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Original Article

Overexpression of TRIM66 functions as an oncogene in lung cancer progression

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Abstract: Tripartite motif (TRIM) family is identified as an E3 ubiquitin ligases that is implicated in critical progression of cancer. This study was in an effort to reveal the expression and functional mechanisms of TRIM66 in lung cancer. The expression of TRIM66 was examined in lung cancer tissues and two lung cancer cell lines were picked up for further experiments. Effects of silencing TRIM66 on cell proliferation, cell cycle, cell apoptosis and metastasis were analyzed respectively. And relative protein levels of apoptosis and epithelial-to-mesenchymal transition (EMT) pathway were detected using Western blot. Our data suggested that expression of TRIM66 was significantly higher in lung cancer tissues compared to normal tissues. Silencing of TRIM66 repressed proliferation, cell cycle and metastasis, but facilitated cell apoptosis of lung cancer cell lines. Suppressed Bcl-2 and promoted Bax, caspase3 and caspase9 were examined in TRIM66 silencing cells. Additionally, the proteins related to EMT pathway including MMP-2, MMP-9, Snail and Twist, was inhibited by TRIM66 knocking down. In conclusion, our study demonstrated that TRIM66 was highly expressed in lung cancer, and functioned as an oncogene in lung cancer development.

Keywords: TRIM66, lung cancer, Bcl-2, Bax, EMT pathway

Introduction

Lung cancer is identified as the most common malignant tumor among all cancers. Moreover, the morbidity and mortality rates of lung cancer have increased persistently during recent decades [1]. The diagnosed lung cancer cases are classified into two histological types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC accounts for approximately 15% of lung cancer, which is highly sensitive to initial treatment [2-4]. NSCLC, consisting of squamous cell carcinoma, adenocarcinoma and pulmonary large cell carcinoma, accounts for nearly 85% of diagnosed lung cancer cases [5]. Although therapies for lung cancer including surgery, chemotherapy and radiotherapy, have developed rapidly through past decades. The long-term survival rate of SCLC or NSCLC is still very low. Most patients ultimately die of recurrent diseases [6-8]. Therefore, deep understanding of knowledge about molecular and functional mechanisms of lung cancer initiation and progression is essential for improving impactful therapeutic strategies and new biomarkers of early diagnosis.

Members of the tripartite motif (TRIM) proteins share an N-terminal tripartite motif containing a Ring-finger domain, one or two B-Box and a coiled coil [9]. The TRIM family counts more than 70 distinct members in human and mouse [10]. TRIM66, also known as transcriptional intermediate factor 1δ (TIF1δ) [11], is located on chromosome 11 in human and belongs to the TIF1 sub-family that contains other members, TRIM24, TRIM28 and TRIM33 [12, 13]. However, unlike other members, TRIM66 just contains two B-Boxes in N-terminal without a Ring domain. And in C-terminal, it consists of a PHD-finger domain and a BROMO domain [14]. Most TRIM proteins are involved in a broad range of biological processes, such as regulation of cell proliferation, cell cycle, apoptosis and tumor occurrence [15]. Many researches have suggested that altered expression and specific function of TRIM proteins relates to the progression of cancers. TRIM19 is involved in chromosome translocation which is specific for acute promyelocytic leukemia [16]. And highly expressed TRIM28 is detected in many kinds of cancers containing colorectal cancer, NSCLC and gastric cancer with promoting p53 inactiva-

tion [17]. Over expression of TRIM24 and TRIM25 are both examined in breast cancer and ovarian cancer, respectively [18-20]. However, the expression pattern and biological function of TRIM66 in cancer cells are still poorly understood.

In our study, we profiled the expression pattern of TRIM66 in lung cancer and further detected the biological function of TRIM66 in two lung cancer cell lines to reveal the involved mechanisms. Our data suggested that TRIM66 was highly expressed in lung cancer tissues as comparing to adjoining normal tissues. And in the following experiments on two lung cancer cell lines, we found that knocking down of TRIM66 via RNA interference inhibited the proliferation, cell cycle progression and metastasis of cancer cells, but promoted cell apoptosis. Sequentially, Western blot analysis showed that silencing of TRIM66 induced cell apoptosis by down-regulation of cellular Bcl-2 level following with up-regulation protein levels of Bax, caspase3 and caspase9, and suppressed tumor metastasis through decreasing the levels of proteins that was relative to epithelial-to-mesenchymal transition (EMT) pathway. In summary, this study demonstrated that TRIM66 functioned as an oncogene in lung cancer.

Materials and methods

Patients and tissue samples

30 lung cancer patients who were treated at The First Affiliated Hospital of Jiaxing University (Zhejiang, China) were enrolled in this study. The clinicopathologic data of each individual was reviewed based on the medical records. Tumor tissue and adjacent normal lung tissue were obtained as experiments and controls respectively. Ethical approval for this study was provided by the independent ethics committee, The First Affiliated Hospital of Jiaxing University. Informed and written consent were obtained from all patients or their advisers according to the ethics committee guidelines.

Cell culture

Human pulmonary carcinoma cell lines H460, H446, Spc-A-1, H1975 and A549 were purchased from Shanghai Cell Bank, Chinese Academy of Science (Shanghai, China). All cell lines were cultured in RPMI 1640 (Hyclone) supplemented with a 10% fetal bovine serum (GIBCO), 100 U/ml penicillin, and 100 μg/ml streptomycin and maintained at 37°C in a humidified 5% CO_2 atmosphere.

siRNA transfection

TRIM66 siRNA were synthesized and transfected into A549 and H446 cells. Negative control (NC) was transfected with a non-specific scramble siRNA. The RNA interference (RNAi) was processed using Lipofectamine 2000 (Invitrogen) according to the instruction of manufacture. The targeting position of siRNA was: 3372-3794, and the sequence of Trim66 siRNA was: 5'-GCGACGACAUGGAGAAU-3'. Relative assays were performed 48 h after transfection.

