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Newcastle disease virus expressing an angiogenic inhibitor exerts an enhanced therapeutic efficacy in colon cancer model

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Abstract

Numerous studies demonstrate that the NDV-mediated gene therapy is a promising new approach for treatment of cancer. VEGF-Trap plays a vital role in anti-angiogenesis. Therefore, we hypothesize that a recombinant NDV (rNDV) expressing VEGF-Trap would be an ideal agent for the colon cancer therapy. In this study, VEGF-Trap gene was incorporated into the genome of rNDV (named rNDV-VEGF-Trap). rNDV-VEGF-Trap reduced cell growth ratio by 85.37% and migration ratio by 87.9% in EA.hy926 cells. *In vivo* studies, treatment with rNDV-VEGF-Trap reduced tumor volume of CT26-bearing mice by more than 3 folds and tumor weight by more than 4 folds. Immunohistochemistry analysis of CD34 showed rNDV-VEGF-Trap significantly decreased the number of vascular endothelial

cells in the tumor tissues of the tumor-bearing mice. Moreover, Western blot analysis demonstrated that treatment with rNDV-VEGF-Trap significantly decreased the phosphorylation levels of AKT, ERK1/2 and STAT3 and increased the expression levels of P53, BAX and cleaved caspase-3 in the tumor tissue. In addition, to evaluate the toxicity of VEGF-Trap, serum chemistries were analyzed. The results showed that rNDV-VEGF-Trap caused insignificant changes of creatinine levels, alanine aminotransferase (ALT) and aspartate transaminase (AST). Furthermore, administration of rNDV-VEGF-Trap did not cause the diarrhoea, decreased appetite, weight decrease and haemorrhage of the experimental mice. These data suggest that rNDV-VEGF-Trap exhibits an enhanced inhibition of CT26-bearing mice by enhancing anti-angiogenesis and apoptosis. rNDV-VEGF-Trap is a potential candidate for carcinoma therapy especially for colon cancer.

Keywords: rNDV, VEGF-Trap, Gene therapy, Anti-angiogenesis, Colon cancer

Introduction

As one of the main causes of human cancer death, the mortality and morbidity of colon cancer continued to rise¹. Currently, surgery, radiotherapy and chemotherapy are the major strategies for colon cancer, but the high recurrence rate and adverse reactions decreased overall survival rate². Colon cancer, which is similar to most other malignancies, has the risk of malignant invasion and metastasis^{3,4}. One-half of patients with colon cancer will develop liver or lung metastasis, which are associated with abundant vascular distribution⁵. Therefore, optimizing the treatment strategy is a major challenge to improve the survival rate⁶⁻⁸. Besides traditional methods, immunotherapy has become a very hot field, which includes oncolytic viruses, tumor vaccines and immune checkpoint inhibitors etc⁹⁻¹⁴. As the development of genetic engineering and related disciplines, oncolytic viruses have been widely applied to treat colon cancer in recent years¹⁵.

Newcastle disease virus (NDV) is affiliated to the family Paramyxoviridae, which is an avian paramyxovirus type I virus¹⁶⁻¹⁷. The 15186 nucleotides of NDV genome encode six structural proteins, which are nucleocapsid protein (NP), phosphoprotein (P), RNA-dependent RNA polymerase (L), matrix protein (M), hemagglutinin-neuraminidase (HN) and fusion protein (F)^{18,19}. NDV causes severe infection in avian species, but there is no significant symptoms to humans²⁰. It exhibits oncolytic ability through mainly replicates in tumor cells rather than normal cells^{21,22}. The oncolytic potency relies on the ability to induce apoptosis in infected cancer cells^{23,24}. However, it is not enough to treat tumor only depending on the oncolytic ability of NDV^{25,26}.

Angiogenesis plays a crucial role in tumor growth and metastasis^{27,28}. The growth, local tissue invasion and distant metastasis of colon cancer are highly dependent on angiogenesis^{29,30}. Colon cancer is one of the ideal angiogenesis-dependent solid tumors. In tumor cells, the activation of oncogenes and inactivation of tumor suppressor genes lead to the upregulated expression of VEGF^{31,32}. Overproduction of VEGF leads to abnormal changes in tumor vessels, such as distortion, fragmentation, and lack of pericytes. These dysfunctional and leaky vessels

