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Knockdown of TRIM66 inhibits malignant behavior and epithelial-mesenchymal transition in non-small cell lung cancer

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ABSTRACT

Objective: The tripartite motif 66(TRIM66) is an important member of the TRIM protein superfamily, which can participate in the expression of multiple proteins, and is closely associated with the behaviors of non-small cell lung cancer (NSCLC). In this study, we aimed to explore the effect of TRIM66 in this process in vitro using NSCLC cell lines, and the role of TRIM66 in regulating epithelial-mesenchymal transition(EMT) in NSCLC.

Methods: Western blotting was used to detect the TRIM66 protein expression levels in NSCLC cell lines and normal lung epithelial cells BEAS-2B. We silenced its expression in A549 cells by transient siRNA transfection to ascertain the function of TRIM66 in NSCLC cells. Western blotting was used to detect the expression of EMT-related proteins.

Results: TRIM66 protein content was highest in NSCLC cell line A549, compared with BEAS-2B, it showed that the TRIM66-siRNA group lung cancer cell proliferation was significantly reduced after knockdown of TRIM66, and knockdown of TRIM66 also suppressed invasion, migration and clonogenic ability of A549 cells. Finally, we found that siRNA-mediated TRIM66 silencing suppressed EMT by downregulating expression of N-cadherin and vimentin and upregulating that of E-cadherin in NSCLC cells, which could effectively reduce the invasive, migratory, and proliferative capacities of lung cancer cells.

Conclusion: Silence TRIM66 expression suppressed NSCLC cell proliferation, invasion, and migration. The siRNA-mediated TRIM66 silencing could block the occurrence of EMT. TRIM66 could be a promising novel target for future NSCLC treatments.

1. Introduction

Morbidity and mortality associated with lung cancer have been increasing annually in recent years, making it the leading malignancy in these respects [1]. Every year, there are approximately 1.8 million new lung cancer cases worldwide, accounting for 13% of all new cancer diagnoses [2]. Non-small cell lung cancer (NSCLC) is the most common pathological type, representing around 80% of all lung cancers [3]. Unfortunately, the majority of NSCLC patients are at an advanced stage at the time of diagnosis, and the 5-year survival rate for early NSCLC patients after surgery is poor [4]. Therefore, the identification of effective prognostic markers and potential therapeutic targets in this malignancy is a key focus of current research.

The tripartite motif (TRIM) protein superfamily plays a vital role during cell growth, differentiation, inflammation, apoptosis, oncogenesis, and development. As an important member of the TRIM family, TRIM66 can participate in the expression of multiple proteins and is closely associated with the behaviors of many types of malignant tumor. Our previous studies demonstrated that in NSCLC, TRIM66 may be aberrantly expressed, can promote lymph node and distant metastases, and is a marker of very poor prognosis [5]. Epithelial–mesenchymal transition (EMT) is a critical stage during the metastasis and progression of malignant tumors [5], and a crucial step in NSCLC metastasis [6]. EMT is characterized by loss of the epithelial adhesion molecule E-cadherin, increased migratory and invasive behavior, and enhanced expression of the mesenchymal markers vimentin and N-cadherin [7].

Nevertheless, the mechanism by which TRIM66 promotes NSCLC metastasis remains unclear. In this study, we aimed to explore the role of TRIM66 in this process in vitro using NSCLC cell lines. The malignant phenotype of such cells and their expression of EMT-related molecules were tested following TRIM66 knockdown to investigate the effects of TRIM66 on NSCLC cell proliferation, invasion, and migration, as well as the underlying mechanism.

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2. Methods

2.1. Cell culture

A549, H292, H1975, and H460 human lung cancer cells and BEAS-2B normal bronchial epithelial cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Culture took place in a 37 °C incubator in an atmosphere containing 5% CO_2, and the medium was replaced every 3 days.

2.2. Transient transfection

Transfection was conducted using a Lipofectamine 2000 transfection kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, the diluted Lipofectamine was mixed with siRNA for 15 min. The sequence of the siRNA targeting TRIM66 as follow: siRNA-TRIM66-1 is GCAGAGCCTTCGAGATAAA; siRNA-TRIM66-2 is CCCATTACTGGGAGAGCAA. A scrambled siRNA construct was also generated as the control treatment, and untreated cells comprised the blank control group. Transfection efficiency was determined by observing GFP-derived fluorescence under a fluorescence microscope (Carl Zeiss Meditec, Jena, Germany) after 48 h of transfection, and reduced TRIM66 expression was confirmed by western blotting. Cells were divided into 4 groups, namely, the blank control group (untreated), scrambled siRNA group (transfected with the empty vector), siRNA-TRIM66-1 group (transfected with TRIM66 siRNA-1) and siRNA-TRIM66-2 group (transfected with TRIM66 siRNA-2).

