Osthole suppresses the proliferation and accelerates the apoptosis of human glioma cells via the upregulation of microRNA-16 and downregulation of MMP-9

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Abstract. Osthole (7-methoxy-8-isoamyl alkenyl coumarin) has been reported to exhibit marked anticancer effects on several types of cancer. The expression levels of matrix metalloproteinase-9 (MMP-9) are closely associated with the pathogenesis of glioma. Furthermore, it is reported that the upregulation of microRNA-16 (miR-16) by the MMP-9 signaling pathway can restrain the proliferation of cancer cells. To examine whether osthole increases the anticancer effect on human glioma cells in the present study, the common glioma cell line, U87, was treated with osthole at concentrations of 0, 50, 100 and 200 μM. The effects of osthole on cell viability were determined using a 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide assay. The rate of cellular apoptosis was analyzed by measuring the activity of caspase-3 and using flow cytometry. The expression of MMP-9 was determined using gelatin zymography assays and the expression of miR-16 was determined using reverse transcription-quantitative polymerase chain reaction. The results demonstrated that osthole significantly suppressed the proliferation and accelerated the apoptosis of the U87 cells. Furthermore, increased expression levels of miR-16 and reduced protein expression levels of MMP-9 were found in the U87 cells. In addition, miR-16 was found to regulate the expression of MMP-9 in the U87 cells through transfection of miR-16 precursor and anti-miR-16 into the U87 cells. In conclusion, these observations indicated that osthole suppressed the proliferation and accelerated the apoptosis of human glioma cells through upregulation of the expression of miR-16 and downregulation of the expression of MMP-9.

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Introduction

It is well known that glioma accounts for 44.6% of tumors of the central nervous system and has the characteristics of a high recurrence rate and high mortality rate (1). Although certain surgical comprehensive treatments can significantly improve the survival rates of patients with glioma, the prognosis remains poor due to the occurrence of chemotherapy drug resistance in glioma therapy (2).

Matrix metalloproteinases (MMPs) are reported to interact with the cellular adhesive molecular degradation of the extracellular matrix, promoting the growth of tumor cells into the surrounding brain tissue (3). Cell proliferation may be affected by regulating MMP-9 activity, and MMP-9 activity is controlled by several factors, including the quantity and activation of enzymes (4). MMP-9 is correlated with glioma and it has been demonstrated that the expression levels of MMP-9 directly reflect the prognosis of patients with glioma and is the preferred predictor of invasive glioma cell growth (5,6).

MicroRNAs (miRNAs) are a series of non-coding, small molecule RNAs, which regulates gene expression via sequence complementation. These small RNAs consist of between 19 and 25 nucleotides (7). Following transcription, they exert inhibitory effects on gene expression and are involved in several physiological processes, including cell differentiation, apoptosis and metabolism (8). Previous studies have demonstrated that miR-16 is important in tumors of various origins (9-11). In glioma growth and invasiveness, the importance of miR-16 as a tumor suppressor gene and a novel mechanism of miR-16 regulation through inhibition the nuclear factor (NF)-κB1/MMP-9 signaling pathway has been reported (11).

Osthole is a natural coumarin isolated from umbelliferae plant monomers (ripe fruit). A previous modern pharmacological investigation demonstrated that osthole has anti-inflammatory, antioxidant and other pharmacological properties (12,13). In addition, it was also revealed that osthole markedly decreases the activity and protein content of MMP-9, suggesting that this protective effect at the molecular level may be due to downregulation of the MMP-9 pathway (14). The aim of the present study was to investigate the anticancer potential of osthole against glioma cells and examine whether

this mechanism is dependent on the upregulation of miR-16 and downregulation of MMP-9 expression.

Materials and methods

Reagents and chemicals. The chemical structure of osthole (Sigma-Aldrich, St. Louis, MO, USA; purity, ≥95%) is shown in Fig. 1. Osthole was dissolved in physiological saline (50-200 μM). Roswell Park Memorial Institute-1640 (RPMI-1640) medium, fetal calf serum (FCS) 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). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Double Staining kit was purchased from BestBio (Shanghai, China).

