Apigenin inhibits glioma cell growth through promoting microRNA-16 and suppression of BCL-2 and nuclear factor-κB/MMP-9

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Abstract. The present study aimed to investigate the effect of apigenin on glioma cells and to explore its potential mechanism. U87 human glioma cells treated with apigenin were used in the current study. Cell Counting Kit-8 solution and Annexin V-fluorescein isothiocyanate/propidium iodide Apoptosis Detection kit were used to analyze the effect of apigenin on U87 cell viability and apoptotic cell death. Reverse transcription-quantitative polymerase chain reaction analysis was also used to determine microRNA-16 (miR-16) and MMP-9 gene expression levels. Nuclear factor-κB (NF-κB) and B-cell CLL/lymphoma 2 (BCL2) protein expression levels were determined using western blot analysis. An anti-miR-16 plasmid was constructed and transfected into U87 cells. The current study demonstrated that apigenin significantly decreased cell viability and induced apoptotic cell death of U87 cells in a dose-dependent manner. Additionally, it was demonstrated that apigenin significantly increased miR-16 levels, suppressed BCL2 protein expression and suppressed the NF-κB/MMP9 signaling pathway in U87 cells. Furthermore, downregulation of miR-16 using the anti-miR-16 plasmid reversed the effect of apigenin on cell viability, BCL2 protein expression and the NF-κB/MMP-9 pathway in U87 cells. The results of the present study suggested that apigenin inhibits glioma cell growth through promoting miR-16 and suppression of BCL2 and NF-κB/MMP-9. In conclusion, the present study demonstrated the potential anticancer effects of apigenin on glioma cells.

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Introduction

Glioma is most commonly observed in adults, and has high severity and fatality rates. Due to the heterogeneity and abnormality of the tumor cells, glioma is rarely curable using current therapeutic methods, which include surgery and chemoradiotherapy (1). During the past decade, the median lifetime for patients with glioma is ≤12 months. The majority of previous studies on glioma were conducted from the perspectives of histopathology and molecular biology (2). Recently, large amounts of evidence has demonstrated that in addition to glioma tumor cells that exhibit strong multiplication capacity and invasiveness, a set of cells, termed glioma stem cells, are also present in glioma tissue and exhibit similar characteristics to neural stem cells, such as infinite proliferation, self-renewing capacity and multi-lineage differentiation (3).

Studies on microRNAs (miRNAs) began early in 1993. The first miRNA was identified in *Caenorhabditis elegans* (4). miRNAs are a type of non-coding single stranded RNA with length of 19-25 nucleotides (5). With the progressing of molecular biology, studies demonstrated that miRNAs are involved in the occurrence and development of tumors (6). miRNAs may function as tumor suppressor genes to lower the activities of oncogenes, and also may oncogenes that lower the activities of tumor suppressors (6). miRNAs can regulate the relative expressions gene associated with tumor development (7). Mutations, deficiency, translocation and abnormality of mutual regulation of miRNAs may cause abnormal expression of miRNA target genes. miRNA-16 (miR-16) is located in chromosome 13ql4, which has been demonstrated to be associated with human tumors in a previous study (8). As a tumor suppressor gene, miR-16 expression is downregulated by regulating BMI1 proto-oncogene in mantle cell lymphoma side population cells, reducing tumor volume (9). miR-16 reduces the degree of malignancy of glioma by downregulating nuclear factor-κB (NF-κB) and matrix metalloproteinase (MMP-9), inhibiting invasiveness of glioma cell lines. Upregulation of miR-16 inhibits the expression of B-cell lymphoma 2 (BCL2), to promote tumor cell apoptosis (10).

Apigenin is a plant flavonoid compound present in numerous fruits, vegetables, beans and tea leaves (11).

Previous studies demonstrated that it exerts various biological activities and pharmacological effects, including anticancer, anti-inflammation, antioxidant, anti-viral and immunoregulatory effects (12,13). Previous studies of apigenin reported that it exerts cytotoxic effects on various cancer cells, including breast, lung, liver and prostate cancer (11,14-16). In the present study, the *in vitro* effects of apigenin on glioma were investigated and the results indicate that apigenin may provide a novel therapeutic approach for the treatment of glioma.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Gibco (Thermo Fisher Scientific, Inc.). Cell Counting Kit-8 (CCK-8) solution was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide Apoptosis Detection kit was purchased from BestBio Biotechnology Co., Ltd. (Shanghai, China). Protein lysis buffer and Bradford protein assay were purchased from Zhongshan Jinqiao Biotechnology Co., Ltd., (Beijing, China).

