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Original Article

Expression and function of TAK1 in osteosarcoma tissue

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Abstract: Objective: To investigate the expression and function of transforming growth factor β-activated kinase 1 (TAK1) in osteosarcoma (OS) tissue. Methods: TAK1 protein expression in OS and adjacent normal tissues was detected by immunohistochemistry. The association of TAK1 expression with clinicopathological features and prognosis in OS patients was analyzed. In vitro cell culture experiments were performed using a selective TAK1 inhibitor, 5Z-7-oxozeaenol, to determine the effects of TAK1 on proliferation and apoptosis in human U2OS cells. Results: The positive expression rate of TAK1 was higher in OS tissue (73.6%) than in adjacent normal bone tissue (19.8%, $P < 0.05$). TAK1 expression was unrelated to age, tumor site, gender, or subtype of the OS patients ($P > 0.05$). However, TAK1 expression was closely associated with the presence or absence of OS metastasis and Enneking stage ($P < 0.05$). TAK1-positive patients had shorter 5-year survival times than TAK1-negative patients ($P < 0.05$). A mechanistic study revealed that the TAK1 inhibitor 5Z-7-oxozeaenol inhibited proliferation and promoted apoptosis in an OS cell line ($P < 0.05$). Western blotting analysis showed that 5Z-7-oxozeaenol suppressed p-TAK1 (Thr87), cyclin D1, and intranuclear p65 expression levels, whereas it increased cleaved-caspase 3 protein levels in OS cells ($P < 0.05$). Conclusion: TAK1 expression was significantly associated with clinical stage and prognosis in OS patients, and this protein may represent a new target for the treatment of OS.

Keywords: Osteosarcoma, TAK1

Introduction

Osteosarcoma (OS) is the most common type of malignant bone tumor, and it primarily occurs in adolescents, with a high mortality. However, the molecular mechanisms underlying OS development and progression have not been fully elucidated, leading to a lack of specific therapeutic clinical drugs [1]. Therefore, in-depth studies of the pathological mechanisms underlying OS development and progression could lead to the development of targeted therapeutic drugs, potentially improving the prognosis of OS patients. Recent studies have found that transforming growth factor β-activated kinase 1 (TAK1) plays an essential role in the development and progression of various tumor tissues, including esophageal cancer [2], breast cancer [3], ovarian cancer [4], liver cancer [5], pancreatic cancer [3], and colon cancer [6]. However, little is known about the expression and role of TAK1 in OS. In the present study, we first analyzed the expression and clinical significance of TAK1 in OS tissue. Then, we used in vitro cell culture experiments and a selective TAK1 inhibitor, 5Z-7-oxozeaenol (5Z70), to assess the role of TAK1 in OS development and progression. Our results reveal novel insights into the role of TAK1 in OS and point to new approaches for the treatment of this disease.

Materials and methods

General data

In total, tumor and peritumoral normal bone tissue samples were selected from 106 patients with OS. These patients underwent surgical treatment in our hospital between August 2000 and August 2010, and OS was confirmed by postoperative pathological diagnosis. This study was approved by the Hospital Ethics Committee. Each patient or guardian signed an informed consent form prior to surgery. The following information was collected: patient name, age, preoperative and postoper-

ative auxiliary examination results, pathological stage, and therapeutic conditions. The study excluded patients who had received radiotherapy or chemotherapy prior to surgery. Patients were followed up with every three months, either by telephone or hospital visit. The follow-up duration was five years.

