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Evaluation of the oncolytic property of recombinant Newcastle disease virus strain R2B in 4T1 and B16-F10 cells *in-vitro*

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ABSTRACT

Recombinant Newcastle disease virus vectors have gained a lot of interest for its oncolytic virus therapy and cancer immune therapeutic properties due to its selective replication to high titers in cancer cells. The aim of this study was to find out the oncolytic effects of mesogenic recombinant NDV strain R2B-GFP on murine mammary tumor cell line 4T1 and murine melanoma cell line B16-F10. The anti-tumor effects of R2B-GFP virus were studied *via* expression of virus transgene GFP in cancer cells, evaluating its cytotoxicity and cell migration efficacies by MTT and wound healing assays respectively. In addition, the underlying apoptotic mechanism of R2B-GFP virus was estimated by TUNEL assay, colorimetric estimation of Caspase-3, 8 and 9 and the estimation of Bax to Bcl-2 ratio. The results showed a significant decrease in viability of both 4T1 and B16-F10 cells infected with R2B-GFP virus at 0.1 and 1 MOI. R2B-GFP virus could significantly induce apoptosis in the 4T1 and B16-F10 cells as compared to the uninfected control. Further, a flow cytometry analysis on apoptotic cells percentage and mitochondria membrane permeability test was also studied in R2B-GFP virus treated 4T1 and B16-F10 cell lines. The R2B-GFP virus caused an increase in loss of mitochondrial membrane permeability in both 4T1 and B16-F10 cells indicating the involvement of mitochondrial regulated cell death. Thus, the recombinant virus R2B-GFP virus proved to be a valid candidate for oncolytic viral therapy in 4T1 and B16-F10 cells.

1. Introduction

Oncolytic viruses can selectively replicate and kill a cancer cell. The wild type and recombinant Newcastle disease viruses (NDV) have been evaluated for its cancer-killing property. Taxonomically NDV belongs to genus Avian *Orthoavulavirus* of sub-family *Avulavirinae* under family *Paramyxoviridae* and is commonly known as Avian paramyxovirus 1 (Dimitrov et al., 2019). NDV genome comprises of single-stranded negative-sense RNA of size approximately 15 kb encoding six genes namely, nucleoprotein (NP), phosphoprotein (P), large polymerase (L), haemagglutinin neuraminidase (HN), fusion (F) and matrix (M) proteins with an RNA editing process during P gene transcription resulting in two additional non-structural proteins, V and W (Steward et al., 1993). NDV exerts its oncolytic property directly by replicating in the tumor cell, activating both intrinsic and extrinsic pathways of apoptosis (Elankumaran et al., 2006; Ravindra et al., 2008a). Viral proteins like HN, M, P and F play a vital role in apoptosis. The virus can also destroy the cancer cells in an immune-mediated pathway during which the tumor associated antigens of the killed cells are exposed to the immune regulatory cells of host, thus upregulating cytokine binding ligands like TNF alpha, TRAIL, nitric oxide and release of damage-associated molecular patterns in both *in-vitro* and *in-vivo* models (Washburn et al., 2003; Krysko et al., 2012). The oncolytic property of NDV has been established in cancer cell lines of both murine and human origin, originally derived from ectoderm, endoderm, mesoderm cells and their respective stem cells (Elankumaran et al., 2006). Based on the replication pattern in a cancer cell, different pathotypes of NDVs are classified as non-lytic or lytic viruses. The lentogenic NDVs are non-lytic whereas mesogenic and velogenic pathotypes are lytic in nature.

Clinical evaluation with natural viral strains such as PV701, 73-T and MTH-68/H are in different phases of clinical trials (Schirrmacher and Fournier, 2009). The mesogenic NDV, R2B is used as a vaccine strain in many Asian countries to control Newcastle disease in poultry. Recent evidence on the oncolytic property of R2B is restricted to colon cancer

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cell line (Sharma et al., 2017). Reverse genetics system allows genomic manipulation and construction of a potent cancer virotherapy vector and till date the oncolytic property of recombinant NDV strain R2B has not been established. The recombinant NDV strain R2B was rescued by Chellappa et al., 2017 and has been used as a vaccine vector in recent times (Saikia et al., 2019; Debnath et al., 2020). The present study aims to ascertain the oncolytic property of recombinant NDV strain R2B expressing GFP protein in two murine cancer cell lines namely, murine melanoma B16-F10 and murine mammary adenocarcinoma 4T1.