Cell culture. Human U87 glioma cells were obtained from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and were cultured in DMEM supplemented with 10% FBS and 100 U penicillin/ml, 100 mg streptomycin/ml at 37˚C in a humidified atmosphere of 5% CO_2.

Cell viability. The effect of apigenin on U87 cell viability was determined using the CCK-8 kit. U87 cells were seeded onto 96-well plates ($1x10^3$ cells/well) and then incubated for 24 h following treatment with different concentrations (0, 1, 5, 10, 20, 30 and 40 μg/ml) of apigenin (Nanjing Pu Yi Biological Technology, Co., Ltd., Nanjing, China). Following treatment with apigenin, 10 μl thawed CCK-8 solution was added to each well and incubated for 4 h at 37˚C. Subsequently, cell viability of was measured using the Varioskan Flash Multimode reader (Thermo Fisher Scientific, Inc.) at 450 nm with a reference wavelength of 600 nm.

Cell apoptosis. The effect of apigenin (0, 10, 20 and 30 μg/ml) on U87 apoptotic cell death was determined using the Annexin V-FITC/propidium iodide Apoptosis Detection kit according to the manufacturer's instructions and were analyzed by flow cytometry (Beckman Coulter, Inc., Brea, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of miR-16 and MMP-9 expression. Total RNA was isolated from U87 cells treated with apigenin using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was generated from 2 μg RNA using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) at 42˚C for 1 h and 72˚C for 5 min. miR-16 and MMP-9 relative expression levels were examined using SYBR Green RT-qPCR (LightCycler 480 Roche, Switzerland). The primers used are presented in Table I. The thermocycling conditions were 30 cycles of denaturation at 94˚C for 15 sec, annealing at 57˚C for 15 sec and extension at 72˚C for 30 sec. The expression of miR-16 was quantified using the $2^{-\Delta\Delta Cq}$ method (17).

Table I. Oligonucleotide of primers of target genes.

Gene	Primer (5'-3')
miR-16	
Sense	GCGGCA ACCCGTAGATCCGAA
Antisense	GTGCAGGGTCCGAGGT
U6 RNA	
Sense	CTCGCTTCGGCAGCACA
Antisense	AACGCT TCACGAATTTGCGT
MMP-9	
Sense	CCCTGCGTATTTCCATTCAT
Antisense	ACCCCACTTCTTGTCAGCGTC
β-actin	
Sense	AAGCCTAAGGCCAACCGTGAAAAG
Antisense	TCAATGAGGTAGTCTGTCAGGT

miR-16, microRNA-16; MMP-9, matrix metalloproteinase-9.

Western blot analysis of NF-κB and BCL-2 expression. U87 cells treated with apigenin were washed with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold lysis buffer (Zhongshan Jinqiao Biotechnology, Ltd.) for 30 min. The samples were centrifuged at 23,000 x g at 4˚C for 10 min. The supernatant was collected and the protein concentration was determined using the Bradford protein assay. Total protein (50 μg) was separated with 12% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk then incubated with anti-NF-κB (cat. no. sc-109) and BCL-2 (cat. no. sc-783) primary antibodies at a dilution of 1:1,000 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight. Subsequently, the membrane was washed with 0.1% Tween 20 in PBS and incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000; Beyotime Institute of Biotechnology, Haimen, China; cat. no. A0208) for 2 h at room temperature. The protein bands were developed using horseradish peroxidase (Beyotime Institute of Biotechnology) and expression was determined using densitometry with Fujifilm Multi Gauge software, version 3.0 (Fujifilm, Tokyo, Japan).