2.3. Western blotting

Cell lysis buffer (Wuhan Zhongzhi Biotechnologies Co., Ltd., Wuhan, China) was used to extract cell proteins. Equivalent amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were then blocked by immersion in 5% skim milk blocking solution for 2 h. Rabbit anti-TRIM66 (diluted 1:100; Proteintech Group, Inc., Chicago, IL, USA) and mouse anti-human E-cadherin, N-cadherin, Vimentin and Snail (Abcam, Cambridge, MA, USA) primary antibodies were incubated with the membranes at 4 °C. GAPDH (the antibody for which was supplied by Santa Cruz Biotechnology, Heidelberg, Germany) was used as the internal reference. Subsequently, the membranes were exposed to a goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, CA, USA), and an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, San Francisco, CA, USA) was used for development. Grayscale analysis was conducted using Quantity One software (Bio-Rad Laboratories, Philadelphia, PA, USA).

2.4. Cell proliferation analysis

Cell proliferation was measured using a 3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide (MTT) assay. Cells at the logarithmic phase were collected to prepare single-cell suspensions, which were then inoculated at a density of 1000–10,000 cells/well into 96-well plates with a well volume of 200 μL. Twenty microliters of MTT solution (Bio-Rad, Hercules, CA, USA) was added to each well after 3–5 days of culture, and the plates were subsequently incubated for 4–6 h at 37 °C. The supernatant was then removed using a sterile pipette, and 150 μL dimethyl sulfoxide (Bio-Rad) was added to each well. Optical density was measured at 450 nm using a Bio-Rad microplate reader.

2.5. Transwell assay

Cell invasive and migratory capacities were determined using Transwell inserts (You Nikang Biological Technology Co., Ltd., Beijing, China). Matrigel was melted and diluted in serum-free Dulbecco's modified Eagle medium (DMEM), before being placed in the upper chambers of the 24-well plate, which was then incubated at 37 °C for 4 h. Cells were collected and suspended in DMEM at a density of 5×10^5 cells/mL. These suspensions (100 μL/well) were transferred to the upper chambers, and 700 μL DMEM containing 10% FBS was added to the lower chambers. After 24 h, the inserts were removed and exposed to 4% methanol and 0.1% crystal violet for fixation and staining of cells, respectively. Images of cells were taken under a microscope (Olympus Corporation, Tokyo, Japan) after staining, and the cells in each group having traversed the Transwell membranes were counted. This experiment was repeated 3 times, and means were calculated.

2.6. Clonogenic assay

Colony formation by single cells was measured using a clonogenic assay. First, 0.25% trypsin was used to collect cells, the concentration of which was adjusted to be 300 cells/culture dish. Subsequently, 2 mL preheated medium was added to the cells, which were then cultured at 37 °C in 5% CO_2 for 2–3 weeks. The formation of colonies was allowed to continue until they could be seen with the naked eye. Finally, the cells were washed twice with phosphate-buffered saline, fixed with 4% paraformaldehyde for 15 min, and treated with Giemsa stain for 10 min. Colonies were counted under a microscope and colony formation efficiency was calculated according to the following formula: colony formation efficiency = (number of colonies/number of cells inoculated) × 100%.

2.7. Statistical methods

Statistical analyses were conducted using SPSS 18.0 software (SPSS, Chicago, IL, USA). Data are expressed as means ± standard deviations. Differences between groups were analyzed using a paired or unpaired *t*-test or one-way analysis of variance. Differences associated with a P-value less than 0.05 were deemed statistically significant.

3. Results

3.1. TRIM66 expression in NSCLC cell lines

TRIM66 expression in NSCLC cell lines was detected using western blotting. Levels of this protein in A549, H1975, and H460 NSCLC cells were higher than in normal cells of the BEAS-2B line (Fig. 1). Notably, TRIM66 expression was highest in A549 cells; therefore, this line was used in subsequent experiments.

3.2. Transfection and knockdown efficiency

To ascertain the function of TRIM66 in NSCLC cells, we silenced its expression in A549 cells by transient siRNA transfection. Proteins were extracted from the transfected cells, and levels of TRIM66 protein in the cell lysates were analyzed using western blotting. As shown in Fig. 2, TRIM66 protein expression was remarkably reduced after TRIM66 siRNA transfection (siRNA-1 and siRNA-2). Given this satisfactory knockdown effect, the results of subsequent experiments could be interpreted with confidence.

3.3. Cell proliferation

The role of TRIM66 in A549 cell proliferation was determined using an MTT assay. After effective silencing of TRIM66 expression in A549 NSCLC cells, their ability to proliferate was notably reduced (Fig. 3).