2. Materials and methods

2.1. Cell lines and SPF eggs

The Vero cells and B16-F10 murine melanoma cell line were obtained from NCCS, Pune, India and 4T1 murine mammary tumor cell line was provided by Dr. Rituraj Konwar, CDRI, Lucknow, India. Vero and B16-F10 cells were grown in high glucose DMEM and 4T1 cells were grown in RPMI-1640 medium, both supplemented with 10% FBS, maintained at 37°C with 5% CO2. For trypsinization of cancer cell lines, TrypLE select enzyme (Gibco, USA) was used. Fertile specific pathogens free eggs were procured from Venky's India Pvt. Ltd., Pune, India and incubated at 37°C with 86% relative humidity.

2.2. Propagation and detection of recombinant R2B-GFP virus

The infectious clone of NDVR2B-GFPvirus along with the three support plasmids pNP, pP and pL were used for rescue and propagation of the recombinant virus was carried out as described previously (Chellappa et al., 2017). Following viral propagation for 10 serial passages in Vero cells, clear cell culture supernatant was inoculated through allantoic route into 9–11 days old embryonated SPF eggs. After incubation at 37°C for 72 h the allantoic fluid was harvested and the virus was identified by hemagglutination assay as per the method described by Alexander and Chettle (1977) using 1% chicken RBCs. RNA isolation was carried out from the allantoic fluid and presence of virus was confirmed by RT-PCR using established protocol. Further to a confluent monolayer of 4T1 and B16-F10 cells, 0.01 MOI of recombinant NDV R2B-GFP virus was infected and incubated at 37°C. After 24 h post infection (hpi) the cells were washed with PBS and fixed with 4% paraformaldehyde (Affymetrix Inc., Cleveland, USA) for 30 min at room temperature. The cells were visualized under a fluorescent microscope (Leica Microsystems, Switzerland).

2.3. Titration of recombinant R2B-GFP virus

The concentration of the recombinant R2B-GFP virus was determined by titration and expressed as 50% tissue culture infectious dose ($TCID_{50}$). The end point of the virus in Vero cells was calculated as per standard procedure (Reed and Muench, 1938).

2.4. In vitro studies of recombinant R2B-GFP virus in tumor cell lines

2.4.1. MTT cytotoxicity assay

4T1 and B16-F10 cells were seeded at a density of 2×10^4 cells into 96-well plate with complete media and incubated for 24 h with 5% CO_2 at 37°C. Subsequently both 4T1 and B16-F10 cells were infected respectively with 0.01,0.1 and 1 MOI of recombinant R2B-GFP virus and incubated for 1 h. The media was removed and renewed by addition of RPMI and DMEM for 4T1 and B16-F10 cells respectively. At 48 hpi 20 µl MTT solution (5 mg/ml in sterile PBS) was added to each well and incubated for 4 h at 37°C. The media was removed and 150 µl of DMSO was added to each well and kept for shaking for 10 min until the formazan crystals were dissolved. The absorption values at 570 nm (OD570) were measured on a microplate reader (Microscan-MS5605A, India). The comparative cytotoxicity was quantified by calculating the percentage viable cells using Mosman's formula: % viability = OD of treatment group/OD of control group × 100% (Mosmann, 1983).

2.4.2. Cell migration assay

Cells were seeded onto a six-well plate at a density of 2×10^5 cells/ml in complete medium and incubated for 24 h. After the cells have attained confluency a horizontal scratch was made with sterile 200 µl microtip, to create a wound. The cells were washed twice with DPBS and incubated with medium containing 5% serum with or without 0.1 MOI R2B-GFP virus that served as positive and negative controls respectively. Migrations of cells from the edges of the wound were photographed at 0, 24 and 48 hpi using an inverted microscope (Leica Microsystems, Switzerland).

2.5. Colorimetric estimation of caspase-3, 8 and 9.

4T1 and B16-F10 cells were seeded onto T-25 flask (2×10^5 cells/flask), and infected with 0.1 MOI of virus. At 48 hpi, cells were harvested and the activation of caspase-3, 8 and 9 was estimated using caspase-3, caspase-8 and caspase-9 colorimetric assay kits (Bio vision Inc., USA) respectively according to manufacturer's instructions. At absorption of 405 nm, the samples were analyzed by fold increase of OD values in caspase activity of infected samples to the level in control sample.

2.6. Determination of apoptosis by Annexin V-Cy3 assay

Cells were seeded at a density of 5×10^5 cells in 6-well plates, and were infected with 0.1 MOI of virus. At 48 hpi, cells were washed with sterile DPBS, trypsinized and washed again in cold DPBS. Both infected and control cells were resuspended at a density of 2×10^5 cells/ml for estimation of the percentage apoptosis in 4T1 and B16-F10 cells using Annexin V-Cy3 apoptosis detection kit (Biovision Inc., USA). Quantification of was done in a flow cytometer (BD FACS Calibur™, USA) with appropriate settings using CellQuest Pro software.

