Salvianolic acid B suppresses cell proliferation and induces apoptosis in osteosarcoma through p38-mediated reactive oxygen species generation

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Abstract. The present study aimed to investigate the potential anticancer effect and mechanisms of salvianolic acid B on osteosarcoma. Salvianolic acid B suppressed osteosarcoma cell proliferation and induced apoptosis in the osteosarcoma MG63 cell line, and activated the expressions of cleaved caspase-3, phosphorylated-tumor protein (p)38 mitogen-activated protein kinase (p-p38 MAPK) and phosphorylated-p53 (p-p53) proteins in the MG63 cells. Additionally, Salvianolic acid B also increased the level of reactive oxygen species (ROS) generation in the MG63 cells. The silencing of p38 expression inhibited the anticancer effect of salvianolic acid B on the levels of cell proliferation, p-p53 protein expression and ROS generation level in the MG63 cells. All these data supported the hypothesis that the anticancer effect of salvianolic acid B includes the suppression of cell proliferation and induces apoptosis in MG63 cells, and that p38 is important in the anticancer effect of salvianolic acid B on osteosarcoma cells due to the direct regulation of ROS generation. These data suggest that salvianolic acid B is important in the proliferation of osteosarcoma cells due to the direct regulation of p38-mediated ROS signaling.

Introduction

Osteosarcoma is a type of primary malignant tumor that exhibits the highest morbidity of all neoplasms in the human skeletal system, and often presents in the metaphysis of the long tubal bones (1). Osteosarcoma often affects young people between the age of 20 and 30 years old. The mortality rate is high (2) and ~20% of patients exhibit pulmonary metastasis prior to diagnosis. Subsequent to diagnosis, the majority of patients succumb to the disease within 2 years (2). At present, there are no effective therapeutic treatments for early osteosarcoma (3). Therefore, it is important to investigate the causes of osteosarcoma occurrence, development and invasion, the mechanisms of osteosarcoma oncogenesis, and to identify effective diagnostic and therapeutic techniques. The development of gene therapy has created novel research targets in oncotherapy, including the identification of a target gene (4).

Mitogen-activated protein kinase (MAPK) is the one of the most important types of signal conduction pathway in humans, and interacts with multiple signaling pathways (5). The tumor protein (p)38 MAPK pathway is activated through phosphotyrosine and threonine and inflammatory and growth factors, and activates downstream transcription factors on target genes, increases the initiation of cancer cell development, promotes protein synthesis, regulates cell surface receptors and regulates the invasion and transfer of tumor cells (6).

Reactive oxygen species (ROS) are secondary products in the process of aerobic metabolism and include oxygen ions, peroxide and oxygen radical molecules (7). The increase in the level of intracellular ROS may promote cellular proliferation and differentiation to some extent. However, excessive levels of ROS results in damage to lipids, proteins and DNA, destroying numerous normal cell signaling pathways, inducing apoptosis and autophagic death, and ultimately results in cell death (8). Notably, previous research demonstrates that the base value of ROS in lung cancer cells is higher than in normal cells, and may be associated with oncogene activation, high metabolic status and disordered mitochondrial functions (9). ROS in splenoma cells are easily induced by external factors, such as inflammation, or bacterial infection, resulting in cellular damage (9). In addition, ROS affects multiple other cell signaling pathways, such as MAPK (10). p38MAPK is one of the important members of the MAPK family, and participates in numerous important cell events subsequent to activation by extracellular signals, such as cell proliferation, differentiation and transfer, including apoptosis and autophagy (11). Therefore, the p38MAPK pathway may be a worthwhile target in tumor cells.

