



Correlation between TRAIL and caspase-8 expression and their relationship with cell proliferation and apoptosis in human osteosarcoma

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ABSTRACT. Osteosarcoma is a common malignant bone tumor that mainly affects children and adolescents. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor superfamily. Caspase-8 appears in the upstream of apoptosis signaling pathway among caspases. We investigated TRAIL and caspase-8 levels in osteosarcoma patients to determine their correlation with cell proliferation and apoptosis. Osteosarcoma and osteochondroma patients receiving surgery in our hospital were selected. TRAIL and caspase-8 expression levels in tissue were determined by immunohistochemistry, and protein levels in cells were evaluated by western blotting. Human osteosarcoma cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The osteosarcoma

and osteochondroma cell cycles and apoptosis were investigated by flow cytometry. Correlation analysis was applied to TRAIL and caspase-8 levels during cell apoptosis. Positive TRAIL and caspase-8 expression rates in osteosarcoma tissue were significantly lower than in the controls ($P < 0.05$). TRAIL (0.114 ± 0.002) and caspase-8 (0.352 ± 0.124) levels in experimental cells were obviously lower than in the controls ($P < 0.05$). Osteosarcoma cells in the experimental group demonstrated higher proliferation and lower apoptosis at 24, 48, and 72 h ($P < 0.05$). The experimental cell number increased in the G1 stage and decreased in the S stage ($P < 0.05$). TRAIL and caspase-8 proteins showed positive correlation with apoptosis in osteosarcoma ($P < 0.05$). Human osteosarcoma presented reduced TRAIL and caspase-8 levels with enhanced cell proliferation and reduced apoptosis. TRAIL and caspase-8 expression levels were positively correlated with apoptosis in osteosarcoma.

Key words: TRAIL; Caspase-8; Osteosarcoma

INTRODUCTION

Osteosarcoma mostly appears in children and adolescents. It is a common malignant bone tumor accounting for 8.9% of all cancer deaths. Adjuvant and neoadjuvant chemotherapies for osteosarcoma have been extensively developed and applied in clinical settings in recent years, greatly increasing the survival rate. However, owing to the side effects and serious toxicity of chemotherapy drugs, many patients do not complete chemotherapy, which leads to tumor recurrence and metastasis. Amputation is necessary in many patients, and the prognosis is poor (Wafa and Grimer, 2006). Osteosarcoma cell proliferation is related to an imbalance in apoptosis, which results in malignant hyperplasia (Ottaviani and Jaffe, 2009). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a type-II transmembrane protein. It has a homologous trimer subunit structure at the extracellular domain C-terminal that can be hydrolyzed from the cell surface by a specific protease to form a soluble TRAIL monomer. TRAIL is widely expressed in the spleen, thymus, small intestine, colon, placenta, ovaries, and prostate, but not in the brain, liver, or testes. TRAIL is important because it participates in the induction of tumor cell apoptosis, although it has no effect on normal cells (Bellail et al., 2009). Caspases-8 is an important protease that is involved in regulating cell apoptosis (Shabanzadeh et al., 2015). Death receptor activation can trigger caspase-8, which directly activates effector caspase-3 through a FAS-related protein, thereby inducing apoptosis. The proteins belonging to the caspase family are the main executors of apoptosis; caspase-8 is an initiator caspase that plays an important role in death receptor-mediated cell apoptosis. In this study, we collected tissue for cell isolation from selected osteosarcoma patients. We determined TRAIL and caspase-8 levels, and evaluated cell proliferation and apoptosis for correlation analysis.

MATERIAL AND METHODS

Material

General information

We selected a total of 30 osteosarcoma patients who had received surgical treatment

between January 2014 and January 2015 at the affiliated hospital of Guizhou Medical University. They comprised 16 males and 14 females with a mean age of 51.2 ± 3.3 years (20-70 years). Another 30 osteochondroma patients who had received surgery in the corresponding period at the same hospital were enrolled as controls. They comprised 15 males and 15 females with a mean age of 49.7 ± 4.1 years (25-70 years). Surgical specimens from the tumor surfaces and section areas were diagnosed by two pathologists. No significant differences in age, height, or weight existed between the two groups ($P > 0.05$).

