RESEARCH ARTICLE

Oxaliplatin Sensitizes OS Cells to TRAIL-induced Apoptosis Via Down-regulation of Mcl1

Tao Huang[1]*, Wei-Hua Gong[2], Xiu-Cheng Li[1], Chun-Ping Zou[1], Guang-Jian Jiang[2], Xu-Hui Li[2], Hao Qian[1]

Abstract

Purpose: To investigate the killing effect on OS cells of a combination of oxaliplatin and TRAIL and related molecular mechanisms. Methods: TRAIL and oxaliplatin were applied to OS732 cells singly or jointly and survival inhibition rates were measured by MTT assay, changes of cellular shape being assessed with inverted phase contrast and fluorescence microscopy. Apoptotic rates were analyzed by flow cytometry (FCM) and immunocytochemistry was used to examine Mcl1 expression of OS732 cells. Results: The survival inhibition rate of combined application of 100 μg/ml TRAIL and 1 μg/ml oxaliplatin on OS-732 cells was significantly higher than that of either agent singly ($p<0.01$). Changes of cellular shape and apoptotic rates also indicated apoptosis-inducing effects of combined application to be much stronger than those of individual application. Oxaliplatin had the effect of down-regulating Mcl1 expression and sensitizing OS cells to TRAIL-induced apoptosis. Conclusion: A combination of TRAIL and oxaliplatin exerts strong killing effects on OS-732 cells which might be related to down-regulation of Mcl1 expression.

Keywords: Osteosarcoma - TRAIL - oxaliplatin - apoptosis

Asian Pacific J Cancer Prev, **13**, 3477-3481

Introduction

Osteosarcoma is the most common primary tumor of bone, and early pulmonary metastasis is liable to occur with bad prognosis (Brunat-Mentigny and Kohler, 1993; Trieb and Kotz, 2001; Kubista et al., 2004; Yuan et al., 2011; Zhang et al., 2011). Although the chemotherapeutic agents like oxaliplatin has obvious killing effect on osteosarcoma cells, the resistance and toxic side effects after long-term application remain the main obstacle for clinical doctors (Bruland and Pihl, 1997; Arlt et al., 2011; Lin et al., 2011). TRAIL(TNF related apoptosis inducing ligand) is a member of TNF family which has drawn attention extensively due to its apoptosis-inducing effect on the tumor cell without doing harm to the normal cell (Van Valen et al., 2003; Locklin et al., 2007; Takeda et al., 2007). But its clinical application is also limited for the poor sensitivity of some tumor cells to TRAIL. It is a prospective anticancer method to combine TRAIL with chemotherapeutic drugs to increase the sensitivity of some tumor cells. Currently, there are some reports about the combination of cisplatin, carboplatin or other platinum chemotherapy drugs with TRAIL, however, there are only occasional reports about the lastest products of platinum drugs such as oxaliplatin combined with TRAIL. In addition, Little is known about the relationship between synergeistic effect of TRAIL combined with chemotherapeutic agents and Mcl1 expression .Therefore, in our research, TRAIL and oxaliplatin were used on the OS732 cells individually or jointly so as to explore whether oxaliplatin could sensitize OS cells to TRAIL-induced apoptosis. Moreover, we also examined whether the synergistic effect was related with down-regulating the expression of Mcl1 on tumor cells so as to analyze its anti-tumor mechanism and provide theoretic evidence for fighting against cancer.

Materials and Methods

The osteosarcoma OS732 cell line was bought from Beijing Jishuitan hospital.RPMI-1640 powder were from the Gibco company. Trypsin, MTT and Rnase A were from Huamei biological company. Multi-clone antibody of Mcl1 was purchased from Santa Cruz company. TRAIL was purchased from Peprotech company and oxaliplatin by Jiangsu Hengrui medicine company.

