

# Ophiopogonin-D suppresses MDA-MB-435 cell adhesion and invasion by inhibiting matrix metalloproteinase-9

YUANYUAN ZHANG, YUWEI HAN, KEFENG ZHAI, MINHUI SUN, JIHUA LIU,  
BOYANG YU and JUNPING KOU

Department of Complex Prescription of Traditional Chinese Medicine, State Key Laboratory of Natural Medicines,  
China Pharmaceutical University, Nanjing, Jiangsu 211198, P.R. China

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**Abstract.** Ophiopogonin-D is one of steroidal saponins isolated from the root of the Chinese medicinal plant *Ophiopogon japonicus*. It has been claimed to possess anti-inflammatory and anti-oxidant properties. The present study was the first to examine the anti-tumor metastasis properties of ophiopogonin-D. An MTT assay showed that ophiopogonin-D inhibited the proliferation of MDA-MB-435 melanoma cells, and decreased invasion was demonstrated using a Transwell invasion assay. Furthermore, adhesion of MDA-MB-435 cells to human umbilical vascular endothelial cells and to fibronectin was inhibited by ophiopogonin-D. Gelatin zymography and western blot analysis showed that ophiopogonin-D inhibited the expression and secretion of matrix metalloproteinase-9 (MMP-9), but not that of MMP-2. Inhibition of phosphorylation of p38 by ophiopogonin-D indicated its inhibition of the mitogen-activated protein kinase pathway. Overall, the results suggested that ophiopogonin-D may be considered as a candidate drug for treating or preventing tumor metastasis.

## Introduction

Malignant neoplastic disease is one of the most common causes of mortality worldwide. ~90% of all cancer mortalities are the result of metastases, rather than of the primary tumors (1). The major risk factors for malignant melanoma are personal or family history of melanoma, exposure to intense and intermittent ultraviolet irradiation, phenotypic characteristics, and multiple nevi (2). The process of tumor metastasis involves tumor cell proliferation, expansion, reorganization, degradation and migration through the surrounding stroma's

microenvironment and the extracellular matrix (ECM), into the circulation to invade other tissues (3).

The process of tumor metastasis is accompanied with changes in gene expression, including mutation, overexpression, loss, activation or inactivation of numerous genes (4). The matrix metalloproteinase (MMP) family is a large group of secreted proteinases which require zinc for their catalytic activity. MMPs, which are proteolytic enzymes in the extracellular matrix (ECM), mainly contribute to cell motility, tumor invasion, angiogenesis and metastasis (5-7). Among these MMPs, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are mainly involved in cancer metastasis and invasion. They predominantly degrade gelatin and type IV, V, XI and XVI collagen, the major structural component of basement membrane, which appears to be crucial in tumor cell invasion and metastasis (8-10). A previous study reported that oroxylin A could inhibit migration and invasion of human breast MDA-MB-435 cancer cells via the inhibition of MMP-2 and MMP-9 (11). Thus, MMP-2 and MMP-9 have been considered as targets in the development of drugs against tumor invasion and metastasis (12,13).

The mitogen-activated protein kinase (MAPK) pathway is associated with tumor proliferation and survival, motility and invasion (14). In melanoma, MAPK signalling cascades are critical and constitutively activated by a variety of mechanisms, making it a target for pathway targeting therapies. The MAPK signalling pathway is one of the most important cellular mechanisms responsible for melanoma metastasis by promoting cell proliferation, survival, invasion and tumor angiogenesis. Extracellular signal-regulated kinase 1 and 2 (ERK1/2) and p38/MAPK are major MAPKs, which are associated with tumor metastasis (15). They have a central role in regulating the expression of MMPs. The up- or downregulation of MAP kinases and their phosphorylation are involved in the regulation of MMP-9 expression in cancer cells (16,17).

