

Original Article

Recombinant avirulent newcastle disease virus expressing the rabies virus glycoprotein (rL-RVG) inhibits migration of lung cancer cells

Yu-Lan Yan^{1*}, Hui Jin^{1*}, Xiao-Mei Shao², Sha Liu², Ying-Hai Zhao², Xue-Feng Bu³

Departments of ¹Respiratory Medicine, ³General Surgery, Affiliated People's Hospital of Jiangsu University, Zhenjiang 212002, Jiangsu, P. R. China; ²Department of Internal Medicine, Clinical Medicine College of Jiangsu University, Zhenjiang 212013, Jiangsu, P. R. China. *Co-first authors.

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Abstract: The aim of the study is to demonstrate the effect of recombinant avirulent newcastle disease virus expressing the rabies virus glycoprotein (rL-RVG) on the migration of lung adenocarcinoma A549, LA795 and SCLC H446 cells and to explore its possible mechanism. The group directly infected by rL-RVG was determined as the experimental group accompanied with Newcastle disease virus (NDV) group and a control group with PBS. The expression of RVG and NDV was observed by immunofluorescence assay. MTT was used to identify the optimal concentration of NDV and rL-RVG. Scratch test and Transwell assay tested the migration of lung adenocarcinoma A549, LA795 and SCLC H446 cells infected by rL-RVG or NDV. Western blot and immunofluorescence assay were used for detection of E-cadherin and MMP2 protein expression. NDV was expressed in lung cancer cells infected by both rL-RVG and NDV, but RVG was expressed only in rL-RVG-infected cells. Compared with the control group, rL-RVG and NDV on the certain dilution concentration inhibited the cell proliferation ($P < 0.05$), the inhibitory effect of rL-RVG was more obvious than NDV. The migration distance and the number of cell migration in rL-RVG group declined significantly ($P < 0.05$). Furthermore, the expression level of E-cadherin protein was up-regulated ($P < 0.05$) and the expression of MMP2 protein was down-regulated ($P < 0.05$) in rL-RVG group. In conclusion, the rL-RVG can inhibit the migration of human lung cancer cell lines A549, LA795, H446 by influencing E-cadherin and MMP2 in the process of epithelial mesenchymal transition (EMT).

Keywords: rL-RVG, NDV, migration, EMT, lung cancer

Introduction

Lung cancer is the most common malignant tumor in the human respiratory system, and the 5 year survival rate of lung cancer is lower than other malignant tumors, which have a great impact on human life [1]. About 60% of patients with stage IIIA lung cancer had recurrence and metastasis within two years after the operation [2].

Newcastle disease virus (NDV) belongs to Paramyxoviridae virus family and Paramyxoviridae virus subfamily of paramyxovirus genera with non segmented single stranded negative strand RNA virus [3]. NDV can produce cytotoxic effect on tumor cells directly and specifically, combing viral protein with tumor cell membrane fragments or intact cells which improves

the immunogenicity of tumor antigen, so as to enhance the ability of host cellular and humoral immune antitumor [4, 5].

The recombinant avirulent newcastle disease virus expressing the rabies virus glycoprotein (rL-RVG) having been constructed with the reverse genetics technique, acted as vector for gene therapy of cancer. In the early studies, it has been demonstrated that the insertion of extraneous gene affected neither the main features of NDV replication nor its tumor selectivity. Further, RVG expression could promote NDV intercellular communication and enhance their capacity to reproduce [6].

At present, a lot of researches have indicated that epithelial-mesenchymal transition (EMT) of malignant tumors is the key step of tumor

metastasis [7, 8]. EMT can decrease the adhesion of normal epithelial cells, which is the core of the EMT [9]. It is worth pointing out that the reduced or missing expression of E-cadherin is considered to be an important marker of EMT phenomenon in lung cancer and other malignant tumors at present [10]. Existing studies show that these signal transduction pathways often acted as signaling upstream of a transcription factor on the EMT process control [11, 12].

In our previous study, we found rL-RVG could strongly inhibit the growth and advance the apoptosis of human lung adenocarcinoma A549 cells [13, 14]. However, the effect of rL-RVG on the migration of lung cancer cells and its potential mechanism has not yet to be elucidated. So in this experiment, we aim to reveal the role of rL-RVG on A549, LA795 and SCLC H446 cells, including cell migration ability and its mechanism.

