TRIM37 promotes tumor cell proliferation and drug resistance in pediatric osteosarcoma

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Abstract. Osteosarcoma (OS) is among the most frequently occurring bone tumors, particularly in children. Clinical treatment of OS is limited due to several factors including resistance to chemotherapy drugs and metastasis, and the underlying molecular mechanisms remain unclear. In the present study, tripartite motif containing 37 (TRIM37) expression levels were upregulated in tumor samples and associated with the development of drug resistance in OS. Furthermore, chemotherapy drug treatment (doxorubicin, cisplatin and methotrexate) induced TRIM37 expression in OS cells in vitro. TRIM37 mRNA and protein were upregulated in 41 pediatric osteosarcoma clinical specimens. To further elucidate the effect of TRIM37, gain and loss-of-function analysis was performed. Overexpression of TRIM37 induced cell proliferation and drug resistance ability of OS cells, whilst TRIM37 knockdown suppressed cell growth rate and restored chemosensitivity. TRIM37-regulated genes were subsequently analyzed by expression microarray and gene set enrichment analysis. Using the Wnt/β-catenin inhibitor XAV-939, the present study demonstrated that TRIM37-induced chemoresistance is partially dependent on the activation of the Wnt/β-catenin signaling pathway. Collectively, the results of the present study suggest that TRIM37 may have a key role in the development of OS and in the ability for the cells to acquire drug resistance, thus it may be a novel target for the treatment of OS.

Introduction

Osteosarcoma (OS) is one of the most common human bone malignancies and a leading cause of tumor-associated mortalities in children and adolescents, which comprises ~2.4% of all tumor types in pediatric patients (1,2). Emerging evidence suggests that osteosarcoma is a type of differentiation disease, which initiates in the regions where bone growth and repair is activated (3). The clinical treatment for osteosarcoma is limited given that the mortality rate in amputated patients is high due to pulmonary metastases (4). Since the use of surgery and neoadjuvant chemotherapy, the 5-year survival rate of osteosarcoma patients has dramatically improved to ~60% (5). However, there remains a significant proportion of patients who may relapse, and distant metastasis may occur due to poor responses to chemotherapy. The acquisition of chemoresistance is a major reason for poor prognosis (6). Therefore, the identification of the underlying mechanisms that are responsible for chemoresistance is critical for improvements in prognosis and therapeutic strategies.

Tripartite motif containing 37 (TRIM37), belongs to the tripartite motif family and contains zinc-binding, RING finger region, B-box motif and coiled-coil domains (7). The encoded TRIM37 gene is located at 17q22-23, and a number of novel mutations have been reported (8,9). During tumorigenesis and development, TRIM37 has essential roles in regulating the expression of oncogenes due to its E3 ligase activity in the ubiquitin-proteasome degradation system (10,11). Upregulated TRIM37 expression has been reported in a number of tumor types, including hepatocellular and pancreatic carcinoma, and breast cancer (11,12). However, the expression pattern and the function of TRIM37 in OS remain unclear.

The role of Wnt/β-catenin signaling pathway as a major oncogenic pathway in a number of cancer types, including OS, has been well established (13,14). β-catenin is a key molecule in the Wnt/β-catenin signaling pathway and is re-localized to the nucleus and forms a complex with T-cell factor (TCF) to regulate gene expression upon Wnt ligand stimulation (15). Studies have demonstrated that Wnt/β-catenin is able to regulate a number of downstream targets including cyclin D1, Myc proto-oncogene protein and mitogen-activated protein kinase 8, which regulates cell proliferation, migration and
stemness (16-18). Notably, previous studies have demonstrated that TRIM37 may interact with β-catenin and be involved in aberrant activation of the Wnt/β-catenin signaling pathway (19). However, the precise mechanisms underlying the association between TRIM37 and Wnt/β-catenin signaling pathway activation and the potential effect on chemoresistance in OS cells remain to be elucidated.

