Ampelopsin suppresses TNF-α-induced migration and invasion of U2OS osteosarcoma cells

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Abstract. Ampelopsin has been suggested as a novel anticancer agent, however, there is no evidence regarding its direct effect on the migration and invasion of osteosarcoma cells. The aims of the present study were to investigate the influence of ampelopsin on the migration and invasion of osteosarcoma cells and to clarify the underlying mechanisms. Scratch wound healing and Transwell assays were used to measure the migratory and invasive activities of the cells, respectively. The protein and RNA levels of matrix metalloproteinase-2 (MMP-2) were detected with western blot and RT-qPCR, respectively, following stimulation with tumor necrosis factor-α (TNF-α) and ampelopsin. The expression levels of phospho- and total-p38MAPK were detected using western blot analysis. Additionally, SB203580, an inhibitor of p38MAPK, was used to investigate the effect of TNF-α and ampelopsin. The results demonstrated that TNF-α upregulated the expression level of MMP-2 and promoted the migration and invasion of osteosarcoma cells. TNF-α also activated the p38MAPK pathway, and SB203580 significantly inhibited the effect of TNF-α on MMP-2 expression. The application of ampelopsin abolished the effects of TNF-α on the activation of the p38MAPK pathway and the expression of MMP-2, and downregulated the migration and invasion of the osteosarcoma cells. These results demonstrated that ampelopsin inhibits the TNF-α-induced migration and invasion of osteosarcoma cells, and that the effect of ampelopsin was mediated by p38MAPK/MMP-2 signaling.

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Introduction

Osteosarcoma is one of the most common malignant bone tumors and predominantly occurs in the long bones, including the humerus, ulna, radius, femur, fibula and pelvis, of adolescents and children. Although the mortality rate of osteosarcoma has declined and the survival rate has increased dramatically over the past decade with the improvement of chemotherapy and aggressive surgical techniques (1), the prognosis of patients with metastasis remains poor, with only a 20% 5-year survival rate (2). Thus, it is important to further clarify the underlying molecular signaling mechanisms that regulate osteosarcoma, and to develop effective strategies to control osteosarcoma carcinogenesis and metastasis.

The mechanisms of osteosarcoma metastasis are complex, requiring multiple processes and various physiological changes. Evidence has demonstrated that the degradation of the extracellular matrix (ECM) is a pivotal process in the migration and invasion of osteosarcoma cells. Numerous factors are associated with the process of ECM degradation, such as matrix metalloproteinase-2 (MMP-2), which enables cancer cells to degrade the ECM facilitating the migration and invasion of tumor cells toward the bloodstream. MMP-2 production and activation is induced by various cytokines, of which tumor necrosis factor-α (TNF-α) is one of the most important mediators (3,4).

Ampelopsin (3,5,7,3'4'5'-hexahydroxyl 2,3 dihydrogen flavonol), one of the most common flavonoids, is derived from the tender stem and leaves of the plant species *Ampelopsis grossedentata*, which has been widely used in traditional Chinese medicine (5,6). Ampelopsin has been used in the treatment of numerous diseases, including inflammation (5), oxidative stress (5), liver injury (7) and hyperlipidemia (8). Previous studies have investigated the anticancer effect of ampelopsin in prostate (9), ovarian (10) and breast (11) cancer. However, to the best of our knowledge there are no reports of the anticancer activity of ampelopsin on osteosarcoma cells. The present study aimed to investigate whether ampelopsin may exert an effect against osteosarcoma cell migration and invasion.

Materials and methods

Reagents. Ampelopsin and recombinant human TNF-α were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse

monoclonal MMP-2 and GAPDH antibodies were purchased from Abcam (Cambridge, UK; cat. nos. ab2462 and ab8245, respectively). Rabbit monoclonal total-p38 mitogen activated protein kinase (MAPK) and phospho-p38MAPK were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA; cat. nos. 8690 and 4511, respectively). SB203580, a selective inhibitor of the p38MAPK signaling pathway, was purchased from Sigma-Aldrich.

Cell culture. U2OS osteosarcoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA). U2OS cells were seeded and maintained at 37°C in a humidified atmosphere of 5% CO_2 in Hyclone Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% Hyclone fetal bovine serum (FBS; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich).

