Abstract. Thyroid hormone receptor-interacting protein 6 (TRIP6), a member of the zyxin family of Lin-Isl-Mec (LIM) proteins, is an adaptor protein primarily expressed in epithelial cells. TRIP6 can regulate a variety of cellular responses, such as actin cytoskeletal reorganization and cell adhesion. However, to the best of our knowledge, the role of TRIP6 in osteosarcoma (Os) has not been previously reported. Therefore, the present study investigated the role of TRIP6 in the occurrence and development of Os, and the potential of utilizing TRIP6 as a therapeutic target in Os. The present results suggested that the expression levels of TRIP6 were significantly increased in Os cells and clinical tissue specimens compared with normal osteoblasts and adjacent non-tumor tissue. Moreover, the present results suggested that overexpressing TRIP6 significantly increased proliferation, migration and invasion, while inhibiting apoptosis in Os cells. However, silencing TRIP6 decreased proliferation, migration and invasion, while activating apoptosis in Os cells. The present results suggested that overexpression of TRIP6 increased NF-κB activation by decreasing the protein expression levels of inhibitor of κBα, and increasing total and phosphorylated P65 levels. The present results indicated that TRIP6 silencing decreased NF-κB activation. Collectively, the present results suggested that TRIP6 may play a role in promoting Os cell proliferation, migration and invasion, while inhibiting cell apoptosis. Furthermore, TRIP6 may be utilized as a novel prognostic biomarker and therapeutic target in Os.

Introduction

Osteosarcoma (Os) is one of the most common primary malignant bone tumors in pediatrics and adolescents, and originates from interstitial cells (1). Os accounts for ~20% of primary malignant bone tumors and 0.5% of malignant tumors (2). Os tends to occur in the epiphyseal region where there is an abundant blood supply, thus the incidence of hematogenous metastasis is high, occurs early and progresses quickly (3). Previous studies have shown that, if not treated in time, 80% of patients with Os will develop lung metastases, which is often fatal (4–6). Currently, the drugs used to treat Os include doxorubicin, methotrexate, cisplatin and ifosfamide (7). Some patients have a poor prognosis or drug resistance after chemotherapy, both of which are difficult to improve despite alternative treatment combinations (8). In addition, high doses of chemotherapeutic agents have side effects (9). Therefore, understanding the molecular mechanism underlying the occurrence and development of Os could aid in the discovery of novel targets for Os treatment (10–12).

Thyroid hormone receptor-interacting protein 6 (TRIP6), a member of the zyxin family of LIM proteins family, is an adaptor protein that regulates a variety of cellular responses, such as actin cytoskeletal reorganization and cell adhesion, and is predominantly expressed in epithelial cells (13,14). Many TRIP6 binding partners have been identified, including the tyrosine phosphatase (PTP)-BL and the adaptor protein reversion-induced LIM, indicating that these proteins have regulatory effects on the actin cytoskeleton and cell viability (15,16). Previous studies have reported that TRIP6 is overexpressed in nasopharyngeal cancer, non-Hodgkin lymphoma and Ewing sarcoma (17–19). Miao et al (17) showed that overexpression of TRIP6 can reverse the cell adhesion-mediated drug resistance phenotype by decreasing the phosphorylation of P27 in non-Hodgkin lymphoma. In addition, Lai et al (20) found that TRIP6 overexpression in glioblastoma inhibits cell apoptosis and causes resistance to Fas-mediated cell invasion by enhancing NF-κB activity. Therefore, TRIP6 may play an important role in cancer progression and development (21). However, the clinical significance and biological role of TRIP6 in human Os remains unknown. Whilst TRIP6 has been reported in other cancer types, it has not been reported in Os; therefore, the present study investigated the effect of TRIP6 on Os. In addition, TRIP6 has been suggested to be
involved in the regulation of the NF-κB signaling pathway, but further investigation is required to understand whether TRIP6 affects the occurrence and development of Os via the NF-κB signaling pathway.

