Knockdown of TRAF4 expression suppresses osteosarcoma cell growth in vitro and in vivo

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Abstract. Tumor necrosis factor (TNF) receptor-associated factor 4 (TRAF4) is an adapter molecule that is overexpressed in certain cancers. TRAF4 is overexpressed in osteosarcoma tissues and osteosarcoma cells. Using the technique of RNA interference, the expression of TRAF4 in the human osteosarcoma Saos-2 cell line was shown to be downregulated. The proliferation, cell cycle arrest and apoptosis ability of Saos-2 cells were examined, as was tumor development in a xenograft mouse model. The results showed that the TRAF4 knockdown exerts inhibitory effects on the proliferation ability of Saos-2 cells and tumor development in a xenograft mouse model. Simultaneously, it was found that TRAF4 knockdown led to cell cycle arrest in the G1 phase and promoted Saos-2 cell apoptosis. Following TNF-α treatment, the expression of nuclear factor κB was significantly reduced in the TRAF4-small interfering RNA group. These results indicate that TRAF4 regulated osteosarcoma cell growth in vitro and in vivo, and offers a candidate molecular target for osteosarcoma prevention and therapy.

Introduction

Osteosarcoma is an aggressive bone tumor characterized by osteoblastic differentiation and malignant osteoid production (1). It is the most common type of primary malignant bone tumor affecting children and adolescents, and the overall 5-year survival rate of osteosarcoma patients is 68% (2). The underlying mechanisms in osteosarcoma carcinogenesis have been investigated in the past few decades, however, the progress has been slow and the survival rate of patients has reached a plateau (3,4). The current treatment of osteosarcoma requires multidisciplinary therapy, incorporating surgery and systemic chemotherapy (5). However, the therapies all have different side-effect profiles and a poor prognosis. Thus, there is a requirement for an improved understanding of the pathogenesis of osteosarcoma, and the development of more effective therapeutic targets for this disease.

The tumor necrosis factor (TNF) receptor-associated factor (TRAF) family were originally discovered as signaling adaptors that couple the cytoplasmic regions of receptors of the TNF-R super-family (6). There are seven known members of the TRAF family (TRAF1 to 7) in mammals, and these play an important role in regulating cell survival, proliferation and stress responses (7). The distinctive feature of all the TRAF proteins is a C-terminal TRAF domain, which is composed of a C-terminal β-sandwich (TRAF-C) and an N-terminal coiled-coil region (TRAF-N) (8). Different members of the TRAF family mediate different signals. TRAF4 was the first member of the TRAF protein family found to be upregulated in human carcinomas. Unlike other canonical TRAFs, TRAF4 only interacts with limited TNFR-family members, including the p75 neurotrophin receptor, lichenoid tissue reaction and glucocorticoid-induced TNFR (GITR) (9), and was originally identified as a protein localized in the nucleus of breast carcinoma cells (10). TRAF4 has also been found in 43% of 623 human tumor samples from the prostate, ovary, lung and colon, among others (11). Therefore, TRAF4 protein overexpression is a common characteristic of numerous human cancers. In the latter study, TRAF4 mRNA was overexpressed in small cell lung carcinoma, lung adenocarcinoma, colon, ovary and prostate carcinomas (11). The amplification and overexpression of TRAF4 indicated that it is not only a marker of human carcinomas, but also a candidate oncogene. However, the role and mechanism of TRAF4 in osteosarcoma remains unclear.

In the present study, the aim was to examine TRAF4 expression in osteosarcoma tissue and osteosarcoma cells by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis and to observe the biological function of Saos-2 cells following TRAF4 knockdown by the RNA interference technique. These data may provide information for prognosis prediction and targeted therapy for osteosarcoma.

Materials and methods

Specimens. Primary osteosarcoma tissues were obtained from biopsies in 38 patients prior to the administration of...
Reagents. Mouse anti-TRAF4 antibody, mouse anti-B-cell lymphoma 2 (Bel-2), mouse anti-Bax, mouse anti-nuclear factor κB (NF-κB) were purchased from purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The antibodies against β-actin were from Good HER Biotech Inc. (Hangzhou, China). The horseradish peroxidase-conjugated goat anti-mouse secondary antibodies were obtained from Abgent Biotechnology Co., Ltd. (Suzhou, China).

