TRAF4 Enhances Osteosarcoma Cell Proliferation and Invasion by Akt Signaling Pathway

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TRAF4, or tumor necrosis factor receptor-associated factor 4, is overexpressed in several cancers, suggesting a specific role in cancer progression. However, its functions in osteosarcoma are unclear. This study aimed to explore the expression of TRAF4 in osteosarcoma tissues and cells, the correlation of TRAF4 to clinical pathology of osteosarcoma, as well as the role and mechanism of TRAF4 in osteosarcoma metastasis. The protein expression levels of TRAF4 in osteosarcoma tissues and three osteosarcoma cell lines, MG-63, HOS, and U2OS, were assessed. Constructed TRAF4 overexpression vectors and established TRAF4 overexpression of the U2OS cell line. Cell proliferation, cell invasion, protein levels, and TRAF4 phosphorylations were assessed following TRAF4 transfection, as well as the effects of TRAF4 siRNA on cell proliferation and invasion. The results show that TRAF4 protein levels in osteosarcoma tissues were significantly higher than that in normal bone tissues. Importantly, an obvious upregulation of TRAF4 was found in carcinoma tissues from patients with lung metastasis compared with patients without lung metastasis. Consistently, a similar increase in TRAF4 mRNA and protein was also demonstrated in the osteosarcoma cell lines MG-63, HOS, and U2OS compared to normal bone cells, hFOB1.19. When TRAF4 was overexpressed in U2OS cells, cell proliferation was significantly enhanced, accompanied by an increase in Ki67 expression and colony formation. Compared with the control and vector-treated groups, TRAF4 transfection increased the invasion potential of U2OS cells (p < 0.05). Interestingly, TRAF4 transfection significantly enhanced the phosphorylation of Akt. After blocking Akt with its specific siRNA, TRAF4-induced cell proliferation and invasion were dramatically attenuated. In summary, our findings demonstrated that TRAF4 enhances osteosarcoma cell proliferation and invasion partially by the Akt pathway. This work suggests that TRAF4 might be an important target in osteosarcoma.

Key words: Osteosarcoma; TRAF4; Cell proliferation; Cell invasion; Akt signaling pathway

INTRODUCTION

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents, with a peak incidence at the age of 15–19 years (1,2). Osteosarcoma is strongly invasive and has a high tendency to metastasize early (3). Unfortunately, treating metastatic osteosarcoma is a challenge in oncology and is the major cause of treatment failure and death (4).

Tumor necrosis factor receptor-associated factors (TRAFs) were initially discovered as adaptor proteins that regulate cell life and death (5). So far, seven members (TRAF1-7) have been identified, and each member shares a common structural domain at the C-terminus (5,6). TRAFs form a complex network in which each TRAF is able to interact with and influence the function of many signaling molecules, either directly or through interactions with other TRAF family members (6). TRAF4 is a unique member of the TRAF protein family (7); it was initially identified in breast carcinoma (8). TRAF4 is required during embryogenesis in key biological processes including the formation of the trachea, the development of the axial skeleton, and the closure of the neural tube (9). TRAF4 deficiency leads to severe developmental alterations (10). TRAF4 is likely to be involved in signal transduction as it directly or indirectly mediates the downstream signal molecules. For instance, TRAF4 downregulates innate immune responses through its direct binding to NOD2 and inhibits NOD2-induced NF-κB activation (11) and restricts IL-17-mediated pathology and signaling processes (12). TRAF4 stimulates MEKK4 kinase activity to regulate JNK activation (13).

Recently, TRAF4 has been considered as an oncogene as it is overexpressed in a wide range of human malignancies, including breast cancer, lung cancer, colon adenocarcinomas, melanomas, neurogenic tumors, and lymphomas (14). Accumulating evidence indicates that TRAF4 plays critical role in breast cancer, such as antiapoptotic (15), and promotes cell migration (16). It has been reported
that TRAF4 interacts with PIPs and acts as a negative regulator of tight junctions and increases cell migration (17). Moreover, TRAF4 promotes TGF-β receptor signaling and drives breast cancer metastasis (18) and TRAF4 overexpression-induced degradation of p53 leading to poor prognosis in breast cancer patients (19). TRAF4 is also involved in maintaining tumorigenic properties in lung cancer; TRAF4 is required for EGF-induced Akt activation, and its deficiency markedly impairs the activity of Akt signaling and Akt-mediated lung cancer glycolysis (20). However, few studies have focused on the function of TRAF4 in osteosarcoma.