Reverse transcription and real-time PCR

The total RNA from human lung cancer samples and lung cancer cell lines was isolated using the TRIzol (Invitrogen) reagent according to the manufacturer's instructions. Reverse transcription was conducted with cDNA Synthesis Kit (Fermentas). For quantitative expression of TRIM66 in cells, amplification was performed in an ABI-7300 Real-Time PCR System (ABI) and was visualized with SYBR Green PCR Kit (Fermentas). The cycling conditions were 10 min at 95°C, 40 cycles of 15 s at 95°C and 45 s at 60°C, followed by 1 min at 60°C, 15 s at 95°C and 15 s at 60°C. The internal control was served with GADPH. The primer sequences were as follow: Tirm66 (NM_001170912.1) Primer Forward: 5'-AGTCTCCTCAGCATCTTC-3', and Primer Reverse: 5'-AGCAGCAGTGTTCTTG-TC-3'; GADPH (NM_008084.2) Primer Forward: 5'-ATCACTGCCACCCAGAAG-3', and Primer Reverse: 5'-TCCACGACGGACACATTG-3'.

Western blot

After conditional treatment, cells were harvested and washed twice with PBS. Cell extract was processed in ice-cold radio immunoprecipitation assay buffer containing 0.01% protease inhibitor cocktail (Sigma), and the concentrations of proteins were determined by BCA (Thermo). The proteins (20-30 μg) were separated by SDS-PAGE and then transferred to a polyvinylidene fluoride membrane. The proteins were detected by the respective primary antibodies: Trim66 (1:1000, Abcam), Bcl-2 (1:200, Santa), Bax (1:300, Santa), Caspase3 (1:500, Abcam), Caspase9 (1:1000, Abcam), MMP2 (1:1000, Abcam), MMP9 (1:500, Abcam), Twist

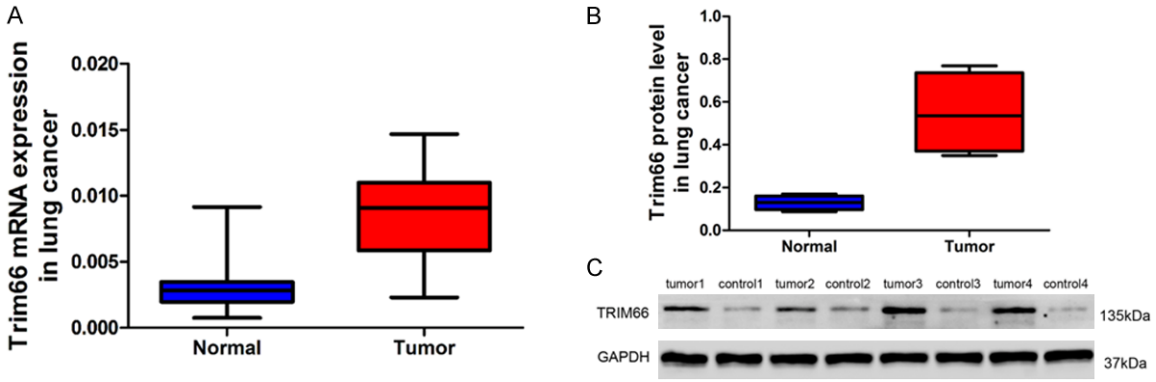

Figure 1. TRIM66 was highly expressed in lung cancer tissues. A. Expression of TRIM66 mRNA in sample tissues (n=30) P<0.00001. And the mRNA level of TRIM66 was obviously higher in lung cancer tissues than in adjacent normal tissues. B and C. Protein level of TRIM66 in lung cancer and normal tissues (n=4) P<0.05.

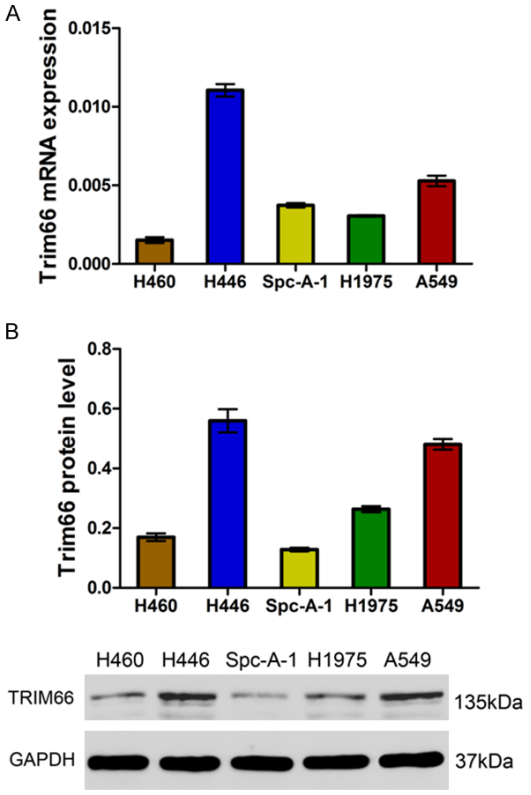

Figure 2. Expression of TRIM66 in 5 lung cancer cell lines. A. mRNA expression of TRIM66 in 5 lung cancer cell lines examined using RT-PCR (n=3). B. Cellular protein level of TRIM66 in lung cancer cell lines examined using Western blot (n=3). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (as comparing to controls).