promote the development of tumor tissue microenvironment that limits the delivery of anti-tumor drugs to the targets³³. VEGF and its signaling pathway represent attractive targets for the treatment of cancer, as this growth factor plays a role in various cancer types as an important pro-angiogenic cytokine³⁴. A previous study describes anti-angiogenic therapy using vascular endothelial growth factor trap (named VEGF-Trap or aflibercept), which is an effective protein that blocks the tumor-associated VEGF signaling pathway. VEGF-Trap, which is a fusion protein, is composed of some extracellular domains of VEGFR-1 and VEGFR-2 and FC segment of human IgG antibody³⁵. VEGF-Trap blocks the signal transduction of VEGF/VEGFR and inhibit angiogenesis and the tumor growth by binding with VEGF³⁶. However, repeated injections of VEGF-Trap lead to adverse reactions such as AST increase, serum creatinine increase, ALT increase, diarrhea, decreased appetite, weight decrease and haemorrhage of the patients³⁷. Therefore, the treatment of VEGF-Trap still needs to overcome these limitations.

In the present study, we generated rNDV-VEGF-Trap and evaluated its anti-angiogenesis, antitumor efficacy and security *in vitro* and *in vivo*. These data suggest that the virus possesses a significant inhibiting effect against vascular endothelial cells line EA.hy926 and exhibits an effective anti-angiogenesis, oncolytic activity and security in the CT26 tumor mouse model.

Materials and methods

Virus and Cell lines

The strain *E.coli* DH5 α , strain *E.coli* STBL2, rNDV vector , VEGF-Trap gene, auxiliary plasmid (PTM-NP, PTM-P and PTM-L), human colon cancer cell line (HCT116), human umbilical vein endothelial cell line (EA.hy926), mouse colon cancer cell line (CT26), mouse breast cancer cell line (4T-1), hamster kidney cell line (BHK-21), were supplied by northeast agricultural university biological pharmaceutical teaching and research section. CT26 and 4T1 were maintained in RPMI 1640 supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin. BHK-21 were maintained in DMEM containing 10% new-born calf serum (NCS) and 1% penicillin/streptomycin. HCT116 was maintained in McCoy's5A supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin. All cell lines were authenticated using Short Tandem Repeat profiling, tested for mycoplasma contamination and grown at 37 °C humidified incubator with 5% CO₂ atmosphere.

Other reagents

DMEM, McCoy's5A, RPMI 1640 were purchased from GIBCO company, and fetal bovine serum was purchased from PAN-Biotech; Restriction enzymes (Pme I , Sac II), rTaq DNA polymerase, T4 DNA ligase purchase since NEB company; DNA Marker, PMD18-T and dNTPs were purchased from TaKaRa Company. Lipofectmine 3000 transfection reagent purchased from Invitrogen company; The plasmid extraction kit was purchased from OMEGA; DNA recovery kit and PCR product purification kit were purchased from Qiagen Company. DNA PCR primers were synthesized by Shanghai Sangon. Anti-human IgG4 Fc (HRP) antibodies, anti-mouse Bcl-2, VEGF-Trap and CD34 antibodies were purchased from Abcam. Antimouse

β -actin, caspase-3, BAX, STAT3, AKT, p44/42MAPK(ERK1/2), phospho-STAT3 (named P-STAT3), phospho-AKT (named P-AKT) and phospho-p44/42MAPK(ERK1/2) (named P-ERK1/2) antibodies were purchased from cell signaling technology.

Recombinant Newcastle disease virus

The rclone30 strain was used to provide a backbone for construction of the recombinant virus. The specific operation steps follow the existing methods in the laboratory. The rNDV viruses were generated as previously described³⁸ and sequenced by reverse transcription PCR for fidelity. rNDV and rNDV-VEGF-Trap which express VEGF-Trap were kept in our lab.

Analysis of viral growth in CT26 cell line

Viral growth was determined in the CT26 cell line. Cells planted in 24-well plates were infected with 0.1 MOI rNDV or rNDV-VEGF-Trap. The supernatants were collected at 12, 24, 36, 48, 60 and 72h post infection. The viral concentration was measured at end-point titration on CT26 cells and calculated as 50% tissue culture infective dose (lgTCID₅₀) per mL.

Expression of VEGF-Trap by tumor cells infected with rNDV-VEGF-Trap

CT26 cells (5×10^6 cells) were infected with 1 MOI rNDV-VEGF-Trap and rNDV prespectively and the control group received PBS. The methods were accorded to description of Ying An's paper²⁴. After 24h incubation, cells were collected and washed twice with cold PBS by centrifugation at $500 \times g$ for 5 min at 4 °C. The pellet was resuspended in lysis buffer supplemented with proteases inhibitor and the supernatant was stored at -20 °C. For western blotting analysis, samples were separated by 10% sodium dodecylsulfate-poly acrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane. The blot was visualized by chemiluminescence and autoradiography using X-ray film. Each gel was tested with mouse anti-human IgG4 Fc (HRP) antibody to identify VEGF-Trap.