The NF-κB signaling pathway is activated by extracellular stimulation (22). Extracellular signaling factors bind to receptors on the cell membrane and initiate a cascade of downstream pathways (23). Receptor proteins first activate IkB kinase (IKK) upon stimulation (24). IKK then phosphorylates serine at the regulatory site of the IkB subunit on the intracellular NF-κB/IkB compound, which allows the IkB subunit to be ubiquitinated and degraded by the proteasome to release the NF-κB dimer (25-29). With the degradation of IkB, free P65 is phosphorylated by protein kinase A at serine 276 in the cytoplasm, and then phosphorylated P65 enters the nucleus and binds to corresponding binding sites on genes, which initiates transcription (30). NF-κB also activates the expression of the inhibitor of κBα (IkBα) gene, and the newly formed IkBα inhibits the activity of NF-κB, resulting in a spontaneous negative feedback loop (31). IkB is an inhibitory protein of NF-κB. The IkB family consists of eight members, including P100, P105, IkBα, IkBβ, IkBγ, IkBa, Bcl-3 and IkB-R (32). During resting state, IkBα and the NF-κB subunits P65 and P50, exist in the cytoplasm in an inactive state (33). When upstream signaling factors activate IKK, IkBα is ubiquitinated, phosphorylated and degraded, converting the two subunits of NF-κB from the inactive to the active state and translocating the subunits from the cytoplasm to the nucleus. NF-κB then binds to corresponding inflammation-related genes, and initiates the transcription of inflammatory cytokines and induces inflammation (34).

A previous preliminary study found that TRIP6 was overexpressed in a large number of human Os samples (data not shown). The present results suggested that overexpression of TRIP6 significantly promoted cell proliferation, migration and invasion, and inhibited apoptosis of Os cells. However, silencing TRIP6 inhibited proliferation, migration and invasion, and promoted apoptosis in Os cells. The present results suggested that TRIP6 may play a role as an oncoprotein in the progression of Os cells, providing novel insights into the regulatory mechanism of the NF-κB signaling pathway.

Materials and methods

Cell culture and transfection. Human Os cell lines U2OS and MG63, and the normal osteoblast cell line hFOB1.19 were purchased from The Cell Bank of The Chinese Academy of Sciences. U2OS and MG63 were cultured for 24 to 48 h in DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (Gibco; thermo Fisher Scientific, Inc.) at 37°C with 5% CO2. hFOB1.19 cells were cultured for 72 to 96 h in DMEM with F12, 0.3 mg/ml G418 and 10% FBS at 33.5°C with 5% CO2. Culture media was changed regularly and cells were split to maintain growth in the logarithmic growth phase. MG63 and U2OS cells were harvested and seeded in 6-well plates (2x10^5 cells/well). Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect cells according to the manufacturer’s instructions. The following plasmids (Shanghai GenePharma Co., Ltd.; 2.5 ng/well) were used: i) pcDNA Flag TRIP6 or shNC (non-coding shRNA)/pGPU6/GFP/Neo plasmids as the negative control; ii) TRIP6-small interfering RNA (siRNA; 5'-GAAAGCTGGTTCACGACATGAA-3') or TRIP6-negative control (NC); iii) pcDNA Flag P65 or shNC/pGPU6/GFP/Neo plasmids as the negative control; and iv) P65−siRNA (5'-GAACACAATGGCCACTTGCC-3') or P65-NC. After transfection, the cells were cultured for 24-48 h before further experiments were performed.

