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Apigenin inhibits proliferation, migration, invasion and epithelial mesenchymal transition of glioma cells by regulating miR-103a-3p/NEED9/AKT axis

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Abstract

Apigenin is one kinds of natural flavonoids from plants. Many researches showed this compound has potential antioxidant, anti-inflammatory and anticancer functions. This study aimed to investigate the effects of apigenin on proliferation, migration, invasion, epithelial mesenchymal transition (EMT) in glioma cells, and to reveal the underlying mechanism of apigenin. MTT assay indicated the proliferation rate of glioma cells U251 was significantly decreased with the treatment of apigenin in a dose-dependent manner. In addition, apigenin could inhibit the cell invasion, wound healing and decrease the expression of EMT-related proteins, NEDD9, p-FAK and p-AKT. Quantitative real-time PCR showed miR-103a-3p was remarkably increased in the presence of apigenin. Inhibition of miR-103a-3p attenuated the effects of apigenin on U251 cells. Dual-luciferase assay and western blot further demonstrated that NEDD9 was a direct target of miR-103a-3p. Moreover, knockdown NEDD9 almost reversed the activities of silenced miR-103a-3p in transwell invasion, wound healing and regulation of NEDD9/FAK/AKT axis. In conclusion, apigenin could inhibit glioma cell proliferation, migration, invasion and EMT via activating of miR-103a-3p and targeting NEDD9/FAK/AKT pathway.

Keywords: apigenin; proliferation; migration; invasion; epithelial mesenchymal transition; glioma.

Practical Application: This study has provided a basis for the application of apigenin to treatment of glioma.

1 Introduction

Glioma is the main malignant tumor of central nervous system, accounting for about 80% of primary brain malignant tumors (Wen & Reardon, 2016). According to the WHO classification, gliomas are classified into four histological grades (Louis et al., 2007). Clinical therapies for glioma mainly include surgery, radiotherapy, chemotherapy, immunotherapy and targeted therapy (Weller et al., 2013; Chen et al., 2020). However, the prognosis of gliomas is poor and the recurrence rate is high, especially for high-grade gliomas. In low-grade glioma patients, about 70% of patients have in-situ recurrence, while the probability of recurrence increases to about 90% in high-grade glioma patients. The 5-year survival rate of grade IV glioma patients is less than 5% (Brem et al., 1995; Kreisl et al., 2009; Chen et al., 2020). Therefore, it is urgent to find a more effective treatment for glioma as well as reduce the recurrence rate.

Flavonoids have been considered to have good anticancer potential for a long time. Apigenin is one of natural flavonoid from plant, which is rich in a variety of fruits and vegetables. Apigenin is reported has anti-inflammatory, antibacterial, antioxidant, anti-tumor and other biological effects (Ali et al., 2017). Compared with chemotherapeutic drugs, apigenin can selectively inhibit and kill tumor cells but have a few harmful effects on normal cells (Xu et al., 2011; Bai et al., 2014). Previous studies have shown that apigenin can inhibit glioma cell proliferation (Chen et al., 2016), migration (Wang et al., 2021a; Zhao et al., 2021) and promote apoptosis (Sung et al., 2016). However, its underlying mechanism remains to be further investigated.

MicroRNAs (miRNAs) are small noncoding RNAs, consisting of 20 to 25 nucleotides. It usually plays important roles in posttranscriptional gene regulation by targeting 3' TUR of mRNA (Bushati & Cohen, 2007). miR-103a-3p is proved to inhibit cell proliferation in many human cancers, including gnon-small cell lung cancer (She et al., 2020), prostate cancer (Ge et al., 2021) and colorectal adenocarcinoma (Wilson et al., 2016). Researches also show that miR-103a-3p is an important role in the process of glioblastoma and it is downregulated in glioma stem cells and glioma tissue (He et al., 2019; Yu et al., 2017). Moreover, Su et al. (2020) proved that miR-103a-3p is negatively regulated by lncRNA FGD5-AS1 and affects the proliferation, migration and invasion of Glioblastoma Cells.

Neural precursor cell-expressed developmentally down-regulated 9 (NEDD9), which is also known as Human enhancer of Filamentation 1 (HEF1) or CrK-associated substrate lymphocyte Type (CAS-L). It belongs to the CrK-related protein subfamily. NEDD9 is a scaffold protein that is localized in focal adhesion and interacts with focal adhesion kinase (FAK) and non-receptor tyrosine kinase C-SRC to regulate a variety of cellular signaling pathways, participating in integrin-dependent adhesion, migration, invasion and apoptosis (Gu et al., 2019; Zhou et al., 2017a). Previous study also indicates NEDD9 is regulated by several miRNAs, for example, microRNA-125b could inhibit cell proliferation and promote apoptosis by targeting NEDD9 and regulating PI3K/AKT signaling pathway (Xue et al., 2020).

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In the present study, we investigate apigenin could inhibit the proliferation, invasion, migration and EMT of glioma cells. The potential mechanism is that apigenin could promote the expression of miR-103a-3p, which directly targets the 3'UTR of NEDD9, and block the pathway of p-FAK and p-AKT signaling. It might give some new clues for a potential treatment of glioma.

2 Materials and methods

2.1 Cell culture

Glioma cells U251 were obtained from National Collection of Authenticated Cell Cultures. U251 cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS, Gibco, 10091155), 2mM L-Glutamine, 1% Streptomycin and penicillin solution (Hyclone, SV30010) at 37 °C in a water-saturated atmosphere of 5% CO2. Cells were subcultured every 2-3 days according to the degree of cell convergence.

2.2 MTT assay

U251 cells were cultured in 96-well plates (7000 cells/well) overnight at 37 °C. Then the cells were treated with indicated concentration of Apigenin (MCE HY-N1201) and incubated for 48 h. Subsequently, cell viability assay was conducted by the 3-(4,5-Dimethyl-2-thiazolyl)-2,5- diphenyltetrazoliumbromide (MTT) reagent (Invitrogen, M6494). After adding 20 μL of 5 mg/mL MTT into each well of 96-well plates and incubating for 4 h, the supernatant was discarded and the purple formazan was dissolved in 150 μL DMSO. The plate was vibrated for 10 min and the absorbance of 490 nm wavelength was checked by micro plate reader. Cell viability was manifested by normalizing the absorbance to that of control group.

2.3 Cell invasion assay

One day before the experiment, the matrix was thawed at 4 °C. The matrix was diluted 5 times with serum-free medium before using. 50ul of diluted matrix was added to each Transwell chamber and placed in a 37 °C incubator for 30min. Meanwhile, the cells were digested, washed once with PBS and suspended in serum-free medium with the density of 1×10^5/ mL. 200 μL of cell suspension was added into Transwell chamber while 600 μL of completed medium was added into the lower chamber of 24-well plate and cultured at 37 °C. 24h later, the Transwell chamber was washed with PBS and fixed with 4% PFA. Crystal violet staining was performed for 5 min and then the Transwell chamber was washed with PBS again. The cells on the upper surface of the filter were erased with cotton swabs. Finally, the cells on the lower surface of the filter were frozen and counted with microscope using a 20 X objective.

2.4 Wound healing assay

Cell migration was carried out by wound healing assay. Briefly, 5×10^5 cells/well were seeded into 6-well plates and treated with indicated condition. When the density of cells was about 90%, a scratch was made at the bottom of plates with a 10 μL pipette tip. The cells were washed with PBS and then incubated in FBS-free medium with or without apigenin. Photographs of wound healing were taken at 0 h, 6 h, 24 h, and 48 h using a microscope (Olympus, Japan) at 10 X objective. The relative migration distance was measured by ImageJ (Rasband, USA).

