Original Article

Inhibition of tumor cell adhesion, invasion and migration in human lung cancer cells by silencing of TRIM27

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Abstract: TRIM27 belongs to the family of tripartite motif (TRIM)-containing proteins. Alternative in TRIM proteins have been implicated in several malignancies. The aim of this study was to evaluate the significance of TRIM27 expression in patients with lung cancer and to define its exact role in human lung cancer proliferation, adhesion, invasion and migration. Here, TRIM27 expression was higher in lung cancer tissues more than in adjacent lung tissues. Silencing of TRIM27 using siRNA suppressed proliferation, adhesion, invasion and migration of A549 and H460 cells. Meanwhile, silencing of TRIM27 decreased mRNA and protein expression levels of proliferating cell nuclear antigen (PCNA), cell division cycle 45 (CDC45), matrix metalloprotein 9 (MMP9) and twist family bHLH transcription factor 1 (TWIST1) in A549 and H460 cells. In conclusion, TRIM27 may act as an oncogene through suppressing proliferation and motility in lung cancer carcinogenesis and may serve as potential therapeutic target in lung cancer.

Keywords: Lung cancer, TRIM27, MMP9, TWIST

Introduction

Lung cancer is one of the leading causes of cancer death and one of the most common cancers worldwide, accounting for more than a million deaths yearly [1]. Of lung cancer, non-small cell lung cancer (NSCLC) is the most common type and accounts for approximately 85% of all cases of lung cancer [2]. NSCLC is further divided into adenocarcinoma, squamous cell carcinoma and large-cell carcinoma. Although recent advances in clinical and experimental oncology, the survival of lung cancer is still poor, with a 5-year survival time of approximately 11% [3]. In order to develop and improve more effective therapies, it is important to understand the mechanisms underlying lung cancer development and progression. Recently, accumulating evidence has shown that genetic alterations may involve in cancer pathogenesis, providing new insights into disease biology.

TRIM27 is a member of the family of tripartite motif (TRIM)-containing proteins. TRIM proteins, members of the RING family of E3 ligases, are well known for their role in regulation of innate immune response during viral infection [4, 5]. Accumulating evidence demonstrates that TRIMs play a crucial role in several cellular processes and have been associated with various pathologies including cancer [6]. For example, upregulated expressions of TRIM24, TRIM25 and TRIM28 were found in breast cancer [7-9]. TRIM33 expression has been reported to be reduced in chronic myelomonocytic leukemia [10]. TRIM27 mRNA levels were higher in several human cancers including uterus, colon, lung and rectum cancer, where predicts poor prognosis [11]. However little is known about the mechanisms underlying TRIM27 involved in lung cancers.

Metastasis is a leading cause of cancer-related deaths and consists of multiple processes. Matrix metalloproteinases (MMPs) play a key role in tumor metastasis mainly by degrading extracellular matrix (ECM) proteins such as collagen and fibronectin [12, 13]. MMP9 acts as a member of MMPs expressed abundantly in various cancers and induced by inflammatory cytokines and epidermal growth factors thus play an important role in cancer cell invasion and
migration [14, 15]. Additionally, TWIST-1 is a basic helix-loop-helix transcription factor regulates genes that are essential for cell migration. Previous study suggests that TWIST-1 involved in cancer metastasis induced by STAT3 activation [16].

In the present study, we aimed to investigate the expression and function of TRIM27 in lung cancer. Finally, we demonstrated that TRIM27 promotes lung cancer processes associated with proliferation, adhesion, invasion and migration.

Material and methods

Patients and tissue samples

35 paired human lung cancer and adjacent tissues from patients admitted to The First Affiliated Hospital of Jiaxing University were enrolled in this study. All patients have complete clinical and pathological follow-up data. These normal lung tissues were resected within at least 5 cm of the tumor margin when the patients underwent definitive surgery. Human lung cancer and adjacent tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until total RNA was extracted. The study protocol was approved by the ethics committee of The First Affiliated Hospital of Jiaxing University. Written informed consents were obtained from all participants in this study. All the research was carried out in accordance with the Helsinki Declaration of 1975.

Cell culture

Human lung cancer cell lines (H1975, A549, PC-9, H460, 95-D and H446) were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in RPMI-1640 (Invitrogen Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin, and incubated in a humidified atmosphere at 37°C with 5% CO₂.

RNA interference (RNAi) and construction of stable cell lines

TRIM27 siRNA was designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Cells were transfected with TRIM27 siRNA (siTRIM27, 40 nM) by using the Lipofectamine 2000 (Invitrogen, Shanghai, China) following the manufacturer’s protocol. A scramble siRNA was used as negative control (NC), and the selective silencing of TRIM27 was confirmed by Real-time PCR analysis. Cells were analyzed 48 h after transfection.

