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Original Article

TRIM9 is up-regulated in human lung cancer and involved in cell proliferation and apoptosis

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Abstract: TRIM proteins, members of the RING family of E3 ligases, are well known for their role in several cellular processes and associated with cancer progression. We aimed to investigate the role of TRIM9 in human lung cancer tissues and cell lines. In this study, TRIM9 expression levels were significantly increased in human lung cancer tissues compared with normal lung tissues. Knockdown of TRIM9 by transfected with shRNA (shTRIM9) in human lung cancer cell lines A549 and H446 revealed proliferation inhibition, cell cycle arrest and apoptosis. Furthermore, knockdown of TRIM9 in A549 and H446 cells regulated production of proteins associated with cell proliferation and apoptosis, such as PCNA, VEGFA, Bcl-2, caspase-7 and caspase-9. These results show that knockdown of TRIM9 inhibits proliferation and induces apoptosis of human lung cancer cell lines by regulating proteins associated with these processes. Thus, TRIM9 expression is a modifier of disease incidence and progression relevant to the development of human lung cancer and is a potential target for intervention in human lung cancer.

Keywords: Human lung cancer, TRIM9, gene therapy, proliferation, apoptosis

Introduction

Cancers arise from epigenetic and genetic alteration. Accumulation of such alterations combined with natural selection leads to complex changes in cell behavior that underlie the development and progression of cancer [1, 2]. Although the number of genetic alterations involved in driving cancer development is probably larger than originally expected, many alterations observed in tumors may not contribute to cancer formation, but merely be as a neutral consequence of genomic and proteomic deregulation [3]. Therefore distinguishing whether alterations drive cancer development or arise as neutral is critical for our understanding of the cellular processes leading to cancers as well as for the design of interfering strategies.

Tripartite motif family protein 9 (TRIM9) belongs to the TRIM family and that, has been identified as an ubiquitin ligase (E3), plays important roles in various cellular processes [4]. The TRIM family consists of more than 70 members, such as TRIM8, 27, 29, 40 and 69, are known to be involved in oncogenesis or tumor progression by affecting specific signaling pathways including Wnt/β-catenin [5], IFN [6] and p53 [7]. Specifically for lung cancers, TRIM29 has been shown to upregulate MMP-9 to promote lung cancer cell invasion by activating ERK and JNK pathways [8]. TRIM28 contributes to EMT via regulation of E-cadherin and N-cadherin in lung cancer cells [9]. TRIM44 facilitates migration and invasion of lung cancer cells via NF-κB signaling pathway [10]. TRIM9 protein is known as a brain-specific E3 ligase expressed in the human brain neurons and associated with neurological disorders such as Parkinson's disease, Alzheimer's disease, epilepsy and stroke [11-13]. However, there have been no reports on possible correlation between TRIM9 and human lung cancer carcinogenesis.

The aim of the present study was first to investigate the TRIM9 expression in human lung cancer tissues and cell lines and second to clarify the role of TRIM9 in proliferation, cell cycle and

Table 1. Primes sequences used in this study

Gene	Sequences
TRIM9-forward	5'-AATGGGCAAGGGTCAAAGAG-3'
TRIM9-reverse	5'-CGTGGTATCATAGCAGCATCAG-3'
PCNA-forward	5'-GCCTGACAAATGCTTGCTGAC-3'
PCNA-reverse	5'-TTGAGTGCCTCCAACACCTTC-3'
VEGFA-forward	5'-TTACTGCTGTACCTCCACC-3'
VEGFA-reverse	5'-GCTCATTCTCTCTATGTGCTG-3'
Bcl-2-forward	5'-CCACCTGTGGTCCATCTGAC-3'
Bcl-2-reverse	5'-CAATCCTCCCCCAGTTCACC-3'
Caspase-7-forward	5'-ACCTATCCTGCCCTCACATC-3'
Caspase-7-reverse	5'-TCTTCTCCTGCCTCACTGTC-3'
Caspase-9-forward	5'-CCTCACCCTGCCTTATCTTG-3'
Caspase-9-reverse	5'-TCCCTCTTCCTCCACTGTTC-3'
GAPDH-forward	5'-CACCCACTCCTCCACCTTTG-3'
GAPDH-reverse	5'-CCACCACCCTGTTGCTGTAG-3'

apoptosis of human lung cancer cell lines and explored its utility as a novel biomarker for lung cancer diagnosis.