Materials and methods

Materials

Newcastle disease virus (NDV) LaSota strain and rL-RVG were provided by Harbin Veterinary Research Institute. Goat anti-rabbit antibody of beta-actin was acquired from Santa Cruz (Santa Cruz, CA, USA). MTT Kit was taken from Sigma. Goat anti-rabbit E-cadherin and MMP2 antibody were from Boster (Wuhan, Hubei, China). HRP labeled goat anti-rabbit IgG secondary antibody was purchased from Beijing ComWin Biotech Co, Ltd. Human lung adenocarcinoma A549 cells and LA795 cells were preserved in the institute of basic medical science, Jiangsu University. SCLC H446 cells were purchased from Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China.

Methods

MTT assays: rL-RVG and LaSota NDV strain were diluted 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} times by serum-free DMEM. A549, LA795 and H446 cells in the logarithmic growth phase were seeded at 5×10^4 cells/ml in 96-well plates, infected by rL-RVG or NDV, incubated overnight. DMEM supplemented with 2% fetal bovine serum (FBS) was added to the cells of rL-RVG and NDV groups on the following day. Phosphate-buffered saline (PBS) group was used as control group. Next, cells were placed in quintuplicate wells in the presence of rL-RVG, NDV and PBS, respectively. 20 μ l MTT (5 mg/ml) was then added into each well. After 4 h of incubation at 37°C, 150 μ l DMSO solution was added to each well and plates were agitated for 10 min. Finally, the absorbance at 490 nm wavelength was read using standard spectrophotometer. The above MTT experiment was repeated for three times and the cell viability was calculated by the following formula. Cell viability = (the average A of the experimental group/control group A) $\times 100\%$.

Protein extraction and western blot analysis: Lung cancer cells were plated at the six-well plates (5×10^4 cells/ml), infected by rL-RVG or NDV, while the control group was treated with PBS. 1×10^6 cells per group were lysed with 100 μ l radioimmunoprecipitation assay buffer after 24 h culture. Subsequently, the supernatant was mixed with loading buffer, then heated the protein for 5 min. Next, equivalent amounts of protein were separated by 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, CA, USA). The membrane was blocked in TBST with 5% BSA for 1 h and incubated with RVG, NDV and beta-actin antibody overnight respectively at 4°C, and washed with Tris-buffered saline containing 1% Tween-20, followed by incubation 2 h with goat anti-mouse IgG conjugated to HRP and rabbit anti-chicken IgG conjugated to HRP at RT, and washed as indicated above. Finally, the membrane was visualized using an enhanced chemiluminescence development solution by the Typhoon 9400 scanner. E-cadherin, MMP2 detection method was same as above.

Cell scratch migration assay: Lung cancer cells were inoculated into 24-well plates (1×10^5 cells/ml), and the high glucose medium containing 10% FBS medium was used to culture for 24 h. With sterile 200 μ l gun head in cell layer in vertical crossed to form a uniform width of no wound cell model. Add rL-RVG, NDV (10^{-5} dilution) and PBS respectively to above different group cells which then cultured for 24 h. Finally, the widths of the scratches were observed under optical microscope.