Cancer cell lines. The U87 glioma cell line was purchased from the Animal Experiments of Clinical School of Taishan Medical University (Taian, China). The U87 cells were cultured in RPMI-1640 medium, supplemented with 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin, at 37°C and 5% CO_2.

MTT viability assay. The effect of osthole on the proliferation of U87 cells was measured using an MTT assay. The U87 cells ($5.0x10^3$ cells/well) were seeded into 96-well culture plates at 95% confluence and incubated at 37°C and 5% CO_2 in a humidified incubator for 24 h. Following incubation, the cells were treated with different concentrations of osthole (0, 50, 100 or 200 μM) for 0, 24, 48 or 72 h. MTT (~10 μl of 10 mg/ml) was added into each well and incubated at 37°C and 5% CO_2 for 4 h. Subsequently, 150 μl dimethyl sulfoxide was added to each well and incubated for 20 min at room temperature with agitation. The absorbance of the plates was detected using an CM2600d spectrometer (Bio-Tek Instruments, Inc., Winooski, VT, USA) at 570 nm.

Measurement of caspase-3 activity. The activity of caspase-3 in the cells was measured using a Colorimetric Caspase-3 Assay kit (Beyotime Institute of Biotechnology). Following treatment with 100 μM osthole for 48 h, the U87 cells ($1.0-2.0x10^6$ cells/well) were centrifuged at 12,300 x g for 20 min at 4°C. The supernatant was collected and the protein concentration was quantified using a bicinchoninic acid (BCA) protein assay kit (Sangon Biotech, Shanghai, China). The protein extract (~50 μg) was incubated and added to a reaction buffer containing 90 μl 1X assay buffer and 10 μl Ac-DEVD-pNA caspase-3 substrate at 37°C for 6 h. The protein extract (~50 μg) was incubated at 37°C and added to a reaction buffer containing 90 μl 1X assay buffer (Beyotime Institute of Biotechnology) and 10 μl Ac-DEVD-pNA caspase-3 substrate (Beyotime Institute of Biotechnology) at 37°C for 6 h. The change was calculated using a CM2600d spectrometer at a wavelength of 405 nm (Bio-Tek Instruments, Inc.).

Annexin V/PI flow cytometric analysis. The apoptotic rates of the U87 cells were determined using flow cytometric analysis (BD Biosciences, Franklin Lakes, NJ, USA) using an Annexin V-FITC/PI apoptosis kit. Following treat-

ment with 100 μM osthole for 48 h, the U87 cells were collected and washed twice with phosphate-buffered saline. Annexin V-FITC (10 μl) was added to the U87 cells, following which the cells were stained with binding buffer (BestBio) for 30 min in the dark, according to the manufacturer's instructions. PI (10 μl) was added to the glioma cell samples, which were then incubated for 30 min at room temperature in the dark. Immediately following incubation, the samples were analyzed using flow cytometry.

MMP-9 measurement. To determine whether 100 μM osthole induced the expression of MMP-9 in the U87 cells, gelatin zymography assays were used. Following treatment with osthole for 48 h at 37°C, the U87 cells were harvested and the concentration of protein was determined using a BCA protein assay kit (Sangon Biotech). Equal quantities of protein were extracted and were subsequently electrophoresed on 10% SDS-PAGE gels (Beyotime Institute of Biotechnology), containing 1% gelatin. Following electrophoresis, the gel was rinsed in 2% Triton X-100 (Nanjing Senbeijia Biotech Company, Nanjing, China) for 1 h and subsequently washed in water. The gels were incubated in radioimmunoprecipitation assay buffer (pH 8.0; Sigma-Aldrich) at 37°C for 12 h and the gel was stained with 0.2% Coomassie Blue R-250 (Beyotime Institute of Biotechnology) for 1 h. The protein expression levels of MMP-9 were quantified using a MiniBis system (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel) and prestained SDS-PAGE standards (Houbio Tech Co., Ltd., Fan Ling, Hong Kong).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of the expression of miR-16. The present study investigated whether 100 μM osthole induced the expression of miR-16 in the U87 cells using RT-qPCR. Following treatment with osthole for 48 h at 37°C, the total RNA was extracted from the cells using TRIzol reagent (Invitrogen Life Technologies), according to manufacturer's instructions. Approximately 1 μg total RNA from U87 cells-treated was used to perform the first-strand cDNA synthesis. Subsequently, 2 ml RNA was transcribed to cDNA using random hexamers (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cycling conditions were as follows: 10 min at 95°C, 40 cycles of 45 sec at 95°C, 45 sec at 58°C and 45 sec at 72°C. The quantification of the miR-16 level was conducted by real-time PCR using TransStart™ SYBR Green qPCR Supermix (TransGen Biotech, Beijing, China) and U6 small nuclear RNA was regarded as an endogenous reference gene. The U6 primer sequence was as follows: Forward 5'-CTCGCTTCGGCA GCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'. The miR-16 primer sequence was as follows: Forward 5'-TTC CATGCTGTTTTGGTCCC-3' and reverse 5'-TGGGTGGAG GTTTGTTCGGA-3'. Primers were provided by Sangon Biotech Co., Ltd. (Shanghai, China). Relative quantification was carried out using the $2^{-\Delta\Delta Ct}$ cycle threshold method.