Water-soluble components in Danshen serve a role in cardiovascular disease and exhibit antineoplastic effects, as

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they reduce blood pressure, provide anti-thrombotic activity, expand the blood vessels, increase capillary permeability (12). Clinically, injections of Danshen may expand blood vessels, increase coronary artery blood flow and promote the recovery of ischemic or damaged myocardium, and may treat multiple types of cardiovascular disease (13). In terms of antineoplastic research, the water-soluble components of Danshen may inhibit the growth of multiple types of tumor, promote tumor cell apoptosis and inhibit tumor angiogenesis (14). Salvianolic acid B, as illustrated in Fig. 1, is a low-polymer compound with a relative molecular weight of 718.59 and comprises 1 molecule of caffeic acid and 3 molecules of danshensu, with a molecular formula of $C_{36}H_{36}0_{16}$ (15). Salvianolic acid B possess anti-oxidative and anti-inflammation effects (16). At present, salvianolic acid B has been demonstrated to exhibit the strongest pharmacological activity in Danshen water-soluble substances (14). Recently, salvianolic acid B was the focus of a study investigating myocardial regeneration, angiogenesis, reversion ventricular remodeling and ischemic peripheral vascular disease (17). However, the molecular mechanism underlying the salvianolic acid B-induced anticancer effect on osteosarcoma is not fully understood, and the effect of salvianolic acid B on osteosarcoma cells has not previously been determined. Based on these data, the present study examined whether salvianolic acid B suppresses cell proliferation and induces apoptosis of osteosarcoma through p38-mediated ROS generation.

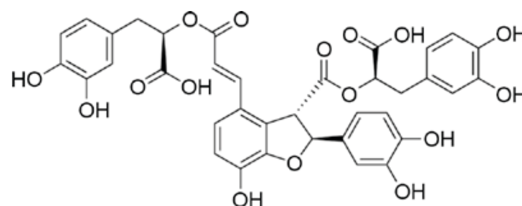

Figure 1. Chemical structure of salvianolic acid B.

Materials and methods

Cell culture. The osteosarcoma MG63 cell lines were purchased from Gansu University of Chinese Medicine (Lanzhou, China) and cultured in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences), 100 mg/ml streptomycin (Hyclone; GE Healthcare Life Sciences) and 100 IU/ml penicillin (Hyclone; GE Healthcare Life Sciences) in 5% CO_2 at 37˚C.

Small interfering (si)RNA and transfection. Si-p38 was as follows: Forward, 5'-AUGAAUGAUGGACUGAAAUGGUCUG-3', reverse, 5'-CAGACCAUUUCAGUCCAUCAUUCAU-3' (Sangon Biotech Co., Ltd, Shanghai, China). The cells were transfected with Si-p38 siRNA (100 nM) or control (100 nM) together with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 24 h.

Cell viability assay. The cells were plated in 96-well microplates and cultured with 1, 10, 50 and 100 μM salvianolic acid B for 24 h or with 50 μM salvianolic acid B for 0, 12, 24 and 48 h. The cellular viability was assessed by MTT assay. A total of 20 μl of 5 mg/ml MTT was added to each culture well, and the cells were incubated for 4 h at 37˚C. DMSO was then added into each well for 20 min at 37˚C and the cell viability was quantified at 490 nm using an ELISA reader (Infinite® 200 PRO; Tecan Schweiz, Männedorf, Switzerland). The control group was treated with DMSO alone.

Flow cytometry. The cells were plated in 6-well microplates and cultured with 50 μM salvianolic acid B or Si-p38 siRNA for 24 h. The cells were resuspended in binding buffer and stained with 10 μl Annexin V-fluorescein isothiocyanate (Beyotime Institute of Biotechnology, Haimen, China) for 30 min in the dark. The cells were then stained with 10 μl propidium iodide (Beyotime Institute of Biotechnology) for 5 min in the dark. Cell apoptosis was immediately detected using an EPICS® ALTRA™ flow cytometer (Beckman Coulter, Inc., Brea, CA, USA).

Western blot analysis. The cells were plated in 6-well microplates and cultured with 50 μM salvianolic acid B or Si-p38 siRNA for 24 h at 37˚C. The cells or tissues were harvested and lysed using ice-cold radioimmunoprecipitation analysis buffer (Beyotime Institute of Biotechnology). The proteins in the supernatants were collected subsequent to centrifugation at 20,000 x g for 10 min at 4˚C and quantified with a bicinchoninic acid assay protein assay kit (Beyotime Institute of Biotechnology). A total of 50 μg protein was separated using 10% SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, Chalfont, UK). The membranes were blocked with 5% nonfat milk for 1 h at 37˚C and incubated with anti-cleaved caspase-3 (Asp175; cat. no., 9579; dilution, 1:3,000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phosphorylated-p38 mitogen-activated protein kinase (p-p38 MAPK; cat. no., 9211; dilution, 1:4,000; Cell Signaling Technology, Inc.) anti-phosphorylated-p53 (cat. no., 2527, ; dilution, 1:2,000, Cell Signaling Technology, Inc.) and anti-GAPDH (cat. no., 5014; dilution, 1:2,000, Cell Signaling Technology, Inc.) at 4˚C overnight. The membranes were incubated with horseradish peroxidase-linked goat anti-rabbit IgG secondary antibodies (cat. no., ab6721; dilution, 1:10,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The bands were visualized using the enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and quantified using the Filmentwickler CP1000 Processor (AGFA, Mortsel, Belgium).

