TRIM37 targets AKT in the growth of lung cancer cells

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Background: TRIM37 is an ubiquitin E3 ligase. Growing evidence has demonstrated the high value of TRIM37 as a potential biomarker for diagnosis of certain cancers. However, the biological function of TRIM37 in lung cancer is still unknown.

Materials and methods: In order to gain a deep insight into the function of TRIM37 in lung cancer cells, in the present study lentiviral vector was used to mediate RNA interference and overexpression of TRIM37 in lung cancer cells (H292, H358, and H1299). In addition, a specific AKT inhibitor LY294002 was utilized to examine the correlation between the expression of TRIM37 and AKT.

Results: TRIM37 acts as a positive regulator of cell proliferation in lung cancer cells. Moreover, cell apoptosis analyses showed the antiapoptosis function of TRIM37, which was mainly dependent on the regulation of BCL2 and BAX. Our results also indicated that AKT might be a target of TRIM37 in lung cancer cells.

Conclusion: This research not only helps in understanding the molecular mechanisms of TRIM37 in detail but also provides evidence to develop novel biomarkers for lung cancer diagnosis.

Keywords: TRIM37, cell proliferation, apoptosis, BCL2, BAX, AKT

Introduction

Lung cancer is one of the most commonly diagnosed cancers worldwide and has been ranked as the top killer cancer all over the world.¹² Though the traditional treatment (resection and radiotherapy) can contribute to fighting against the recurrence of lung cancer, the outcome usually remains poor. Therefore, gaining a deep insight into the molecular mechanism of lung cancer would help to develop new markers and therapy for this disease.

TRIM37 is one of the members of TRIM family, which is mapped on chromosome 17q22-23.³ TRIM37 serves as an ubiquitin E3 ligase and plays an essential role in oncogenesis. A previous report has shown that TRIM37 is upregulated in human colorectal cancer (CRC) and contributes to the development of CRC.⁴ Moreover, the expression of TRIM37 is significantly upregulated in pancreatic cancerous tissues, which indicates the oncogenic role of TRIM37 in pancreatic cancer.⁵ In addition, high expression of TRIM37 is observed in osteosarcoma.⁶ Growing evidence has indicated that TRIM37 participates in the tumorigenesis of several cancers. However, the biological function of TRIM37 in lung cancer is less well understood.

BCL2 mediates the intrinsic apoptosis pathway. A previous report has shown that BCL2 is also essential for cell cycle progression.⁷ The dysregulation of BCL2 not only damages normal development but also contributes to tumor progression.⁸ The high level of BCL2 suppresses apoptosis in human lung cancer cells, which serves as the antiapoptotic factor in this cancer.⁹ In addition, BAX belongs to the BCL2 family, which
functions as proapoptotic marker. Therefore, the occurrence of cell apoptosis depends on the ratio of BAX/BCL2. Dysregulation of the ratio of BAX/BCL2 may lead to carcinogenesis. Taken together, BCL2 family members have potential value as critical targets in lung cancer treatment.\textsuperscript{11,12}

The AKT pathway plays an essential role in various biological functions, which is commonly abnormal in human cancer.\textsuperscript{13} The activity of AKT protein relies on its phosphorylation. A previous report has indicated that the AKT phosphorylation (p-AKT) on Thr308 correlates with the activity of AKT protein in human non-small-cell lung cancer.\textsuperscript{14} The PI3K/AKT pathway is involved in cell cycle and apoptosis of lung cancer cells.\textsuperscript{15} Some evidence has demonstrated that the major function of TRIM37 in glioma cells is the inactivation of PI3K/AKT signaling pathway.\textsuperscript{16} Therefore, further studies are needed to examine the relationship between TRIM37 and AKT in lung cancer cells.

Materials and methods

Tissue specimens and cell culture

A total of 30 pairs of lung cancer tissues and adjacent non-cancerous tissues were obtained from patients at the First Affiliated Hospital of Soochow University. Samples were snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) for further analysis. All patients provided written informed consent. This research was approved by the independent ethics committee of the First Affiliated Hospital of Soochow University and was carried out in accordance with the Declaration of Helsinki.