Cell viability assay

Cell viability was quantified by a short-term microculture tetrazolium (MTT) assay²⁴. Approximately $1-2 \times 10^4$ CT26, 4T1, HCT116 and EA.hy926 cells were plated into 96-well plates in complete medium. Then, these cells were infected with rNDV-VEGF-Trap, rNDV at the dose of 0.01 MOI, 0.1 MOI, 1 MOI, and 10 MOI in triplicate. According to Ying An's paper²⁴, 20 μ L MTT solutions (5 mg/mL in sterile phosphate-buffered saline) were added to the cell after 48h of incubation. After 4 hours, the MTT solution in the wells was discarded, then 150 μ L dimethyl sulfoxide (DMSO) was added. The absorption was measured by a microplate reader at 490 nm (OD₄₉₀). The cell viability was converted and expressed as the percentage of the control. The cells without any treatment were used as negative control.

Inhibition rate = (control group OD - treatment group OD)/control group OD $\times$ 100%

Migration assay

The effect of rNDV-VEGF-Trap on cell migration was measured by wound healing experiments³⁹. The same EA.hy926 cell numbers were respectively seeded into 6-well plates as control and treatment groups. Then, a cell-free area of confluent

monolayer cells was created by scratching with a 1 mL pipette tip. After washing the isolated cells, monolayer cells were cultured in a maintenance medium containing 2% FBS. The cells were incubated with 1 MOI rNDV or rNDV-VEGF-Trap for 24h and 48h, then washed with PBS and photographed. The representative images were captured under 100 magnification with a camera (Nikon, Japan) attached to a microscope. For comparing the migration rate, scratches were photographed in three separate fields, and then the scratch area was measured by ImageJ. The experiment was repeated three times.

Analysis of tumor growth in colon cancer model

All procedures involving animals were according to the guidelines issued by National Institute of Health and the Institutional Animal Care and Use Committee of Northeast Agriculture University. Healthy 6-week-old female BALB/c mice with a weight of 19 ± 1 g were permitted to feed and drink freely. CT26 cells (1×10^6) were subcutaneously implanted into the right groin of mice and tumors were allowed to grow until the average diameter reached 5–8 mm. Reference to previous publications^{23,24}, Mice were randomly divided into different groups ($n = 10$), and intratumorally injected with allantoic fluid or 1×10^7 pfu of the indicated viruses every two day. Tumor volumes were calculated using the following formula: tumor volume (V) = $\frac{4}{3} \times \pi \times S^2/2 \times L/2$, where S is the smallest measured diameter and L is the largest diameter. Animals were humanely culled when tumor size reached 18 mm in any dimension or at defined experimental time points. After the treatment, animals of each group were sacrificed, their tumors and spleens were excised, weighted, frozen, or fixed in 4% paraformaldehyde.

Histological analysis and Immunohistochemical (IHC) assay

According to the method of Elham Assareh paper⁵⁸, the tumor tissues of tumor-bearing mice were fixed with 4% formalin at room temperature for 2 days, treated with graded concentrations of ethanol and xylene, and embedded in paraffin. The sections of 4-5 mm were mounted on adhesive glass slides. Tumor tissue sections were stained with Hematoxylin and eosin (HE) for investigation of morphological changes in treatment and control groups. IHC staining analysis was performed to localize specific tissue antigens. The sections were incubated at 4 °C with the primary mouse monoclonal antibodies CD34 (1:2500) for overnight. Antigens is detected with 3,3-diaminobenzidine (DAB). All images were analyzed using Image J software.

Western blot analysis

CT26 tumor tissues were lysed with RIPA buffer containing protease and phosphatase inhibitors (Beyotime, China) as described by Maryam Farizaneh Behelgard report⁵⁹. After centrifugation, the protein samples were treated with 5 × loading buffer (Beyotime, China). Protein concentrations were determined by the BCA quantitative kit (Beyotime, China). Equal amounts of total proteins were added into 12% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis. Proteins were transferred to polyvinyl difluoride (PVDF) membranes followed by blocking with 5% non-fat milk for 2h. The primary antibodies including anti-P-AKT, anti-total-AKT, anti-P-STAT3 (1:2000), anti-P-ERK1/2, anti-total-STAT3, anti-total-ERK1/2, anti-P53, anti-BAX, anti-Bcl-2 and anti-caspase-3 (1:1000) were

incubated overnight at 4 °C. After three times washing in Tris Buffered Saline-Tween (TBS-T), the membranes were incubated with an appropriate HRP-conjugated secondary antibody for 1 h at room temperature. Protein bands were visualized using ECL reagent.