2.5 Reverse transcription-quantitative real time PCR (qRT-PCR)

Total cellular RNA of each group was extracted by TRIZOL reagent (Invitrogen, 15596-026) according to the manufacturer's protocol. The RNA concentration and optical density (OD) ratio at 260 nm and 280 nm was measured by NanoDrop (Thermo Fisher Scientific, USA). Total RNA was reverse transcribed into cDNA with PrimeScriptTM RT reagent Kit (TaKaRa, RR037A) according to the manufacturer's instructions. qRT-PCR analysis was conducted using a one-step SYBR PrimeScript plus RT-PCR kit (Takara, HRR096A) by ABI 700 Real-Time PCR Detection system (Thermo Fisher Scientific, USA). U6 was used as an internal control for target miRNA expression levels. The relative miRNA expression level was calculated with the $2^{-\Delta\Delta Cq}$ method. The stem loop sequence for miRNA reverse transcription is 5'-GTCGTATCCAGTGC-GTGTCGTGGAGTCGGCAATTGCACTGGATACGAC-3'. qPCR primer sequences were as follows: universal reserve primer is 5'- CAGTGCGTGTCGTGGAGT-3', miR-103a-3p forward primer is 5'- TACAGGGCTATGAGTCGT -3', miR-125a-5p forward primer is 5'- TGAGACCCTTTAACCTGTG -3', miR-145-5p forward primer is 5'- AGTTTTCCCAGGAATCCC -3', miR-203a-3p forward primer is 5'- AGTTTTCCCAGGAATCCC -3', The stem loop sequence for U6 reverse transcription is 5'-GAATTTGCGTGTCATCCTTG-3'. U6 forward primer is 5'- TCGCTTCGGCAGCACAT-3', and reverse primer is 5'-TTGCGTGTCATCCTTGC -3'.

2.6 Cell transfection

For miRNA transfection, NC inhibitor and miR-103a-5p inhibitor (GenePharma, China) were transfected with Lipofectamine RNAiMAX regent (Invitrogen, 13778150) at a final concentration of 100 nM according to the manufacturers' protocols. For siRNA transfection, NC siRNA and NEDD9 siRNA (GenePharma, China) were also transfected with Lipofectamine RNAiMAX regent (Invitrogen, 13778150) at a final concentration of 20 nM according to the manufacturers' protocols. NC siRNA sequence is 5'UUCUCCGAACGUGUCACGUdTdT3', and NEDD9 siRNA sequence is 5'-GAUGGGAUCAACCGAUUGUdTdT-3'(Zhao et al., 2019).

2.7 Luciferase assay

We identify the putative binding sites of miR-103a-3p at the 3′ untranslated region (UTR) of NEDD9 with online tool TargetScan (TargetScanHuman, 2022). Wild-type (WT) sequence of NEED9 3'UTR containing the predicted binding sites of miR-103a-3p site, as well as the corresponding mutant type (MUT) sequence, were constructed into pmirGLO plasmid respectively. The constructed plasmids were co-transfected with NC mimics or miR-103a-3p mimics ((GenePharma, China)) into HEK-293T cells by Lipofectamine 2000 regent (Invitrogen, 11668019) according to the manufacturers' protocols. After 48 h of transfection, the cells were lysed and the luciferase activity

was measured using a Dual-Luciferase Reporter Assay System (Promega, E1910) by microplate reader. The relative luciferase activity was showed by the ratio of firefly luciferase activity and Renilla luciferase activity.

2.8 Western blot analysis

Cells of each group were digested using 0.25% trypsin and washed with PBS. Total cellular protein was extracted by RIPA Lysis Buffer (Beyotime, P0013) at 4 °C for 30 min and then centrifuged at 10000 g for 5 min. Protein concentrations were measured by BCA assay kit (Beyotime, P0012) follow the principle of manufacturers' protocols. Then 30 μg of total protein was used for SDS-PAGE and separated proteins were subsequently transferred to a nitrocellulose membrane with the wet transfer method. The membrane was blocked with 5% non-fat milk for 2 h at room temperature. The primary antibody was incubated at 4 °C overnight at a concentration as following: Anti-E-cadherin (1:1000, cat. no. 20874-1-AP, Proteintech), anti-MMP9 (1:1000, cat. no. 10375-2-AP, Proteintech), anti-Vimentin (1:1000, cat. no. 10366-1-AP, Proteintech), anti-GAPDH (1:20000, cat. no. 60004-1-Ig, Proteintech), anti-NEDD9 (1:1000, cat. no. ab88584, Abcam), anti-p-FAK (1:1000, cat. no. ab81298, Abcam), anti-FAK (1:1000, cat. no. ab76496, Abcam), anti-p-AKT (1:1000, cat. no. 9271S, Cell Signaling), anti-AKT (1:1000, cat. no. 9272S, Cell Signaling). The secondary antibody was incubated at room temperature for 1 h with a dilution of 1:5000. The target protein was detected using Pierce™ ECL Western (Thermo Scientific, 32209) and photographed by the chemiluminescence imaging analysis system (Bio-Rad, USA). The relative gray value was quantified by ImageJ (Rasband, USA). GAPDH was used as the internal standard.

2.9 Statistical analysis

All the experiments were repeated at least three times and the data were showed by mean ±SD. The data were analyzed by SPSS 17.0 and photographed by GraphPad Prism 5. One-way ANOVA was used for three or more group data analysis while Student's t test was used for two group data analysis. The P value < 0.05 represented as statistically significant.

3 Results

3.1 Apigenin inhibits proliferation, invasion and migration of U251 cells

The MTT assay was conducted to investigate the effect of apigenin on glioma cells U251. As is shown in Figure 1A, the survival rate of U251 cells was gradually decreased with the increasing concentration of apigenin. According to the MTT assay, we chose the concentration of 40 μM for the subsequent experiment. After treated with 40 μM apigenin, transwell assay was used to study cell invasive ability. The amount of invasive cell was significantly decreased and the cell morphology became abnormal compared to untreated control (Figure 1B and 1C). Meanwhile, wound healing assay also suggested apigenin inhibit migration of U251 cells at the time point of 24 h and 48 h (Figure 1D and 1E).

3.2 Apigenin prevents EMT and NEDD9/p-FAK/p-AKT expression

Epithelial mesenchymal transformation (EMT) is often related to the invasion and migration of cells. Considering that apigenin could inhibit the invasion and migration of U251 cells, we checked the expression of EMT-related proteins E-cadherin, MMP9 and Vimentin. Compared to untreated control, the expression of E-cadherin protein was increased in U251 cells while the expression of MMP9 and Vimentin protein was decreased under apigenin treatment (Figure 2A and 2B), which confirmed that apigenin could reduce EMT of U251 cells. NEDD9 regulates cancer cell migration and progression in many previous studies. We investigated the expression of NEDD9, p-FAK and p-AKT in the absence or presence of apigenin. Conformably, the pathway of NEDD9/ p-FAK /p-AKT was inhibited by apigenin treatment (Figure 2C and 2D).

3.3 Apigenin activates the expression of miR-103a-3p

Numerous researches verified miRNAs play important roles in cell proliferation, migration and invasion, therefore, it may be some miRNAs participate in the regulating mechanism of apigenin. miRNAs that may regulate NEDD9 were analyzed by TargetScan. And the miRNAs that may inhibit proliferation, migration and invasion of tumor cells was screened through previous study. Finally, we got four candidate miRNAs which were including miR-103a-3p, miR-125a-5p, miR-145-5p and miR-203a-3p. qRT-PCR was examined to confirm whether these miRNAs were respond to the treatment of apigenin. The result suggested the expression of miR-125a-5p and miR-203a-3p was almost unchanged while the expression of miR-145-5p was slightly upregulated in the treatment of apigenin compared to the control group (Figure 3B, 3C and 3D). However, we found the level of miR-103a-3p was significantly increased in the presence of apigenin (Figure 3A). Therefore, our result indicated apigenin could activate the expression of miR-103a-3p.

3.4 Inhibition of miR-103a-3p attenuates the function of apigenin

In order to investigate whether the inhibition of cell invasion and migration by apigenin via miR-103a-3p, we downregulated the level of miR-103a-3p by miRNA inhibitor. In accord with our suppose, miR-103a-3p inhibitor could promote U251 cells invasion and attenuate the effect of apigenin in cells invasion (Figure 4A and 4B). At the same time, the decline in cell migration by apigenin was also weakened by miR-103a-3p inhibitor (Figure 4C and 4D). The next, effect of miR-103a-3p inhibitor on EMT was evaluated. Briefly, the U251 cells were transfected with NC or miR-103a-3p inhibitor in the presence or absence of apigenin and harvested to do by western blot analysis. The result manifested the suppression of EMT by apigenin could reversed by miR-103a-3p inhibitor (Figure 5A and 5B). In addition, the same effect was observed in the expression of NEDD9, p-FAK and p-AKT (Figure 5C and 5D). Taken together, inhibition of miR-103a-3p could block the function of apigenin.