In the present study, we aimed to assess the expression of TRAF4 in osteosarcoma tissue and cell lines, that is, MG-63, HOS, and H2OS. The role and the underlying mechanism of TRAF4 in osteosarcoma cell proliferation and invasion were also explored.

**MATERIALS AND METHODS**

**Specimens**

Fresh osteosarcoma tissue specimens were collected from 62 patients who underwent osteosarcoma resection operation in our hospital between July 2012 and March 2014. In addition, 22 normal bone tissue specimens were collected. Patients with osteosarcoma were matched for gender, age, and lung metastasis (Table 1). The patients with osteosarcoma included 42 males and 20 females; 44 cases were younger than 23 years old, while 20 patients were older than 23 years old. Without any preoperative treatment, all 36 cases were pathologically diagnosed with osteosarcoma postoperatively. There were 41 cases with lung metastases, while 21 others had no metastases. The specimens were preserved in liquid nitrogen immediately for subsequent testing. The specimen collection procedure was performed with informed patient consent and the approval of the Medical Ethics Committee.

**Cell Lines and Cell Culture**

The immortalized human osteoblast cell line hFOB1.19 and the human osteosarcoma cell lines MG-63, HOS, and U2OS were purchased from American Type Culture Collection (ATCC). Three osteosarcoma cell lines (MG63, U2OS, and HOS) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin at 37°C in a humidified 5% CO2 incubator. The immortalized human osteoblast cell line (hFOB1.19) was grown in DMEM/Ham’s F-12 containing 10% and genetin (400 µg/ml) at a temperature of 34°C. These cells were maintained in the appropriate medium and passaged every 3 days.

**Construction of Expression Vectors and Cell Transfection**

Total RNA from U2OS cells were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), based on the manufacturer’s instructions. The cDNA was synthesized by reverse transcription of total RNA, using the PrimeScript® RT reagent kit (Takara, Dalian, China) with oligo-dT primers, according to the manufacturer’s protocol. Then, the open reading frame of TRAF4 cDNA was cloned and inserted into the pcDNA3.1 vector (Invitrogen) to construct the recombinant pcDNA3.1–TRAF4 expression vector. Control cells and pcDNA3.1 vector-transfected cells were also prepared. For cell transfection, cells were cultured to 60% confluence, and transfection was performed using the FuGENE HD transfection reagent (Roche, Indianapolis, IN, USA) method, as suggested by the manufacturer.

**siRNA Transfection**

Akt siRNA and control siRNA (con-siRNA) were purchased from Cell Signaling (Beverly, MA, USA). For transfection, 5 × 10^4 cells were seeded in each cell of 24-well microplates, grown for 24 h to reach 30–50% confluence, and then incubated with a mixture of siRNA and Lipofectamine 2000 reagent (Invitrogen) in 100 µl of serum-free OPTI-MEM according to the manufacturer’s instructions. The transfection efficiency was examined by real-time PCR and Western blotting.

**Western Blotting**

The proteins were extracted from tissue and cells using RIPA lysis buffer (Beyotime, Nantong, China). The protein