Measurement of ROS production. The cells were plated into 6-well microplates and cultured with 50 μM salvianolic Acid B or Si-p38 siRNA for 24 h. The cells incubated with dichlorofluorescein diacetate for 1 h at 37˚C in the dark and washed with PBS. ROS production was detected using the fluorescence intensity of 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) using an EPICS® ALTRA™ flow cytometer (Beckman Coulter, Inc.).

Statistical analysis. SPSS 19.0 (IBM SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis. Data are presented as the mean ± standard deviation (n=3). Statistical evaluation

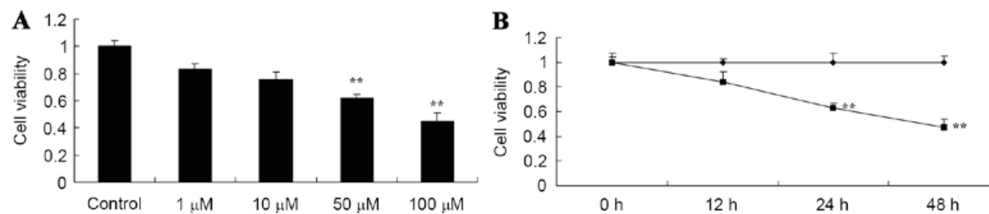

Figure 2. Salvianolic acid B suppresses cell proliferation of osteosarcoma MG63 cell line. (A) MG63 cell were treated with 1, 10, 50, and 100 μM salvianolic acid B for 24 h. (B) 50 μM salvianolic acid B for 0, 12, 24 and 48 h vs. control. Control, control group, 0 μM salvianolic acid B, top ine on figure. **P<0.01 vs. control group.

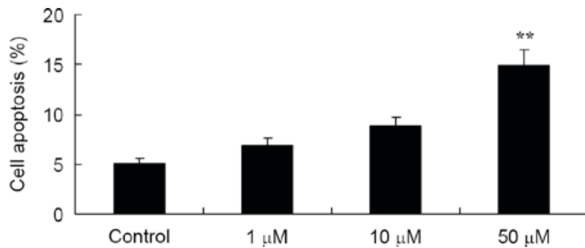

Figure 3. Salvianolic acid B induces apoptosis in osteosarcoma MG63 cell line. Control, control group. **P<0.01 vs. control group.

of the data was performed by one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Salvianolic acid B suppresses cell proliferation in the osteosarcoma MG63 cell line. To investigate the anticancer effect of salvianolic acid B on osteosarcoma MG63 cells, the cells were treated with 1, 10, 50 and 100 μM salvianolic acid B for 24 h or with 50 μM salvianolic acid B for 0, 12, 24 and 48 h and cell viability was measured by MTT assay. The cell proliferation of osteosarcoma MG63 cell was suppressed by treatment with salvianolic acid B in dose-dependent manner compared with the control cells, as illustrated in Fig. 2A. Subsequent to treatment with 50 μM salvianolic acid B for 12, 24 and 48 h, the cell proliferation of the osteosarcoma MG63 cells was suppressed in a time-dependent manner, as demonstrated in Fig. 2B.

Salvianolic acid B induces the apoptosis of osteosarcoma MG63 cells. To determine whether the growth inhibition effect of salvianolic acid B on the osteosarcoma MG63 cells was mediated through the induction of apoptosis, flow cytometry was used to examine levels of apoptotic cell death in the MG63 cells. As demonstrated in Fig. 3, the rate of apoptotic cells significantly increased with the treatment of 50 μM salvianolic acid B compared with the control cells.