Anti-miR-16 plasmid transfection. Anti-miR-16 plasmid (5'-CGCCAAUAUUUACGUGCUGCUA-3') and negative control plasmid (5'-CAGUACUUUUGUGUAGUACAA-3'). were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and transfected into U87 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Statistical analysis. Data are presented as the mean ± standard error and statistical analysis was performed where appropriate with the two-tailed Student's t-test using SPSS software

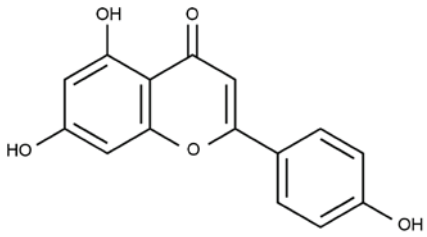

Figure 1. The chemical structure of apigenin.

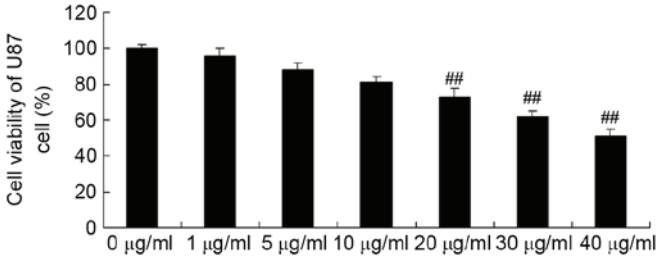

Figure 2. Apigenin inhibits the viability of U87 cells. ##P<0.01 vs. 0 μg/ml apigenin group. Data are presented as the mean ± standard error.

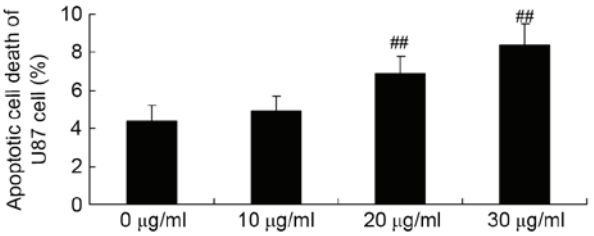

Figure 3. Apigenin induces apoptotic cell death of U87 cells. ##P<0.01 compared with 0 μg/ml apigenin group. Data are presented as the mean ± standard error.

(version 17.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Apigenin inhibits U87 cell viability. The chemical structure of apigenin is presented in Fig. 1. The effects of apigenin on U87 cell viability were determined. As demonstrated in Fig. 2, apigenin (20, 30, and 40 μg/ml) significantly decreased cell viability in a dose-dependent manner compared with the control group (P=0.0081, P=0.005 and P=0.0004, respectively).

Apigenin induces apoptotic cell death of U87 cell. Flow cytometry analysis was used to investigate the effect of apigenin on apoptotic cell death of U87 cells. The results demonstrated that 30 and 40 μg/ml apigenin significantly induced apoptotic cell death of U87 cells in a dose-dependent manner compared with the 0 μg/ml apigenin group (P=0.0032 and P=0.0007, respectively; Fig. 3).

Apigenin promotes miR-16 expression in U87 cells. To determine the underlying mechanism that mediates the effect of apigenin on U87 cells, the effect of apigenin on miR-16

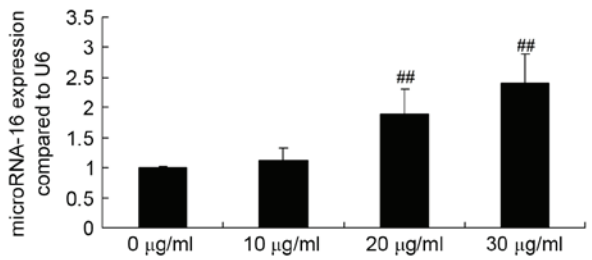

Figure 4. Apigenin promotes microRNA-16 expression in U87 cells. ##P<0.01 vs. 0 μg/ml apigenin group. Data are presented as the mean ± standard error.

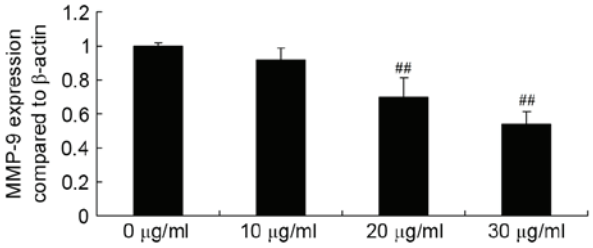

Figure 5. Apigenin inhibits MMP-9 expression of U87 cell. ##P<0.01 vs. 0 μg/ml apigenin group. Data are presented as the mean ± standard error. MMP-9, matrix metalloproteinase-9.

expression in U87 cells was examined. Briefly, U87 cells were treated with 0, 10, 20 and 30 μg/ml apigenin for 24 h, then subjected to RT-qPCR analysis. When U87 cells were treated with 20 or 30 μg/ml of apigenin, miR-16 expression was significantly increased compared with the control group (P=0.0005 and P<0.0001, respectively; Fig. 4).