Real-time RT-PCR

Total RNAs were extracted from human lung cancer and adjacent tissues and cell lines with TRIZOL reagent (Invitrogen Life Technologies) and stored at -80°C. Complementary DNA was synthesized with cDNA synthesis kit (Thermo Fisher Scientific, Rockford, IL, USA). Real-time PCR was performed using a standard SYBR Green PCR kit protocol on ABI7300 (Applied Biosystem, Shanghai, China) thermal cycler. GAPDH was used as internal controls for indicated genes. The ∆∆Ct method for relative quantification of gene expression was used to determine mRNA expression levels. The primers sequences (sense/antisense) used were as followed, TRIM27, 5'-TGGAGAGGGAGAAGAT-TG-3' (forward) and 5'-CAAGAGAACTGGGTA-G-3' (reverse); PCNA, 5'-GCCTGACAAATGCTTGAC-3' (forward) and 5'-TGTAGTGCTTCACA-CCTTC-3' (reverse); CDC45, 5'-ACCATACCG-ATTGACTAA-3' (forward) and 5'-ATAAGCACA-TCTGAAGAC-3' (reverse); MMP9, 5'-AAGGGCTCTGATGTGTTAG-3' (forward) and 5'-AGCATGGCGTCCGTGGATG-3' (reverse); TWIST, 5'-AGTCCGAGTGCTTACGAG-3' (forward) and 5'-GCCTGGCTATCCTTGGAGT-3' (reverse); GAPDH, 5'-CACCCACCTCCTCCACCTTTG-3' (forward) and 5'-CCACACCTCGGTCTGCTGTA-3' (reverse).

Western blot analysis

Human lung cancer tissues and cell lines were harvest and washed twice with PBS and lysed in ice-cold radio immunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China) and incubated on ice for 30 min. Cell lysis was centrifuged at 13,000 rcf for 10 min at 4°C and the supernatant (20-30 μg of protein) was run on 10% SDS-PAGE gel and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Shanghai, China). The blots were blocked with 5% skim milk, followed by incubation with antibodies specific against TRIM27, CDC45, MMP9 and TWIST (Abcam, Cambridge, MA, USA), and antibodies against PCNA and GAPDH were purchased from Cell Signaling Technology, Inc. (Beverley, MA, USA).
TRIM27 and human lung cancer

The RNA interfering effects of the TRIM27 siRNA (siTRIM27) on the A549 and H460 cells were determined by Cell Count Kit-8 (CCK-8, Dojindo, Rockville, MD, USA) assay. In brief, 5×10^4 cells were dispensed within 96-well culture plates in 100 mL volumes. CCK-8 reagent was added to each well at 1 hour before the endpoint of incubation. The optical density (OD) 450 nm values in each well were determined by a microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Cell proliferation assay

Cell treated with siTRIM27 were plated in the top chamber of the transwell (Greiner Bio-One, Frickenhausen, Germany) precoated with Matrigel (BD, San Diego, CA, USA) for invasion assay and without Matrigel for migration. In both assays, the lower chamber was filled with 600 μL DMEM containing 10% FBS. After 48 h incubation, cells on the upper well were wiped off by the Q-tip. The cells attached to the lower surface were washed with PBS, fixed in 4% paraformaldehyde and stained by 0.5% crystal violet. Images of the cells were captured and cell numbers were counted under a microscope (Olympus Corporation) with magnification of ×200.

Cell invasion and migration assays

Cell treated with siTRIM27 were plated in the top chamber of the transwell (Greiner Bio-One, Frickenhausen, Germany) precoated with Matrigel (BD, San Diego, CA, USA) for invasion assay and without Matrigel for migration. In both assays, the lower chamber was filled with 600 μL DMEM containing 10% FBS. After 48 h incubation, cells on the upper well were wiped off by the Q-tip. The cells attached to the lower surface were washed with PBS, fixed in 4% paraformaldehyde and stained by 0.5% crystal violet. Images of the cells were captured and cell numbers were counted under a microscope (Olympus Corporation) with magnification of ×200.

Statistical analysis

The data was presented as the mean value ± SD. Statistical significance was determined by the unpaired, two-tailed Student's t-test and One-way ANOVA analysis. SPSS 10.0 software was used to perform the statistical analyses. All the experiments were performed at least thrice in triplicates. P<0.05 was considered significant.
Results

TRIM27 expression was up-regulated in lung cancer tumor tissues and cell lines

We first examined expression levels of TRIM27 in 35 paired human lung cancer tumor and adjacent tissues by Real-time PCR using GAPDH as reference. We found that the mRNA level of TRIM27 in cancer tissues was significantly higher than that in adjacent lung tissues (Figure 1A). To further validate the above mRNA results, we also examined TRIM27 expression in 5 randomly selected lung cancer tumor tissues by Western blotting. The results showed a statistically significant elevation of TRIM27 expression in tumors, as compared to the adjacent-normal lung cancer tissues using GAPDH as the reference (Figure 1B).

