JBUON 2020; 25(6): 2592-2599
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

MiR-489 inhibits proliferation and apoptosis of glioblastoma multiforme cells *via* regulating TWIST1 expression

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Summary

Purpose: *To investigate the influences of micro ribonucleic acid (miR)-489 on the proliferation and apoptosis of glioblastoma multiforme (GBM) and the relationship between miR-489 and twist-related protein 1 (TWIST1) expression.*

Methods: *The GBM cells were isolated and cultured in vitro, and then transfected with miR-489 inhibitor, miR-489 mimics and miR-negative control (NC) or TWIST1-small interfering RNA (siRNA) and TWIST1-NC. The expression levels of miR-489 and TWIST1 gene in the cells were measured via quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and the proliferative capacity of cells in each group was detected by cell counting kit-8 (CCK-8) assay. Besides, the target gene TWIST1 of miR-489 was predicted to construct the luciferase reporter gene vectors of TWIST1 containing miR-489 target sites.*

Results: *The expression level of miR-489 in GBM tissues and GBM cells isolated and cultured in vitro was remarkably lower than that in normal tissues and cells ($p<0.01$). The proliferative capacity of GBM cells was enhanced notably after inhibiting the expression of miR-489 ($p<0.01$), while it was obviously weakened by overexpressed miR-489 or TWIST1-siRNA ($p<0.01$). Moreover, the apoptosis rate was increased from 2.3±0.4% to 19.6±1.2% following miR-489 overexpression. TWIST1-siRNA could markedly down-regulate the expression level of TWIST1 ($p<0.01$) but evidently up-regulate the protein expression levels of Caspase-3 and Caspase-8 ($p<0.01$). The results of luciferase reporter assay manifested that miR-489 mimics significantly repressed TWIST1 ($p<0.01$).*

Conclusions: *MiR-489 can repress the proliferation and promote the apoptosis of glioma cells by targeting TWIST1.*

Key words: *glioblastoma multiforme, miR-489, TWIST1, cell proliferation, cell apoptosis*

Introduction

Gliomas can be classified into astrocytomas, oligodendrogliomas and mixed gliomas, of which glioblastoma multiforme (GBM), a type of astrocytoma, accounts for over 80% of all the brain tumors, becoming the most common malignant tumor in the central nervous system [1-3]. Neuroglioma is the most ubiquitous invasive malignant primary tumor in human central nervous system [4,5]. Despite great progress in treatment techniques of glioma, the recurrence rate of brain glioma is still fairly high after treatments such as primary resection, and the disease develops rapidly [6]. Better comprehending the potential molecular pathology and signaling pathways involved in neuroglioma progression may help discover new potential targets, thus designing innovative therapies for preventing neuroglioma recurrence and prolonging survival time [7].

Micro ribonucleic acids (miRs), a category of small non-coding RNAs composed of 18-25 nucleotides, play crucial regulatory roles in cell proliferation and apoptosis [8-14]. Formed by transcription of deoxyribonucleic acid (DNA) sequences, miRs are processed into precursor and mature miRs in

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Received: 08/10/2020; Accepted: 07/11/2020

animals and plants. Moreover, miRs complementarily bind to the messenger RNAs (mRNAs) of target genes to promote mRNA degradation or inhibit mRNA translation, thereby negatively regulating gene expression [15]. Large quantities of studies have elucidated that miRs participate in multiple biological processes, including cell cycle, proliferation, differentiation, apoptosis and metabolism, and they exert important effects in the occurrence and development of various cancers [16-18]. Given the causality between miRs and cancer development, the interaction between miRs and their target has attracted the attention of researchers [19]. More importantly, the dysregulated expression pattern of miRs may serve as promising biomarkers for cancer diagnosis and prognosis. The analysis of miR expression profiles in GBM revealed that the expression of a variety of miRs is increased or decreased [19]. Zhang et al [1] found that LINC01446 plays a vital role in the progression of GBM mainly through the miR-489-3p/tumor protein, translationally-controlled 1 (TPT1) axis, and overexpressed miR-489-3p can suppress the proliferation of GBM.

In this research, the action mechanism of miR-489 in regulating the proliferation and apoptosis of glioma cells was investigated, so as to better understand the pathogenic mechanisms of miR-489 and TWIST1 in GBM.