The inclusion criteria were: diagnosis by pathology; absence of connective tissue disease or immune system disease; absence of serious liver disease, renal disease, or autoimmune disease; normal blood routine, urine routine, liver function, and renal function; and no history of radiotherapy, chemotherapy, immunotherapy, freezing, or laser treatment.

The study protocol was approved by the Research Ethics Committee of the affiliated hospital of Guizhou Medical University, and all patients gave their informed consent before study commencement.

Reagents and instruments

Rabbit antihuman TRAIL and caspase-8 polyclonal antibodies, an SP general kit, a DAB kit, polylysine, citric acid antigen retrieval buffer, phosphate-buffered saline (PBS), hydrogen peroxide, xylene, anhydrous ethanol, paraffin wax, hematoxylin, and neutral balsam were obtained from ZSbio (Beijing, China). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, and fetal bovine serum (FBS) were obtained from Gibco. Trizol reagent was obtained from Invitrogen.

The following is a list of the instruments used in this study: a Benchtop (Formal 205, USA) inverted microscope (Olympus, Japan), a tissue-embedding machine (Sakura, Japan), a dehydrator (Tiyoda, Japan), an ultramicrotome (Leica, Germany), a thermostatic oscillator (Jinghong, China), electric HCB-Turn equipment (Shanghai Shengxin Scientific Instruments Co., Ltd., China), a computer image analysis system (Hewlett-Packard, USA), an incubator (Thermo Fisher Scientific, USA), a carbon dioxide incubator and a -80°C refrigerator (Sanyo Electric Co., Ltd., USA), and a low-temperature, high-speed centrifuge (Beckman Coulter Inc., USA).

Experimental methods

Cell isolation and cultivation

Osteosarcoma and osteochondroma tissue samples from patients were cut into pieces and soaked in a solution containing penicillin-streptomycin for 15 min under aseptic conditions. After washing with Hank's buffer, the tissue was digested with 1% collagenase for 20 min. The supernatant was removed and the reaction was stopped with DMEM and 10% FBS. The cells were suspended in DMEM containing 10% FBS at $10^4/\text{mL}$ and cultured in 5% CO_2 at 37°C . The cells were then passaged at 1:2 every other day, investigated using a microscope, and counted.

Immunohistochemistry

The tissue was fixed in formalin and subjected to dehydration, hyalinization, embedding, sectioning, dewaxing, dehydration, and high-pressure heat repair. Next, the tissue

was blocked with 3% hydrogen peroxide for 10 min, and sealed with goat serum at room temperature for 10 min. After incubation in 50 μ L primary antibody at room temperature for 1 h, the sections were further incubated in 50 μ L secondary antibody for 10 min. Finally, 50 μ L streptavidin-peroxidase was added to the sections for 10 min. After dying with hematoxylin and eosin (H&E) and differentiating with hydrochloric acid/alcohol solution, the sections were investigated using a microscope.

Western blotting

Osteosarcoma and osteochondroma cells in the logarithmic phase were collected to extract protein. The protein was separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After blocking at room temperature for 1 h, the membrane was incubated in primary antibody (1:200, β -actin 1:500) at 4°C overnight. The membrane was then washed with TBS-T solution and incubated in secondary antibody (1:2000) for 1 h. The membrane was developed by chemiluminescence and analyzed using the Quantity One software. The experiment was repeated twice.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Osteosarcoma and osteochondroma cells in the logarithmic phase were cultured in 2% FBS for 24 h, then cultured in DMEM with 10% FBS. After adding 20 μ L MTT solution (5 mg/mL), the cells were further cultured for 4 h. The plate was read at 570 nm after adding 150 μ L dimethyl sulfoxide for 10 min. The experiment was repeated twice.

Flow cytometry

An Annexin V-FITC detection kit was used to determine the rate of cell apoptosis. The cells were digested and collected at 10^6 /L. Next, 490 μ L buffer, 5 μ L Annexin V-FITC, and 5 μ L propidium iodide (PI) were added to the cells, which were kept in the dark for 10 min. The cell cycle and apoptosis rates were investigated by flow cytometry. The experiment was repeated twice.