Animal safety assessment

Based on the exposure of VEGF-Trap in clinical treatment of various adverse reactions. The diarrhoea, appetite, weight and haemorrhage of the experimental mice were recorded throughout the treatment phase. At the end of the animal experiment, whole blood was collected through the eyeballs of the experimental mice. Then the serum was isolated from whole blood for determination of serum concentrations of AST, ALT and creatinine.

Statistical analysis

The statistical significance of quantitative data between different groups was determined with GraphPad prism software (Version 5.01, GraphPad Software Inc., La Jolla, California). All data were expressed as mean $\pm$ SEM (standard error of the mean), significance was determined by performing one- or two-sided Student's *t* tests and defined as a *p* value < 0.05, *p* value < 0.01.

Results

Generation of rNDV-VEGF-Trap

The VEGF-Trap gene was incorporated at the position between the P and M genes of the rNDV genome (Fig. 1A). The incorporated plasmid was transfected into BHK-21 cells. The viral population was amplified by inoculating to the cell cultures and then transferred into 10 days SPF embryo chicken eggs²⁴. After 3 days, the allantoic fluid was harvested and analyzed in a HA test²⁴. The successful generation of rNDV-VEGF-Trap virus were evaluated by the high titers of virus.

Growth and VEGF-Trap protein expression of rNDV-VEGF-Trap

In order to verify the proliferation ability of rNDV-VEGF-Trap in tumor cells, CT26 cells were infected with 0.1 MOI rNDV or rNDV-VEGF-Trap. The supernatant was harvested at different time points. Then the viral titers in the supernatants were determined in triplicate. As shown in Fig. 1B, compared with the parental virus, rNDV-VEGF-Trap has no significant difference in the kinetics of replication. To investigate the expression of VEGF-Trap protein, CT26 cells were treated with 1 MOI rNDV-VEGF-Trap. Western blotting analysis Fig. 1C showed that the VEGF-Trap expression was detected the tumor cells infected with rNDV-VEGF-Trap.

rNDV-VEGF-Trap suppresses tumor cells and vascular endothelial cells growth

The direct cytotoxic activity of rNDV-VEGF-Trap was tested in CT26, 4T1, HCT116, EA.hy926 cells in this study. As shown in Fig. 2A, rNDV-VEGF-Trap induced cell apoptosis in a dose-dependent manner during 48 h treatment. When the cells were infected with rNDV-VEGF-Trap or rNDV of 10 MOI for 48h, the inhibition rates for EA.hy926 were respectively 85.37% $\pm$ 3.20% and 74.26% $\pm$ 2.12%. However, there is no significant difference between rNDV and rNDV-VEGF-Trap in inhibiting the growth of the other three cell types. In summary, these data indicate that rNDV-VEGF-Trap significantly inhibits the growth of

EA.hy926 cells compared with rNDV.

Effects of rNDV-VEGF-Trap on EA.hy926 cell migration

Scratch test was used to investigate changes in the migration of EA.hy926 cells after treatment with rNDV and rNDV-VEGF-Trap for 48h. As shown in Fig. 2B, the migration rate of EA.hy926 cell in PBS group, rNDV group and rNDV-VEGF-Trap were respectively 81.79%, 26.5% and 12.1% at 48h. The results showed that rNDV-VEGF-Trap significantly inhibited EA.hy926 cell migration compared with rNDV.

rNDV-VEGF-Trap suppresses tumor growth in mice

When the tumor size reached about 100 mm^3 , the mice were divided into three groups. Their tumor volume and weight were recorded every 2 days. The average tumor size for the model group, rNDV group and rNDV-VEGF-Trap group were $1889.18 \pm 408.16 \text{ mm}^3$, $728.49 \pm 287.41 \text{ mm}^3$ and $351.1 \pm 142.58 \text{ mm}^3$ (Fig. 3A). After necropsy, tumors excised from each group showed that rNDV-VEGF-Trap significantly ameliorates tumor texture and vascular density compared to both model and rNDV groups (Fig 3B). Moreover, the average tumor weight of each group were respectively $1.456 \pm 0.459 \text{ g}$, $0.603 \pm 0.311 \text{ g}$ and $0.169 \pm 0.154 \text{ g}$ (Fig. 3C). The average rates of tumor/weight in each group were respectively 5.34 ± 0.81 , 2.22 ± 0.33 and 0.64 ± 0.24 (Fig. 3D). Taken together, these data demonstrate that treatment with rNDV-VEGF-Trap significantly inhibited tumor growth compared to treatment with rNDV in the colon cancer model.