The mRNA and protein expressions of TRIM27 were higher in A549 and H460 cells than in the four other human lung cancer cell lines, as shown in Figure 1C. Therefore, the siRNA targeted TRIM27 was transfected into the A549 and H460 cells. The scramble siRNA (NC) transfected into the A549 and H460 cells were used as negative controls. The A549 and H460 cells without transfection were used as controls.

Effects on cell proliferation by TRIM27 siRNA transfection

With a TRIM27 siRNA (siTRIM27), the expressions of TRIM27 mRNA were successfully reduced to approximately 54% and 68% of the levels in the negative control siRNA (NC)-transfected A549 and H460 cells at 48 h after transfection (Figure 2A). At the same time, the
expression of TRIM27 remained at the same levels as in control A549 and H460 cells without transfection. The expression of TRIM27 protein were also examined by Western blot and showed significantly decreased levels of TRIM27 in siTRIM27-transfected A549 (reduced to approximately 44%) and H460 cells (reduced to approximately 59%) at 48 h after transfection compared with NC-transfected A549 and H460 cells (Figure 2B).

CCK-8 assay revealed that the siTRIM27 significantly inhibited the cell proliferation in a time-dependent manner in siTRIM27-transfected A549 and H460 cells, decreased by 14%, 39% and 56% of that in NC-transfected A549 cells at 24, 48 and 72 h, respectively (Figure 2C), and decreased by 6%, 33% and 38% of that in NC-transfected H460 cells at 24, 48 and 72 h, respectively (Figure 2D). These data suggest that silencing of TRIM27 in A549 and H460 cells by siRNA transfection significantly inhibited cell proliferation and A549 cells rather than H460 cells were sensitivity to siTRIM27 transfection.

Effects of adhesion, invasion and migration with TRIM27 siRNA transfection

Carcinoma cell adhesion to extracellular matrix and basement membranes is regarded as an initial step in the invasive process for metastatic tumor cells. The effects of TRIM27 siRNA on lung cancer cell adhesion were identified by the
cell adherence as previously described. As shown in Figure 3A and 3B, siTRIM27 could notably suppress adhesion of A549 and H460 cells by 34% and 52%, respectively, in comparison with the NC-transfected A549 and H460 cells. The number of A549 and H460 cells transfected with siTRIM27 which invaded through matrigel was significantly decreased by 36% and 66% compared with NC-transfected A549 and H460 cells (Figure 3C and 3D). The ability to migrate assessed in chambers without a matrix was also significantly reduced by 33% and 59% in the case of siTRIM27-transfected A549 and H460 cells (Figure 3E and 3F).

Effects on gene expression levels with TRIM27 siRNA transfection

To provide more insight regarding how TRIM27 may regulate the proliferation, invasion and migration of lung cancer cells, Real-time PCR and Western blot were performed to measure the expression of genes at both mRNA and protein levels. The mRNA expression of genes involved in cell proliferation and cycle, including PCNA and CDC45 were significantly decreased by 47% and 59% in siTRIM27-transfected A549 cells compared with NC-transfected A549 cells (Figure 4A). At the same time, the mRNA expression of genes involved in cell invasion and migration, including MMP9 and TWIST were significantly decreased by 71% and 38% in siTRIM27-transfected A549 cells compared with NC-transfected A549 cells. Similar to Real-time PCR analysis, the Western blot assay also showed markedly decreased protein expression of these genes in siTRIM27 assay also showed markedly decreased protein expression of these genes in siTRIM27-transfected A549 cells, but not in NC-transfected A549 cells (Figure 4B). H460 cells rather than A549 cells were sensitivity to siTRIM27 transfection and revealed 63%, 69%, 81 and 66% reduced invasion, migration and proliferation in comparison with NC-transfected H460 cells.

Figure 4. Changes in PCNA, CDC45, MMP9 and TWIST1 expression in A549 and H460 cells transfected with TRIM27 siRNA. The expression levels of PCNA, CDC45, MMP9 and TWIST1 were significantly decreased in A549 cells transfected with siTRIM27, as compared to the cells transfected with NC, detected by Real-time PCR (A) and Western blot (B). The expression levels of PCNA, CDC45, MMP9 and TWIST1 were significantly decreased in H460 cells transfected with siTRIM27, as compared to the cells transfected with NC, detected by Real-time PCR (C) and Western blot (D). GAPDH was used as loading control. *P<0.001 compared with NC. *P<0.01 compared with NC.
mRNA expression of PCNA, CDC45, MMP9 and TWIST in siTRIM27-transfected cells compared with NC-transfected cells, respectively (Figure 4C). In agreement with Real-time PCR analysis in H460 cells, the decreased protein expressions of these genes were also found in siTRIM27-transfected H460 cells, but not in NC-transfected H460 cells (Figure 4D).