Material and methods

Patients and tissue samples

To investigate the difference of TRIM9 expression between normal and human lung cancer tissue, 35 paired normal and human lung cancer tissues were obtained from Northern Jiangsu People's Hospital. None of these patients had received radiotherapy or chemotherapy. Human lung cancer and normal tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until total RNA was extracted. Tumor samples were at least 80% composed of viable-appearing tumor cells on histological assessment. The pathological stage, grade were appraised by an experienced pathologist. Ethical approval for the study was provided by the independent ethics committee, Northern Jiangsu People's Hospital. Informed and written consent was obtained from all patients or their advisers according to the ethics committee guidelines.

Cell culture

All culture media were supplemented with 10% fetal bovine serum (FBS), 100 mg/mL penicillin G, and 50 mg/mL streptomycin (Life Technologies, Gaithersburg, MD, USA). All of the human lung cancer cell lines (H1975, PC-9, H460, A549, MRC-5 and H446) were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in DMEM (Life Technologies) and incubated in a humidified atmosphere at 37°C with 5% CO_2.

RNA interference (RNAi) and construction of stable cell lines

A short hairpin RNA (shRNA) targeting human TRIM9 mRNA was cloned into the pLVX-AcGFP-C1 lentiviral vector (Sangon BiotechCo., Ltd., Shanghai, China). The scramble shRNA was cloned into the pLVX-AcGFP-C1 lentiviral vector used as negative control (shNC). The constructs were subsequently transfected into HEK-293T cells with lentiviral packaging vectors using Lipofectamine 2000 (Invitrogen Life Technologies), according to the manufacturer's instructions. Viruses were collected 48 h after transfection and used to infect A549 and H446 cells at an MOI of 20 in the presence of 8 mg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA). Assay was performed 48 h after infection.

Real-time RT-PCR

Total RNAs were extracted from normal and human lung cancer tissues and cells 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). The DyNAmo Flash SYBR Green qPCR kit (FinnzymesOy, Espoo, Finland) was used according to the manufacturer's instructions. Real-time PCR was performed to detect mRNA levels of indicated genes. The primers sequences (sense/antisense) used were listed in **Table 1**. Relative quantification of the signals was performed by normalizing the signals of different genes with the GAPDH signal.

Western blot analysis

Fifty micrograms of protein from human lung cancer tissues and cell lines were subjected to SDS-PAGE using a Bio-Rad miniature slab gel apparatus and electrophoretically transferred onto a nitrocellulose sheet. Membranes were washed and incubated with respective secondary antibodies and were visualized by enhanced chemiluminescence (Millipore, Beijing, China)