Western blot analysis. To clarify the effect of TNF-α on the expression of MMP-2, U2OS cells were cultured in 6-well plates, and were randomly divided into 5 groups as follows: Control, physiological saline of equal volume; 1, 10, 50 and 100 ng/ml TNF-α for 24 h. Furthermore, U2OS cells were pretreated with ampelopsin (5, 10 and 50 µM) for 2 h prior to TNF-α treatment (100 ng/ml) for 24 h, and the relative expression of MMP-2 was determined. To ascertain the effect of TNF-α on the p38MAPK signaling pathway, U2OS cells were cultured and stimulated with TNF-α (100 ng/ml) for 5, 15, 30, 60 and 120 min. A different set of cells were treated with 50 µM ampelopsin for 2 h prior to TNF-α treatment (100 ng/ml) for 5, 15, 30, 60 and 120 min, and the phosphorylation of p38MAPK was detected using western blot analysis. In addition, SB203580 (10 mmol/l), an inhibitor of the p38 pathway, was used to pre-treat another set of U2OS cells for 2 h prior to TNF-α (100 ng/ml) treatment for 24 h.

For detection of the target proteins, ampelopsin, SB203580 or TNF-α-treated cancer cells were collected and lysed for 30 min in a lysis buffer containing phenylmethylsulfonyl fluoride, EDTA, pepstatin and leupeptin as protein inhibitors (Beyotime Institute of Biotechnology, Haimen, China). Lysates were then centrifuged at 21,000 x g at 4°C for 15 min to remove the insoluble material. The concentrations of the extracted proteins were measured with a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) following the manufacturer's instructions. The protein lysates (10 µg) were subjected to 10% SDS-PAGE according to the concentrations at 90 V. Following electrophoresis, the proteins were electrotransferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA) at 200 mA for 2 h. In order to minimize the non-specific binding, the blots were blocked in 5% non-fat milk for 2 h at room temperature. The blots were then incubated with antibodies against MMP-2 (1:1,000), total-p38MAPK (1:1,500), phospho-p38MAPK (1:1,500) and GAPDH (1:10,000) overnight at 4°C. The blots were washed and exposed to horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies, Inc., Rockville, MD, USA; cat. nos. ZDR-5306 and ZDR-5307, respectively) for 2 h, and the levels of the target proteins were detected by the FluorChem HD2

electrochemiluminescence detection system (Alpha Innotech Corporation, Sa Jose, CA, USA) and an enhanced chemiluminescence reagent (EMD Millipore). The quantification of the scanned blots was performed using ImageJ software (imagej.nih.gov/ij/) and the results are expressed as fold change relative to the control. Three independent experiments were duplicated for each reaction.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. U2OS cells were cultured in 6-well plates, and were randomly divided into 5 groups as follows: Control, physiological saline of equal volume; 1, 10, 50 and 100 ng/ml TNF-α for 24 h. Another set of cells were pretreated with SB203580 (10 mmol/l) or ampelopsin (5, 10 and 50 µM) for 2 h prior to TNF-α treatment for 24 h, and the expressions of MMP-2 at the mRNA level were detected. Total RNA was extracted from the U2OS osteosarcoma cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA), and measured using a SmartSpec 3000 UV/Vis spectrophotometer (Bio-Rad Laboratories, Inc., Beijing, China). RT was performed using oligo (dT) primers (Boshang Biology Technology Ltd., Shanghai, China; MMP-2 primers cat. no. H187580; 18s primers cat. no. H179060) and the RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) at 37°C for 1 h according to the manufacturer's instructions. Briefly, reverse transcription was performed at 37°C for 10 min using 1 µg RNA, 1 µl oligo (dT) primers and 10 µl RNase-free water. Mixture was incubated at 70°C for 10 min and 4 µl 5X reaction buffer, 1 µl ribonuclease inhibitor and 2 µl dNTP were added. Following incubation at 37°C for 5 min, 2 µl reverse transcriptase were added to the mixture, and the final 20 µl reaction mixture was cultured at 37°C for 1 h. The enzymatic activity was inactivated by heating at 65°C for 10 min at the end of the incubation period.

RT-qPCR was subsequently performed with the SYBR Green I kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions and analyzed using a LightCycler (Roche Diagnostics, Mannheim, Germany) with 18sRNA as a reference gene. The thermal cycling conditions used for qPCR were as follows: denaturing at 94° for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec, a total of 37 cycles. The primer sequences used for RT-qPCR were as follows: MMP-2, forward 5'-GGATGATGCCTTTGCTCG and reverse 5'-GGAGTCCGTCCTTACCGT (12); 18sRNA, forward 5'-CTTAGTTGGTGGAGCGATTTG-3' and reverse 5'-GCTGAACGCCACTTGTCC-3' (13). The melting curves were assessed and the comparative $2^{-\Delta\Delta Cq}$ method was used to normalize the relative expression levels of the products generated by each set of primers (14).

