

Paeoniflorin inhibits proliferation and induces apoptosis of human glioma cells via microRNA-16 upregulation and matrix metalloproteinase-9 downregulation

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Abstract. Paeoniflorin is one of the active ingredients of the commonly used herbal medicine derived from *Paeonia*, which exhibits anticancer properties. MicroRNA-16 (miR-16) is upregulated in CD133⁺ cells, but downregulated in CD133⁺ cells from glioma tissue. Matrix metalloproteinase-9 (MMP-9) expression in glioma tissue samples is significantly higher than that in healthy brain tissue samples. Therefore, miR-16 and MMP-9 expression may be associated with glioma pathogenesis. In the present study, the effects of paeoniflorin on glioma were analyzed. U87 cells were treated with paeoniflorin at 0, 5, 10 and 20 μ M concentrations. The results suggested that paeoniflorin inhibited U87 cell proliferation and accelerated cell apoptosis. In the present study paeoniflorin treatment increased miR-16 expression and reduced MMP-9 protein expression in U87 cells. Additionally, the results of the present study suggested that miR-16 may regulate MMP-9 expression in miR-16-transfected U87 cells. Furthermore, anti-miR-16 antibodies were used in order to investigate the apoptotic effects of paeoniflorin on U87 cells. The results demonstrated that paeoniflorin inhibits proliferation and induces apoptosis of human glial cells, via miR-16 upregulation and MMP-9 downregulation.

Introduction

Glioma is the most common type of intracranial neuroepithelial tumor and the most aggressive primary tumor, exhibiting rapid growth rates (1). Furthermore, the 2-year survival rate for

patients with poorly differentiated glioma is only 10% (2,3). Glioma accounts for 44.6% of tumors in the central nervous system, with high recurrence and mortality rates (4). Survival times are low and mortality rates are high in patients with glioma, and the disease is associated with poor prognosis (5). Furthermore, the outcomes of radiotherapy treatment combined with chemotherapy do not improve patient prognosis (6).

Expression profiling analysis is an effective method used to demonstrate abnormalities in miRNA expression patterns (7). Glioma exhibits a unique miRNA expression profile, which distinguishes it from the surrounding healthy brain tissue. Furthermore, miRNA expression profiles vary between the different stages of glioma (8). Compared with healthy brain tissue, 17 miRNAs, including miR-21, -221, -222, -125b and -10b, are overexpressed in glial cells, whereas 33 miRNAs, including miR-181a/b/c, -124, -137, -7 and -128, are downregulated in glial cells (9). Furthermore, six miRNAs (miR-16, -107, -185, -425, -451 and -486) are upregulated in CD133⁺ cells, but downregulated in tumorous glial stem cells (CD133⁺). Research has demonstrated that miR-16 expression is markedly decreased in glioma cell lines compared with healthy cells and that the upregulation of miR-16 may suppress glioma growth and invasiveness (10,11).

Transcription and expression levels of matrix metalloproteinase (MMP)-2 and -9 are associated with the degree of malignancy in glioma (12). MMP-2 and -9 may be used as an indicator of malignant human brain glioma (13). MMP expression predominantly modulates the local invasiveness of glial cells. Therefore, MMP-2 and -9 expression may reflect the degradation of glioma matrix (14). Studies have demonstrated that U251 multiform glioblastoma expresses MMP-9 during cancer cell invasion (15,16). In addition, MMP tissue inhibitor treatment is capable of decreasing percentage cell invasion from 42 to 10% (17).

Paeoniflorin is an active ingredient of the commonly used herbal medicine derived from *Paeonia* (18). Pharmacological studies have demonstrated that paeoniflorin prevents free radical damage, inhibits intracellular calcium-overload and exhibits anticancer activities, as well as exhibiting a number of biological effects, such as inhibiting cancer cell

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proliferation, improving microcirculation, and prevents oxidation and convulsion (19). Paeoniflorin treatment induces human cervical cancer cell apoptosis via the upregulation of the pro-apoptotic genes, Bax and caspase-3, and the down-regulation of the anti-apoptotic gene, Bcl-2 (20). Paeoniflorin inhibits H₂O₂-induced apoptosis in SH-SY5Y cells, reducing H₂O₂-induced MMP expression changes. Paeoniflorin treatment inhibits cluster of differentiation 147 expression in THP-1 cells and reduces MMP-9 secretion. In a previous study, the levels of transforming growth factor β 1 and thymidylate synthetase were significantly higher in healthy samples compared with paeoniflorin-treated samples, which corresponded with an improvement in sample histology. By contrast, MMP-2 and -9 expression levels demonstrated the opposite results (21).