(1:500, Abcam), Snail (1:1000, Abcam) and followed by incubation with secondary antibodies (Beyotime) respectively. Signals were performed with enhanced chemiluminescence (ECL). Staining intensities were analyzed by Image J (NIH). Antibody to GADPH (CST) was served as the internal control.

Cell proliferation assay

A549 and H446 cells were seeded into 96-well plates for proliferation assay. After the RNA interference as above, the proliferation was examined by Cell Counting Kit-8 (7seaBiotech) at 0 h, 24 h, 48 h and 72 h according to the manufacturer's instructions. Briefly, 10 μl CCK-8 was added to each well, and the cells were further maintained from light at 37°C, 5% CO_2 for 2 hours. Absorbance was measured with excitation at 450 nm to calculate the relative growth.

Cell cycle assay

At 48 h post siRNA transfection, A549 and H446 cells were harvested and resuspended with PBS, followed by fixed with 70% ethanol at -20°C for at least 2 hours. After washed with PBS, the cells were treated with ribonuclease for 15 min at 37°C and then incubated with propidium iodide (PI, 0.05 mg/ml, Sigma) at room temperature in the dark for 30 min. DNA content was detected using flow cytometer (BD Biosciences) and cell cycle distribution was analyzed using FlowJo cell cycle analysis software.

Cell apoptosis assay

The percentage of cells actively undergoing apoptosis was analyzed using commercial Annexin V Apoptosis Detection Kit APC (eBioscience). A549 and H446 cells were harvested at 48 h after siRNA transfection, and then double

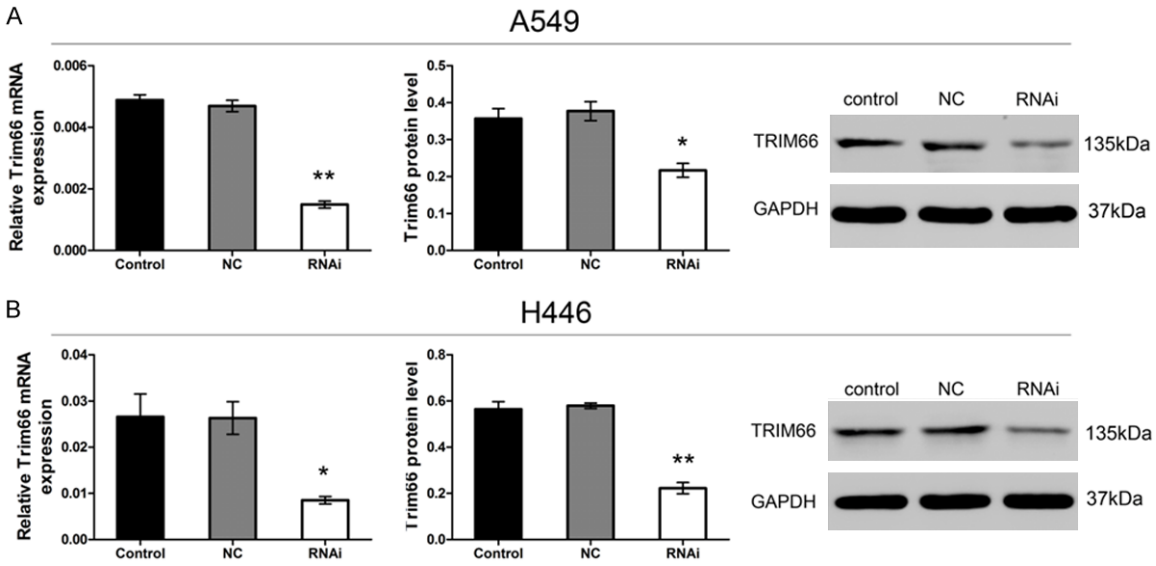

Figure 3. Silencing of TRIM66 expression post RNA interference. Results were examined by RT-PCR and Western blot. A. Depletion of TRIM66 in A549 after siRNA transfection (n=3). B. Depletion of TRIM66 in H446 after siRNA transfection (n=3). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (as comparing to controls).

stained with Annexin V-fluorescein isothiocyanate (FITC) and PI. The stained cells were detected by flow cytometry (BD Biosciences). At least 20,000 cells were obtained for each sample.

Transwell assay

Cell transwell assay was performed using a 24-well Transwell chamber (Trueline) with a pore size of 8 μm. In migration assays, the medium added with 30% fetal bovine serum was placed in the lower chamber. Cells post siRNA transfection were serum starved for 24 h and then seeded in upper chamber for 24 h of incubation. The migrating cells from upper side were fixed with 4% formaldehyde and stained with 0.05% crystal violet (Solarbio). Finally, the stained cells on the lower surface of membrane were counted under the microscope.

Cell invasion assays were performed in transwell chambers containing a polycarbonate filter. And the inserts of upper chambers were coated with 50 μl Matrigel (dilution at 1:2; BD Biosciences). The rest of the assay was processed as the migration assay described above.

Statistical analysis

Each experiment was carried out at least three independent duplicates and data were analyzed by a SPSS 13.0 statistical package (SPSS, Inc, Chicago, IL). All data were expressed as mean (± SD) and analyzed with Student's t-tests for multiple comparisons. *P*-value less than 0.05 was considered statistically significant.

Results

High expression of TRIM66 in lung cancer tissues

To verify the TRIM66 expression in lung cancer, we detected the expression of TRIM66 mRNA in 30 lung cancer patients using RT-PCR. As shown in **Figure 1A**, TRIM66 mRNA was notably over expressed in tumor tissues compared to their adjacent normal tissues. And the TRIM66 protein expression in tumor and adjacent normal tissues was further investigated (**Figure 1B** and **1C**). Our data showed significantly high expression of TRIM66 protein in tumor tissues. These results indicated that TRIM66 was highly expressed in lung cancer tissues.