Patient information and tissue specimens. The present study was conducted on a total of 55 paraffin-embedded archived Os samples which had been histopathologically and clinically diagnosed in The Department of Orthopedics at The Second Affiliated Hospital of Anhui Medical University from June 2008 to June 2018. All the experiments performed using human tissues were from the 55 paraffin-embedded archived samples (males, 36; females 19; age range, 8-62 years; mean age, 25±8.4 years). Enneking stages: 19 cases were in I stage, in II stage were 17 cases and in III stage were 19 cases. Osteosarcoma was found in the following sites in patients: In the femur in 21 patients, in the tibia in 24 patients and in other sites in ten patients. The tumor diameter was <5 cm in 19 cases and ≥5 cm in 36 cases. There were 15 osteoblastoma cases, 12 cases of chondroblastoma, 18 cases of fibroblastoma and ten cases involving other factors. There were 28 cases with metastasis and 27 cases without metastasis. Inclusion criteria included patients who were pathologically diagnosed with Os. Clinical and clinicopathological stage was determined according to American Joint Committee on Cancer criteria (35). For the use of clinical materials for research, written informed consent was obtained from each patient or their relatives prior to surgery. The present study was approved by The Institutional Research Ethics Committee. The clinical information for patient samples is summarized in Tables SI and SII.

Western blotting. Western blotting was performed according to standard methods (26). Total protein from cell lines was extracted using RIPA lysis buffer (Thermo Fisher Scientific, Inc.) and quantified using the Bradford method (36). According to paraffin-embedded tissue protein extraction kit (BestBio; cat. no. BB-3164-2) instructions, protein extracted from paraffin embedding organization was used to analyze TRIP6 protein expression level in the adjacent non-tumor tissue and Os tissue. Paraffin-embedded adjacent non-tumor samples were also obtained from the same patient. In total, 60 µg protein were separated by 10% SDS-PAGE and transferred to a PVDF membrane (EMD Millipore). The membranes were blocked using 5% milk at 37°C for 1 h. Membranes were incubated overnight at 4°C with the following antibodies: Anti-TRIP6 (1:200; ab70747; Abcam), anti-P65 (1:5,000; ab109458; Abcam), anti-P65 (1:16,000; ab6503; Abcam), anti-IkBα (1:1,000; ab32518; Abcam), anti-GAPDH (1:10,000; ab181602; Abcam). The membranes were then incubated with the following secondary antibodies for 2 h at 37°C: Horse radish peroxidase-conjugated goat anti-mouse (1:1,000; Abcam; ab6789) and horse radish peroxidase-conjugated goat anti-rabbit (1:1,000; Abcam; ab6721). Bound proteins were visualized using enhanced chemiluminescent kit (Thermo Fisher Scientific, Inc.) and detected using a BioImaging...
Cells were plated in 6-well plates at a density of 2x10^3 cells/well in 200 µl of complete or serum-free media (Gibco; Thermo Fisher Scientific, Inc.). At each time point (0, 24, 48, 72 and 96 h) cells were stained with 200 µl sterile MTT dye (0.5 mg/ml; Sigma-Aldrich; Merck KGaA) for 5 h at 37˚C, followed by removal of the culture medium and addition of 100 µl of DMSO (Sigma-Aldrich; Merck KGaA). Absorbance was measured at 570 nm, with 655 nm used as a reference wavelength. Experiments were performed in triplicate.

Colonies were plated in 6-well plates at a density of 4x10^4 cells/well and cultured for 12 days at 37˚C. Colonies were stained with 1% crystal violet for 30 sec after fixation with 4% formaldehyde for 5 min, 20-25˚C. The number of colonies of more than 10 cells (>0.1 mm diameter) was counted under a light microscope (x400). Data are presented as the fold change compared with the control group. Experiments were performed in triplicate.

Flow cytometric analysis of apoptosis. Apoptosis was measured using the Annexin V-FITC (50 mM Tris; 100 mM NaCl; 1% BSA; 0.02% sodium azide; pH 7.4) Apoptosis Detection kit (Invitrogen; Thermo Fisher Scientific, Inc.). After cells were harvested, washed twice with PBS and resuspended in 450 µl binding buffer (Annexin V-FITC cell apoptosis assay kit; Beyotime Institute of Biotechnology), Annexin V-FITC was added (5 µl/well). Cells were stained for 15 min under light protection at room temperature, followed by resuspension in 450 µl binding buffer (Annexin V-FITC cell apoptosis assay kit; Beyotime Institute of Biotechnology). Cells were then stained with propidium iodide (PI) in the dark (10 µl, 20-25˚C, 10-20 min). Cell apoptosis was analyzed on a flow cytometer (Attune Nxt; BD Biosciences) and the percentage of apoptotic cells was analyzed using CellQuest Pro analysis software (version 5.1; BD Biosciences; Attune Nxt; FACs Calibur). Experiments were performed in triplicate. The calculation method of apoptotic rate was the sum of early and late apoptotic rate.