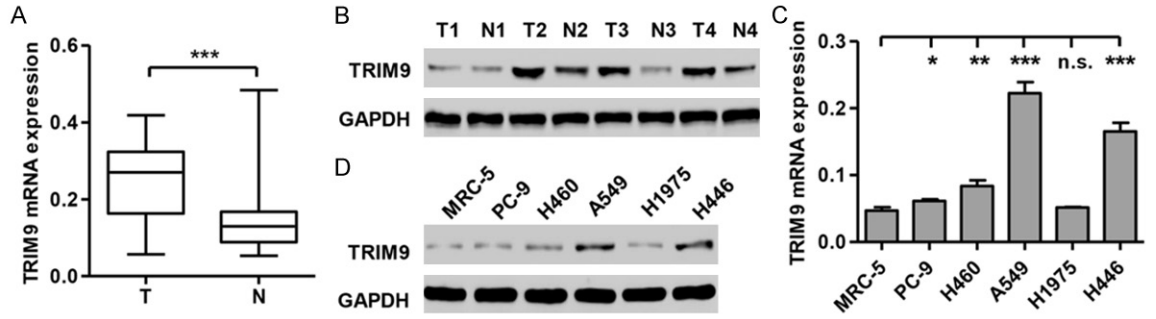

Figure 1. TRIM9 expression in human lung cancer tissues and cell lines. A. TRIM9 mRNA expression was assessed using Real-time PCR containing 35 paired human lung cancer and normal tissues. B. TRIM9 protein expression was assessed using Western blot containing 5 paired human lung cancer and normal tissues. C, D. TRIM9 expression was assessed using Real-time PCR and Western blot containing five human lung cancer cell lines and a lung fibroblast cells MRC-5 used as control. GAPDH was used as loading control. T, tumor tissues. N, normal tissues. n.s. no significance, **P*<0.05, ***P*<0.01, ****P*<0.001.

according to the manufacturer's instructions. Antibodies for TRIM9, VEGFA, caspase-7 and caspase-9 were purchased from Abcam (Cambridge, MA, USA). Antibody for Bcl-2 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies for PCNA and GAPDH were purchased from Cell Signaling Technology, Inc. (Beverley, MA, USA).

Cell proliferation assay

The RNA interfering effects of the TRIM9 shRNA (shTRIM9) on the A549 and H446 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. Subsequently the cell proliferation was evaluated by CCK-8 following the manufacturer's instructions. The absorbance at wavelength 450 nm was measured for the supernatant of each well using a microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Cell cycle and apoptosis detected by flow cytometery

For cell cycle analysis, cells were collected after shTRIM9 transfection for 48 h. The percentages of cells in the different phases of cell cycle were evaluated by determining the DNA content after propidium iodide (PI) staining (BiovisionInc, Mountain View, CA, USA) as previously described [14]. Data acquisition was done by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) using Cell Quest software. For cell apoptosis analysis, cells were collected after shTRIM9 transfection for 48 h, cells were stained with annexin V-fluorescein isothiocyanate and apoptosis rates were analysed using a flow cytometer.

Statistical analysis

SPSS 10.0 software was used to perform the statistical analyses. All the experiments were performed at least thrice in triplicates. Bars and error bars on the graphs as well as data in the text represent the mean ± SD. An unpaired two-tailed t test analysis was used to analyze the data from different groups. $P<0.05$ was considered significant.

Results

TRIM9 expression in human lung cancer

Previous studies have shown an increase in TRIM9 expression in specific caners [15]. We investigated whether enhanced TRIM9 expression may be detectable in other types of cancer and how widely this may be seen across cancer cell lines. We isolated mRNA samples from 35 paired normal and human lung cancer tissues of patients from Northern Jiangsu People's Hospital. Then Real-time PCR was performed to analysis of TRIM9 expression in human lung cancer tissues. GAPDH was used as control. Analysis of normalized data across all samples revealed statistically significantly increased mean expression of TRIM9 in cancers compared with normal tissues (TRIM9 expression relative to GAPDH: cancers vs. normal, mean = 0.26 vs. mean = 0.15, $P<0.001$; **Figure 1A**).

