# Ophiopogonin D inhibits cell proliferation and induces apoptosis of human laryngocarcinoma through downregulation of cyclin B1 and MMP-9 and upregulation of p38-MAPK signaling

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**Abstract.** The pharmacological actions of Ophiopogonin D include resistance to cardiovascular and cerebrovascular diseases, anti-aging effects, improvement in learning deficit and dysmnesia, anti-tumor, anti-radiation and anti-inflammatory effects, immunoregulation, and the relief of cough and hepatopulmonary pathological lesions. However, the efficacy of Ophiopogonin D on human laryngocarcinoma remains to be elucidated. The present study therefore investigated whether the anti-cancer effect of Ophiopogonin D inhibits cell proliferation and induces apoptosis of human laryngocarcinoma. In the present study, it was found that Ophiopogonin D inhibited cell proliferation, promoted cytotoxicity, induced apoptosis and increased caspase-3/9 activity in human laryngocarcinoma cells. Ophiopogonin D significantly suppressed cyclin B1 and matrix metalloproteinase-9 (MMP-9) protein expression, and upregulated p-p38 MAPK protein expression in human laryngocarcinoma cells. Together, these results suggest Ophiopogonin D inhibits cell proliferation and induced apoptosis in human laryngocarcinoma cells through downregulation of cyclin B1 and MMP-9 and upregulation of the p38 MAPK signaling pathway. Therefore, Ophiopogonin D may be a potential therapy for the treatment of human laryngocarcinoma.

## Introduction

Laryngocarcinoma is a common head and neck malignant tumor that mostly presents as squamous cell carcinoma (1). In addition, the morbidity rate in northern China is increased compared with that in southern China (2). The morbidity rate has increased over the past 40 years, and laryngocarcinoma results in a serious effect on the quality of life and human health. At present, surgery remains the primary therapeutic method for laryngocarcinoma. However, lymphatic metastasis is an essential factor that affects the prognosis of laryngocarcinoma (3). Supraglottic and glottic cancer account for the vast majority of cases of laryngocarcinoma (3), and supraglottic laryngocarcinoma is more prone to metastasize than glottic laryngocarcinoma (1). Generally, the lower the differentiation degree of the tumor, the earlier the occurrence of neck lymph node metastasis will be. The course of lymphatic metastasis is consistent with the flow of laryngopharyngeal lymph (4). The lymphatic vessels at in the supraglottic region are abundant, and the differentiation degree of supraglottic laryngocarcinoma is low; thus, the incidence rate of cervical nodal metastases is high (1).

Cell cycle regulation is closely associated with tumor development. The process of cell cycle regulation is complicated (5), and this regulation is involved in almost all biological effects of oncogenes and cancer suppressor genes (5). Cell cycle cytokines may reflect the progression of tumors from the aspect of cell cycle kinetics (6). Numerous oncogenes and cancer suppressor genes are directly involved in cell cycle regulation, and are main components of the cell cycle regulation checkpoints. Cell cycle regulation occurs mainly via two key limiting points, G1/S and G2/M (7). The main regulation factors at the G2/M phase include cyclin B, cyclin-dependent kinase 1, also termed cell division cycle (Cdc) 2, cyclin-dependent kinase 2 and Cdc25C (8).

Matrix metalloproteinases (MMPs) are a type of proteolytic enzyme closely associated with tumor invasion and metastasis (9). MMP-2 and MMP-9 are important members of the MMP family (9). They can effectively degrade collagen IV, the main component of the extracellular matrix and basilar membrane, thereby destroying the integrity of the basilar membrane (10). This process is closely associated with tumor infiltration and metastasis (11). Previous studies have shown that MMP-2 and MMP-9 are overexpressed in human malignancies, and promote the infiltration and metastasis of tumor cells; this is associated with the prognosis of the patient (9,11). A previous study also demonstrated that MMP-2 and MMP-9 are overexpressed in laryngocarcinoma tissue, and they are associated with lymphatic metastasis and poor prognosis (12).