Salvianolic acid B activates cleaved caspase 3 protein expression in the osteosarcoma MG63 cells. To determine the activation of apoptosis of salvianolic acid B, a western blot analysis was used to examine Asp175 expression levels in the MG63 cells. Treatment with 50 μM salvianolic acid B significantly enhanced the levels of Asp175 protein expression in the MG63 cells compared with the control cells, as illustrated in Fig. 4.

Salvianolic acid B activates p-p38 protein expression in the osteosarcoma MG63 cells. To determine the role of p-p38 in salvianolic acid B-induced apoptosis, the level of p-p38 protein expression was measured using a western blot analysis. The results from the western blot analysis revealed that treatment with 50 μM salvianolic acid B significantly increased the level of p-p38 protein in MG63 cells compared with the control, as demonstrated in Fig. 5.

Salvianolic acid B activated p-p53 protein expression in the osteosarcoma MG63 cells. To quantify the activation of p-p53 protein expression in the MG63 cells exposed to salvianolic acid B, western blotting was used to detect the expression of p-p53 protein in the MG63 cells. The results demonstrated that salvianolic acid B increased the expression of p-p53 in a dose-dependent manner, as illustrated in Fig. 6. in particular, 50 μM salvianolic acid B significantly increased the levels of p-p53 protein in the MG63 cells compared with the control cells, as demonstrated in Fig. 6.

Salvianolic acid B-activated ROS generation in the osteosarcoma MG63 cells. To confirm the antitumor effect of salvianolic acid B on ROS generation in the osteosarcoma MG63 cell line, ROS generation was measured using H2DCFDA. As demonstrated in Fig. 7, there was a significant increase in ROS generation levels in the 50 μM salvianolic acid B treated group compared with the control group.

Salvianolic acid B suppresses cell proliferation in osteosarcoma MG63 cells subsequent to the knockdown of p38. To confirm that salvianolic acid B acts via p38 to mediate its effects on osteosarcoma *in vitro*, si-p38RNAs were transfected into MG63 cells to suppress the protein expression of p38 and influence the anticancer effect of salvianolic acid B on osteosarcoma. As illustrated in Fig. 8A, si-p38 significantly suppressed the p38 protein expression in the MG63 cells treated with 50 μM salvianolic acid B compared with the control cells. Additionally, si-p38 significantly inhibited the anticancer effects of salvianolic acid B on the cell proliferation of osteosarcoma MG63 cell compared with the 50 μM salvianolic acid B treated group, as illustrated in Fig. 8B.

Salvianolic acid B activated p53 protein expression in osteosarcoma MG63 cells subsequent to knockdown of p38. Salvianolic acid B acted via a P-38 mediated mechanism to

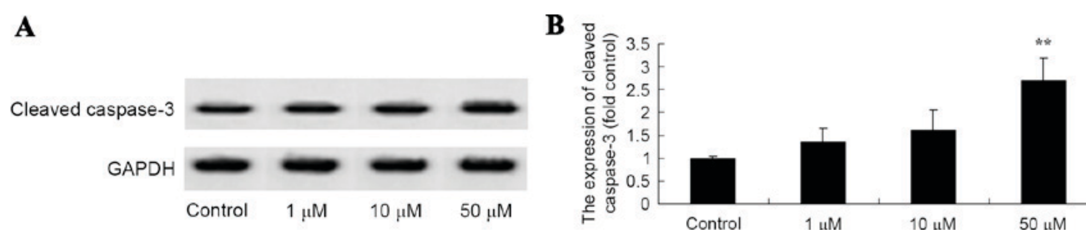

Figure 4. Salvianolic acid B activated cleaved caspase-3 protein expression of osteosarcoma MG63 cell. Salvianolic acid B activated cleaved caspase-3 protein expression using (A) western blot analysis and (B) statistical analysis of cleaved caspase-3 protein expression in the osteosarcoma MG63 cell line. Control, control group (0 μM salvianolic acid B). **P<0.01 vs. control group.