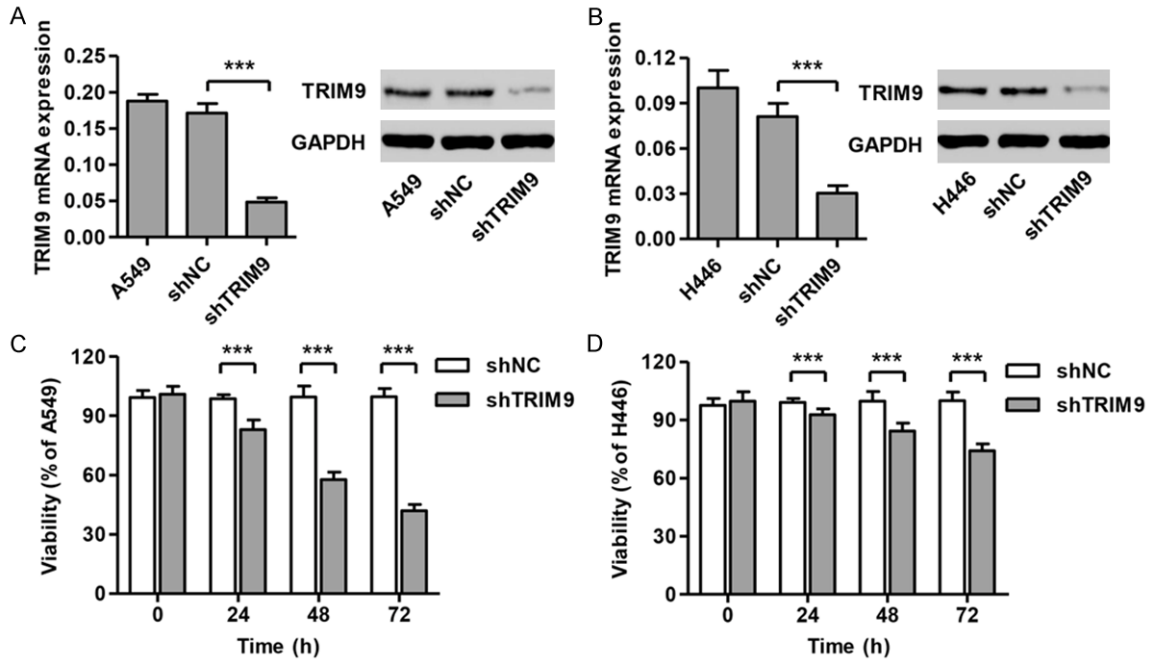

Figure 2. Knockdown of TRIM9 inhibits cell proliferation. A, B. TRIM9 expression was assessed using Real-time PCR and Western blot in shTRIM9-transfected A549 and H446 cells. C, D. Cell proliferation was assessed using CCK-8 assay and inhibitory proliferation was showed in shTRIM9-transfected A549 and H446 cells. GAPDH was used as loading control. ***P<0.001.

Next, we randomly selected five paired normal and human lung cancer tissues for detectable of TRIM9 expression. As shown in **Figure 1B**, the TRIM9 expression was significantly increased in human lung cancers compared with normal tissues at protein levels. These results suggest that TRIM9 overexpression in human lung cancer tissues and may be an oncogene involved in lung cancer.

To determine if TRIM9 expression was increase in human lung cancer cells, we examined five human lung cancer cell lines, including PC-9, H460, A549, H1975 and H446, and a lung fibroblast cell MRC-5 used as a control. A statistically significant increase in TRIM9 expression was observed among the four cell lines, with H1975 exception, compared with MRC-5 cell, and was comparable to the degree by which TRIM9 transcript and protein levels were altered in human lung cancer cell lines tested (**Figure 1C** and **1D**). Furthermore, with the highest TRIM9 expression detected in A549 and H446 cells, these cells were therefore used for subsequent experiments. These findings support the notion that TRIM9 may act as an oncogene in lung cancer.

Knockdown of TRIM9 by shRNA inhibits cell proliferation in human lung cancer cell lines

To explore the biological significance of TRIM9 in human lung cancer tumorigenesis, we sustainably knocked down TRIM9 using transfection of human lung cancer cell lines, A549 and H446, with shRNA (shTRIM9). Decreased expression of shTRIM9 was confirmed in A549 (P<0.001; **Figure 2A**) and H446 cells (P<0.001; **Figure 2B**) after transfected with shTRIM9 but not in cells transfected with shNC, measured by Real-time PCR and Western blotting. We found that knockdown of TRIM9 significantly decreased the proliferation of A549 (P<0.001; **Figure 2C**) and H446 cells (P<0.001; **Figure 2D**), in a time-dependent manner, compared with their corresponding shNC controls.