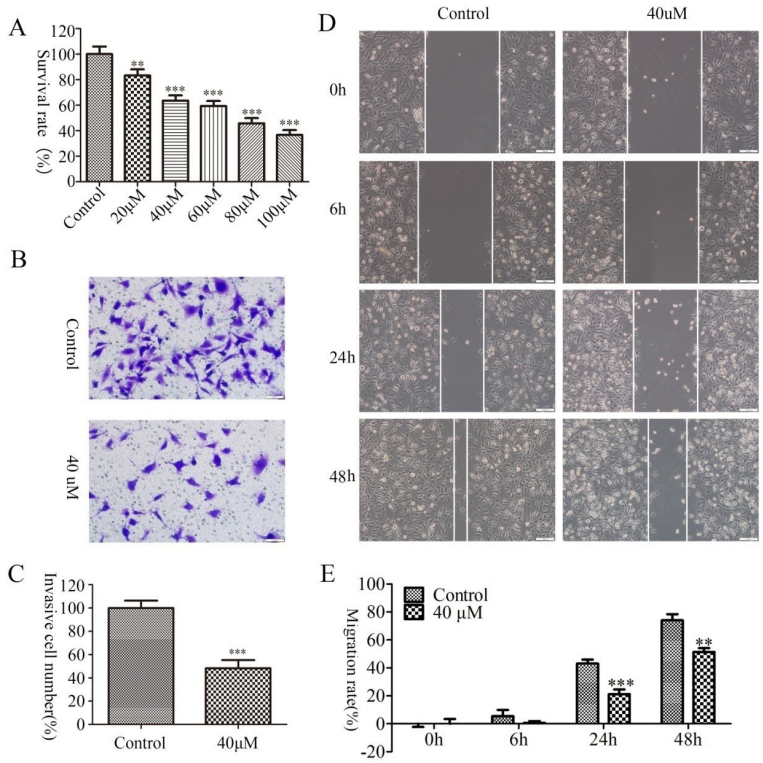

Figure 1. Apigenin inhibits proliferation, invasion and migration of U251 cells. (A) U251 cells were cultured in 96-well plates and treated with indicated concentration of apigenin for 48h. The survival rate of each group was checked by MTT assay. (B) U251 cells were treated with 40 μM apigenin. After 48 h, transwell analysis was performed to detect the invasion ability of U251 cells. (C) Data analysis of transwell experiment. (D) Wound healing assay of U251 cells in the presence or absence of 40 μM apigenin. (E) Data analysis of wound healing assay. Data are showed as mean ± SD. **P < 0.01, ***P < 0.001 (n = 3).

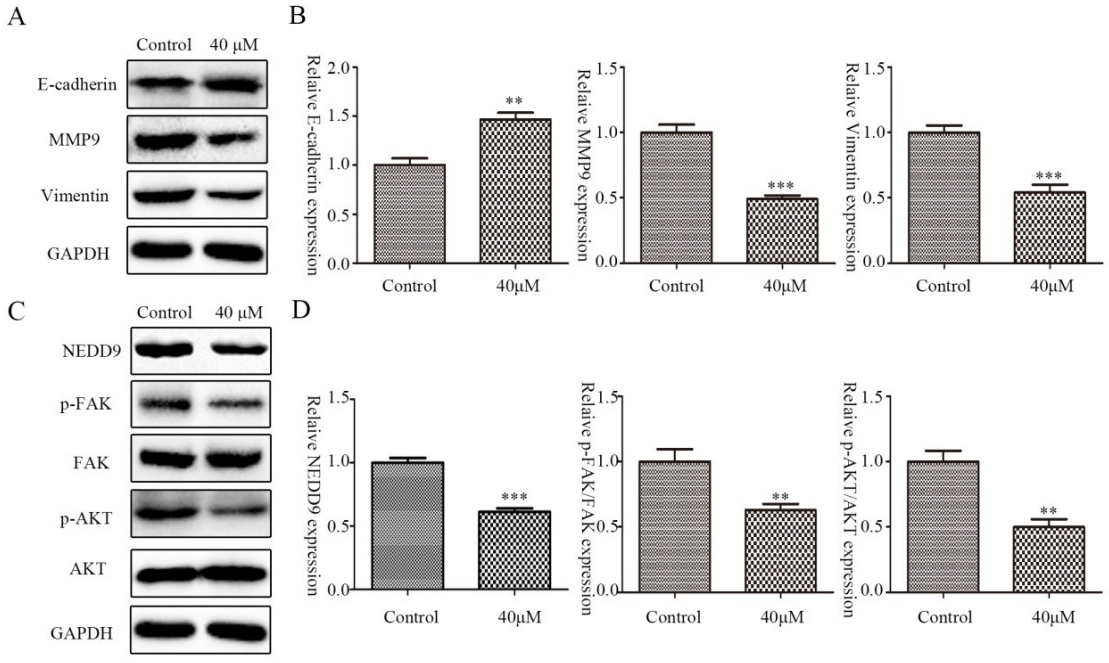

Figure 2. Apigenin prevents EMT and NEDD9/p-FAK/p-AKT expression. (A) U251 cells were cultured in 6cm dishes with 40 μM apigenin for 48 h and western blot analysis of E-cadherin, MMP9, Vimentin was conducted. (B) Relative protein levels of E-cadherin, MMP9 and Vimentin were quantified by image J. (C) Western blot analysis of NEDD9, p-FAK, FAK, p-AKT and AKT. (D) Relative protein levels of E-cadherin, MMP9 and Vimentin were quantified by image J. GAPDH was used as an internal control. Data are showed as mean ± SD. **P < 0.01, ***P < 0.001 (n = 3).

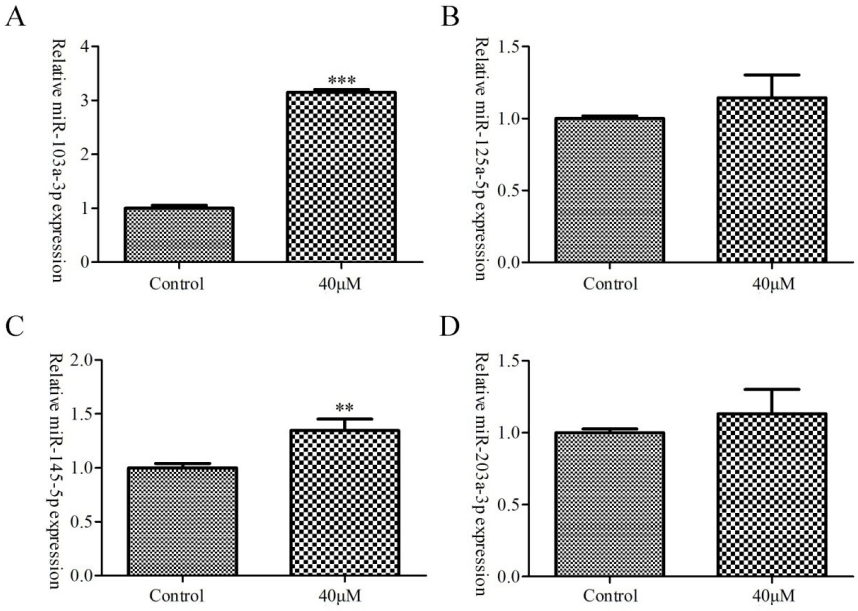

Figure 3. qRT-PCR analysis of miRNAs in U251 cells treated with apigenin . (A) The relative RNA expression of miR-103a-3p. (B) The relative RNA expression of miR-125a-5p. (C) The relative RNA expression of miR-145-5p. (D) The relative RNA expression of miR-203a-3p. Data are showed as mean ± SD. **P < 0.01, ***P < 0.001 (n = 3).

