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Mechanism of action of KIAA1456 gene on the proliferation and apoptosis of alveolar epithelial cells

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Abstract. – OBJECTIVE: To explore the mechanism by which KIAA1456 acts on alveolar epithelial cells through lentiviral transfection.

MATERIALS AND METHODS: After constructing a KIAA1456 gene vector, 293T cells were co-transfected with lentiviral vectors and after incubation cells were examined by fluorescence microscopy. CCL-149 cells were transfected with LV-KIAA1456 and were examined by fluorescence microscopy. The proliferation capacity of transfected CCL-149 cells was evaluated using flow cytometry. The effect of KIAA1456 overexpression on CCL-149 cells proliferation was studied using the CCK-8 method.

RESULTS: The expression level of KIAA1456 in the LV- KIAA1456 group was significantly higher compared with the LV-Con group and the blank group. Compared with the LV-Con and the blank groups, the proportion of responding cells in G2/M phase showed statistically significant differences. Viable cells had adarker color and higher OD value measured by ELISA. Compared with the control and the blank groups, the growth and proliferation in the CCL-149 transfection group were significantly slower.

CONCLUSIONS: KIAA1456 gene inhibited the proliferation of CCL-149 cells by negative regulation of the G2/M cell cycle. We suggest that it can be used as a specific target for the treatment of alveolar epithelium.

Key Words:
Alveolar epithelium, KIAA1456 gene, Cell cycle.

Introduction

Chronic obstructive pulmonary disease (COPD) is a type of obstructive lung disease characterized by long-term poor airflow. COPD is a chronic bronchitis and/or emphysema that can further evolve into pulmonary heart disease and respiratory failure[1,2]. The severe consequences caused by COPD result in high morbidity and mortality[1-4]. COPD which is a disease related to smoking and air pollution is gradually becoming a serious public health risk. Several pathological processes for COPD have been observed[3-7]: (i) Limited airflow takes on progressive development, and the airway and lungs shows more intensive chronic inflammatory reactions; (ii) Chronic bronchitis and emphysema: bronchial epithelium necrosis, degeneration, desquamation of cilia, epithelial cell repair and hyperplasia, granuloma formation, and bronchial gland hyperplasia; (iii) Inflammatory infiltration process of bronchial wall cells: an increase in the levels of neutrophils, lymphocytes secretion and collagen, along with trachea malignant remodeling; (iv) Emphysema: lung hyperinflation, compliance decrease, disappearance of alveolar cavity structure, rupture expansion, blood supply reduction, ventilation/perfusion imbalance and dead space expansion.

Prior studies showed that KIAA1456 may play an important role in the proliferation and apoptosis of colorectal cancer cells[8]. CCL-149 cells belong to rat alveolar epithelial cell line[9]. CCL-149 cells (belong to rat alveolar epithelial cell line) *in vitro* studies can usually simulate the proliferation and apoptosis of alveolar epithelial cells as well as a variety of biological activities. We in this work took a look at the mechanism by which KIAA1456 acted on alveolar epithelial cells through lentiviral transfection. We provided a theoretical foundation for identifying new alveolar epithelium therapeutic targets.

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Materials and Methods

Experimental Materials

Virus packaging system, a three-plasmid system composed of pspax2, pMD2G, pLVX-IRES-ZsGreen1/pLVX - shRNA2 (the ZsGreen1 expression box on plasmid was able to express green fluorescent protein); dNTPs (Gene Craft, Munster, Germany); 75% alcohol (Sigma, St. Louis, MO, USA); SYBR Green PCR kit (Qiagen, Dusseldorf, Germany); Primer (Eurofins MWG Operon, Ebersberg, Germany), dNTPs (Gene Craft, Munster, Germany) and Triazole reagents (Invitrogen, Carlsbad, CA, USA).

Experimental equipment: gel-imaging system (Siemens, Berlin, Germany); precision PH meter (Thermo, Waltham, MA, USA), PCR amplification (Bio-Rad, Hercules, CA, USA), refrigerator (Thermo, Waltham, MA, USA), low temperature refrigerator (Thermo, Waltham, MA, USA), electronic balance (OHAUS, New Jersey, USA); thermostat water bath (Thermo Fisher, Waltham, MA, USA); micro-pipette (Eppendorf, Hamburg, Germany); high pressure sterilization pan (Market Forge, Orange, CA, USA), super-clean worktable (Labconco, Kansas City, MO, USA), inverted fluorescence microscope (OLYMPUS, Tokyo, Japan), and microscope camera system (Lumenera, Ottawa, Canada).