miR-16 and anti-miR-16 transfection. The miR-16 precursor and the anti-miR-16 were obtained from KeyGen Biotech Co., Ltd. (Nanjing, China). The U87 cells ($5x10^5$ cells/well) were cultured in six-well plates and transfected with either miR-16 precursor or anti-miR-16 using Lipofectamine 2000 for 6 h at 37°C. The transfection media was replaced with RPMI-1640
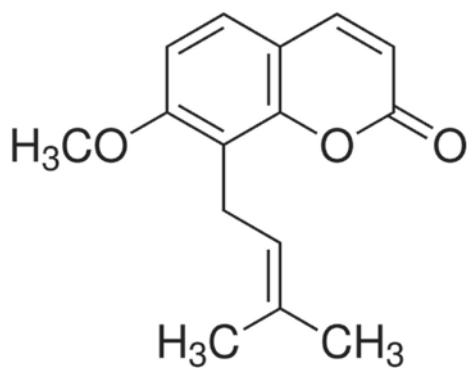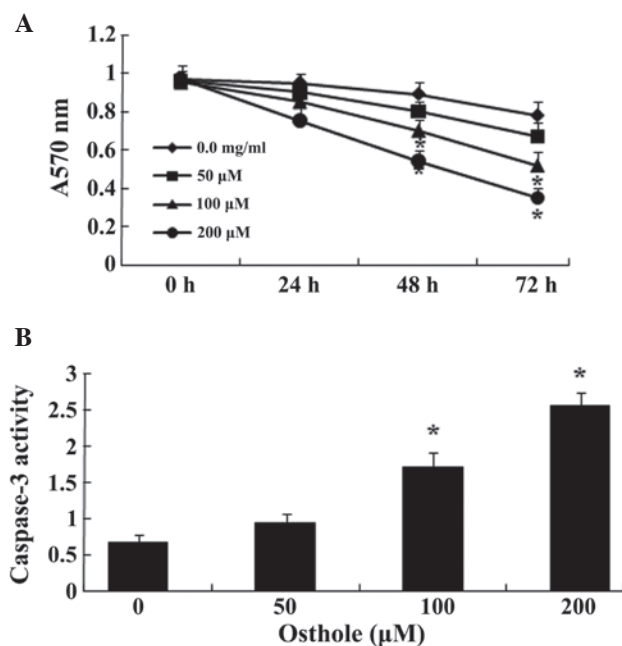
Figure 1. Chemical structure of osthole.

Figure 2. Osthole inhibits U87 cell growth and increases capsase-3 activity. A 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide assay was used to measure cell proliferation and the activity of caspase-3 was determined. Treatment with osthole (A) dose-dependently inhibited the growth of U87 cells and (B) significantly increased the activity of caspase-3 Data are presented as the mean ± standard deviation (*P<0.05, compared with the 0 μM osthole treatment group).

containing 10% FCS without antibiotic in a humidified atmosphere at 37°C with 5% CO_2 for 18 h.

