

Original Article

TRIM26 acts as a tumor suppressor in non-small-cell lung cancer

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Abstract: The family of tripartite motif (TRIM) represents a kind of pattern recognition receptors, which are known to be required for the restriction of virus infection. TRIMs are involved in many biological process as well as diverse pathological conditions, such as development disorders, neurodegenerative diseases, and viral infections. Innate immunity is essential for anti-tumor effects; however, the effects of TRIM26 on tumor development remain to be explored. In the present study, using lung cancer patients' samples and related cell lines, we evaluated the role of TRIM26 in human NSCLC development and found that the expression of TRIM26 decreased in 36 patient samples as well as NSCLC cell lines. Further studies showed that overexpression of TRIM26 can inhibit cell growth and induce cell apoptosis through downregulating BCL-2. Collectively, our study demonstrates that TRIM26 limits lung cancer growth, suggesting a potential therapeutic target to fight against cancer.

Keywords: TRIM26, lung cancer, apoptosis

Introduction

Lung cancer is one of the most common human malignancies all over the world and a major cause of death in many developed countries. Non-small-cell lung cancer (NSCLC) accounts for at least 80% of all lung cancer case [1, 2]. Only 15% of NSCLC patients live 5 years or more after diagnosis [3]. Rapidly metastasis and spread are important reasons for the low 5-year survival rate of NSCLC [4]. The prediction of clinical prognosis still depends on conventional pathologic variables such as tumor size, tumor grade, lymph node, and clinical stage. It is of great clinical value to find sensitive and specific early biomarkers for the diagnosis and prognosis of this malignancy, as well as novel therapeutic strategies.

The tripartite motif (TRIM) family is a diverse family of RING finger domain-containing proteins, which are involved in a variety of cellular functions. The TRIM family has more than 70 members in humans [5, 6]. They contain an N-terminal RBCC motif composed of a RING domain, one or two B-boxes, a coiled-coil domain as well as C-terminal regions with different structures. The B-box is a zinc-binding motif but its function is unclear while the coil-

ed-coil domain mediates protein interactions. Although the C-terminal regions differ among individual members, the majority of the TRIM proteins contain a PRY or SPRY domain or both domains, which mediates protein-protein interactions [7]. Recent studies have indicated that some members of the TRIM proteins function as important regulators for carcinogenesis. Loss of heterozygosity of the TRIM3 locus can be found in about 20% of human glioblastomas and TRIM3 may block tumor growth by sequestering p21 and preventing it from facilitating the accumulation of cyclin D1-cdk4 [8]. TRIM16 has significant effects on neuroblastoma proliferation and migration in vitro and tumorigenicity in vivo. TRIM16 also plays a role in cell cycle progression through changes in cyclin D1 and p27 expression [9]. Loss of TRIM29 expression in normal breast luminal cells can contribute to malignant transformation and lead to progression of ER+ breast cancer in premenopausal women [10]. TRIM44-overexpressing tumors had a worse overall rate of survival than those with non-expressing tumors in both intensity and proportion expression-dependent manner [11].

However, little is known about the role of TRIMs in NSCLC. TRIM26 contains three zinc-

binding domains, RING, B-box type 1 and B-box type 2, and a coiled-coil region [12]. But the exact function of TRIM26 still remains unknown. Here we tested the expression of TRIM26 in NSCLC patient samples and overexpressed TRIM26 in several cell lines so as to determine its effect on NSCLC. Our data demonstrate that TRIM26 is a tumor suppressor in non-small-cell lung cancer.

Materials and methods

Patient samples and cell lines

Human NSCLCs and their matched normal tissues were obtained from 36 patients at The First People's Hospital of Jingmeng. Tissues were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. Written informed consent was obtained from each patient and this study was approved by the Medical Ethics and Human Clinical Trial Committee at The First People's Hospital of Jingmeng. Four NSCLC cell lines (A549, SK-MES-1, H1299 and H1650) and a normal lung bronchus epithelial cell line BEAS-2B were purchased from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. All cells were incubated in 5% CO₂ humid atmosphere at 37°C.

RNA extraction and qRT-PCR

Total RNA was isolated from tissues and cell lines using Rneasy kit (Qiagen, Valencia, CA), and cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) from total RNA. Real-time PCR was performed with LightCycle 2.0 system using the Light-Cycler-FastStart DNA Master SYBR Green Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. For PCR amplification, the following primers were used: TRIM26 forward primer, 5'-AGTTTGAGCAGGGTCATCAG-3', TRIM26 reverse primer, 5'-CGTTGCAGAGAGAGGAGTTTAT-3', β -actin forward primer, 5'-AGTGTGACGTGGACATCCGCAAAG-3'; and β -actin reverse primer, 5'-ATCCACATCTGCTGGAAGGTGAC-3'. Relative transcript levels were normalized to the reference gene, β -actin. The results are the mean \pm SE of the three independent experiments performed at least in duplicate.