The tumor tissue treated with rNDV-VEGF-Trap show antineoplastic activity and anti-angiogenesis

As shown in Fig. 4A, H&E staining tumor sections showed that tumors derived from rNDV-VEGF-Trap demonstrated significant suppression to tumor cell viability than rNDV and model group. Then we evaluated the inhibition of rNDV-VEGF-Trap on vascular endothelial cell proliferation in the tumor tissue by CD34 assay. Compared with model group and rNDV group, we found that the expression of CD34 was significantly decreased by treatment with rNDV-VEGF-Trap (Fig. 4B). These data suggest that rNDV-VEGF-Trap exhibits an antiangiogenic role.

rNDV-VEGF-Trap modulates the related protein expression of angiogenesis and apoptosis

To elucidate the antiangiogenic mechanisms of rNDV-VEGF-Trap, the related proteins of VEGF signaling pathway were analyzed by Western blot. As shown in Fig. 5A, rNDV-VEGF-Trap treatment significantly reduced the expression levels of P-AKT, P-ERK and P-STAT3, while there was no difference in expression levels of AKT, ERK, and STAT3. The results showed that rNDV-VEGF-Trap has the ability to inhibit the related protein activation of VEGF/VEGFR downstream signaling pathways. Then the related protein expression of tumor apoptosis were measured by Western blot. As show Fig.5B, rNDV-VEGF-Trap significantly increased the expression levels of P53, BAX and cleaved caspase-3 and significantly downregulated the expression level of Bcl-2 compared with rNDV. The results show that rNDV-VEGF-Trap significantly enhances tumor apoptosis. These data suggest that rNDV-VEGF-Trap further promotes apoptosis through anti-angiogenesis,

consequently inhibiting the growth of tumor tissue.

rNDV-VEGF-Trap demonstrates therapeutic safety

Previous papers demonstrate that administration of VEGF-Trap exhibits common adverse events in clinical, including diarrhoea, decreased appetite, weight loss, haemorrhage, deteriorated creatinine levels, AST and ALT. In this study, our results showed these common adverse events were not significantly observed in each group (normal, model, rNDV, rNDV-VEGF-Trap) of the CT26-bearing mice (Table1, Fig. 6). These results suggest that rNDV-VEGF-Trap exhibits therapeutic safety.

Discussion

As one of the oncolytic viruses, NDV represents a new class of therapeutic agents of cancer therapy⁴⁰. Some clinical trials of phase I-IV colon cancer based on NDV vectors have shown encouraging results⁴¹. At present, VEGF-Trap, bevacizumab, cetuximab and panitumumab show promising in antiangiogenic therapy, in which VEGF-Trap has the advantages of significant therapeutic effect and long Half-Life compared with others^{42,43}. However, VEGF-Trap inevitably arises multiple adverse effects in the clinical treatment of colon cancer³⁷. Previous study showed that researchers used recombinant AAV2 as a delivery vehicle to achieve long-lasting expression of VEGF-Trap protein in a mouse model, simultaneously suppressing primary tumor growth and preventing the metastases of tumors⁴⁴. However, the therapeutic effect of AAV2 vector was not significant. These problems indicate that it is important for reducing the adverse reactions of VEGF-Trap and enhancing colon cancer apoptosis and anti-angiogenesis through VEGF-Trap gene was integrated into NDV genes.

In this study, we generated rNDV-VEGF-Trap using reverse genetics. As anticipated, the kinetics of replication of rNDV-VEGF-Trap did not show a significant change and rNDV-VEGF-Trap successfully expressed VEGF-Trap protein in the CT26 tumor cells. MTT results showed that the EA.hy926 cells were infected with the virus at 10 MOI for 48h, the apoptosis rate induced by rNDV-VEGF-Trap was 85.37% and rNDV was 74.26%. The results showed that rNDV-VEGF-Trap enhanced pro-apoptotic activity of the virus. Scratch test results show that the EA.hy926 cells were infected with the virus at 1 MOI for 48h, the relative mobility rate induced by rNDV-VEGF-Trap was 12.1% and rNDV was 26.5%. The results showed that rNDV-VEGF-Trap enhanced the ability of the virus to inhibit the migration of EA.hy926 cells. The results are similar to the previously report that VEGF-Trap protein inhibits vascular endothelial cell proliferation and migration³⁶. In summary, rNDV expressing VEGF-trap was successfully generated in this study. *In vitro*, VEGF-Trap protein expressed from rNDV-VEGF-Trap inhibits the proliferation and migration of vascular endothelial cells.