Statistical analysis. All data are presented as the mean ± standard deviation. The data were analyzed using Student's t-test. Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Osthole suppresses proliferation and increases caspase-3 activity in the U87 cells. To determine whether there is an association between osthole and U87 cells, the viability of U87 cells following treatment with different concentrations of osthole (0, 50, 100 or 200 μM) was determined using an

MTT assay. As shown in Fig. 2A, the analysis revealed that treatment with 50 μM osthole for 72 h significantly reduced U87 cell viability, and treatment with 100 and 200 μM for 48 h or 72 h significantly reduced U87 cell viability. In addition, the decrease in U87 cell viability was found to occur in a time- and concentration-dependent manner in the osthole-treated cells. The activity of caspase-3 in the U87 cells was analyzed following treatment with osthole (0, 50, 100 or 200 μM) using a caspase-3 assay. Following treatment with 100 or 200 μM osthole for 48 h, the activity of caspase-3 was significantly increased in the U87 cells (P<0.05), and this increase in the activity of caspase-3 occurred in a concentration-dependent manner in the osthole-treated cells (Fig. 2B).

Flow cytometric analysis for the detection of cellular apoptosis. The U87 cells were exposed to osthole at concentrations of 0, 50, 100 or 200 μM for 48 h, and the effect of osthole on the augmentation of apoptosis was investigated. The results of this analysis using flow cytometry demonstrated that osthole inhibited the growth of the U87 cells in a dose-dependent manner (Fig. 3). Treatment with 100 and 200 μM osthole for 48 h significantly increased the apoptosis of U87 cells.

Inhibition of MMP-9 by osthole. To examine a potential association between the effect of osthole (0, 50, 100 of 200 μM) on the U87 cells and MMP-9, the protein expression levels of MMP-9 were determined using gelatin zymography assays. The data indicated that osthole inhibited the protein expression levels of MMP-9 in a dose-dependent manner (Fig. 4A). As shown in Fig. 4B, treatment with 100 and 200 μM of osthole for 48 h significantly reduced the protein expression levels of MMP-9 in the U87 cells.

Osthole activates the expression of miR-16. The present study aimed to determine the possible correlations between osthole (0, 50, 100 or 200 μM) and the expression levels of miR-16, which was assessed using RT-qPCR. The data indicated that osthole promoted the expression levels of miR-16 in a dose-dependent manner. As shown in Fig. 5, treatment with 100 and 200 μM osthole for 48 h significantly promoted the expression of miR-16 in the U87 cells (P<0.05).

Overexpression of miR-16 inhibits the protein expression of MMP-9. The protein expression levels of MMP-9 were examined following transfection of the U87 cells with miR-16. The results demonstrated that the protein expression levels of MMP-9 were reduced by overexpression of miR-16 in the U87 cells treated with 100 μM osthole. As shown in Fig. 6A and B, the miR-16 overexpressing cells inhibited the protein expression of MMP-9.