Saos-2 cells culture. The human osteosarcoma cell line, sarcoma osteogenic (Saos-2), was purchased from the Shanghai Academy of Life Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (Pierce, Rockford, IL, USA) and supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine at 37°C under a humidified atmosphere of 5% CO₂.

TRAF4 knockdown by small interfering RNA (siRNA). Saos-2 cells were divided into three groups: Control (treated with Lipofectamine 2000 2000 only), vector (treated with Lipofectamine 2000 and control siRNA), and TRAF4-siRNA group (treated with Lipofectamine 2000 and TRAF4 siRNA). The Saos-2 cells were seeded into 6-well plates and incubated overnight, and were subsequently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, with slight modifications. The TRAF4 sense sequence was: 5'-CACCAGCACATTGGAAGCGA-3' (GeneChem Co., Ltd., Shanghai, China) (12). For every 1x10⁵ cells, 0.5 μg TRAF4 siRNA or control siRNA was diluted and mixed with 3 μl transfection reagent. After mixing and incubating 30 min, the transfection mixture was added to the cells. After 6 h, the medium was changed to growth medium (13).

Cell proliferation. The MTS assay was used to determine cell proliferation, as previously described with a few modifications (14). Approximately 1x10⁵ Saos-2 cells were seeded in each well of a 96-microwell plate. After incubation for 48 h, Cell Titer 96™ AQueous One Solution Reagent (Hitachi, Tokyo, Japan), which is composed of the novel tetrazolium compound MTS and an electron-coupling reagent, phenazine methosulfate (PES, a redox intermediary), was added to each well according to the manufacturer's instructions. After 3 h in culture, the cell viability was determined by measuring the absorbance at 490 nm using an ELISA microplate reader (Invitrogen).

In vivo tumor growth. Athymic nude mice (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were divided into two groups (n=3) and injected in the right flank with control or TRAF4-siRNA Saos-2 cells (3x10⁶). The (a) tumor diameter and the (b) shortest track were measured using a vernier caliper every five days. The tumor volume (in cubic millimeter) was calculated according to the formula V=ab²/2 (15). On day 20, the tumors were removed and weighed. All the studies were performed in compliance with the Guide for the Care and Use of Laboratory Animals of Henan Province, China.

Determination of cell cycle by flow cytometry. Cell cycle analyses were performed as previously described, with a few modifications (16). The Saos-2 cells were cultured in serum-free medium for 24 h to complete synchronization; subsequently, cells were cultured in complete medium for 24 h. The Saos-2 cells were digested by trypsin, washed in PBS, and fixed by 70% cold ethanol at -20°C. The next day, Saos-2 cells were washed with citrate phosphate buffer, followed by PBS, before the Saos-2 cells were incubated with RNase solution (100 μg/ml) for 30 min at 37°C. Subsequently, the Saos-2 cells were incubated in propidium iodide (PI) solution (100 μg/ml in PBS) at room temperature for 30 min. The cell cycle was detected by flow cytometry (Invitrogen). The experiment was repeated three times.

Determination of cell apoptosis by flow cytometry. Cell apoptosis analyses were performed as previously described, with a few modifications (17). Saos-2 cells were detached by trypsinization and washed twice in PBS, centrifuged at 1000 x g for 5 min and resuspended in 195 μl Annexin V-fluorescein isothiocyanate (FITC)-binding buffer. A volume of 5 μl Annexin V-FITC was added and the solution was mixed. Subsequently, Saos-2 cells were stained in the dark for 10 min at room temperature. Following this, Saos-2 cells were centrifuged at 1000 x g for 5 min and resuspended in 190 μl Annexin V-FITC-binding buffer. Finally, 10 μl propidium iodide-staining solution was added and mixed. Saos-2 cells were maintained on ice in the dark and immediately subjected to flow cytometric analysis. The data were analyzed using the CellQuest software (BD Biosciences, San Jose, CA, USA). The experiment was repeated three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The tissue sample was cryopreserved in liquid nitrogen. Total RNA was extracted from cultured cells and tissue samples using the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RT-qPCR was performed with a SuperRT One-Step RT-PCR kit (Jiangsu Jiangnan Biotechnology Co., Ltd., Jiangsu, China) according to the manufacturer's instructions. The primer sequences used for RT-qPCR were as follows: TRAF-4 forward, 5'-CTGGCTAA ACCACAGCAGTTC-3'; and reverse, 5'-TCGCTTCGAAT GTCTCG-3' (18). The 25 μl reaction mixtures contained 12.5 μl 2X One Step RT-qPCR buffer, 0.5 μM reverse primer, 0.5 μM forward primer, 0.9 μl enzyme, 90 ng RNA template and 0.5 μM probe. PCR conditions for the reverse transcription used to obtain cDNA were as follows: 45°C for 10 min, pre-denaturation at 95°C for 10 min and subsequently 45 cycles.
at 95°C for 15 sec and 60°C for 45 sec. This was performed using the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Relative quantification of gene expression was performed using the 2^\DeltaΔCt method and with β-actin mRNA as an internal control (19).