2.7. Immunocytochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The confluent monolayer of 4T1 and B16-F10 cells were infected with 0.1 MOI of recombinant virus. Both control and infected cells were harvested after 48 hpi. Cells were trypsinized, washed with sterile PBS and pelleted. The cells were resuspended in 100 µl PBS and added on APES (3-Aminoproply triethoxysilane) coated slides. The TUNEL assay was performed using DeadEnd™ Colorimetric TUNEL system (Promega, USA), following manufacturer's protocol.

2.8. Mitochondrial membrane potential ($\Delta\Psi m$) test (MMPT)

For measuring the loss of mitochondrial membrane potential (MMP) of the infected and control cells, MitoTell™ Orange dye available in the Cell Meter™ Mitochondrion Membrane Potential Assay Kit, AAT Bioquest, Inc., USA was used. 4T1 and B16-F10 cells were seeded at density of 5×10^5 cells in 6-well plates, and infected with 0.1 MOI of the recombinant virus. At 48 hpi, the cells were washed with sterile DPBS, trypsinized and washed again in cold DPBS. Cells were resuspended to a concentration of1×10^6 cells/ml to which 2 µl of 500× MitoTell™ Orange was added and incubated at 37°C, 5% CO_2 for 15 min. The cells were resuspended and analyzed in a flow cytometer with appropriate setting using CellQuest Pro software BD (BD FACS Calibur™, USA).

2.9. Realtime quantification of apoptosis

Total RNA was isolated from both infected and uninfected cell lines and cDNA was prepared as described earlier (Dey et al., 2014). The level of mRNA expression was quantified by quantitative real time PCR, for apoptotic related genes Bax and Bcl-2 in a CFX96 Thermal Cycler (Bio-

Rad, USA). The following primer sets were used:

Bax sense: 5′- GGAGCAGCTTGGGAGCG-3′,
Baxanti-sense:5'-AAAAGGCCCCTGTCTTCATGA-3'.
Bcl-2sense: 5'-ACTTCGCAGAGATGTCCAGTCA-3′,
Bcl-2 anti-sense: 5'-TGGCAAAGCGTCCCCTC-3'.
GAPDH sense: 5'-TGGTGAAGAAGGCATCTGAG-3′,
GAPDH anti-sense: 5' TGCTGTTGAAGTCGCAGGAG-3'.

The reaction mixture was carried out in a 20 µl volume with the cyclical conditions as 95°C for 5 min followed by 40 cycles of 95°C for 45 s, 60°C for 35 s and 72°C for 45 s. Non-templated control was included in every run. The ratio of mRNA expression of pro and anti-apoptotic genes *viz.*, Bax/Bcl-2 were analyzed on both cell lines at 72 hpi with uninfected control cells. Comparative CT method was used for quantifying relative gene expression and ΔCT was calculated by the difference between CT value of Bax/Bcl-2 and the CT value of GAPDH.

2.10. Statistical analysis

All statistical analysis was performed in Graph Prism version 5.01 (GraphPad Software, CA, USA). A one-way ANOVA was used to compare the means among the groups with $*P < 0.0001$ considered as significant.

3. Results

3.1. Detection of R2B-GFP virus

Expression of green fluorescent protein in 4T1 and B16-F10 cells was observed under fluorescent microscope 24 hpi (Fig. 1). Under UV 405 nm, bright green florescence in both the cells indicated the ability of R2B-GFP virus in infecting cancer cells.

3.2. Cytotoxicity effect of R2B-GFP virus

MTT reduction assay determined the level of cytotoxic effect of recombinant virus on 4T1 and B16-F10 cells at 0.01, 0.1 and 1MOI respectively. Mitochondrial enzymes are reduced by MTT dye in metabolically active cells and the level of activity is measured as cell viability. In this study, the viability of 4T1 cell declined to 45% and 35% at MOI of 0.1 and 1.0 respectively whereas the cell viability declined to 42% and 30% respectively in B16-F10 infected cells (Fig. 2). The results indicate that NDV reduces cell viability in both the cell lines in a MOI dependent manner.

3.3. R2B-GFP virus inhibits cell migration in-vitro

The cell migration inhibitory effect of R2B-GFP virus on 4T1 and B16-F10 cells was performed by conducting a wound healing assay. There was a significant regression of cell migration 48 hpi in both 4T1 and B16-F10 cell lines (Fig. 3).