It is hypothesized that paeoniflorin may be useful for the treatment of glioma. The present study investigated the molecular mechanisms underlying the effects of paeoniflorin on glial cells. In order to test this hypothesis, the effects of different concentrations of paeoniflorin treatment on human glioma cells were analyzed.

Materials and methods

Primary reagents. The chemical structure of paeoniflorin is indicated in Fig. 1. Paeoniflorin (98%; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in physiological saline solution. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum and Lipofectamine 2000® were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). 3-[4,5-dimethylthiazol-2-thiazolyl]-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Beyotime Institute of Biotechnology (Haimen, China).

Cancer cell lines. The U87 glioma cell line was purchased from the cell bank of the Chinese academy of sciences (Shanghai, China). U87 cells were cultured in DMEM, supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C and 5% CO₂.

MTT viability assay. U87 cells (5.0x10³ cells/well) were seeded in 96-well culture clusters and incubated at 37°C and 5% CO₂ in a humidified incubator, for 24 h. Following treatment with different concentrations of paeoniflorin (0, 5, 10 and 20 μ M), cell viability was measured using an MTT assay. MTT (~10- μ l; 10 mg/ml) was added into each well and the wells were incubated at 37°C and 5% CO₂, for 4 h. Subsequently, 200 μ l dimethylsulfoxide was added to each well. The wells were then agitated for 10 min at room temperature. Viable cells were detected using an enzyme-linked immunosorbent assay reader (SpectraMax® M5e, BioTek, USA) at 570 nm.

Caspase-3 activity measurement. U87 cells (5.0x10³ cells/well) were seeded in 96-well culture clusters and incubated at 37°C and 5% CO₂ in a humidified incubator for 24 h. Following treatment with paeoniflorin, A549 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were centrifuged at 16,000 x g for 15 min at 4°C. Caspase-3 activity of cells was measured using a colorimetric caspase-3 assay kit (Beyotime Institute of Biotechnology). Protein extracts

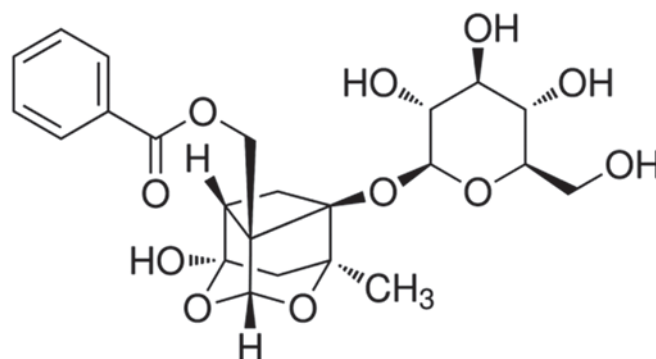


Figure 1. Chemical structure of paeoniflorin.

(50- μ g) were obtained from U87 cells and were incubated and added to a reaction buffer (Tianjin Hualida Biotechnology Co., Ltd., Tianjin, China), containing 85 μ l assay buffer and 10 μ l caspase-3 substrate (Ac-DEVD-pNA) at 37°C for 4-6 h. The change was calculated at 405 nm using a microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Apoptosis assay. Flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) was conducted in order to investigate whether paeoniflorin treatment induced U87 cell apoptosis. Following treatment with paeoniflorin, A549 cells were collected and washed twice with phosphate-buffered saline (PBS). Annexin V-fluorescein isothiocyanate (FITC; 5 μ l; BD Pharmingen, San Diego, CA, USA) was added to the A549 cells and stained using a binding buffer for 30 min in the dark according to the manufacturer's instructions. Subsequently, 10 μ l propidium iodide (PI) was added to the cells and incubated for 15 min at room temperature in the dark. Samples were then analyzed using flow cytometry (FACS Calibur; BD Biosciences).