Scratch wound healing assay. A scratch wound healing assay was used to assess the migratory ability of the U2OS osteosarcoma cells. U2OS cells were cultured in 6-well plates (1x10⁶ cells/well; Corning Incorporated, Corning, NY, USA) with DMEM supplemented with 10% FBS. A pipette tip was used to make straight scratches of the same width in the monolayer of U2OS cells on the surface of each well. U2OS cells were treated as follows: Control, physiological saline; TNF-α, 100 ng/ml for 24 h; TNF-α + ampelopsin, 50 µM

ampelopsin for 2 h prior to TNF-α (100 ng/ml) incubation for 24 h; ampelopsin, 50 μM treatment for 2 h prior to physiological saline of equal volume for 24 h. Images were captured to measure the wound healing under the Olympus IX71 microscope (Olympus Corporation, Tokyo, Japan) (10).

Transwell assay. A Transwell assay was used to assess the effect of ampelopsin on the invasive ability of U2OS osteosarcoma cells. Briefly, U2OS cells ($2x10^5$ cells/well) were cultured in modified Boyden chambers with 8-μm pore filter inserts (Corning Incorporated). The upper chamber contained cells in DMEM supplemented with 1% FBS, while the lower chamber contained DMEM supplemented with 10% FBS. Following re-suspension in the upper chamber, U2OS cells were treated as described above for the control, TNF-α, TNF-α + ampelopsin and ampelopsin groups. The cells remaining on the upper surface were gently wiped away with a cotton swab and the cells on the lower surface were fixed with 95% ethanol for 30 min and stained with 0.1% hexamethylpararosaniline following methanol fixation (Sigma-Aldrich) at 37°C for 30 min (10). The number of cells on the lower surface of the membranes was quantified using the Olympus IX71 microscope.

Statistical analysis. All data in the present study were evaluated with SPSS predictive analytics software, version 18.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to analyze the normally distributed data, while Mann-Whitney U test was used to analyze the non-parametric variables with post-hoc test used for multiple comparisons between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

TNF-α upregulates MMP-2 expression at the protein and mRNA levels. In a previous study, MMP-2 was correlated with aggressive tumor progression and low survival rates (15). Thus, the present study investigated the effect of TNF-α on the expression levels of MMP-2 in osteosarcoma cells. U2OS cells were cultured in 6-well plates and randomly divided into 5 groups, which were treated with 1, 10, 50 or 100 ng/ml TNF-α for 24 h, while treatment with physiological saline of equal volume was used as the control group. As demonstrated in Fig. 1A, TNF-α increased MMP-2 protein expression levels in a concentration-dependent manner. A significant effect was observed at a concentration of 50 ng/ml compared with the control group (P=0.0021) and peak MMP-2 expression was reached at 100 ng/ml TNF-α treatment (Fig. 1B; P=0.0017). The results indicated that TNF-α may induce the migration of U2OS cells via the increased expression levels of MMP-2.

Additionally, the same TNF-α stimulations were performed and RT-qPCR was used to examine the mRNA levels of MMP-2. As demonstrated in Fig. 1C, following TNF-α treatment, the relative expression of MMP-2 mRNA was increased in a concentration-dependent manner. The MMP-2 mRNA expression level was significantly increased compared with the control group following stimulation with 50 and 100 ng/ml TNF-α (P<0.00001).

Similar changes to MMP-2 protein and mRNA levels were observed, and thus, the data suggested that TNF-α induces

MMP-2 production in a concentration-dependent manner at the protein and mRNA levels.

TNF-α increases the phosphorylation of p38MAPK, with the effect abolished by ampelopsin. The current study aimed to elucidate the mechanism by which TNF-α induced the expression of MMP-2. In previous studies, the p38MAPK pathway was demonstrated to be an important regulator of MMP-2 following stimulation with a variety of cytokines in multiple cell types (16-19). TNF-α was previously reported regulate a number of biological functions via the activation of the p38MAPK pathway (20-22). Therefore, it was speculated that TNF-α may stimulate the expression of MMP-2 via the phosphorylation of p38MAPK. Following stimulation with TNF-α (100 ng/ml) for 5, 15, 30, 60 and 120 min, the proteins were extracted from U2OS cells. As demonstrated in Fig. 2A, the relative expression of phospho-p38 was significantly upregulated, compared with the control group, after 15 min stimulation (P=0.0014). Maximal activity was observed at 30 min of stimulation (P=0.0011), which gradually decreased after 60 and 120 min of stimulation (P=0.0018 and P=0.0237, respectively). Additionally, 50 μM ampelopsin was cultured with U2OS cells for 2 h prior to TNF-α stimulation (100 ng/ml) for 5, 15, 30, 60 and 120 min. As demonstrated in Fig. 2B, ampelopsin markedly inhibited the enhancement of p38 phosphorylation that was induced by TNF-α stimulation, and no significant differences in the levels of phospho-p38 were observed compared with the control group (P>0.05). These results demonstrated that TNF-α increased phosphorylation of p38MAPK, and that ampelopsin abolished the effect.

