Knockdown of tripartite motif 59 (TRIM59) inhibits tumor growth in prostate cancer

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Abstract. – OBJECTIVE: Members of the tripartite motif (TRIM) protein family contain a highly conserved N-terminal really interesting new gene (RING) domain that is involved in regulating transcriptional factors and tumor suppressors. In this study, the effects of TRIM59 expression on tumor growth were investigated in prostate cancer.

MATERIALS AND METHODS: The expression of TRIM59 in prostate cancer tissues (n = 15) and prostate cancer cell lines was determined by quantitative reverse transcriptase-PCR (qRT-PCR), Western blotting, and immunohistochemistry. A specific shRNA targeting TRIM59 was employed to knockdown TRIM59 expression in the prostate cancer cell lines PC3 and DU145. The effects of TRIM59 knockdown on cell proliferation were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and colony formation assays. The effects on cell cycle progression were determined by flow cytometry, and a xenograft mouse model of prostate cancer was generated to determine the in vivo effects of TRIM59 knockdown. The effects on cell cycle regulators were determined by Western blotting.

RESULTS: TRIM59 was highly expressed in prostate cancer tissues. Knockdown of TRIM59 significantly inhibited cell proliferation and colony formation, and cell cycle analysis showed that TRIM59-depleted cells accumulated in S-phase. TRIM59 knockdown was shown to inhibit tumorigenesis in mice. In addition, the cell cycle regulators CDC25A, CDC2, and cyclin B1 were decreased by TRIM59 shRNA-mediated knockdown.

CONCLUSIONS: Our study suggests that TRIM59 promotes prostate cancer cell proliferation, possibly through its effects on cell cycle progression.

Key Words:
TRIM59, Prostate cancer, shRNA, Proliferation, Cell growth.

Introduction

Prostate cancer (PCa) is one of the most common non-cutaneous malignancies and the second leading cause of tumor-related deaths in males, accounting for nearly 30,000 deaths annually in the United States of America alone. In China, the morbidity of PCa was 9.92 cases per 100,000 people in 2012 and the number of cases is increasing each year. In recent decades, great progress has been made towards understanding the disease; however, the detailed mechanisms of prostate tumorigenesis remain obscure. According to recent studies, the incidence of PCa increases with age. Therefore, it is an emerging priority to find novel molecular biomarkers to diagnose and treat PCa in its early stages.

The human tripartite motif (TRIM) protein family has more than 77 members, of which most possess E3 ubiquitin ligase activity associated with the conserved really interesting new gene (RING) domain. They are involved in a variety of cellular processes, including transcriptional regulation, membrane repair, cytoskeleton remodeling, and oncogenesis. Of note, TRIM13, TRIM19, and TRIM 25 have been implicated in leukemia, breast, and prostate cancers through the regulation of transcriptional factors, demonstrating the crucial roles of TRIM proteins in tumorigenesis. The biological importance of TRIM59, a surface molecule belonging to the TRIM family, has already been described in some tumors. Zhou et al reported that TRIM59 was markedly increased in gastric cancer and was strongly associated with poor outcomes for patients. Moreover, TRIM59 interacts with p53, leading to p53 ubiquitination and degradation, which promotes tumor growth, cell proliferation,
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Immunohistochemistry (IHC) Analysis
Prostate cancer samples and their adjacent non-cancerous tissues were obtained from 15 patients treated at Shanghai Seventh People’s Hospital, fixed in formalin, and sectioned into 4 μm slices. After deparaffinization, the slices were incubated in 3% H2O2 solution for 10 min at room temperature. Antigen retrieval was performed by heating the sections in 0.1 M citric acid buffer (pH 6.0) for 10 min. The sections were then incubated with TRIM59 primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. After washing three times with TBS, the sections were incubated with secondary antibody (Epitomics, Inc., Burlingame, CA, USA) for 1 h at room temperature. For negative controls, normal goat serum was used in place of the specific primary antibody.

Cell Culture and Western Blot Analysis
Human prostate cancer cell lines PC3, 22Rv1, LNCaP, and DU145 were purchased from American Type Culture Collection (ATCC) and cultured in MEM medium (Gibco, Los Angeles, CA, USA) supplemented with 10% FBS (Gibco). Protein extractions for subsequent immunoblot analysis were performed when cells had reached approximately 80% confluence. Briefly, 50 μg of total protein was loaded into each well of a 12% SDS-PAGE gel and GAPDH was included as an internal control. Immunoreactivity was determined with enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA) using the LAS-3000 imaging system (Fuji Film, Tokyo, Japan).