Testing Methods

To construct lentivirus vector, specific amplification in the upstream and downstream was designed, and enzyme cleavage sites were introduced. For PCR (high-fidelity KOD enzyme, with a mutation rate of 0% within 3K), the coding sequence (CDS) of the target gene was obtained from the template (cDNA library) and was ligated to the T-carrier. CDS zone was excised and separated from T-carrier and loaded into the lentivirus overexpressed plasmid vector. DNA region corresponding to siRNA was synthetized and linked to the plasmid vector; lentivirus shuttle plasmid and its auxiliary packaging original vector plasmid were prepared, high-purity extraction with no endotoxin was carried out, and 293T cells were co-transfected. Complete culture medium was used at 6 h after transfection and supernatant (rich in lentivirus particles) were collected 24 and 48 hours after cultivation. Viral particles were concentrated using ultracentrifugation.

Cell Transfection

Cells were inoculated into a 6-hole culture plate (10^5 per hole), one day before the experiment. Culture medium (2 ml) was added to each hole, and cell fusion rate was evaluated at 50% in the course of virus infection. The medium was replaced and 2 ml culture medium was added after the supernatant was removed. The virus was then added to each hole (MOI=50, 50 ml, 10^8 TU/ml). Polybrene (final concentration = 5 mg/ml) was added into each hole, and cells were incubated. Fresh culture medium was added after 24 hours. The fluorescent microscope was used to observe the fluorescent expression 72 hours after infection.

Flow Cytometry

Cell suspension with high activity was prepared and the concentration of CCL-149 cell was adjusted between $5x10^6$ to $1x10^7$ /ml with 10% FCS Roswell Park Memorial Institute-1640 (RPMI-1640). A tube containing specific McAb (5 to 50 ml) was prepared, then 40 ml centrifuged cell suspension was added (50 ml, 1:20). Dulbecco's Phosphate Buffered Saline (DPBS) was then used for dilution. Rabbit serum was inactivated (4°C, 30 min) and rinsed with washing buffer twice (2 ml) and samples were then centrifuged at 1000 rpm for 5 minutes. The supernatant was collected and 50μl rabbit anti-rat fluorescent marker was added and samples were shaken for 30 minutes at 4°C. 2 ml fluid was added followed by centrifugation at 1000 rpm for 5 min. Stationary buffer (1 ml) was added before observation under a

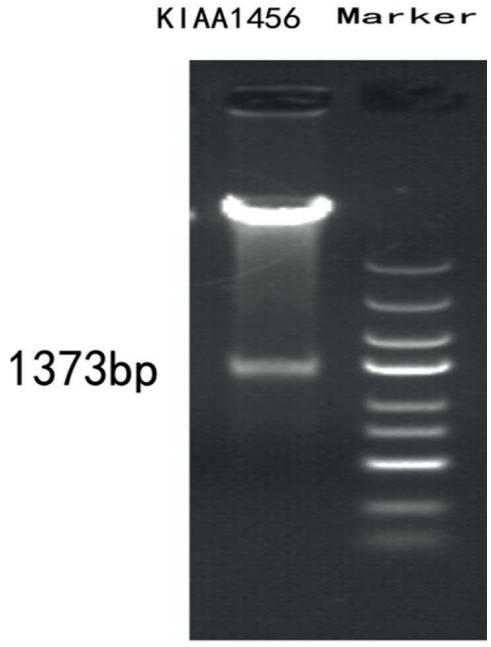

Figure 1. The PCR product of the target gene.

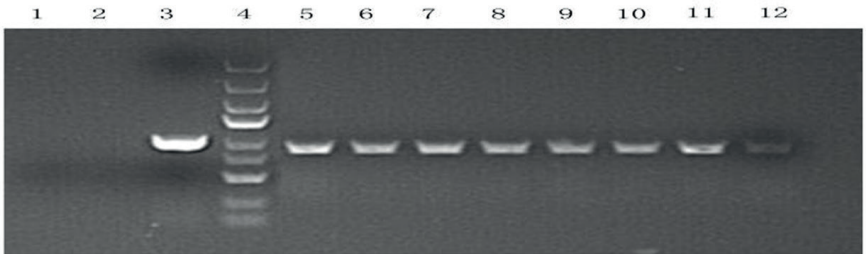

Figure 2. Identification of positive clone.

fluorescence microscope. 100 µl to 500 µl stationary buffer was added according to different cell concentrations. The observation was conducted under a fluorescence microscope.