TNF-α-induced expression of MMP-2 is mediated by the p38MAPK pathway and suppressed by ampelopsin. In order to further understand the importance of the p38MAPK pathway the upregulation of MMP-2 expression levels by TNF-α, SB203580 (10 mmol/l), a specific inhibitor of p38, was used to pre-treat U2OS cells for 2 h prior to TNF-α treatment for a further 24 h. As presented in Fig. 3A and B, pre-treatment with SB203580 inhibited the TNF-α-induced production of MMP-2, compared with the TNF-α only treatment group, at the protein (P=0.0191) and mRNA (P<0.00001) level, which strongly suggested that TNF-α increases MMP-2 production via the p38MAPK signaling pathway.

Additionally, ampelopsin was used to investigate whether it inhibits the effects of TNF-α. U2OS cells were pretreated with various concentrations of ampelopsin (5, 10 and 50 μM) for 2 h before TNF-α (100 ng/ml) was added to the cells for a further 24 h. As demonstrated in Fig. 3C and D, the enhanced MMP-2 expression level induced by TNF-α was gradually reduced with increasing ampelopsin concentrations. At 50 μM ampelopsin, the expression of MMP-2 mRNA and protein was significantly reduced compared with the TNF-α group (P<0.00001 and P=0.0072, respectively). The results confirmed the importance of the p38MAPK pathway and the inhibitory effect of ampelopsin on TNF-α-stimulated MMP-2 production.

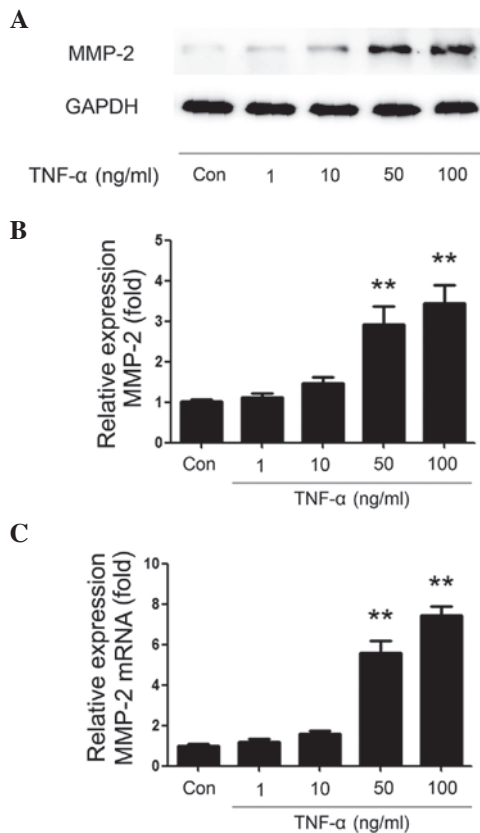*TNF-α induces the migration and invasion of osteosarcoma cells.* Wound healing and Transwell assays were used to assess

Figure 1. Increased expression of MMP-2 following TNF-α treatment. U2OS osteosarcoma cells were treated with 0, 1, 10, 50 or 100 ng/ml TNF-α for 24 h, with the same volume of physiological saline used as the control group. (A) Western blotting was used to observe the changes of MMP-2 protein expression. (B) Quantification of western blot. (C) Reverse transcription-quantitative polymerase chain reaction was used to detect the mRNA expression level of MMP-2. **P<0.01 vs. the control group. Data are presented as the mean ± standard error from three independent experiments in duplicate. TNF-α, tumor necrosis factor-α; MMP-2, matrix metalloproteinase-2; Con, control.

the effects of TNF-α on the migration and invasion of U2OS osteosarcoma cells, respectively. U2OS cells were cultured with 100 ng/ml TNF-α for 24 h in the TNF-α group, or physiological saline of equal volume was used in the control group. As presented in Fig. 4A, the wound healing assay demonstrated that TNF-α significantly enhanced the healing of the scratch wound compared with the control group (P<0.00001). The results indicated that TNF-α promotes the migration of U2OS cells. Additionally, as presented in Fig. 4B, the Transwell assay also demonstrated that treatment with TNF-α significantly increased the invasion cells compared with the control group (P=0.0087).