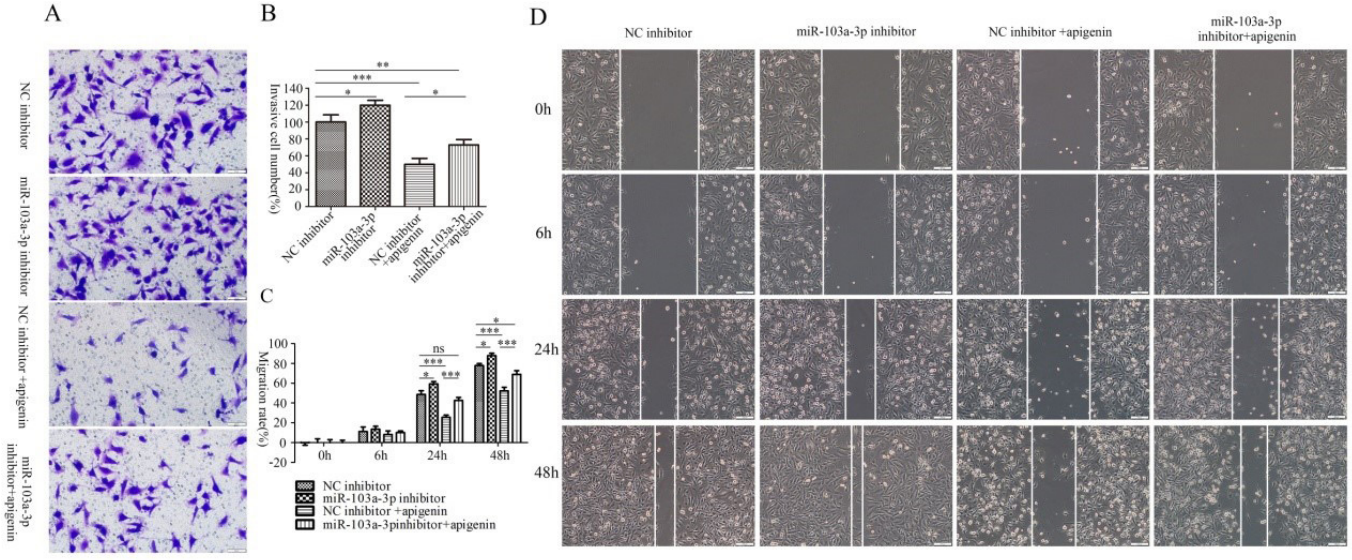

Figure 4. Inhibition of miR-103a-3p attenuated the inhibition effects of apigenin in U251 cells. U251 cells were transfected with NC inhibitor or miR-103a-3p inhibitor in the presence or absence of 40 μM apigenin. (A) Transwell analysis was performed to detect the invasion ability of U251 cells in each group. (C) Data analysis of transwell experiment. (D) Wound healing assay of U251 cells. Data are showed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 (n =3).

3.5 NEDD9 is a direct target of miR-103a-3p

To explore the molecular mechanisms of miR-103a-3p in U251 cells, we used online tool TargetScan to analysis the putative binding sites of miR-103a-3p at the 3′ untranslated region (UTR) of NEDD9. As shown in Figure 6A, NEDD9 3'UTR region contains the putative binding sites of miR-103a-3p. This predicted result was further verified by dual-luciferase assay. miR-103a-3p mimics significantly declined the relative luciferase activity in the NEED9 3'UTR WT group. However, luciferase reporter activity in NEED9 3'UTR MUT was nearly unaffected by miR-103a-3p mimics (Figure 6B). In order to further confirm the direct regulation of NEED9 by miR-103a-3p, NC mimics or miR-103a-3p was transfected separately and NEED9 expression level was examined by western blot assays. The result showed miR-103a-3p mimics could directly reduce the expression of NEED9 compared to NC

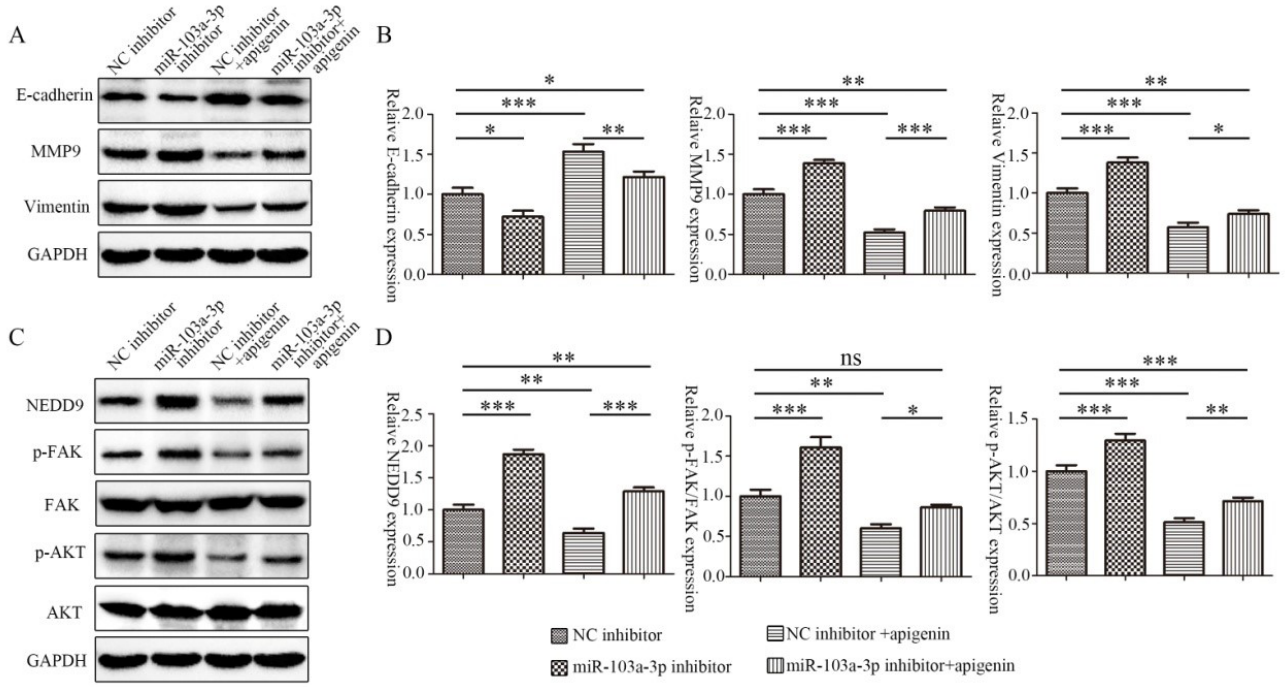

Figure 5. Inhibition of miR-103a-3p partly reversed the regulation effects of apigenin in EMT and NEDD9/p-FAK/p-AKT pathway. U251 cells were transfected with NC inhibitor or miR-103a-3p inhibitor in the presence or absence of 40 μM apigenin. (A) Western blot analysis of E-cadherin, MMP9 and Vimentin (B) Relative protein levels of E-cadherin, MMP9 and Vimentin were quantified by image J. (C) Western blot analysis of NEDD9, p-FAK, FAK, p-AKT and AKT. (D) Relative protein levels of E-cadherin, MMP9 and Vimentin were quantified by image J. GAPDH was used as an internal control. Data are showed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 (n = 3).