Cell migration and invasion assay. Transwell inserts were used to detect cell migration and invasion. After transfection, cells in logarithmic phase were harvested according to the manufacturer's instructions for the 24 wells BD basement membrane matrix (dilution, 1:3; BD Biosciences) invasion Transwell chamber. A cell suspension of 5x10^4 cells/ml was seeded in the upper chamber, with or without Matrigel, for 24 h at 37°C). The upper chamber was filled with serum-free DMEM culture media (Gibco; Thermo Fisher Scientific, Inc.), and the lower chamber was filled with DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS. Plates were then incubated at 37°C with 5% CO2 for 24 h. After culturing the cells for 24 h, the non-invaded cells in the inner layer of the compartment membrane were wiped with cotton swabs. The membranes were then fixed for 10-20 min at 20-25°C, with 3% paraformaldehyde, stained with 0.1% crystal violet for 5 min at 20-25°C, washed with water and dried. The number of cells in the experimental group was measured by placing the Transwell chamber (Costar; Corning, Inc.) on a microscope slide and acquiring images using a light microscope (x400; Olympus Corporation). Each experiment was performed in triplicate.

Statistical analysis. Statistical analyses were performed using the SPSS version 13.0 statistical software package (SPSS, Inc.). Statistical tests for data analysis included log rank test, χ2 test, Spearman-rank correlation test, Student's 2-tailed t-test and one-way ANOVA followed by Newman-Keuls test. Multivariate statistical analysis was performed using a Cox regression model. Data are presented as the mean ± SD. P<0.05 was considered to indicate a statistically significant difference.

Results

Overexpression of TRIP6 correlates with the progression and poor prognosis of Os. To investigate the carcinogenic effect of TRIP6 in Os, the present study evaluated the protein expression level of TRIP6 in Os cell lines and human Os tissue. TRIP6 was significantly increased in U2OS and MG63 cells, compared with the normal osteoblast cell line hFOB1.19 (Fig. 1A and B). Comparative analysis results suggested that the expression level of TRIP6 was significantly increased in the Os samples compared with matched adjacent non-tumor tissues (Fig. IC and D). The present results suggested that that TRIP6 expression levels were significantly increased in Os cells and human Os tissues compared with normal osteoblasts and matched adjacent non-tumor tissues. Therefore, the present results suggested that TRIP6 is overexpressed in Os.

Furthermore, the present results indicated that TRIP6 overexpression was correlated with the Enneking stage and metastasis (P<0.05), suggesting that TRIP6 overexpression is associated with the clinical progression of human Os (Table SI). Moreover, based on the varying expression levels of TRIP6 in different paraffin-embedded Os samples, patients with Os could be separated into high- and low-expression groups. The present results suggested that survival time was significantly different between patients with Os with low and high TRIP6 expression (P=0.01; Fig. 1E). In addition, univariate and multivariate analyses results indicated that TRIP6 expression level may function as an independent prognostic factor in Os (Table SII). Therefore, the present results suggested that TRIP6 may be a potential prognostic indicator for Os.

TRIP6 promotes Os cell proliferation and apoptosis. The present study investigated the effect of TRIP6 on Os cell proliferation by overexpressing or silencing TRIP6. Western blotting results suggested that TRIP6 was significantly knocked down in the U2OS-TRIP6-siRNA cells compared with the U2OS-NC cells (Fig. 2A and B). Cell proliferation was significantly decreased in the U2OS-TRIP6-siRNA group compared with the U2OS-NC group. After TROP6 overexpression, TRIP6 expression level was significantly increased in the MG63-TRIP6-flag group compared with the MG63-vector group (Fig. 2C and D). The MTT and colony formation assay results suggested that, compared with the U2OS-NC group, cell
TRIP6 regulates osteosarcoma cells by activating the NF-κB signaling pathway.

