TRIM16 suppresses the progression of prostate tumors by inhibiting the Snail signaling pathway

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Abstract. Prostate carcinoma is a devastating disease which is characterized by insidious early symptoms, rapid progression and a poor prognosis. Tripartite motif-containing protein 16 (TRIM16) was identified as an estrogen- and antiestrogen-regulated gene in epithelial cells stably expressing estrogen receptors. The protein encoded by this gene contains two B-box domains and a coiled-coiled region that are characteristic of the B-box zinc finger protein family. Proteins belonging to this family have been reported to be involved in a variety of biological processes including cell growth, differentiation and pathogenesis. TRIM16 expression has been detected in most tissues. However, the functions of this gene remain to be elucidated. In the present study, immunohistochemical staining revealed that the expression of TRIM16 was decreased in prostate adenocarcinoma compared with that in normal prostate tissues. The patients with high TRIM16-expressing tumors had a significantly greater survival than those with low TRIM16-expressing tumors. Western blot analysis showed that TRIM16 was downregulated in distant metastatic cancer tissues compared with that in non-distant metastatic cancer tissues. The overexpression of TRIM16 inhibited the migration and invasion of prostate cancer cells as well as inhibiting the epithelial-to-mesenchymal transition process, whereas TRIM16 depletion enhanced these processes. Moreover, TRIM16 inhibited the Snail signaling pathway. The silencing of Snail by small interfering RNA was performed in order to determine the role of Snail in the TRIM16-mediated tumor phenotype. Taken together, these findings suggest that TRIM16 may be an important molecular target which may aid in the design of novel therapeutic agents for prostate cancer.

Introduction

Prostate cancer is highly malignant and characterized by an early diagnosis rate and poor treatment efficacy (1). The pathologic stages of prostate cancer begin with abnormal epithelial proliferation and prostatic intraepithelial neoplasia with progression to invasive carcinoma and eventually, metastatic disease (2). Early localized disease is usually curable whereas once progression to invasive and metastatic disease has occurred, the survival rate drops to 34% (3, 4). Surgery is currently the only possible cure for prostate cancer, involving radical removal of the tumor (5, 6). However, due to the highly heterogeneous nature of prostate cancer, unraveling the molecular and biological processes that contribute to the development and progression of prostate cancer remains a challenging task.

The tripartite motif-containing protein (TRIM) family is named from its structure which contains three conserved domains known as the RING domain, B-box domain and coiled-coil domain (7). Since XNF7, the first member of TRIM family to be cloned and identified in 1997 (8), over 70 TRIM proteins have been found in the human genome to date (9). TRIM proteins play differing roles in cellular function including differentiation, transcription, cell cycle regulation, innate immunity and cell migration (10). Several TRIM proteins have been implicated in cancer, which demonstrates that they may have potential applications as novel targets for cancer therapies or as prognostic markers (11).

TRIM16, also known as estrogen-responsive B-box protein (EBBP), is a member of the TRIM family and the functions are not yet fully elucidated (12). TRIM16 is a positive transcriptional regulator of the retinoic acid receptor β2 (13). It plays an important role in many different types of cancers, including neuroblastoma, non-small cell lung cancer (NSCLC) and melanoma (12). Overexpression of TRIM16 reduced neuroblastoma cell growth, enhanced retinoid-induced differentiation and decreased tumorigenicity in vivo (14, 15). The expression of TRIM16 was markedly decreased in NSCLC and correlated with tumor metastasis (16). Upregulation of TRIM16 significantly inhibited epithelial-to-mesenchymal transition (EMT) and metastasis of NSCLC cells (16). In conclusion, TRIM16 may act as a tumor suppressor, but whether TRIM16 plays a role in the development, EMT and metastasis of prostate tumors remains unknown.

In this study, using immunohistochemistry and western blot analysis, we demonstrated that TRIM16 expression was
decreased in prostate tumor tissues compared with that in normal tissues. Furthermore, high TRIM16 expression was associated with the extended survival of patients with prostate cancer. In addition, silencing TRIM16 expression significantly enhanced the migratory ability and invasiveness of prostate tumor cells. By contrast, upregulation of TRIM16 significantly inhibited the migratory ability and invasiveness of prostate tumor cells. We also found that TRIM16 played a role in EMT of prostate tumor cell and this may be partly associated with the Snail pathway. Taken together, these findings demonstrate the importance of TRIM16 in prostate cancer and suggest that it may be a potential therapeutic target in prostate tumors.