Medicinal plants have been used as traditional remedies for hundreds of years. *Radix Ophiopogon japonicus* is an important medicinal herb widely used for the treatment of various tumors, inflammatory diseases, hepatitis and diarrhea in East Asian countries, including China, Korea, Taiwan, and Japan (18-21). The plant has been reported to contain a large number of steroidal saponins. Ophiopogonin-D (ruscogenin 1-*O*- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 3)- $\beta$ -D-fucopyranoside) (Fig. 1) is one of the steroidal

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*Correspondence to:* Professor Junping Kou, Department of Complex Prescription of Traditional Chinese Medicine, State Key Laboratory of Natural Medicines, China Pharmaceutical University, 639 Longmian Road, Nanjing, Jiangsu 211198, P.R. China  
E-mail: junpingkou@cpu.edu.cn

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saponins isolated from the root of *Ophiopogon japonicus* (22). According to previous studies, the ophiopogonin-D processes anti-inflammatory and anti-oxidant properties, including prevention of H<sub>2</sub>O<sub>2</sub>-induced injury in primary human umbilical vascular endothelial cells (HUVECs) (23), anti-thrombotic activity (24), anti-inflammation (25) and suppressed leukocyte migration *in vivo* (26). However, to the best of our knowledge, the anti-tumor activity of ophiopogonin-D has not been evaluated *in vitro*.

The present study investigated the molecular mechanisms by which ophiopogonin-D suppresses the migration and invasion of MDA-MB-435 cells *in vitro*. Furthermore, the underlying mechanism of the effect of ophiopogonin-D on MDA-MB-435 cells was explored by assessing its effect on MMP activity and the MAPK pathway. The present study provided evidence that ophiopogonin-D is suitable for use in the treatment of metastasis of melanoma.

## Materials and methods

**Chemicals and reagents.** Ophiopogonin-D was isolated from the root of *Ophiopogon japonicus* according to the procedure of a previous study (27). Compounds were dissolved in dimethyl sulfoxide (DMSO; Nanjing Sunshine Biotechnology Ltd., Nanjing, China) and diluted with Dulbecco's medium Essential medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) without serum prior to each experiment. The final concentration of DMSO in the culture medium never exceeded 0.1% (v/v), a concentration known not to affect cell proliferation, and control groups were always treated with 0.1% DMSO in the corresponding experiments. MTT, Rose Bengal and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). High-glucose DMEM was purchased from Gibco-BRL (Invitrogen Life Technologies). Antibodies specific for phosphorylated (p-)ERK and p-p38/MAPK or total ERK, p38/MAPK,  $\beta$ -actin, MMP-2 and MMP-9 were obtained from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA, USA). The cell culture plate and Transwell plate (8- $\mu$ m pore size, 6.5-mm diameter) were obtained from Corning Costar Corporation (Corning, NY, USA). Matrigel was purchased from BD Biosciences (La Jolla, CA, USA).

**Cells lines and culture.** The human breast carcinoma cell line MDA-MB-435 was obtained from Keygene Corporation (Nanjing, China), grown in high-glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin and 3.7 g/l sodium bicarbonate (all purchased from Nanjing Sunshine Biotechnology Ltd.). HUVECs were obtained from Shanghai FuMeng Gene Biotechnology Co., Ltd. (Shanghai, China). The cells were maintained under a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C and used for experiments whilst in logarithmic phase.

**Cell adhesion of HUVECs and fibronectin.** The tumor cell adhesion with HUVECs was detected according to the method by Jones *et al* (28) with certain modifications. HUVECs were seeded in 96-well plates in complete HUVEC medium at a density of 10<sup>4</sup> cells per well for 24 h. The HUVEC medium

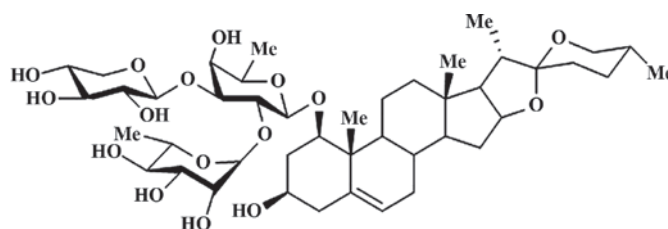


Figure 1. Chemical structure of ophiopogonin-D.

was then removed and cells were washed with phosphate-buffered saline (PBS). MDA-MB-435 cells were trypsinized and suspended at a final concentration of 5x10<sup>5</sup> cells/ml with various concentrations of ophiopogonin-D (5, 10, 20, 40 and 80  $\mu$ M) dissolved in serum-free DMEM. After 3 h, the medium was removed and the undetached cells were washed with PBS twice and incubated with Rose Bengal for 5 min. The cells were washed with PBS twice and fixed with 100  $\mu$ l mixed solution (95% ethanol/PBS 1:1) per well for 30 min at room temperature. The ratio of adhesion was detected by measuring the absorbance of Rose Bengal at a wavelength of 600 nm using an ELISA reader (Bio-Tek Instruments, Winooski, VT, USA).