Mitogen-activated protein kinase (MAPK) is an important signaling pathway in cells, mainly involving c-Jun

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NH2-terminal kinase/stress-activated protein kinase, extracellular signal regulated protein kinase and p38-MAPK (13). This pathway has a close association with cell proliferation, differentiation and apoptosis. The p38-MAPK signaling pathway has complex effects on tumors (14). For example, transient activation of p38-MAPK can promote tumor cell proliferation (15). Maintaining a long-term activated state of phosphorylated p38-MAPK induces tumor cells apoptosis via the tumor necrosis factor-α pathway (15).

*Ophiopogon japonicus* is a herb used as a Traditional Chinese Medicine, and is considered a yin-nourishing drug (16). With the deepening of investigation into *O. japonicus*, the pharmacological actions of Ophiopogonin D, the major active component in *O. japonicus*, have been identified (17). Ophiopogonin D has significant pharmacological effects and has anti-aging properties, improves learning deficit and dysmnesia, increases resistance to cardiovascular and cerebrovascular diseases, and has anti-tumor, anti-inflammatory and immunoregulatory effects (16,18,19). The aim of the present study was to investigate whether the anti-cancer effect of Ophiopogonin D inhibits cell proliferation and induced apoptosis of human laryngocarcinoma, and may therefore be a potential therapy for the treatment of laryngocarcinoma.

**Materials and methods**

*Cell culture.* The human laryngocarcinoma AMC-HN-8 cell line was purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in complete RPMI-1640 with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin, 100 mg/ml streptomycin and 1 mM sodium pyruvate (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C and in a 5% $CO_2$ atmosphere. Ophiopogonin D (≥98%; Fig. 1) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

*MTT assay and lactate dehydrogenase (LDH) assay.* AMC-HN-8 cells ($5x10^6$ cells/well) were plated into a 96-well plate for 24 h at 37°C. The cultured cells were treated with various concentrations of Ophiopogonin D (0, 12.5, 25 and 50 $\mu$mol/l) for 12 or 24 h. Subsequently, 20 $\mu$l MTT (5 mg/ml) was added daily to each well and incubated for an additional 4 h at 37°C. Dimethyl sulfoxide (200 $\mu$l) was added to each well and dissolved for 10 min, and the absorbance at 490 nm was measured using a plate reader (Synergy-HT; BioTek Instruments, Inc., Winooski, VT, USA).

*LDH assay.* AMC-HN-8 cells ($5x10^6$ cells/well) were plated onto a 96-well plate for 24 h culture, and were then treated with various concentrations of Ophiopogonin D (0, 12.5, 25 and 50 $\mu$mol/l) for 12 or 24 h. Subsequently, 60 $\mu$l LDH (Beyotime Institute of Biotechnology, Haimen, China) was added daily to each well and incubated for an additional for 30 min at room temperature, and the absorbance at 490 nm was measured using a plate reader (Synergy-HT; BioTek Instruments, Inc.).

*Apoptosis assay.* AMC-HN-8 cells ($5x10^6$ cells/well) were plated onto a 6-well plate for 24 h and were treated with
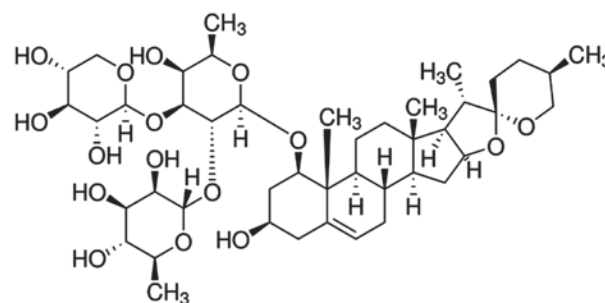


Figure 1. Chemical structure of Ophiopogonin D (C44H70O16).

various concentrations of Ophiopogonin D (0-50 $\mu$mol/l) for 24 h. AMC-HN-8 cells was stained with Annexin V (BD Biosciences, Franklin Lakes, NJ, USA) and propidium iodide (PI) (BD Biosciences) in darkness for 30 min. Apoptosis was quantitated with the Guava EasyCyte™ flow cytometer (Becton Dickinson, Bedford, MA).