Based on the encouraging *in vitro* data, we investigated the therapeutic efficacy of rNDV-VEGF-Trap in CT26-bearing mice. The results show that treatment with rNDV-VEGF-Trap reduced tumor volume of CT26-bearing mice by more than 3 folds and tumor weight by more than 4 folds compared to rNDV. The results indicate that rNDV-VEGF-Trap effectively inhibits tumor growth. Previous studies have shown

that tumor tissue angiogenesis is caused by excessive and abnormal proliferation of vascular endothelial cells^{45,46}. As surface markers of vascular endothelial cells, CD34 is commonly applied to detect the situation of vascular proliferation^{47,48}. Immunohistochemistry results of CD34 showed rNDV-VEGF-Trap significantly reduced the number of vascular endothelial cells in the tumor tissues of the tumor-bearing mice. The results indicate that rNDV-VEGF-Trap has a significant antiangiogenic ability in tumor tissue than rNDV. In summary, rNDV-VEGF-Trap significantly enhances the inhibition effect on the tumor growth of the CT26 tumor-bearing mice and VEGF-Trap protein expressed by rNDV-VEGF-Trap effectively inhibits the proliferation of vascular endothelial cells in tumor tissues.

Based on the results of *in vivo* experiments, we have shown that rNDV-VEGF-Trap has the ability to inhibit tumor growth and angiogenesis in the tumor. However, the mechanism of rNDV-VEGF-Trap inhibiting angiogenesis and tumor growth is not clear. Previous studies have shown that VEGF-Trap binds VEGF and prevents its binding to and activation of VEGFR³⁶. As a pivotal and pervasive pro-angiogenic factor, VEGF regulates multiple facets of tumor angiogenesis through VEGFR⁴⁹. In response to its binding, VEGFR initiates signaling through the Raf-MEK-ERK, PI3K-AKT kinase cascades⁵⁰⁻⁵² and STAT3⁵³. Signaling through these pathways promotes proliferation, survival and chemotaxis in endothelial cells and ultimately produces the characteristic effects of VEGF on vessels, such as increased vascular permeability and angiogenesis^{54,55}. In general, these signaling pathways in endothelial cells are not activated in normal mouse organs, but only in blood vessels of tumor tissues. In addition, these signaling pathways were activated not only by VEGF-VEGFR, but also by other angiogenic promoters (such as basic FGF) and thus, may serve as general indicators of angiogenic activation^{56,57}. To determine these signaling pathways are blocked in tumor vessels *in vivo* by rNDV-VEGF-Trap, we employed Western blot for detection of ERK (P-ERK), AKT (P-AKT) and STAT3 (P-STAT3) in tumor tissue in mice. The results showed that treatment with rNDV-VEGF-Trap significantly reduced the phosphorylation levels of AKT, ERK and STAT3 compared with rNDV or PBS. This suggests that rNDV-VEGF-Trap inhibits tumor tissue angiogenesis by blocking the activation of these signaling pathways. Previous studies demonstrated that VEGF-Trap indirectly induces apoptosis of tumor cells by inhibiting angiogenesis³⁶. In order to demonstrate that rNDV-VEGF-Trap enhances virus-mediated tumor tissue apoptosis, we detected the expression levels of P53, BAX, Bcl-2 and cleaved caspase-3 in tumor tissues by Western blotting. The results showed that the expression levels of P53, BAX, and cleaved caspase-3 in rNDV-VEGF-Trap treated tumor tissues were significantly increased and the expression levels of Bcl-2 were significantly decreased compared with rNDV. This indicates that rNDV-VEGF-Trap significantly promotes the apoptosis of tumor tissue. In this part, we preliminarily confirmed the mechanism by which rNDV-VEGF-Trap inhibits angiogenesis and tumor growth. First, rNDV-VEGF-Trap inhibits the activation of AKT, ERK and STAT3 signaling pathways by blocking VEGF and VEGFR binding, leading to a reduced tumor angiogenesis. Secondly, the VEGF-Trap effectively enhanced the viral-vector-

mediated tumor tissue apoptosis by inducing the expression of apoptotic proteins P53, BAX, and cleaved caspase-3.

In previous studies, purified VEGF-Trap proteins were applied to cancer therapy, and the main problem was the side effects³⁷. In order to evaluate the adverse reactions of rNDV-VEGF-Trap *in vivo*, a toxicity study was performed. We have detected the AST, creatinine, ALT, diarrhoea, appetite, weight and haemorrhage of the experimental mice. The results showed that these parameters in rNDV-VEGF-Trap treated mice were all within the normal range, indicating that rNDV-VEGF-Trap was relatively safe in the treatment of colon cancer.