Western blot analysis. Western blot analyses were performed as previously described (20). Tissue sample or cells were homogenized in lysis buffer and centrifuged at 4°C for 30 min at 16,000 x g. The supernatant was collected and the same amount of protein from each sample was separated by sodium SDS-PAGE on a 12% gel and transferred to a nitrocellulose membrane. The following anti-TRAF4 or anti-β-actin was used and, subsequently, horseradish peroxidase-conjugated secondary antibodies were added (Invitrogen). The proteins were briefly incubated with an enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA) and visualized on X-ray film.

Statistical analysis. The SPSS 19.0 software (IBM Corp., Armonk, NY, USA) was used to analyze the associated data with a t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

TRAF4 mRNA and protein expression in osteosarcoma tissues and osteosarcoma cells. To verify the expression of TRAF4 in osteosarcoma, the protein and mRNA levels of TRAF4 in osteosarcoma tissue and in a human osteosarcoma cell line array were determined. As shown in Fig. 1A and B, the TRAF4 protein and mRNA expressions in osteosarcoma tissues were significantly higher compared to normal bone tissues (P<0.05). Consistent with observations from samples, the protein and mRNA expressions of TRAF4 were higher in the osteosarcoma cells, Saos-2, 143B and MG63, compared to the normal human osteoblastic cells, hFOB1.19 (Fig. IC and D). These results indicated that TRAF4 may be a critical molecule in osteosarcoma development.

Determination of transfection effects. On the basis of the observations, it was hypothesized that TRAF4 may affect the tumorigenic properties in osteosarcoma. Thus, the stable knock-down TRAF4 Saos-2 line was generated. To test the efficiency of TRAF4 transfection, western blotting and RT-qPCR were employed to determine the expression level of the protein and mRNA. As shown in Fig. 2, expression levels of the TRAF4 protein and mRNA were significantly decreased in the TRAF4 siRNA-transfected group. There was no significant difference in the expression level of TRAF4 protein and mRNA between the control and vector groups, which also demonstrated that...
Lipofectamine and control siRNA did not affect the expression of TRAF4 protein and mRNA. Collectively, these data also demonstrated that the TRAF4 protein and mRNA were inhibited in Saos-2 cells.

**Effects of TRAF4 on Saos-2 cells in vitro and in vivo.** The impact of TRAF4 on Saos-2 cell proliferation was determined via an MTS assay every 24 h after transfection, for up to 72 h. The results revealed that the viability of the cell was, to a certain extent, inhibited by TRAF4 in a time-dependent manner. As shown in Fig. 3, the TRAF4-transfected group grew more slowly compared to the control and vector groups. Furthermore, xenograft growth of Saos-2 cells in athymic nude mice was also attenuated following the knockdown of TRAF4 (Fig. 3B). These results demonstrated that the downregulation of TRAF4 inhibited the tumorigenic properties of Saos-2 cells.

**Effects of TRAF4 on cell cycle progression in Saos-2 cells.** Flow cytometry cell cycle analysis showed that the Saos-2 cells in the TRAF4-siRNA group had significantly more cells in the G1 phase and significantly fewer cells in the S and G2 phases compared to the control and vector groups (P<0.05) (Fig. 4). These results indicated that the downregulation of TRAF4 may affect the cell cycle distribution of Saos-2 cells.

**Effects of TRAF4 on apoptosis in Saos-2 cells.** Apoptosis of Saos-2 cells was detected via PI staining and the Annexin V method after 48 h of TRAF4 transfection, followed by flow cytometry. As shown in Fig. 5A, there was an extremely low level (10.6 and 10.5%) of Saos-2 cell apoptosis in the control and vector groups. Furthermore, the percentage of apoptotic cells did not differ significantly between the control and vector groups (P<0.05).