Ampelopsin suppresses TNF-α-induced increases to osteosarcoma cell migration and invasion. Subsequently, to further clarify the effect of ampelopsin on the migration and invasion of osteosarcoma cells, U2OS cells were pretreated with 50 μM ampelopsin prior to TNF-α stimulation. As demonstrated in Fig. 4A, the wound healing assay demonstrated that ampelopsin significantly inhibited the effect of TNF-α on wound healing areas compared with the TNF-α group (P=0.0032). As presented in Fig. 4B, the Transwell assay also demonstrated

that pretreatment with ampelopsin significantly decreased the invasion of U2OS cells compared with the TNF-α group (P=0.0069). These results indicated that ampelopsin suppresses the migration and invasion of osteosarcoma cells that is promoted by TNF-α. The current study also investigated the effects of ampelopsin on the osteosarcoma cell migration and invasion when used alone, without TNF-α stimulation. The results presented in Fig. 4 demonstrated that ampelopsin treatment alone also had the ability to suppress U2OS cells migration compared with the control group (P=0.0251) or the TNF-α group (P<0.0000), and invasion compared with the control group (P=0.0376) or the TNF-α group (P<0.0000).

Discussion

Cancer metastasis is a complex biological process requiring multiple steps, including degradation of the ECM, activation of cell adhesion, migration and angiogenesis. The interactions between cells, cytokines, proteinases and the ECM that result in cell migration and tumor invasion are not fully clarified, however, TNF-α has been demonstrated to be important in inflammatory reactions, angiogenesis, cell proliferation and apoptosis via the regulation of various signaling pathways, including the phosphorylation of MAPKs (23,24). TNF-α has been demonstrated to promote tumor angiogenesis, invasion and metastasis via upregulating the expression of angiogenic factors (25) and stimulating multiple cytokines and chemokines that accelerate the mobilization of tumor cells and their colonization to other tissues (26). Previous clinical reports have observed that the TNF-α concentration in serum was increased in patients with various types of cancer, including chronic lymphocytic leukemia (27), ovarian (28) and prostate (29) cancer. Simultaneously, chemotherapy significantly decreased the serum TNF-α concentration (28,29). The ability of TNF-α to promote tumor progression has also been confirmed in animal models. Additional pathways associated with TNF-α upregulation of tumor growth include the vascular endothelial growth factor (VEGF) (25), hepatocyte growth factor (30), epithelial-mesenchymal transition (31), macrophage migration-inhibitory factor, chemoattractant protein-1 (24) and MMP (32) pathways. However, TNF-α was also reported to promote cancer cell death and exhibit an anti-oncogenic effect on certain tumors (33). Thus, the present study aimed to clarify the specific effects of TNF-α on the metastasis of osteosarcoma.

The human osteosarcoma U2OS cell line, which was initially derived from a moderately differentiated sarcoma of a 15-year-old female in 1964, has been widely used in biomedical research. The U2OS cell line has the lowest level of chromosomal numerical variations compared with other osteosarcoma cell lines. In addition, according to previous reports, only 2% of U2OS cells have multipolar mitoses, which is similar to normal control fibroblasts (34). Thus, in the present study, the U2OS cell line was selected to represent osteosarcoma *in vitro*. The results confirmed that TNF-α significantly induced the migration and invasion of U2OS osteosarcoma cells. This result was in accordance with an investigation by Mori *et al* (35), which also demonstrated that TNF-α was required for the tumorigenesis of AX osteosarcoma cells. It is suggested that although TNF-α exerts a cytotoxic effect
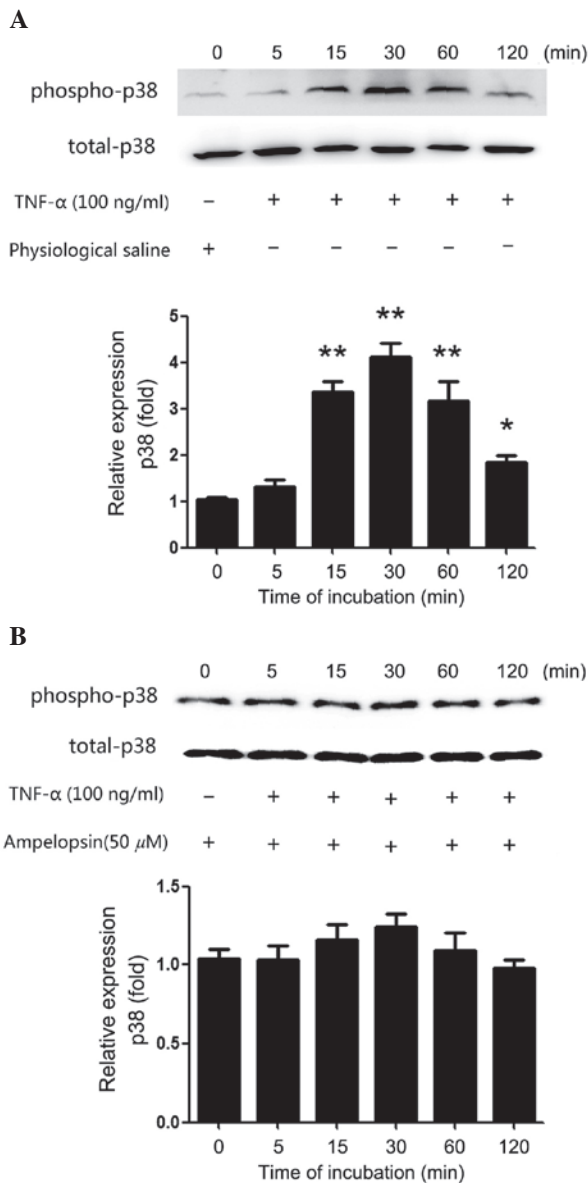
A