| Table 1. Correlation of TRAF4 Expression With Pathologic Parameter of Osteosarcoma |
|---------------------------------------------------------------|
| Pathologic Parameter | Case | Positive Rate (%) | \( \chi^2 \) | \( p \) |
|----------------------|------|-------------------|---------|------|
| Normal tissues       | 22   | 2 (9.10%)         | 31.466  | 0.000|
| Carcinoma tissues    | 62   | 48 (77.42%)       |         |      |
| Gender               |      |                   |         |      |
| Male                 | 42   | 32 (76.19%)       | 0.010   | 0.919|
| Female               | 20   | 15 (75.00%)       |         |      |
| Age                  |      |                   |         |      |
| <23                  | 44   | 34 (77.27%)       | 0.388   | 0.533|
| >23                  | 20   | 14 (70.00%)       |         |      |
| Lung metastasis      | 41   | 38 (92.69%)       | 11.038  | 0.001|
| Nonmetastasis        | 21   | 12 (57.14%)       |         |      |
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Concentration in the lysates was determined using a BCA protein assay kit (Beyotime, Nantong, China). Western blotting was performed according to standard methods. Proteins were separated by SDS-PAGE and then transferred onto nitrocellulose membranes (GE Healthcare, Germany) in a semidyed cellulose membrane apparatus using transfer buffer. Membranes were blocked with 10% defatted milk in PBS at 4°C overnight and incubated with the primary antibodies for 60 min. The primary antibodies are as follows: mouse anti-TRAF4 antibody (Santa Cruz, CA, USA), mouse anti-β-actin antibody (KangChen Bio-tech, Shanghai, China), rabbit anti-Akt antibody, and rabbit anti-phospho-Akt (p-Ser473) antibody (Cell Signaling Technology, Danvers, MA, USA). After washing with TBST buffer, the membranes were incubated for 1 h at room temperature with HRP-conjugated secondary antibodies (KangChen Bio-tech, Shanghai, China). ECL reagent (Beyotime) was used for detection. All experiments were performed in triplicate, and the results were normalized according to β-actin.

Real-Time Quantitative PCR

Total RNA and cDNA was obtained as described above. Real-time quantitative PCR (RT-PCR) reactions were performed on a Bio-Rad iQ5 real-time thermal cycler using SYBR® Premix Ex Taq™ II kit (Takara, Dalian, China). The PCR primers specific for the TRAF4 were designed as previously reported (21): 5'-AGGAG TTGCTTTTGACACCATC-3' (forward), 5'-CTTTGGA ATGGGCAAGGAC-3' (reverse); those for β-actin were 5'-CAACCTGATGTGAGGCTGGT-3' (forward) and 5'-ACTTTATTGGTCTCAAGTCAGTGTA CAG-3' (reverse). These primers were all synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The PCR procedure was as follows: polymerase activation for 30 s at 95°C; 40 cycles of amplification each consisting of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. All reactions were performed in triplicate, and the results were represented as the relative mRNA expression calculated according to the 2−ΔΔCT method (22).

MTT Assay

Cell proliferation was determined by the MTT assay. Cells were seeded into 96-well plates, washed twice with PBS, and 10 µl of MTT was added to each well. Then, the cells were incubated at 37°C for 2 h, and 100 µl DMSO was added to dissolve the formazan crystals. Absorbance was measured at 560 nm with a SpectraMax Paradigm Multi-Mode Reader (Molecular Devices, Austria).

Soft Agar Assay

The soft agar assay was performed according to a previous report (23). The control group, the vector-transfected group, and the TRAF4-transfected group cells were evenly seeded into 100-mm petri dishes with soft agar. The cells were allowed to grow for 3 weeks until colonies formed. Colonies were stained with crystal violet and counted under a microscope using OpenCFU 3.8-BETA software (24).

Cell Invasion Assay

The in vitro invasive properties of cells were evaluated using a Transwell chamber assay. According to the manufacturer’s protocol, chambers coated with Matrigel (50 µl per filter) (BD, USA) are incubated at 37°C for 30 min to create a Matrigel membrane, and divide the chamber into upper and lower compartments. Serum-free DMEM was added to the upper chambers, and DMEM containing 10% fetal calf serum was added to the lower chambers. The control, the vector transfected, and the TRAF4-transfected cells were cultured for 48 h and transferred to the upper chambers (1 × 10⁵ cells per Transwell). After 24 h of incubation, cells that had migrated to the lower surface were fixed in 90% alcohol and stained with 0.08% crystal violet.

Statistical Analysis

The SPSS version 19.0 software was used to analyze the related data with chi-square test or t test. The results were considered statistically significant if p < 0.05.

RESULTS

The Expression of TRAF4 in Human Osteosarcoma Tissue

To investigate whether TRAF4 was highly expressed in osteosarcoma tissue, we analyzed the protein level of TRAF4 in normal and different osteosarcoma patient tissues by Western blotting. The results showed that the TRAF4 expression level in osteosarcoma tissue was more than a 2.5-fold increase compared to normal bone tissue. Moreover, TRAF4 expression in osteosarcoma with lung metastatic tissue was more than twofold higher compared to tissue without lung metastases (Fig. 1). These results indicate that TRAF4 might be critical in osteosarcoma.