Knockdown of TRIM9 by shRNA arrests cell cycle in human lung cancer cell lines

Subsequently, the potential inhibitory effect of TRIM9 knockdown on cell cycle progression was investigated. As shown in **Figure 3**, the suppression of TRIM9 knockdown resulted in a higher number of cells in the G1 phase (A549, mean = 75.43; H446, mean = 64.1) compared

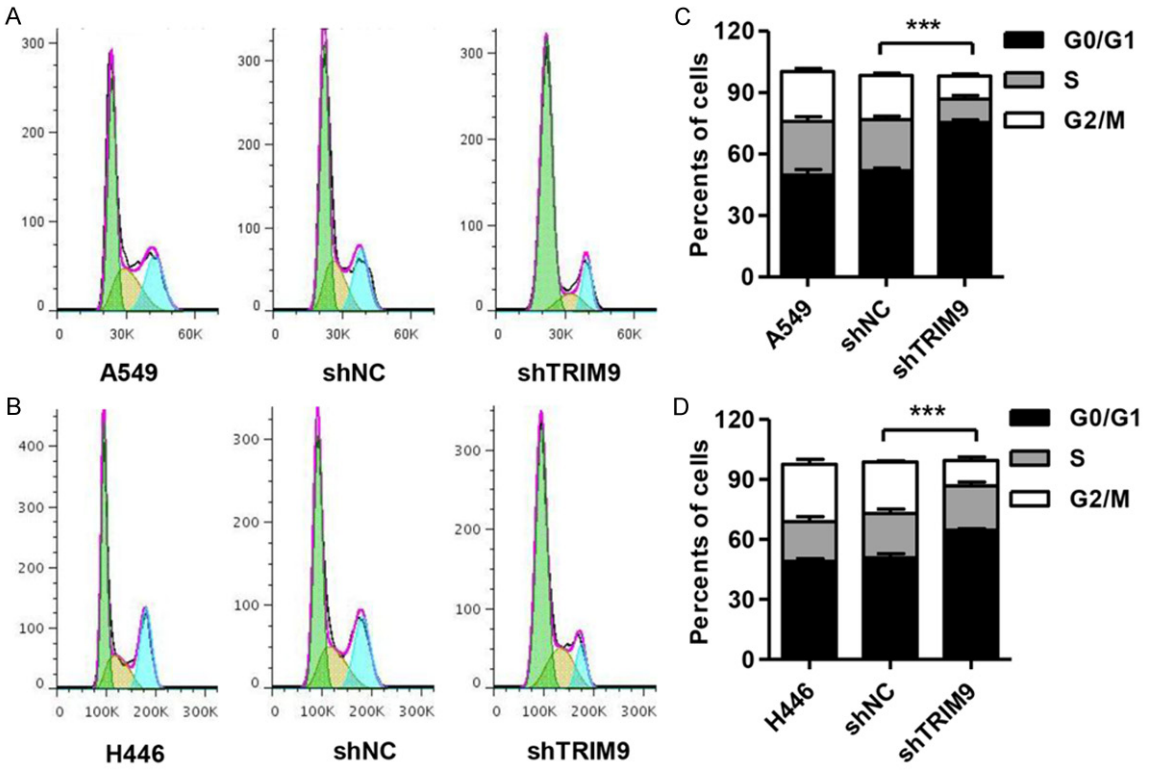

Figure 3. Knockdown of TRIM9 arrests cell cycle. A, B. Cell cycle was assessed using propidium iodide (PI) staining and flow cytometry. C, D. The graph depicts the percentages of cells in the G1, S and G2-M phases for each sample at 48 h following shTRIM9 transfection of A549 and H446 cells. ***P<0.001.