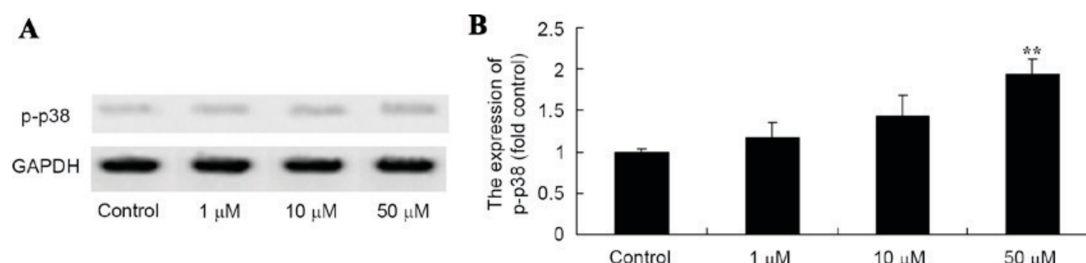

Figure 5. Salvianolic acid B activated p-p38 protein expression in the osteosarcoma MG63 cell line. Salvianolic acid B activated p-p38 protein expression using (A) western blot analysis and (B) statistical analysis of p-p38 protein expression of osteosarcoma MG63 cell. Control, control group (0 μM salvianolic acid B); o-p38, phosphorylated tumor protein 38. **P<0.01 vs. control group.

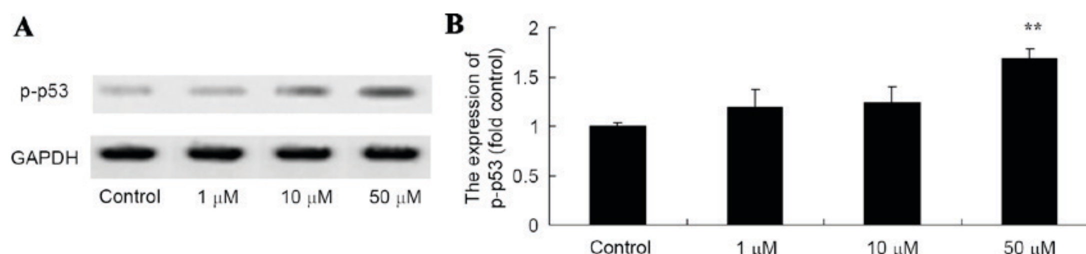

Figure 6. Salvianolic acid B activated p-p53 protein expression in osteosarcoma MG63 cell lines. Salvianolic acid B activated p-p53 protein expression using (A) western blot analysis and (B) statistical analysis of p-p53 protein expression in osteosarcoma MG63 cells. Control, control group (0 μM salvianolic acid B). **P<0.01 vs. control group.

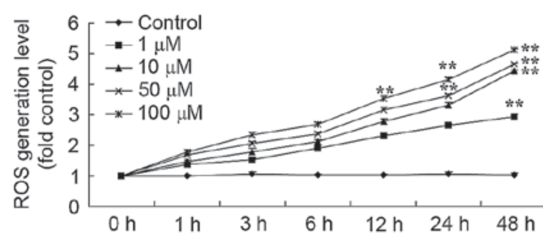

Figure 7. Salvianolic acid B activated ROS generation in osteosarcoma MG63 cell lines. Control, control group (0 μM salvianolic acid B). **P<0.01 vs. control group.

alter levels of p-p53 protein in osteosarcoma MG63 cells. Knockdown of p38 expression significantly suppressed the levels of p-p53 protein phosphorylation in MG63 cells treated with 50 μM salvianolic acid B compared with 50 μM salvianolic acid B treated group that was not knocked down (Fig. 9).

Salvianolic acid B activated ROS generation in osteosarcoma MG63 cells subsequent to knockdown of p38. To examine the p38-mediated mechanism of salvianolic acid B on the ROS generation in osteosarcoma MG63 cells, ROS generation was detected using H2DCFDA subsequent to knockdown of p38. The knockdown of p38 expression significantly reduced ROS generation in osteosarcoma MG63 cells treated with 50 μM salvianolic acid B, as compared with the 50 μM salvianolic acid B treated group that was not knocked down, as illustrated in Fig. 10.