Transfection and establishment of stable cell lines

Transfection was performed in the absence of serum, with cells being 50-70% confluent.

Lipofectamine 2000 was used for transfection according to the manufacturer's protocol. Stable cell lines were selected and maintained in G418.

Western blotting

Proteins were extracted with RIPA buffer with protease inhibitors and quantified by the BCA method. Lysates (25 μ g) were separated on SDS-PAGE and then electrotransferred to nitrocellulose membranes. Membranes were blocked for 2 h at room temperature with 5% nonfat dried milk solution and then immunoblotted overnight at 4°C with primary antibodies against TRIM26 and β -actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA). After washing, the membranes were probed with HRP-conjugated secondary antibodies. Signals were visualized with Enhanced Chemiluminescence Plus Kit (GE Healthcare).

Growth and colony formation assays

For growth assays, 1000 cells were seeded per well in a 24-well plate in triplicate. Cells were incubated at 37°C in a humidified incubator for different periods of time. After incubation, cells were washed with PBS and trypsinized and counted. For colony formation assay, 5000 cells were seeded in a p100 plate in triplicate and incubated at 37°C in a humidified incubator for 7-10 days. After incubation, cells were washed with PBS once and stained with 0.5% crystal violet for 1 min at room temperature. Cells were washed with PBS, and images were scanned using an Epson Expression 1680 scanner. 10% acetic acid was added to each plate and incubated with shaking for 20 min. One-half ml of supernatant was diluted 1:4 in water and its absorbance measured at 590 nm.

Cell apoptosis analyses

Cell apoptosis assays were performed using ssDNA apoptosis ELISA kit (Chemicon). 1×10^4 cells were plated on 96-well plates and the assay was performed according to the manufacturer's protocol.

Statistical analysis

The 2-tailed Student's t test was used for all statistical analyses in this study using SPSS. A value of $P < 0.05$ is defined as statistically significant.

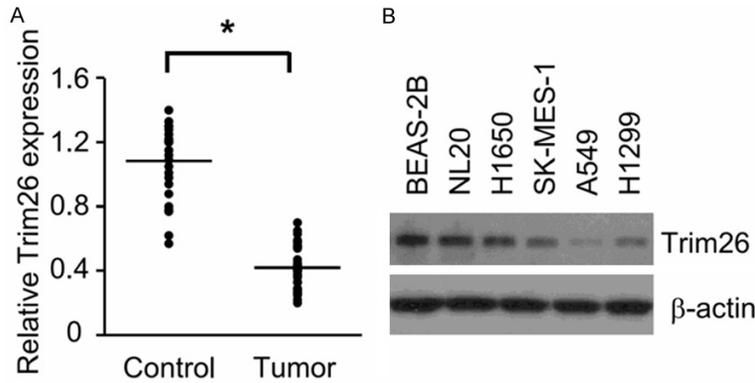


Figure 1. TRIM26 is downregulated in NSCLC tissues and cell lines. A. The expression levels of TRIM26 in 36 pairs of NSCLC tissues and peritumoral lung tissues were measured by qRT-PCT. Data are presented as mean ± SD of three independent experiments. The 2-tailed Student's t test was used to analyse the data. * $P < 0.05$. B. Cell lysates from cell lines as indicated were used to perform Western blot analysis with TRIM26-specific antibody. The images shown are representative of three independent experiments.

Results

Expression of TRIM26 is decreased in NSCLC tissues and cell lines

To assess the role of TRIM26 in non-small-cell lung cancer, we first examined its expression in tumors and normal tissues. Total RNA were extracted from 36 lung tumor issue or peritumoral tissue, and RT-PCR assays were used to measure the levels of TRIM26. As shown in **Figure 1A**, our data demonstrated that TRIM26 mRNA was largely decreased in NSCLC tissues compared to normal tissues, suggesting the feasible regulation role in lung cancer cellular behavior. Then we used a TRIM26-specific antibody to test the levels of the TRIM26 protein in A549, SK-MES-1, H1299, and H1650 by western blot. The expression of TRIM26 is dramatically lower in all these four cell lines that of control cell line (**Figure 1B**). So our data indicate that expression of TRIM26 is decreased in non-small cell lung cancer and TRIM26 may play a role in inhibiting the growth of cancer cells.