*DAPI assay.* AMC-HN-8 cells ($5x10^6$ cells/well) were plated into a 6-well plate for 24 h and were treated with various concentrations of Ophiopogonin D (0, 12.5, 25 and 50 $\mu$mol/l) for 24 h at 37°C. AMC-HN-8 cells was stained with DAPI assay (Beyotime Institute of Biotechnology) for 1 h. Images were captured using a DP70 fluorescence microscope (magnification, x20; Olympus, Tokyo, Japan).

*Caspase-9/3 activity analysis.* AMC-HN-8 cells ($5x10^6$ cells/well) were plated into a 6-well plate for 24 h and cultured were treated with different concentrations of Ophiopogonin D (0-50 $\mu$mol/l) for 24 h. Cell pellets were collected and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors cocktail. The supernatant was collected after centrifuged at 13,000 x g for 10 min at 4°C and the protein concentration measured using a bicinchoninic acid (BCA) kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Equal concentrations of protein were incubated with Ac-DEVD-pNA (caspase-3) and Ac-LEHD-pNA (caspase-9) at 37°C for 2 h in the dark, and the absorbance at 405 nm was measured using a plate reader (Synergy-HY; BioTek Instruments, Inc.).

*Western blot analysis.* AMC-HN-8 cells ($5x10^6$ cells/ well) were plated into a 6-well plate for 24 h and cultured were treated with various concentrations of Ophiopogonin D (0, 12.5, 25 and 50 $\mu$mol/l) for 24 h at 37°C. Cell pellets were collected and lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology) containing 0.5 mM PMSF and protease inhibitor cocktail. The supernatant was collected after centrifugation at 13,000 x g for 10 min at 4°C and the protein concentration was measured using a BCA kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Equal protein amounts from each sample (50 $\mu$g) were electrophoresed on 8-15% SDS-PAGE gels and electrotransferred onto a nitrocellulose membrane (0.22 mm; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Nitrocellulose membrane was incubated with antibodies
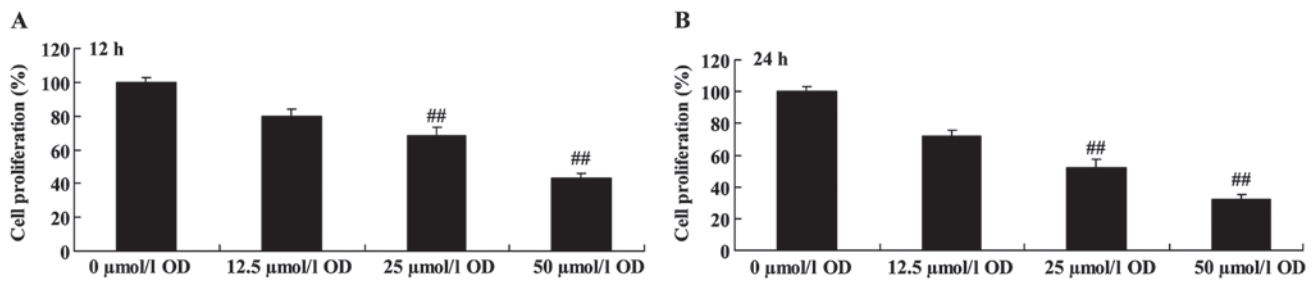
Figure 2. Ophiopogonin D inhibits cell proliferation of human laryngocarcinoma AMC-HN-8 cells subsequent to (A) 12 h and (B) 24 h of treatment. ##P<0.01 compared with the 0 $\mu$mol/l OD group; 0 $\mu$mol/l OD, 0 $\mu$mol/l Ophiopogonin D; 12.5 $\mu$mol/l OD, 12.5 $\mu$mol/l Ophiopogonin D; 25 $\mu$mol/l OD, 25 $\mu$mol/l Ophiopogonin D; 50 $\mu$mol/l OD, 50 $\mu$mol/l Ophiopogonin D.
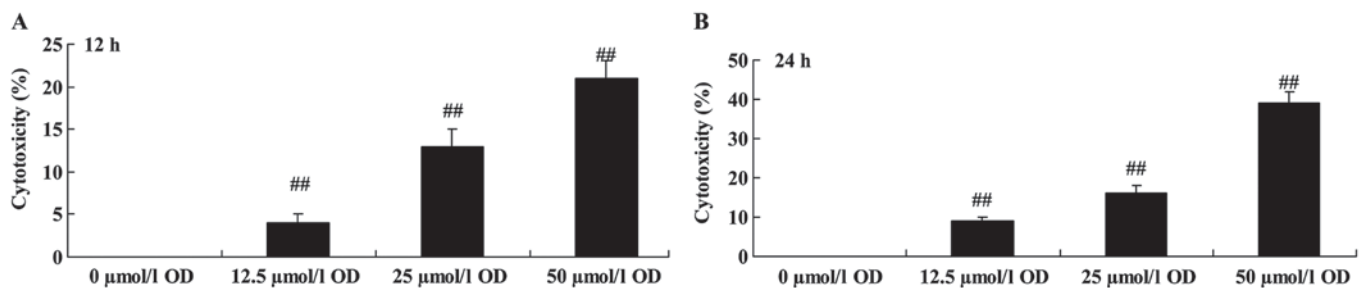