Recombinant Lentiviral Constructs and shRNA Interference
For knockdown of TRIM59, a specific shRNA was chemically synthesized by GenePharma (Shanghai, China) with the sequence described previously. After insertion into the LV3-GFP vector, the recombinant plasmid was cotransfect-
ed into HEK 293T cells with the packaging vectors. Lentivirus particles were harvested 48 h after transfection and purified by ultracentrifugation. PC3 and DU145 cells were then transduced with the packaged TRIM59-shRNA lentivirus. 96 h after infection, fluorescence microscopy was used to determine the viral titer.

**Cell Proliferation Assay**
PC3 and DU145 cells were inoculated into 96-well plates (3000 cells/well) and allowed to grow overnight. Cells were treated with 10 μM shRNA and incubated in MEM medium for another 72 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (2 mg/mL) was added to each well. After incubation for 4 h at 37°C, the medium was removed and 200 μL DMSO was added to each well. The plate was shaken for 5 min and the optical density was measured at 570 nm.

**Colony Formation Assay**
PC3 and DU145 cells were cultured in 10 cm plates and treated with TRIM59 shRNA lentivirus. After 72 h incubation, transduced cells were washed with PBS, recultured in new dishes (800 cells/well), and allowed to form natural colonies. After 10 days of incubation, treated PC3 and DU145 cells were stained with crystal violet (Sangon Biotech, Shanghai, China). Subsequently, cells were washed twice and fixed with paraformaldehyde. After washing with PBS, fixed cells were treated with crystal violet, washed with ddH2O and photographed with an inverted microscope. Colonies containing more than 50 cells were manually counted.

**Cell Cycle Analysis**
PC3 and DU145 cells were harvested after DEB treatment for cell cycle analysis. Cells were fixed in 70% pre-chilled ethanol and incubated at 4°C overnight. After centrifugation and resuspension in staining buffer (Beyotime, Nanjing, China), cells were incubated with propidium iodide (PI) at 37°C for 30 min. The FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) was used to measure DNA content and Modfit 2.0 software was used to determine the percentage of cells in G0/G1, S, and G2/M phases.

**Mouse Xenograft Model of Prostate Cancer**
Twelve male BALB/c-nu mice (six weeks old) were purchased from SLRC Laboratory Animal Co. (Shanghai, China). Mice were randomly divided into the shRNA group (n = 6) and the negative control (NC) group (n = 6). Mice were housed with ad libitum access to water and food in a temperature- and light-controlled environment. A total of 1 × 10^6 PC3 cells, stably expressing either TRIM59-specific shRNA or control shRNA, were injected into the left flank of each mouse accordingly. Tumor dimensions were measured once a week and tumor size was calculated using the function TV = (L × W^2)/2, where TV represents tumor volume, L represents the longer dimension, and W represents the smaller dimension. Eight weeks after the injection, all mice were sacrificed and each tumor was dissected for subsequent analysis. The animal experimental protocol was approved by the Institutional Animal Care of Shanghai Seventh People’s Hospital. All efforts were made to minimize suffering.

**Statistical Analysis**
Results are presented as mean ± standard deviation (SD). Statistical analysis was carried out using Student’s t-test. p < 0.05 was considered significant. All experiments were performed in triplicate.

**Results**
**TRIM59 was Overexpressed in Prostate Cancer Samples and Cell Lines**
To determine the importance of TRIM59 in PCa, we began by analyzing the expression of TRIM59 in 15 clinical cases. IHC analysis showed that TRIM59 was highly expressed in PCa tissues while it was barely detected in the adjacent non-cancerous tissues. TRIM59 staining was observed in the cell membrane, cytoplasm, and nucleus (Figure 1A). Quantitative reverse transcriptase PCR (qRT-PCR) analysis showed that the average level of TRIM59 mRNA in PCa tissues was approximately twice of that seen in adjacent non-cancerous tissues (Figure 1B). Expression of TRIM59 was also determined in a series of PCa cell lines, including RWPE-1, PC3, 22Rv1, LNCaP, and DU145. TRIM59 was differentially expressed in these five cell lines with the highest level detected in PC3 cells and DU145 cells. RWPE-1 cells exhibited the lowest expression level of TRIM59 (Figure 1C). These data suggest that TRIM59 is highly expressed in PCa.
High Transduction Efficiency of TRIM59-Specific shRNA Lentivirus