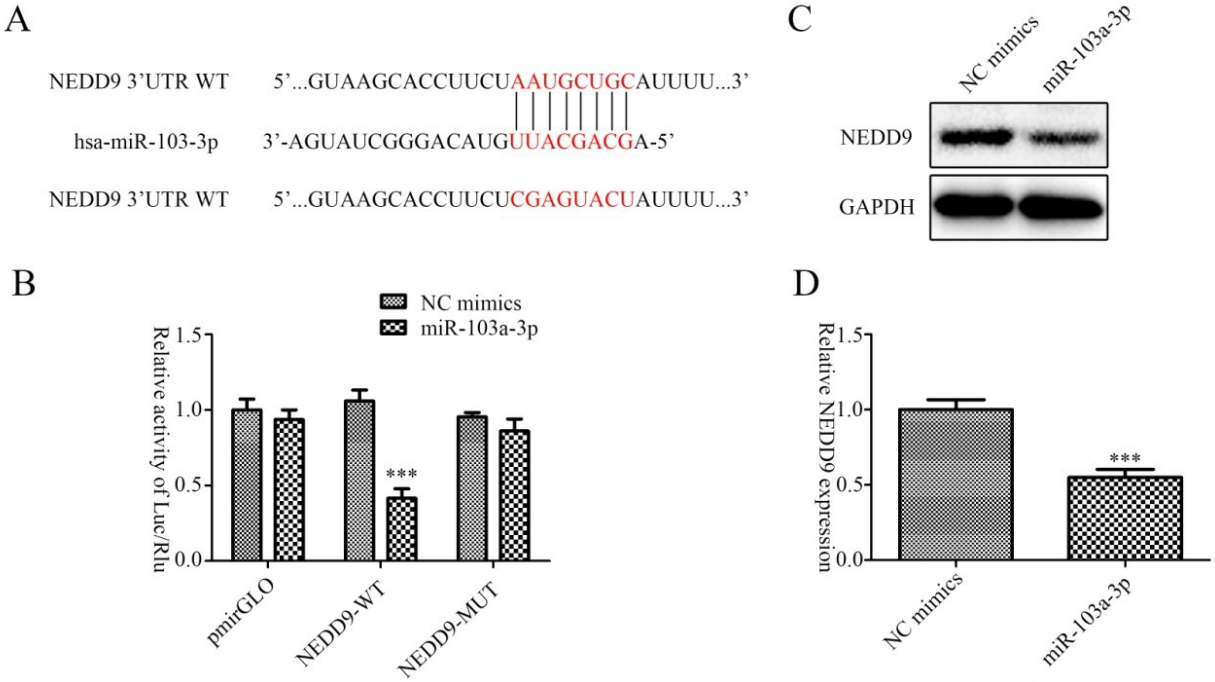

Figure 6. NEDD9 is a direct target of miR-103a-3p. (A) Binding site of miR-103a-3p in NEDD9 3'UTR predicted by TargetScan. (B) Luciferase activity was detected by dual-luciferase assay. pmirGLO empty vector, NEDD9-WT or NEDD9-MUT was co-transfected with NC mimics or miR-103a-3p mimics in U251 cells. (C) U251 cells were transfected with NC mimics or miR-103a-3p mimics and western blot analysis for NEDD9 expression. (D) Relative protein level of NEDD9 was quantified by image J. Data are showed as mean ± SD. ***P < 0.001.

mimics (Figure 6C and 6D). Collectively, these data verify miR-103a-3p may inhibit NEDD9 expression by binding to its 3'-UTR.

3.6 Function of apigenin is through miR-103a-3p and targeting NEED9/FAK/AKT pathway

To further determinate the underlying mechanism of apigenin in glioma, U251 cells were transfected with miR-103a-3p inhibitor or miR-103a-3p inhibitor+ siNEDD9 in the absence or presence of apigenin. Obviously, compared with control group, the invasive ability of U251 cells was restrained by apigenin treatment, but it was partly reserved in the presence of miR-103a-3p inhibitor. However, the inhibition effect of apigenin in cell invasion was almost recovered by co-transfection of miR-103a-3p inhibitor and siNEDD9 (Figure 7A and 7C). The wound healing assay also showed that silence of miR-103a-3p attenuated the function of apigenin in preventing cell migration, which was reversed by siNEDD9 (Figure 7B and 7D). Furthermore, Western blot analysis demonstrated that blocking the expression of NEDD9 by siNEED9 could recover the inhibiting regulation of apigenin on FAK/AKT pathway when miR-103a-3p was inhibited (Figure 8A and 8B). Collectively, the underlying mechanisms of apigenin in glioma maybe promote miR-103a-3p expression which targeting NEED9/FAK/AKT pathway.

4 Discussion

Many extracts and compounds from plants have good anti-tumor effects (Li et al., 2022a; Li et al., 2022b; Tian et al., 2022). Glioma is a common primary tumor of central nervous system with strong invasion ability, easy to metastasis and recurrence, and poor prognosis. Therefore, it is urgent to find a more effective way for glioma treatment. Previous study has found that, the extract of *Rumex japonicus* houtt roots has the inhibitory effect on glioma cells (Wu et al., 2022). Apigenin has lower endogenous toxicity and selectively kills tumor cells (Gupta et al., 2001). The anti-tumor mechanisms of apigenin mainly includes inhibition of proliferation, promotion of apoptosis

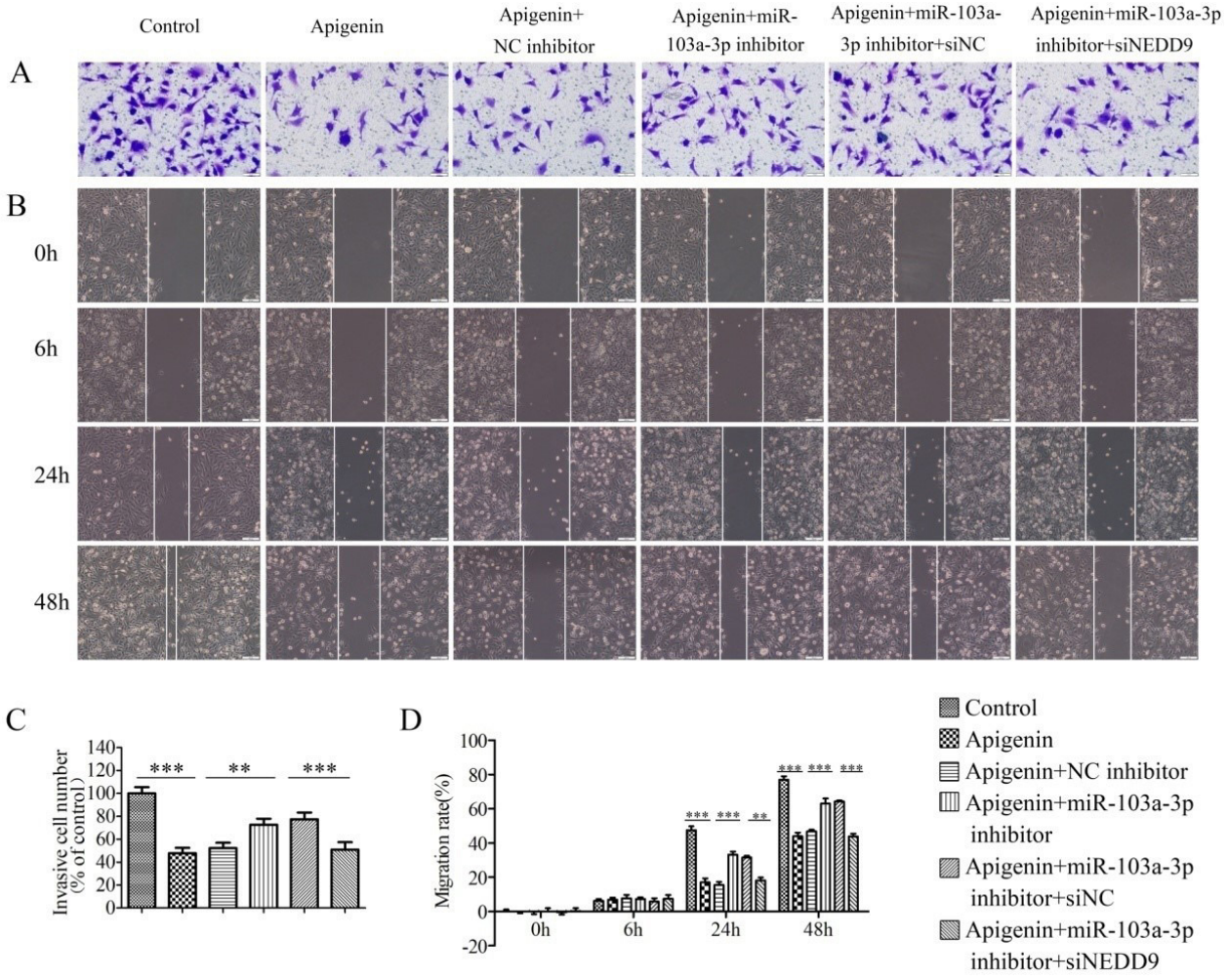

Figure 7. Co-expression of miR-103a-3p inhibitor and siNEDD9 recovers the inhibition of invasion and migration by apigenin. U251 cells were transfected with miR-103a-3p inhibitor or miR-103a-3p inhibitor+siNEDD9 in the absence or presence of 40 μM apigenin. (A) Transwell analysis was performed to detect the invasion ability of U251 cells in each group. (C) Data analysis of transwell experiment. (D) Wound healing assay of U251 cells. Data are showed as mean ± SD. **P < 0.01, ***P < 0.001 (n = 3).