In conclusion, our study shows that rNDV-VEGF-Trap can effectively inhibit the tumor growth and angiogenesis of the CT26-bearing mice. Moreover, treatment with rNDV-VEGF-Trap reduced the phosphorylation levels of AKT, ERK1/2 and STAT3, increased the expression levels of P53, BAX and cleaved caspase-3 in the tumor tissue. In addition, rNDV-VEGF-Trap effectively reduces the adverse reactions induced by the treatment of purified VEGF-Trap. Thereby, this study provides a potential candidate for the treatment of colon cancer.

Availability of data and materials

The data analyzed during the current study may be available upon reasonable request.

Ethics statement

This study was approved by the ethics committee of Northeast Agriculture University. All experimental protocols involving animals followed the guidelines issued by National Institute of Health and the Institutional Animal Care and Use Committee of Northeast Agriculture University.

Conflicts interest

The authors declare that there are no conflicts of interest.

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Author' contributions statement

FR, M. YK, C performed the research, and analyzed the data. **H, S. LM, T** and **JR, Y** participated in data collection and analysis. **W, X** and **DS, Li** contributed to the initial and consequent project discussion, manuscript discussion and revision. All the authors approved the final version of the manuscript.

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Figure

Table1

Diarrhoea, appetite, weight and haemorrhage situation of CT26-bearing mice throughout the treatment phase.

	Normal	Model	rNDV	rNDV-VEGF-Trap
Diarrhoea	no	no	no	no
Decreased appetite	no	no	no	no
Weight (g)	26 ± 1.3	25 ± 0.5	24 ± 1.6	25 ± 0.8
Haemorrhage	no	no	no	no

Data was presented from ten mice per group. no: No clinical symptoms were observed during the experimental period.

Fig.1 Schematic representation of the genomes, characterization and VEGF-Trap protein of rNDV-VEGF-Trap (A) Diagram showed insertion of the VEGF-Trap gene into the NDV genomer at the position between the P and M genes. (B) Growth curves of rNDV-VEGF-Trap. CT26 cells were infected with rNDV and rNDV-VEGF-Trap at 0.1 MOI. Cell monolayers were lysed at 12, 24, 48, 60 and 72h post-infection for intracellular titer measurement by TCID50. (C) The expression of VEGF-Trap proteins in CT26 cells. CT26 cells were respectively infected with 1 MOI rNDV-VEGF-Trap or rNDV and the control group received PBS. After 24h incubation, cells were washed twice with cold PBS and collected by centrifugation at 500 × g for 5 min at 4 °C. The cells were resuspended in lysis buffer supplemented with proteases inhibitor and after centrifugal the supernatant was used to western blot.

Fig.3 rNDV-VEGF-Trap enhances inhibition of tumor growth of CT26-bearing mice. Six-week-old female BALB/c mice were injected with 10^6 CT26 cells in the right groin. When the tumor size reached about 100 mm^3 , the mice were intratumorally treated with rNDV or rNDV-VEGF-Trap at 10^7 pfu. The mice were euthanized and necropsy was performed after 15 days of treatment. (A) The tumor volume of CT26-bearing mice was measured in two dimensions every other day using digital calipers. (B) Tumor collected in the end of the experiment. (C) Tumor Weight. (D) Tumor to body weight ratio. Model: mice treated with allantoic fluid. $n=10$; ns, no significant; $*P < 0.05$; $**P < 0.01$.

Fig.4 rNDV-VEGF-Trap suppression tumor cell viability and angiogenesis. Six-week-old female BALB/c mice were injected with 10^6 CT26 cells in the right groin. When the tumor size reached about 100 mm^3 , the mice were intratumorally injected with rNDV or rNDV-VEGF-Trap at 10^7 pfu. The mice were euthanized and necropsy was performed after 15 days of treatment. (A) H&E staining of CT26 tumor section after treatment. (B) CD34 in the tumor tissues were visualized by IHC assay. The result were shown in brown. The little the brown area, the less the proliferation of vascular endothelial cells was. Model: mice treated with allantoic fluid. N=3. Magnification: 100 $\times$, 200 $\times$, 400 $\times$; scale bar = 20 μm , 50 μm , 100 μm .

Fig.5 rNDV-VEGF-TRAP inhibits the phosphorylation of the VEGF signaling pathway related proteins AKT, ERK1/2, STAT3 and mediates the expression of the apoptosis-related proteins P53, Bcl-2, BAX, caspase-3. Six-week-old female BALB/c mice were injected with 10^6 CT26 cells in the right groin. When the tumor size reached about 100 mm^3 , the mice were intratumorally treated with rNDV or

rNDV-VEGF-Trap at 10^7 pfu. The mice were euthanized and necropsy was performed after 15 days of treatment. CT26 tumor tissue (each group 0.5 g) was homogenized, the supernatant was collected after centrifugation. The expression of the VEGF signaling pathway related proteins and the apoptosis-related proteins in supernatant was detected by Western blot. (A) The expression level of AKT, P-AKT, ERK1/2, P-ERK1/2, STAT3 and P-STAT3. (B) The expression level of P53, Bcl-2, BAX and caspase-3. β -actin was used as reference to quantify the protein bands by densitometry using ImageJ software. All data were expressed as mean $\pm$ SEM, N = 3, (ns, no significant; * P < 0.05; ** P < 0.01) compared to the model.

