration and morphological analysis

A genetically modified NDV La Sota strain, a genotype II class II NDV, expressing GFP (AL-GARIB et al., 2003), was kindly provided by Dr. Muhammad Munir (Lancaster University, England). Virus titers were obtained by calculating the median tissue culture infectious dose per ml (TCID₅₀/ml) using Reed and Muench method (REED; MUENCH, 1938). Briefly, M25 cells were seeded at 3,000/well in 96 well plates containing 100 μl of supplemented media as described. After 24h, media was removed and each well was washed with PBS. The cells were exposed to different concentrations of the virus (10⁻¹¹ a 10⁻¹). After 90 minutes, 100 μl of medium supplemented with 2% of FBS and 1%
antibiotic/antimycotic were added in each well. The cytopathic and morphologic effects were evaluated daily for 5 days. Pictures were taken with optical and fluorescence microscopy using ZEISS—Axio Vert A1 with a camera Axio Can 503 attached using a 520 nm wavelength filter for green color (ZEISS, GER).

**DNA sequencing of the NDV fusion gene**

DNA sequencing was done to confirm the cleavage site sequence. Viral RNA purifications were performed with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to manufacturer’s instructions, followed by RT-PCR using SuperScript III One-Step RT-PCR System with Platinum I Taq DNA Polymerase (Life Technologies, Carlsbad, CA, USA) and previously described 4331F/5090R primers (MILLER et al., 2015). Amplicons were visualized in 2% of SYBR Safe (Life Technologies, Carlsbad, CA, USA) low Melting Point Agarose (Life Technologies, Carlsbad, CA, USA). Products were purified using Illustra GFX PCR DNA and Gel Band Purification Kits (GE Health Care and Life Sciences, Buckinghamshire, England). DNA sequencing was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) in ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA USA) at CEGH-CEL facility (IB-USP). The obtained sequences were evaluated for quality using the Sequence Scanner™ Software 2 (Applied Biosystems, Foster City) and edited by MEGA7 (KUMAR; STECHER; TAMURA, 2016). Afterwards, the obtained sequences and sequences available in GenBank were aligned using Clustal W (THOMPSON; HIGGINS; GIBSON, 1994) and Bioedit Sequence Alignment software version 7.2.5. (HALL, 1999). The cleavage site was deduced based on criteria utilized by OIE to assess virulence of NDV isolates (OIE, 2012).

**Virus infection and replication in cell lines**

The CMC cells and canine embryonic fibroblasts were seeded at 5,000 cells per well in 96 well plates containing 100 µl of supplemented media as described. After 24h, media was removed, each well was washed with PBS and new culture media containing $10^2$ to $10^5$ MOI of NDV-GFP was added. After 90 minutes, 100 µl of media supplemented with 2% of FBS and 1% antibiotic/antimycotic was added in each well. Then, cell lines were evaluated up to 72 hours for the detection of GFP expression due to virus replication and gene expression.
**NDV cytotoxic assay**

The CMC cells and canine embryonic fibroblasts were seeded at 5,000 cells per well in 96 well plates containing 100 µl of supplemented media as described. After 24h, media was removed, each well was washed with PBS and new culture media containing different concentrations of MOI (10² to 10⁻⁵) was added using the NDV-GFP. After 90 minutes, 100 µl of media supplemented with 2% of FBS and 1% antibiotic/antimycotic was added in each well. After 72h, cells were fixed using 4% paraformaldehyde for 10 minutes and stained with 2% crystal violet for 20 minutes. Acetic acid (10%) was added to dissolve the crystals. Optical density at 540 nm was measured in a Fluorstar Optima (BMG Labtech, Germany). The selectivity index (SI) was calculated using the half maximal inhibitory concentration (IC50) of each cancer cell line in comparison to the non-tumorigenic cell line.

**Cell death assay**

The CMC cells and canine embryonic fibroblasts were seeded at 5,000 cells per well in 96 well plates. After 24h, cells were treated with the NDV-GFP IC50 for 72h. A dye mix containing 100 µg/ml of acridine orange and 100 µg/ml of ethidium bromide was added to cells and observed for fluorescence emission using ZEISS—Axio Vert A1 with a camera Axio Can 503 attached using a 520 nm and 620 nm wavelength filter for green and red colors, respectively. Analysis were performed in triplicate, counting at least 100 cells each. The results were analyzed based on the arrangement of chromatin to differentiate apoptotic, oncotic and live cells (Ribble et al., 2005). Live cells have normal nuclei staining which presents green chromatin with organized structures. Apoptotic cells contain condensed or fragmented chromatin (green or orange) and oncotic cells have similar normal nuclei staining as live cells except the chromatin is orange instead of green (Ribble et al., 2005).