TRIM26 inhibits NSCLC cell proliferation in vitro

As TRIM26 expression in NSCLC tissues and cell lines is significantly lower, so we assumed that TRIM26 negatively regulated the growth of lung cancer cells. Proliferation contributes to the cell growth; we next determined to test whether overexpression would regulate the pro-

liferation in cell lines, which is more stable and repeatable. We transfected A549 and H1299 cells with plasmid expressing wild-type TRIM26, and our results confirmed the efficiency of transfection (**Figure 2A**). Then we assessed the cell growth rate by measure the proliferation with MTT assay on a daily basis for 5 days. As shown in **Figure 2B**, cells overexpressing TRIM26 grew significantly slower than control cell line. In order to strengthen the effects, we used clonal forming assay to measure the cell proliferation in which we plated cells at very low density

and measured the ability to form colonies after 10 days. The colonies were stained with 0.5% crystal violet and quantified, and our results showed that control cells formed colonies much more efficiently than TRIM26-overexpressing cells (**Figure 2C**). Overall, these results demonstrate that TRIM26 plays an important role in inhibiting NSCLC cell proliferation.

TRIM26 induces NSCLC cell apoptosis by down-regulating BCL-2

As accelerated apoptosis sometimes displays the slower proliferation, so we then analysis the apoptosis in NSCLC cell lines if overexpression of TRIM26. Using A549 and H1299 cells, we performed the overexpression of TRIM26, and then we monitored the apoptosis. Our data showed that overexpression of TRIM26 in these cells induced the apoptosis significantly (**Figure 3A**). This result may explain the effects of TRIM26 on NSCLC cell proliferation. As Bcl-2 is one essential component working against apoptosis, and downregulation of Bcl-2 contributes to apoptosis in various environments. In cell lines of A549 and H1299 cells, overexpression of TRIM26 substantially decreased the expression of Bcl-2 in both cell lines (**Figure 3B**). Simultaneously overexpression of Bcl-2 in the cells partially reverse the TRIM26-mediated the apoptosis, additionally suggesting the Bcl-2 is involved in the TRIM26-induced the apoptosis (**Figure 3A**). Taken together, our results indicate that TRIM26 induces NSCLC cell apoptosis through down-regulating Bcl-2.