The present study demonstrated that TRIM37 expression is induced by treatment with chemotherapy drugs, potentially promoting chemoresistance in patients with pediatric osteosarcoma. TRIM37-induced activation of the Wnt/β-catenin signaling pathway may be responsible for the chemoresistance. Therefore the current study hypothesized that decreasing TRIM37 levels combined with a Wnt/β-catenin signaling pathway inhibitor may be an optimal therapeutic strategy in pediatric OS.

Materials and methods

Patient samples. The present study was approved by the Jining Medical University Affiliated Hospital Ethics Committee (Jining, China) and written informed consent was obtained from all patients. A total of 41 OS tissues were collected from patients (<21 years old; median, 17; range, 14-21) and the clinicopathological features of patients are displayed in Table I. Tissue samples were stored at -80°C.

Cell culture and transfection. Human OS cell lines (MG-63, SaOS-2, U-2 OS and SOSP-9901) and osteoblasts (hFOB1.19) were obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin-streptomycin solution (Gibco; Thermo Fisher Scientific, Inc.). The cells were incubated at 37°C with 5% CO₂ in a humidified incubator. The small interfering RNAs (siRNAs) targeting TRIM37 were designed and synthesized by Chang Jing Bio-Tech, Ltd. (Changsha, China; siRNA sequences, 5'-ATT TGT ATG GAG AAA TTG C-3' and reverse, 5'-AGT CCT TCC ACG GAC A-3'). The sequence of the control siRNA was 5' -TTC TTC GAA CGT GTC ACG TT-3'.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from tissues samples or cultured cells using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and subsequently converted to single stranded cDNA using the PrimeScript RT Master kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. The RT-qPCR was performed on a 7500 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR-Green PCR mix (Takara Biotechnology Co., Ltd.). The PCR conditions were as follows: 95°C 30 sec; 95°C 30 sec; 60°C 30 sec (40 cycles). The gene expression was normalized to GAPDH by 2-ΔΔCT method as previously described (20). The primer sequences used were as follows: TRIM37 forward, 5’-TCA GCT GTA TTA GGC GC-3' and reverse, 5’-ATC CTA TAC TCA GGT TAC-3'; and housekeeping gene GAPDH forward, 5’-CATGGAG TATGACAACGCT-3' and reverse, 5’-AGTCTCTCAGCG ATACCAAGT-3’.

Western blotting. The cells were harvested and lysed in radio-immunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) supplemented with phosphatase inhibitor and protease inhibitor. Total protein concentration was measured by bichoninic acid assay. Protein (60 μg) was separated by 10% SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes. Following blocking with 5% bovine serum albumin (Sangon Biotech Co., Ltd., Shanghai, China), the membrane was incubated with the appropriate primary antibody at 4°C overnight, followed by washing and incubation with horseradish peroxidase goat-anti-rabbit Immunoglobulin G secondary antibody (dilution, 1:2,000; no. ab6721; Abcam, Cambridge, MA, USA) for 1 h at room temperature and detection using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.). The primary antibodies used were as follows: Anti-TRIM37 (dilution, 1:1,000; cat. no. sc-49548; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Tubulin (dilution, 1:1,000; cat. no. sc-9104; Santa Cruz Biotechnology, Inc.) and anti-cleaved poly(ADP-ribose) polymerase (PARP; dilution, 1:1,000; anti-cleaved PARP; cat. no. 5625; Cell Signaling Technology, Inc., Danvers, MA, USA).

Immunohistochemical staining. The osteosarcoma tissues were fixed in 4% formaldehdy for 30 min at room temperature, embedded in paraffin and sectioned (4 μm). The slides were

Table I. Clinicopathological features of 41 patients with pediatric osteosarcoma.

| Clinicopathological feature | n |
|-----------------------------|---|
| Age, years                  |   |
| ≤15                        | 24 |
| >15                        | 17 |
| Sex                        |   |
| Male                       | 22 |
| Female                     | 19 |
| Tumor size, cm             |   |
| <5                         | 30 |
| ≥5                         | 11 |
| Tumor location             |   |
| Femur                      | 20 |
| Tibia                      | 16 |
| Humerus                    | 3  |
| Other                      | 2  |
| Metastasis                 |   |
| Present                    | 15 |
| Absent                     | 26 |
deparaffinized with xylene and rehydrated in graded ethanol, and immersed in 3% H$_2$O$_2$ to block endogenous peroxidase activity. Following antigen retrieval, the slides were blocked by 10% goat serum (Sangon Biotech Co., Ltd.) and incubated with primary anti-TRIM37 antibody overnight at 4˚C. Visualization was performed by adding biotinylated goat-anti-rabbit secondary antibody (dilution, 1:2,000; no. SV0002; Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 30 min at room temperature, followed by the 3,3’-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin.