rL-RVG inhibits migration of lung cancer cells

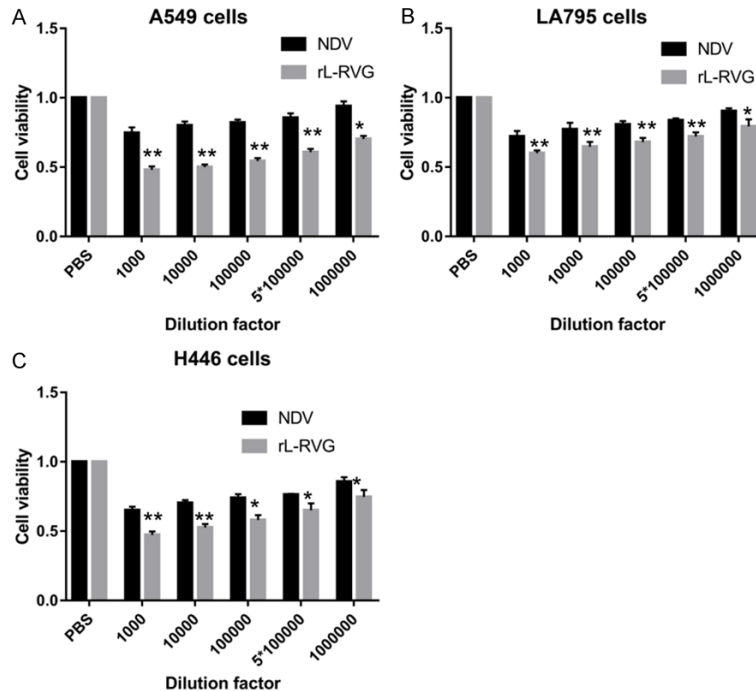


Figure 1. rL-RVG inhibited the cell viability of A459, LA795 and H446 cells. The cells were infected by six different dilution factor of viruses and MTT was used to detect the cells viability. In (A-C), the cells viability decreased obviously at 10^{-3} , 10^{-4} , 10^{-5} dilution factor (**, $P < 0.01$) in A549, LA795 and H446 cells. And the cell viability also decreased at 5×10^{-5} dilution factor in A549 and LA795 cells (**, $P < 0.01$). In (C), the cell viability also decreased at 5×10^{-5} dilution factor in H446 cells (*, $P < 0.05$). The cell viability decreased at 10^{-6} dilution factor in the three cell lines (*, $P < 0.05$).

Transwell assay: Lung cancer cells concentration in the logarithmic phase was diluted by serum free DMEM to 1×10^5 cells/ml. The cells were plated in transwell upper chamber of 24-well plates. Transwell lower room was added DMEM containing rL-RVG or NDV, 37°C cultured for 24 h. Wipe off the Transwell with a cotton swab on the surface of cells, Wash 2 times in PBS, the room would be placed in the 4% poly formaldehyde and fixed 15 min, Washed 2 times in PBS, stain with crystal violet 20 min, and then washed 2 times in PBS. Finally, the average value of the through membrane cell count ($200\times$) was calculated by 5 different fields of vision under the inverted fluorescence microscope.

Immunofluorescence

After NDV and rL-RVG treatment, cells in a 24-well plates were fixed in 4% paraformaldehyde for 20 min at room temperature, washed three times in PBS for 5 min, and permeabilized for 1 hour at 37°C by 0.3% TritonX-100 and bovine

serum albumin (BSA) $400 \mu\text{l}$, washed three times in PBS, and then probed in sequence with primary MMP2, E-cadherin antibodies and secondary antibodies, washed 3 times in PBS. The stained cells were monitored using fluorescence microscope.

Statistical analysis

Data were analyzed by SPSS V16.0 software and the results were expressed as mean \pm standard deviation. Multiple groups comparison by ANOVA from the MTT assay, and one-way analysis of variance for Immunofluorescence data. $P < 0.05$ and $P < 0.01$ was considered to indicate a statistically significant difference between values.

Results

rL-RVG inhibited cell viability

MTT assay detected the inhibition of rL-RVG in A549, LA795 and H446 cells. It showed that the cell viability was 76.8%, 80.8%, 84.7%, 86.8%, 96.8% after NDV infected lung cancer A549 cells at the different concentrations 10^{-3} , 10^{-4} , 10^{-5} , 5×10^{-5} and 10^{-6} 24 h. At the same time, the cell viability was 48.7%, 34.7%, 60.3%, 64.8%, 75.4% respectively after rL-RVG infected 24 h, which showed that rL-RVG and NDV inhibited cell proliferation when concentration was greater than 5×10^{-5} . The LA795 cells viability was 76.1%, 81.5%, 83.0%, 85.3%, 92.7% infected by NDV and the cell viability was 60.3%, 65.6%, 68.9%, 72.2%, 78.6% infected by rL-RVG at the above six different dilution factor. H446 cells viability was 65.8%, 72.1%, 74.6%, 76.3%, 86.6% after infected by NDV and the cell viability was 47.0%, 53.7%, 55.9%, 60.6%, 65.4% infected by rL-RVG. As in **Figure 1**, the cells viability decreased obviously at 10^{-3} , 10^{-4} , 10^{-5} dilution factor in A549, LA795 and H446 cells. And the cell viability also decreased at 5×10^{-5} dilution factor in A549, LA795 cells. So, we selected the 5×10^{-5} as our effective concentration.