TRAF4 Is Upregulated in Human Osteosarcoma Cell Lines

To further verify the expression of TRAF4 in normal bone and osteosarcoma cells, we used RT-PCR to detect TRAF4 mRNA levels and Western blotting to analyze the protein levels in hFOB1.19, MG-63, HOS, and U2OS cell lines. RT-PCR analysis showed that the mRNA levels of TRAF4 in three osteosarcoma cell lines were higher than that in normal cells (Fig. 2A). Consistent with the results of mRNA levels, the protein levels in different osteosarcoma cells were higher than that in normal cells (Fig. 2B). It is noteworthy that both the mRNA and protein levels of TRAF4 in U2OS cells were higher than those in MG-63 and HOS cells.
Expression Levels of TRAF4 in TRAF4-Transfected U2OS Cells

On the basis of our observations that TRAF4 is more highly expressed in U2OS cells than other two osteosarcoma cell lines, MG-63 and HOS (Fig. 2), we generated TRAF4-overexpressing U2OS cells to investigate the function of TRAF4 in osteosarcoma cells. After TRAF4 transfection, the mRNA and protein levels of TRAF4 were significantly increased (Fig. 3). Therefore, these results confirm that TRF4-overexpressing cells were successfully established.

The Effect of TRAF4 Overexpression on Cell Growth

In order to investigate the role of TRAF4 in osteosarcoma cell growth, cell proliferation was assessed in TRAF4-transfected U2OS cells. The MTT analysis revealed that TRAF4-transfected cells possessed almost twofold higher cell proliferative ability than the other two groups ($p < 0.05$) (Fig. 4A). No differences were detected between the control and vector control cells. Ki67 is a nuclear protein that is expressed in proliferating cells (25) and may be required for maintaining cell proliferation. It had been used as a marker for cell proliferation in cancers
including osteosarcoma (26–28). We further analyzed the Ki67 protein expression levels and revealed that TRAF4 overexpression promoted the Ki67 protein expression (Fig. 4B). Additionally, colony formation was also detected by the soft agar assay. We found that U2OS cells transfected with TRAF4 yielded more colonies than the control and vector-transfected cells \( (p < 0.05) \) (Fig. 4C).

No significant difference was detected between the control and vector-transfected groups.

The Effect of TRAF4 Overexpression on Cell Invasion

To further assess the function of TRAF4 on osteosarcoma cells, U2OS cell invasion was detected after TRAF4 transfection. As shown in Figure 5, forced
TRAF4 expression notably upregulated the number of invading cells from 120 to 197 compared to the control group. No difference was found between the control and vector groups (p < 0.05). Hence, these results indicate that TRAF4 is a positive regulator of osteosarcoma cell invasion.

**TRAF4 Enhances Osteosarcoma Cell Viability and Invasion Partially by the Akt Signaling Pathway**

The Akt signaling plays a major role in tumorigenesis by regulating cell proliferation, colony formation, cell cycle, cell survival, cell invasion, and metabolism (23,29–31). The fact that TRAF4 enhanced osteosarcoma cell growth and cell invasion was demonstrated in our previous results. To further investigate the underlying mechanism involved in this process, we investigated Akt signaling. Interestingly, the TRAF4-transfected cells exhibited higher Akt phosphorylation levels than the other two groups (p < 0.05) (Fig. 6A). To further explore the function of Akt signaling in TRAF4-induced osteosarcoma cell proliferation and invasion, Akt siRNA was used to knock down Akt level in U2OS cells. After silencing Akt signaling with its specific siRNA, the effect of TRAF4 on enhanced cell viability (p < 0.05) (Fig. 6B) and invasion (p < 0.05) (Fig. 6C) was obviously decreased. These results indicate that TRAF4 enhances osteosarcoma cell proliferation and invasion partly through the Akt signaling pathway.