All the cell lines (A549, H1299, H1975, H358, H292, and 16HBE) used in this study were obtained from the cell bank of Shanghai Institute for Biological Sciences (Shanghai, People’s Republic of China). FBS (10%; Thermo Fisher Scientific, Waltham, MA, USA) was mixed into all culture media along with 2 mM L-glutamine and 1% penicillin/streptomycin (Solarbio, Beijing, People’s Republic of China). Cells were grown in DMEM (TrueLine, Nashville, TN, USA) and were incubated in a 5% CO\(_2\) atmosphere at 37\(^\circ\text{C}\).

Immunohistochemistry

In brief, all tissue samples were fixed on 10% formalin and embedded with paraffin. A set of xylene baths and graded alcohols were used to deparaffinize and rehydrate samples. Antigen retrieval was carried out through steam heating for 15 minutes in 0.01 M citrate buffer at pH 6.0, and slides were rinsed three times with PBS (0.02 M) after natural cooling. Then, the activity of endogenous peroxidase was suppressed using Tris-buffered saline with Tween 20 containing 3% hydrogen peroxide for 10 minutes. The sections were incubated with a primary antibody (TRIM37; Novus, St Charles, MO, USA) overnight at 4\(^\circ\text{C}\) followed by biotin-labeled secondary antibody (Longislandbio, Shanghai, People’s Republic of China) at room temperature for half an hour. Then, hematoxylin was used for nuclear counterstaining after slides were reacted with diaminobenzidine substrate.

RNA isolation and real-time PCR

Total RNA from all samples was extracted using TRIzol reagent (Thermo Fisher Scientific). After that, cDNA synthesis kit (Thermo Fisher Scientific) was used to synthesize cDNA according to the instruction of the manufacturer. The conditions of real-time PCR were as follows: 95\(^\circ\text{C}\) for 10 minutes followed by 40 cycles of 95\(^\circ\text{C}\) for 15 seconds and 60\(^\circ\text{C}\) for 45 seconds. The expression was normalized to that of GAPDH. The relative gene expression was calculated by the 2\(^{-}\Delta\Delta\text{Ct}\) method. All data represented the average of three replicates. The primer sequences used in this study are listed in the Supplementary materials.

Lentiviral vector-mediated RNA interference and overexpression of TRIM37

Lentiviral plasmids (pLKO.1) containing three shRNAs directed to various regions of human TRIM37 (NM_015294.5) and a negative control shRNA (siNC) were synthesized (Major, Shanghai, People’s Republic of China), respectively. Lentiviral plasmid (pLVX-puro) containing the full length of human TRIM37 cDNA sequence and the mock plasmid served as the negative control (oeNC). All of them were transiently transfected into lung cancer cells as indicated above using Lipofectamine 2000 (Thermo Fisher Scientific) according to the instruction of the manufacturer. Experiments were performed 48 hours after transfection. Detailed sequence information about siTRIM37s is provided in Table S1.

Western blot

Whole-protein lysates were extracted from indicated cells (H292, H358, and H1299) using RIPA lysis buffer (JRDUN, Shanghai, People’s Republic of China) with EDTA-free

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protease inhibitor cocktail (Roche, Basel, Switzerland). The protein concentration was estimated using an enhanced BCA protein assay kit (Thermo Fisher Scientific). An equal amount of total protein (25 µg) was fractionated on a 10% SDS-PAGE gel. Then, the gel was transferred to a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA) overnight. After being blocked with 5% nonfat dry milk for 1 hour at room temperature, the membrane was probed at 4°C overnight with primary antibodies followed by secondary antibody anti-mouse IgG (1:1,000; Beyotime, Haimen, People’s Republic of China) for 1 hour at 37°C. An enhanced chemiluminescence system (Tanon, Shanghai, People’s Republic of China) was used for detecting protein expression value. The detailed information of primary antibodies is provided in Table S2.