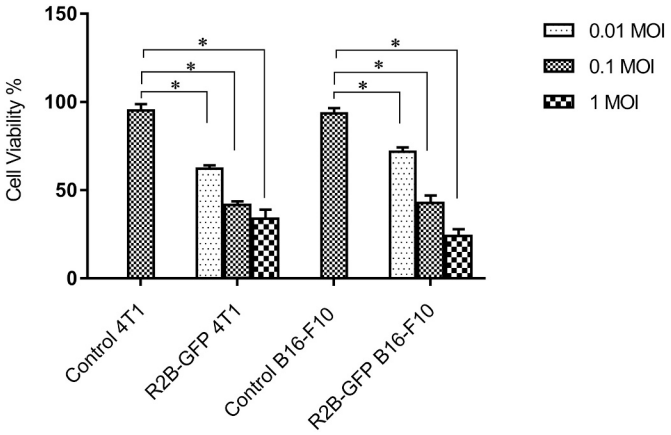

Fig. 2. Cytotoxicity effect of R2B-GFP virus on 4T1 and B16-F10 cells was evaluated by MTT assay. At 48 hpi 0.1 MOI can significantly reduce the viability of 4T1 and B16-F10 cells as to untreated control. $P < 0.0001$(*) indicates statistically significant difference between MOIs 0.01, 0.1 and 1 MOI when compared with respective control group by one-way ANOVA. In both the cell lines, 0.1 MOI of virus could reduce 50% cell viability. Data are presented as mean ± S.E.M. of at least three independent experiments.

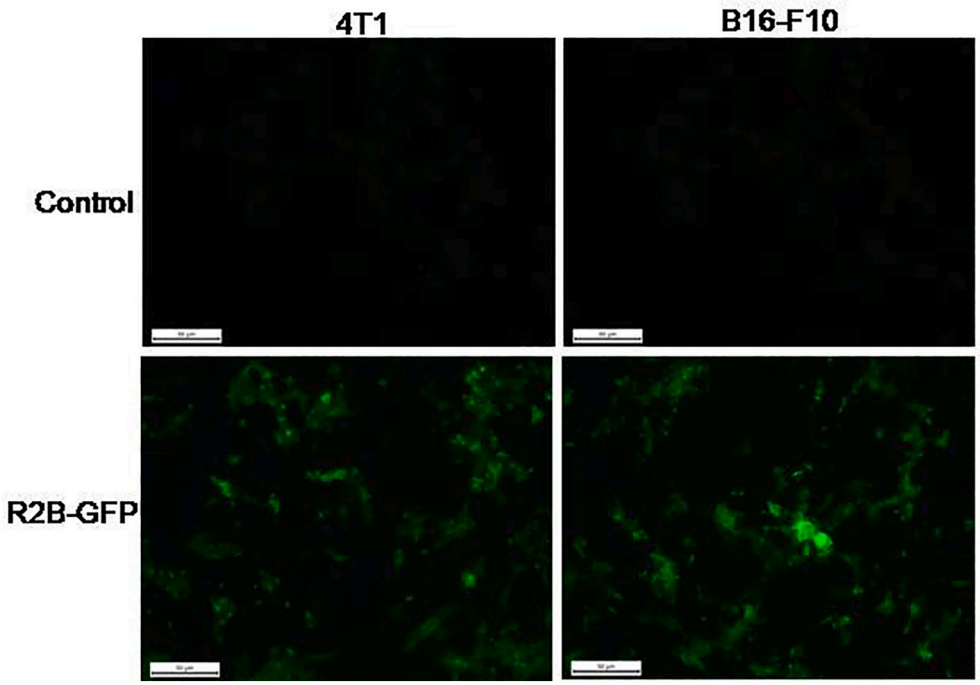

Fig. 1. Expression of green fluorescent protein by R2B-GFP virus infected 4T1 and B16-F10 at 24 hpi 40 ×. Control cells showing no fluorescence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