**Total RNA extraction, RNA-sequencing and data analysis**

Four cell lines (E20, CF41.Mg, M25 and M5 but not E37 and control cells) were analyzed by RNA-seq before exposure to the virus to evaluate the possible biological processes associated with the oncolytic effects of the virus. The total RNA of triplicates of 10⁶ cells of each of the four CMC cell lines was extracted using RNeasy Mini Kit (QIAGEN, UK). The RNA quality and quantity were assessed using automated capillary gel electrophoresis on a Bioanalyzer 2100 with RNA 6000 Nano Labchips according to the
manufacturer’s instructions (Agilent Technologies, Ireland). Only samples that presented
an RNA integrity number (RIN) higher than 8.0 were considered to the sequencing. RNA
libraries were constructed using the TruSeq™ Stranded mRNA LT Sample Prep Protocol,
than each library were analyzed by bioanalyzer for quality and sequenced on Illumina
HiSeq 2500 equipment in a HiSeq Flow Cell v4 using HiSeq SBS Kit v4 (2x100pb).
Sequencing quality was evaluated using the software FastQC
(https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and no additional filter
was performed. Sequence alignment against the canine reference genome (CanFam3.1)
was performed using STAR (DOBIN et al., 2013), according to the standard parameters
and including the annotation file (Ensembl release 89). Secondary alignments, duplicated
reads and reads failing vendor quality checks were removed using Samtools (LI et al.,
2009). Alignment quality was confirmed using Qualimap (GARCÍA-ALCALDE et al.,
2012). Gene expression was estimated by read counts using HTseq (ANDERS; PYL;
HUBER, 2015). Count were normalized by Variance-stabilizing Transformation (VST)
and differential expression analysis (DE) was performed using DeSeq2 package (LOVE;
HUBER; ANDERS, 2014) and the Benjamini-Hochberg procedure was used to calculate
the false discovery rate (FDR) and transcripts presenting FDR ≤ 0.01 and log-fold change
(LogFC) > 1 were considered differential expressed.

Statistical Analysis

The IC50 was calculated by a nonlinear regression and the apoptotic index was analyzed
using Two-way ANOVA. GraphPad Prism version 8.0.0 for Mac (GraphPad Software,
USA) was used for statistical analysis and graph preparation.

Results

NDV-GFP titration and cleavage site sequencing

The obtained titer of NDV-GFP in M25 cells was $10^{5.86}$ TCID<sub>50</sub>/ml after 5 days post-
fection. The cleavage site sequence from the virus confirmed to be $^{113}$RQGR*L$^{117}$,
which is specific for lentogenic viruses based on criteria utilized by OIE to assess
virulence of NDV isolates.
Morphological effects of NDV-GFP in Canine Mammary Cancer cell lines

The time that GFP was visualized varied between cell lines, being the M5 cell line the first where GFP (20h) was visualized and the E20 the last (48h, table 1). The other 3 cell lines (M25, Cf41.Mg and E37) and normal cells (fibroblasts) have GFP detected at 24h after the exposure to NDV-GFP. The viral infection didn’t induce the formation of syncytia in the cell lines as expected since the virus is a lentogenic strain.

Cytotoxicity of NDV-GFP in CMC cell lines

The cytotoxic potential of NDV-GFP in all CMC cell lines and normal cells was evaluated by the comparison of IC50 for each cell line (Table 1). As evidenced by the selectivity index, all CMC cell lines were more susceptible to NDV-GFP than the normal cells ranging from ~3.1x to ~78.7x. The oncolytic effect of NDV-GFP was more evident in the cell lines with mesenchymal-like morphology (M5, M25 and CF41.Mg) instead the cell lines with epithelial-like phenotype (E20 and E37).