B

Figure 2. Phosphorylation of p38MAPK increased following TNF-α treatment, while ampelopsin abolished the effect. (A) U2OS osteosarcoma cells were treated with TNF-α (100 ng/ml) for 5, 15, 30, 60 and 120 min, with the same volume of physiological saline used as the control group. Western blot assay was performed to observe the change of phospho-p38MAPK protein expression. (B) Ampelopsin (50 μM) was cultured with U2OS cells for 2 h prior to TNF-α (100 ng/ml) addition to the supernatant for 5, 15, 30, 60 or 120 min, with the same volume of physiological saline used as the control group. Western blot was performed to measure the change to phospho-p38MAPK protein expression. *P<0.05 and **P<0.01 vs. the control group. Data are presented as the mean ± the standard error from three independent experiments in duplicate. Phospho, phosphorylated; p38MAPK, p38 mitogen-activated protein kinase; TNF-α, tumor necrosis factor-α.

on osteosarcoma, osteosarcoma cells may be resistant to the cytotoxicity.

The mechanisms of osteosarcoma metastasis are complex, involving multiple signaling pathways and various physiological changes. MMP-2 is an important member of the MMP super-family, the members of which act by digesting ECM molecules and promoting the invasion and metastasis of various types of cancer. The present study addressed one aspect of the multiple interactions of TNF-α during tumorigenesis
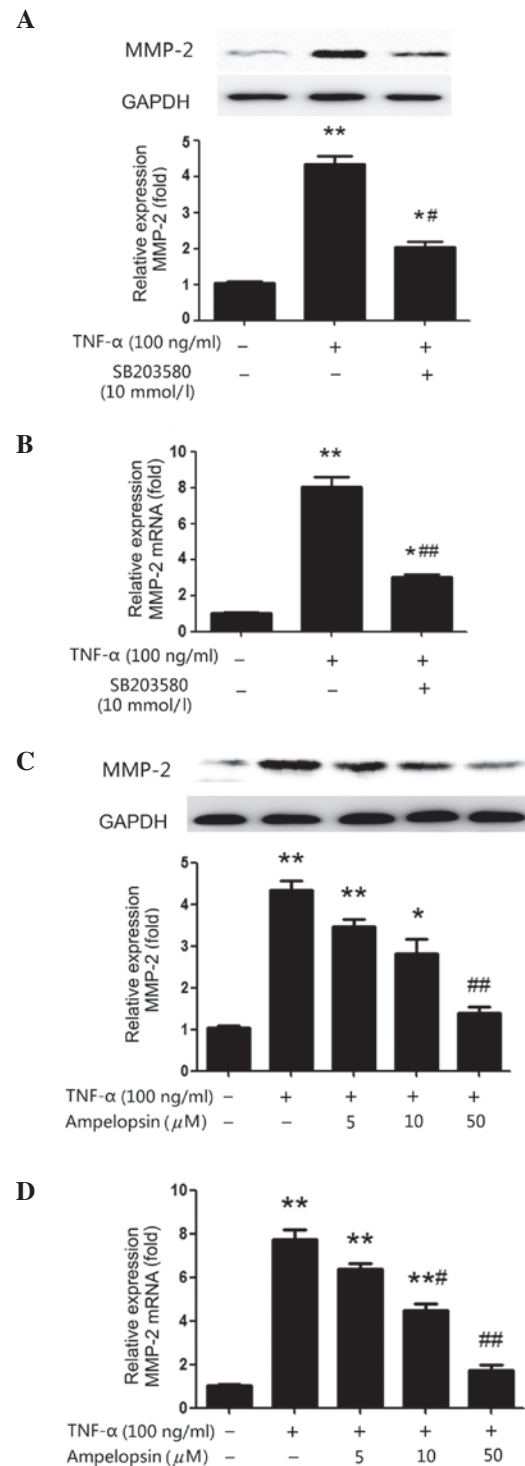
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D