Anti-tumor effects of TRIM26

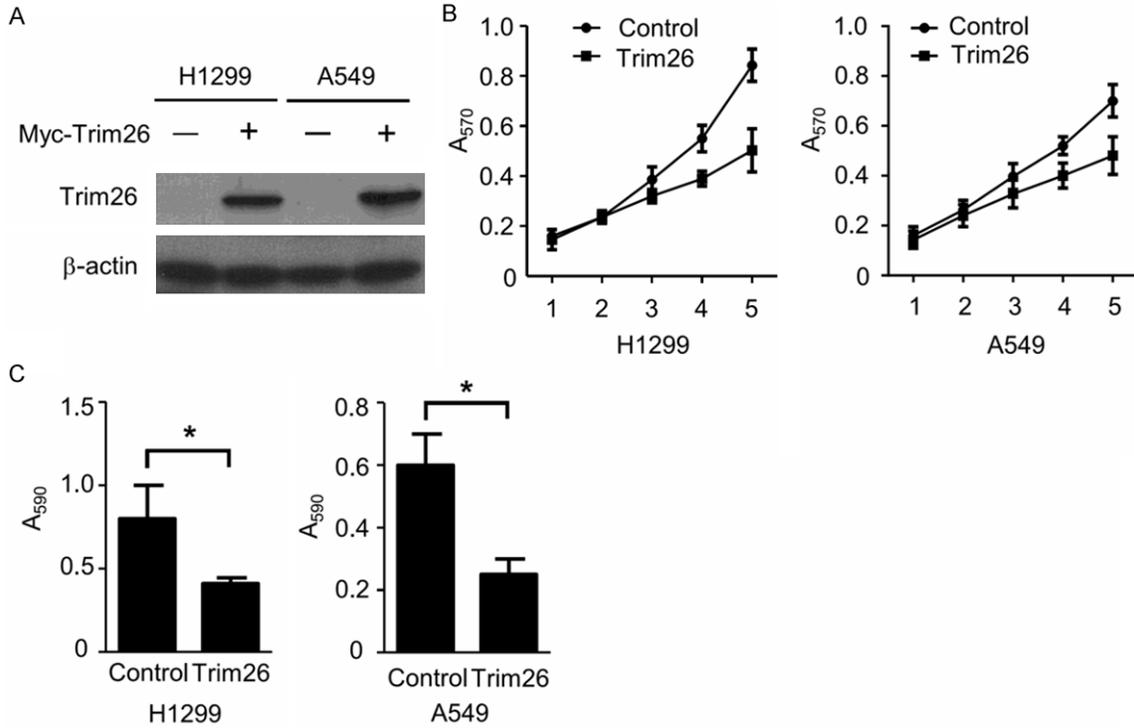


Figure 2. TRIM26 overexpressing in NSCLC cell lines decreases cell growth. (A) A549 and H1299 cells were transiently transfected with expression plasmids for TRIM26. Whole cell lysates of transfected cell were subjected to western blot. The images shown are representative of three independent experiments. (B) Growth rates of control cells and TRIM26 overexpressing cells were determined. One thousand cells per well were plated in 24-well plates in 18 wells on day 0. Three wells were harvested by trypsin, collected, and counted each day. (C) Colony formation assays were conducted on control and TRIM26 overexpressing cell lines. For quantification, colonies were stained with crystal violet and then washed with 10% acetic acid. The absorbance was measured at 570 nm. Data are presented as mean \pm SD of three independent experiments. The 2-tailed Student's t test was used to analyse the data (B and C). * $P < 0.05$.

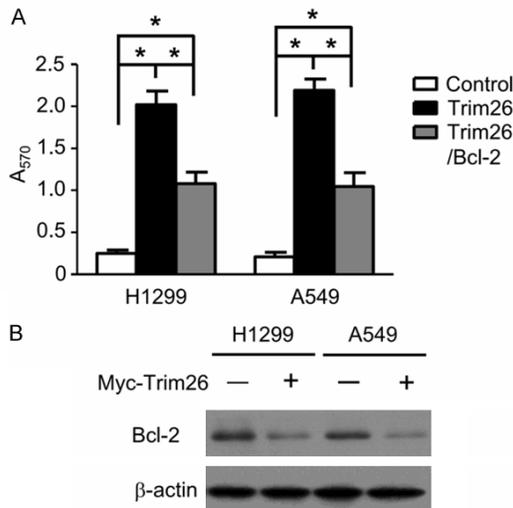


Figure 3. Overexpression of TRIM26 induces cell apoptosis through downregulating BCL-2. A. Cell apoptosis assays were performed using ssDNA apoptosis ELISA kit and was performed according to the manufacturer's instructions. The quantitative data

was presented as mean \pm SD from three independent experiments. The 2-tailed Student's t test was used to analyse the data. * $P < 0.05$. B. Cell lysate of two TRIM26-overexpressing cell lines as well as the control cell line were detected for BCL-2 by western blot.

Discussion

TRIM family proteins are involved in a broad range of biological processes and their alterations are associated with diverse pathological conditions, such as development disorders, neurodegenerative diseases, viral infections and cancer [13, 14]. Most of the TRIM proteins function as E3 ubiquitin ligases, and several TRIM family members are involved in various oncogenic processes, such as transcriptional regulation, cell proliferation and apoptosis. Here we found that TRIM26 can induce NSCLC cell apoptosis but it is unknown whether the ligase activity of TRIM26 is involved in this pro-

cess or not. It would be interesting to test this by using some mutants of TRIM26 without the E3 ligase activity. Though we found that overexpression of TRIM26 in NSCLC cell lines lead to the downregulation of BCL-2, it still remains unknown about the exact molecular mechanism. If TRIM26 interact with BCL-2 directly, then the chances are that TRIM26 is the specific E3 ubiquitin ligase of BCL-2.

Most of the TRIM proteins are overexpressed in cancer cells which indicate that they positively regulate oncogenesis and tumor progression [15-18]. However, there are still some TRIM proteins downregulated in cancer cells. TRIM33 was almost undetectable in leukemic cells of 35% of chronic myelomonocytic leukemia patients. This downregulation was related to the hypermethylation of CpG sequences and specific histone modifications in the gene promoter [19]. TRIM40 is highly expressed in normal gastrointestinal epithelia but that TRIM40 is downregulated in gastrointestinal carcinomas and chronic inflammatory lesions of the gastrointestinal tract. Knockdown of TRIM40 in the small intestinal epithelial cell line IEC-6 caused NF- κ B activation followed by increased cell growth [20]. In this study, we found that the expression of TRIM26 decreases in NSCLC tissues as well as cell lines. Our findings also proved that TRIM protein can negatively regulate tumor progression. However, it is still unknown why TRIM26 downregulated in NSCLC cells.

In summary, our study demonstrated that TRIM26 is a tumor suppressor in non-small cell lung cancer. TRIM26 inhibits NSCLC cell proliferation and induces apoptosis of NSCLC cell by downregulation BCL-2.

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Disclosure of conflict of interest

None.

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