**DISCUSSION**

Osteosarcoma is a leading malignant tumor in childhood and adolescence. It is a highly aggressive tumor that metastasizes to the lung (4). TRAF4 was first identified overexpressed in breast cancer in 1995 (8). In the following years, accumulating evidence indicated that TRAF4 is highly expressed in a range of human malignancies and tumors (32). However, its role and mechanism in osteosarcoma remains undefined. In this study, our results showed that TRAF4 was highly expressed not only in osteosarcoma tissue but also in different osteosarcoma cell lines such as MG-63, HOS, and U2OS, indicating a potential critical role of TRAF4 in the development of osteosarcoma.

Cell proliferation in cancer is associated with cancer development and prognosis. In order to explore the role

![Figure 5. The effect of TRAF4 overexpression on cell invasion. Number of invaded cells in control cells, cells stably transfected with empty pcDNA3.1 vector, and cells stably transfected with pcDNA3.1–TRAF4 expression vector. *p < 0.05 compared with control.](image)

![Figure 6. TRAF4 enhance osteosarcoma cell viability and invasion partially by Akt signaling pathway. (A) The phosphorylated Akt (p-Akt) and total Akt protein levels in control cells, cells stably transfected with empty pcDNA3.1 vector, and cells stably transfected with pcDNA3.1–TRAF4 expression vector. H2OS cells stably transfected with control siRNA (con-siRNA), Akt siRNA, and pcDNA3.1–TRAF4 vector, MTT (B) and Transwell chamber (C) assay were performed. *p < 0.05.](image)
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...of TRAF4 in osteosarcoma cell proliferation, we generated TRAF4-overexpressing U2OS cells and assessed cell proliferation. The results showed that TRAF4 overexpression dramatically promoted cell proliferation. We also detected the expression of protein Ki67, which is a marker for proliferating cells (33). Consistent with speculation, TRAF4 promoted Ki67 protein expression. Moreover, we also found that TRAF4 overexpression promoted colony formation. Our data demonstrate that TRAF4 plays an important role in promoting osteosarcoma cell growth.

A common feature of osteosarcoma is that it metastasizes primarily to the lung (34). Invasion of cancer cells into surrounding tissue and the vasculature is an initial step in tumor metastasis (35). Thus, the invasion ability of tumor cells is closely related to tumor metastasis. Previous studies reported that TRAF4 promotes TGF-β receptor signaling and drives breast cancer metastasis (18); moreover, TRAF4 modulates tight junctions and promotes cell migration (17). Our results reveal that TRAF4 expression was higher in osteosarcoma tissue from a patient with lung metastases than in those without metastases, indicating that TRAF4 is a critical molecule during osteosarcoma metastasis. Further in vitro analysis confirmed that TRAF4 overexpression enhanced cell invasion, providing evidence of the positive role of TRAF4 in osteosarcoma metastasis.

The serine/threonine kinase Akt is a central node in cell signaling downstream of growth factors, cytokines, and other cellular stimuli (30). As a key molecule of this pathway, Akt is relevant to cancer cell survival, proliferation (increased cell number), and growth (increased cell size) (30,36,37). To further clarify the underlying mechanism involved in TRAF4-induced osteosarcoma cell proliferation and invasion, we detected Akt phosphorylation after TRAF4 transfection. As expected, TRAF4 upregulation promoted the activation of the Akt signaling pathway. When this signaling was blocked with Akt siRNA, cell proliferation induced by TRAF4 was decreased. It has been reported that Akt can promote cell invasion in prostate cancer (38) and by increased motility and metalloproteinase production in the HT1080 cell line (39). To further explore the mechanism of TRAF4 in promoting osteosarcoma cell invasion, we analyzed Akt function on TAFR4-induced cell invasion. The results show that Akt siRNA impaired the TAFR4-dependent enhancement of osteosarcoma cell invasion. Together, these results suggest that TRAF4 enhances osteosarcoma cell proliferation and invasion partially by the Akt signaling pathway.

In summary, TRAF4 was confirmed in this study to be overexpressed in human osteosarcoma. Furthermore, TRAF4 may facilitate osteosarcoma progression by enhancing osteosarcoma cell proliferation and invasion partially through the Akt signaling pathway. Our research suggests that TRAF4 plays a critical role in osteosarcoma and may serve as a potential target against osteosarcoma.

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