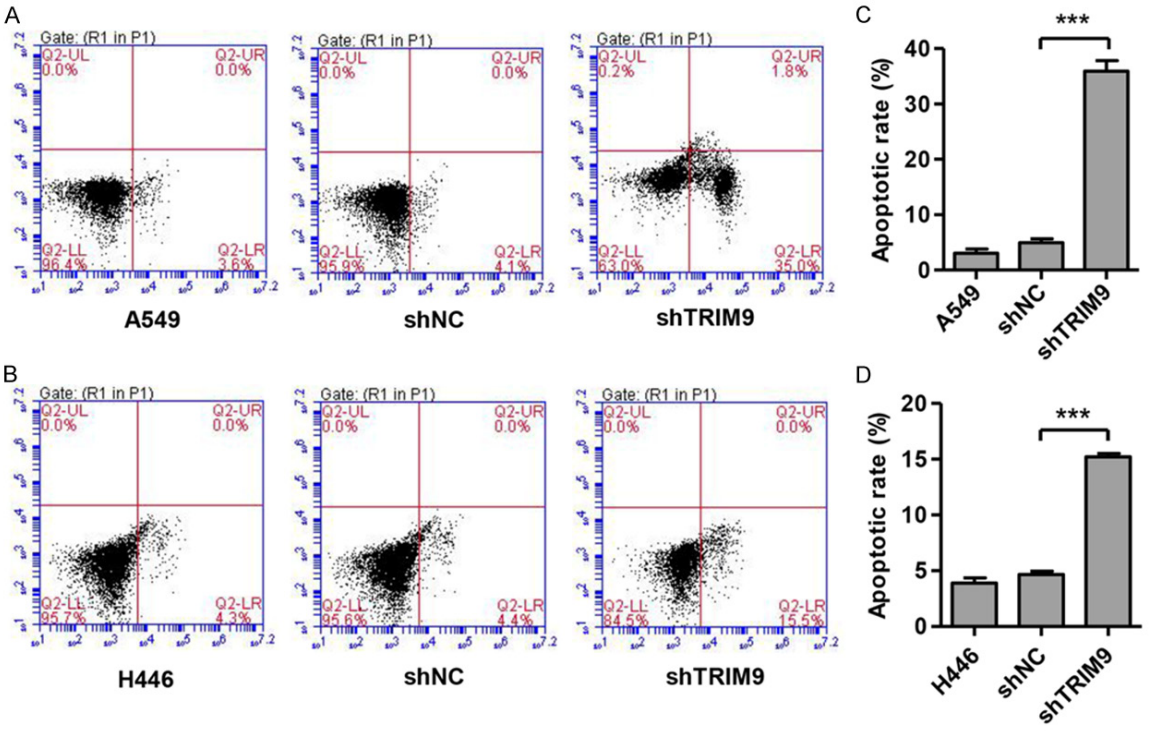

Figure 4. Knockdown of TRIM9 induces cell apoptosis. A, B. Cell apoptosis was assessed using annexin V-FITC/PI staining performed staining and flow cytometry. C, D. The graph depicts the apoptotic rate for each sample at 48 h following shTRIM9 transfection of A549 and H446 cells. ***P<0.001.