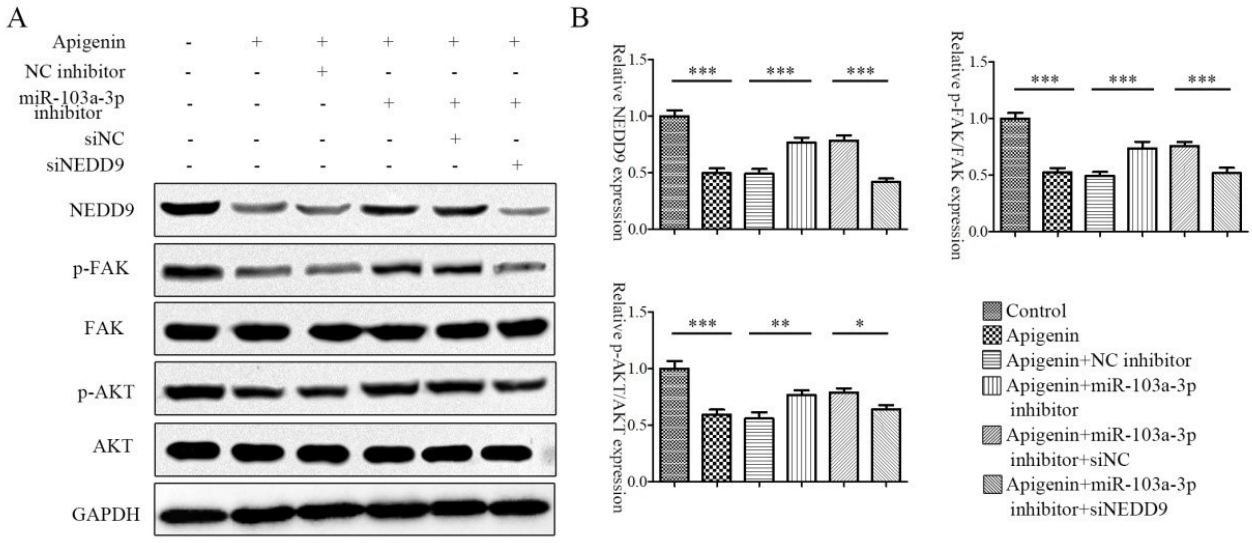

Figure 8. Co-expression of miR-103a-3p inhibitor and siNEDD9 recovers the inhibition of p-FAK/p-AKT signaling by apigenin. U251 cells were transfected with miR-103a-3p inhibitor or miR-103a-3p inhibitor+siNEDD9 in the absence or presence of 40 μM apigenin. (A) Western blot analysis of NEDD9, p-FAK, FAK, p-AKT and AKT. (B) Relative protein levels of E-cadherin, MMP9 and Vimentin were quantified by image J. GAPDH was used as an internal control. Data are showed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

and inhibition of cell migration and invasion (Chen et al., 2016; Sung et al., 2016; Wang et al., 2021a; Zhou et al., 2017b).

In this study, we firstly explored the effect of apigenin on proliferation of glioma cells U251. The observation suggests apigenin could suppress cell growth in a dose-dependent matter. Many studies have shown that glioma has strong invasion ability, easy to metastasis and recurrence. If the invasion and migration of glioma can be inhibited, the poor prognosis of glioma may be improved. The next, we found that apigenin treatment reduced cell invasion and migration by transwell invasion and wound healing assay. EMT refers to the loss of epithelial polarity and the acquisition of mesenchymal phenotype, mainly manifested as decreased expression of E-cadherin, increased expression of N-cadherin, Vimentin, MMP2, MMP9, Snail, etc. EMT often leads to epithelial tumor invasion and malignant transformation (Tong et al., 2019; Wang et al., 2021b). After apigenin treatment, we also found that apigenin inhibited cell EMT by promoting the expression of E-cadherin and inhibiting the expression of MMP9 and Vimentin. Therefore, our results suggest that apigenin may be a good candidate for the treatment of glioma.

NEDD9, also known as HEF1 or CAS-L, is a scaffold protein that is localized on focal adhesion and interacts with focal adhesion kinase (FAK) and non-receptor tyrosine kinase C-SRC to regulate multiple cellular signaling pathways. Many studies have shown that NEDD9 is closely related to cell migration and invasion (Dai et al., 2016; Meng et al., 2019; Gu et al., 2019; Zhou et al., 2017a). In addition, downregulation of NEDD9 by apigenin suppressing proliferation, migration and invasion in several kinds of cancer cells was reported, such as colorectal cancer cells (Dai et al., 2016) and lung cancer cells (Zhou et al., 2017b). As a protein kinase, FAK can promote the occurrence and metastasis of tumors and its high expression is closely related to tumor occurrence (Xie et al., 2019). Previous studies have shown that FAK/AKT signaling pathway is related to the proliferation, invasion and metastasis of glioma (Chen et al., 2021; Zhu et al., 2021). Therefore, we supposed that apigenin reduces glioma cell proliferation, migration and invasion, and EMT transformation may be though inhibiting the NEDD9/FAK/AKT pathway. Subsequent western blot experiment confirmed that the expression of NEDD9, the phosphorylation levels of FAK and AKT also decreased after apigenin treatment, indicating that apigenin can inhibit the NEDD9/FAK/AKT signaling pathway.

In consideration of NEDD9 is reported to be regulated by miRNAs (Han et al., 2015; Speranza et al., 2012; Xue et al., 2020), Putative candidate miRNAs which may target NEDD9 were predicted by TargetScan database. We selected four miRNAs that may not only target NEDD9 but also inhibit the invasion and migration of tumor cells (especially glioma cells). They are miR-103a-3p (Su et al., 2020; Yu et al., 2017), miR-125a-5p (Tang et al., 2019), miR-145-5p (Han et al., 2015; Wang et al., 2019) and miR-203a-3p (Wang et al., 2018). qRT-PCR was conducted to explore whether the expression of these miRNAs was affected by apigenin treatment. The result suggested only miR-103a-3p was significantly increased in the presence of apigenin. Thus, miR-103a-3p might be a tumor-suppressive miRNA and the candidate which mediates the expression of NEED9 under apigenin treatment.

For further study, we investigated whether inhibition of miR-103a-3p could reverse the effects of apigenin. As expected, U251 cells invasion and migration ability were partly recovered in the presence of apigenin and miR-103a-3p inhibitor compared with apigenin treatment alone. Western blot also showed the inhibition of EMT by apigenin was blocked with the downregulation of miR-103a-3p. Moreover, NEDD9 was upregulated by miR-

103a-3p inhibitor as well as the level of p-FAK and p-AKT. Collectively, it was clarified that the function of apigenin in U251 cell was mediated by miR-103a-3p.

Previous study indicated miR-145 (Han et al., 2015; Speranza et al., 2012), miR-25-5p (Wang et al., 2020) and miR-125b (Xue et al., 2020) suppress invasion and migration by targeting NEDD9. However, the relationship between miR-103a-3p and NEDD9 in glioma is still unknown. Bioinformatics analysis showed there was a binding site of miR-103a-3p in the 3'UTR of NEDD9. Subsequently, dual luciferase reporter assay demonstrated that miR-103a-3p could target NEDD9. This targeted regulation was also verified by western blot assay. Furthermore, we showed siNEDD9 could rescue the function of apigenin which was inhibited by miR-103a-3p inhibitor on the migration, invasion and FAK/AKT pathway in U251 cells. These results suggest that the effects of apigenin is promoting miR-103a-3p and targeting NEED9/FAK/AKT pathway in glioma cells.

5 Conclusions

Our study proved miR-103a-3p/NEDD9 was the downstream of apigenin in restraining glioma cells proliferation, migration, invasion and EMT via FAK/AKT signaling pathway. Apigenin is a plant-derived flavonoid with a variety of biological functions. Compared with other flavonoids, apigenin has lower endogenous toxicity and selectively kills tumor cells (Gupta et al., 2001), and it is easily to obtain from daily food. Therefore, it may provide new prospect for the prevention and treatment of glioma.