MMP-9 expression. Gelatin zymography assays were used in order to investigate whether paeoniflorin inhibits MMP-9 expression in U87 cells. Following treatment with paeoniflorin, U87 cells were harvested and MMP-9 protein was electrophoresed on a 10% SDS-PAGE, containing 1% gelatin. Following gel electrophoresis, the gel was washed in 1.5% Triton X-100 (Shanghai Biological Co., Ltd., Shanghai, China) for 0.5-1 h and then washed in water. Gels were incubated in buffer (pH 8.0) at 37°C for 12 h. Gels were then stained with 0.2% Coomassie Brilliant Blue R-250 dye (Qingdao Jacob Chemical Reagent Sales Co., Ltd., Shandong, China) for 1 h. MMP-9 protein expression was then quantified using a MiniBis system (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel) and prestained SDS-PAGE standards (Hou-Bio Tech. Ltd., Shandong, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of miR-16 expression. RT-qPCR was used in order to investigate whether paeoniflorin treatment induced miR-16 expression in U87 cells. Following treatment with paeoniflorin, total RNA was extracted from the cells using TRIzol® reagent according to manufacturer's instructions (Invitrogen Life Technologies). SuperScript® III Reverse Transcriptase (Invitrogen Life Technologies) was used to

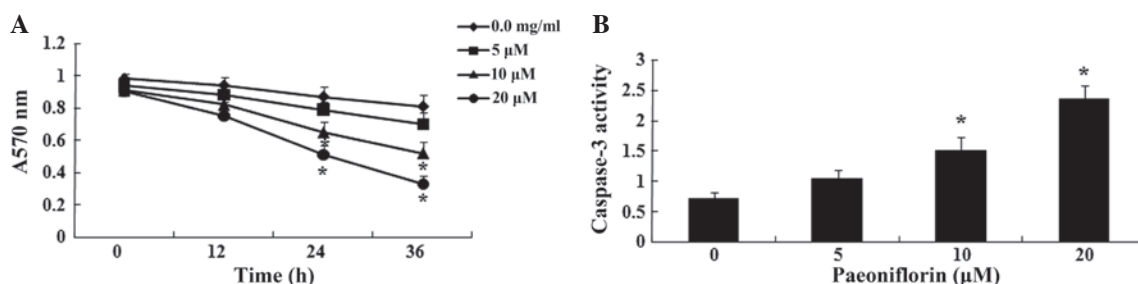


Figure 2. 3-(4,5-Dimethylthiazol-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide analysis and caspase-3 activity. (A) Paeoniflorin treatment inhibited U87 cell growth in a dose-dependent manner and (B) paeoniflorin treatment led to a significant increase in caspase-3 activity. * $P < 0.05$ compared with the 0 μM paeoniflorin treatment group.