Apigenin inhibits MMP-9 expression in U87 cells. Subsequently, the effect of apigenin on MMP-9 gene expression in U87 cells was determined by performing RT-qPCR analysis. The assay results demonstrated that exposure of U87 cells to 20 and 30 μg/ml apigenin for 24 h resulted in an significant decrease in MMP-9 gene expression levels compared with untreated control cells (P=0.0057 and P=0.0022, respectively; Fig. 5).

Apigenin inhibits NF-κB expression in U87 cells. To determine the effect of apigenin on NF-κB levels, western blotting was performed to detect NF-κB protein expression in U87 cells. The results of western blot analysis demonstrated that NF-κB protein expression in U87 cells was significantly reduced by treatment with 20 and 30 μg/ml of apigenin for 24 h compared with the control group (P=0.0042 and P=0.0013, respectively; Fig. 6).

Apigenin inhibits BCL2 expression of U87 cell. The loss of BCL2 protein expression is an early event in apoptosis. Thus, the present study assessed the effect of apigenin on BCL2 protein expression using western blot analysis. As demonstrated in Fig. 7, when U87 cells were incubated with 20 and 30 μg/ml apigenin for 24 h, BCL2 protein expression was significantly suppressed compared with the control group (P=0.0031 and P=0.0009, respectively).

Anti-miR-16 reverses the effect of apigenin on U87 cell viability. Whether anti-miR-16 reverses the effect of apigenin on U87

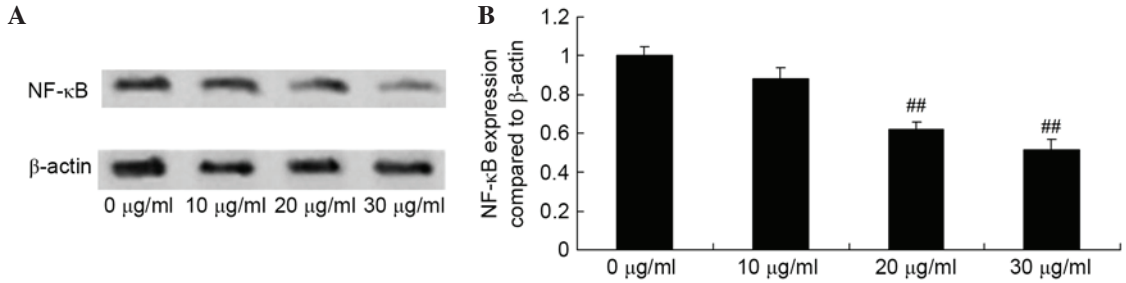

Figure 6. Apigenin inhibits NF-κB expression in U87 cells. (A) Apigenin inhibits NF-κB protein expression demonstrated using western blotting analysis and (B) statistical analysis of NF-κB protein expression in U87 cells. ##P<0.01 vs. 0 μg/ml apigenin group. Data are presented as the mean ± standard error. NF-κB, nuclear factor-κB.

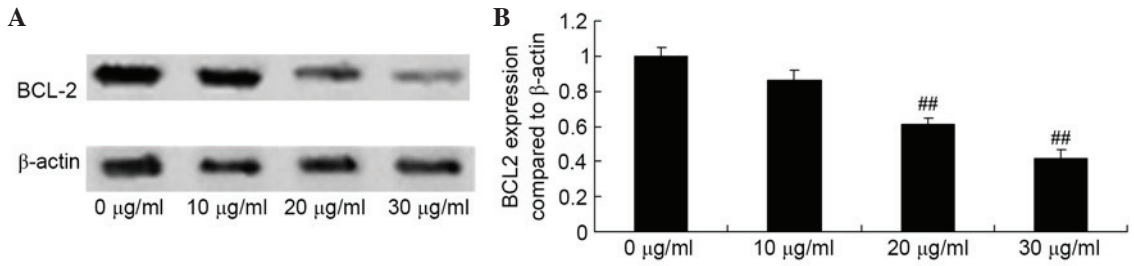

Figure 7. Apigenin inhibits BCL2 expression in U87 cells. (A) Apigenin inhibits BCL2 protein expression, demonstrated using western blotting analysis and (B) statistical analysis of BCL2 protein expression in U87 cell. ##P<0.01 vs. 0 μg/ml apigenin group. Data are presented as the mean ± standard error. BCL2, B-cell CLL/lymphoma 2.