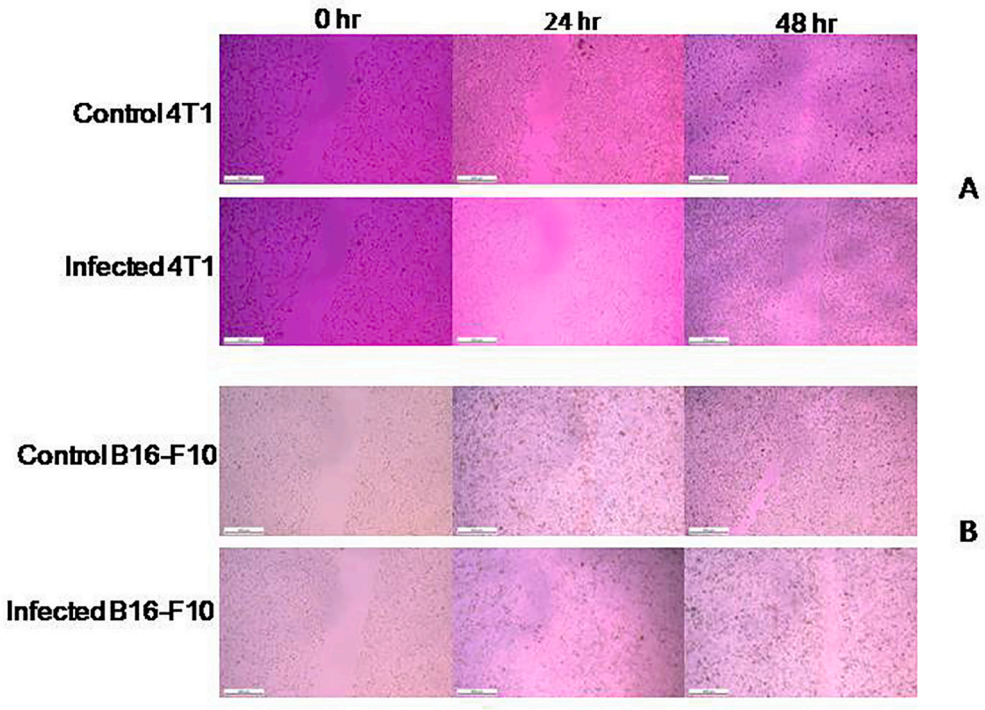

Fig. 3. Effect of R2B-GFP virus on 4T1 (A) and B16-F10 (B) cells on cell migration by wound healing assay. Confluent monolayer was infected with 0.1 MOI of the virus and the migratory inhibition was evaluated at 0, 24, 48 h and compared with control uninfected cells. At 48 hpi in both cells showed decreased cell migration in the wound area when compared with the respective control in which cells have fully migrated in to the wound area.

3.4. Establishing the apoptotic effect of R2B-GFP virus on cancer cells

3.4.1. Determination of R2B-GFP induced apoptosis in 4T1 and B16-F10 cells by TUNEL assay

The results revealed that the number of apoptotic cells was much higher in both 4T1 and B16-F10 cells, indicating DNA fragmentation when compared to the uninfected control cells (Fig. 4A).

3.4.2. Determination of caspase-3, 8 and 9 activities

The activation of the effector (caspase-3) and initiator (caspases-8 and 9) caspases was established by colorimetric assay 48 hpi. There was a significantly increased activity of caspase upon infection in both the cell lines as compared to their respective controls (Fig. 4B). Comparative absorbance of cleaved executor caspase-3 was found to be higher in 4T1 cells than in B16-F10 cells.

3.4.3. Determination of R2B-GFP virus induced apoptosis by Annexin V-CY3 assay

To assess the apoptosis, 4T1 and B16-F10 cells were monitored by staining the cells with Annexin V-CY3 and analyzed by flow cytometry. The findings showed that the percentages of apoptotic cells were significantly higher in R2B-GFP virus infected 4T1 and B16-F10 cells in comparison to their respective uninfected controls (Fig. 4C).

3.5. R2B-GFP virus modulates apoptotic regulatory genes

The relative quantification of Bax/Bcl-2 genes in both the cell lines was estimated to understand the correlation between viral infection and induction of apoptosis in cancer cells. The Bax gene was up-regulated in R2B-GFP virus infected 4T1 cells and B16-F10 cells respectively whereas Bcl-2 gene was significantly down regulated in the treated cells as compared to control uninfected cells (Fig. 4D). The mean delta CT value for Bax to Bcl-2 ratio showed a significant up regulation in both the infected cancer cells (Fig. 4E).

3.6. Effects of R2B-GFP virus on mitochondrial membrane potential ($\Delta\Psi m$).

To elucidate the mechanism of apoptosis by R2B-GFP virus on 4T1 and B16-F10 cells, mitochondrial membrane potential was examined by flow cytometry and the results indicated a significant loss of the mitochondrial membrane potential in R2B-GFP virus infected 4T1 and B16-F10 cells, as compared to its respective uninfected controls (Fig. 5A and B).