Cell culture and research methods

The osteosarcoma OS732 cell line was placed in the 1640 culture solution with 10% fetal bovine serum and cultured in incubator at 37 °C in a humidified 5% CO_2 atmosphere. The cells that entered the logarithmic growth period were selected for experiment. We selected the concentration of 10 ng/ml, 50 ng/ml, 100 ng/ml, 1000 ng/

*[1]Department of Orthopedics, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning, China, [2]Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, USA *For correspondence: huangtao@mail.cmu.edu.cn*

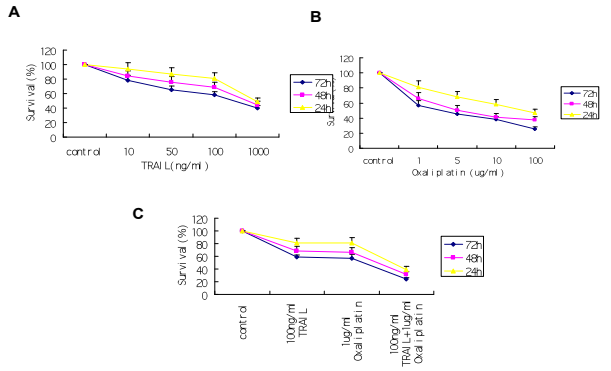

Figure 1. The Inhibition Effect on OS732 after 24h, 48h, 72h Measured by MTT. (A) The survival rate of OS732 with different concentration of TRAIL. (B) The survival rate of OS732 with different concentration of oxaliplatin. (C) The survival rate of OS732 with combination of 100 ng/ml TRAIL and 1 μg/ml oxaliplatin

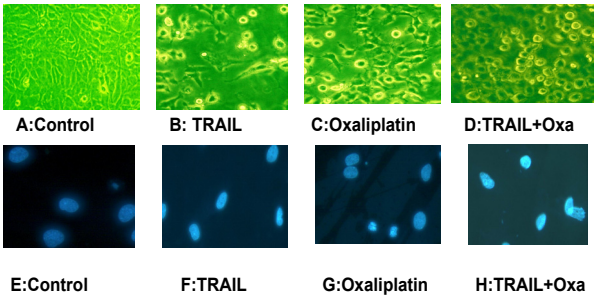

Figure 2. Morphological Changes and Fluorescent Staining of OS732 Cells After 72h with Different Drugs. (A)Morphological appearance of OS732 under inverted phase contrast microscope ×400. (B) Morphological changes of OS732 treated with 100ng /ml TRAIL under inverted phase contrast microscope ×400. (C) Morphological changes of OS732 treated with 1 μg/ml oxaliplatin under inverted phase contrast microscope ×400. (D) Morphological changes of OS732 treated with combination of 100 ng/ml TRAIL and1 μg/ml oxaliplatin under inverted phase contrast microscope ×400. (E) Fluorescent staining of OS732 cells under the fluorescence microscope ×400. (F) Fluorescent staining of OS732 cells treated with 100 ng/ml TRAIL under the fluorescence microscope ×400. (G) Fluorescent staining of OS732 cells treated with 1 μg/ml oxaliplatin under the fluorescence microscope ×400. (H) Fluorescent staining of OS732 cells treated with 100 ng/ml TRAIL and 1 μg/ml oxaliplatin under the fluorescence microscope ×400

ml for the TRAIL group , the concentration of 1 μg/ml, 5 μg/ml, 10 μg/ml, 100 μg/ml for the oxaliplatin group, and 100 ng/ml TRAIL with 1 ug/ml oxaliplatin for the combined group, meanwhile setting PBS blank control group. The experiment time were as follows : 24 h, 48 h, 72 h, after the treatment with different drugs.

Measurement of the survival rates of tumor cells with MTT method

5×10^5/ml Cells were seeded in a 96-well plate with 200 μl per well. After culturing for 24 hours, newly prepared 5 mg/ml MTT was added to each well at 37 °C for 4 hours. The supernatant was discarded, followed by dissolution in 150 μl DMSO. The absorption was measured at a wavelength of 540 nm. Survival rate of tumor cells (%) = experimental group A value/control group A value × 100%.