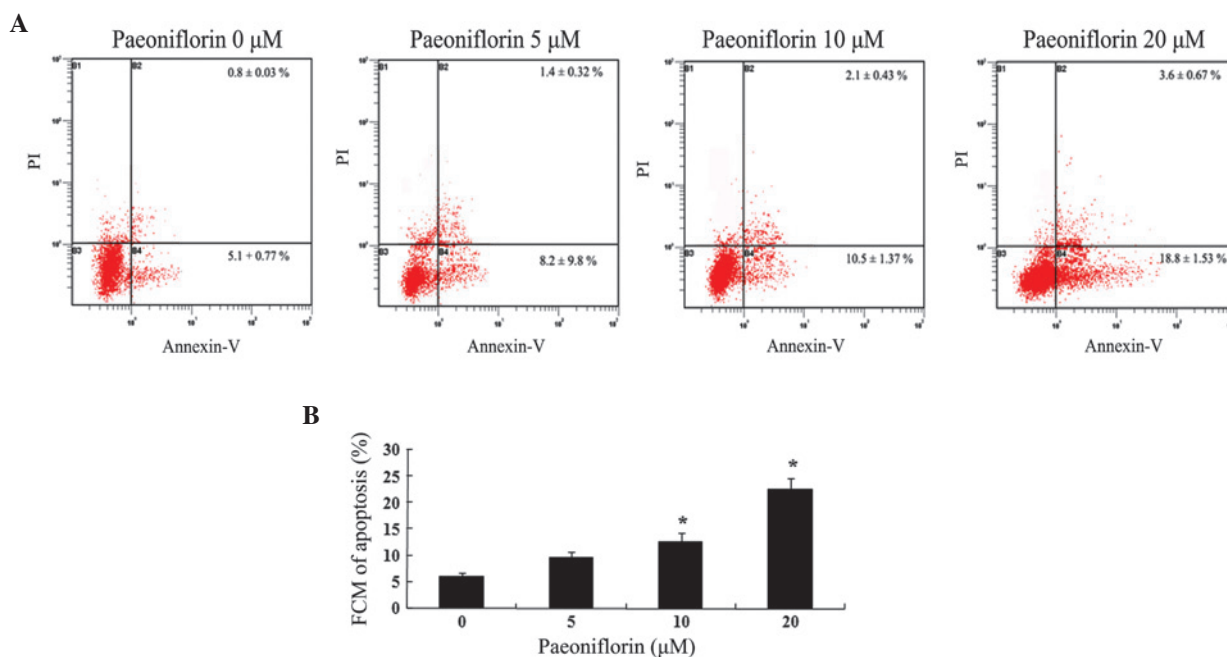


Figure 3. Flow cytometric analysis of cell apoptosis. (A) U87 cell apoptosis increased following paeoniflorin treatment for 24 h in a dose-dependent manner, according to Annexin V-fluorescein isothiocyanate/PI and (B) U87 cell apoptosis following paeoniflorin treatment. * $P < 0.05$ compared with the 0 μM paeoniflorin treatment group. PI, propidium iodide.

analyze cDNA. and subsequently, SYBR[®] Green PCR Master mix (Life Technologies, Grand Island, NY, USA) was used to obtain the final cDNA. miR-16 mRNA expression was quantified using an RT-PCR kit (Invitrogen Life Technologies) according to the manufacturer's instructions and an 7900HT Real-time PCR detection system. The following primers were used: 5'-TAGCAGCACGTAATATTGGC-3' for miR-16; 5'-TGCTGTCGTGGAGTCG-3' for β -actin; U6, forward 5'-CGCTTCGGCACATATACTA-3' and reverse 5'-CGCTTCACGAATTTGCGTGTCA-3'. The cycling conditions were as follows: 94°C for 10 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, followed by 73°C for 5 min.

miR-16 expression and anti-miR-16 transfection. miR-16 precursor and anti-miR-16 (Ambion Life Technologies, Carlsbad, CA, USA) were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). U87 cells (5×10^5 cells/well) were cultured in 6 well plates and transfected with miR-16 precursor/anti-miR-16 (Ambion Life Technologies) using

Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) for 6 h, following treatment with 10 μM paeoniflorin for 24 h. Subsequently, the transfection medium was replaced with DMEM containing 10% fetal bovine serum without antibiotic (Beijing Genetic Company, Beijing, China) in a humidified atmosphere at 37°C with 5% CO_2 for 18 h.

Statistical analysis. Experiments were performed at least three times and data are provided as the mean \pm standard error. Data were analyzed by Student's t-test using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

MTT analysis and caspase-3 activity. In order to determine the effects of paeoniflorin on U87 cells, U87 cell viability was analyzed following treatment with paeoniflorin (0, 5, 10 and 20 μM), using MTT assays. As shown in Fig. 2A, treatment with 10 and 20 μM paeoniflorin for 24 or 36 h significantly reduced

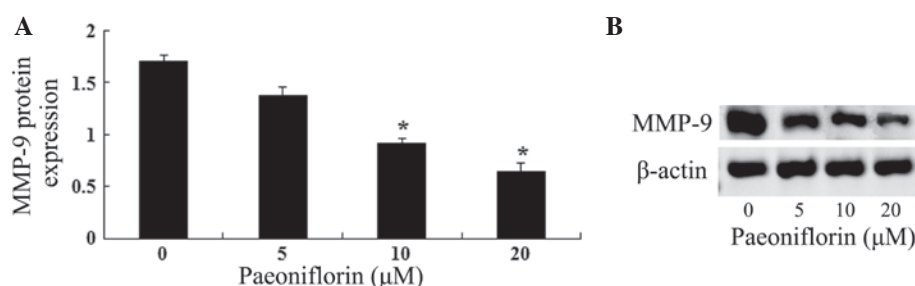


Figure 4. Paeoniflorin-induced inhibition of MMP-9 expression. (A) According to gelatin zymography assays, MMP-9 activity was reduced in U87 cells following treatment with paeoniflorin for 24 h in a dose-dependent manner. (B) MMP-9 protein expression. * $P < 0.05$ compared with the 0 μM paeoniflorin treatment group. MMP-9, matrix metalloproteinase-9.