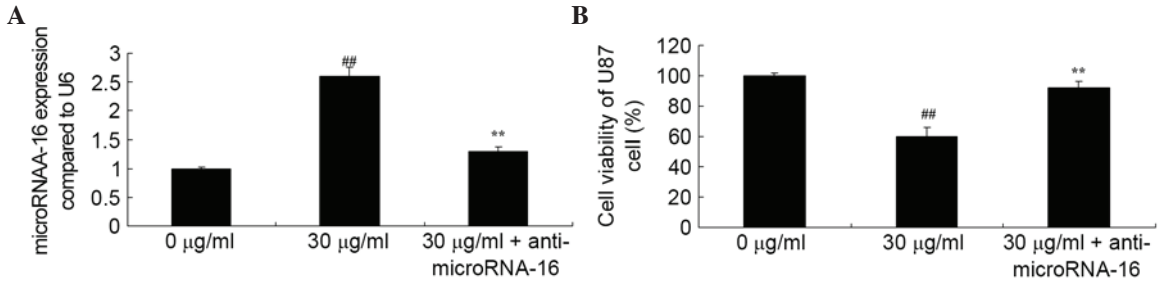

Figure 8. Anti-microRNA-16 reverses the effect of apigenin on U87 cell viability. (A) Anti-microRNA-16 effect on microRNA-16 gene expression and (B) can reverse the effect of apigenin on cell viability of U87 cell. ##P<0.01 vs. 0 μg/ml apigenin group and **P<0.01 vs. 30 μg/ml apigenin group. Data are presented as the mean ± standard error.

cell viability was investigated. The results demonstrated that anti-miR-16 inhibited apigenin-induced miR-16 gene expression (P=0.0027; Fig. 8A) and increased apigenin-suppressed viability of U87 cells compared with 30 μg/ml apigenin treatment (P=0.0047; Fig. 8B).

Anti-miR-16 reverses the effect of apigenin on BCL2 expression in U87 cells. To determine the molecular events involved in BCL2 expression in apigenin-induced apoptosis of U87 cells, the effect of anti-miR-16 on the level of BCL2 protein expression in U87 cells was analyzed. The results demonstrated that anti-miR-16 significantly increased BCL2 protein expression in U87 cell compared with 30 μg/ml apigenin treatment (P=0.0022; Fig. 9).

Anti-miR-16 reverses the effect of apigenin on NF-κB/MMP-9 expression in U87 cells. To identify whether miR-16 is involved in the effect of apigenin on NF-κB/MMP-9 expression in U87 cells, the effect of abti-miR-16 on the protein expression levels of NF-κB and MMP-9 were determined. As demonstrated in Fig. 10, apigenin-inhibited NF-κB/MMP-9 levels were significantly increased by anti-miR-16 compared with 30 μg/ml apigenin (P=0.0072 and P=0.0041; Fig. 10).

Discussion

Glioma is a common tumor with high severity (18). Compared with malignant tumors in other locations, glioma is rarely transferred to sites out of the central nervous system (1). Additionally, glioma exhibits strong invasion capacity, and can infiltrate from primary lesions to other cerebral tissue or metastasize to distant sites far from the primary lesions (19). As a result, the edges of the primary tumor are unclear. Combined with its strong invasive capacities, mortality rates of patients with glioma are high (20). Consequently, even following treatment, including surgery and chemoradiotherapy, survival periods