**Cell proliferation assay**

Cell Counting Kit-8 (CCK-8) assay (SAB, College Park, MD, USA) was performed to examine cell proliferation according to the protocol of the manufacturer. Briefly, cells transfected as indicated were seeded in 96-well plates and cultured for 0, 24, 48, and 72 hours. CCK-8 solution (1:10) was mixed to each well and incubated for 1 hour. A microplate reader (Pulangxin, Beijing, People’s Republic of China) was used to measure the OD values at a wavelength of 450 nm. Independent triplicate experiments were carried out for each time point.

**Cell apoptosis assay**

In brief, H292, H358, and H1299 cells were collected and stained with Annexin V-fluorescein isothiocyanate apoptosis detection kit (Beyotime) according to the instructions of the manufacturer at 48 hours after viral infection. A flow cytometer (BD, Franklin Lakes, NJ, USA) was used to determine cell apoptosis.

**Migration assay**

Briefly, the indicated TRIM37 siRNA cells were serum-starved for 24 hours followed by seeding in the upper chamber, while the medium supplemented with 30% FBS (Thermo Fisher Scientific) was placed in the lower chamber. After 24 hours of incubation, cells on the upper side of the filters were removed and the remaining cells were fixed in 4% formaldehyde and stained with 0.01% crystal violet (Solarbio). Then, cells in the lower chamber were stained with crystal violet and counted under a microscope at ≥200 magnification (Caikon, Shanghai, People’s Republic of China). All the procedures were performed using Boyden chambers (Costar; Corning, Corning, NY, USA).

**Invasion assay**

This assay was also performed using Boyden chambers containing a polycarbonate filter coated with Matrigel on the upper surface. The procedure was performed as indicated above.

**Xenograft model**

This assay was carried out according to the institute’s guidelines for animal experiments and was approved by the independent ethics committee of the First Affiliated Hospital of Soochow University, People’s Republic of China, and all animals were treated in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines. An equal number of siNC- and siTRIM37-transfected H358 cells (n=2×10⁶) were subcutaneously injected into the right flank of 4- to 6-week-old nude mice (n=26 for each group; Shanghai Laboratory Animal Company, Shanghai, People’s Republic of China). The length and width of the tumor were examined every 3 days for 33 days after injection. The volume of the tumor was calculated as length×(width/2). Six weeks after injection, six mice from the siNC- and siTRIM37-injected groups were sacrificed by cervical dislocation and tumor tissues were fixed in 4% formalin for further analysis. A total of 20 nude mice in each injected group as indicated were used to measure mouse survival rate. Mouse survival was monitored daily for a total of 100 days.

**Statistical analysis**

GraphPad Prism software Version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA) was utilized for statistical analyses. Data were expressed as mean ± SD of at least three samples. Statistical significance was determined by one-way ANOVA for multiple comparisons. A P-value <0.05 was considered to indicate statistical significance.

**Results**

**The level of TRIM37 was upregulated in lung cancer tissues and cell lines**

The mRNA expression of TRIM37 was examined by quantitative real-time PCR in 30 pairs of lung cancer samples and matched para-cancerous tissues. In addition, Western blot was utilized to examine the protein level of TRIM37 in eight pairs of lung cancer samples and matched normal tissues. As shown in Figure 1A and B, both the mRNA and protein levels of TRIM37 in lung cancer tissues were significantly
upregulated as compared with normal tissues. Moreover, immunohistochemistry was used to further investigate the protein level of TRIM37 in lung cancer and normal tissues. The results also suggested that TRIM37 was upregulated in tumor tissues (Figure 1C). Then, the mRNA and protein expression level of TRIM37 was detected in five lung cancer cell lines, including H292, H358, H1299, A549, and H1975, using a human bronchial epithelial cell line (16HBE) as normal control. As shown in Figure 1D and E, the mRNA and protein expression levels of TRIM37 were significantly upregulated in lung cancer cell lines, especially in H292 and H358 cells, compared with 16HBE cells. On the other hand, the H1299 cells showed relatively lower level of TRIM37 than other lung cancer cell lines. Based on these results, H292, H358, and H1299 cell lines were chosen for further study.