4. Discussion

Oncolytic virotherapy is a novel way to treat cancer cells allowing live viruses to selectively replicate in them and induce cell lysis with minimal toxic effect to normal tissues (Jhawar et al., 2017; Wei et al., 2010). NDV strains are one of the underrated oncolytic viruses and are reported to be still in various stages of clinical trials for human cancer therapy. Extensive studies have been reported on lentogenic strains of the virus as compared to moderately virulent mesogenic strains. However, the mesogenic NDV strains have shown to be more effective for its oncolytic property. Usually, a lentogenic strain is chosen as an anti-tumor agent to minimize the environmental hazard in countries where NDV is not endemic in spite of the limitations in its tumor cytotoxicity. In general, the mesogenic and the very virulent velogenic NDV strains, also known as lytic viruses demonstrate a lytic replication cycle in human tumor cells leading to amplification of viral load (Elankumaran et al., 2006), whereas the non-lytic, lentogenic NDV strains enhance the cancer therapeutic efficacy (Vigil et al., 2008). Furthermore, the development of reverse genetics system for NDV strain R2B will enable us to introduce apoptotic genes to further enhance its oncolytic activity.

The recombinant R2B-GFP virus is a mesogenic NDV whose oncolytic activity has not been studied earlier. The main objective of this study is to understand and evaluate the oncolytic property of recombinant R2B-GFP virus on tumor cell lines 4T1 and B16-F10. Being murine cell lines, both have been used in earlier studies as a model for studying human

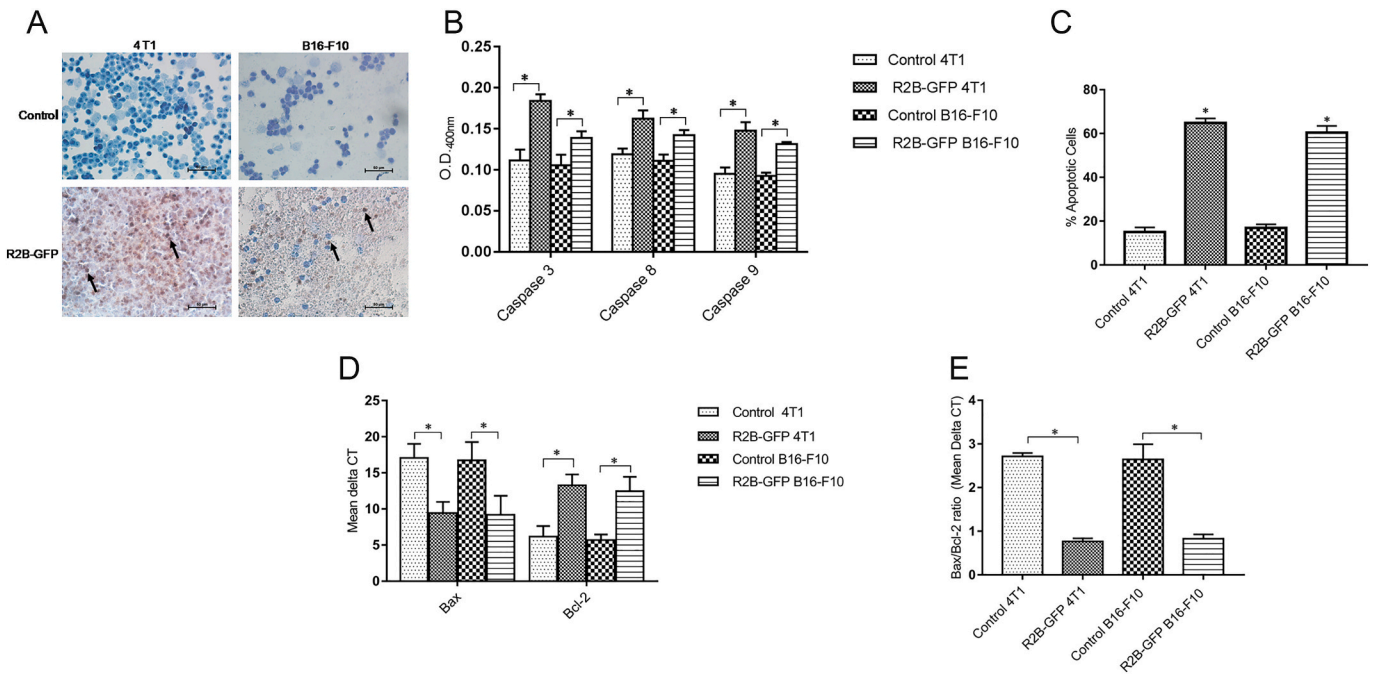

Fig. 4. Assessment of apoptosis in 4T1 and B16-F10 cells following an *in vitro* assay were performed with 0.1 MOI of R2B-GFP virus and statistical analysis with one-way AVOVA was done with significant value of * indicating ($P < 0.0001$).