rL-RVG inhibits migration of lung cancer cells

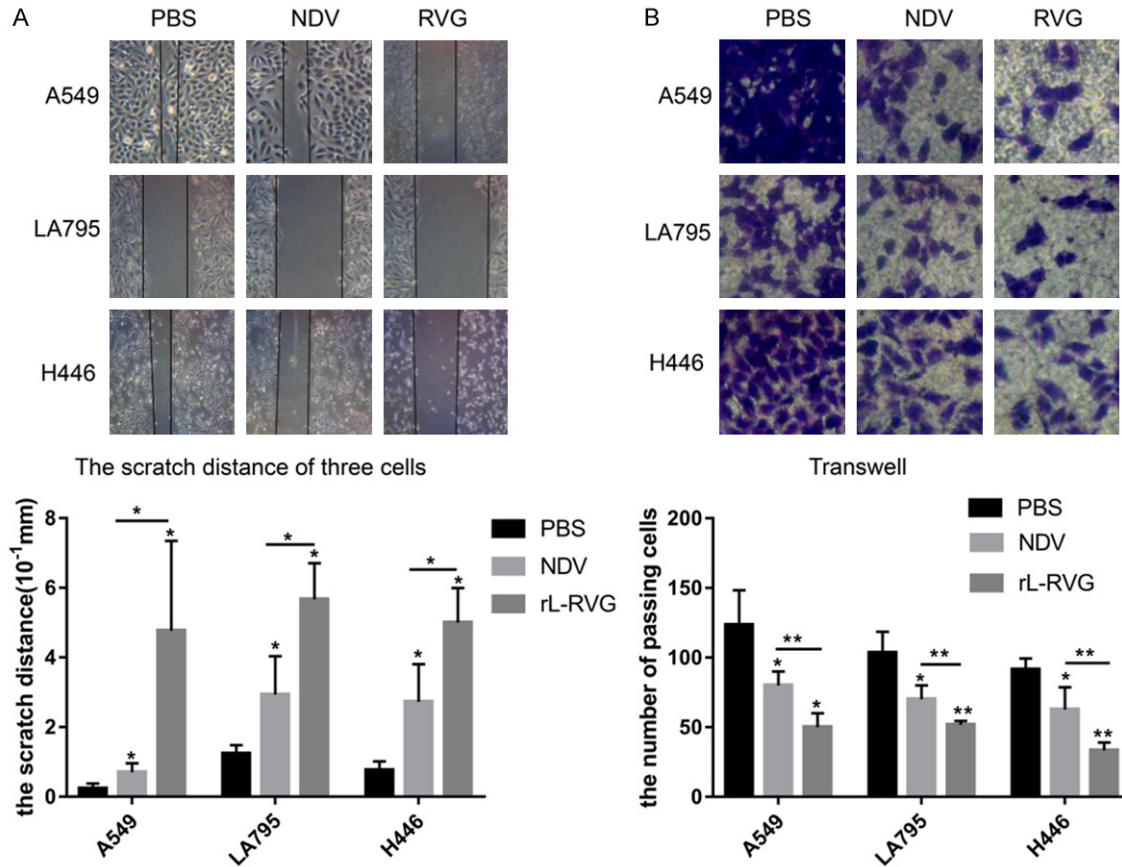


Figure 2. rL-RVG inhibited the migration of A549 and H446 cells, in (A), the scratch distance from edge to the center was obviously bigger in rL-RVG or NDV group than PBS group (*, $P < 0.05$), and the rL-RVG group scratch distance was obviously bigger than NDV group (*, $P < 0.05$) both in A549, LA795 and H446 cells. In (B), the number of A549, LA795 and H446 cells passing the Transwell chamber decreased infected by rL-RVG (**, $P < 0.01$) or NDV (*, $P < 0.05$) compared with PBS group. And the number of passing cells in rL-RVG group was less than NDV group (**, $P < 0.01$).

rL-RVG inhibited the migration ability of lung cancer cells

In this study, we have detected the migration activity of the three cell lines in vitro infected by rL-RVG or NDV. The scratch distance from the edge to the center was $465 \pm 39.9 \mu\text{m}$ in A549 cells infected by rL-RVG in Cell scratch migration assay, on the other hand, it was $778.6 \pm 6.7 \mu\text{m}$ and $1405 \pm 12.6 \mu\text{m}$ in NDV group and PBS group, respectively. At the same time, LA795 and H446 cells had the same phenomenon that the scratch distance in rL-RVG and NDV group became bigger than PBS group and the rL-RVG group scratch distance was obviously bigger than NDV group both in A549, LA795 and H446 cells (**Figure 2A**), which suggested that rL-RVG could inhibit the migration of lung cancer cells.