Knockdown and overexpression of TRIM37 in lung cancer cells

To silence the expression of TRIM37, three shRNAs targeting human TRIM37 gene (siTRIM37; RNAi1-1, RNAi1-2, RNAi1-3) and a negative control shRNA (siNC) were synthesized and transfected to H292 and H358 cell lines, respectively. The untreated cells acted as blank control (BLANK). As shown in Figure 2A and B, all the TRIM37-siRNAs were able to strongly reduce endogenous TRIM37. Cells transfected with RNAi1-1 and RNAi1-2 showed lower TRIM37 level than RNAi1-3. Therefore, RNAi1-1 and RNAi1-2 were chosen for further study. In addition, H1299 cells were transfected with a plasmid overexpressing TRIM37 (oeTRIM37) and a mock plasmid (oeNC), respectively. As shown by the TRIM37 mRNA and protein levels in Figure 2C and D, a remarkable overexpression...
of TRIM37 was observed in oeTRIM37-transfected cells. Hence, the oeTRIM37 cells were chosen for the following TRIM37 overexpression analysis.

Suppression of TRIM37 inhibited cell development and metastasis

The function of TRIM37 in the proliferation of lung cancer cells was examined through CCK-8 assays. siNC, RNAi1-1, and RNAi1-2 were transfected to H358 and H292 cells, respectively. As shown in Figure 3A, there was no obvious difference between BLANK and siNC groups in cell proliferation of the two lung cancer cell lines. However, the cell growth rate of the two cell lines in siTRIM37 group was significantly decreased at 24, 48, and 72 hours compared with siNC group. Thus, these data suggested that TRIM37 is a positive regulator of cell proliferation of lung cancer cells. Next, Annexin V-FITC/propidium iodide staining assay was performed to investigate the effects of TRIM37 on cell apoptosis of lung cancer cells. As shown in Figure 3B, the cell apoptosis ratio of the two cell lines was obviously higher in siTRIM37 group than in siNC group. These results might reflect the interaction between AKT and TRIM37.

Knockdown of TRIM37 affected the activity of BCL2, BAX, and AKT

As shown in Figure 3E and F, the mRNA and protein expression level of BCL2 was remarkably downregulated in TRIM37 siRNA cells, which was contrary to the expression pattern of BAX. These results were consistent with our expectation. Moreover, the AKT level showed no obvious difference among all transfected cells as indicated above. However, the protein level of p-AKT was highly decreased in siTRIM37 cells, which was contrary to the expression pattern of BAX. These results were consistent with our expectation. Moreover, the AKT level showed no obvious difference among all transfected cells as indicated above. However, the protein level of p-AKT was highly decreased in siTRIM37 cells, which indicated that the activity of AKT was deeply suppressed when TRIM37 was knocked down. These results might reflect the interaction between AKT and TRIM37 in lung cancer cells.

The function of TRIM37 was inhibited by a specific PI3K/AKT inhibitor LY294002

A specific AKT inhibitor LY294002 (10 µM) was used to further determine the relationship between TRIM37 and AKT. As shown in Figure 4A, the cell proliferation rate of oeTRIM37 was significantly upregulated as compared with that of oeNC cells. However, a remarkable decrease in cell proliferation rate was observed after the cells were treated with LY294002. Moreover, the cell apoptosis rate was significantly inhibited by oeTRIM37 in lung cancer cells. However,
this suppression effect of TRIM37 was partly reduced after oeTRIM37 cells were treated with LY294002 (Figure 4B). Additionally, the cell migration and invasion ability analysis also showed similar results (Figure 4C).

Western blot was performed to analyze the protein level of BCL2, BAX, AKT, and p-AKT in oeNC, oeNC + LY294002, oeTRIM37, and oeTRIM37 + LY294002 cells, respectively. As shown in Figure 4D, the BCL2 level was
Knockdown of TRIM37 inhibited tumorigenicity of lung cancer cells in vivo

In order to investigate the function of TRIM37 in tumorigenicity in vivo, an equal number of H358 cells transfected with siRNA and siTRIM37 were hypodermically injected into nude mice (n=6 for each group) and tumor formation was determined every 3 days for 33 days (starting at day 12). As shown in Figure 5A and B, although both the injected cells were capable of developing tumors, it was easily identified that the TRIM37-siRNA cells significantly suppressed the tumor growth rate. The volume and weight of TRIM37-siRNA tumors showed highly significant reduction as compared with that of siNC tumors. In addition, the siTRIM37-transfected cells contributed to prolong the survival length of nude mice (Figure 5C).