TRIM66 expression in lung cancer cell lines

As described above, a notable difference was showed between tumor and normal tissues. To further investigate the biological role of TRIM66 in lung cancer, we chose five lung cancer cell lines H446, H460, Spc-A-1, H1975 and A549. The mRNA expression and protein levels of TRIM66 in these cell lines were measured by

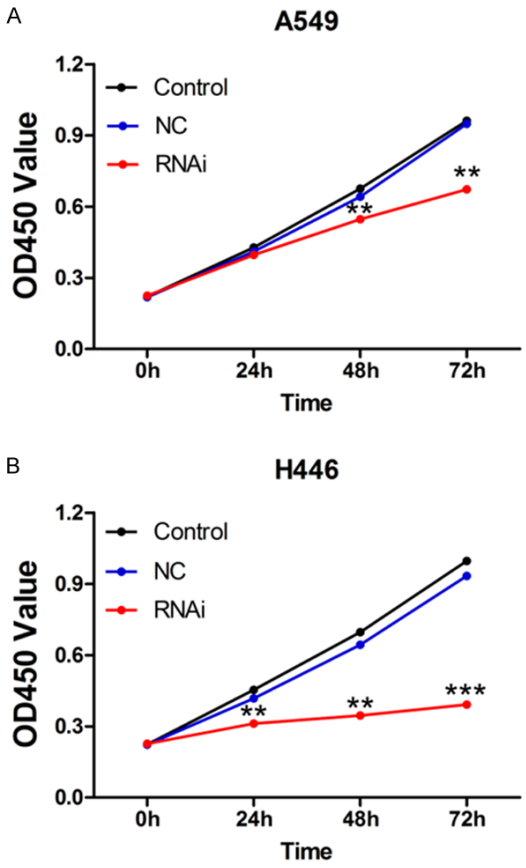

Figure 4. Knocking down of TRIM66 inhibited cell proliferation. Cell proliferation was analyzed by CCK-8 assay as described in materials and methods. A. Cell proliferation of A549 examined at 24 h, 48 h and 72 h after siRNA interference (n=3). B. Cell proliferation of H446 examined at 24 h, 48 h and 72 h after RNA interference (n=3). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (as comparing to controls).

RT-PCR and western blot respectively. As shown in **Figure 2A** and **2B**, mRNA and protein expression of TRIM66 in two cell lines, A459 and H446, were obviously higher than other cell lines. As a result, A549 and H446 cell lines were selected to carry out further experiments.

Silencing of TRIM66 expression via siRNA transfection

In an effort to analysis the biological function of TRIM66 in lung cancer, an siRNA targeting human TRIM66 was transfected into A549 and H446 to silence TRIM66 expression. And a non-specific scramble siRNA was transfected as NC treatment. RT-PCR and Western blot assays were processed at 48 h after siRNA transfection. As **Figure 3** showed, endogenous TRIM66 expression was declined significantly after siRNA transfection. The decrease ratio of mRNA expression and protein level was 61.3% and 39.2% in A549, 68.0% and 60.6% in H446, respectively. Whereas, silencing of TRIM66 was not detected in scramble siRNA transfected cells.

Inhibition of proliferation by TRIM66 silencing

To analysis the effect of TRIM66 in growth of lung cancer cell lines, we examined the proliferation of A549 and H446 cells at 0 h, 24 h, 48 h and 72 h post RNA interference using CCK-8 assay. As shown in **Figure 4**, the proliferation of both A549 and H446 cells remained unaffected post scramble siRNA transfection. Whereas, an obvious decrease of A549 cell growth was examined at 48 h and 72 h after RNA interference (**Figure 4A**). And a more significant inhibition of H446 proliferation was occurred progressively over 72 h after siRNA transfection (**Figure 4B**). Consequently, these results suggest that TRIM66 may promote the proliferation of lung cancer cell lines.

G1 phase arrest and cell apoptosis induced by TRIM66 silencing

As described above, silencing of TRIM66 inhibited the proliferation of lung cancer cells. This result indicated that TRIM66 may involve in the regulation of cell cycle and cell apoptosis. So we examined the cell cycle and cell apoptosis of A549 and H446 cells at 48 h after siRNA transfection. The cell cycle was measured by flow cytometry post PI staining. As shown in **Figure 5A** and **5B**, depletion of TRIM66 significantly increased the proportion of G0/G1 phase compared to control, with increased ratio: 27.8% and 53.8% of A549 and H446 respectively. Simultaneously, knocking down of TRIM66 decreased the proportions of S and G2/M phase notably (decreased ratio: 29.8% of A549 and 60.4% of H446 in S phase; 32.5% of A459 and 44.5% of H446 in G2/M phase).