Figure 3. Ophiopogonin D promotes cytotoxicity of human laryngocarcinoma AMC-HN-8 cells subsequent to (A) 12 h and (B) 24 h of treatment. ##P<0.01 compared with the 0 $\mu$mol/l group; 0 $\mu$mol/l OD, 0 $\mu$mol/l Ophiopogonin D; 12.5 $\mu$mol/l OD, 12.5 $\mu$mol/l Ophiopogonin D; 25 $\mu$mol/l OD, 25 $\mu$mol/l Ophiopogonin D; 50 $\mu$mol/l OD, 50 $\mu$mol/l Ophiopogonin D.

against cyclin B1 (sc-594), MMP-9 (sc-10737), p-p38 MAPK (sc-101759) and GAPDH (sc-25778), purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), at a dilution of 1:500 overnight at 4°C. Membranes were blocked with 5% skim milk powder, and incubated with horseradish peroxidase-conjugated secondary antibody (sc-2004, 1:5,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37°C for 1 h. The relative protein expression was observed with chemiluminescence kit (GE Healthcare Life Sciences) and analyzed using an Odyssey Two-Color Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

*Statistical analysis.* Statistical data are expressed as the mean ± standard error. Comparisons were made using one/two-way analysis of variance followed by the Tukey post hoc test for multiple comparisons. P<0.05 was used to indicate a statistically significant difference.

## Results

*Ophiopogonin D inhibits cell proliferation of human laryngocarcinoma AMC-HN-8 cells.* To investigate the effect of Ophiopogonin D on laryngocarcinoma cell proliferation, AMC-HN-8 cells were treated with different concentrations of Ophiopogonin D (0-50 $\mu$mol/l) for 12 or 24 h. After treatment with Ophiopogonin D, the inhibition of cell proliferation in human laryngocarcinoma AMC-HN-8 cells was found to be time and dose-dependent (Fig. 2). In particular, Ophiopogonin D (25 and 50 $\mu$mol/l) significantly suppressed the cell proliferation in human laryngocarcinoma AMC-HN-8 cells at 12 and 24 h, compared with the control (0 $\mu$mol/l Ophiopogonin D; Fig. 2).
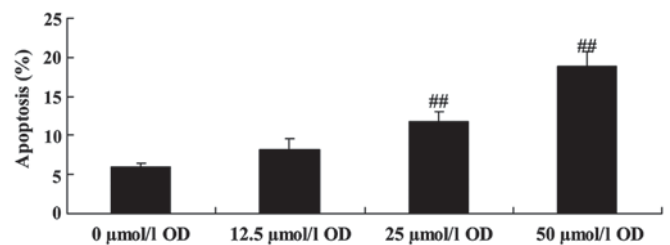