Materials and methods

Patients and tissue samples. A total of 49 prostate cancer and normal para-cancerous tissues used for immunohistochemical analysis and another 7 non-distant metastatic prostate cancer and 6 distant metastatic prostate cancer tissues for western blot analysis were randomly collected from the Affiliated Hospital of Weifang Medical University (Weifang, China). Follow-up data were summarized at the end of May 2015, with a median observation time of 61.3 weeks. The study was approved by the Ethics Committee of Weifang Medical University and informed consent was obtained from all subjects prior to beginning the study. No patients had received prior blood transfusions, chemotherapy or radiotherapy. All patients received neoadjuvant chemotherapy and underwent wide resection of the tumor. Tumor biopsies were collected prior to neoadjuvant chemotherapy and the tissues were fresh frozen and stored at -80°C.

Cell culture. Human prostate cancer cell lines (LNCaP, Du145, PC3) and a non-cancerous prostate epithelial cell line (RWPE-1) were obtained from the American Type Culture Collection (ATCC: Manassas, VA, USA). The cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) (both from Invitrogen, Carlsbad, CA, USA).

Immunohistochemical staining. Paraffin-embedded sections of prostate tissues were deparaffinized, blocked and incubated with 1:200 anti-TRIM16 antibody at 4°C overnight. Horseradish peroxidase-conjugated secondary antibody (1:500) was then added and further incubated for 1 h at room temperature. The sections were developed using a 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 1-5 min and then counterstained with hematoxylin. The proportion of stained cells (lower, <10% staining; higher, ≥10% staining) was semiquantitatively determined according to published protocols (17).

Western blot analysis. Samples and cells were solubilized in lysis buffer (50 mM Tris, pH 7.4, 2 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 0.5% Triton X-100). Whole protein was extracted by centrifugation (14,000 x g) for 15 min at 4°C. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) which was blocked in 5% bovine serum albumin (BSA). The membrane was then probed overnight at 4°C in blocking buffer with primary antibodies followed by washing in TBST (0.02 M Tris pH 7.6, 0.8% NaCl, 0.1% Tween-20) and incubated in TBST with secondary antibodies (1:10,000) for 1 h at RT. After washing in TBST again, the chemiluminescence liquid (Millipore) was added and fluorescence was captured on photographic film (Kodak, Tokyo, Japan). Mouse monoclonal TRIM16 (ab194498), E-cadherin (ab76055), α-catenin (ab51032), N-cadherin (ab19348), vimentin (ab16700), transforming growth factor-β1 (TGF-β1; ab92486), Snail (ab55319), Slug (ab10677), ZEB1 (ab203829) and ZEB2 (ab138222) antibodies were purchased from Abcam (Cambridge, UK). Mouse monoclonal β-actin antibody (#3700) was obtained from Cell Signalling Technology (Danvers, MA, USA).

RNA extraction and quantitative PCR (qPCR). Total cellular RNA was extracted from the cells using the RNaseasy Plus Mini Kit (Qiagen, Valencia, CA, USA). qPCR was carried out using SYBR-Green PCR Master Mix (Applied Biosystems) in a total volume of 20 µl on a 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 60 sec. The sequences of the primer pairs were as follows: TRIM16 forward, 5′-TGCACACAGAAGAGTGACC-3′ and reverse, 5′-TATTTTGGCTGAACAACCGGC-3′; and GAPDH forward, 5′-ATAGCAACCCTTGTGAACACT-3′ and reverse, 5′-CACCTTCTACAAATGAGTCGTTG-3′. GAPDH was used as the reference gene. The relative levels of gene expression were represented as ΔΔCt - Ct gene - Ct reference, and the fold change of gene expression was calculated using the 2-ΔΔCt method. Experiments were repeated in triplicate.