U87 cell viability compared with control cells ($P < 0.05$). Cell viability decreased in a time- and concentration-dependent manner. Following paeoniflorin treatment (0, 5, 10 and 20 μM), caspase-3 activity in U87 cells was analyzed using a caspase-3 assay kit. As shown in Fig. 2B, treatment with 10 and 20 μM paeoniflorin for 24 h significantly increased caspase-3 activity in U87 cells, compared with 0 μM treatment ($P < 0.05$). Caspase-3 activity increased in a concentration-dependent manner in U87 cells.

Flow cytometric analysis and cell apoptosis. In order to investigate the effect of paeoniflorin on cell apoptosis, U87 cells were treated with different concentrations of paeoniflorin for 24 h. Flow cytometry assays demonstrated that paeoniflorin exerted a dose dependent inhibitory effect on U87 cell growth (Fig. 3A). As demonstrated in Fig. 3B, treatment with 10 and 20 μM paeoniflorin for 24 h significantly increased the U87 cell apoptosis compared with the 0 μM paeoniflorin-treated group ($P < 0.05$).

Paeoniflorin-induced inhibition of MMP-9. In order to investigate the association between paeoniflorin-induced U87 cell growth inhibition and MMP-9 protein expression induction, gelatin zymography assays were conducted. As shown in Fig. 4A, the results of gelatin zymography assays suggested that paeoniflorin inhibited MMP-9 protein expression in a dose-dependent manner. As shown in Fig. 4B, treatment with 10 and 20 μM paeoniflorin for 24 h significantly reduced the MMP-9 protein expression in U87 cells compared with control cells ($P < 0.05$).

Paeoniflorin induces miR-16 expression. As shown in Fig. 5, paeoniflorin treatment promoted miR-16 expression levels in a dose-dependent manner. Treatment with 10 and 20 μM paeoniflorin for 24 h significantly increased miR-16 expression levels in U87 cells, compared with control cells ($P < 0.05$).

Overexpression of miR-16 and MMP-9 expression levels. In order to investigate the association between miR-16 expression and paeoniflorin-induced MMP-9 protein expression, an miR-16 precursor was transfected into U87 cells. As shown in Fig. 6A and B, miR-16 upregulation led to significant inhibition of MMP-9 protein expression.

Anti-miR-16 reverses the antitumor effects of paeoniflorin. An anti-miR-16 antibody was transfected into the U87 cells. The

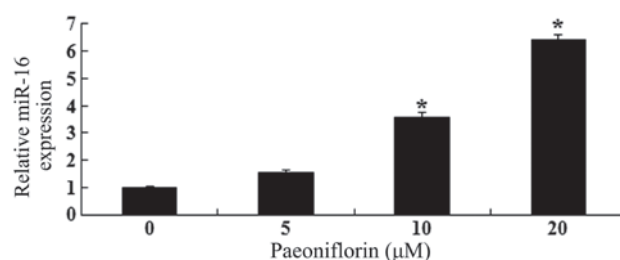


Figure 5. Paeoniflorin activated miR-16 expression. Paeoniflorin treatment increased the expression of miR-16 in a dose-dependent manner. * $P < 0.05$ compared with the 0 μM paeoniflorin treatment group. miR, microRNA.