Discussion

Lung cancer is a highly invasive, rapidly metastasizing, and prevalent cancer all over the world.1 Much attention has been paid to explore the effective methods for its treatment. Although the tumorigenic mechanism of lung cancer is still not fully understood, some factors have been found to have high value as biomarkers for diagnosing it at an early stage.

In this study, we systematically analyzed the function of a positive tumor regulator TRIM37 in lung cancer cells. To obtain reliable results, RNA interference was used to silence the expression of TRIM37. On the other hand, lentiviral vector was used to mediate overexpression of TRIM37. The results from those two analyses were consistent, which showed that our results were reliable.

The CCK-8 assay suggested TRIM37 as a positive regulator of cell proliferation of lung cancer cells. A previous report indicated that TRIM37 mediated cell proliferation in pediatric osteosarcoma.4 In addition, TRIM44 and TRIM59 have been reported to promote proliferation of lung cancer cells.17,18 Therefore, we suspected that all the TRIM proteins contribute to proliferation of lung cancer cells.

Figure 3 Knockdown of TRIM37 inhibited cell proliferation and apoptosis.

Notes: (A) Cell proliferation was detected at 0, 24, 48, and 72 hours after transfection with siNC, siTRIM37-1, and siTRIM37-2 in H358 and H292 cells. ***P<0.001 vs siNC, and ****P<0.001 vs siNC. (B) Cell apoptosis profile of cells as indicated above. ****P<0.001 vs siNC. (C and D) H292 and H358 cells transfected with indicated TRIM37 siRNAs, and cell migration and invasion were analyzed as described in the “Materials and methods” section, respectively. Magnification, ×200. ***P<0.001 vs siNC group. (E) The mRNA expression level of BCL2 and BAX in cells as indicated above. ****P<0.001 vs siNC. (F) The protein level of BCL2, BAX, AKT, and p-AKT in cells as indicated above. ****P<0.001 vs siNC.
**Figure 4** The function of TRIM37 was inhibited by a specific AKT inhibitor LY294002.

Notes: (A) Cell proliferation was detected at 0, 24, 48, and 72 hours after transfection with oeNC, oeNC + LY294002, oeTRIM37, and oeTRIM37 + LY294002 in H1299 cells. *P* < 0.05 vs oeNC, **P* < 0.01 vs oeNC, and ***P* < 0.001 vs oeNC. (B) Cell apoptosis profile of cells as indicated above. **P* < 0.01 vs oeNC and ***P* < 0.001 vs oeNC. (C) The cell migration and invasion data of H1299 cells transfected with oeNC and oeTRIM37 as well as treated with LY294002. **P* < 0.01 vs oeNC and ***P* < 0.001 vs oeNC. (D) The protein level of BCL2, BAX, AKT, and p-AKT in cells as indicated above. **P* < 0.01 vs oeNC and ***P* < 0.001 vs oeNC.

**Figure 5** Knockdown of TRIM37 in lung cancer cells inhibited tumor growth in vivo.

Notes: H358 cells transfected with siRNA control (siNC) or siTRIM37 were subcutaneously injected into athymic nude mice. (A and B) The tumor volume and weight in nude mice injected with H358 cells transfected with siNC and siTRIM37. *P* < 0.05 vs siNC, **P* < 0.01 vs siNC, and ***P* < 0.001 vs siNC. (C) The survival rate of nude mice injected with H358 cells transfected with siNC and siTRIM37.
A previous study showed that knockdown of TRIM9 induced apoptosis of lung cancer cells through mediating BCL2 and other related proteins. In the present study, the cell apoptosis results indicated the antiapoptosis function of TRIM37 in lung cancer cells. Moreover, our results showed that the protein level of a pair of cell apoptosis factors (BCL2 and BAX) was deeply influenced when TRIM37 was knocked down or overexpressed in lung cancer cells, which indicates that TRIM37 can serve as an apoptotic marker of primary lung cancer. Hence, we suspected that the antiapoptosis function of TRIM37 was mainly through the regulation of BCL2 and BAX. Moreover, we also evaluated the function of TRIM37 in cell migration and invasion in lung cancer cells. Our results indicated that the level of TRIM37 was positively correlated with the ability of migration and invasion of lung cancer cells. Previous reports have demonstrated that TRIM37 promoted cell metastasis in pancreatic cancer cells. Our results also insisted this conclusion, which suggested that TRIM37 is a positive regulator of cell metastasis.