The number of A549 cells passing the Transwell chamber was 37.2 ± 3.7 infected by rL-RVG compared with the PBS group (104.8 ± 17.2) and NDV group (62.8 ± 6.7) in transwell assay which showed that rL-RVG reduced the number of A549 cells significantly. On the other hand, the number of H446 cells passing the Transwell chamber in rL-RVG group also distinctly decreased (**Figure 2B**). The Transwell assay results were consistent with the results of the Scratch test which further proved that rL-RVG could inhibit the migration of lung cancer cells.

rL-RVG upregulated RVG and NDV protein

Immunofluorescence was used to detect the expression of RVG and NDV protein. The red fluorescent protein was RVG protein which was

rL-RVG inhibits migration of lung cancer cells

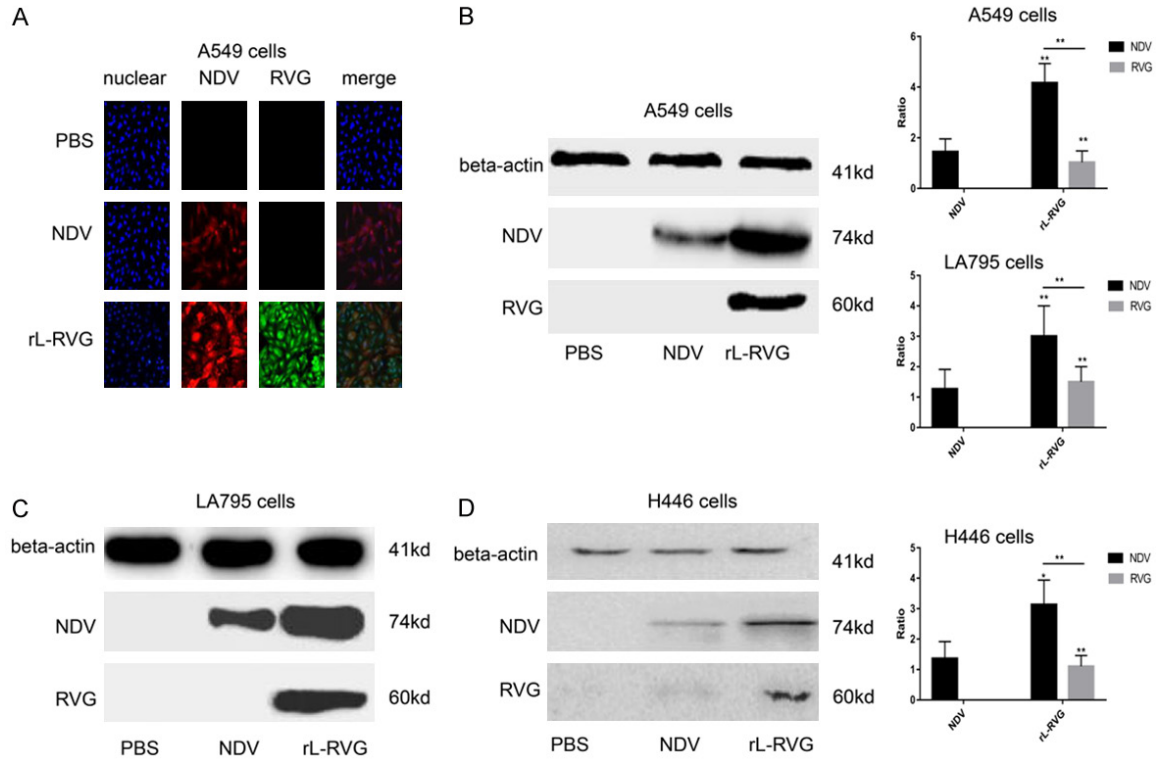


Figure 3. rL-RVG upregulated RVG and NDV protein. In (A), the green fluorescent protein was RVG protein which was expressed only in infected rL-RVG cells. At the same time, the red fluorescent protein was NDV protein which was expressed in lung adenocarcinoma cells infected by both rL-RVG and NDV in A549 cells, and control group was short of NDV and RVG expression. In (B-D), Western blot showed that the NDV protein was expressed both in rL-RVG and NDV group (**, $P < 0.01$), RVG protein was expressed only in rL-RVG group (**, $P < 0.01$). What's more, the expression of NDV protein increased in rL-RVG group than NDV group (**, $P < 0.01$) both in A549, LA795 and H446 cells.

expressed only in infected rL-RVG cells. At the same time, the green fluorescent protein was NDV protein which was expressed in lung adenocarcinoma cells infected by both rL-RVG and NDV group, and control group was short of NDV and RVG expression (**Figure 3A**).