Cell adhesion to fibronectin was assayed as described previously with a few alterations (29). Briefly, 96-well plates were coated with fibronectin (Merck, Whitehouse Station, NJ, USA) at 4°C overnight. MDA-MB-435 cells, which were trypsinized and suspended at a final concentration of 5x10<sup>5</sup> cells/ml in serum-free DMEM with various concentrations of ophiopogonin-D and cultured for 3 h. The unattached cells were removed by discarding the media and washing with PBS twice. A total of 100  $\mu$ l MTT (0.5 mg/ml in PBS) was added to the wells and the cells were cultured for another 3 h. Following discarding the MMT solution, DMSO was then added to the wells. After agitation for 10 min at room temperature, the cytotoxicity was determined by measuring the absorbance of the converted dye at a wavelength of 570 nm and a reference wavelength of 650 nm in an ELISA reader.

**Cell invasion assay.** The cell invasion assay was determined as described previously with a few modifications (30). Briefly, the Transwell membrane (8  $\mu$ m pore size, 6.5 mm diameter; Corning Costar Corporation) was pre-coated with Matrigel. The MDA-MB-435 cells, which were trypsinized and suspended at a final concentration of 5x10<sup>5</sup> cells/ml in serum-free DMEM with various concentrations of ophiopogonin-D, were added to the upper well, while 500  $\mu$ l DMEM containing 10% FBS was added to the lower well. After 20 h, assays were stopped by removal of the medium from the upper wells. The cells on the membrane were fixed with 90% ethanol and cells on the upper side of the membrane, which had not transgressed through the membrane, were subsequently wiped off using cotton buds. The cells on the lower side of the membrane were stained with 0.1% methyl violet. After 10 min, the chamber was washed with PBS and images of the membrane were captured using a microscope (Axiovert40CFL; Carl Zeiss, Oberkochen, Germany). Finally, the chamber was extracted with 10% acetic acid, and the absorbance of the extract was measured at 570 nm in an ELISA reader.