Next, the effects of shRNA-mediated TRIM59 knockdown on PCa growth and proliferation were assessed. Lentiviral vectors expressing shRNA against TRIM59 or scramble shRNA were constructed and transduced into PC3 and DU145 cells. Figure 2A shows the high transduction efficiency for both cell lines (approximately 95%). After transduction, we extracted total RNA and protein to perform qRT-PCR and Western blot analyses. Following transduction with TRIM59-specific shRNA lentivirus, TRIM59 mRNA levels were significantly suppressed in both PC3 and DU145 cell lines, by 80% and 60%, respectively (Figure 2B). Furthermore, TRIM59 protein levels were decreased by more than 80% compared with the control group, while levels of the internal control GAPDH remained stable (Figure 2C). These observations confirmed the high efficiency of lentiviral shRNA-mediated RNA interference.

TRIM59 Knockdown Inhibited Cell Proliferation and Colony Formation in vitro

TRIM59 functions to promote cell proliferation and growth in gastric cancer; consequently, we examined the role of TRIM59 in prostate cancer. An MTT assay showed that there were no significant differences in cell proliferation within the first three days between cell lines (PC3 or DU145) transduced with control and TRIM59 shRNA. However, by the fourth day, the proliferation rate of TRIM59 shRNA-transduced PC3 cells was significantly lower than that of the control cells, and this difference was increased by the fifth day. Similarly, significant discrepancies between the two groups were observed by the fifth day in DU145 cells. The cell proliferation rate was suppressed by nearly 25% on the fifth day following transduction of DU145 cells with TRIM59 shRNA (Figure 3A). A colony formation assay further confirmed that knockdown of TRIM59 with specific shRNA caused a marked

Figure 1. Tripartite motif 59 (TRIM59) was overexpressed in prostate cancer (PCa) samples and cell lines. (A) Representative images show the high expression of TRIM59 in clinical PCa samples. (B) Quantitative reverse transcriptase-PCR (qRT-PCR) analysis of 15 PCa tumors and their paired adjacent non-cancerous tissues. The relative mRNA level of TRIM59 in the tumor tissues was significantly higher than that in the adjacent tissues \( (p = 0.013) \). (C) Western blot analysis of TRIM59 expression in five PCa cell lines. TRIM59 was differentially expressed in these cell lines.
decrease of colony numbers for both prostate cancer cell lines (Figure 3B). Approximately 90 colonies were formed in the control shRNA-infected PC3 cells, while approximately 30 colonies were seen in the TRIM59 shRNA-infected PC3 cells (Figure 3C, left panels). Similarly, in the DU145 cell line, 80 colonies were formed in the control group and 25 colonies were formed in the TRIM59 shRNA group (Figure 3C, right panels). These data suggest that knockdown of TRIM59 inhibits prostate cancer cell proliferation in vitro.

**TRIM59 knockdown Caused cell Cycle Arrest in Prostate Cancer Cell Lines**

To determine the effect of TRIM59 knockdown on the cell cycle distribution of PC3 and DU145 cell lines, we performed cell cycle analysis. After incubation for 24 h, cells with and without TRIM59 knockdown were collected and subjected to flow cytometry following PI staining. As shown in Figure 4, TRIM59 knockdown increased the percentage of PC3 cells in S-phase from 36% to 48% and decreased the percentage of cells in G2/M phase from 18% to 5%. Similarly, in the TRIM59 shRNA-transduced DU145 cell line, the percentage of cells in S-phase increased from 26% to 40% while the percentage of cells in G2/M phase decreased from 16% to 2%. These results suggest that knockdown of TRIM59 arrested cell cycle progression leading to the accumulation of cells in G0/G1 and S-phase, in both PC3 and DU145 cell lines.

![Figure 2](image-url)
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Next, we established a nude mouse model of prostate cancer. Mice were injected with PC3 cells expressing either TRIM59 shRNA or scramble shRNA. After approximately one week, mice began to bear tumors that increased in size over time. However, tumor volumes were higher in the control mice than in the TRIM59 shRNA group. By the seventh week, the average tumor size in the shRNA group was significantly smaller than that in the control group (Figure 5). Analysis of dissected tumors confirmed this (Figure 5B). These data strongly suggest that TRIM59 knockdown can decrease tumor growth.