Discussion

Osteosarcoma is the most common type of primary malignant bone tumor in adolescents (18). According to statistical data from China, the morbidity of osteosarcoma is the highest of all primary malignant bone tumors (5). Levels of malignancy in osteosarcoma are high, patient prognosis is poor, and patients may exhibit lung metastases within several months. Subsequent

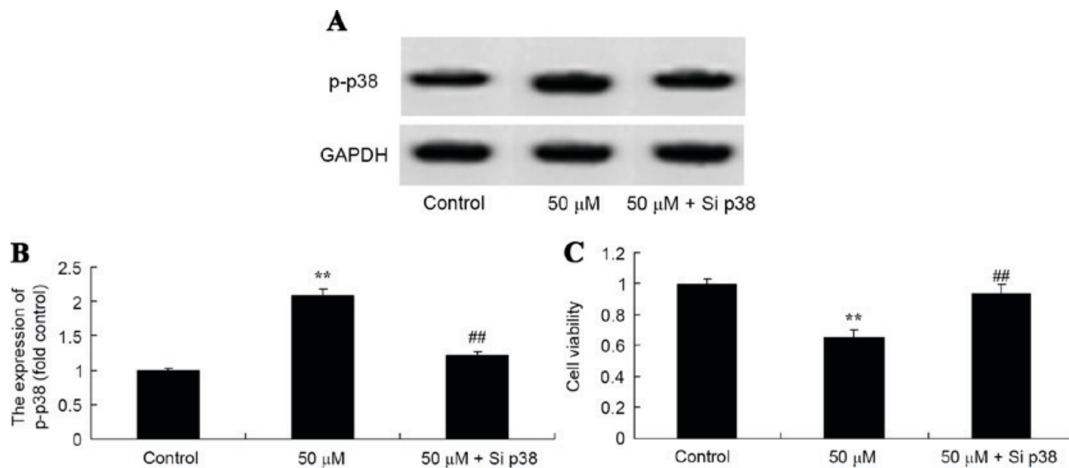

Figure 8. Salvianolic Acid B suppresses cell proliferation in osteosarcoma MG63 cell lines subsequent to knockdown of p38. p-p38 protein expression using (A) western blot analysis and (B) statistical analysis of p-p38 protein expression in osteosarcoma MG63 cells. (C) Salvianolic acid B suppresses cell proliferation in osteosarcoma MG63 cells subsequent to knockdown of p38. Control, control group (0 μM salvianolic acid B); si, small interfering; p38, tumor protein 38; p-p38, phosphorylated p38. **P<0.01 vs. control group; ##P<0.01 vs. 50 μM salvianolic acid B group.

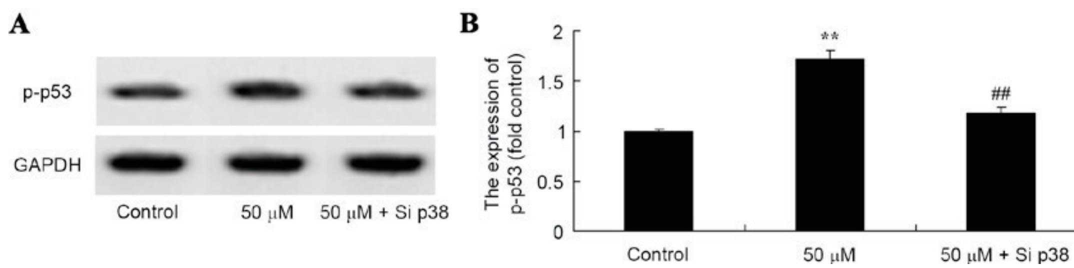

Figure 9. Salvianolic acid B activated p53 protein expression in osteosarcoma MG63 cell lines subsequent to knockdown of p38. p-p53 protein expression using (A) western blot analysis and (B) statistical analysis p53 protein expression of osteosarcoma MG63 cells subsequent to knockdown of p38. Control, control group (0 μM salvianolic acid B); p53, tumor protein 53; p-p53, phosphorylated p53. **P<0.01 vs. control group; ##P<0.01 vs. 50 μM salvianolic acid B group.