Observation of the morphology of apoptotic cells

The morphology, number, and adherence of tumor cells were directly observed by inverted phase contrast microscopy. A cover slide was placed in the 6-well plate with OS732 cells seeded, fixed for 10 min, and stained with 0.5 ml Hoechst33258 staining solution for 5 min. Then images were taken by fluorescence microscopy.

Measurement of the proportion of apoptotic cells with flow cytometer(FCM)

OS cells were collected, washed by PBS, centrifuged, and then 70% cold ethanol was added to fix over night. The cells were then centrifuged to remove the ethanol, washed twice with PBS, and stained with 100 μl PI at 4 °C for 1 hour in the dark. The fluorescence intensity was measured by FACScan flow cytometry. The wavelength of activated light was 488 nm, and the apoptotic rates were measured with Cell Quest analysis software.

Immunocytochemistry to detect Mcl1 expression of OS732 cells

2×10^5/ml digestive cells were placed in a 6-well culture plate with pre-treated cover slide in each well.cultured for 24 h and then supernatant were discarded, added with medicine, continue to culture for 24 hours ,meanwhile set blank control group,The cover slide were removed, fixed with acetone at 4 °C for 10 min and stained with SP method according to manual. The brown yellow cytoplasm indicated positive, and the expression intensity of Mcl1 was inversely determined by the average gray value obtained with image analysis system ,which means the more is the average gray value, the less is the Mcl1 level.

Statistic method

Experimental data, compared among different groups, were given as mean ± SD with ANOVA. T-tests were used for statistical analysis between the two groups. $P<0.05$ was considered statistically different. All results were analyzed with SPSS 13.0 for Windows.

Results

Changes of survival rates of tumor cells

When the concentration of TRAIL was 10 ng/ml, 50 ng/ml, 100 ng/ml and 1000 ng/ml, the cell growth was inhibited in a dose-dependent manner. There were significant differences between different groups ($P<0.05$) (Figure 1A). Similarly, when the concentration of oxaliplatin was 1μg/ml,5μg/ml,10μg/ml and 100μg/ml, the cell growth was also suppressed between different groups ($P<0.05$) (Figure 1B). With the combination of 100ng/ml TRAIL and 1μg/ml carboplatin, the survival rate was significantly lower ($P<0.01$) (Figure 1C), compared with the respective use of 100 ng/ml TRAIL or 1 μg/ml oxaliplatin, showing that the combined use of TRAIL and oxaliplatin may have stronger inhibition effect than the single agent. More importantly, we found that cell growth was inhibited in a time-denpendent manner since the survival rate of OS732 cells were the lowest at 72 h and the highest at 24 h.

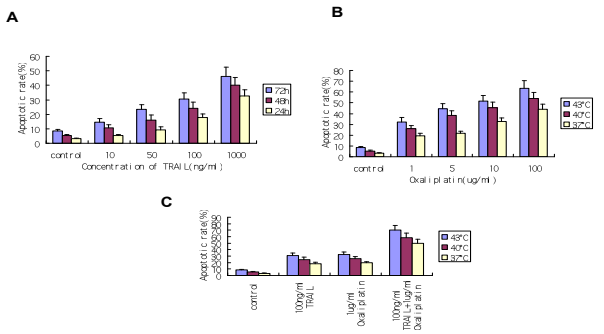

Figure 3. Apoptotic Effect of OS732 Cells After 24h, 48h, 72h Treated with Different Drugs. (A) Apoptotic rate of OS732 treated with different concentration of TRAIL. (B) Apoptotic rate of OS732 treated with different concentration of oxaliplatin. (C) Apoptotic rate of OS732 treated with combination of 100 ng/ml TRAIL and 1 μg/ml oxaliplatin