Immunohistochemistry and hematoxylin & eosin staining

Surgical specimens of OS and adjacent normal tissues were fixed in 10% formalin and embedded in paraffin. Specimens were then sliced into 4-μm-thick sections using a microtome and baked in an oven at 60°C for 8 h prior to use. Immunohistochemical (IHC) detection was performed as follows: paraffin sections were deparaffinized and rehydrated. After washing with phosphate-buffered saline (PBS), specimen antigens were retrieved by treatment with an EDTA buffer at 100°C for 20 min, followed by natural cooling to room temperature. The samples were washed with distilled water, and peroxidase was added dropwise to the block solution. Specimens were incubated at room temperature, and after washing the specimens with PBS, non-immune animal serum was added dropwise. The specimens in solution were incubated at room temperature for 10 min and then decanted. Anti-TAK1 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:50 dilution) was added dropwise, and the specimens were incubated at room temperature for 60 min. PBS was substituted for the primary antibody as a negative control. After washing the specimens with PBS, a biotin-labeled goat anti-rabbit IgG secondary antibody (ZsBio, Beijing, China) was added, and the specimens were incubated at room temperature for 10 min. Following a PBS wash, a streptavidin-biotin-peroxidase solution was added dropwise, and the specimens were incubated at room temperature for 10 min. After washing the specimens with PBS, a colorimetric reaction was performed using the DAB chromogenic reagent. After washing the specimens with tap water, they were counter-stained with hematoxylin and then washed with tap water, dehydrated with alcohol, cleared with xylene, and mounted with neutral gum. Hematoxylin & eosin (HE) staining was performed according to standard procedures.

Three experienced pathologists independently interpreted the IHC results, as previously described [7]. Briefly, the double-blind method was used to count cells within 15 high-magnification (× 400) fields of view for each section specimen. The average number of TAK1-positive cells per 100 cells was defined as the positive rate for TAK1. Positive IHC data were interpreted using guidelines in the literature, which were based on two criteria. The first criterion was the intensity of positively stained cells: negative was scored as 0 points, pale yellow as 1 point, yellow as 2 points, and brown as 3 points (final interpretation: 0-2 points as negative and 3-7 points as positive). The second criterion was the percentage of positive cells: 0%-5% was scored as negative, 6%-25% as weakly positive, 26%-75% as moderately positive, and > 76% as strongly positive (final interpretation: weakly positive, moderately positive, and strongly positive were defined as a positive result).

Cell culturing and grouping

The human OS cell line U2OS was provided by the Cell Bank of Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences. After they were thawed, the cells were seeded into culture flasks and incubated at 37°C under 5% CO_2. Logarithmic phase U2OS cells were divided into four groups for the experiments: blank, control (solvent), low-dose 5Z7O treatment (500 nM), and high-dose 5Z7O treatment (1000 nM). Thirty-nine replicate wells were set for each group. 5Z7O was purchased from Sigma-Aldrich (St. Louis, MO, USA).

MTT cell proliferation assay

After 5Z7O treatment, culture plates were placed in a cell incubator at 37°C under 5% CO_2 for 24, 48, or 72 h. Four hours before the end of the incubation, 20 μL of an MTT solution was added to each well. At the end of the incubation period, the supernatant from each well was aspirated, and 150 μL of DMSO was added to each well. The plates were oscillated for 10 min, and the absorbance was measured at 570 nm (A570) using an ELISA analyzer. The results are expressed as the mean value from six replicate wells. The cell proliferation inhibition rate for each group was calculated as follows: inhibition rate = (1-OD of the experimental group/OD of the blank control) × 100%.