**Colony formation and cell viability assay.** For colony formation and cell viability assay, cells were seeded in 6-well plates (1,000 cells/well) and cultured at 37˚C with 5% CO$_2$ for 7 days. Following washing, the plates were stained with crystal violet. For cell viability assay, cells were seeded in 96-well plates (2,000 cells/well). Following treatment with chemotherapy drugs [10 ng/µl doxorubicin (Dox; Sigma-Aldrich; Merck KGaA), 20 µM cisplatin (Cis; Sigma-Aldrich; Merck KGaA), 10 ng/µl methotrexate (Mtx; Sigma-Aldrich; Merck KGaA), 10 µM XAV-939 (Selleck Chemicals, Shanghai, China) or dimethylsulfoxide], the cells were incubated with 5 mg/ml MTT for 3 h. The medium was subsequently removed and dimethylsulfoxide was added to solubilize the crystals. Absorbance was detected using a spectrometer at a wavelength of 590 nm. Clone number (%) was calculated by randomly selecting 5 fields in which the cells were counted. The control group was 100% and test groups were calculated according to this control.

**Bioinformatics analysis.** Microarray analysis was used to analyze the genes where expression was altered following the knockdown of TRIM37 in OS cells. SaOS-2 cells were treated with siRNA to knockdown levels of TRIM37. Total RNA was extracted from these cells, as previously stated, and sent to Shanghai Biomedical Laboratory, Co., Ltd., (Shanghai, China) to be analyzed using the Affymetrix Human U133 Plus 2.0 array. Gene Set Enrichment Analysis (GSEA) was performed using the GSEA program (Broad Institute, Boston, MA; http://www.broadinstitute.org/gsea/index.jsp). GSEA was used to analyze signaling pathways in which TRIM37-regulated genes were enriched.

**Statistical analysis.** Statistical analysis was performed using SPSS version 21.0 (IBM SPSS, Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Differences between the mRNA levels in tumors and non-cancerous tissue were determined using the Wilcoxon signed-rank test. Two-tailed Student's t-test was used to analyze the significance between the experimental group and the control. Data are presented as the mean ± standard deviation in ≥3 independent experiments. *, P<0.05 was considered to indicate a statistically significant difference.

**Results**

Upregulation of TRIM37 is induced by chemical drug treatment in OS cells. To investigate the possible role of TRIM37, the expression of TRIM37 in OS cells following treatment with chemotherapy drugs (doxorubicin, cisplatin and methotrexate) was examined. Notably, all three chemotherapy drugs markedly increased TRIM37 mRNA and protein expression in MG-63 and SaOS-2 cells (Fig. 1A and B). Furthermore, transcription inhibitor cycloheximide was able to reduce
chemotherapy drug-induced TRIM37 expression in SaOS-2 cells (Fig. 1C, left panel). MG132, which is able to inhibit proteasome activity, was employed to further analyze TRIM37 expression following chemotherapy drug treatment. TRIM37 expression was increased following MG132 treatment (Fig. 1C, right panel). These results indicated that the upregulation of TRIM37 may be dependent on transcriptional activation and protein stability. In addition, it was observed that the upregulation of TRIM37 in OS cells (MG-63 and SaOS-2) was time dependent in the case of doxorubicin (Fig. 1D), whilst this effect was not observed in cells with cisplatin and methotrexate treatment. Collectively, the present findings indicated that TRIM37 was upregulated when OS cells were treated with chemotherapy drugs.