Figure 3. TNF-α induced the expression of MMP-2, which was mediated by the p38MAPK pathway, while ampelopsin suppressed the effect. SB203580 (10 mmol/l) was used to pre-treat U2OS cells for 2 h prior to TNF-α treatment for another 24 h, the same volume of physiological saline was used as the control group. (A) Western blot and (B) RT-qPCR demonstrated that SB203580 significantly reversed the effect of TNF-α on MMP-2 expression. U2OS cells were pre-treated with 5, 10 or 50 μM ampelopsin for 2 h prior to TNF-α (10 ng/ml) addition to the cells for a further 24 h, the same volume of physiological saline was used as the control group. (C) Western blot and (D) RT-qPCR demonstrated that pre-treatment with ampelopsin concentration-dependently reversed the effect of TNF-α on MMP-2 expression. *P<0.05, **P<0.01 vs. the control group; #P<0.05 and ##P<0.01 vs. the TNF-α group. Data are presented as the mean ± the standard error from three independent experiments in duplicate. TNF-α, tumor necrosis factor-α; MMP-2, matrix metalloproteinase-2; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
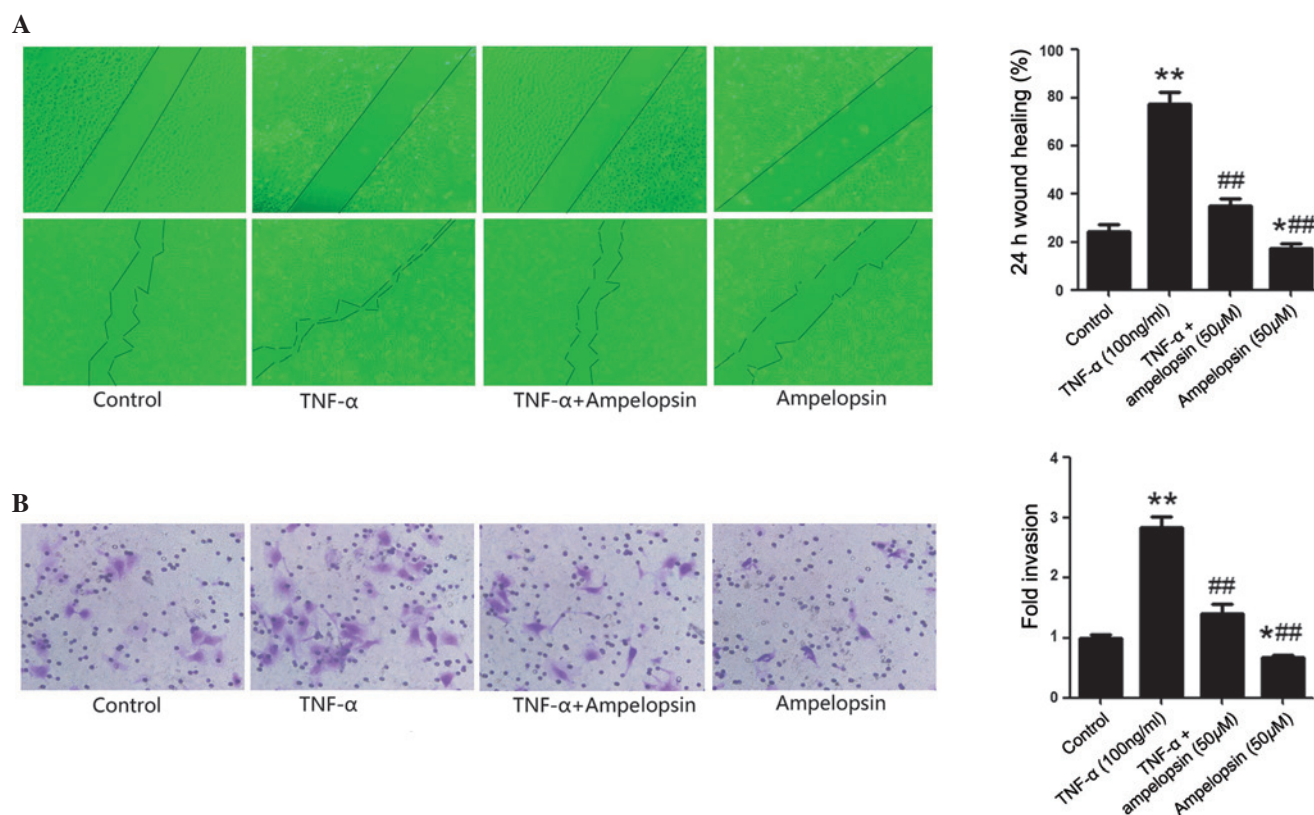
Figure 4. Ampelopsin inhibits TNF-α-induced migration and invasion of U2OS osteosarcoma cells. U2OS osteosarcoma cells were treated with TNF-α, TNF-α + ampelopsin (pre-treatment for 2 h) or ampelopsin only for 24 h, the same volume of physiological saline was used as the control group. (A) The effect of TNF-α and ampelopsin on the migration of U2OS osteosarcoma cells was assessed by a wound healing assay. (Top-panel) A pipette tip was used to make straight scratches of the same width in the monolayer of U2OS cells on the surface of each well. (Bottom panel) Following treatments images were captured to measure the wound healing. (B) The effect of TNF-α and ampelopsin on the invasion of U2OS osteosarcoma cells was assessed by a Transwell assay. $^*P<0.05$ and $^{**}P<0.01$ vs. the control group; $^{##}P<0.01$ vs. TNF-α group. Data are presented as the mean ± the standard error from three independent experiments in duplicate. TNF-α, tumor necrosis factor-α.