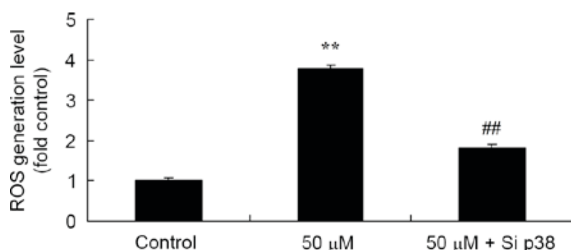

Figure 10. Salvianolic acid B activated ROS generation of osteosarcoma MG63 cell subsequent to knockdown of p38. Control, control group (0 μM Salvianolic Acid B); ROS, reactive oxygen species; p38, tumor protein 38. **P<0.01 vs. Control group; ##P<0.01 vs. 50 μM salvianolic acid B group.

to amputation, the 3-5-year survival rates are only 60% (19). The present study observed that salvianolic acid B suppressed cell proliferation, induced apoptosis and activated cleaved caspase 3 of the osteosarcoma MG63 cell. Wang *et al* (12) suggested that salvianolic acid B induced apoptosis through p38-mediated ROS generation in human glioma U87 cells and suppressed the growth and angiogenic potential of oral squamous carcinoma cells (20) and neuroblastoma SH-SY5Y cell lines (16).

MAPK is a type of serine/threonine protein kinase, is one of the most important types of signal transduction molecules in humans, is involved in cell signal regulation through acting on target genes by activating multiple nuclear transcription factors and is equipped with multiple regulatory functions for participating in cell proliferation, differentiation and intercellular functional synchronization (21).

Gene sequencing analysis demonstrated that p38MAPK is a tyrosine phosphorylation protein kinase comprising 360 amino acids (22). Previous studies revealed that the p38 MAPK pathway may be activated through the double phosphorylation of tyrosine and threonine, act on multiple downstream factors and possess an important role in regulating tumor cell invasion (22,23). Through the p38MAPK signal transduction pathway, vascular endothelial growth factor induces metastasis of tumor cells. p38 proteins are present in invasive colorectal cancers (24). The p38MAPK pathway induces expression of MMPs promotes the invasion of osteosarcoma cells. However, data indicate that p38 serves a protective role in apoptosis caused by ultraviolet light, restrains p38MAPK and promotes and induces keratinocyte apoptosis in healthy individuals (24). Activated p38MAPK improves the stability and phosphorylation of p53 and promotes the concentration of p53 in the cytoplasm (25). In the present study, it was revealed that salvianolic acid B activated p-p38 and p-p53 protein expression in the MG63 cell line.

Additionally, the silencing of p38 expression inhibited the anticancer effects of salvianolic acid B on cell proliferation of MG63 cells. p38 was also indicated to be a target of salvianolic acid B on the MG63 cell line through the regulation of p53 expression in osteosarcoma cells. Hung *et al* (26) suggested that salvianolic acid B induces neointimal cell apoptosis through p53 expression in a rabbit angioplasty model. Ma *et al* (17) reported that salvianolic acid B inhibits tumor necrosis factor-α-induced human coronary artery endothelial cells through matrix metalloproteinase-9 and p38 activity.

The generation of ROS may trigger multiple biological process, including apoptosis and programmed cell death (27). In a previous study on apoptosis caused by ultraviolet A (UVA) light, it was demonstrated that UVA triggers ROS-mediated apoptosis (27). In addition, the authors hypothesized that singlet oxygen is the dominant ROS. A previous study also revealed that antioxidants may effectively restrain ROS generation and apoptosis caused by UVA (28). The generated ROS activates the upstream activating agent and apoptosis signal conditioning kinase 1 (AsKI) of p38 and c-Jun N-terminal kinase, so as to activate p38, indicating that ROS-ASKI-p38MAPK pathway is an important signal transduction pathway in the carcinogenic effects of ultraviolet light (29). In the present study it was revealed that salvianolic acid B increased ROS generation levels and the silencing of p38 expression inhibited the anticancer effect of salvianolic acid B on the level of ROS generation in the MG63 cell line.

In conclusion, the present study demonstrated the salvianolic acid B inhibits cell proliferation and tumor growth via inducing apoptotic cell death in the osteosarcoma MG63 cell line. Additionally, these results suggest that salvianolic acid B may act as a novel type of chemotherapy in osteosarcoma therapy via the p38-mediated generation of ROS.

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