Judgment standard

A known positive sample slice was used as the positive control, and PBS instead of the primary antibody was used as the negative control. The H&E-stained slices were examined.

A sample was judged to be positive for TRAIL and caspase-8 if brown or tan particles were present in the cell membrane or cytoplasm instead of the nucleus. Staining was graded as follows: negative (-), stained cell number $\leq 10\%$; weak positive (+), stained cell number 11-25%; positive (++) , stained cell number 26-50%; and strong positive (+++) , stained cell number $>50\%$. Five fields in each slice were randomly selected for image analysis and recording.

Statistical analysis

All data analysis was performed using the SPSS 17.0 software. The data are reported as means \pm standard deviation. Enumeration data were compared using the chi-square test,

whereas measurement data were compared using the *t*-test. The correlation test was performed using logistic analysis. $P < 0.05$ was considered statistically significant.

RESULTS

TRAIL and caspase-8 expression in tissue

Immunohistochemistry was performed to detect TRAIL and caspase-8 expression in osteosarcoma tissue, in para-carcinoma tissue, and in the controls. The results showed that TRAIL and caspase-8 proteins were present in the cytoplasm in osteosarcoma and osteochondroma tissue. TRAIL and caspase-8 positive expression rates in the osteosarcoma tissue, para-carcinoma tissue, and the controls were 7.5 and 13.3%, 13.3 and 16.7%, and 50 and 80%, respectively. TRAIL and caspase-8 positive expression rates in the osteosarcoma tissue were significantly lower than in the controls ($P < 0.05$) (Table 1 and Figure 1).

Table 1. TRAIL and caspase-8 expression in tissue detected by immunohistochemistry.

Group	Cases	Trail				Caspase-8			
		-	+--+	+++	Positive rate	-	+--+	+++	Positive rate
Experimental group									
Osteosarcoma tissue	30	27	3	0	7.5% ^{*#}	26	4	0	13.3% ^{*#}
Para-carcinoma tissue	30	26	4	0	13.3% ^{&}	25	5	0	16.7% ^{&}
Control	30	15	10	5	50%	6	3	21	80%

TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; ^{*} $P < 0.05$, compared with para-carcinoma tissue; [#] $P < 0.05$, compared with control; [&] $P < 0.05$, compared with control.

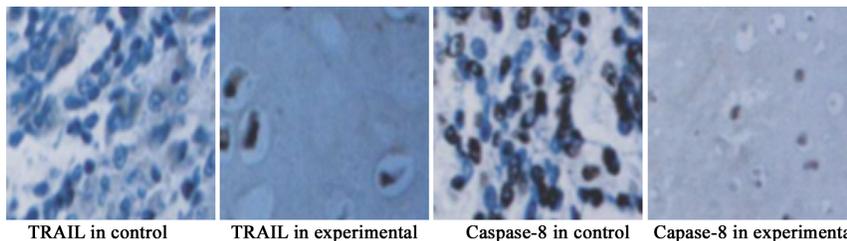


Figure 1. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and caspase-8 expression in tissue detected by immunohistochemistry.

TRAIL and caspase-8 protein levels in cells

Western blotting showed that TRAIL protein (0.114 ± 0.002) and caspase-8 protein (0.352 ± 0.124) levels in the experimental cells were obviously lower than in the controls ($P < 0.05$) (Table 2 and Figure 2).

Table 2. TRAIL and caspase-8 expression in cells.

Group	Trail	Caspase-8
Control	0.892 ± 0.011	0.787 ± 0.016
Experimental group	$0.114 \pm 0.002^*$	$0.252 \pm 0.124^*$

TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; ^{*} $P < 0.05$, compared with control.