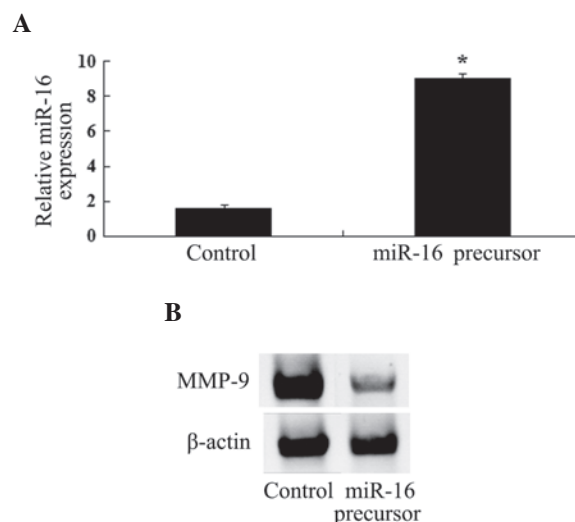


Figure 6. miR-16 overexpression and MMP-9 expression. (A) miR-16 precursor significantly elevated the expression of miR-16 and (B) transfection of miR-16 precursor led to a decrease in MMP-9 protein expression levels. * $P < 0.05$ compared with the 0 μM paeoniflorin treatment group. miR, microRNA.

results indicated that miR-16 expression was significantly lower in anti-miR-16-transfected U87 cells compared with control cells (Fig. 7A). The anti-miR-16 antibody significantly reduced the anticancer effects of paeoniflorin treatment (10 μM) on U87 cell proliferation (Fig. 7B) and U87 cell apoptosis (Fig. 7C) at 24 h. The results of the present study suggested that anti-miR-29b may influence the anticancer effects of paeoniflorin (10 μM) via the downregulation of MMP-9 expression (Fig. 7D).

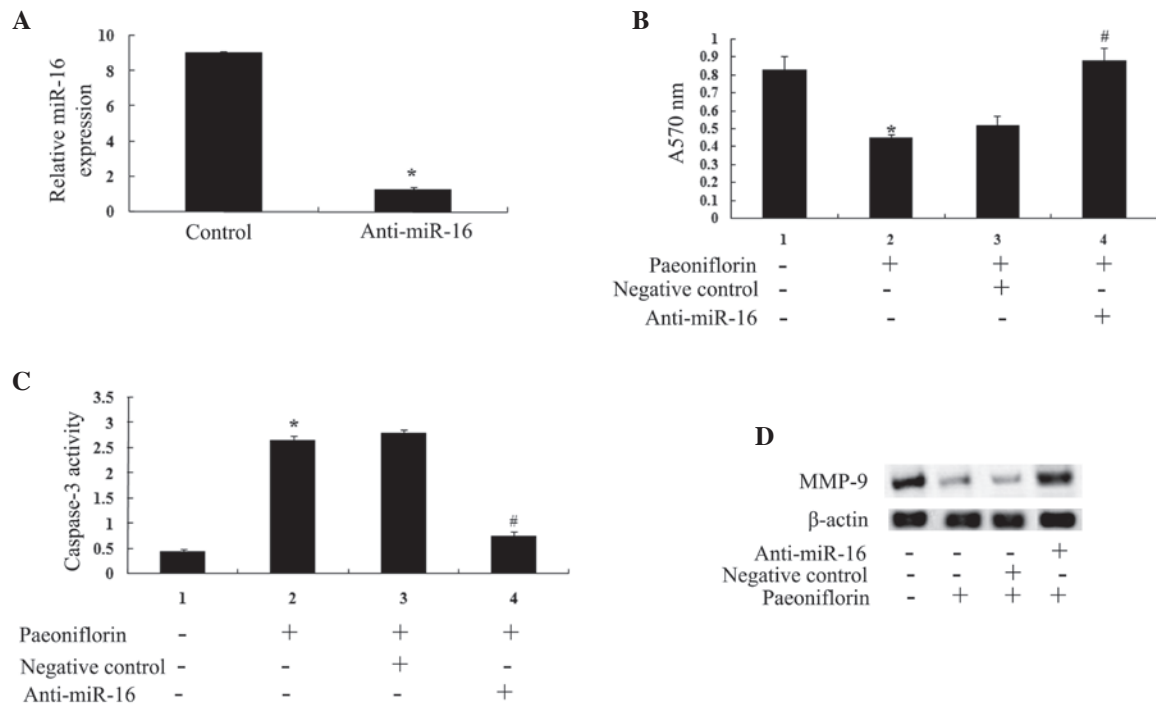


Figure 7. Anti-miR-16 reverses the antitumor effects of paeoniflorin. (A) Anti-miR-16 could significantly diminish the expression of miR-16 in U87 cells; (B) following treatment with paeoniflorin (10 μ M) for 24 h, anti-miR-29b promoted cell proliferation; (C) following treatment with 10 μ M paeoniflorin for 24 h, anti-miR-29b inhibited U87 cell apoptosis; and (D) anti-miR-16 significantly increased MMP-9 activity in U87 cells following 10 μ M paeoniflorin treatment at 24 h. * $P < 0.05$ compared with the 0 μ M paeoniflorin treatment group, and # $P < 0.01$ compared with the paeoniflorin-treated group transfected with negative control. miR, microRNA.