Generation of cell lines with overexpression/knockdown of TRIM16. A retroviral construct containing human pBabeTRIM16 cDNA, and pSuper with shRNAs against human TRIM16 (#1: GCTCGGTATCTATGTAAACTT, #2: GCAGTAACCGGCAG-3′, #3: CCGGATGATACCAACGTAC-3′) were prepared as previously described (17). The generation of retrovirus supernatants and the transfection of cells were conducted as described in a previous study (18). Infected cells were selected by the addition of 2 µg/ml puromycin (Sigma, St. Louis, MO, USA) to the culture medium for 48 h and then maintained in complete medium with 1 µg/ml puromycin. The expression of TRIM16 was confirmed by western blot analysis.

Transient transfection with Snail siRNA. DU145-shTRIM16#-transfected cells were transiently transfected with either 20 nM control siRNA (Sigma) or siRNA specific to Snail (Sigma) using Lipofectamine 2000 (Thermo Fisher Scientific) as the transfection agent. The sequence for Snail siRNA was TTGTACCTCAAAAGAAGGTGCC.

Wound-healing assay. RWPE-1 and Du145 cells were cultured and transfected with pBabe-TRIM16 and pSuper-shTRIM16, respectively, or empty vectors, seeded in 6-well plates and allowed to grow into a cell monolayer overnight. A wound was created using a yellow sterile pipette tip on the surface of the plates, and the suspended cells were gently cleared with
phosphate-buffered saline (PBS). Cells in the plates were cultured in serum-free medium. Images of the wound were captured under a phase-contrast microscope (Leica DM750; Leica Microsystems, Wetzlar, Germany) at 0, 24 and 48 h. The distance was measured using Image Pro-Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). For each experiment, 5 visual fields and 2 repeated wells were measured with three replications.

**Assays of cell invasion and migration.** Twenty-four-well Transwell inserts (8-µm pore size; Millipore) were used to perform a migration assay and a Matrigel invasion assay. The Matrigel-coated chamber was prepared according to the manufacturer's instructions. For the migration assay, 24 h after transfection, the concentration of cells was adjusted to 2.5x10^5/ml using Dulbecco's modified Eagle's medium (DMEM) FBS-free medium, and cell suspension (100 µl) was loaded into the top chamber of the Transwell insert with non-coated membrane. For the invasion assay, cell suspension (100 µl) was loaded into the upper Matrigel-coated chamber instead. In both assays, 600 µl DMEM with 10% FBS was added into the bottom chamber. Cells were then allowed to migrate or invade for 48 h at 37˚C. The cells that migrated or invaded into the bottom chamber were fixed in paraformaldehyde, permeabilized in methanol and stained with crystal violet (v5265, Sigma). Images were captured and the number of migrating/invading cells that penetrated through the non-coated membrane or Matrigel-coated filters, respectively, was counted under a light microscope (Leica DM500; Leica Microsystems). The average cell number of four random fields was the final result.

**Confocal immunofluorescence microscopy.** Cell lines were plated on culture slides (Costar, Manassas, VA, USA). After 24 h, the cells were rinsed with PBS and fixed with 4% paraformaldehyde, and the cell membrane was permeabilized using 0.5% Triton X-100. These cells were then blocked for 30 min in 10% BSA and then incubated with primary antibodies overnight at 4˚C. After three washes in PBS, the slides were incubated for 1 h in the dark with FITC-conjugated secondary antibodies (Invitrogen, Grand Island, NY, USA). After three further washes, the slides were stained with 4',6-diamidino-2-phenylindole (DAPI; #268298, Sigma) for 5 min in order to visualize the nuclei, and examined using a confocal imaging system (LSM 780; Carl Zeiss, Jena, Germany).

**Statistical analysis.** The results were analyzed with SPSS 13.0 statistical software. The survival probability was estimated by the Kaplan-Meier method, and the comparison of survival curves between groups was performed using the log-rank test. A two-tailed t-test was used to determine statistical significance. The results are presented as the means ± SD. P-values <0.05 were considered to indicate a statistically significant difference.