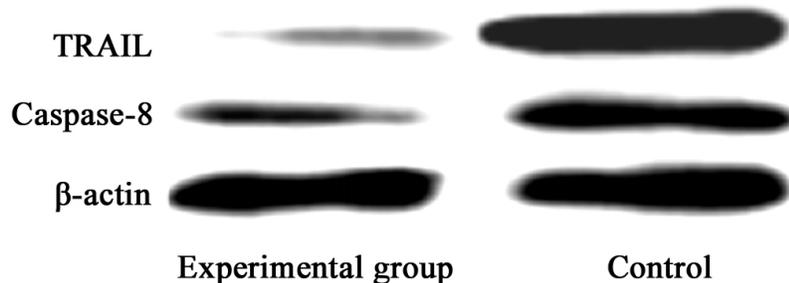


Figure 2. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and caspase-8 expression in cells.

Cell viability compared by MTT assay

Human osteosarcoma cell in the experimental group demonstrated higher proliferation at 24, 48, and 72 h compared with the controls ($P < 0.05$) (Table 3).

Table 3. Cell viability compared by MTT assay.

Group	24 h	48 h	72 h
Control	0.125 \pm 0.012	0.151 \pm 0.045	0.223 \pm 0.051
Experimental group	0.106 \pm 0.153*	0.122 \pm 0.028* [#]	0.996 \pm 0.026* ^{#&}

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; * $P < 0.05$, compared with control; [#] $P < 0.05$, compared with 24 h; [&] $P < 0.05$, compared with 48 h.

Human osteosarcoma and osteochondroma cell cycle and apoptosis rate comparison

An Annexin V-FITC detection kit was used to determine the human osteosarcoma and osteochondroma cell apoptosis rate. The results demonstrated that the cell apoptosis rate in the experimental group was obviously lower than in the control ($P < 0.05$) (Table 4).

Table 4. Cell apoptosis rate detected by Annexin V-FITC.

Group	Cell apoptosis rate (%)			
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 4
Control	2.4	23.5	37.9	43.2
Experimental	0.7*	2.2*	73.8*	10.3*

* $P < 0.05$, compared with control.

Cell cycle detection revealed that the experimental cell number increased in the G1 stage and decreased in the S stage compared with the control ($P < 0.05$) (Table 5 and Figure 3).

Table 5. Cell cycle changes detected by flow cytometry.

Group	Cell cycle			Apoptotic peak	Apoptosis index
	G1	S	G2/M		
Control	74.7 \pm 0.5	18.7 \pm 1.3	7.6 \pm 1.1	0	24.5 \pm 2.5
Experimental group	92.6 \pm 1.2*	7.6 \pm 0.8*	0.3 \pm 0.7*	1.1 \pm 0.5*	17.8 \pm 1.8*

* $P < 0.05$, compared with control.

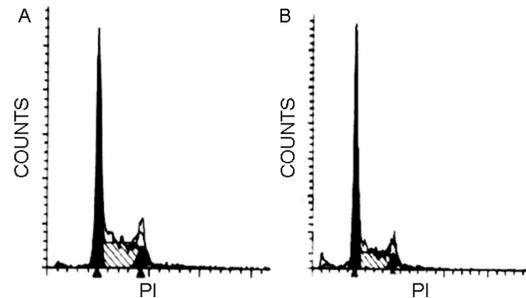


Figure 3. Cell cycle changes detected by flow cytometry. **A.** Experimental group; **B.** control group.

TRAIL and caspase-8 correlation with apoptosis rate in osteosarcoma

Correlation analysis showed that TRAIL was positively correlated with caspase-8 protein ($\gamma = 0.799$, $P < 0.01$). TRAIL and caspase-8 proteins showed positive correlation with apoptosis in osteosarcoma ($\gamma = 0.596$, $P < 0.01$; $\gamma = 0.547$, $P < 0.01$).

DISCUSSION

Tumor occurrence and development is related to unlimited and uncontrollable cell proliferation. The purpose of malignant tumor treatment is to selectively trigger tumor cell death (Hanahan and Weinberg, 2000). Because malignant tumors constantly strive to prevent cell death and survive, the process often leads to apoptosis resistance and chemotherapy resistance (Sharifi et al., 2015). TRAIL is an important member of the tumor necrosis factor superfamily that can induce tumor cell apoptosis. It has a lethal effect on tumor cells but has no significant toxic effect on normal cells (Zhao et al., 2012). Caspase-8 is a cysteine protease that is mainly expressed in bone and cartilage tissues and cells. It has a death effect domain that can bind with the Fas-associated protein with death domain (FADD), enabling its participation in cell apoptosis (Kavanagh et al., 2015; Polanski et al., 2015).