Western blot was also used to observe the expression of RVG and NDV protein. It showed that the NDV protein was expressed both in rL-RVG and NDV group, but RVG protein was expressed only in rL-RVG group. What's more, the expression of NDV protein increased in rL-RVG group than NDV group (**Figure 3B-D**).

rL-RVG decreased the expression of MMP2 and increased the expression E-cadherin in A549, LA795 and H446 cells

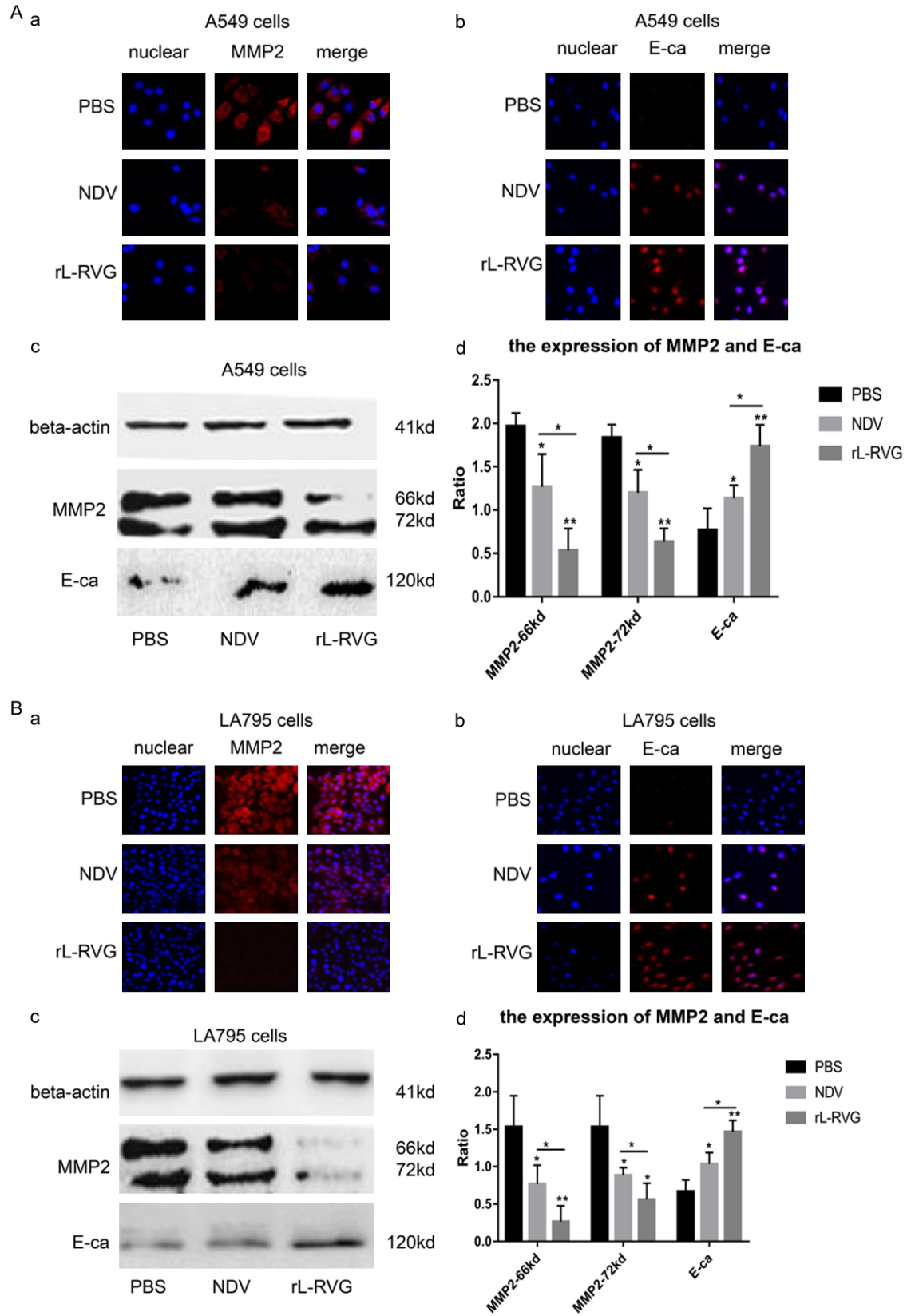
Immunofluorescence was used to detect the expression of MMP2 and E-cadherin. In **Figure 4Aa, 4Ba, 4Ca**, the fluorescence intensity of MMP2 on the cell membrane and the fluorescent particles in the cytoplasm both decreased in A549, LA795 and H446 cells in rL-RVG group