TRIM37 is upregulated in clinical OS samples and OS cell lines. Expression of TRIM37 in pediatric OS was detected by RT-qPCR and immunohistochemical analysis in patient tissue specimens (n=41; Fig. 2). The results indicated that TRIM37 expression was significantly increased at the mRNA level and markedly increased at the protein level (Fig. 2B and C). Immunohistochemical staining demonstrated relatively high TRIM37 expression in the cytoplasm and cell nucleus in 80.5% (33/41) of OS specimens, whilst low expression was observed in normal tissues (Fig. 2A). Furthermore, TRIM37 expression was examined in OS cell lines (n=5) and hFOB1.19 cells. As shown in Fig. 2C, expression of TRIM37 was increased in OS cell lines (MG-3, SaOS-2, U-2 OS, SOSP9901 and KHOS) compared with the osteoblast cell line hFOB1.19. Taken together, the results of the present study indicate that TRIM37 expression was increased in OS tissues and cell lines and may contribute to tumor progression.

TRIM37 promotes cell proliferation and inhibits chemotherapy-induced cell apoptosis. To investigate whether TRIM37 expression affects proliferation and survival of OS cells, gain and loss-of-function analysis was performed. Endogenous TRIM37 was knocked down by co-transfection with 2 siRNAs targeting TRIM37. As shown in Fig. 3A, transfection with TRIM37 siRNAs in SaOS-2 cells led to a marked decrease in TRIM37 protein levels. TRIM37 knockdown reduced the proliferation rate as determined by colony formation assay; the number of colonies significantly decreased by ~60-85%, compared with control cells (Fig. 3B). Next, the effect of TRIM37 knockdown on chemotherapy-induced cell death in OS cells was examined. As indicated by the cell viability assays, significant increases in apoptotic rates were observed following TRIM37 knockdown (Fig. 3C). This finding suggested that TRIM37 knockdown may increase the sensitivity of OS cells to doxorubicin, cisplatin and methotrexate. Furthermore, western blotting was used to analyze the expression of the apoptosis marker cleaved PARP and it was demonstrated that the knockdown of TRIM37 resulted in an increase in the expression of cleaved PARP protein. Notably, cleaved PARP expression was markedly increased.
when TRIM37 knocked down cells were treated with doxorubicin, cisplatin and methotrexate, compared with no treatment (Fig. 3D).

The effect of TRIM37 overexpression was also examined in an OS cell line (MG-63; Fig. 3E). Colony formation and cell viability assays indicated that overexpression of TRIM37 significantly increased the cell proliferation rate and significantly decreased the apoptotic rate (Fig. 3F and G). The chemotherapy drugs induced C-PARP expression, a marker of cell apoptosis, which was attenuated by overexpression of TRIM37 (Fig. 3H). Collectively, these data suggested that TRIM37 promoted cell proliferation and increased the resistance of OS cells to chemotherapy drugs.

Wnt/β-catenin inhibitor abrogates TRIM37-induced chemoresistance. To further investigate the mechanism by which
TRIM37 affects OS proliferation and chemoresistance, genes regulated by TRIM37 were identified by microarray analysis. GESA was subsequently performed to investigate the potential downstream signaling pathways that may be regulated by TRIM37. GESA indicated that the Wnt/β-catenin signaling pathway was enriched, which was verified in the present study by RT-qPCR detection for the downstream genes (Fig. 4A and B). This notable finding was consistent with the finding of a previous study, which demonstrated that TRIM37 interacts with β-catenin and induces transcriptional activity of β-catenin/TCF (12,19). Notably, Wnt/β-catenin aberrant activation has been associated with chemoresistance in different types of tumors, including hepatocellular carcinoma, neuroblastoma and prostate cancer (21-23). Therefore, the TRIM37-transfected OS cells (MG-63 and SaOS-2) were treated with a specific Wnt/β-catenin pathway inhibitor XAV-939 (10 µM), in combination with doxorubicin, cisplatin and methotrexate. Western blotting was used to confirm the inhibition of TRIM37-induced Wnt/β-catenin signaling (Fig. 4C, upper panel). As expected, 48 h following transfection, XAV-939 significantly abrogated TRIM37-induced chemoresistance in MG-63 and SaOS-2 cells (Fig. 4C, lower panel). These results further indicated that the Wnt/β-catenin signaling pathway may be a major target of TRIM37 and mediates TRIM37-induced chemoresistance.