Table 1. Cytotoxic effects of NDV in cancer cell lines and fibroblasts.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Time to detect GFP in cell culture</th>
<th>IC50 (MOI)</th>
<th>Selectivity index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M5</td>
<td>~20h</td>
<td>0.12 ± 0.04</td>
<td>~78.7x</td>
</tr>
<tr>
<td>M25</td>
<td>~24h</td>
<td>0.17 ± 0.04</td>
<td>~56.1x</td>
</tr>
<tr>
<td>Cf41.Mg</td>
<td>~24h</td>
<td>1.54 ± 0.17</td>
<td>~6.2x</td>
</tr>
<tr>
<td>E37</td>
<td>~24h</td>
<td>2.03 ± 0.49</td>
<td>~4.7x</td>
</tr>
<tr>
<td>E20</td>
<td>~48h</td>
<td>3.08 ± 0.51</td>
<td>~3.1x</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>~24h</td>
<td>9.51 ± 2.39</td>
<td>~1x</td>
</tr>
</tbody>
</table>

* In comparison to normal cells (fibroblasts)

Cell death induced by NDV-GFP

We also analyzed which type of cell death the virus induced in cancer cells by double staining with acridine orange and ethidium bromide, which discriminates between apoptosis and oncolysis. The cell lines exposed to each IC50 of NDV-GFP (including the
control cells) cells died exclusively by apoptosis ($p<0.0001$, fig.1) with no difference between cells lines ($p=0.61$) due to the use of the IC50 for each cell line.

Figure 1. Induction of apoptosis in CMC cell lines by NDV-GFP. Cell lines were exposed to the IC50 and non-exposed (controls) were evaluated after 72 hours. The NDV exposure significantly induced cell death by apoptosis in all cells regardless of type (* $p<0.0001$).

Selectivity of NDV-GFP is inversely related to interferon pathway expression

Global gene expression PCA analysis showed that some cells are clearly different from each other (Figure 2A) being M25 and M5 more similar than CF41.Mg and E20. Next we selected representative genes from 4 biological processes associated with virus infection and cell response: metabolism of sialic acid (4 genes), trypsin-like proteases (9 genes), interferon pathway (4 genes) and cell-death by apoptosis (19 genes). When we analyzed the expression of these 36 genes at the same time, cell types clustered according to the susceptibility to oncolytic effects (M5>M25>CF41.Mg>E20, being the M5 the most susceptible) and a clusterization of mesenchymal-like cells occurred in opposition to the epithelial-like cancer cells (Fig. 2B). The four cell lines expressed similarly the genes of metabolism the sialic acid (Fig. 2E) but the majority of the trypsin-like proteases have low expression in these cancer cells (Fig. 2D) apart from the SP1 protease which was highly expressed in all cell lines. On the other hand, the less sensitive cell line (E20) to NDV oncolysis expressed more two proteases (TMPRSS4 and TMPRSS11E) than the more sensitive cell lines. The expression of genes from the interferon pathway response clustered the cell lines according to the oncolytic effects. Clearly the more sensitive cell line (M5) expressed significantly less $IFNA$, $IL29L$ and also $STAT1$ than the more
resistant cell line (E20, Figure 2C). Therefore, we proposed the IFN pathway as the most important for the oncolytic effects of NDV in canine mammary cancer cells.

**Figure 2.** Transcriptome analysis of CMC cell lines. (A) Principal component analysis of transcriptome from 4 CMC cell lines. Triplicates of each CMC cell line were analyzed. This analysis considered all genes expressed in all CMC cell lines and showed distinction between cell lines. Clearly, the E20 cell line was distinct from the mesenchymal cell lines. (B) Heatmap from selected genes of 4 major pathways related to virus infection and cell response. This analysis clustered all mesenchymal-like cells (CF41.Mg, M5 and M25) in opposition to the epithelial-like cell line (E20) and supported the selectivity index to NDV oncolysis. (C-F) Heatmaps based on genes related to metabolism of sialic acid (4 genes), trypsin-like proteases (9 genes), interferon pathway (4 genes) and cell-death by apoptosis.
(19 genes). Only the Interferon-pathway genes (C) clustered cells according to the selectivity index (M5>M25>Cf41.Mg>E20, being the M5 the most susceptible). The interferon genes were more expressed in the E20 cell line, the most resistant to oncolytic effects of NDV.

Discussion

In the present study, we studied the oncolytic effects of one lentogenic NDV strain in five canine mammary cancer cell lines and one non-tumorigenic fibroblast cell line, regarding cell viability, cell death, selectivity index, morphology and transcriptome analyses. We demonstrated a remarkable oncolytic effect of NDV-GFP in the mammary cancer cell lines and showed that the interferon pathway response was the most important for the susceptibility of NDV-GFP in cancer cells. Also, a strong selectivity index in more tumorigenic, mesenchymal-like cancer cells was demonstrated in comparison to other less tumorigenic and non-tumorigenic cells. Hence, these results support this NDV strain as a promising therapeutic option for clinical trials in canine patients.