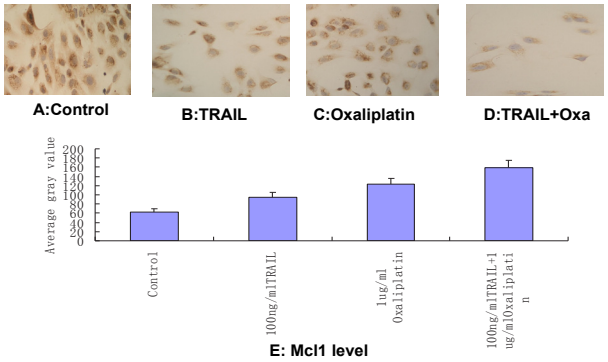

Figure 4. Mcl1Expression of OS732 Cells After 72h with Different Drugs. (A) Mcl1 expression of OS732 by immunocytochemistry ×400. (B) Mcl1 expression of OS732 treated with 100 ng/ml TRAIL by immunocytochemistry ×400. (C) Mcl1 expression of OS732 reated with 1 μg/ml oxaliplatin immunocytochemistry ×400. (D) Mcl1 expression of OS732 treated with combination of 100 ng/ml TRAIL and 1 μg/ml oxaliplatin by immunocytochemistry ×400. (E) Quantitative analysis of Mcl1 level by comparing the average gray value of different groups with Meta Morph automatic image analyzer

Morphological changes of apoptosis of OS-732 cells

Under the inverted phase contrast microscopy, The normal OS-732 cells were attached to the dish and were rhombus-like and angular (Figure 2A). With individual application of TRAIL (100 ng/ml) or oxaliplatin (1 μg/ml), only part of the cells became small and round (Figure 2B, 2C), However, with the combined application of TRAIL (100 ng/ml) and oxaliplatin (1 μg/ml), chromatin and cytoplasm were condensed, and many cells became nonadherent and suspended in the culture medium (Figure 2D). Under fluorescence microscopy, OS cells were lightly-stained (Figure 2E), whereas with individual application of TRAIL (100 ng /ml) or oxaliplatin (1 μg/ml), only a few cells showed condensed and flared fluorescence (Figure 2F, 2G). With the combination of TRAIL (100 ng/ml) and oxaliplatin (1 μg/ml), obvious condensed and flared fluorescence was observed, revealing the presence of many apoptotic cells (Figure 2H).

Comparison of apoptotic rates of OS-732 cells

After the treatment of the cells for 24h, 48h and 72h, with various concentration of TRAIL or oxaliplatin, the apoptotic rate increased in a dose-dependent manner. There were significant differences between different groups (P<0.05) (Figure 3A,3B). Furthermore, the apoptotic rate of the combination of TRAIL (100ng/ml) and oxaliplatin(1μg/ml) is significantly higher than the individual application of TRAIL (100ng/ml) or oxaliplatin(1μg/ml) , (P<0.01) (Figure 3C).

Mcl1 expression of OS732 by Immunocytochemistry

In the control group, deformed osteosarcoma cells are clearly visible and all Vision are covered with deepest staining (Figure 4A); We observed a lighter staining effects in OS732 cells with 100 ng/ml TRAIL (Figure 4B) or 1μg/ml oxaliplatin (Figure 4C) and the lightest staining in the cytoplasm of OS732 cells with the combination of 100 ng/ml TRAIL and 1 μg/ml oxaliplatin (Figure 4D). we further measured the Mcl1 level quantitatively with Meta Morph automatic image analyzer by comparing average gray value which is Inversely proportional to the Mcl1 expression (Figure 4E). All of the above results revealed Mcl1 expression of OS cells in the combination group are lower than those in the individual group.