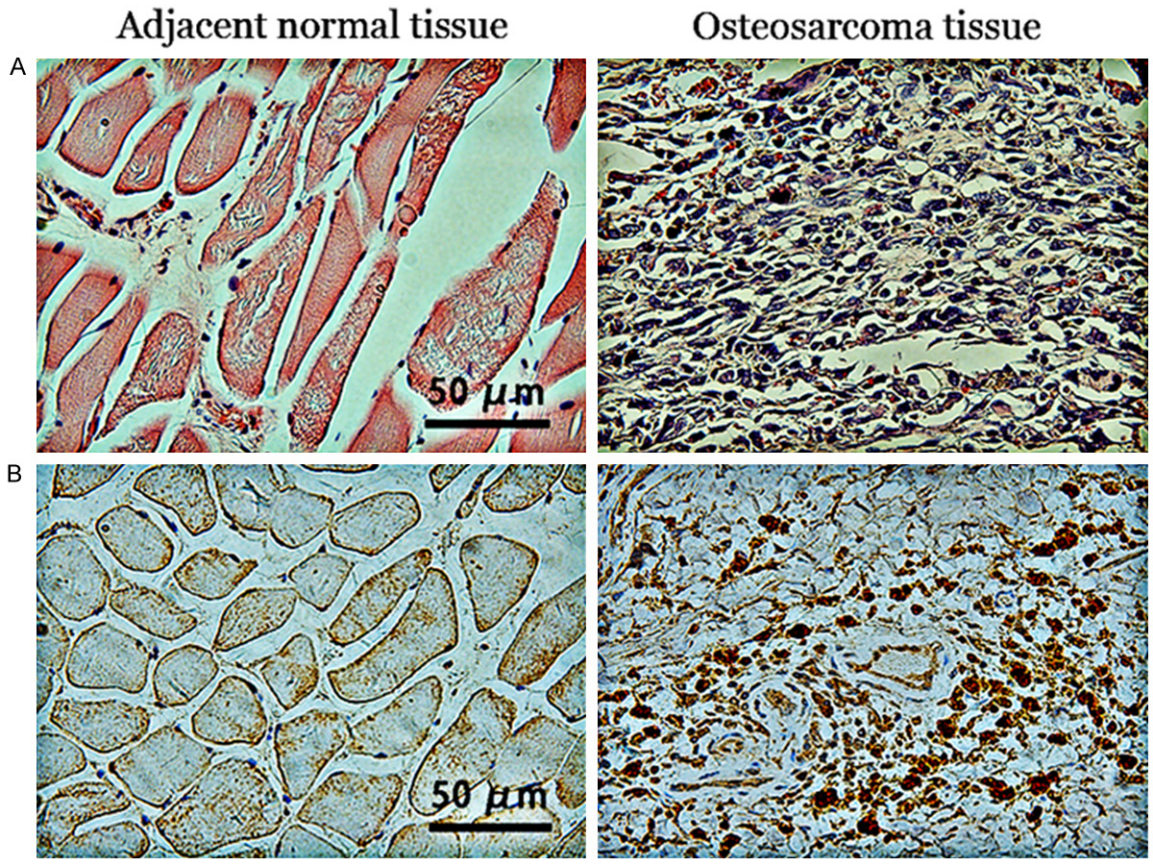

Figure 1. HE staining of and TAK1 expression in osteosarcoma and adjacent normal tissues (typical images).

Flow cytometry assay for measuring apoptosis

Apoptosis in human U2OS cells was detected using an Annexin V-FITC kit (Biovision, San Francisco, CA, USA), according to the manufacturer's instructions. Cells harvested at 24, 48, or 72 h were digested with trypsin and then resuspended in complete medium. After centrifugation, the supernatant was discarded. Following a PBS wash, 1 × binding buffer was added dropwise to the cells. Each sample was then treated with 5 μL Annexin V-FITC and 10 μL PI. The samples were thoroughly mixed before analysis.

Western blotting assay of cell proliferation

Human OS cells were harvested after 48 h of treatment. Protein was extracted using a cell total protein and nuclear protein extraction kit (Beyotime, Nantong, China), according to the manufacturer's instructions. The BCA method was used for protein quantitation. Thirty micrograms of each protein sample was separated by electrophoresis and transferred to a membrane. The membranes was blocked with blocking solution (Beyotime) for 1 h and then incubated with the diluted primary antibodies against β-actin, H3, p65, p-TAK1 (Thr187), and cleaved-caspase-3 (CST, Beverly, MA, USA) at 4°C overnight. After they were washed, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody diluted in 5% milk. After the membranes were washed, a luminescence solution was added dropwise to the membrane. Images were acquired using a gel imager, and gray scale data analysis was performed using Image J.

Statistical analysis

Data were processed using the SPSS 19.0 Statistics software program (IBM SPSS, Somers, NY, USA). Count data were analyzed using the χ^2 test. Means were compared among multiple groups using one-way ANOVA with post-hoc multiple comparisons by LSD-t test. The

Table 1. Association of TAK1 with osteosarcoma clinicopathological stage

Group	Cases	TAK1		P
		Positive cases	Negative cases	
Gender				
Male	69	49	20	0.41
Female	37	29	8	
Age (year)				
< 18	78	55	23	0.23
≥ 18	28	23	5	
Tumor site				
Femur	51	38	13	
Tibia	47	34	13	0.64
Others	10	6	4	
Subtype				
Osteoblastic	81	59	22	0.75
Others	25	19	6	
Metastasis				
Yes	58	53	5	< 0.001
No	48	25	23	
Enneking				
I	40	26	14	0.03
II	29	19	10	
III	37	33	4	