Fig. 6 rNDV-VEGF-Trap demonstrates therapeutic safety. At the end of the animal experiment, whole blood was collected through the eyeballs of the experimental mice. Then the serum was isolated from whole blood for determination of serum concentrations of AST, ALT and creatinine (A). The serum biochemistry data of the experimental mice values were expressed as mean $\pm$ SEM. ALT, alanine aminotransferase; AST, aspartate transaminase; CREA, creatinine; Normal, normal mice. (ns, no significant).

Figures

Figure 1

Schematic representation of the genomes, characterization and VEGF-Trap protein of rNDV-VEGF-Trap (A) Diagram showed insertion of the VEGF-Trap gene into the NDV genomer at the position between the P and M genes. (B) Growth curves of rNDV-VEGF-Trap. CT26 cells were infected with rNDV and rNDV-VEGF-Trap at 0.1 MOI. Cell monolayers were lysed at 12, 24, 48, 60 and 72h post-infection for intracellular titer measurement by TCID50. (C) The expression of VEGF-Trap proteins in CT26 cells. CT26 cells were respectively infected with 1 MOI rNDV-VEGF-Trap or rNDV and the control group received PBS. After 24h incubation, cells were washed twice with cold PBS and collected by centrifugation at 500 × g for 5 min at

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Figure 2

In vitro studies (A) Cytotoxic effects of rNDV-VEGF-Trap on cancer cells and vascular endothelial cells. 4T-1, CT26, HCT116, and EA.hy926 cells incubated with PBS were used as controls. MTT method was used to measure the cell density. The data are the means ± SEM. of triple samples (*P < 0.05; **P < 0.01). (B)

Inhibition of cell migration by recombinant virus. Wound healing assay was performed on EA.hy926 cells, the cells were infected with 1 MOI rNDV-VEGF-Trap or rNDV and cell migration was evaluated at regular time points post-infection. The number of migrated cells were assessed by microscope. The assay was performed in triplicate. The data are the means $\pm$ SEM of triple samples (ns, no significant; *P < 0.05; **P < 0.01).

Figure 3

rNDV-VEGF-Trap enhances inhibition of tumor growth of CT26-bearing mice. Six-week-old female BALB/c mice were injected with 106 CT26 cells in the right groin. When the tumor size reached about 100 mm³, the mice were intratumorally treated with rNDV or rNDV-VEGF-Trap at 107 pfu. The mice were euthanized and necropsy was performed after 15 days of treatment. (A) The tumor volume of CT26-bearing mice was measured in two dimensions every other day using digital calipers. (B) Tumor collected in the end of the experiment. (C) Tumor Weight. (D) Tumor to body weight ratio. Model: mice treated with allantoic fluid. n=10; ns, no significant; *P < 0.05; **P < 0.01.

Figure 4

rNDV-VEGF-Trap suppression tumor cell viability and angiogenesis. Six-week-old female BALB/c mice were injected with 106 CT26 cells in the right groin. When the tumor size reached about 100 mm³, the mice were intratumorally injected with rNDV or rNDV-VEGF-Trap at 107 pfu. The mice were euthanized and necropsy was performed after 15 days of treatment. (A) H&E staining of CT26 tumor section after treatment. (B) CD34 in the tumor tissues were visualized by IHC assay. The result were shown in brown. The little the brown area, the less the proliferation of vascular endothelial cells was. Model: mice treated with allantoic fluid. N=3. Magnification: 100×, 200×, 400×; scale bar = 20 μm, 50 μm, 100 μm.

Figure 5

rNDV-VEGF-TRAP inhibits the phosphorylation of the VEGF signaling pathway related proteins AKT, ERK1/2, STAT3 and mediates the expression of the apoptosis-related proteins P53, Bcl-2, BAX, caspase-3. Six-week-old female BALB/c mice were injected with 106 CT26 cells in the right groin. When the tumor size reached about 100 mm³, the mice were intratumorally treated with rNDV or rNDV-VEGF-Trap at 10⁷ pfu. The mice were euthanized and necropsy was performed after 15 days of treatment. CT26 tumor

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