Detecting cell Proliferation Condition by CCK 8

Cells were cultured until the convergence degree reached 50% to 90%. Cells were rinsed with phosphate buffered saline (PBS), and complete culture medium was added. Cells were centrifuged at 400 g, followed by resuspension in fresh culture medium. Cells were counted and adherent cells were inoculated into a 96-pore plate with 200 ml medium in each hole ($3x10^3$ per hole). The plate was placed in a CO_2 incubator overnight to allow cell adherence. On the second day, three pores were chosen for NC and ESR1 groups respectively. After removing the culture media, 100 ml fresh media and 10 ml CCK-8 reagent were added into each hole. 100 ml fresh culture medium and 10 ml CCK-8 reagent were added to the non-inoculated hole as blank control. After 1 to 4 hours incubation at 37°C, absorbance was measured at 450 nm. Afterwards, detection was performed every two days.

Statistical Analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). p values < 0.05 were considered statistically significant.

Results

PCR on the target gene KIAA1456 produced a 1373 bp fragment (Figure 1). Using that, a fragment with the size of 881bp was amplified. Clones producing a fragment with consistent size were considered as positive clones. Figure 2 shows the result of PCR on positive colonies. Sequencing results were consistent with Gen Bank (NM_020844) sequence, suggesting that the recombinant vector Ubi-KIAA1456-EGFP-Puromycin was successfully constructed. 293T cells were co-transfected with lentiviral vectors and then cultured in a 5% CO_2 incubator at 37°C for 48 h before observation under a fluorescence microscope (Figure 3). To calculate

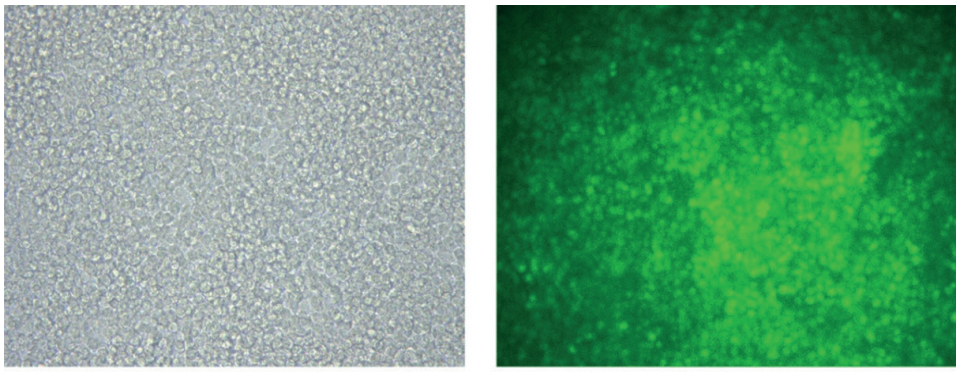

Figure 3. Fluorescent expression of plasmid transfected 293T cells for 24 h (100X).

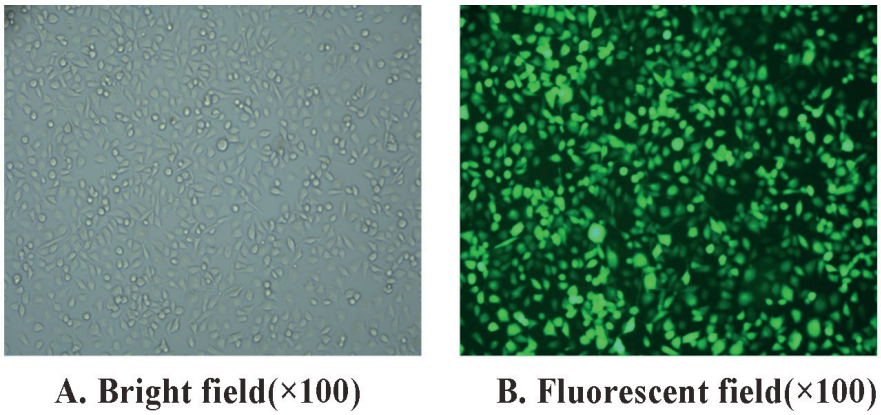

Figure 4. Fluorescent expression examination of transfected CCL-149 cells.

the virus titer, we used the following formula: Virus titer (TU/μl) = (100×number of cells in the view) / dilution

LV-KIAA1456 titer after concentration was about 2×10^8 TU/ml, and LV-Con titer after concentration was about 4×10^8 TU/ml. We observed green fluorescent expression in CCL-149 cells 72 h after transfection with lentivirus LV-KIAA1456. The transfection efficiency was around 95% (Figure 4). The expression level of KIAA1456 in the LV- KIAA1456 group was higher compared with the LV-Con group and the blank group ($p<0.05$) (Figure 5).