Figure 4. Ophiopogonin D induces apoptosis of human laryngocarcinoma AMC-HN-8 cells. ##P<0.01 compared with the 0 $\mu$mol/l OD group; 0 $\mu$mol/l OD, 0 $\mu$mol/l Ophiopogonin D; 12.5 $\mu$mol/l OD, 12.5 $\mu$mol/l Ophiopogonin D; 25 $\mu$mol/l OD, 25 $\mu$mol/l Ophiopogonin D; 50 $\mu$mol/l OD, 50 $\mu$mol/l Ophiopogonin D.

*Ophiopogonin D is cytotoxic in human laryngocarcinoma AMC-HN-8 cells.* To analyze the anti-cancer effect of Ophiopogonin D on laryngocarcinoma cells, the LDH assay was performed. Following treatment with Ophiopogonin D (50 $\mu$mol/l) for 12 h, the cytotoxicity rate of human laryngocarcinoma AMC-HN-8 cells was effectively promoted, compared with the control (0 $\mu$mol/l Ophiopogonin D; Fig. 3). Subsequent to treatment with 25 and 50 $\mu$mol/l Ophiopogonin D for 24 h, the cytotoxicity rate of human laryngocarcinoma AMC-HN-8 cells was also increased, compared with the control (0 $\mu$mol/l Ophiopogonin D; Fig. 3).

*Ophiopogonin D induces apoptosis of human laryngocarcinoma AMC-HN-8 cells.* Annexin V/PI staining was performed to qualify the efficacy of Ophiopogonin D on AMC-HN-8 cells apoptosis. As shown in Fig. 4, 25 or 50 $\mu$mol/l Ophiopogonin D treatment significantly induces apoptosis of

human laryngocarcinoma AMC-HN-8 cells, compared with the control (0 $\mu$mol/l Ophiopogonin D).

*Ophiopogonin D induces degradation of the nucleolus in human laryngocarcinoma AMC-HN-8 cells*. The anti-cancer effect of Ophiopogonin D on the nucleoli of AMC-HN-8 cells was detected using DAPI assay. Compared with the control 0 $\mu$mol/l Ophiopogonin D, cell apoptosis was observed in the nucleoli of AMC-HN-8 cells in the 5 or 50 $\mu$mol/l Ophiopogonin D treated group (Fig. 5).

*Ophiopogonin D induces caspase-3/9 activity in human laryngocarcinoma AMC-HN-8 cells*. Commercially available PEDF ELISA kits were used to analyze the effect of Ophiopogonin D on caspase-3/9 activity in of AMC-HN-8 cells. Treatment with Ophiopogonin D (25 or 50 $\mu$mol/l) significantly induced caspase-3/9 activity in AMC-HN-8 cells, compared with the control (0 $\mu$mol/l Ophiopogonin D; Fig. 6).

*Ophiopogonin D downregulates cyclin B1 and MMP-9 protein expression in human laryngocarcinoma AMC-HN-8 cells*. Furthermore, in order to investigate whether the molecular mechanism of Ophiopogonin D on human laryngocarcinoma AMC-HN-8 cells, cyclin B1 protein expression was measured using western blot analysis. Ophiopogonin D (25 or 50 $\mu$mol/l) significantly suppressed the protein of cyclin B1 and MMP-9 expression level in AMC-HN-8 cells, compared with control 0 $\mu$mol/l of Ophiopogonin D (Fig. 7).

*Ophiopogonin D upregulates p38-MAPK protein expression in human laryngocarcinoma AMC-HN-8 cells*. To analyze whether Ophiopogonin D upregulates p38-MAPK protein expression of human laryngocarcinoma AMC-HN-8 cells, the present study also examined p-p38-MAPK protein expression of AMC-HN-8 cells. As shown in Fig. 8, 25 or 50 $\mu$mol/l of Ophiopogonin D treatment significantly activated p-p38-MAPK protein expression of AMC-HN-8 cells, compared with the control 0 $\mu$mol/l of Ophiopogonin D.