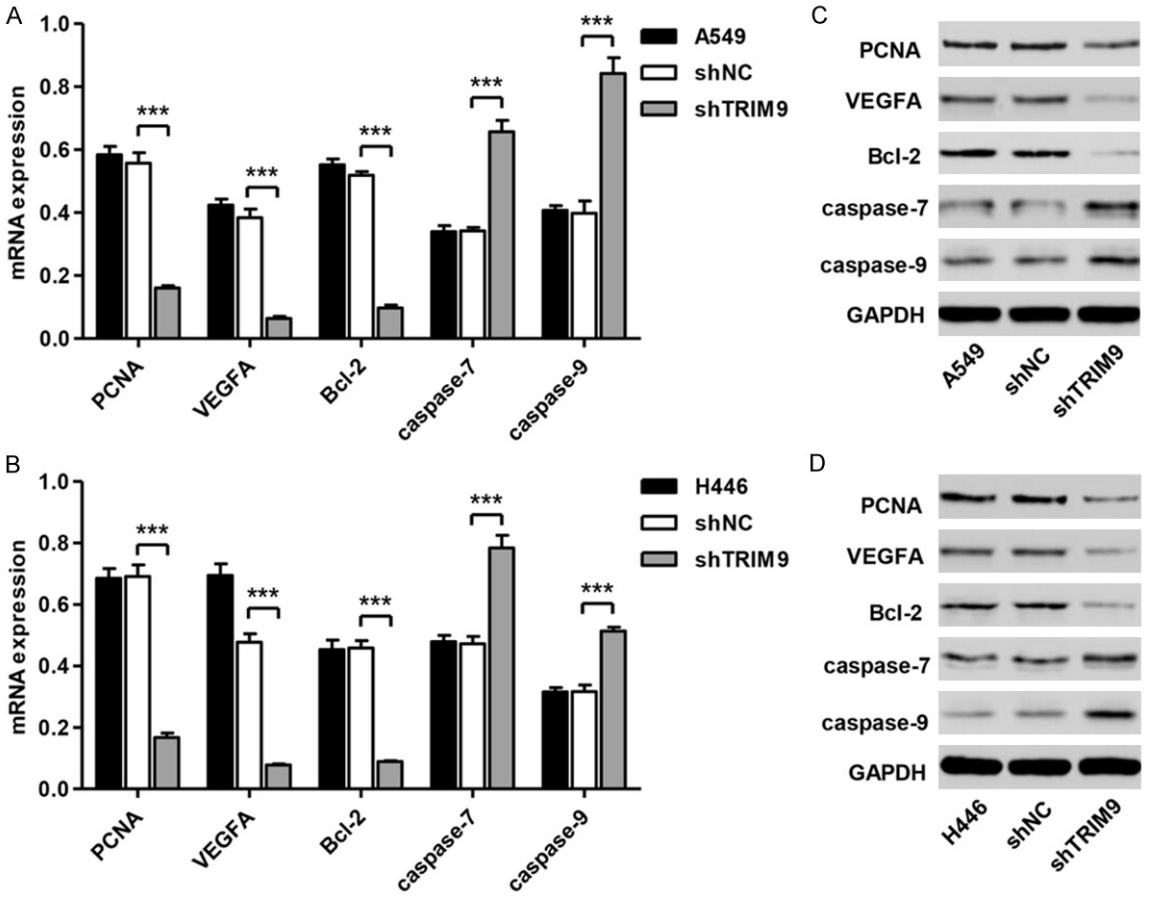

Figure 5. Knockdown of TRIM9 regulates proteins associated with proliferation and apoptosis. A, B. The mRNA expression of PCNA, VEGFA, Bcl-2, caspase-7 and caspase-9 was assessed using Real-time PCR in A549 and H446 cells. C, D. The production of these proteins was assessed using Western blot in A549 and H446 cells. GAPDH was used as loading control. ***P<0.001.

with their corresponding shNC controls (A549, mean = 51.81; H446, mean = 50.77; P<0.001). There was a concomitant reduction in the number of cells in the S and G2/M phases in A549 cells transfected with shTRIM9, but a concomitant reduction in the number of cells in G2/M but not S phase in H446 cells transfected with shTRIM9. These data suggest that knockdown of TRIM9 induces cell cycle arrest in G1 phase in human lung cancer cell lines, which may be associated with the inhibition of cell proliferation in shTRIM9-transfected cells.

Knockdown of TRIM9 by shRNA induces apoptosis in human lung cancer cell lines

To assess the effects of MAP4K4 on cell apoptosis, annexin V/PI staining was performed (**Figure 4A** and **4B**). The apoptotic rate was significantly increased to the mean of 35.9% and 15.2% in shTRIM9-transfected A549 cells compared with the cells transfected with shNC (A549, mean = 4.93%; H446, mean = 4.67%, P<0.001; **Figure 4C** and **4D**). These results indicate that TRIM9 may fulfil an antiapoptotic role in human lung cancer cell lines, and highly sensitive of TRIM9 knockdown was in A549 cells rather than in H446 cells.

Knockdown of TRIM9 by shRNA regulates the production of proteins associated with cell proliferation and apoptosis

We next examined the regulation of proteins that are associated with cell proliferation and apoptosis signaling pathways by Real-time PCR and Western blotting. Knockdown of TRIM9 in both A549 (**Figure 5A**) and H446 cells (**Figure 5B**) resulted in decreased mRNA expression of PCNA, VEGFA and Bcl-2 and increased expression of capsase-7 and caspase-9 compared with untransfected cells (P<0.001). We also

observed the decreased production of PCNA, VEGFA and Bcl-2 and increased production of capsase-7 and caspase-9 in shTRIM9-transfected A549 (**Figure 5C**) and H446 cells (**Figure 5D**) compared with untransfected cells (P<0.001). These results demonstrate that knockdown of TRIM9 inhibits proliferation and induces apoptosis through regulating the proteins involved.