**TRIM59 Knockdown Inhibited Tumor Growth in a Mouse Model of Prostate Cancer**

As shown in Figure 6, the cyclin family proteins CDC25A, CDC2, and cyclin B1 were downregulated following TRIM59 knockdown, while levels of the internal control GAPDH remained stable. The regulation of cyclins by TRIM59 might underlie the TRIM59-mediated cell cycle arrest in PCa cells.

**Discussion**

Prostate cancer originates from the prostate, a gland of the male reproductive system. Malignant prostate cancer cells often spread to the bones and lymph nodes, leading to serious outcomes including difficulty urinating, blood in the urine, pelvic pain, and death\(^1\). Approximately 99% of prostate cancers occur in patients over 50 years old, making prostate cancer one of the most hazardous diseases among older male patients. However, there are no established pre-
There-Therefore, the discovery of novel diagnostic and prog-nostic molecular markers and an improved un-derstanding of the mechanisms underlying prostate cancer remain urgent problems.

The present work provides evidence that TRIM59 is overexpressed in both human prostate cancer tissues and cultured prostate cancer cells. However, the role of TRIM59 in prostate cancer development is far from clear. This study showed that the cell proliferation rate was significantly lower in cells treated with specific TRIM59 shRNA than in those treated with control shRNA. The inhibitory effects of TRIM59 knockdown on tumor growth were also observed in a xenograft mouse model of prostate cancer. This showed that the introduc-tion of TRIM59-depleted prostate cancer cells led to the development of smaller tumors than those seen with the introduction of control cells. These in vitro and in vivo data suggest a prominent tumorigenic function for TRIM59 in prostate cancer. Moreover, following cell cycle analysis, we observed that prostate cancer cells accumulated predominantly in G0/G1 and S-phase when TRIM59 was depleted. These find-ings indicated that TRIM59 functions as an activator of cell division. In support of this, TRIM59 knockdown led to significant down-regulation of the key cell cycle regulators CDC25A, CDC2, and cyclin B1. These findings suggest that TRIM59 may contribute to tumor growth by regulation of cell cycle progression in prostate cancer.

Cyclins were originally named because their concentrations vary in a cyclical fashion during the cell cycle. CDC25A is proto-oncogene in humans and has been shown to be overex-pressed in various cancers. In addition, CDC25A is involved in the process of apoptosis mediated by the p53-dependent and ATF3 signaling pathways. CDC2, also known as CDK1, is a small and highly conserved protein. Human CDC2 can form complexes with its cyclin partners that phosphorylate various target substrates, leading to cell cycle progression. The cyclin B1-CDC2/CDK1 complex is in-volved in many of the early events of mitosis, including chromosome condensation and spindle pole assembly. The main role of cyclin B1 is to facilitate the transition of cells from G2 to M phase, and dysregulation of this process is implicated in human tumorigenesis. In the present report, we found that TRIM59 knock-down significantly inhibited the expression of CDC25A, CDC2, and cyclin B1 in the prostate cancer cell lines PC3 and DU145. However, the detailed mechanisms by which TRIM59 regu-

Figure 4. Knockdown of tripartite motif 59 (TRIM59) caused cell cycle arrest in S-phase in prostate cancer cell lines. (A) Cells were starved for 24 h and then treated with scramble shRNA (control group) or TRIM59-specific shRNA (shRNA group) for another 24 h. (B–C) Cell distributions in PC3 cells (B) and DU145 cells (C) treated with control or TRIM59 shRNA. Data shown represent the mean ± standard deviation (SD) of three independent experiments. **p < 0.01, shRNA vs. control.
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Recent studies have elucidated expression of these cell cycle regulators remain unclear. TRIM59 has been reported to be involved in NF-κB- and IRF3/IRF7-mediated signaling pathways. Furthermore, c-Myc overexpression and DNA promoter hypermethylation repress the expression of TRIM59 in cancers. These reports suggest several possible mechanisms by which TRIM59 may regulate cell cycle progression in prostate cancer, and further work is required to explore these.

Conclusions

We demonstrated that TRIM59 was highly expressed in human prostate cancer. Knockdown of TRIM59 with specific shRNA inhibited in vitro cancer cell proliferation and in vivo tumor growth, and led to cell cycle arrest in S-phase. Regulation of cell cycle progression may underlie TRIM59-mediated prostate cancer development. Our evidence provides novel clues that may aid prostate cancer diagnosis and treatment in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.
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