Cell apoptosis was analyzed by flow cytometry after double stained by Annexin V-FITC and PI. As **Figure 5C** showed, as comparing to controls, nearly 8-fold increase and 15-fold increase of cell apoptosis was found in A549 and H446 post RNA interference, respectively. However, only slight increase was noted in scramble siRNA transfected cells which did not reach sta-

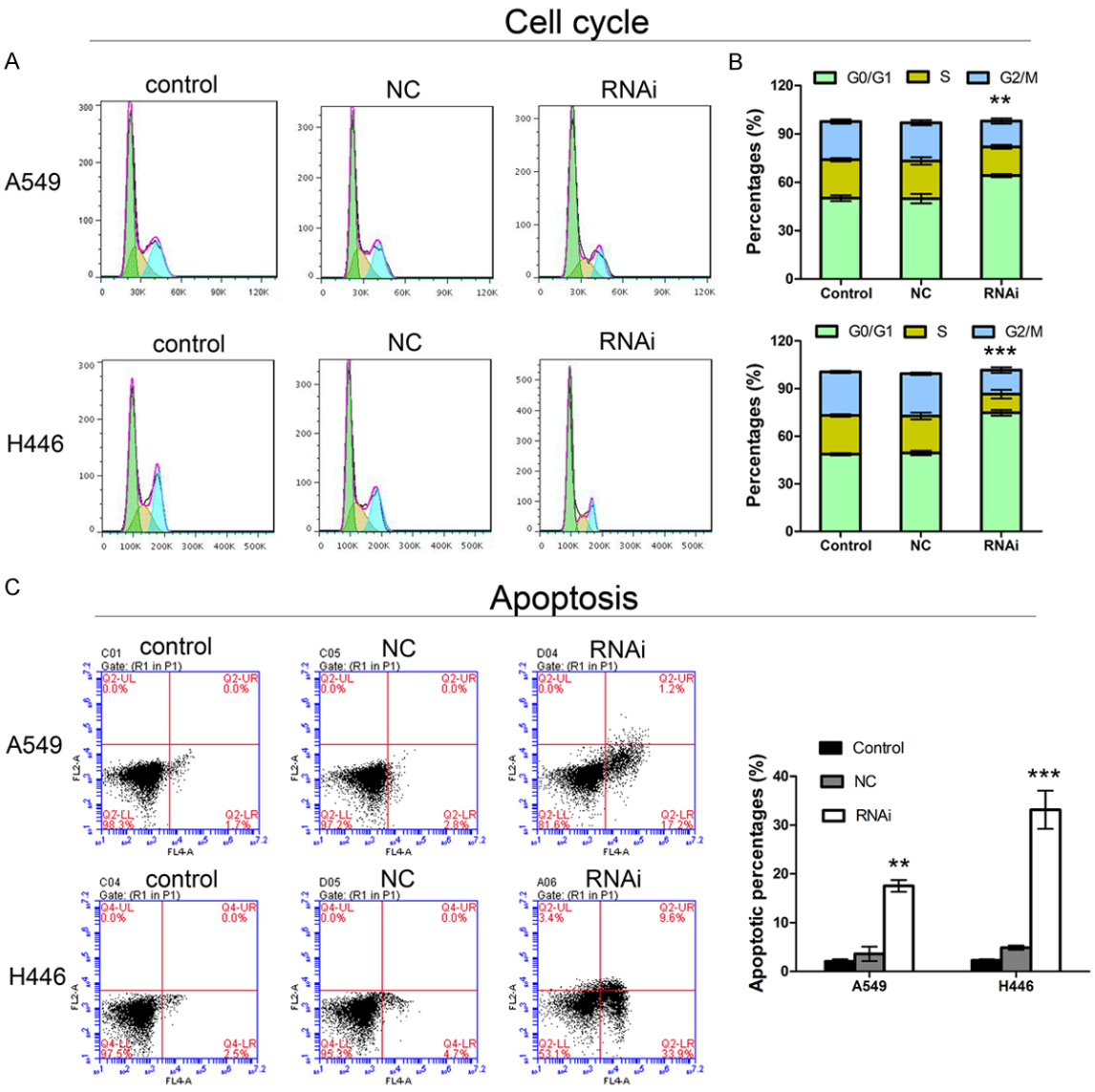

Figure 5. Depletion of TRIM66 promoted G1 phase arrest of cell cycle and cell apoptosis in lung cancer cell lines. Cell cycle and cell apoptosis were examined at 48 h after siRNA transfection using flow cytometry. A and B. Cell cycle profile of A549 and H446 (n=3). C. Cell apoptotic percentages of A549 and H446 analyzed post Annexin V-FITC and PI double stained (n=3). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (as comparing to controls).

tistical significance. All these results indicated that silencing of TRIM66 increased cells arresting in G0/G1 phase and facilitated cell apoptosis, which may lead to the depression of cell proliferation.

Inhibition of migration and invasion induced by TRIM66 silencing

To examine the effect of TRIM66 on tumor metastasis, we performed transwell assay and recorded the number of migrated and invaded cells. As shown in **Figure 6A** and **6B**, the numbers of migrated cells in A549 and H446 was reduced to 73±1 and 63±2 respectively after siRNA transfection. Whereas, the similar numbers of migrated cells were remained in control and NC treatment, which were notably higher than RNA interference treatment (A549: Control, 112±4; NC, 109±3; H446: Control, 120±3; NC, 119±1). Additionally, a same result was brought out in invasion assay. As **Figure 6C** and **6D** showed, the invasive ability of lung cancer cells was inhibited obviously by TRIM66 knock-