compared with control group which suggested that rL-RVG could reduce the expression of MMP2. At the same time, the expression of E-cadherin increased in rL-RVG infected cells which suggested that rL-RVG up-regulated the expression of E-cadherin (**Figure 4Ab, 4Bb, 4Cb**). Furthermore, Western blot was also used to test the expression of MMP2 and E-cadherin protein. E-cadherin expression increased but MMP2 expression decreased in A549, LA795 and H446 cells infected by rL-RVG or NDV group compared with that in control groups. Meanwhile, the expression of E-cadherin increased in NDV group infected A549, LA795 and H446 cells, but was weaker than that in RVG group. On the other hand, the expression of MMP2 stronger than that in RVG group (**Figure 4Ac, 4Ad, 4Bc, 4Bd, 4Cc, 4Cd**), which showed that rL-RVG could affect the EMT of lung cancer cells.

Discussion

According to the surveys in recent years, the annual new lung cancer showed a rising trend

rL-RVG inhibits migration of lung cancer cells



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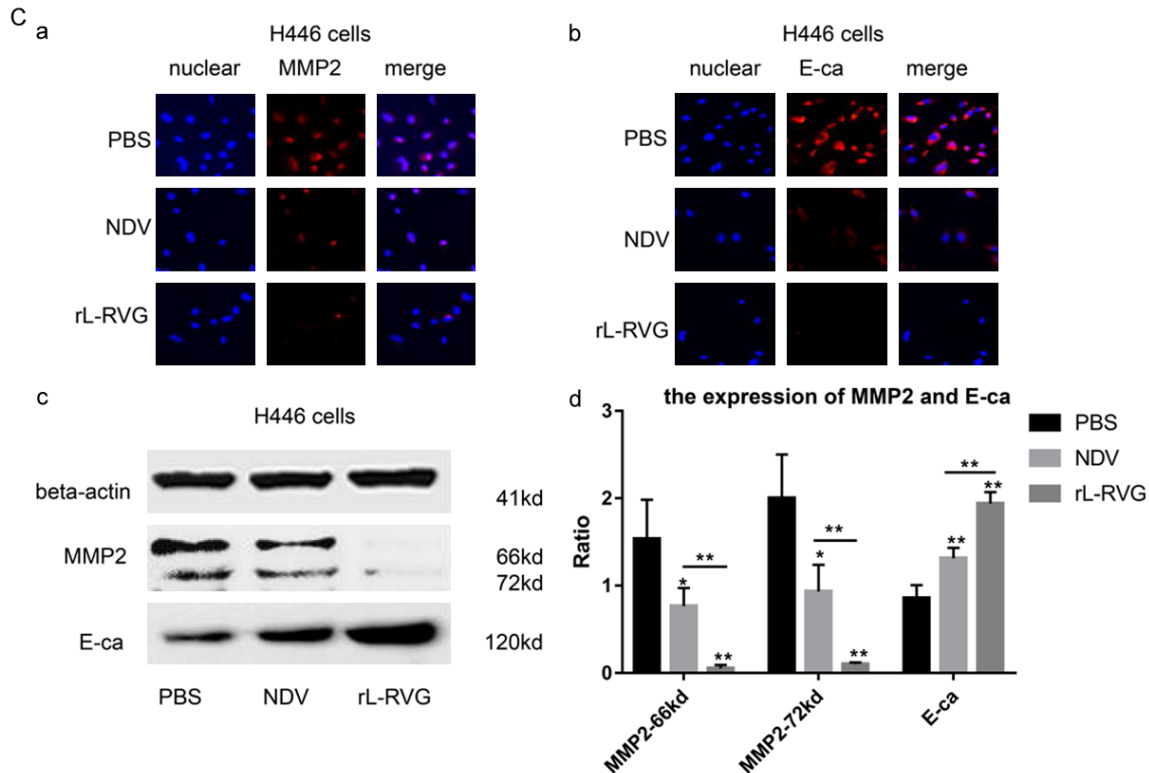


Figure 4. (A-C) The expression of MMP2 and E-cadherin in A549, LA795 and H446 cells infected by rL-RVG. In (Aa, Ba, Ca), the red fluorescent was the MMP2 protein, the expression of MMP2 decreased both in NDV and rL-RVG group which showed that the expression of MMP2 reduced more in NDV group than rL-RVG group. In (Ab, Bb, Cb), the red fluorescent was the E-cadherin protein, the expression of E-cadherin increased both in NDV and rL-RVG group, and the expression of E-cadherin increased more in rL-RVG group than NDV group. Western blot showed that E-cadherin expression increased but MMP2 expression decreased in A549, LA795 and H446 cells infected by rL-RVG (**, $P < 0.01$) or NDV group (*, $P < 0.05$) compared with that in control groups. Meanwhile, the expression of E-cadherin increased in NDV group (*, $P < 0.05$) infected A549, LA795 and H446 cells but weaker than that in RVG group (**, $P < 0.01$). On the other hand, the expression of MMP2 stronger than that in RVG group (*, $P < 0.05$).