## Discussion

Laryngocarcinoma is a common malignant tumor of the ear, nose and throat, head and neck. It is most commonly diagnosed as squamous cell carcinoma (3). Laryngocarcinoma is divided into supraglottic, glottic, subglottic and across glottis types. Supraglottic laryngocarcinoma is most frequently observed in certain regions of China (Eastern, Southern and Northern) (20). Studies on the genesis and development mechanism of laryngocarcinoma, particularly fundamental studies on laryngocarcinoma, are of great significance for clinical effective prevention and control (1,20). The present data indicate that Ophiopogonin D inhibits cell proliferation, promotes cytotoxicity, induces apoptosis and increases the caspase-3/9 activity of human laryngocarcinoma cells. Furthermore, the present study provided evidence that Ophiopogonin D may be a potential antitumor compound in human laryngocarcinoma cells.

Cyclin B1 protein, as a regulative subunit of the kinase Cdc2, forms active compounds, including mitosis-promoting factor (also termed M phase-promoting factor), with Cdc2 (21).

These compounds mainly participate in transformation regulation at the G2/M phase and regulate the activation and distribution of multiple microtubule germinal-associated proteins, such as CENP-E and Eg5 (22). The compound also participates in spindle formation, chromosome segregation and further produces a series of actions. Cyclin then degrades and inactivates via dependent protease hydrolysis (23). The present results have shown that Ophiopogonin D significantly suppressed the level of cyclin B1 protein expression in AMC-HN-8 cells. Zang *et al* (18) suggested that Ophiopogonin D inhibits MCF-7 cell growth through downregulation of cyclin B1 at the G2/M phase. Therefore, the effect of Ophiopogonin D on AMC-HN-8 cells may be associated with cyclin B1 expression.

MMPs belong to an endopeptidase family dependent on zinc. This family is named due to its activity on ECM endopeptidase (24). MMP-2 and MMP-9 are important members of the family. MMP-2 and MMP-9 can be expressed on a variety of cells, mainly consisting of inflammatory cells, mesenchymal cells in tumor tissues and tumor cells (25). Numerous studies have shown that MMP-2 and MMP-9 are overexpressed in the majority of human malignancies, which is associated with the infiltration and metastasis of tumor cells and prognosis of the patient (24,25). Previous studies have shown that MMP-2 and MMP-9 are also overexpressed in laryngocarcinoma tissue, and they are associated with lymphatic metastasis and poor prognosis (10). The present results demonstrated that Ophiopogonin D significantly downregulates MMP-9 protein expression in AMC-HN-8 cells. Zhang *et al* (26) suggested that Ophiopogonin-D suppresses MDA-MB-435 cell adhesion and invasion through inhibition of phosphorylation of the p38 and MMP-9 pathway, not the MMP-2 pathway. Therefore, MMP-9 expression may have a crucial role in Ophiopogonin-D-induced apoptosis within laryngocarcinoma AMC-HN-8 cells.

Studies show that the occurrence of laryngocarcinoma is associated with MAPK signal transduction. p38-MAPK is an important member of the MAPK family (27). It participates in cell proliferation, apoptosis and differentiation, and has an important role on cell apoptosis process (15). The genesis and development, rapid proliferation and indeterminate expression of p38-MAPK in laryngocarcinoma are closely associated with metastasis (28). p38-MAPK participates in the process of laryngocarcinoma and carcinogenic process of certain organics. These results suggest that Ophiopogonin D treatment significantly activated p-p38-MAPK protein expression in AMC-HN-8 cells. Zhang *et al* (26) suggested that Ophiopogonin D suppresses MDA-MB-435 cell adhesion and invasion through inhibition of phosphorylation of p38 and MMP-9 pathway. P38-MAPK pathways may also be associated with the antitumor activity of Ophiopogonin D on laryngocarcinoma. Additional studies are required to examine the detailed pharmacological mechanisms that underlie the action of Ophiopogonin D *in vivo*.