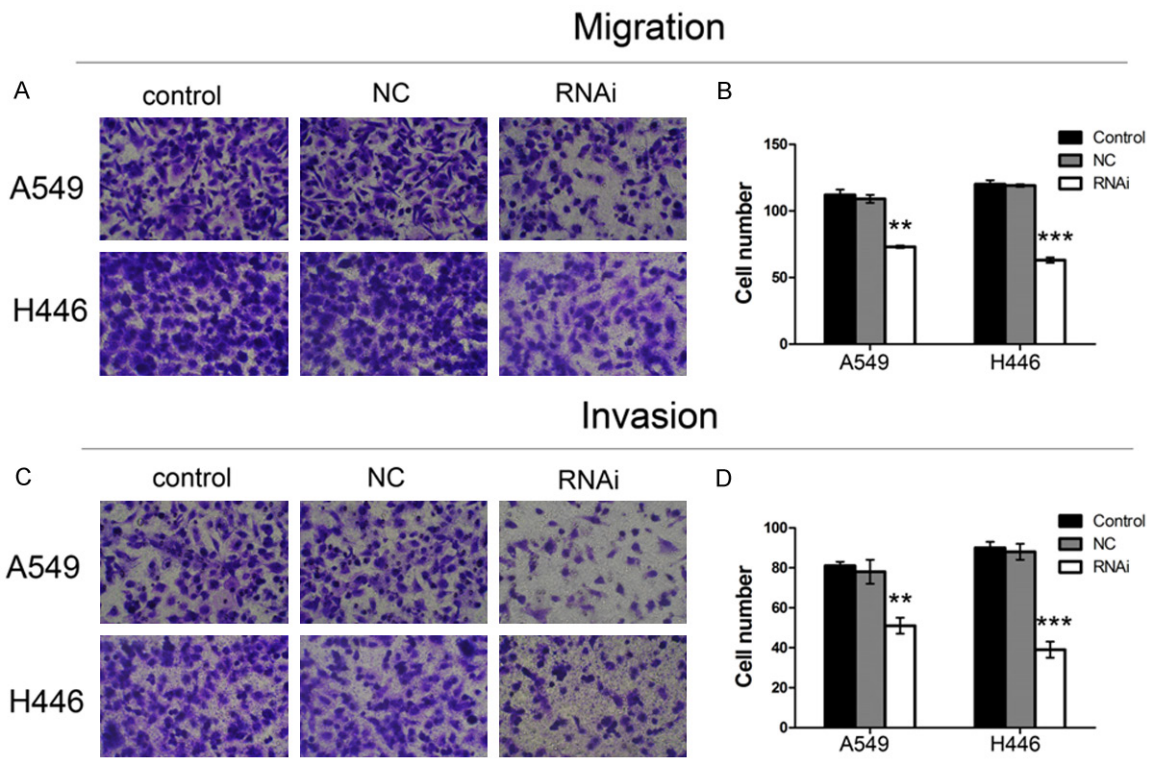

Figure 6. Silencing of TRIM66 inhibited metastasis of lung cancer cells. Two cancer cell lines were analyzed using transwell assay at 24 h post RNA interference. And cell numbers of migration and invasion were detected at 48 h after siRNA transfection. A-D. Depletion of TRIM66 significantly repressed cell migration and invasion in A549 and H446 (n=3). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (as comparing to controls).

ing down (A549: Control, 81±2; NC, 78±6; RNAi, 51±4; H446: Control, 90±3; NC, 88±4; RNAi, 39±4). These data indicated that TRIM66 may promote the migration and invasion of lung cancer cell lines.

Effects of TRIM66 knockdown on apoptotic protein levels

As repressing of TRIM66 promoted the apoptosis of lung cancer cells, we then analyzed the mechanism of cell apoptosis activation by examined the protein levels of Bcl-2, Bax, caspase3 and caspase9 using Western blot. As shown in **Figure 7A** and **7B**, compared to control, the protein level of Bcl-2 was significantly reduced by knocking down of TRIM66 (decrease ratios: A549, 73.0% and H446, 43.4%). And the protein levels of Bax (increase ratios: A549, 69.9% and H446, 182.3%), caspase3 (increase ratios: A549, 56.6% and H446, 120.1%) and caspase9 (increase ratios: A549, 79.8% and H446, 108.8%) were increased obviously.

Effects of TRIM66 knockdown on EMT pathway protein levels

To further indicate the mechanism of metastasis promotion by TRIM66, we measured the protein expression of MMP2, MMP9, Snail and Twist, which were played essential roles in EMT signaling pathway. The endogenous protein levels were examined by Western blot at 48 h after RNA interference. The results showed in **Figure 8A** and **8B** indicated that, the expression of EMT pathway proteins were significantly depressed by depletion of TRIM66 compared to control. And the decrease ratios were: MMP2, 33.9%; MMP9, 66.6%; Twist, 76.0% and Snail, 60.8% of A549 cells; MMP2, 52.6%; MMP9, 81.8%; Twist, 77.9% and Snail, 47.2% of H446 cells.

Discussion

More than 70 known TRIM proteins comprise a large family which has been involved in a broad range of cellular processes, such as cell prolif-