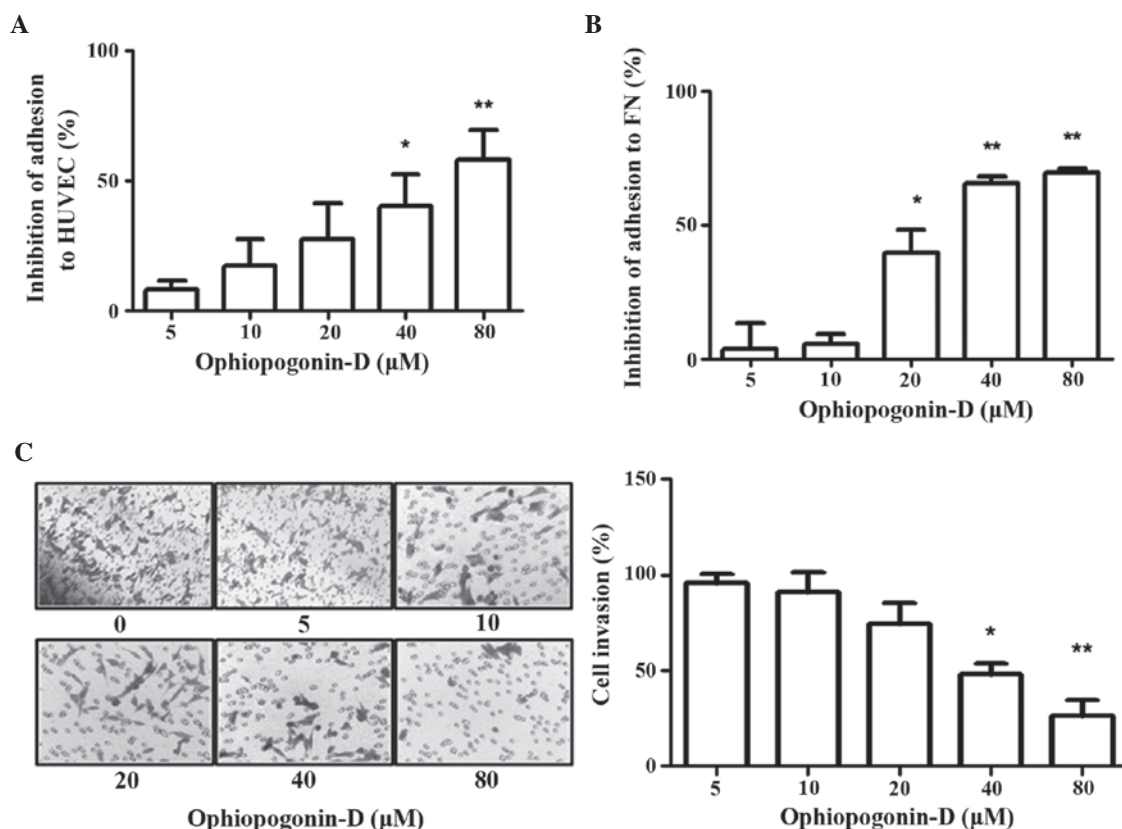


Figure 2. Effect of ophiopogonin-D on the adhesion and invasion of MDA-MB-435 cells. (A) Ophiopogonin-D inhibited the adhesion of MDA-MB-435 cells to HUVECs at 40 and 80  $\mu$ M, as detected by Rose Bengal staining. (B) Ophiopogonin-D inhibited the adhesion of MDA-MB-435 cells to FN at 20, 40 and 80  $\mu$ M, as detected by MTT assay. (C) Ophiopogonin-D inhibited the invasion of MDA-MB-435 cells *in vitro* as shown by a Transwell invasion assay. Values are expressed as the mean  $\pm$  standard deviation of three experiments (magnification, x400). \* $P$ <0.05, \*\* $P$ <0.01 vs. the control group. FN, fibronectin; HUVEC, human umbilical vein endothelial cell.

**Gelatin zymography.** The activities of MMP-2 and MMP-9 were assayed by gelatin zymography (31,32). Gelatin zymography was performed in 10% SDS-polyacrylamide gel containing 0.1% gelatin. After treatment with various concentrations of ophiopogonin-D (5, 10, 20, 40 and 80  $\mu$ M) for 24 h, the supernatants were collected. Samples were mixed with loading buffer and electrophoresed. Gels were then washed twice in zymography washing buffer (2.5% Triton X-100, 50 mmol/l Tris-HCl, 5 mmol/l CaCl<sub>2</sub>; pH 7.6) for 45 min at room temperature to remove SDS, followed by rinsing twice in washing buffer for 20 min. The gels were then incubated at 37°C for 40 h in zymography reaction buffer (50 mmol/l Tris, 150 mmol/l NaCl, 10 mmol/l CaCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, pH 7.5) and stained with 0.05% Coomassie blue R-250 (diluted in 30% methanol and 10% acetic acid) for 3 h and destained with destaining solution (10% methanol and 5% acetic acid in H<sub>2</sub>O<sub>2</sub>). The field of the destained band was quantified using the Quantity One System (Bio-Rad, Hercules, CA, USA).

**Western blot analysis.** Activation of MMP-2/9 and MAPK were assessed by western blot analysis. MDA-MB-435 cells were pre-incubated with various concentrations of ophiopogonin-D for 20 h, and cells were collected. Cells were then lysed in lysis buffer, and the equal amount of proteins was separated by SDS-PAGE and transferred onto the polyvinylidene difluoride membranes. Membranes were blocked with blocking buffer for 1.5 h at room temperature, followed by overnight incubation

at 4°C in the relevant primary antibody (1:1,000), and finally blocked for 1 h with a secondary antibody HRP-conjugate (1:2,000). The band detection was revealed by enhanced chemiluminescence (ECL) using ECL western blotting detection reagents (Vazyme Biotech Co., Ltd., Nanjing, China) and exposure to ECL hyperfilm on Kodak film (Eastman Kodak, Rochester, NY, USA) and analyzed using the Quantity One System.

**Statistical analysis.** Values are expressed as the mean  $\pm$  standard deviation. The experimental data were analyzed using GraphPad Prism 5 software (GraphPad Inc., La Jolla, CA, USA). All comparisons were made relative to the control groups and significance of differences was indicated as \* $P$ <0.05 and \*\* $P$ <0.01.