Anti-miR-16 reverses the effect of osthole. To confirm the functional role of osthole and of miR-16 in mediating the effects on the viability of the U87 cells, the present study examined the activity of caspase-3 and the protein expression levels of MMP-9 following transfection of the cells with anti-miR-16. The results indicated that transfection with the anti-miR-16 antibody efficiently penetrated into the U87 cells and significantly reduced the expression of miR-16 in the U87 cells (Fig. 7A). Following treatment with 100 μM osthole for
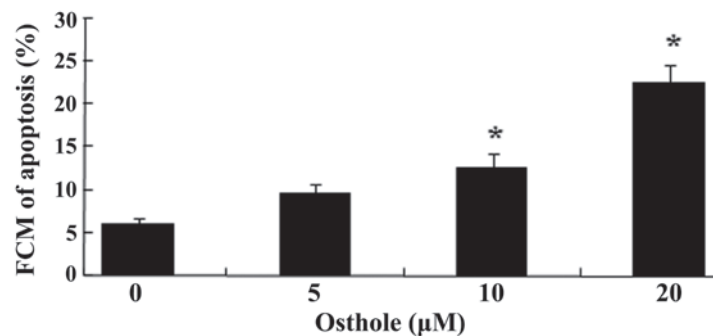
Figure 3. FCM analysis to detect levels of cellular apoptosis. Statistical analysis of the apoptosis of U87 cells. Data are presented as the mean ± standard deviation (*P<0.05, compared with the 0 μM osthole treatment group). FCM, flow cytometric analysis.
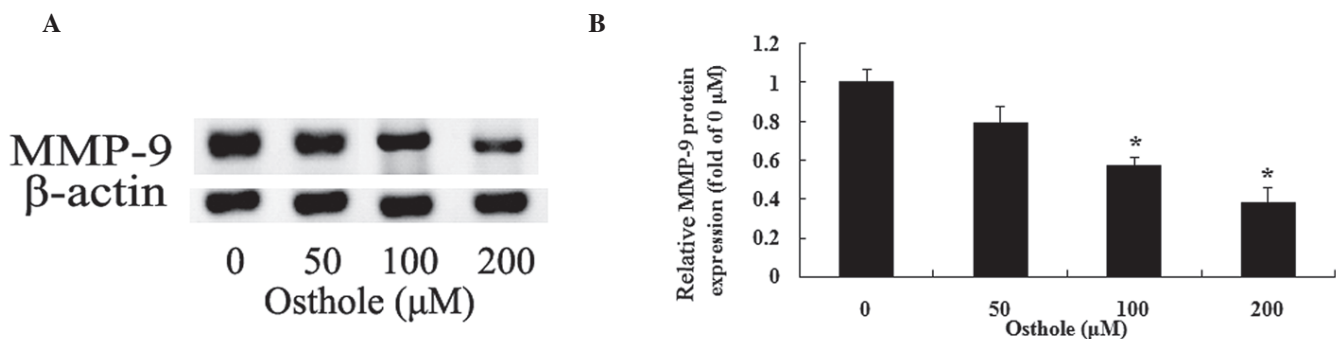

Figure 4. Osthole inhibits the expression of MMP-9. (A) Expression levels of MMP-9 were reduced in a dose-dependent manner in the U87 cells following treatment with 100 μM osthole for 48 h, determined using a gelatin zymography assay. (B) Protein bands were quantified and statistical analysis of the protein expression levels of MMP-9 was performed. Data are presented as the mean ± standard deviation (*P<0.05, compared with the 0 μM osthole treatment group). MMP-9, matrix metalloproteinase-9.

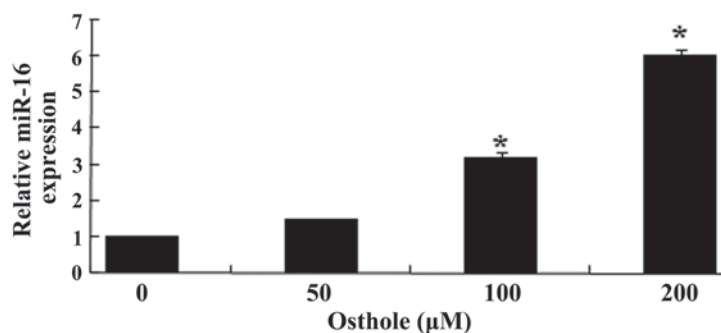
Figure 5. Osthole activates the expression of miR-16. Treatment with 100 μM osthole for 48 h increased the expression levels of miR-16 in a dose-dependent manner Data are presented as the mean ± standard deviation (*P<0.05, compared with the 0 μM osthole treatment group). miR, microRNA.

48 h, transfection with the anti-miR-16 antibody neutralized the effect of miR-16 on the U87 cells (Fig. 7B), inhibited the apoptotic effect on the U87 cells, indicated by downregulation in capsase-3 activity (Fig. 7C), and neutralized the inhibitory effect of osthole through downregulating the expression of MMP-9 (Fig. 7D).