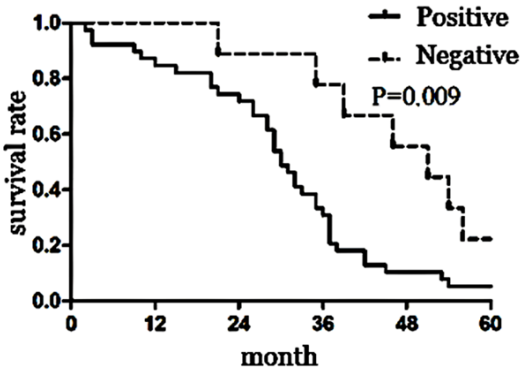

Figure 2. Association of TAK1 protein expression with 5-year survival rate in OS patients.

survival rate of OS patients with different characteristics was estimated using the Kaplan-Meier method, and univariate analysis was performed using the log-rank test. Multivariate analysis of prognostic factors for OS patients was conducted using the Cox proportional hazards regression model. Statistical tests were conducted with a significance level of $\alpha = 0.05$, and a value of $P < 0.05$ was considered significant.

Results

HE staining of OS and adjacent normal tissues

All clinical OS specimens used in the study were confirmed by postoperative pathological diagnosis. Typical HE staining results from the OS samples are shown in **Figure 1A**. The OS cells were interspersed with the trabecular or sheet-like structure of the osteoid tissue. Morphologically, the OS cells had clear spindle or polygonal shapes. The nuclei of these cells varied in shape, although they were large and intensely stained with prominent nucleoli.

Expression and significance of TAK1 protein in OS tissue

In the OS tissue, TAK1 staining was primarily cytoplasmic and appeared as pale yellow, yellow, or brown staining. TAK1-positive cells were markedly less common in the adjacent normal tissue (**Figure 1B**) compared with the OS tissue. The positive expression rate of TAK1 was 73.6% in the OS tissue, whereas it was only 19.8% in the adjacent normal bone tissue, which was significantly different.

Association of TAK1 protein expression with the pathological stage of OS

The positive expression rate of TAK1 was unrelated to the gender, age, tumor site, and subtype of the OS patients ($P > 0.05$). However, TAK1 expression was strongly associated with the presence or absence of metastasis and Enneking stage ($P < 0.05$, **Table 1**).

Association of TAK1 protein expression with clinical prognosis

Statistical analysis using the Kaplan-Meier method revealed that (**Figure 2**) among the TAK1-positive OS patients, 37 died within five years, with a median survival time of 30 months. By contrast, among the TAK1-negative OS patients, seven died within five years, with a median survival time of 51 months. Therefore, TAK1-negative OS patients had a significantly higher 5-year survival rate than TAK1-positive OS patients ($P = 0.009$).

Effects of the TAK1 inhibitor 5Z7O on OS cell proliferation and apoptosis

Tables 2 and **3** show that the 5Z7O-treated groups showed significantly lower cell prolifera-

Table 2. Cell proliferation in different groups

Group	n	24 h	48 h	72 h
Blank	10	0	0	0
Control	10	3.1±1.12	4.9±1.38	5.8±1.92
Low-dose 5Z-7-oxozeaenol	10	20±3.12*	24±3.79*	29±4.68*
High-dose 5Z-7-oxozeaenol	10	35±4.59#,*	39±6.78#,*	41±6.21#,*

*P < 0.05, compared with the control group; #P < 0.05, compared with the low-dose group.

Table 3. Cell apoptosis in different groups

Group	n	24 h	48 h	72 h
Blank	10	2.78±1.22	4.01±2.11	4.45±2.09
Control	10	3.2±1.12	4.1±2.01	4.5±1.96
Low-dose 5Z-7-oxozeaenol	10	31.1±4.56*	36.8±5.03*	41.1±6.12*
High-dose 5Z-7-oxozeaenol	10	41.6±7.12#,*	47.8±8.89#,*	54.1±9.81#,*

*P < 0.05, compared with the control group; #P < 0.05, compared with the low-dose group.