References

Ali, F., Rahul, Naz, F., Jyoti, S., & Siddique, Y. H. (2017). Health functionality of apigenin: a review. *International Journal of Food Properties*, 20(6), 1197-1238. http://dx.doi.org/10.1080/10942912.2016.1207188.

Bai, H., Jin, H., Yang, F., Zhu, H., & Cai, J. (2014). Apigenin induced MCF-7 cell apoptosis-associated reactive oxygen species. *Scanning*, 36(6), 622-631. http://dx.doi.org/10.1002/sca.21170. PMid:25327419.

Brem, H., Piantadosi, S., Burger, P. C., Walker, M., Selker, R., Vick, N. A., Black, K., Sisti, M., Brem, S., Mohr, G., Muller, P., Morawetz, R., & Schold, S. C., & The Polymer-brain Tumor Treatment Group (1995). Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas. *Lancet*, 345(8956), 1008-1012. http://dx.doi.org/10.1016/S0140-6736(95)90755-6. PMid:7723496.

Bushati, N., & Cohen, S. M. (2007). microRNA functions. *Annual Review of Cell and Developmental Biology*, 23, 175-205. http://dx.doi.org/10.1146/annurev.cellbio.23.090506.123406. PMid:17506695.

Chen, W., Lei, C., Liu, P., Liu, Y., Guo, X., Kong, Z., Wang, Y., Dai, C., Wang, Y., Ma, W., & Wang, Y. (2020). Progress and prospects of recurrent glioma: a recent scientometric analysis of the web of science in 2019. *World Neurosurgery*, 134, e387-e399. http://dx.doi.org/10.1016/j.wneu.2019.10.078. PMid:31639500.

Chen, X. J., Wu, M. Y., Li, D. H., & You, J. (2016). Apigenin inhibits glioma cell growth through promoting microRNA-16 and suppression of BCL-2 and nuclear factor-κB/MMP-9. *Molecular Medicine Reports*, 14(3), 2352-2358. http://dx.doi.org/10.3892/mmr.2016.5460. PMid:27430517.

Chen, X., Guo, Z. Q., Cao, D., Chen, Y., & Chen, J. (2021). MYC-mediated upregulation of PNO1 promotes glioma tumorigenesis by activating THBS1/FAK/Akt signaling. *Cell Death & Disease*, 12(3), 244. http://dx.doi.org/10.1038/s41419-021-03532-y. PMid:33664245.

Dai, J., Van Wie, P. G., Fai, L. Y., Kim, D., Wang, L., Poyil, P., Luo, J., & Zhang, Z. (2016). Downregulation of NEDD9 by apigenin suppresses migration, invasion, and metastasis of colorectal cancer cells. *Toxicology and Applied Pharmacology*, 311, 106-112. http://dx.doi.org/10.1016/j.taap.2016.09.016. PMid:27664007.

Ge, J., Mao, L., Xu, W., Fang, W., Wang, N., Ye, D., Dong, Z., Guan, H., & Guan, C. (2021). miR-103a-3p suppresses cell proliferation and invasion by targeting tumor protein D52 in prostate cancer. *Journal of Investigative Surgery*, 34(9), 984-992. http://dx.doi.org/10.1080/08941939.2020.1738602. PMid:32166986.

Gu, Y., Lu, J., Chen, C., & Zheng, F. (2019). NEDD9 overexpression predicts poor prognosis in solid cancers: a meta-analysis. *OncoTargets and Therapy*, 12, 4213-4222. http://dx.doi.org/10.2147/OTT.S205760. PMid:31213839.

Gupta, S., Afaq, F., & Mukhtar, H. (2001). Selective growth-inhibitory, cell-cycle deregulatory and apoptotic response of apigenin in normal versus human prostate carcinoma cells. *Biochemical and Biophysical Research Communications*, 287(4), 914-920. http://dx.doi.org/10.1006/bbrc.2001.5672. PMid:11573952.

Han, T., Yi, X. P., Liu, B., Ke, M. J., & Li, Y. X. (2015). MicroRNA-145 suppresses cell proliferation, invasion and migration in pancreatic cancer cells by targeting NEDD9. *Molecular Medicine Reports*, 11(6), 4115-4120. http://dx.doi.org/10.3892/mmr.2015.3294. PMid:25646678.

He, Q., Zhao, L., Liu, X., Zheng, J., Liu, Y., Liu, L., Ma, J., Cai, H., Li, Z., & Xue, Y. (2019). MOV10 binding circ-DICER1 regulates the angiogenesis of glioma via miR-103a-3p/miR-382-5p mediated ZIC4 expression change. *Journal of Experimental & Clinical Cancer Research*, 38(1), 9. http://dx.doi.org/10.1186/s13046-018-0990-1. PMid:30621721.

Kreisl, T. N., Kim, L., Moore, K., Duic, P., Royce, C., Stroud, I., Garren, N., Mackey, M., Butman, J. A., Camphausen, K., Park, J., Albert, P. S., & Fine, H. A. (2009). Phase II trial of single-agent bevacizumab followed by bevacizumab plus irinotecan at tumor progression in recurrent glioblastoma. *Journal of Clinical Oncology*, 27(5), 740-745. http://dx.doi.org/10.1200/JCO.2008.16.3055. PMid:19114704.

Li, H., Wang, T., Cui, W., Gao, Z., & Che, Z. (2022a). Effect of ginsenoside Rg3 on proliferation and apoptosis of 786-0 cells and AktmTORSTAT3 signaling in renal carcinoma. *Food Science and Technology*, 42, e124121. http://dx.doi.org/10.1590/fst.124121.

Li, K., Yuan, D., Chen, W., Ma, R., & Xian, Y. (2022b). (S)-(-)-N-[2-(3-Hydroxy-2-oxo-2,3-dihydro-1H-indol-3-yl)-ethyl]-acetamide inhibits colon cancer growth via the STAT1 pathway. *Food Science and Technology*, 42, e49121. https://doi.org/10.1590/fst.49121.

Louis, D. N., Ohgaki, H., Wiestler, O. D., Cavenee, W. K., Burger, P. C., Jouvet, A., Scheithauer, B. W., & Kleihues, P. (2007). The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathologica*, 114(2), 97-109. http://dx.doi.org/10.1007/s00401-007-0243-4. PMid:17618441.

Meng, H., Wu, J., Huang, Q., Yang, X., Yang, K., Qiu, Y., Ren, J., Shen, R., & Qi, H. (2019). NEDD9 promotes invasion and migration of colorectal cancer cell line HCT116 via JNK/EMT. *Oncology Letters*, 18(4), 4022-4029. http://dx.doi.org/10.3892/ol.2019.10756. PMid:31516604.

She, Y., Han, Y., Zhou, G., Jia, F., Yang, T., & Shen, Z. (2020). hsa_circ_0062389 promotes the progression of non-small cell lung cancer by sponging miR-103a-3p to mediate CCNE1 expression. *Cancer*

Genetics, 241, 12-19. http://dx.doi.org/10.1016/j.cancergen.2019.12.004. PMid:31962276.

Speranza, M. C., Frattini, V., Pisati, F., Kapetis, D., Porrati, P., Eoli, M., Pellegatta, S., & Finocchiaro, G. (2012). NEDD9, a novel target of miR-145, increases the invasiveness of glioblastoma. *Oncotarget*, 3(7), 723-734. http://dx.doi.org/10.18632/oncotarget.547. PMid:22869051.

Su, D., Ji, Z., Xue, P., Guo, S., Jia, Q., & Sun, H. (2020). Long-noncoding RNA FGD5-AS1 Enhances the viability, migration, and invasion of glioblastoma cells by regulating the miR-103a-3p/TPD52 axis. *Cancer Management and Research*, 12, 6317-6329. http://dx.doi.org/10.2147/CMAR.S253467. PMid:32848452.

Sung, B., Chung, H. Y., & Kim, N. D. (2016). Role of apigenin in cancer prevention via the induction of apoptosis and autophagy. *Journal of Cancer Prevention*, 21(4), 216-226. http://dx.doi.org/10.15430/JCP.2016.21.4.216. PMid:28053955.