Discussion

Glioma comprises 44.6% of tumors of the central nervous system and exhibit characteristics of a high recurrence rate and high mortality rate. With the development of molecular biology techniques, investigating glioma resistance mecha-

nism has been achieved at the molecular gene level and, through increased investigation, a deeper understanding of the resistance mechanism of glioma has been demonstrated (15). Several studies have reported certain natural chemical and compound products, which exert anticancer effects on a wide variety of tumor types (16,17). A previous study reported that osthole induces cell apoptosis and weakens cell migration in brain tumors (18). However, the detailed mechanisms underlying the anticancer effects of osthole on glioma remain to be fully elucidated. The present study demonstrated that osthole suppressed the proliferation and increased the activity of caspase-3 in the U87 cells. These results were consistent with those of previous studies (19-21). For example, Ding *et al* (19)
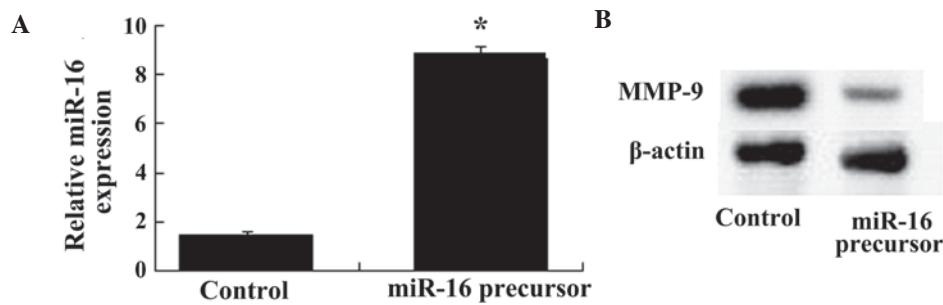
Figure 6. Overexpression of miR-16 and its effect on the expression levels of MMP-9. (A) Transfection with the miR-16 precursor significantly increased the expression levels of miR-16. (B) Transfection with the miR-16 precursor decreased the protein expression levels of MMP-9. Data are presented as the mean ± standard deviation (*P<0.05, compared with the 0 μM osthole treatment group). miR, microRNA; MMP-9, matrix metalloproteinase-9.
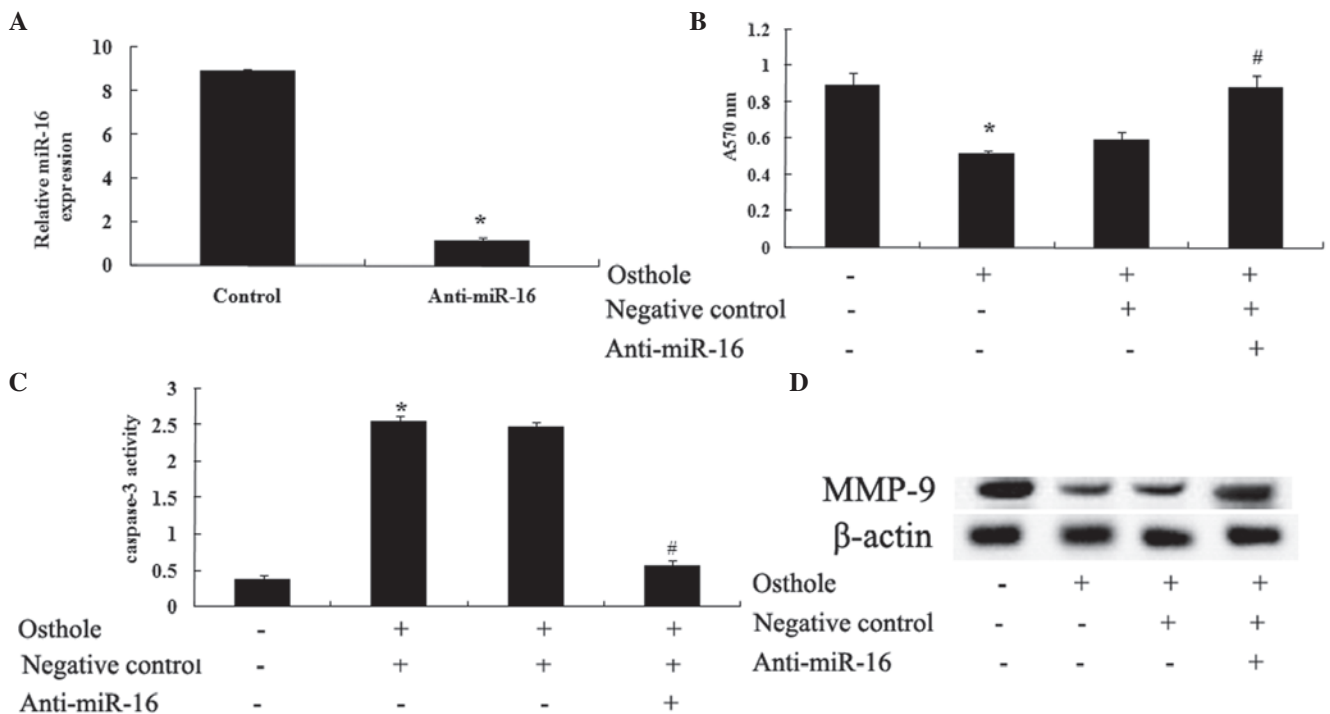