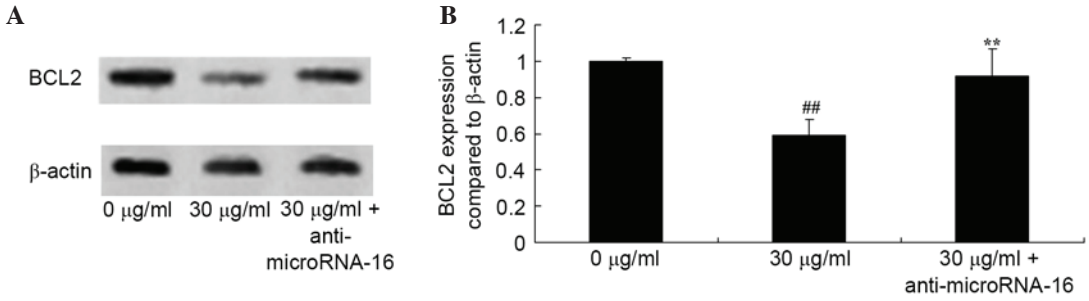

Figure 9. Anti-microRNA-16 can reverse the effect of apigenin on BCL2 expression in U87 cells. (A) Anti-microRNA-16 can reverse BCL2 expression as demonstrated using western blotting analysis and (B) statistical analysis of BCL2 protein expression in U87 cells. ##P<0.01 vs. 0 μg/ml apigenin group, **P<0.01 vs. 30 μg/ml apigenin group. Data are presented as the mean ± standard error. BCL2, B-cell CLL/lymphoma 2.

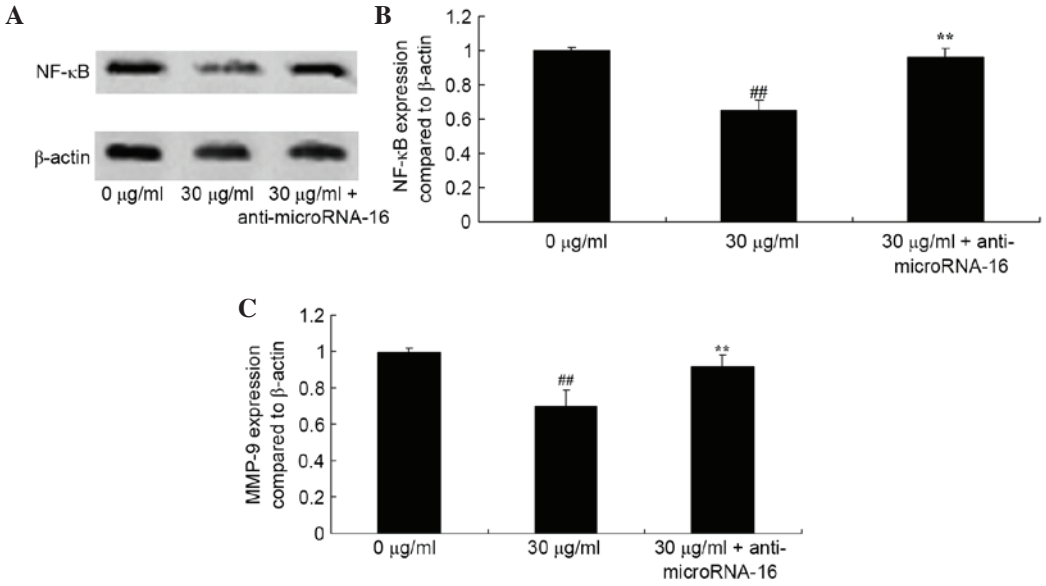

Figure 10. Anti-microRNA-16 can reverse the effect of apigenin on NF-κB/MMP-9 of U87 cells. (A) Anti-microRNA-16 can reverse NF-κB expression demonstrated using western blotting analysis and (B) statistical analysis of NF-κB protein expression and (C) MMP-9 expression. ##P<0.01 vs. 0 μg/ml apigenin group and **P<0.01 vs. 30 μg/ml apigenin group. Data are presented as the mean ± standard error. NF-κB, nuclear factor-κB; MMP-9, matrix matalloproteinase-9.

of patients with glioma ≤15 months (21). It is particularly important to identify novel molecules that participate in the metastasis and invasion of glioma. The present study demonstrated that apigenin significantly decreased cell viability and induced apoptotic cell death of U87 cells in a dose-dependent manner. Previous studies demonstrated that apigenin inhibits the proliferation of human bladder cancer (11), ovarian cancer cells (14) and lung cancer (15).