Effect of TRIP6 on the invasive and migratory abilities of Os cells. TRIP6 was successfully transfected into U2OS and MG63 cells, and Transwell assays were used to measure cell migration and invasion. The present results suggested that the migratory (Fig. 3A-C) and invasive (Fig. 3D-F) ability of cells in the MG63-TRIP6-flag group was significantly increased compared with the MG63-Vector group. The migratory (Fig. 3G-I) and invasive (Fig. 3J-L) ability of cells in the U2OS-TRIP6-siRNA group was significantly decreased compared with the U2OS-NC group. The present results suggested that silencing TRIP6 inhibited, whereas overexpression of TRIP6 promoted, the invasion and migration of Os cells.

TRIP6 downregulates IκBα and activates the NF-κB signaling pathway. Previous studies have shown that TRIP6 plays a role in the NF-κB signaling pathway in non-Hodgkin lymphoma, Ewing sarcoma, nasopharyngeal cancer and hepatocellular carcinoma (18–20). Therefore, the present study investigated whether TRIP6 was involved in the NF-κB signaling pathway in OS cells. The present study knockdown TRIP6 using siRNA in U2OS cells and overexpressed TRIP6 in MG63 cells. Knockdown of TRIP6 in the U2OS-TRIP6-siRNA group, increased the expression level of IκBα and decreased NF-κB P65 activation compared with the control group (Fig. 4A and B). Overexpression of TRIP6 in the MG63-TRIP6-flag group reduced the expression level of IκBα and increased NF-κB P65 activation compared with the MG63-Vector group (Fig. 4A and C). Therefore, the present results suggested that TRIP6 may play a role in activating
Figure 2. TRIP6 plays a role in Os cell proliferation. (A) Western blot analysis of TRIP6 protein expression level in TRIP6 silenced-U2OS cell lines and (B) quantification. (C) Western blot analysis of TRIP6 protein expression level in TRIP6 transduced-MG63 cell lines and (D) quantification. Data were normalized to GAPDH and are presented as the mean ± SD from three independent experiments. MTT assay results from (E) U2OS cells and (F) MG63 cells indicated that growth rates increased in TRIP6-overexpressing cells and decreased in TRIP6-silenced cells. (G) U2OS cells colony formation assay (H) results and (I) MG63 cells colony formation assay (J) results indicated that growth rates increased in TRIP6-overexpressing cells and decreased in TRIP6-silenced cells. Colony formation, classified as colonies >0.1 mm diameter, was quantified after 12 days of culture. Apoptosis of U2-OS and MG63 cells analyzed by flow cytometry after different treatments. (K) The apoptotic rate was decreased in U2OS-NC cells (6.42%) (L) compared with U2OS-TRIP6-siRNA cells (15.61%). (M) The apoptosis rates of (K) and (L) were statistically significant. (N) The apoptotic rate was 7.16% in the MG63-Vector group and (O) 3.48% in the MG63-TRIP6-flag group. (P) The apoptosis rates of (N) and (O) were statistically significant. *P<0.05 vs. control group. Os, osteosarcoma; TRIP6, thyroid hormone receptor-interacting protein 6; NC, negative control; siRNA, small interfering RNA.
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Figure 3. Effect of TRIP6 knockdown and overexpression on migratory and invasive abilities of U2OS and MG63 cells. (A) MG63-Vector and (B) MG63-TRIP6-flag cells migration was measured using a Transwell assay. After staining, five random fields of view were photographed. (C) Number of migrated MG63 cells. Data are presented as the mean ± SD. (D) MG63-Vector and (E) MG63-TRIP6-flag cell invasion was measured using a Transwell assay with Matrigel, cells were allowed to migrate for 48 h. (F) Number of invaded MG63 cells. Data are presented as the mean ± SD. (G) U2OS-NC and (H) U2OS-TRIP6-siRNA cell migration was measured using a Transwell assay and cells were allowed to migrate for 24 h. (I) Number of migrated U2OS cells. Data are presented as the mean ± SD. (J) U2OS-NC and (K) U2OS-TRIP6-siRNA cell invasion was measured using a Transwell assay with Matrigel, cells were allowed to migrate for 48 h. (L) Number of invaded U2OS cells. Data are presented as the mean ± SD. Scale bar, 100 µm. *P<0.05 vs. control group. TRIP6, thyroid hormone receptor-interacting protein 6; NC, negative control; siRNA, small interfering RNA.