In this study, we selected osteosarcoma and osteochondroma patients that had received surgery in our hospital, and detected TRAIL and caspase-8 expression in their tissues. The study showed that TRAIL and caspase-8 are widely distributed in osteosarcoma, but locally distributed in osteochondroma. The TRAIL and caspase-8 positive expression rates in osteosarcoma tissue, in para-carcinoma tissue, and in the controls were 7.5 and 13.3%, 13.3 and 16.7%, and 50 and 80%, respectively. The TRAIL and caspase-8 positive expression rates in osteosarcoma tissue were significantly lower than in the controls. Western blotting revealed that TRAIL protein and caspase-8 protein levels in the experimental cells were obviously lower than in the controls. This suggests that TRAIL and caspase-8 expression decreased in osteosarcoma. Previous studies have demonstrated that TRAIL participates in multiple tumor cell apoptosis without affecting normal cells, including in the gastrointestinal tract, breast, and lung (Soria et al., 2010). Basic research has revealed that TRAIL targets five receptors, one of which is the death receptor expressed in tumor cells, and it can induce apoptosis by binding with that receptor. In contrast, when TRAIL binds to the decoy receptor that is mainly expressed in normal cells, apoptosis may not occur (Dorsey et al., 2009; Na et al., 2010; Piras et al., 2011). Because its effect on cells depends on the distribution of the receptors,

TRAIL has a unique antitumor function (Chen et al., 2010). Caspase enzymes are involved in cell apoptosis; they deliver apoptosis signals and regulate the effects of apoptosis. Caspase-8 can form a death-inducing signaling complex that plays an important role in apoptosis by reflecting the levels of trigger factors and cell apoptosis (Kang et al., 2015; Qi et al., 2015).

In this study, we isolated osteosarcoma and osteochondroma cells from tissue. The MTT assay revealed that human osteosarcoma cell proliferation in the experimental group increased at 24, 48, and 72 h. Annexin V-FITC detection showed that the cell apoptosis rate in the experimental group was obviously lower than in the controls. Flow cytometry demonstrated that the experimental cell number increased in the G1 stage and decreased in the S stage compared with the controls. The authors of a previous study reported that TRAIL protein is downregulated in osteosarcoma, indicating that deletion of the *TRAIL* gene may occur, leading to decreased receptor binding and promotion of osteosarcoma (Himeji et al., 2002). The destructive effect of TRAIL on CHP212 and SY5Y cells is reduced after adding caspase-8 inhibitor, suggesting that TRAIL-induced cell apoptosis is regulated by caspase-8 (Yang and Thiele, 2003). Caspase-8 downregulation prevents TRAIL-induced apoptosis in glioblastoma cell line SC189 (Woods et al., 2008; Bellail et al., 2010).

In this study, we examined the correlation between TRAIL and caspase-8 expression and their relationship with cell apoptosis in osteosarcoma patients. TRAIL was positively correlated with caspase-8 protein, and TRAIL and caspase-8 proteins showed positive correlation with apoptosis in osteosarcoma.

In brief, TRAIL and caspase-8 expression decreased in human osteosarcoma, resulting in increased cell proliferation, elevated numbers of cells in the G1 stage, decreased numbers of cells in the S stage, inhibition of transition from the G1 stage to the S stage, and reduced cell apoptosis. TRAIL and caspase-8 play important roles in the occurrence, development, and prognosis of osteosarcoma. Further in-depth investigation to confirm the specific signaling pathway mechanism between TRAIL and caspase-8 would provide a more detailed theoretical basis for the improvement of the early diagnosis and treatment of osteosarcoma.

Conflicts of interest

The authors declare no conflict of interest.

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