Compared with the LV-Con group and the blank group, the proportion of responding cells in G2/M phase, showed statistically significant differences ($p<0.05$) (Figure 6). The fundamental principle of CCK-8 was basically similar to that of MTT. Viable cells had a darker color and higher OD value. The OD value for CCL-149 cells on day 5 was detected using CCK-8 method and growth curve was drawn for each group. The results indicated that compared with the control group and the blank group, the growth and proliferation in the CCL-149 transfection group were significantly slower. The difference was statistically significant ($p<0.05$) (Figure 7).

Discussion

The exact cause of COPD is still unclear. The occurrence of chronic bronchitis and obstructive pulmonary emphysema may play a role in the pathogenesis of COPD[10-13]. Risk factors can be divided into external factors and internal factors. External factors include smoking, inhalation of dust and chemical substances, respiratory tract infection (RFI), air pollution, poor nutrition and factors related to the social economic status of patient[14,15]. Internal causes include genetic factors, airway high reactivity, poor pulmonary development or growth caused by various reasons[16,17].

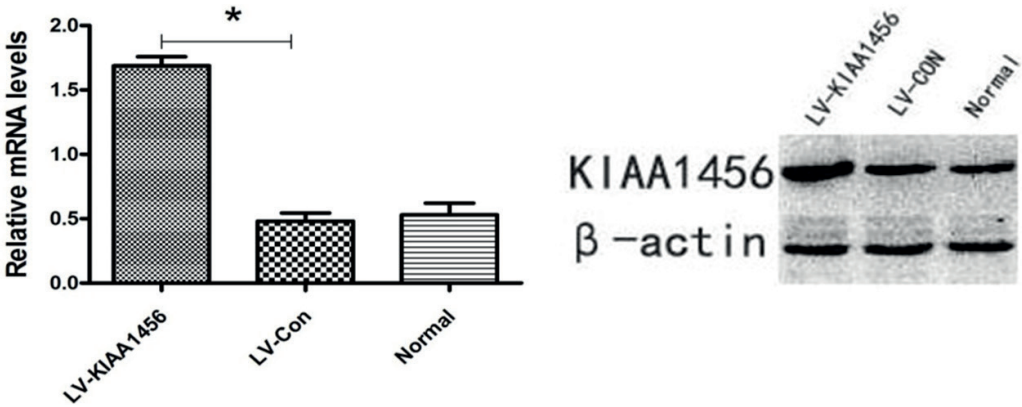

Figure 5. KIAA1456 expressions in CCL-149 cells in all groups.