## Results

**Ophiopogonin-D inhibits MDA-MB-435 cell adhesion and invasion.** In a preliminary experiment, the cytotoxic effects of ophiopogonin-D were determined using an MTT assay (data not shown). Ophiopogonin-D demonstrated no obvious cytotoxicity on MDA-MB-435 cells under 80  $\mu$ M. Concentrations of ophiopogonin-D (5, 10, 20, 40 and 80  $\mu$ M) with no apparent cytotoxicity on MDA-MB-435 cells were selected for all subsequent experiments. As shown in Fig. 2A, ophiopogonin-D was able to inhibit the adhesion

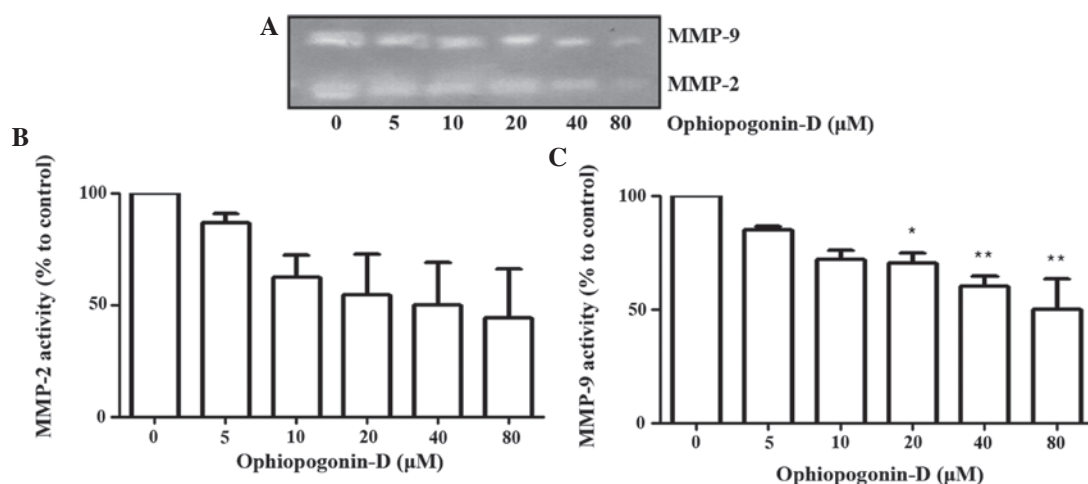


Figure 3. Effect of ophiopogonin-D on the activity of MMP-2/9 detected by (A) gelatin zymography. The relative enzyme activities of (B) MMP-2 and (C) -9 are expressed as percentages relative to control samples, quantified using the Quantity One System. \* $P < 0.05$  and \*\* $P < 0.01$  vs. the control. MMP, matrix metalloproteinase.