To conclude, the present study demonstrated that Ophiopogonin D could inhibit cell proliferation, promoted cytotoxicity, induced apoptosis and increased the activity of caspase-3/9 in human laryngocarcinoma cells. The potential mechanism underlying the antitumor effects of Ophiopogonin D may result from a downregulatory effect
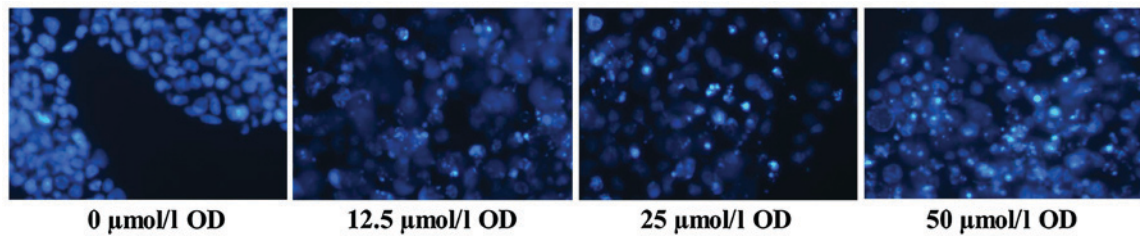
Figure 5. Ophiopogonin D induces degradation of the nucleolus of human laryngocarcinoma AMC-HN-8 cells. 0 µmol/l OD, 0 µmol/l Ophiopogonin D; 12.5 µmol/l OD, 12.5 µmol/l Ophiopogonin D; 25 µmol/l OD, 25 µmol/l Ophiopogonin D; 50 µmol/l OD, 50 µmol/l Ophiopogonin D.
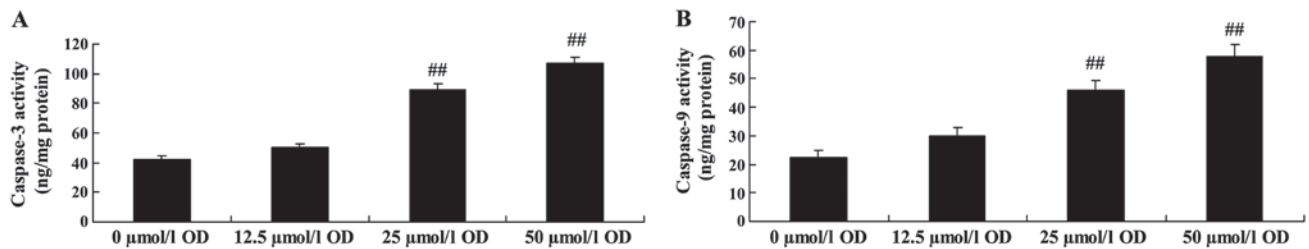