Discussion

Currently, human lung cancer has the highest rates of morbidity and mortality of all malignant tumors worldwide. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer [16]. Since the 5-year survival rate for lung cancer remains poor, it is urgent and important to prevent and treat lung cancer. However, the pathogenesis and development of lung cancer is a complex biological process, in which multiple genes are involved, and is attributed to the activation of an oncogene or the inactivation of a tumor suppressor [17]. These alterations can induce abnormal proliferation, differentiation and apoptosis. Therefore, targeted therapy could be a new method for the prevention and treatment of lung cancer.

We demonstrated here that TRIM9 expression was a contributing factor in the development of human lung cancer. Previous publications have shown enhanced, and possibly aberrant, expression of TRIM9 transcript and protein in specific types of human cancer and cell, including lung cancer [18] and central nervous system [4]. However, the association between TRIM9 and lung cancer processes was not well defined, and role of TRIM9 in cell proliferation, cell cycle and apoptosis in lung cancer cell lines was obscure. Our data showed that TRIM9 expression was increased in human lung cancer tissues compared with normal lung tissues. Further studies on TRIM9 in human lung cancer cell lines may reveal that higher TRIM9 expression was found in four human lung cancer cell lines, including PC-9, H460, A549 and H446 cells, but not inH1975 cells. These results suggest that high expression of TRIM9 is dependent on the types of human lung cell lines.

We then explore the oncogenic roles of TRIM9 in human lung cancer. TRIM9 inactivation by shRNA directed against TRIM9 in A549 and H446 human lung cell lines exhibited suppression of tumor cell proliferation, cell cycle progression and induction of apoptosis. For the underlying mechanism by which TRIM9 promoted cell proliferation and cell cycle, our results demonstrated that TRIM9 knockdown elicited the downregulation of PCNA and VEGFA. PCNA has been found in animal cells that undergo cell division, suggesting a function in cell cycle regulation and/or DNA replication [19]. Decreased PCNA expression in lung cancer showed inhibitory effects of tumor cell proliferation and cell cycle [20, 21]. Angiogenesis plays an essential role in tumor growth and progression. A large body of research literature incriminates VEGFA as the most potential mediator of tumor-induced angiogenesis in lung cancer [22, 23]. Wang et al. has reported that PCNA may participate in the regulation network of VEGFA to play an important role in NSCLC tumorigenesis and serve as a potential molecular marker associated with human lung cancer [24]. For the underlying mechanism by which TRIM9 suppressed apoptosis, our results demonstrated that TRIM9 knockdown elicited the reduction of Bcl-2 expression, and concomitantly activation of caspase-7 and caspase-9. These results could be partially explained by that Bcl-2 is a key apoptotic resistance gene and is upregulated in the majority of lung cancer cells [25]. Apoptosis in lung cancer cells has been showed concomitantly activation of caspase-7 and/or caspase-9 [26, 27]. Also, previous data have shown that caspase-9 is a highly specific protease that only cleaves a few proteins, whereas caspase-7 and caspase-3 contribute to the majority of cleavage that takes place during apoptosis [28].

In summary, we found that TRIM9 was overexpressed in human lung cancer tissues and cell lines. Knockdown of TRIM9 inhibits cell proliferation and cell cycle progression, possibly through downregulation of PCNA and VEGFA. Meanwhile, knockdown of TRIM9 promotes cell apoptosis through downregulation of Bcl-2 and activation of caspase-7 and caspase-9. Further validation with functional analyses of this protein in the context of human carcinogenesis may assist in development of novel therapeutic strategies for lung cancer.

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Disclosure of conflict of interest

None.

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