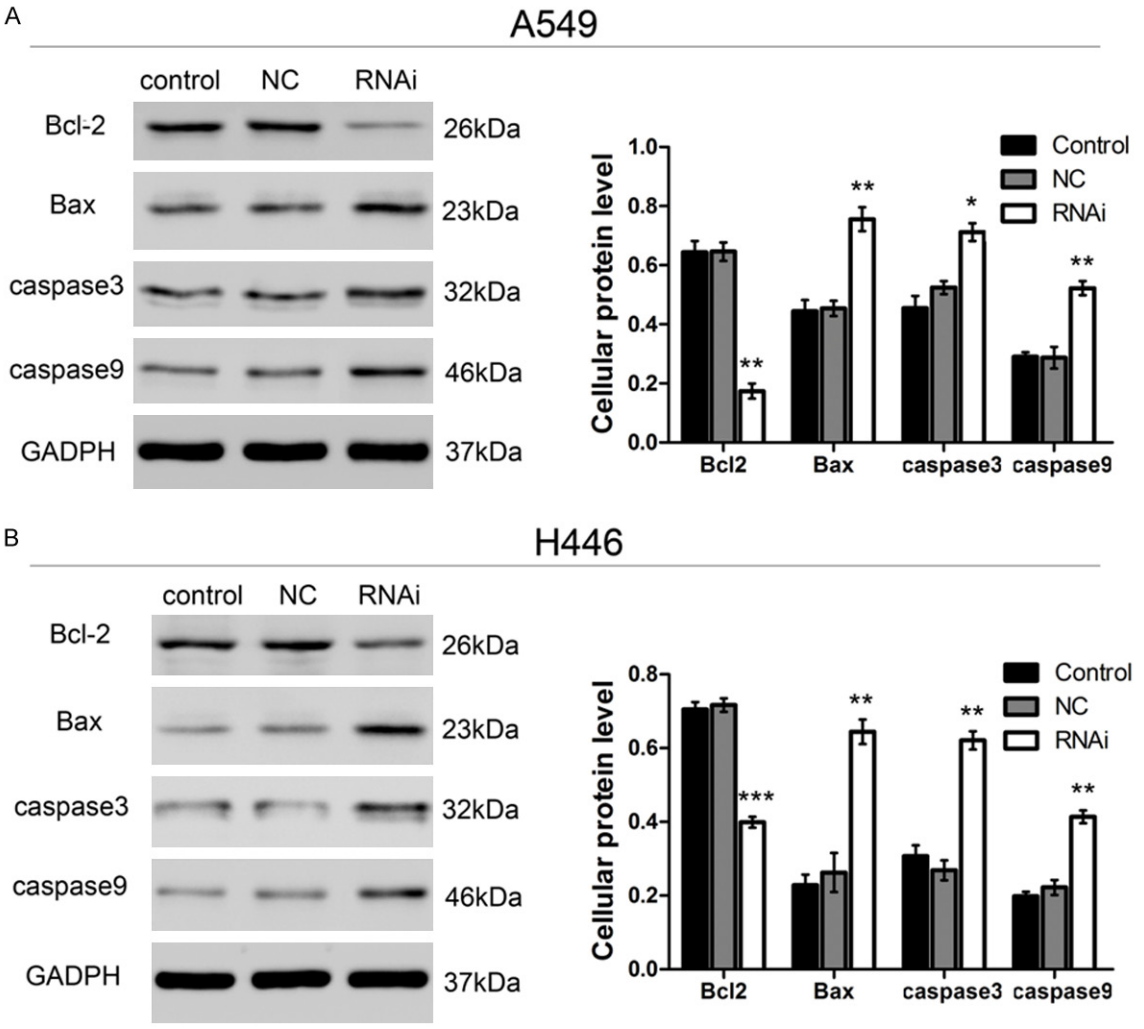

Figure 7. Knocking down of TRIM66 affected cell apoptosis pathway related protein levels in lung cancer cells. Relative protein levels were examined at 48 h after transfection using Western blot. A and B. TRIM66 knockdown decreased cellular Bcl-2 level, but increased the levels of cellular Bax, caspase3 and caspase9 obviously in A549 and H446 respectively (n=3). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (as comparing to controls).

eration and apoptosis, immunity, transcriptional regulation and signaling pathways [21]. Over expression of several TRIM proteins have been implicated in human lung cancer including TRIM28, TRIM24, TRIM44 and TRIM59 [22-25]. And Yu Chen et al. have illuminated that TRIM66 is over expressed in human osteosarcoma tissues and the over expression of TRIM66 promoted cell proliferation and metastasis of osteosarcoma cell lines [26]. However, the expression of TRIM66 as well as its biological functions has been poorly defined in human lung cancer. In this study, we found that TRIM66 was up-regulated significantly in lung cancer tissues compared to corresponding normal lung tissues. And this result may validate TRIM66 as an effective biomarker for diagnosis of lung cancer. The following vitro experiments in A549 and H446 cell lines demonstrated that depletion of TRIM66 inhibited cell proliferation, cell migration and invasion. Moreover, knocking down of TRIM66 induced G1 phase arrest and apoptosis in lung cancer cell lines.

Many researches have demonstrated that TRIM proteins are involved in inhibiting apoptosis of cancer cells. To further indicate the pathway via which TRIM66 impairs cell apoptosis, we examined the protein levels of Bcl-2, Bax, caspase3 and caspase9 in two cell lines. And our data