Figure 7. Anti-miR-16 reverses the effect of treatment with osthole. (A) Anti-miR-16 significantly reduced the expression levels of miR-16 in the U87 cells. Following treatment with 100 μM osthole for 48 h, (B) anti-miR promoted cell proliferation, (C) anti-miR-16 markedly inhibited the apoptosis of the U87 cells and (D) anti-miR-16 significantly decreased the expression levels of MMP-9 (*P<0.05, compared with the 0 μM osthole treatment group; $^#$P<0.01, compared with osthole-treated group transfected with the negative control). miR, microRNA; MMP-9, matrix metalloproteinase-9.

demonstrated that osthole inhibits the proliferation, migration and invasion, and induces the apoptosis of glioma cells.

Osthole, isolated from the rumbrelliferae plant monomers of the ripe fruit, *Cnidium monnieri*, possesses anti-inflammatory properties, improves learning and memory, and inhibits thrombosis (22-24). In the present study, treatment of the U87 cells with osthole significantly increased the apoptosis of the cells. Osthole was previously observed to suppress cell proliferation and induce cellular apoptosis in NCI-H460 lung carcinoma cells, which corresponded with the present study (25).

The degradation of the extracellular matrix by MMPs via type II collagenase AB is associated with the invasive cell growth of glioma, with MMP-9 being the most closely associated with glioma and, to a certain extent, its expression can reflect the extent of glioma invasion within the body (26,27).

The mRNA expression level of MMP-9 is closely associated with the degree of malignant glioma, and it has been reported that the intensity of MMP-9-positive staining is closely associated with the pathological levels of glioma (28). These reports indicate that MMP-9 can be used to determine the human glioma malignant phenotype (29). Using a nude mouse model to investigate the growth of glioma, it was determined that increased expression levels of MMP-9 are more marked, compared with the expression level of MMP-2, and indicates that MMP-9 is important in the growth of glioma (30). Consistently, the results of the present study demonstrated that treatment with osthole significantly reduced the protein expression levels of MMP-9. It was previously revealed that osthole suppresses the levels of MMP-2 and MMP-9 in A549 human lung cancer cells (31). In addition, osthole has been observed to inhibit NF-κB-MMP-9 in human lung adenocarcinoma (14).

In the present study, RT-qPCR was used to identify possible correlations between osthole (0, 50, 100 or 200 μM) and the expression of miR-16. The data indicated that osthole promoted the expression of miR-16 in a dose-dependent manner. The expression levels of miR-16-1 were markedly reduced in the human glioma cell lines, which suggested that miR-16-1 is involved in the proliferative, migratory and invasive abilities of highly-invasive glioma cells (32). The present study demonstrated that upregulation of the expression of miR-16 reduced the protein expression levels of MMP-9 in the (U87 cells. In addition, the results revealed that downregulation of the expression of miR-16, via anti-miR-16 transfection, promoted the protein expression of MMP-9 in the U87 cells and reduced the effect of osthole on the proliferation and apoptosis of the U87 cells. In conclusion, the results of the present study suggested that osthole suppressed the proliferation and accelerated the apoptosis of U87 human glioma cells via upregulation of the expression of miR-16 and downregulation of the expression of MMP-9.

Acknowledgements

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