[18]. The main cause of failure and death in patients with lung cancer is tumor metastasis. Therefore, the search for effective and low toxic anti-lung cancer drugs has become the most important issue to be solved [2, 15].

The dissemination of cancer cells from the primary tumor to a distant organ is metastasis that is the most frequent cause of death for patients with cancer. An initial step in tumor metastasis is the invasion of cancer cells into the surrounding tissue and the vasculature [16-19]. Cell membrane and its attachment to the extracellular matrix steered by protrusive activity consist chemotactic migration of cancer cells. Extracellular matrix architecture and other cell types found in primary tumors are elements of the cancer cell migration in local microenvironment in recent intravital imaging and the development of an *in vivo* invasion assay [2, 16-19].

Epithelial-mesenchymal transitions (EMTs) is a central process during embryonic development and also exists in a variety of chronic inflammation as well as cancer progression of primary tumors towards metastases. It is possible to provide more fundamental and effective cancer therapies by increasing evidence [22, 23]. E-cadherin and MMP2 are the important regulatory factors in the EMT [23].

E-cadherins is expressed by human epidermis and it undergoes continual differentiation and morphogenesis both during embryonic development and throughout life. In culture, E-cadherin has played relative role in epidermal morphogenesis [24]. Some diverse cellular processes such as proliferation, migration, apoptosis or maintenance of epithelial cell polarity were regulated by E-cadherin [25]. The expression of E-cadherin generally exists in poorly differentiated and advanced tumors, so E-cadherin is

regarded as the inhibitors of tumor metastasis [26]. MMP2 is crucial for metastasis and invasion of surrounding tissue. MMP2 can participate in tumor angiogenesis formation, the invasion and metastasis of tumor cells and it is closely related to tumor EMT [22]. Recent studies have confirmed that the expression of MMP2 is significantly increased in small lung cancer in early metastasis and a study indicated that angelicin inhibits NSCLC A549 cell's growth both by reducing MMP2 and increasing E-cadherin expression levels [27, 28].

A large number of domestic and foreign researches show that NDV has anti-tumor effect mainly through specific oncolytic activity on tumor cells, and it can reduce tumor cells proliferation and promote apoptosis [20, 21]. RVG could be integrated into the surface of NDV particles, but didn't change the NDV trypsin dependent infection in mammalian cells and RVG expression would not significantly affect the body cells immune response [6, 29]. An animal study using the mice with lung cancer suggested the NDV could inhibit the migration of the tumor cells in vitro as well as the growth of microvessels and the metastasis of cancer cells, tissues in vivo [30]. In our previous study, we found the recombinant avirulent newcastle disease virus expressing the rabies virus glycoprotein (rL-RVG) could strongly inhibit the growth and promote the apoptosis of human lung adenocarcinoma A549 cells. In the present study, Western blot results showed that RVG protein could be expressed stably in lung adenocarcinoma A549, LA795 as well as SCLC H446 cells and the NDV protein had much more expression in rL-RVG group than the NDV group which suggested that RVG could promote the spread of NDV among the cells. Then, we found rL-RVG could inhibit the migration activity of the lung adenocarcinoma A549, LA795 and SCLC H446 cells. Furthermore, the expression of E-cadherin increased but the expression of MMP2 decreased in lung adenocarcinoma A549, LA795 and SCLC H446 cells which showed that rL-RVG probably played a regulatory role in E-cadherin and MMP2.

In conclusion, rL-RVG could inhibit lung cancer cells migration. Furthermore, rL-RVG inhibited lung cancer cells proliferation and migration by decreasing the expression of MMP2 and increasing the expression of E-cadherin

which played an important role in EMT. Our results suggest that rL-RVG may become a powerful biotherapy on lung cancer but the related profound mechanism of the EMT and its effect caused by rL-RVG need be further explored in the future.

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Disclosure of conflict of interest

None.

Address correspondence to: Xue-Feng Bu, Department of General Surgery, Affiliated People's Hospital of Jiangsu University, 8# Dianli Road, Zhenjiang 212002, Jiangsu, P. R. China. Tel: +86-0511-88915153; E-mail: xuefengbu05@163.com

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