Table 4. Univariate analysis of prognostic factors in patients with osteosarcoma (n = 106)

Factor	Case	Median survival time (months)	χ^2	P
Gender				
Male	69	48.0	2.285	0.131
Female	37	54.5		
Age (years)				
< 60	78	52.0	5.219	0.022*
≥ 60	28	35.0		
Tumor site				
Femur	51	48.0	2.048	0.359
Tibia	47	46.0		
Other	10	53.5		
Subtype				
Osteoblastic	81	52.0	1.669	0.196
Other	25	48.0		
Metastasis				
Yes	58	22.5	9.591	0.002*
No	48	57.0		
Enneking stage				
I	40	52.0	8.252	0.016*
II	29	31.0		
III	37	23.5		
TAK1 expression				
Positive	78	30.0	6.606	0.009*
Negative	28	51.0		

*P < 0.05.

tion and higher apoptosis in OS cells, which occurred in a dose-dependent manner (P < 0.05).

Effects of the TAK1 inhibitor 5Z7O on p-TAK1 (Thr87), intranuclear p65, cyclin D1, and cleaved-caspase 3 expression

As the high-dose 5Z7O group showed the strongest effects, we used this dose in further experiments to determine the mechanisms underlying TAK1 activity in OS. **Table 4** and **Figures 3**, **4** show that relative to the control group, the 5Z7O group showed significantly lower intranuclear p65, p-TAK1 (Thr187), and cyclin D1 expression (P < 0.05). By contrast, cleaved-caspase 3 protein expression was strongly enhanced in the 5Z7O group compared with the control group (P < 0.05).

Univariate analysis of prognostic factors in OS patients

The results of univariate analysis by log-rank test showed significant differences in the survival of OS patients with regard to age, metastasis, Enneking stage, and TAK1 expression (P < 0.05). However, no significant differences were found in the survival of OS patients with regard to gender, tumor site, and subtype (P > 0.05) (**Table 4**).

Multivariate analysis of prognostic factors in OS patients

Multivariate Cox regression analysis revealed that metastasis and TAK1 expression were independent risk factors for a poor prognosis of OS patients (P < 0.05) (**Table 5**).

Discussion

Recent studies have observed abnormally high TAK1 expression in a variety of tumor tissues, and TAK1 has been shown to play an essential role in the development and progression of different tumors. Inhibiting the TAK1 activity can

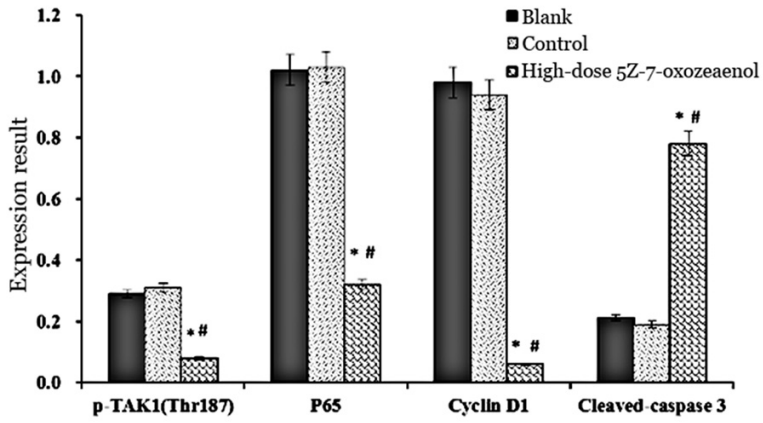

Figure 3. Cell apoptosis in the different groups. *P < 0.05, compared to the blank group; #P < 0.05, compared to the control group.