Figure 6. Ophiopogonin D induced (A) caspase-3 and (B) caspase-9 activity in human laryngocarcinoma AMC-HN-8 cells. ##P<0.01 compared with the 0 µmol/l OD group; 0 µmol/l OD, 0 µmol/l Ophiopogonin D; 12.5 µmol/l OD, 12.5 µmol/l Ophiopogonin D; 25 µmol/l OD, 25 µmol/l Ophiopogonin D; 50 µmol/l OD, 50 µmol/l Ophiopogonin D.
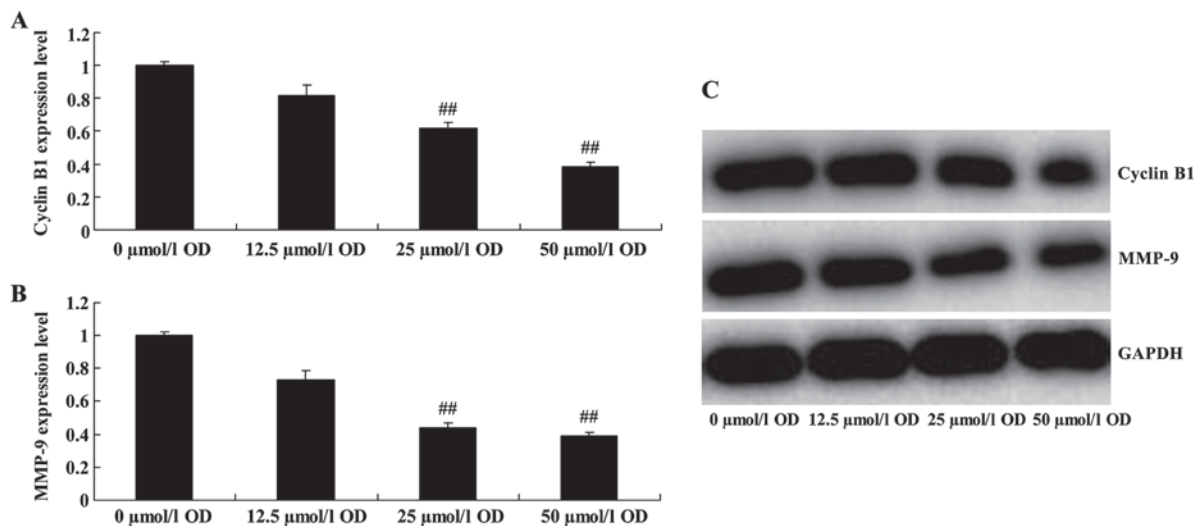


Figure 7. Ophiopogonin D downregulates (A) cyclin B1 and (B) MMP-9 protein expression in human laryngocarcinoma AMC-HN-8 cells, as shown by (A and B) quantitative analysis and (C) western blotting of cyclin B1 and MMP-9 protein expression. ##P<0.01 compared with the 0 µmol/l OD group; 0 µmol/l OD, 0 µmol/l Ophiopogonin D; 12.5 µmol/l OD, 12.5 µmol/l Ophiopogonin D; 25 µmol/l OD, 25 µmol/l Ophiopogonin D; 50 µmol/l OD, 50 µmol/l Ophiopogonin D.
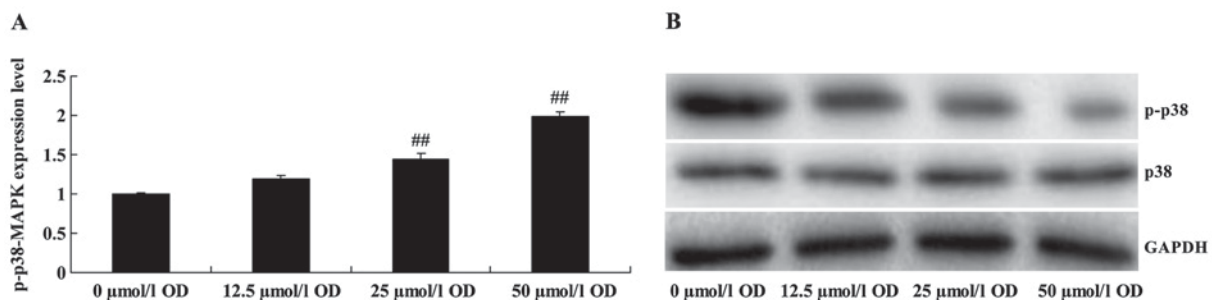


Figure 8. Ophiopogonin D upregulates p38-MAPK protein expression in human laryngocarcinoma AMC-HN-8 cells, as shown by (A) quantitative analysis and (B) western blotting of p-p38-MAPK protein expression. ##P<0.01 compared with the 0 µmol/l OD group; 0 µmol/l OD, 0 µmol/l Ophiopogonin D; 12.5 µmol/l OD, 12.5 µmol/l Ophiopogonin D; 25 µmol/l OD, 25 µmol/l Ophiopogonin D; 50 µmol/l OD, 50 µmol/l Ophiopogonin D.

upon cyclin B1/MMP-9 expression and suppression of p38-MAPK, which ultimately induces cellular apoptosis in AMC-HN-8 cells.

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