Discussion

Increasing evidence indicates that TRIM37 may have a role as an oncogene and be a potential molecular target for therapy in human carcinoma. TRIM37 is highly upregulated in a number of cancer types. Jiang et al (12) demonstrated that elevated expression of TRIM37 promoted migration and metastasis of hepatocellular carcinoma cells by upregulating the β-catenin signaling pathway. Furthermore, a previous study indicated that TRIM37 interacts with β-catenin and recruits the β-catenin/TCF complex to promote pancreatic cancer progression. TRIM37 has been established to mediate downstream target genes of β-catenin/TCF complex transcriptional activation, which is independent of its E3 ligase function.
activity (19). Bhatnagar et al (11) demonstrated the role of TRIM37 as an E3 ubiquitin ligase for histone H2A, and the association of TRIM37 with polycomb repressive complex 2 in breast cancer. In addition, mutations in TRIM37 have been reported which are associated with OS (24,25). Increasing evidence indicate that TRIM37 may have key roles in tumor development and progression. However, limited information has been reported regarding the impact of TRIM37 on the Wnt/β-catenin-TCF axis in pediatric OS and chemotherapy resistance.

In the present study, the role of TRIM37 in pediatric OS tissues and cells was examined. Initially, it was observed that TRIM37 expression at the mRNA and protein level was induced by treatment with chemotherapy drugs (doxorubicin, cisplatin and mitotane). TRIM37 expression was significantly enhanced in 41 pediatric osteosarcoma specimens and cell lines. The data of the present study are also consistent with previous studies that have reported that TRIM37 is upregulated in other types of cancer, thus upregulation of TRIM37 is not tumor type-specific.

For assessing the function of TRIM37, siRNAs were used to specifically knockdown TRIM37. It was observed in the present study that TRIM37 regulates OS cell viability, apoptosis and resistance to chemotherapy drugs. The results of the present study suggested that TRIM37 downregulation increased cleaved-PARP expression, which is an important mediator of cell apoptosis. Furthermore, the pattern of gene expression of TRIM37 knockdown in SaOS-2 OS cells was analyzed by microarray. The GSEA analysis indicated that the genes with altered expression were enriched in the Wnt/β-catenin signaling pathway, which suggest that the inhibition of Wnt/β-catenin signaling caused by downregulation of TRIM37 may be a potential mechanism for chemotherapy drug-induced apoptosis in OS cells.

The involvement of Wnt/β-catenin signaling pathway in cell survival and mobility is well recognized, and the activation of Wnt/β-catenin signaling increases resistance to drug induced apoptosis. Wickström et al (26) reported that the inhibition of β-catenin signaling is able to repress O6-methylguanine-DNA methyltransferase activation and therefore prevents chemoresistance. Hsieh et al (23) demonstrated that microRNA-320 is able to inhibit β-catenin expression, and suppress chemoresistance and tumorigenic abilities in prostate cancer. Furthermore, Ma et al (27) observed that the inhibition of Wnt/β-catenin and notch signaling sensitized OS cells to chemotherapy. These results suggested that the Wnt/β-catenin cascade is upregulated in OS cells. The GSEA demonstrated that the expression of TRIM37-regulated genes is consistent with activation of the Wnt/β-catenin signaling pathway, which is also in concordance with the results of previous studies (28,29). Furthermore, the reverse experiment using XAV-939 to specifically inhibit Wnt/β-catenin signaling confirmed the hypothesis that TRIM37 mediates Wnt/β-catenin signaling, which is involved in chemoresistance of OS cells.

In summary, the present study demonstrated that the effect of TRIM37 upregulation in OS on cellular proliferation and resistance to chemotherapy drugs may be mediated by modulating the expression of specific genes in the Wnt/β-catenin signaling pathway. Thus, TRIM37 may be a potential therapeutic target for the treatment of pediatric OS.

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