Discussion

TRAIL was cloned firstly by Wiley from human cardiomuscular cDNA library in 1995 (Wiley et al., 1995). It is the signal molecule that mediates cellular apoptosis. The selective apoptosis-inducing effect of TRAIL on tumor cells is the significant feature that distinguishes it from conventional chemotherapeutic drugs (Rizza et al., 2011; Seol, 2011; Song et al., 2011; Kim et al., 2012). It is well known that TRAIL is a member of the tumor necrosis factor (TNF) gene superfamily and a promising anticancer cytokine because of its preferential toxicity in cancer cells. However, recent studies have reported that the potential application of TRAIL in cancer therapy is limited, as many cancer cells have been found to be resistant to TRAIL (Locklin et al., 2007), which has become the obstacle for anticancer application of TRAIL (Bouralexis et al., 2003; Locklin et al., 2007; Moon et al., 2011). Our research found that TRAIL may inhibit the proliferation of OS-732 cells, and after 72h of the treatment, when the concentration of TRAIL increased from 10 ng/ml to 1000 ng/ml , the survival rate decreased from 78.59% to 40.03% (Figure1A). Therefore the OS-732 cell is not very sensitive to TRAIL.

Oxaliplatin is a new anticancer agent with exact therapeutic effect (Clairambault et al., 2003; Geoerger et al., 2008; Beaty et al., 2010; Hartmann et al., 2011). However, it often causes toxicity and drug resistance after large-amount or long-term use. Our research demonstrated that oxaliplatin may induce the apoptosis of OS-732 cells. After 72 h of the treatment ,when the concentration of oxaliplatin was 1ug/ml, the survival rate was 56.92%. And the survival rate may just decrease to 25.47% after the concentration of oxaliplatin increased 100 times to 100 ug/ml (Figure1B). However, in the clinical treatment, it is obviously not feasible to increase the anti-tumor effects by magnifying 100 times of the oxaliplatin dose , which will definitely trigger serious toxicity and clinical side effects to osteosarcoma patients..

To our surprise, after 72h of combination of 100

ng/ml TRAIL and 1 ug/ml of oxaliplatin on OS732 cell line, the survival rate dropped sharply to 23.74%. Apparently, the synergistic-killing effect is much stronger than the individual use of 100 ng/ml TRAIL or 1ug/ml oxaliplatin ($P<0.01$, Figure1C). In addition, our research showed the killing effect by the application of TRAIL and oxaliplatin were fulfilled by inducing apoptosis of osteosarcoma cells which were revealed from the cell morphology (Figure2A-2D), cell apoptosis (Figure2E-2H) and FCM analysis (Figure3A-3B). All these results suggested us that oxaliplatin could enhance killing effect of osteosarcoma cells when combined with TRAIL, which means oxaliplatin could sensitize TRAIL to osteosarcoma cells in chemotherapy (El Fajoui et al., 2011). Since there were a great number of reports about side effects on digestive, nervous, blood and other body systems due to application of oxaliplatin during the regular chemotherapy of osteosarcoma patients, we suppose ,combination of TRAIL and oxaliplatin will avoid these side effects of chemotherapy.

Mcl-1 is an important antiapoptotic protein, belonging to the Bcl-2 protein family, which is highly expressed in a variety of malignant tumors playing an important role in the inhibition of apoptosis and promoting the formation of tumors (Rassidakis et al., 2002; Pritchard et al., 2008; Zhou et al., 2011; Gores and Kaufmann, 2012). Mcl-1 is distinct from other Bcl-2 family members in extremely unstable nature, which provides a mechanism for cells to switch into either survival or apoptotic mode in response to various stresses. Our research showed that the expression of Mcl1 was much lower when Oxaliplatin was combined with TRAIL which is consistent with previous research reported by Jani et al. (2010). Furthermore, individual application of TRAIL, to some extent, could also decrease the MCL-1 level. However, this kind of down-regulation of Mcl-1 expression was far less than those of individual application of oxaliplatin (Figure4). Therefore, we deduce the down-regulation of Mcl-1 in the combined group is manily accomplished by oxaliplatin instead of TRAIL, which means oxaliplatin sensitizes OS cells to TRAIL-induced apoptosis via down-regulation of Mcl-1. However, the specific pathway concerning about Mcl1-regulating apoptosis is still unclear, which needs further study.