**Results**

**Expression of TRIM16 is downregulated in human prostate tumor samples.** Firstly, TRIM16 expression level in prostate tumor and normal prostate tissues was analyzed by immunohistochemical staining (Fig. 1A). The results showed that TRIM16 was downregulated in prostate tumor tissues compared with that in normal prostate tissues (Fig. 1B). Kaplan-Meier
survival analysis showed that survival was extended in prostate cancer tissues with high TRIM16 expression compared with that in prostate cancer tissues with low TRIM16 expression (>60%) (Fig. 2). Subsequently, western blot analysis and qPCR were performed. TRIM16 expression in distant metastatic tissues was lower than that in non-distant metastatic cancer tissues (Fig. 3A-C). The TRIM16 protein levels were measured by western blot analysis in the prostate cancer cell line (PC3, Du145, and LNCaP) and the non-cancerous prostate epithelial cell line (RWPE-1) (Fig. 3D).

Establishment of stable TRIM16 transfectants in prostate cell lines. To characterize the functional role of TRIM16 in prostate cancer, we established cell lines with overexpression and knockdown of TRIM16 protein and then studied the effects of TRIM16 overexpression and knockdown on cell proliferation. For this purpose, the TRIM16 inhibition plasmid pSuper-shTRIM16 was first transfected into the Du145 cell line and the TRIM16-overexpressing plasmid pBabe-TRIM16 was transfected into the RWPE-1 cell line. After selection with puromycin, the expression of TRIM16 was assayed by western blot analysis and qPCR. A low level of TRIM16 was expressed in Du145-pSuper-shTRIM16 cells compared with that in the cells transfected with pSuper alone (Fig. 4A and B), whereas TRIM16 was highly expressed in RWPE-1-pBabe-TRIM16 cells compared with that in the cells transfected with pBabe alone (Fig. 4C and D).

Suppression of TRIM16 enhances the migratory ability and invasiveness of prostate cells. Wound-healing, migration and invasion assays were performed in order to examine the effect of TRIM16 knockdown on prostate cancer cells. Du145 cells transfected with pSuper-shTRIM16 exhibited significantly faster closure of the wound area compared with the control cells (Fig. 5A). Furthermore, cell migration and invasion was significantly enhanced in the Du145 cells transfected with pSuper-shTRIM16 (Fig. 5B). On the contrary, RWPE-1 cells transfected with pBabe-TRIM16 exhibited slower closure of the wound area compared with the control cells (Fig. 6A). Cell migration and invasion was significantly inhibited in the RWPE-1 cells transfected with pBabe-TRIM16 (Fig. 6B).

Figure 2. Low expression of tripartite motif-containing protein 16 (TRIM16) is associated with poorer survival in patients with prostate cancer.

Figure 3. Tripartite motif-containing protein 16 (TRIM16) protein and mRNA expression is decreased in distant metastatic tissues (n=6) compared with that in non-distant metastatic tissues (n=7). (A) TRIM16 protein levels in distant metastatic tissues (n=6) and non-distant metastatic tissues (n=7) were measured by western blot analysis. (B) Analysis of TRIM16 protein expression in distant metastatic tissues and non-distant metastatic tissues. (C) TRIM16 expression was measured in distant metastatic tissues and non-distant metastatic tissues by qPCR. (D) TRIM16 protein levels in prostate cancer cell lines (PC3, Du145, and LNCaP) and a non-cancerous prostate epithelial cell line (RWPE-1) were measured by western blot analysis. **P<0.01 is based on the Student's t-test. All results are from three independent experiments. Error bars indicate the means ± SD. Met, metastases.
Figure 4. Transfection efficiency of tripartite motif-containing protein 16 (TRIM16) in prostate cancer cell lines. (A) The transfection efficiency of shTRIM16 was analyzed by performing western blot analysis in order to measure protein levels. (B) The transfection efficiency of shTRIM16 was analyzed by performing qPCR in order to measure transcript levels. GAPDH was used as a loading control. (C) The transfection efficiency of TRIM16 was analyzed by performing western blot analysis in order to measure protein levels. (D) The transfection efficiency of TRIM16 was analyzed by performing qPCR in order to measure transcript levels. GAPDH was used as a loading control. **P<0.01 is based on the Student's t-test. All results are from three independent experiments. Error bars indicate the means ± SD.