To investigate whether TRAF4 induces apoptosis in Saos-2 cells, the possible molecular mechanisms of TRAF4 associated with apoptosis were investigated. Thus, the expression of Bcl-2 and Bax proteins were measured in Saos-2 cells (Fig. 5C). The results showed that the expression of Bcl-2 decreased and the expression of Bax was simultaneously upregulated in the TRAF4-siRNA group compared to the control and vector groups (P<0.05). The apoptosis rate was significantly higher due to the upregulation of pro-apoptotic genes. These data revealed that TRAF4 plays a critical role in promoting apoptosis of Saos-2 cells.

**Effect of TRAF4 on NF-κB expression.** TRAF4 is an important regulatory factor for the expression of NF-κB (28). The expression of NF-κB was examined in the Sao-2 cell line by western blotting. Compared to the control and vector groups, the TRAF4-siRNA group had significantly reduced expression levels of NF-κB following TNF-α treatment (Fig. 6). These results indicate that TRAF4 may promote the activation of NF-κB induced by TNF-α in Saos-2 cells.

**Discussion**

Osteosarcoma is the most common primary malignant bone lesion and a highly malignant tumor with extensively destructive potential (21). Despite great advancements in the diagnosis and treatment of osteosarcoma thus far, substantial improvements in overall survival rate have been elusive and overall survival has remained relatively constant for over 2 decades (22). Thus, establishing the molecular mechanism of tumorigenesis and the progression of osteosarcoma and exploring the effective treatments for osteosarcoma is vital.

TRAF4 was initially identified as an overexpressed gene in human breast carcinoma (23). Overexpression of the TRAF4 protein is the consequence of its gene amplification in approximately one-quarter of human carcinomas (11). Several previous studies have hypothesized that TRAF4 may be involved in apoptosis. For instance, TRAF4 provides
resistance to an apoptotic stimulus in HEK293 cells (24). By contrast, Sax and El-Deiry (25) reported that TRAF4 may play a role in p53-mediated pro-apoptotic signaling in response to cellular stress. However, depending on the study the role of TRAF4 in apoptosis is controversial. Currently, TRAF4 has been found highly expressed in human carcinomas, but its biological functions in tumorigenesis remain unclear.

The results of the present study showed that TRAF4 is overexpressed in osteosarcoma tissues and cell lines. To study
the function of TRAF4, a stable TRAF4 knockdown Saos-2 cell line was generated and the effects of the downregulation of TRAF4 on the proliferation, cell cycle arrest and apoptosis ability in the Saos-2 cell line, as well as tumor development in a xenograft mouse model were examined. The study showed that the knockdown of TRAF4 inhibited the proliferation of the Saos-2 cell line in vitro and slowed down tumor growth in a xenograft mouse model. These results indicated that TRAF4 plays a crucial role in osteosarcoma carcinogenesis. However, TRAF4 as a p53-regulated pro-apoptotic gene in a p53 temperature-sensitive cell line VM10 induced apoptosis and inhibited colony formation (25). Simultaneously, TRAF4 knockdown may lead to cell cycle arrest in the G1 phase and promote Saos-2 cell apoptosis. Knockdown of TRAF4 can downregulate Bcl-2 expression and upregulate Bax expression. TRAF4 knockdown possibly induces Saos-2 cell apoptosis by inhibiting Bcl-2 and activating Bax expression. This apparent discrepancy indicates that the same gene can perform various biological functions that are dependent upon the form of the cell and the type of stimulation.

Previous studies have indicated that TRAF4 positively regulates GITR-induced NF-κB activation (26). NF-κB plays an essential role in preventing TNF-α-induced cell death (27). Therefore, the expression of NF-κB was examined. Following TNF-α treatment, NF-κB exhibited downregulation in Saos-2 cells from the TRAF4-siRNA group compared to the control and vector groups. These data indicate that the downregulation of TRAF4 may be associated with the downregulation of NF-κB, and that the downregulation of NF-κB may be further responsible for the induced apoptotic ability. However, its exact mechanism requires further research.

These results indicate that the knockdown of TRAF4 may play an important role in carcinogenesis and the development of osteosarcoma. Additional studies are required to investigate the specific mechanisms underlying the effects of TRAF4 in the tumorigenesis, xenograft tumor growth, cell cycle arrest and apoptosis of osteosarcoma. These results suggest that TRAF4 is a good molecular target for the prevention and treatment of osteosarcoma.

References