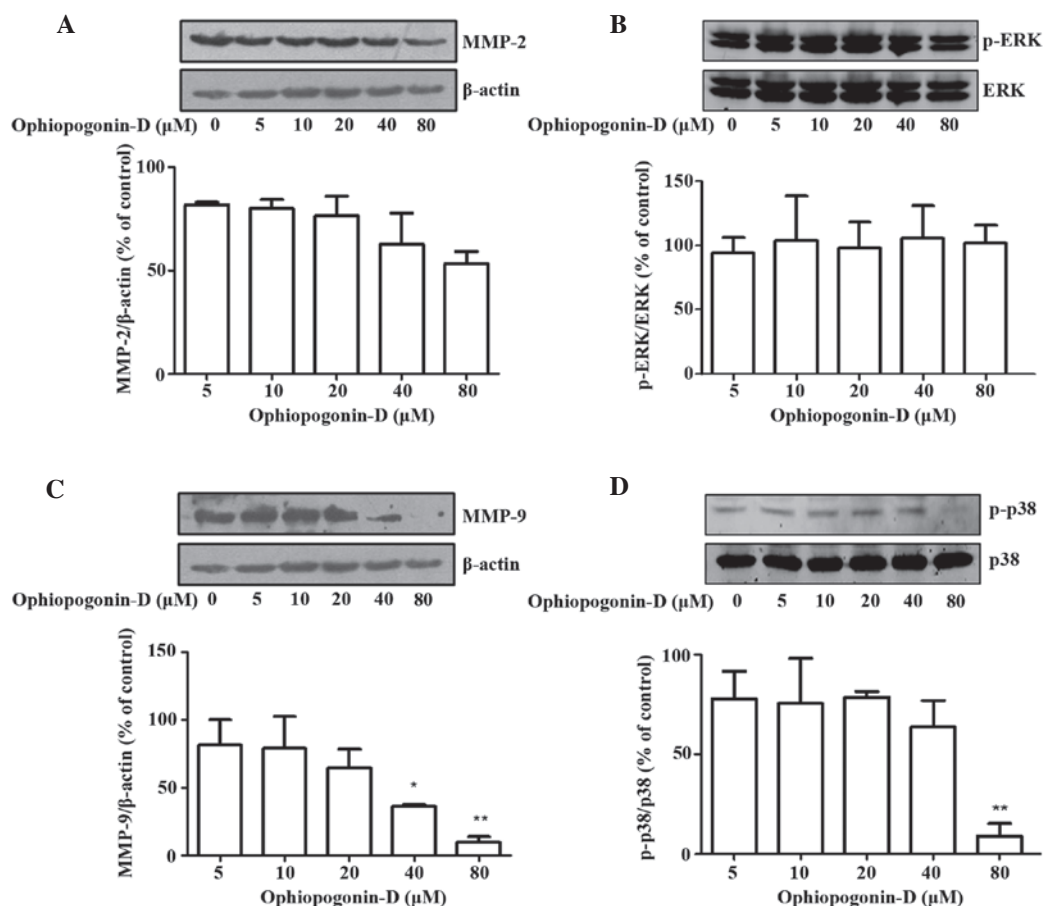


Figure 4. Effect of ophiopogonin-D on protein expression levels of (A) MMP-2, (B) p-ERK/ERK, (C) MMP-9 and (D) p-p38/p38 in MDA-M-435 cells as determined using western blot analysis. The intensity of the bands was densitometrically assessed. Values are expressed as the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$ , vs. control. MMP, matrix metalloproteinase; p-ERK, phosphorylated extracellular signal-regulated kinase.

of MDA-MB-435 cells to HUVECs. Ophiopogonin-D at concentrations of 40 and 80  $\mu$ M significantly reduced the number of MDA-MB-435 cells adhered to HUVECs, and the inhibition rates were ~50 and 65%, respectively. In addition, MDA-MB-435-cell adhesion to fibronectin was detected for more accurate determination of the effect of ophiopogonin-D on the adhesion of melanoma cells. Fig. 2B shows the

inhibitory effect of ophiopogonin-D on the adhesion of MDA-MB-435 cells to fibronectin. At concentrations of 40 and 80  $\mu$ M, ophiopogonin-D significantly inhibited the adhesion of MDA-MB-435 cells to fibronectin at rates of ~64 and 80%, respectively. Furthermore, the effect of ophiopogonin-D on the invasive ability of MDA-MB-435 cells was evaluated using a Transwell assay. Fig. 2C indicates that after treatment



with ophiopogonin-D the invasive capacity of MDA-MB-435 cells was markedly inhibited. The inhibition rate of 80  $\mu$ M of ophiopogonin-D was ~78%.

*Ophiopogonin-D decreases MMP-9, but not MMP-2 enzyme activity.* The expression of MMP-2 and MMP-9 has been reported to have a critical role in degrading the basement membrane in tumor invasion and migration (33). A gelatin zymography assay showed that the activity of MMP-9 was reduced with increasing concentration of ophiopogonin-D (Fig. 3). MMP-9 was obviously decreased following incubation with 40 and 80  $\mu$ M ophiopogonin-D, while there was no obvious decrease in MMP-2. This result indicated that ophiopogonin-D mostly inhibits tumor metastasis through MMP-9, not MMP-2.