(A) TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling assay) at 48 hpi. The brown area indicated with arrow shows higher number of apoptotic cells in the infected cells when compared with control uninfected cells.

(B) Caspase-3, 8 and 9 estimated by caspase colorimetric assay kit. After 48 hpi with R2B-GFP virus both the cells, indicated that infection with 0.1 MOI can significantly increase the cleaved caspase in 4T1 and B16-F10 cells as compared to untreated control. Data are presented as mean ± S.E.M. of at least three independent experiments.

(C) FACS assay of Annexin V-Cy3 staining at 48 hpi led to 62.21% of apoptosis in 4T1 and 57.54% of apoptosis in B16-F10 cells. The percentage of apoptotic cells indicates a significant percentage increase of apoptotic cells in comparison to control uninfected cells. Data are presented as mean ± S.E.M. of at least three independent experiments. Ten thousand events were evaluated per experiment, and cellular debris was omitted from analysis.

(D) Transcriptional levels of Bax and Bcl-2 genes in 4T1 and B16-F10 cells were calculated relative to the GAPDH. Both the cells were infected with R2B-GFP virus at 0.1 MOI 72 hpi. Note the significant decrease in mean delta CT of Bax indicating up-regulation of Bax mRNA expression in R2B-GFP virus infected cells. A significant increase in mean delta CT indicates down-regulation of Bcl-2 mRNA expression in R2B-GFP virus infected cells. Data are presented as mean ± S.E.M. of at least three independent experiments.

(E) Ratio of mRNA expression level of Bax to Bcl-2 in control and R2B-GFP virus infected cells by real time RT-PCR. Note (*) the significant decrease in mean delta CT of R2B-GFP virus 4T1and R2B-GFP B16-F10 groups indicative of up regulated ratio of Bax to Bcl-2 mRNA expression. Data are presented as mean ± S.E.M. of at least three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

triple negative mammary tumor (Yang et al., 2020) and melanoma (Potez et al., 2018) respectively.

The cytotoxicity effect of R2B-GFP virus on 4T1 and B16-F10 cells was estimated by MTT assay. The recombinant virus could effectively lyse the tumor cell at a dosage of 0.1 MOI. The lysis of host cells by NDV could be attributed to the inherent property of the virus to form syncytia during its replication cycle thereby enabling its oncolytic activity. These results find credence to the findings of a NDV strain AF2240 (Ahmad et al., 2015) that induced an effective killing of about 50% of the cancerous cells owing to syncytia formation and an impaired interferon pathway in these cells (Yurchenko et al., 2018).

The results of wound healing assay indicated that R2B-GFP virus would inhibit the 4T1 and B16-F10 cell migration at the wound area when compared to uninfected cells. The findings support the previous results wherein NDV AF2240 strain also decreased the cell migration and proliferation in the wound area of MDA-MB-231 breast cancer cell line *in-vitro* (Ahmad et al., 2015). The R2B-GFP virus further fragmented the DNA as quantified by TUNEL assay supporting the earlier work of Bu et al., 2016.

It has been proven that NDV triggers apoptosis in cancer cells *via* activation of both initiator caspases-8 and 9 and effector caspase-3 (Ravindra et al., 2008b). R2B-GFP virus infected tumor cells revealed an increase in activation of caspases-3, 8 and 9 suggesting that the virus could induce apoptosis by both intrinsic and extrinsic pathways. Ravindra et al., 2008b showed down regulation of caspase-8 in a velogenic NDV induced apoptosis mediated through mitochondrial pathway. However, in this study, the initial event of NDV mediated activation of caspase-9 (intrinsic pathway) follows the activation of caspase-3. The cross-talk in the intrinsic pathway in turn activates the caspase-8 (extrinsic pathway), and leads to the amplification of the apoptosis mechanism in late infection. Similar findings were observed in an earlier study involving Beaudette C, a mesogenic strain of NDV (Elankumaran et al., 2006). During the early stages of cancer, due to structural damage to the plasma membrane, phosphatidylserine translocate to the outer plasma membrane. This event reveals information about initiation of apoptosis (Ravindra et al., 2009). In the present study, a 0.1 MOI of R2B-GFP virus infected cancer cells, at 48hpi showed a significantly higher percentage of annexin-V positive cells when compared to the uninfected cell control.

Bcl-2, belonging to the anti-apoptotic family binds to and inactivates Bax and other pro-apoptotic proteins, there by inhibiting apoptosis (Tzifi et al., 2012). In the present study, an increased Bax to Bcl-2 ratio in both the R2B-GFP virus induced 4T1 and B16-F10 cells indicated a higher level of expression of Bax gene in R2B-GFP virus infected cells as compared to Bcl-2 gene in the uninfected control cells. Following NDV infection, Molouki et al., 2011 showed that level of expression of Bax-gene increased whereas Bcl-2 gene decreased.