Discussion

Malignant glioma is the most predominant type of primary brain tumor in adults with relatively high rates of recurrences (22). Diffuse glioma cells are able to infiltrate the surrounding brain tissue, which is one of the most important characteristics of glioma (23). Therefore, novel approaches for glioma therapy are required.

Paeoniflorin, an active compound derived from the medicinal herb *Paeonia*, has been shown to exhibit a variety of biological effects (24). Paeoniflorin treatment may increase superoxide dismutase (SOD) levels and reduce malondialdehyde (MDA) content in ischemic brain tissue. It has been suggested that paeoniflorin treatment, following cerebral ischemia, may inhibit the production of free radicals, improve SOD activity and decrease MDA content in the brain. Therefore, paeoniflorin treatment may protect the brain from secondary neuron injury in patients with cerebral ischemia (25). A study has reported that paeoniflorin may modulate multidrug resistance of the human gastric cancer cell line, via the inhibition of nuclear factor (NF)- κ B activation (26). Paeoniflorin treatment may decrease MMP-9 expression levels in human liver carcinoma cells. It inhibits human liver carcinoma cell growth, metastasis and invasion (27). The results of the present study suggested that paeoniflorin may be an effective agent for the inhibition of proliferation and induction of apoptosis in U87 cells.

The upregulation of MMP-9 expression and the down-regulation of p16 expression in glioma may be associated with tumor invasiveness. MMP-9 expression was shown to be lower

in non-malignant astrocytoma cells, than in anaplastic astrocytoma and glioblastoma multiforme cells exhibiting high levels of malignancy (28). High levels of MMP-9 expression may reflect the degree of malignancy and invasiveness in brain glioma. High MMP-9 expression and low phosphatase and tensin homolog expression levels are indicators of increased glioma invasiveness. The combination of the two indices may be used as an important reference for diagnosis and prognosis for patients with glioma (14,29). The results of the present study demonstrated that paeoniflorin is associated with the expression of MMP-9 in U87 cells.

miRs are involved in the development of a number of diseases, including cancer. They are typically underexpressed in cancer tissues and the inhibition of the expression of certain miRs may lead to the occurrence of cancer. A small number of miRs are overexpressed in cancer tissues and are associated with tumor genes. However, the majority of miRs are underexpressed in tumor tissues, serving as tumor suppressor genes in cancer (30). A number of experiments have demonstrated the involvement of miR-16 as a tumor suppressor gene in glioma growth, via the inhibition of Bcl2 and the NF- κ B1/MMP-9 signaling pathway (10).

In the present study, treatment of U87 cells with paeoniflorin resulted in a significant increase in miR-16 expression levels. The results of the present study suggested that upregulation of miR-16 promotes MMP-9 expression in U87 cells. Paeoniflorin treatment exerted anticancer effects against human glioma cells via upregulating miR-16 and downregulating MMP-9 expression.

In conclusion, paeoniflorin may be useful for the treatment of human glioma. The results of the present study demonstrated that paeoniflorin treatment may lead to decreased proliferation and increased apoptosis of human glioma cells (31). To the best of our knowledge, the results of the present study support the hypothesis that paeoniflorin may be an effective antitumor agent for the treatment of human glioma (32). Paeoniflorin inhibited MMP-9 protein expression and promoted miR-16 expression in U87 cells. Upregulating miR-16 inhibited MMP-9 protein expression levels in anti-miR-16-transfected U87 cells. Therefore, miR-16 is associated with the downregulation of MMP-9 expression in U87 cells. Paeoniflorin treatment appeared to inhibit proliferation and accelerate apoptosis of human glioma cells via miR-16 upregulation and MMP-9 expression downregulation. To the best of our knowledge this is the first study to suggest that paeoniflorin may inhibit proliferation and accelerate apoptosis of human glioma cells via miR-16 upregulation and MMP-9 downregulation.

Acknowledgements

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