Figure 5. Downregulation of tripartite motif-containing protein 16 (TRIM16) enhances the migratory ability and invasiveness of prostate cancer cells in vitro. (A) Migratory ability was measured by performing a wound-healing assay in Du145 and shTRIM16-transfected Du145 cells. Scale bar, 500 µm. (B) Migratory ability and invasiveness were measured using Transwell assays in Du145 and shTRIM16-transfected Du145 cells. Scale bar, 50 µm. **P<0.01 is based on the Student's t-test. All results are from three independent experiments. Error bars indicate the means ± SD.
These results provide further evidence to support the involvement of TRIM16 in the migration and invasiveness of prostate cancer cells.

**TRIM16 regulates the transition between epithelial and mesenchymal phenotypes in prostate cells.** Epithelial and mesenchymal markers were detected by immunohistochemistry and western blot analysis in both overexpression and knockdown TRIM16 prostate cancer cells to examine whether TRIM16 plays a role in EMT. The expression analyses showed that TRIM16 knockdown decreased the levels of epithelial markers (E-cadherin and α-catenin) and increased the levels of mesenchymal markers (N-cadherin and vimentin) in DU145 cells (Fig. 7A and B). On the contrary, TRIM16 overexpression increased the levels of epithelial markers (E-cadherin and α-catenin) and decreased the levels of mesenchymal markers (N-cadherin and vimentin) in RWPE-1 cells (Fig. 7C and D). These results suggest that TRIM16 affects the expression of epithelial and mesenchymal markers at the transcript level. We concluded that TRIM16 inhibits EMT of prostate cancer cells.

**TRIM16 inhibits tumor metastasis by inhibiting the transcription factor Snail.** Previous studies showed that a number of signaling pathways promote the proliferation, migration and invasiveness of prostate cancer cells as well as stem cell properties (19-21). Thus, we explored whether TGF-β1, Snail and their downstream signaling pathways are induced by TRIM16 in prostate cancer cells. The knockdown of TRIM16 in DU145 cells significantly increased the expression of Snail. However, there was no change in the expression of other proteins (Fig. 8A). On the contrary, the overexpression of TRIM16 in RWPE-1 cells significantly decreased the expression of Snail (Fig. 8B). In order to verify whether Snail mediated TRIM16 functions, we knocked down Snail in DU145-shTRIM16 #1 cells (Fig. 9A). The results showed that Snail knockdown increased the levels of epithelial markers (E-cadherin and α-catenin) and decreased the levels of mesenchymal markers (N-cadherin and vimentin) (Fig. 9B), as well as reducing the migratory ability and invasiveness of DU145-shTRIM16 #1 cells (Fig. 9C). Taken together, these findings suggest that the inhibition of EMT as well as the mobility of prostate cells by TRIM16 may at least in part occur through the transcription factor Snail.

**Discussion**

Prostate cancer is a highly malignant form of cancer, which is difficult to diagnosis and treat. It is estimated that there will be 217,730 new cases of prostate cancer and 32,050 deaths in 2010, ranking prostate cancer as the most frequently diagnosed cancer and the second leading cause of cancer death in the United States (2). A lack of early detection tests when the disease is limited to the prostate and patients do not show clinical symptoms results in late diagnosis of the disease, by which time the cancer has metastasized to other organs (22). Therefore, there is
an urgent need to identify novel molecular factors involved in the invasiveness and metastasis of prostate cancer cells.

The tripartite motif-containing protein (TRIM) family are involved in diverse cellular processes, and are often characterized by critical protein-protein interactions necessary for their functioning (7). A significant number of TRIM proteins act as oncogenes or tumor suppressors by positively or negatively regulating pathways associated with tumor progression and suppression (23). Some TRIMs, such as TRIM13, TRIM8 and TRIM32, function as tumor suppressor proteins by regulating transcription and apoptosis (11). Other TRIM proteins, such as TRIM27/RFP and TRIM24/TIF1α, function normally as tumor suppressor proteins, but acquire oncogenic activity when fused to kinases by tumor-associated chromosomal rearrangements (11). TRIM16 does not have a RING domain but it has two B-box domains which have been suggested to adopt RING-like folds leading to the hypothesis that TRIM16 acts as an ubiquitin ligase (13). The C-terminus of TRIM16 contains an RFP-like or B30.2/SPRY (B30.2) domain. TRIM16 expression is induced in different types of cancer, when the cancer cell is forced to proceed down a differentiation pathway (12,13,24,25); however, the role of TRIM16 in prostate cancer has not yet been clarified.