by determining the effects of TNF-α on an ECM remodeling enzyme, MMP-2. Previous reports have demonstrated that TNF-α activates the expression of MMP-2 in multiple cell types, including synovial fibroblasts, endothelial cells, dermal fibroblasts and corneal epithelial cells (36-38). The data of the present study confirmed the importance of TNF-α on the promotion of MMP-2 expression in U2OS osteosarcoma cells and provided further support for the theory that TNF-α increases MMP-2 activation via the phosphorylation of p38MAPK. In previous studies, the p38MAPK pathway was demonstrated to be an important regulator of MMP-2 following stimulation of several cell types with a variety of cytokines (16-19). The present study confirmed that p38 and MMP-2 proteins were highly expressed in U2OS osteosarcoma cells. Simultaneously, the results demonstrated that stimulation with TNF-α (100 ng/ml) at different time points significantly upregulated the phosphorylation of p38MAPK and exhibited an abnormal time-dependent increase, with the peak activity observed at 30 min. The application of SB203580 (10 mmol/l), a specific inhibitor of the p38 pathway, reduced the protein expression level of MMP-2 that was increased by TNF-α, further confirming the theory that TNF-α promotes the expression of MMP-2 via the p38MAPK signaling pathway. Overall, these results suggested that TNF-α activates p38MAPK and increases MMP-2 expression through this pathway.

Ampelopsin is a type of flavonoid extracted from *Ampelopsis grossedentata*, a plant predominantly distributed in South China. The flavonoid is widely used to treat cold and tinea corporis, and has exhibited multiple biological functions in the processes of inflammation, oxidation, liver injury and hyperlipidemia. In previous studies, ampelopsin was demonstrated to possess anticancer properties in several types of malignant tumors. Ampelopsin has been reported to inhibit the proliferation of prostate cancer cells via the downregulation of B-cell lymphoma 2 expression, which is associated with cell apoptosis, and suppress migration and invasion via the downregulation of chemokine (C-X-C motif) receptor 4 expression (9). Ampelopsin was also observed to reduce breast carcinogenesis by inhibiting the mechanistic target of rapamycin signaling pathway (39). Ampelopsin also exhibits the ability to inhibit the secretion of VEGF and basic fibroblast growth factor, thus, inhibiting the development of hepatocellular carcinoma (40). In a previous study, it was confirmed that ampelopsin reduces the migration and invasion of ovarian cancer cells via inhibiting the epithelial-to-mesenchymal transition (10). Ampelopsin has demonstrated anti-carcinogenesis characteristics, however, direct evidence of ampelopsin involvement in osteosarcoma and the detailed molecular mechanisms have not been reported. The current study provided insight into the mechanisms by which ampelopsin regulates osteosarcoma cell migration and invasion.

In the present study, pretreatment with ampelopsin for 2 h significantly inhibited the increases to MMP-2 expression stimulated by TNF-α in a concentration-dependent manner. Additionally, following pre-treatment with ampelopsin, the changes to the phosphorylation of p38 were no longer clear. As presented in Fig. 3A, it was demonstrated that ampelopsin significantly reduced the effect of TNF-α on the phosphorylation of p38. Therefore, in the current study, ampelopsin was demonstrated to be an inhibitor of the p38 signaling pathway, which mediated the expression of MMP-2. The results suggested that ampelopsin exerts an inhibitory effect on TNF-α-induced MMP-2 production. The present study also investigated the effect of ampelopsin on TNF-α-induced osteosarcoma cell migration and invasion; the results demonstrated that ampelopsin significantly reduced the effect of TNF-α on migration and invasion.

In summary, the present study demonstrated that ampelopsin inhibits TNF-α-induced osteosarcoma cell migration and invasion, and the effect of ampelopsin was mediated by p38MAPK/MMP-2 signaling. According to the results of the present study, the invasive properties of osteosarcoma cells may be reduced by ampelopsin via the inhibition of TNF-α/p38MAPK/MMP-2 signaling, and further *in vivo* studies should be performed in the future to confirm these findings.

Acknowledgements

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