Further, a higher expression of Bax gene and localization on

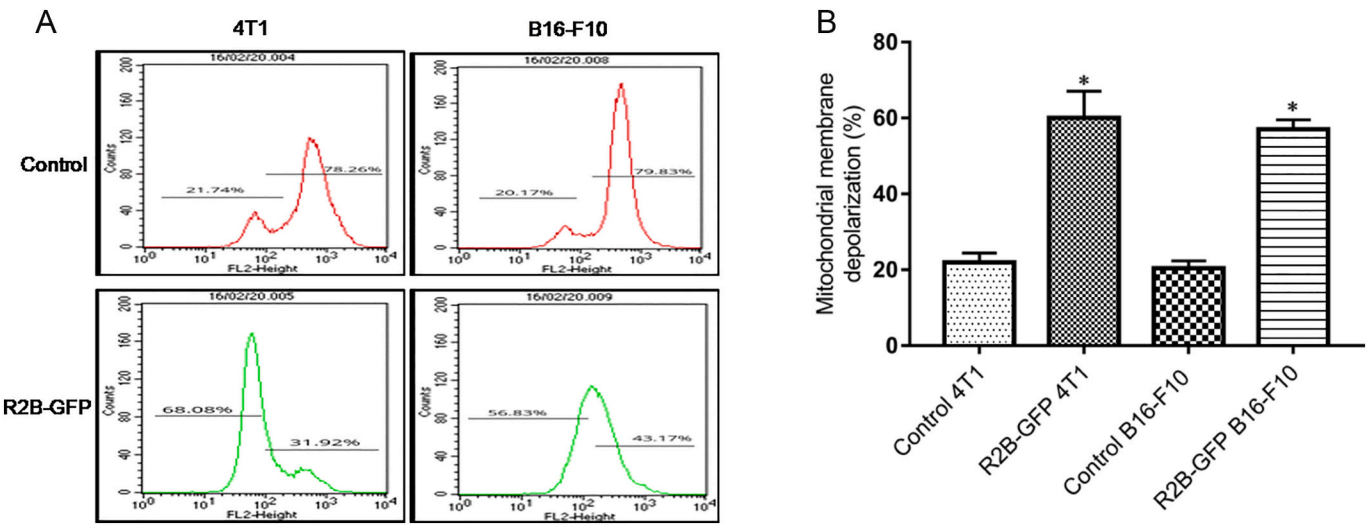

Fig. 5. Mitochondrial membrane potential was determined by flow cytometry using MitoTell™ Orange staining following infection of 0.1 MOI for 48 hpi with R2B-GFP virus in 4T1 and B16-F10 cells and respective uninfected control cells. (**A**) Representative histograms are illustrated. Note the histogram peaks of R2B-GFP virus infected cells shifting towards left due to loss of florescence when compare to their respective control. (**B**) Quantification of the percentage of mitochondrial membrane potential. The mitochondrial depolarization was significantly enhanced in both the cells infected with R2B-GFP virus. Twenty thousand events were evaluated per experiment, and cell debris was omitted from analysis. Data are presented as mean ± S.E.M. of at least three independent experiments. * $P < 0.0001$ compared with uninfected control by one way ANOVA.

mitochondrial outer membrane led to a decrease in mitochondrial membrane potential in the infected tumor cells when compared to the control uninfected cells. The loss of mitochondrial membrane permeability in tumor cells allowed the mitochondrial proteins seep through the pores resulting in loss of fluorescence (Elankumaran et al., 2006).

5. Conclusion

The present study has thrown considerable insights into the replicative potential of NDV-GFP virus in both the tumor cell lines *in-vitro* and the mechanisms underlying the apoptotic potential of the virus. However, the present study needs to be validated in live animal models to assess the therapeutic efficacy of this recombinant virus towards supportive treatment in different types of cancers of livestock and human origin.

Author contributions

S.D., M.M.C., V.N. conceived and designed the experiments; N.R., D. C·P, A.L.D., A.K.M., M.M.C., S.D., performed the experiments; M.M.C., S. D., N.R., analyzed the data; S.D., M.M.C., N.R., D.C.P., wrote the paper; S.D., M.M.C., V.N., A.K.M., N.R., D.C.P., R.B., and A.L.D., read and approved the manuscript.

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Declaration of Competing Interest

Authors hereby declare that there is no conflict of interest in the manuscript.

Acknowledgments

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