Our research revealed that TRAIL and oxaliplatin may kill OS-732 cells synergeticly and effectively. In fact, different tumor cells are differently sensitive to Chemotherapy medicine. Therefore, it needs further research to study the exact effect of combined therapy of TRAIL and oxaliplatin on other tumor cell lines. In conclusion, Oxaliplatin facilitates TRAIL-induced apoptosis in osteosarcoma cells by down-regulation of Mcl1 expression. Since the combined application of TRAIL and oxaliplatin could reduce the use of dose and side effects in clinical application, we deduce, Oxaliplatin-induced sensitivity to TRAIL might be developed as an approach to cancer therapy.

Acknowledgements

This article was supported by Liaoning education foundation and checked by Dr Shavali Shaik from BIDMC, Harvard Medical School.

References

Arlt MJ, Walters DK, Banke IJ, et al (2011). The antineoplastic antibiotic taurolidine promotes lung and liver metastasis in two syngeneic osteosarcoma mouse models and exhibits severe liver toxicity. *Int J Cancer*, **131**, E804-12.

Beaty O, 3rd Berg S, Blaney S, et al (2010). A phase II trial and pharmacokinetic study of oxaliplatin in children with refractory solid tumors: a Children's Oncology Group study. *Pediatr Blood Cancer,* **55**, 440-5.

Bouralexis S, Findlay DM, Atkins GJ, et al (2003). Progressive resistance of BTK-143 osteosarcoma cells to Apo2L/TRAIL-induced apoptosis is mediated by acquisition of DcR2/TRAIL-R4 expression: resensitisation with chemotherapy. *Br J Cancer,* **89**, 206-14.

Bruland OS, and Pihl A (1997). On the current management of osteosarcoma. A critical evaluation and a proposal for a modified treatment strategy. *Eur J Cancer* **33**, 1725-31.

Brunat-Mentigny, M., and Kohler, R. (1993). [Osteosarcoma]. *Rev Prat,* **43**, 2197-203.

Clairambault J, Claude D, Filipski E, Granda T, Levi F (2003). [Toxicity and anti-tumour efficacy of oxaliplatin on Glasgow osteosarcoma induced in mice: a mathematical model]. *Pathol Biol (Paris),* **51**, 212-5.

El Fajoui Z, Toscano F, Jacquemin G et al (2011). Oxaliplatin sensitizes human colon cancer cells to TRAIL through JNK-dependent phosphorylation of Bcl-xL. *Gastroenterology,* **141**, 663-73.

Geoerger B, Doz F, Gentet JC et al (2008). Phase I study of weekly oxaliplatin in relapsed or refractory pediatric solid malignancies. *J Clin Oncol,* **26**, 4394-400.

Gores GJ, Kaufmann SH (2012). Selectively targeting Mcl-1 for the treatment of acute myelogenous leukemia and solid tumors. *Genes Dev,* **26**, 305-11.

Hartmann C, Weinel P, Schmid H, et al (2011). Oxaliplatin, irinotecan, and gemcitabine: a novel combination in the therapy of progressed, relapsed, or refractory tumors in children. *J Pediatr Hematol Oncol,* **33**, 344-349.

Jani TS, DeVecchio J, Mazumdar T, Agyeman A, Houghton JA (2010). Inhibition of NF-kappaB signaling by quinacrine is cytotoxic to human colon carcinoma cell lines and is synergistic in combination with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or oxaliplatin. *J Biol Chem,* **285**, 19162-72.