**Discussion**

To date, only a few studies have implicated TRIM27 in human lung cancer. Zoumpoulidou et al. demonstrated that enhanced, and possibly aberrant, expression of TRIM27 transcript and protein in lung cancer and predicts poor outcome [11]. This follows reports showing that TRIM27 expression is enhanced in several human malignancies, including endometrial [17], seminomas [18] and invasive breast cancer [19]. However, the role of TRIM27 in proliferation, invasion and migration in lung cancer was obscure. Our study investigated TRIM27 expression in lung cancer and the associations of TRIM27 and cell proliferation and mobility. In this study, we detected TRIM27 by Real-time PCR and Western blotting. A strong point of this research is the findings that expression of TRIM27 was significantly increased in lung cancer tissues compared with adjacent lung tissues, and A549 and H460 cells had higher levels of TRIM27 than in other lung cancer cell lines, suggesting that TRIM27 may be a therapeutic target for cancer therapy.

We further demonstrated that TRIM27 was important for cancer proliferation and invasion. Down-regulation of TRIM27 expression by siRNA in A549 and H460 cells lung cancer cells led to decreased cell proliferation and adhesion, consisting with the defined role of TRIM27 in the migration and attachment of epithelial cells. At the same time, the cell growth and cycle-related genes PCNA and CDC45 were also detected in A549 and H460 cells. We found that the expression of these genes was significantly decreased in cells transfection of TRIM27 siRNA. These support growing evidences that TRIM27 promotes proliferation and influences the transformation of tumor cells. In addition, down-regulation of TRIM27 is associated with reduced turnover of the focal adhesion, which is needed for cell invasion and migration [20].

In addition to adhesion, tumor cells need to invade to establish metastasis successfully at a distant site. Tumor cell invasion involves the degradation of the matrix, and this destruction has been attributed to the activity of proteases. MMPs are a member of proteases and that, which implicated in tissue remodeling in normal cells [21], the production of MMPs has been observed in many invasive tumor cell lines and during tumor growth [22]. In some cases, MMP9 has been found in several lung cancer cell lines and surgical specimens, evidencing that MMPs are involved in invasion and angiogenesis in lung cancer [23, 24]. TWIST1 is temporally expressed during normal development, but constitutively expressed in some forms of cancers [25], including breast, liver, prostate and stomach, and the role of TWIST1 in tumor invasion and metastasis has been attracting increasingly greater interest. TWIST1 can enhance cell invasion in gastric cancer both in vitro and in vivo [26]. On the basis of these report, we next investigated the effect of TRIM27 siRNA on MMP and TWIST1 expression. In this study, we showed that down-regulation of TRIM27 inhibited tumor cell invasion through down-regulation of MMP and TWIST1 expression. Thus TRIM27 inhibition may activate a signaling cascade resulting in regulation of transcription of MMP and TWIST1. However, further studies are warranted to explore these possibilities. Whatever the underlying mechanism, the ability of TRIM27 down-regulation to inhibit tumor cell invasion has been clearly shown in this study.

Finally, we detected the effect of TRIM27 siRNA on tumor cell migration, which is an important element in the physiology of a cell in pathologic states. For example, the migration of tumor cells to distant organ sites plays a pivotal role in metastasis [27]. This is further supported by our findings, which show that the decreased tumor cell migration during down-regulation of TRIM27 is accompanied by decreased expression of MMP9 and TWIST1. Recently, inhibition of MMP9 has been demonstrated to inhibit migration in several lung cancer cell lines by suppressing Notch-1 mediated NF-kB and uPA pathways [27], and activating MMP9 transcription potentiates lung cancer cell migration and invasion [28]. Depletion of TWIST1 can suppress the migration in prostate cancer cells. Moreover, TWIST1 also enhance cell migration.
in hepatocellular carcinoma both in vitro and in vivo [29]. These results indicate that TRIM27 may participate in the regulation of cell migration by down-regulation of MMP and TWIST1 expression. Investigation on the mechanisms by which TRIM27 enhances invasive and migratory phenotypes in lung cancer cells and other tumor cell lines is ongoing.

In summary, our study provides experimental evidence that TRIM27 highly expressed in human lung cancer tissues and cell lines, and down-regulation of TRIM27 suppresses cell proliferation, adhesion, invasion and migration in lung cancer cells by inhibition of MMP9 and TWIST1. The present study implies the intriguing possibility of using TRIM27 as an innovative and promising therapeutic target for lung cancer.

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Disclosure of conflict of interest

None.

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