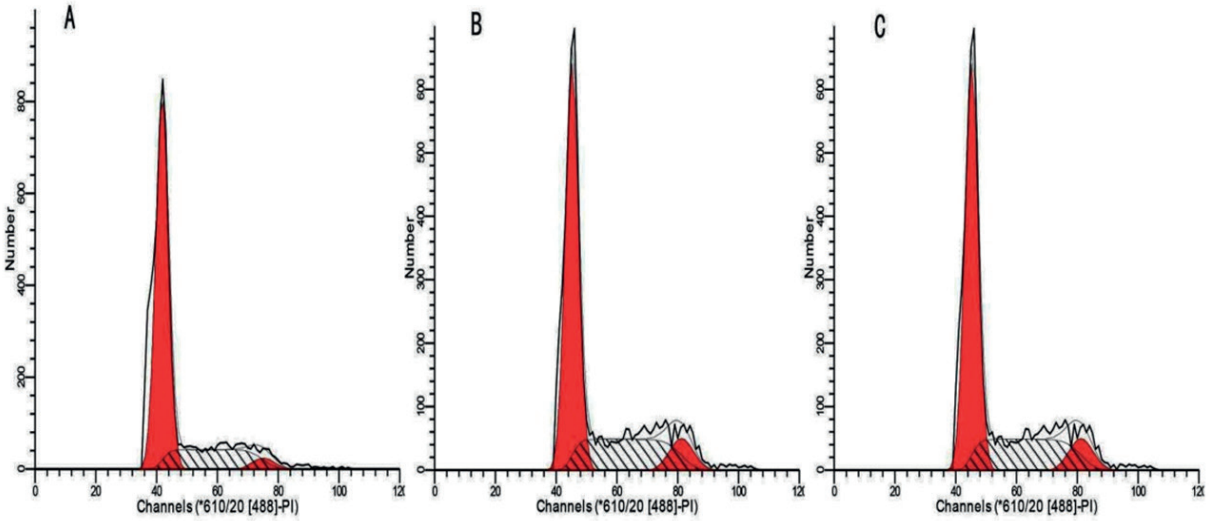

Figure 6. The reproductive capacity of LV- KIAA1456 transfected CCL-149 cells detected by flow cytometry.

Among multiple pathogenic factors, smoking has been shown to be one of the major factors in COPD[18]. It has been reported that more than 90% of COPD cases are associated with smoking. Prior studies on the pathogenies of COPD showed that long exposure to cigarette smoke or a high content of inhalable particles, upregulated the expression of genes involved in endoplasmic reticulum stress (GRP78, CHOP, and AFT4)[6,19]. Upregulation of these genes intensified the synthesis and secretion of unfolded protein products within the respiratory system. Signaling pathways involved in this process were a PERK_eIF2 pathway, Toll-like receptor pathway, and ATF4-CHOP signaling pathway

Through radiation hybridization analysis, Nagase et al[20] confirmed that KIAA1456 gene was located at chromosome 8. Chen et al[21] confirmed that KIAA1456 gene was located in the 8p22 region.

KIAA1456 gene product is a protein with 421 amino acid residues. Results obtained from prior studies showed that KIAA1456 expression level in adult brain tissue was the highest while the lowest level was detected in skeletal muscles, testicles, and ovaries. KIAA1456 expression in fetal brain tissue was lower than that in adult brain tissue. An analysis of the specific region in adult brain indicated that KIAA1456 expression in the cerebellum was the highest.

In the present study, we constructed KIAA1456 lentivirus and transfected CCL-149 cells. The expressions level of KIAA1456 in the LV-KIAA1456 group was higher than that in the LV-Con group and the blank group. Compared with the LV-Con and the blank groups, the proportion of responding cells in G2/M phase in LV-KIAA1456 group was significantly lower. The growth and proliferation in CCL-149 transfection group were slower. Therefore, we suggest that KIAA1456 gene is a regulatory gene involve in the negative control of cell cycle.

Among many chemotherapy drugs used for tumor treatment, cell cycle negative regulatory drugs showed to inhibit the proliferation of alveolar epithelial cells[22]. However, since these chemotherapy drugs are non-specific, beside inhibiting the cell cycle in tumor cells they also affect the growth in normal cells. Development of specific drugs can solve this problem effectively. We, in this work, tried to provide a theoretical basis to explain the biological function of KIAA1456 and targeted therapeutic drugs[18].

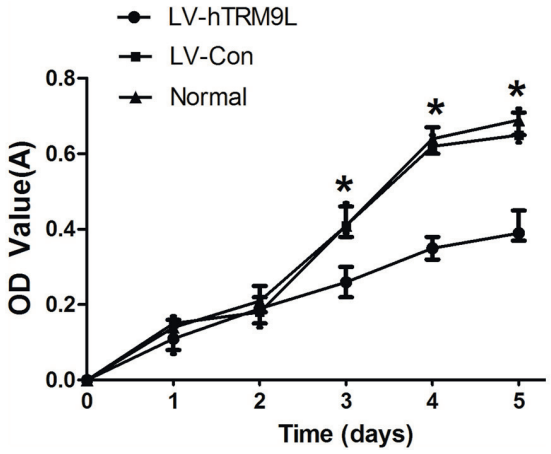

Figure 7. The role of KIAA1456 expression in inhibiting the proliferation of CCL-149 cells detected by CCK-8 method.

Conclusions

KIAA1456 gene inhibited the proliferation of CCL-149 cells by the negatively regulating the G_2/M cell cycle. We suggest that it can be used as a specific target for the treatment of alveolar epithelium.

Conflict of interest

The authors declare no conflicts of interest.

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