Kim J H, Park B, Gupta SC, et al (2012). Zyflamend sensitizes tumor cells to TRAIL-induced apoptosis through up-regulation of death receptors and down-regulation of survival proteins: role of ROS-dependent CCAAT/enhancer-binding protein-homologous protein pathway. *Antioxid Redox Signal,* **16**, 413-27.

Kubista B, Erovic BM, Klinger H, Sulzbacher I, Trieb K (2004). CD9 expression is not a prognostic factor in human osteosarcoma. *Cancer Lett,* **209**, 105-10.

Lin F, Wang Q, Yu W et al (2011). Clinical analysis of Chinese limb osteosarcoma patients treated by two combinations of methotrexate, cisplatin, doxorubicin and ifosfamide. *Asia Pac J Clin Oncol,* **7**, 270-5.

Locklin RM, Federici E, Espina B, et al (2007). Selective targeting of death receptor 5 circumvents resistance of MG-63 osteosarcoma cells to TRAIL-induced apoptosis. *Mol Cancer Ther,* **6**, 3219-28.

Moon MH, Jeong JK, Seo JS, et al (2011). Bisphosphonate enhances TRAIL sensitivity to human osteosarcoma cells via death receptor 5 upregulation. *Exp Mol Med,* **43**, 138-145.

Pritchard DM, Berry D, Przemeck SM, et al (2008). Gastrin increases mcl-1 expression in type I gastric carcinoid tumors and a gastric epithelial cell line that expresses the CCK-2 receptor. *Am J Physiol Gastrointest Liver Physiol,* **295**, G798-805.

Rassidakis GZ, Lai R, McDonnell TJ, et al (2002). Overexpression of Mcl-1 in anaplastic large cell lymphoma cell lines and tumors. *Am J Pathol* **160**, 2309-10.

Rizza SA, Challagundla KB, Natesampillai S, et al (2011). TRAIL dependent fratricidal killing of gp120 primed hepatocytes by HCV core expressing hepatocytes. *PLoS One,* **6**, e27171.

Seol DW (2011). p53-Independent up-regulation of a TRAIL receptor DR5 by proteasome inhibitors: a mechanism for proteasome inhibitor-enhanced TRAIL-induced apoptosis. *Biochem Biophys Res Commun,* **416**, 222-5.

Song S, Choi K, Ryu, SW, Kang SW, Choi C (2011). TRAIL promotes caspase-dependent pro-inflammatory responses via PKCdelta activation by vascular smooth muscle cells. *Cell Death Dis,* **2**, e223.

Takeda S, Iwai A, Nakashima M, et al (2007). LKB1 is crucial for TRAIL-mediated apoptosis induction in osteosarcoma. *Anticancer Res,* **27**, 761-8.

Trieb K, Kotz R (2001). Proteins expressed in osteosarcoma and serum levels as prognostic factors. *Int J Biochem Cell Biol,* **33**, 11-7.

Van Valen F, Fulda S, Schafer KL, et al (2003). Selective and nonselective toxicity of TRAIL/Apo2L combined with chemotherapy in human bone tumour cells vs. normal human cells. *Int J Cancer,* **107**, 929-40.

Wiley SR, Schooley K, Smolak PJ, et al (1995). Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity,* **3**, 673-82.

Yuan JM, Li XD, Liu ZY, et al (2011). Cisplatin induces apoptosis via upregulating Wrap53 in U-2OS osteosarcoma cells. *Asian Pac J Cancer Prev,* **12**, 3465-9.

Zhang G, Li M, Jin J, Bai Y, Yang C (2011). Knockdown of S100A4 decreases tumorigenesis and metastasis in osteosarcoma cells by repression of matrix metalloproteinase-9. *Asian Pac J Cancer Prev,* **12**, 2075-80.

Zhou W, Hu J, Tang H, et al (2011). Small interfering RNA targeting mcl-1 enhances proteasome inhibitor-induced apoptosis in various solid malignant tumors. *BMC Cancer,* **11**, 485.