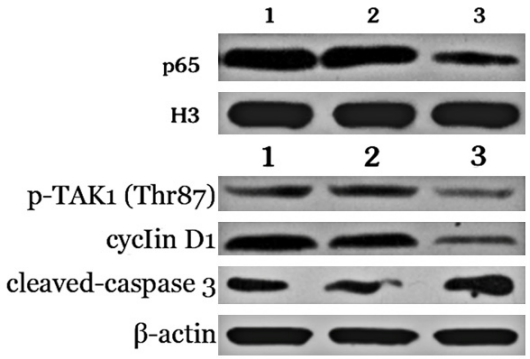

Figure 4. Western blotting analysis of P65, p-TAK1 (Thr187), cyclin D1, and cleaved-caspase 3 expression in the different treatment groups (representative images are presented).

significantly suppress proliferation and promote apoptosis in tumor cells, suggesting that TAK1 could represent a novel target for tumor treatment [8]. TAK1 is located at the intersection of multiple signal transduction pathways. For example, while the activation of TAK1 can trigger the MAPK pathway, it can also lead to the activation of IKK, which causes the degradation of IκBα; NF-κBp65, now freed from inhibition by IκBα, can move from the cytoplasm to the nucleus to bind to specific target gene sequences and regulate the transcription of relevant genes [9]. MAPK expression is significantly elevated in OS tissue, and inhibiting MAPK expression can markedly suppress proliferation and induce apoptosis in OS [10-12]. Similarly, NF-κB shows abnormal activation in OS tissue, and the inhibition of NF-κB activity can strongly suppress proliferation and facilitate apoptosis in OS [13]. These findings suggest that TAK1 plays an essential role in OS development and progression. In the present study, we first analyzed TAK1 expression in tumor tissue samples from clinical OS patients using IHC. The results clearly showed that TAK1 expression was significantly higher in the OS tissue samples than in the adjacent normal tissue samples. Further analysis found no link between TAK1 expression and the age, gender, or tumor site of OS patients. However, TAK1 was significantly associated with the presence or absence of metastasis and Enneking stage, suggesting that TAK1 may be important for tumor progression in OS patients. A Kaplan-Meier survival analysis showed that TAK1-positive patients had a significantly shorter 5-year survival time compared with TAK1-negative patients, again suggesting that TAK1 is a key factor affecting OS patient prognosis. Therefore, postoperative detection of TAK1 expression in OS tissue could have prognostic significance for OS patients.

Our results demonstrate that TAK1 expression is significantly higher in OS tissue and is associated with prognosis in OS patients. Thus, using in vitro cell culture experiments, we treated an OS cell line with a specific TAK1 inhibitor, 5Z7O, to further elucidate the role of TAK1 in OS cell proliferation and apoptosis. Numerous experiments have shown that 5Z7O can significantly inhibit proliferation and promote apoptosis in a variety of tumor cells [14]. The drug dose used in the present study was based on previous studies, and the results showed that 5Z7O significantly inhibited proliferation and promoted apoptosis of the OS cell line in a dose-dependent manner. Thr187 is a key site for TAK1 activation [15], and 5Z7O can strongly inhibit Thr187 phosphorylation, leading to TAK1 inhibition [16]. Indeed, we found that the 5Z7O group showed markedly lower p-TAK1 (Thr187), intranuclear p65, and cyclin D1 expression levels than the control group. By contrast, cleaved-caspase 3 protein expression was markedly increased in the 5Z7O group compared with the control group. Therefore, this may be the mech-

Table 5. Multivariate Cox regression analysis of prognostic factors for patients with osteosarcoma (n = 106)

Independent variable	Coefficient of regression	Standard error	Wald χ^2	*P* value	HR (95% CI)
Metastasis (reference group = no)	0.576	0.260	8.081	0.004*	1.78 (1.06-2.96)
TAK1 expression (reference group = negative)	0.812	0.341	14.972	< 0.001*	2.25 (1.15-4.39)

*P < 0.05.

anism through which 5Z70 inhibits proliferation and promotes apoptosis in OS cells. We do note that the present study shows only that the selective TAK1 inhibitor 5Z70 can suppress proliferation and enhance apoptosis in OS cells in an in vitro setting. Further experiments should be performed using in vivo animal experiments to determine the best methods for treating OS by targeting TAK1.

In summary, TAK1 expression was significantly elevated in OS tissue and was closely associated with clinical stage and prognosis in OS patients. We showed that a specific TAK1 inhibitor can inhibit proliferation and promote apoptosis in OS cells, suggesting that TAK1 may be a promising new target for the treatment of OS.

Disclosure of conflict of interest

None.

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