In this study, the function of TRIM16 in prostate cancer was characterized. Downregulated levels of TRIM16 were found in most of the prostate cancer tissues examined compared with levels of TRIM16 expression in the normal prostate tissues by immunohistochemical staining and western blot analysis. TRIM16 downregulation was also associated with the decreased survival of patients with prostate cancer and high rates of metastasis. Overexpression of TRIM16 was found to inhibit cell migration and invasion as well as the EMT process, whereas TRIM16 knockdown resulted in opposing effects. These findings suggest that TRIM16 is involved in the genesis of prostate cancer for the first time, to the best of our knowledge.
Figure 8. Tripartite motif-containing protein 16 (TRIM16) inhibits tumor metastasis by reducing the expression of the transcription factor Snail. Western blot analysis of transforming growth factor-β1 (TGF-β1), Snail, Slug, ZEB1 and ZEB2 expression in (A) Du145 and shTRIM16-transfected Du145 cells, and in (B) RWPE-1 and TRIM16-transfected RWPE-1 cells.

Figure 9. Verification of the mediation of the functions of tripartite motif-containing protein 16 (TRIM16) by the transcription factor Snail. (A) The transfection efficiency of siSnail was analyzed by performing western blot analysis in order to measure protein levels in Du145 cells transfected with shTRIM16 #1. (B) The expression of epithelial and mesenchymal markers was analyzed by western blot analysis in Du145 cells transfected with both siSnail and shTRIM16 #1 and in Du145 cells transfected with shTRIM16 #1. (C) Migratory ability and invasiveness were measured using Transwell assays in Du145 cells transfected with both siSnail and shTRIM16 #1 and in Du145 cells transfected with shTRIM16 #1. Scale bar, 50 µm. **P<0.01 is based on the Student's t-test. All results are from three independent experiments. Error bars indicate the means ± SD.
The Snail transcript is 2.0 kb and found in the placenta as well as adult heart, lung, brain, liver and skeletal muscle tissues. It codes for a 29.1 kDa protein composed of 264 amino acids. This protein contains three classic zinc fingers and one atypical zinc finger (26). The zinc-finger transcription factor Snail belongs to the Snail superfamily of transcriptional repressors. The Snail family of transcription factors has previously been demonstrated to play a role in the differentiation of epithelial cells into mesenchymal cells (also known as EMT) during embryonic development (26). Snail is a strong repressor of the transcription of the E-cadherin gene and is considered as a marker of malignancy (26). Snail has been implicated in the pathogenesis of a variety of types of human cancers including prostate, hepatocellular, gastric, melanoma and pancreatic cancers (26). In this study, we found that the overexpression of TRIM16 significantly downregulated the expression of Snail, whereas knockdown of TRIM16 produced opposing effects. We also found that TRIM16 inhibited the EMT process through Snail. Moreover, the suppression of Snail signaling also reduced the migratory ability and invasiveness of Du145-super-shTRIM16 #1 cells as well as inhibiting the EMT process. Thus, the downregulation of TRIM16 in prostate cancer may enhance the oncogenic effects of the activated Snail signaling pathway. However, further investigations are warranted into the mechanisms responsible for mediating the expression profile of TRIM16.

In conclusion, we have demonstrated for the first time, to the best of our knowledge, that TRIM16 expression is decreased in prostate cancer tissues and overexpression of TRIM16 inhibits cell migration, invasion and the EMT process in vitro in prostate cancer through the transcription factor Snail. Taken together, these findings suggest that TRIM16 may be an important molecular target which may aid in the design of novel therapeutic agents for prostate cancer.

References