The NF-κB signaling pathway in Os. The expression level of phosphorylated (p)-P65 was related to the phosphorylation of this protein (Fig. 4D and E). The present study investigated the role of P65 in Os cell proliferation by knocking down P65 in U2OS cells and overexpressing P65 in MG63 cells. MTT assay results suggested that the expression level of P65 in U2OS-NC group was decreased compared with the U2OS-NC group. In addition, the expression level of P65 in the MG63-P65-flag group was significantly higher compared with the MG63-Vector group (Fig. 4J-L). The MTT assay results suggested that cell proliferation in the U2OS-P65-siRNA group was decreased compared with the U2OS-NC group (Fig. 4F). Furthermore, MTT assay results suggested that overexpressing P65 in U2OS-TRIP6-siRNA cells increased...
the growth rate of U2OS-TRIP6-siRNA cells (Fig. 4G-I).

Collectively, the present results suggested that P65 may play a role in the proliferative effect of TRIP6 on Os cells.

Discussion

TRIP6 is an adaptor protein primarily expressed in epithelial cells that can mediate several cellular responses, such as actin cytoskeletal reorganization and cell adhesion (13,14). However, the clinical significance and biological role of TRIP6 in Os is not fully understood. Previous studies have shown that TRIP6 is involved in cell growth in Ewing sarcoma and non-Hodgkin lymphoma (17,18). In line with a previous study, the present results suggested that TRIP6 was highly expressed in human Os tissue but weakly expressed in matched adjacent non-tumor tissues (P<0.05) (18). Therefore, the present results supported the hypothesis that TRIP6 may be an oncogenic protein that promotes cell proliferation, migration and invasion, and inhibits apoptosis in Os cells.

The mechanism underlying the effect of TRIP6 in promoting Os cell proliferation, migration and invasion, and inhibiting apoptosis may be via the activation of the NF-κB signaling pathway. The NF-κB signaling pathway is activated by IκB phosphorylation and degradation (37). The present results suggested a novel role for TRIP6 in Os, which may facilitate the development of novel therapeutics for Os.
NF-κB is a family of transcription factor proteins that includes the five subunits Rel, p65, RelB, p50 and p52 (38,39). The inhibitory protein IκBα, which is downregulated in various types of cancer, such as breast cancer, hepatocellular carcinoma and prostate cancer, plays vital roles in multiple biological processes including proliferation, migration, invasion and apoptosis (40-43). Previous studies have reported that the target protein TRIP6 binds to receptors on the cell membrane and initiates a cascade of downstream reactions (44,45). Receptor proteins first activate IKK upon stimulation (46). Then, IKK phosphorylates serine at the regulatory site of the IκB subunit of the intracellular NF-κB/IκB compound, which allows the IκB subunit to be ubiquitinated and degraded by the proteasome to release the NF-κB dimer (47). Then, P65, with the aid of P50, moves from the cytoplasm to the nucleus and binds to specific DNA sequences to regulate the transcription of target genes (48).

The present study was limited to investigating TRIP6 function in the in vitro, in vivo - collected and interpreted the data. WL and JJ drafted the manuscript. WL, LC and JJ were responsible for the conception and design of the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate.

The study protocol was approved by The Ethics Committee of The Second Affiliated Hospital of Anhui Medical University (SL-YX2019-042). Written informed consent was obtained from each patient or their relatives prior to surgery.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