It has been reported that TRIM1 functions in lung cancer cells via enhancing the activity of PI3K/AKT. Previous reports have demonstrated that the oncogenes TRIM22 and TRIM44 contribute to the activity of PI3/AKT in lung cancer cells. Moreover, knockdown of TRIM37 in glioma cells led to the inactivation of PI3K/AKT signaling pathway. In this study, our results indicated that TRIM37 could activate PI3K/AKT. Therefore, we conclude that TRIM37 may promote lung cancer cell development through PI3K/AKT signaling pathway.

Moreover, some evidence has suggested that the activity of AKT is vital for retaining BAX in the cytoplasm. In addition, the AKT protein also functions to upregulate the expression of BCL2. Therefore, we suggest that TRIM37/AKT/BCL2 pathway might be a novel target for regulating cell apoptosis in lung cancer cells.

Conclusion

In the present study, we examined the effect of TRIM37 knockdown and overexpression in lung cancer cells. Our results not only help in better understanding of TRIM37 in lung cancer but also provide evidence that TRIM37 might serve as a novel biomarker in clinical application.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Primer sequence information

**Homo sapiens TRIM37, transcript variant 2, mRNA**
NCBI Reference Sequence: NM_001005207.4
Primer F: 5’ TGGACTTACTGCAAAATG 3’
Primer R: 5’ ATCTGGTGTTGACAAATC 3’
Pos: 1645-1874 C
Amplified product: size: 230 bps, product GC: 39%

**H. sapiens BCL2, apoptosis regulator, transcript variant alpha, mRNA**
NCBI Reference Sequence: NM_000633.2
Primer F: 5’ GCAGTGTGGTCTCCGAATGTC 3’
Primer R: 5’ CATTGCCTCTCCTCACGTTC 3’
Pos: 2277-2521 C
Amplified product: size: 245 bps, product GC: 56%

**H. sapiens BAX, apoptosis regulator, transcript variant 1, mRNA**
NCBI Reference Sequence: NM_001291428.1
Primer F: 5’ CTGAGCAGTGTCTCAAG 3’
Primer R: 5’ CAGCCCATGATGGTCTG 3’
Pos: 244-485 C
Amplified product: size: 242 bps, product GC: 57%

**H. sapiens GAPDH, transcript variant 1, mRNA**
NCBI Reference Sequence: NM_001256799.2
Primer F: 5’ AATCCCATCACCATCCTC 3’
Primer R: 5’ AGGCTGTGTCATACTTC 3’
Pos: 436-653
Amplified product: size: 218 bps

| Table S1 | Homo sapiens TRIM37 (NM_001005207.4) RNAi targeting locus information |
|-----------------|-----------------|-----------------|
| Name            | Locus position (bp) | Sequence         |
|-----------------|-----------------|-----------------|
| RNAi-1          | 595–613         | GCTCCAAACGTGTTGT |
| RNAi-2          | 968–986         | GGAACGTACAGTGTAG |
| RNAi-3          | 2,501–2,519     | GCGAGTGCCTCTGATTA |

| Table S2 | The primary antibodies information |
|-----------------|-----------------|-----------------|
| Antibody name   | Source           | Dilution factor |
|-----------------|-----------------|-----------------|
| TRIM37          | CST, Danvers, MA, USA | 1:1,000         |
| BCL2            | Abcam, Cambridge, UK  | 1:1,000         |
| BAX             | Abcam            | 1:1,000         |
| AKT             | CST              | 1:1,000         |
| p-AKT           | CST              | 1:2,000         |
| GAPDH           | CST              | 1:2,000         |

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