*Effect of ophiopogonin-D on the expression of MMP-2/9 and MAPKs.* MMPs have significant roles in cancer metastasis. Furthermore, the activity of MMP-2/9 is associated with the adhesion, invasion and angiogenesis of cancer metastasis (34,35). Therefore, the effect of ophiopogonin-D on the expression of MMP-2 and MMP-9 was detected by western blot analysis. The results indicated that ophiopogonin-D inhibited the expression of MMP-9 at concentrations of 40 and 80  $\mu$ M (Fig. 4). With increasing ophiopogonin-D concentration, the expression of MMP-9 declined, while there was no obvious decrease in MMP-2 expression. The result of the western blot analysis corresponded with the results of the adhesion and invasion experiments. Furthermore, the expression and phosphorylation of ERK1/2 and p38/MAPK were examined to assess whether the MAPK signaling pathway was involved in the anti-metastatic effect of ophiopogonin-D in melanoma cells (Fig. 4). The phosphorylation of p38 was inhibited by ophiopogonin-D at the concentration of 80  $\mu$ M, while the phosphorylation of ERK was not inhibited by ophiopogonin-D. Further investigation is required to fully elucidate the underlying mechanism of the anti-metastatic effect of ophiopogonin-D on melanoma cells.

## Discussion

Tumor growth, invasion and metastasis are multistep and complex processes that include cell division and proliferation, proteolytic digestion of the extracellular matrix, cell migration through basement membranes to reach the circulatory system, as well as re-migration and growth of tumors at the metastatic sites (36). The present study demonstrated that ophiopogonin-D, derived from the root of *Ophiopogon japonicus*, was able to inhibit the metastatic process in MDA-MB-435 cancer cells. It was found that ophiopogonin-D inhibited the proliferation, adhesion and invasion of MDA-MB-435 cells. Furthermore, the expression of MMP-9 and the activation of p38 were inhibited by ophiopogonin-D.

MMPs have a significant role in cancer metastasis (37). The activities of MMP-2 and MMP-9 are associated with the adhesion, invasion and angiogenesis of cancer metastasis (38,39). Invasiveness inhibitors may act by downregulating extracellular barrier-degrading proteinases, including MMP-2 and/or MMP-9 (40). To further explore the mechanism of inhibition of invasion induced by ophiopogonin-D, the present study

examined the effects of ophiopogonin-D on the expression and activity of MMP-2 and -9 in MDA-MB-435 human breast cancer cells. The results showed that ophiopogonin-D was able to inhibit the expression of MMP-9 and zymographic assays showed full development of the gelatinolytic potential of MMP-9 in the presence of invasion-restraining concentrations of ophiopogonin-D. It was demonstrated that ophiopogonin-D was able to concentration-dependently inhibit the secretion of MMP-9 in MDA-MB-435 cells. However, ophiopogonin-D had no obvious effects on the expression and secretion of MMP-2 in MDA-MB-435 cells. MMP-9, but not MMP-2, has an important role in the mechanism of the anti-tumor metastatic action of ophiopogonin-D. The results of the western blot analysis corresponded with the results of the adhesion and invasion assays.

To assess whether the MAPK signaling pathway is involved in the mechanism of action of ophiopogonin-D, the present study examined the expression and phosphorylation of ERK1/2 and p38 MAPK in ophiopogonin-D-treated cells. The expression of p-p38 was inhibited by ophiopogonin-D, but not the expression of p-ERK. The results indicated that ophiopogonin-D inhibited cell metastasis, likely through inactivation of p38, but not ERK1/2. It has been reported that a p38 pathway inhibitor was able to reduce MMP-9 expression and secretion, as well as *in vitro* invasion of cancer cells (41,42). According to the results of the present study, ophiopogonin-D was able to reduce the expression and secretion of MMP-9 and the expression of p38/MAPK, and inhibit the invasion of MDA-MB-435 cells. Therefore, it is concluded that ophiopogonin-D has the ability to act as a p38 inhibitor to impede tumor metastasis. Further investigation is required to fully elucidate the detailed mechanism of ophiopogonin-D on cancer metastasis, particularly with regard to the MAPK pathway and additional kinases.

In conclusion, the present study demonstrated that ophiopogonin-D, derived from the root of *Ophiopogon japonicus*, was able to inhibit the metastatic capacity of MDA-MB-435 cancer cells. Ophiopogonin-D effectively inhibited the proliferation, adhesion and invasion of MDA-MB-435 cells. Furthermore, the expression of MMP-9 was inhibited by ophiopogonin-D. The present study suggested that ophiopogonin-D inhibits tumor metastasis through downregulation of MMP-9 and suppression of the p38/MAPK pathway. Hence, ophiopogonin-D is suggested to be a therapeutic agent for inhibiting the metastasis and invasion of cancer.

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