Additionally, multiple studies have demonstrated that miRNAs are important in tumor physiological and pathological processes, including growth, proliferation, apoptosis, lipid metabolism, hormone secretion and tumorigenesis (4). miRNAs can be regarded as oncogenes or tumor suppressor genes (7). Particularly, studies have reported that miRNAs are involved in cell differentiation, proliferation and invasion (5). A previous study reported that miR-16 reduced the metastasis and invasion of glioma cells by inhibiting MMP-9 (22). Additionally, another previous report demonstrated that increased miR-16 inhibited the proliferation of glioma and accelerated its apoptosis (23). To the best of our knowledge, the current study demonstrated for the first time that apigenin significantly increased miR-16 levels in U87 cells. Yang *et al* (22) previously reported that miR-16 reduced glioma cell growth via suppression of BCL2 and the NF-κB/MMP9 signaling pathway.

The BCL2 genes are localized at chromosome 18q21 and the encoded protein products can reverse the apoptosis-promoting effects of c-myc proteins, stabilize mitochondrial function and structures, and disrupt the activity of caspase enzymes via antagonism of p53 proteins (24). Thus, BCL2 inhibits cell apoptosis induced by various factors and participates in the dynamic equilibrium that regulates proliferation and apoptosis (25). Abnormal increases in BCL2 expression causes cells with abnormal changes to avoid apoptosis (25). Increased accumulation of genetic abnormal events is a prerequisite of cell transformation and tumorigenesis (26). Infiltrative growth is a distinctive feature of glioma and the invasive capability is proportional to its severity. Studies using glioma cell lines *in vitro* demonstrated that BCL2 promoted the synthesis, secretion and activity of MMPs to increase the infiltrative capacities of glioma cells (27,28). The present study demonstrated that treatment with apigenin significantly suppressed BCL2 protein

expression in U87 cells. Additionally, Shukla and Gupta (16) concluded that apigenin induced apoptosis of DU145 human prostate carcinoma cells by altering the BCL2 associated X protein/BCL2 ratio (16).

Previous findings have demonstrated that NF-κB is a important factor during inflammation (29). As one of most important transcription factors discovered in recent years, NF-κB was named as it sequence-specifically binds with enhancer κB of κ light chain of B cell immunoglobulins (30). NF-κB is involved in vascularization and tumor spread through regulation of vascular endothelial growth factor and interleukin-8; it promotes the transcription of MMPs to degrade extracellular matrix (31). Thus, NF-κB may promote tumor infiltration to surrounding tissues and the metastasis of breast cancer cells (31). The current study identified that apigenin significantly reduced NF-κB protein expression in U87 cells. Chang *et al* (32) demonstrated that apigenin protects against adjuvant-induced arthritis via inhibiting the purinergic receptor P2X 7 /NF-κB pathway.

A study investigating chronic myeloid leukemia by Li *et al* (23) demonstrated that miR-15a and miR-16-1 expression was increased and BCL2 was decreased in glioma cells. miR-15a and miR-16-1 negatively regulate BCL2 expression at the post-transcriptional level. BCL2 was demonstrated to be a target gene of miR-16, which significantly inhibited the 3'UTR region of BCL2 transcripts (22). A previous *in vivo* experiment demonstrated that miR-16 directly inhibited protein expressions of BCL2 to induce early apoptosis of glioma cells in human brain (22). The altered activity of NF-κB/MMP-9 signaling pathways in brain glioma promoted invasion (33). *In vitro* experiments demonstrated that, as a cancer suppressor gene, miR-16 reduced the proliferation and invasion of brain glioma via inhibiting the gene expressions NF-κB and MMP-9 (22). In the present study, apigenin suppressed the NF-κB/MMP9 signaling pathway in U87 cells. Palmieri *et al* (12) reported that apigenin inhibits the TNFα-induced expression of MMP-9. Additionally, the current study demonstrated that anti-miR-16 reversed the effect of apigenin on cell viability, BCL2 protein expression and the NF-κB/MMP-9 pathway in U87 cells. Yang *et al* (22) reported that miR-16 reduced the proliferation of glioma cells via suppression of BCL2 expression and NF-κB/MMP9 signaling.

In conclusion, the present study demonstrated that apigenin reduced glioma viability through increased expression of miR-16, and suppression of BCL-2 and NF-κB/MMP-9. These results suggested that apigenin may represent a valuable cancer therapeutic option for the treatment of glioma.

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