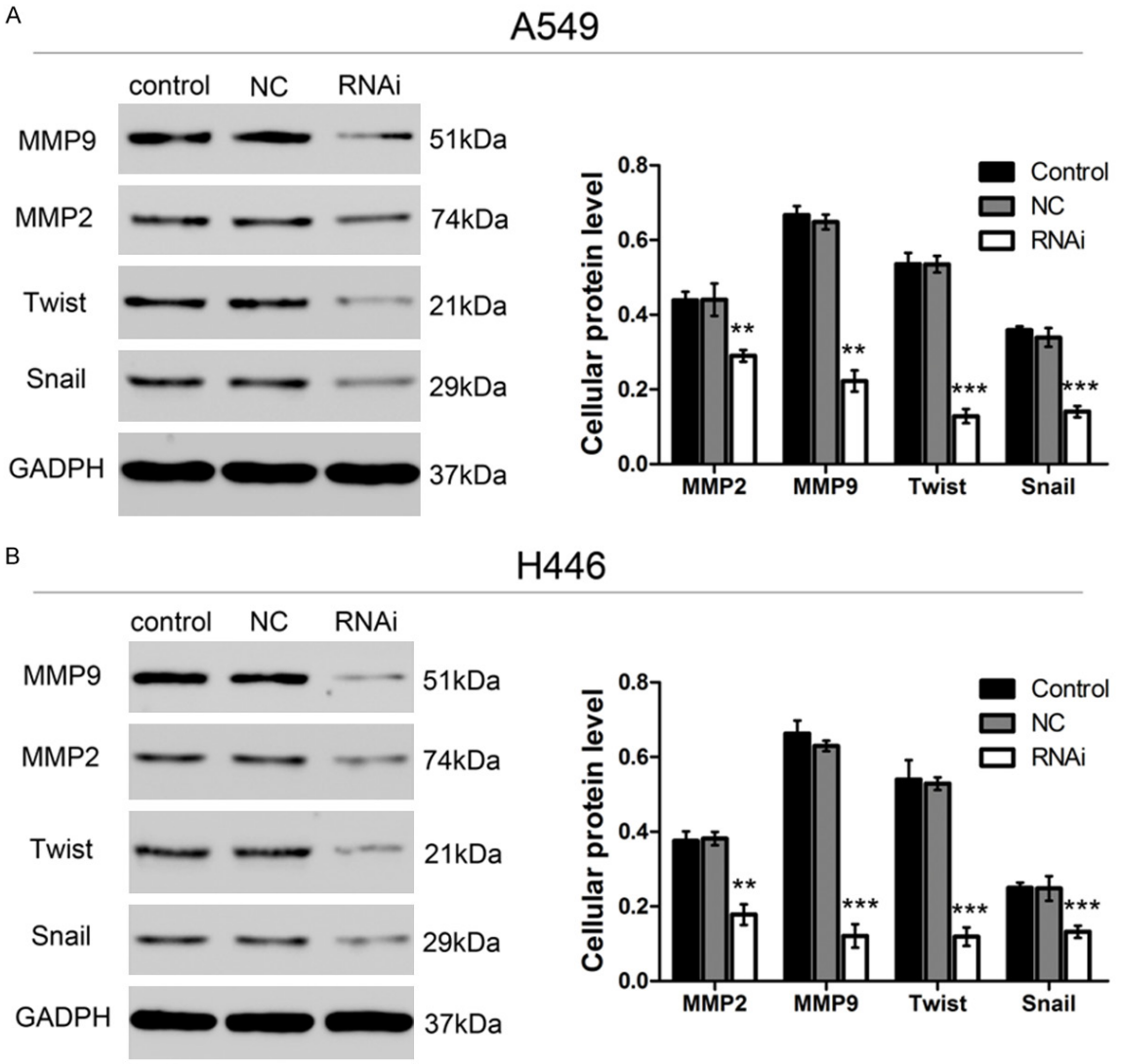

Figure 8. Silencing of TRIM66 affected EMT pathway related protein levels in lung cancer cells. Relative protein levels were examined at 48 h after transfection using Western blot. A and B. TRIM66 depletion decreased levels of cellular MMP2, MMP9, Snail and Twist significantly in A549 and H446 (n=3). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (as comparing to controls).

showed the Bax, caspase3 and caspase9 were up regulated significantly in RNA interference cells, whereas Bcl-2 was obviously decreased post siRNA transfection. Bcl-2 is identified as an anti-apoptosis protein that can inhibit Bax activation [27-29]. While Bax is known as a key effectors in regulating the permeability of outer mitochondrial membrane that may govern the release of mitochondrial cytochrome c [30]. And cytochrome c released in the cytosol via Bax proapoptotic properties activates caspase9 and subsequently trigger activation of caspase3 [31]. Up-regulated Bcl-2 can assume stable Bax/Bcl-2 heterodimer with down-regulated Bax, which prevent the formation of pro-apoptotic homodimer Bax/Bax [32]. The effects of TRIM66 knocking down on cellular target protein levels implicated that TRIM66 may inhibit apoptosis of lung cancer cells through up regulating Bcl-2 in cells.

EMT process is characterized by losing cell-cell adhesions and epithelial markers, while acquiring mesenchymal markers and phenotype. Accumulating studies demonstrate that EMT is very important in processing of tumor invasion and metastasis [33]. Snail and Twist are main transcriptional regulators of EMT. Moreover,

high expression of Snail and Twist promotes EMT progression and are detected in multiple carcinoma types [34]. Over expression of TRIM proteins such as TRIM24 and TRIM28 also contribute to up-regulation of Snail and Twist in hepatocellular carcinoma and lung cancer respectively. MMPs belong to a family of zinc-containing proteolytic enzymes that are involved in tumor metastasis [35, 36]. MMP2 and MMP9 are the most studied MMPs for their degradative capacity of type IV collagen in basement membranes [37, 38]. Researches have suggested that up-regulation of MMP2 and MMP9 is correlative with poor prognosis of lung cancer patients. TRIM29 up regulates MMP9 to promote invasion of lung cancer [39, 40]. And TRIM44 promotes non-small cell lung cancer progression by up-regulating MMP9 expression [24]. Additionally, MMP9 is identified as a EMT marker [41]. In this study, to further revealed the functional mechanism of TRIM66 on promotion of cell migration and invasion, we examined the cellular Snail, Twist, MMP2 and MMP9 using Western blot. And the data showed that depletion of TRIM66 significantly decreased the levels of these four target proteins, which indicated that TRIM66 may promote metastasis of lung cancer cells via effective activation of EMT pathway.

In conclusion, our study implicates that TRIM66 is highly expressed in lung cancer tissues. TRIM66 promotes proliferation and cell cycle transition from G1 to S phase of lung cancer cell lines. Moreover, TRIM66 may inhibit cell apoptosis through up-regulation of cellular Bcl-2 level following with decrease Bax level, and promote cell metastasis via activating EMT progression effectively.

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Disclosure of conflict of interest

None.

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