The NDV is known for its oncolytic effects for a long time (ADAMS; PRINCE, 1957; PRINCE; GINSBERG, 1957a, 1957b), but its use in canine patients was restricted to two studies so far (SANCHEZ. et al., 2014; SÁNCHEZ et al., 2015). There are several advantages to consider NDV a potential canine therapeutic option: the low virulence in dogs, several options of lentogenic strains available, established methods to produce the viral particles, relative cheap production and the possibility of gene editing by reverse genetics (CHENG et al., 2016). The direct oncolysis induced by paramyxoviruses in cancer cells are dependent of at least three different levels: the overexpression of sialic acid-containing sialoglycoproteins in cell surface; virus activation through cancer-specific proteases and; genetic defects of cancer cells that allowed virus replication as the loss of the ability to produce and respond to IFN and the induction of apoptotic pathways (MATVEEVA et al., 2015). In our work, we analyzed these mechanisms in canine cancer cells and showed a correlation of downregulation of IFN pathway and the selective oncolysis of NDV, demonstrating this mechanism as the most prominent for oncolysis by NDV. Interestingly, the less susceptible cell lines (E20 and E37) are less malignant, with an epithelial-like morphology and the more susceptible cell lines (M25, M5 and CF41)
are more malignant, with a mesenchymal-like morphology. The epithelial-mesenchymal transition is a fundamental oncogenic process improving the ability of cancer cells to migrate and metastasize, therefore, increasing the malignant potential of cancer cells. Interestingly, one work showed that EMT augmented the response to oncolytic herpesviral therapy (CHEN et al., 2014), suggesting that cancers exhibiting EMT may be naturally sensitive targets for herpesviral therapy. Although different viruses (herpesvirus x NDV) were used in different species (human x canine), our results supported that mesenchymal-like cancer cells are more susceptible to oncolytic virotherapy.

The two principal component analysis (PCA) explained 92% of the variance between 4 cell lines (E20, CF41, M5 and M25) and clearly demonstrated the epithelial-like cancer cell line (E20) is different than the other mesenchymal-like ones. The epithelial-like cancer cell line (E20) expressed more IFN pathway genes as *IFNA*, *IL29*, and *STAT1* than the mesenchymal-like cells, but also certain apoptotic pathway genes as *P53*, *TNF* and the pro-apoptotic *BAK1* and also expressed two trypsin-like proteases the *TMPRSS4* and *TMPRSS11E*. Apparently, the sialic acid pathway is the less important process for oncolysis by NDV in our model since all cell lines expressed the genes in a similar way. In addition, no clear pattern of expression of trypsin-like proteases by cancer cells was noted since the less susceptible cells to NDV (E20) expressed more *TMPRSS4* and *TMPRSS11E* but was the last cell line to show virus infection (48h) for example. These results suggest that the pathways and genes related to antiviral response and/or virus infection were important to NDV selective oncolysis in our conditions. Thereby, if one ponders the future use of NDV for canine cancer it will probably be as a targeted therapy that will consider a panel of tumor gene expression prior to use due to the highly variable selectivity index.

The NDV-GFP used in this experiment induced apoptosis in a similar way in all cell lines, tumorigenic or not, when using the respective IC50 for each cell line, proving that NDV induced cell death mode of action is by apoptosis independently of the cell type. More exciting, is the high SI found in cancer cells ranging from ≈3x up to ≈79x, especially in the more malignant cell lines; for comparison, a value of SI>2 is generally considered a high degree of selectivity (BADISA et al., 2009). The SI demonstrates the differential activity of a given substance and the greater the SI value is, the more selective
it is. This is a very important property for cancer therapeutics since the majority of the commercial cancer drugs have a very small therapeutic window.

To the best of our knowledge, this is the first description of oncolysis by an NDV strain in canine mammary cancer cells. We also demonstrated specific molecular pathways related to NDV susceptibility in these cancer cells, opening the possibility of using NDV as therapeutic-targeted option for more malignant CMCs. Therefore, these results greatly urge for more studies using oncolytic NDVs, especially considering genetic editing to improve efficacy in dogs and in humans.

References


CORDEIRO, Y. G. et al. Transcriptomic profile reveals molecular events associated to focal adhesion and invasion in canine mammary gland tumour cell lines. Veterinary and Comparative Oncology, v. 16, n. 1, 2018.


XAVIER, P. L. P. et al. ZEB1 and ZEB2 transcription factors are potential therapeutic targets of canine mammary cancer cells. *Veterinary and Comparative Oncology*, 2018.