Tang, L., Zhou, L., Wu, S., Shi, X., Jiang, G., Niu, S., & Ding, D. (2019). miR-125a-5p inhibits colorectal cancer cell epithelial–mesenchymal transition, invasion and migration by targeting TAZ. *OncoTargets and Therapy*, 12, 3481-3489. http://dx.doi.org/10.2147/OTT.S191247. PMid:31190857.

TargetScanHuman. (2022). Retrieved from http://www.targetscan.org/

Tian, Y., Pang, X., & Wang, F. (2022). Isolation of curcumol from zedoary turmeric oil and its inhibitory effect on growth of human hepatocellular carcinoma xenografts in nude mice. *Food Sci Tech*, 42, e46621. http://dx.doi.org/10.1590/fst.46621.

Tong, J., Shen, Y., Zhang, Z., Hu, Y., Zhang, X., & Han, L. (2019). Apigenin inhibits epithelial-mesenchymal transition of human colon cancer cells through NF-κB/Snail signaling pathway. *Bioscience Reports*, 39(5), BSR20190452. http://dx.doi.org/10.1042/BSR20190452. PMid:30967496.

Wang, D., Wang, Z., Dai, X., Zhang, L., & Li, M. (2021a). Apigenin and temozolomide synergistically inhibit glioma growth through the PI3K/AKT pathway. *Cancer Biother Radio*. In press. http://dx.doi.org/10.1089/cbr.2020.4283.

Wang, S., Huang, Y., Luo, G., Yang, X., & Huang, W. (2021b). Cyanidin-3-O-glucoside attenuates high glucose–induced podocyte dysfunction by inhibiting apoptosis and promoting autophagy via activation of SIRT1/AMPK pathway. *Canadian Journal of Physiology and Pharmacology*, 99(6), 589-598. http://dx.doi.org/10.1139/cjpp-2020-0341. PMid:33049148.

Wang, Y., Dan, L., Li, Q., Li, L., Zhong, L., Shao, B., Yu, F., He, S., Tian, S., He, J., Xiao, Q., Putti, T. C., He, X., Feng, Y., Lin, Y., & Xiang, T. (2019). ZMYND10, an epigenetically regulated tumor suppressor, exerts tumor-suppressive functions via miR145-5p/NEDD9 axis in breast cancer. *Clinical Epigenetics*, 11(1), 184. http://dx.doi.org/10.1186/s13148-019-0785-z. PMid:31801619.

Wang, Y., Tao, B., Li, J., Mao, X., He, W., & Chen, Q. (2020). Melatonin inhibits the progression of oral squamous cell carcinoma via inducing miR-25-5p expression by directly targeting NEDD9. *Frontiers in Oncology*, 10, 543591. http://dx.doi.org/10.3389/fonc.2020.543591. PMid:33344223.

Wang, Z., Zhao, Z., Yang, Y., Luo, M., Zhang, M., Wang, X., Liu, L., Hou, N., Guo, Q., Song, T., Guo, B., & Huang, C. (2018). MiR-99b-5p and miR-203a-3p function as tumor suppressors by targeting IGF-1R in gastric cancer. *Scientific Reports*, 8(1), 10119. http://dx.doi.org/10.1038/s41598-018-27583-y. PMid:29973668.

Weller, M., Cloughesy, T., Perry, J. R., & Wick, W. (2013). Standards of care for treatment of recurrent glioblastoma—are we there yet? *Neuro-Oncology*, 15(1), 4-27. http://dx.doi.org/10.1093/neuonc/nos273. PMid:23136223.

Wen, P. Y., & Reardon, D. A. (2016). Progress in glioma diagnosis, classification and treatment. *Nature Reviews. Neurology*, 12(2), 69-70. http://dx.doi.org/10.1038/nrneurol.2015.242. PMid:26782337.

Wilson, R., Espinosa-Diez, C., Kanner, N., Chatterjee, N., Ruhl, R., Hipfinger, C., Advani, S. J., Li, J., Khan, O. F., Franovic, A., Weis, S. M., Kumar, S., Coussens, L. M., Anderson, D. G., Chen, C. C., Cheresh, D. A., & Anand, S. (2016). MicroRNA regulation of endothelial TREX1 reprograms the tumour microenvironment. *Nature Communications*, 7(1), 13597. http://dx.doi.org/10.1038/ncomms13597. PMid:27886180.

Wu, Z., Ameer, K., & Jiang, G. (2022). Isolation and characterization of anti-tumor compounds from ethyl acetate extract of Rumex japonicus houtt roots and their cytotoxic effects. *Food Sci Tech*, 42, e05421. http://dx.doi.org/10.1590/fst.05421.

Xie, Y., Du, J., Liu, Z., Zhang, D., Yao, X., & Yang, Y. (2019). MiR-6875-3p promotes the proliferation, invasion and metastasis of hepatocellular carcinoma via BTG2/FAK/Akt pathway. *Journal of Experimental & Clinical Cancer Research*, 38(1), 7. http://dx.doi.org/10.1186/s13046-018-1020-z. PMid:30621734.

Xu, Y., Xin, Y., Diao, Y., Lu, C., Fu, J., Luo, L., & Yin, Z. (2011). Synergistic effects of apigenin and paclitaxel on apoptosis of cancer cells. *PLoS One*, 6(12), e29169. http://dx.doi.org/10.1371/journal.pone.0029169. PMid:22216199.

Xue, Y., Zhong, Y., Wu, T., Sheng, Y., Dai, Y., Xu, L., & Bao, C. (2020). Anti-Proliferative and Apoptosis-Promoting Effect of microRNA-125b on Pancreatic Cancer by Targeting NEDD9 via PI3K/AKT Signaling. *Cancer Management and Research*, 12, 7363-7373. http://dx.doi.org/10.2147/CMAR.S227315. PMid:32903925.

Yu, M., Xue, Y., Zheng, J., Liu, X., Yu, H., Liu, L., Li, Z., & Liu, Y. (2017). Linc00152 promotes malignant progression of glioma stem cells by regulating miR-103a-3p/FEZF1/CDC25A pathway. *Molecular Cancer*, 16(1), 110. http://dx.doi.org/10.1186/s12943-017-0677-9. PMid:28651608.

Zhao, S., Min, P., Liu, L., Zhang, L., Zhang, Y., Wang, Y., Zhao, X., Ma, Y., Xie, H., Zhu, C., Jiang, H., Du, J., & Gu, L. (2019). NEDD9 facilitates hypoxia-induced gastric Cancer cell migration via MICAL1 related Rac1 activation. *Frontiers in Pharmacology*, 10, 291. http://dx.doi.org/10.3389/fphar.2019.00291. PMid:31019460.

Zhao, Y., Huang, H., Jia, C. H., Fan, K., Xie, T., Zhu, Z. Y., & Xie, M. L. (2021). Apigenin increases radiosensitivity of glioma stem cells by attenuating HIF-1α-mediated glycolysis. *Medical Oncology (Northwood, London, England)*, 38(11), 131. http://dx.doi.org/10.1007/s12032-021-01586-8. PMid:34554338.

Zhou, R. T., He, M., Yu, Z., Liang, Y., Nie, Y., Tai, S., & Teng, C. B. (2017a). Baicalein inhibits pancreatic cancer cell proliferation and invasion via suppression of NEDD9 expression and its downstream Akt and ERK signaling pathways. *Oncotarget*, 8(34), 56351-56363. http://dx.doi.org/10.18632/oncotarget.16912. PMid:28915595.

Zhou, Z., Tang, M., Liu, Y., Zhang, Z., Lu, R., & Lu, J. (2017b). Apigenin inhibits cell proliferation, migration, and invasion by targeting Akt in the A549 human lung cancer cell line. *Anti-Cancer Drugs*, 28(4), 446-456. http://dx.doi.org/10.1097/CAD.0000000000000479. PMid:28125432.

Zhu, Y. G., Lv, Y. X., Guo, C. Y., Xiao, Z. M., Jiang, Q. G., Kuang, H., Zhang, W. H., & Hu, P. (2021). Harmine inhibits the proliferation and migration of glioblastoma cells via the FAK/AKT pathway. *Life Sciences*, 270, 119112. http://dx.doi.org/10.1016/j.lfs.2021.119112. PMid:33508300.