Methods

Main materials

The following materials were used in this study: GBM, SYBR Green reverse transcription (RT) Master Mix kit (TaKaRa, Tokyo, Japan), Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12), Opti-Minimal Essential Medium (MEM), α-minimum Eagle's medium (α-MEM), fetal bovine serum (FBS), 2.5% trypsin + 0.02% ethylene diamine tetraacetic acid (EDTA) and phosphate-buffered saline (PBS) (Gibco, Rockville, MD, USA), Lipofectamine 2000 and TRIzol (Invitrogen, Carlsbad, CA, USA), HiPerFect Transfection Reagent (QIAGEN, Hilden, Germany), antibodies against twist-related protein 1 (TWIST1), Caspase-3 and β-Actin (Abcam, Cambridge, MA, USA), miRNA RT kit (Applied Biosystems, Foster City, CA, USA), luciferase reporter gene assay system (Promega, Madison, WI, USA), bioluminescent plate reader (Modulus™), and 0.22 μm pinhole filter (Millipore, Billerica, MA, USA).

Experimental methods

Acquisition, isolation and culture of human GBM

Human GBM tissues were obtained from patients definitely diagnosed with GBM and treated with tumor resection. The excised tumor samples were stored in sterile tissue preservation solution and sent to the laboratory timely. Moreover, the normal tissues and tumor tissues were separated on a super-clean bench.

Isolation and culture of cells *in vitro*: The tumor tissues and normal para-tumor tissues were washed with PBS for 5-8 times to remove blood and other impurities. Next, the tumor tissues and normal para-tumor tissues were cut into tissue blocks (1 mm^3) and then digested in 2.5% trypsin + 0.02% EDTA in an incubator for 10-30 min. The cell suspension was filtered by a cell sieve, the digestion was terminated by a medium containing 10% FBS, and the cell suspension was centrifuged to eliminate the supernatant. After that, the cells were resuspended in DMEM/F12 + 10% FBS, counted, seeded into 6-well plates at 1×10^5 cells/per well and cultured in the incubator with 5% CO_2 at 37°C. The next day, the liquid was replaced to remove non-adherent cells, and then the liquid was replaced every other day. Later, the cells were subcultured when they reached 90% confluence. After washing with PBS once, the cells were digested with a proper amount of 0.02% EDTA + 2.5% trypsin in the incubator at 37°C for 2-3 min. Finally, the cells were blown scattered carefully after the digestion was terminated by DMEM/F12 + 10% FBS, followed by subculture at 1:3.

Cell transfection with miR-489

The cells in good growth status were inoculated into a 24-well plate (2×10^4 cells/per well) and then transfected separately with miR-489 inhibitor, miR-489 mimics and miR-negative control (NC) upon reaching 90% confluence. Specifically, 20 μM mother solutions of miR-489 inhibitor, miR-489 mimics and miR-NC (5 μL each) were mixed with 250 μL of α-MEM separately and incubated at room temperature for 5 min. Subsequently, the mixed transfection solution was added into the above 24-well plates, shaken well and cultured in the incubator under 5% CO_2 at 37°C. Forty-eight h later, the expression level of miR-489 in each group of cells was detected.

Interference in TWIST1 mRNA

The well-grown cells were seeded into 6-well plates (2×10^4 cells/per well). When the cell confluence reached 90%, 5 μL of TWIST1-small interfering RNA (siRNA) mother solution (20 μM) and 5 μL of TWIST1-NC stock solution were added into 83 μL of serum-free Opti-MEM. After that, the mixture was mixed with 12 μL of HiPerFect transfection reagent, and the transfection complex was added into the 6-well plates in drops after 10 min of incubation at room temperature, followed by gentle shaking and culture in the incubator under 5% CO_2 at 37°C. Forty-eight h later, the expression level of TWIST1 gene was determined.

Extraction of total RNA and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The tissue samples were ground in liquid nitrogen, mixed with TRIzol and cultured. Later, the DMEM was discarded, and the cells were mixed with TRIzol for 5 min, added with 200 μL of chloroform, and centrifuged at 12,000 rpm and 4°C for 10 min. Next, the supernatant was taken away, added with an equal volume of isopropyl alcohol and centrifuged at 12,000 rpm at 4°C for 15 min after standing at room temperature for 10 min. Then, the cells were washed twice with freshly

Table 1. Primer sequences for miR-489, TWIST1 and GAPDH detection

Name	*Sequence*
MiR-489 F	5'-GTGACATCACATATACGGCAGC-3'
MiR-489 R	5'-CACAGCTCGTAGAACAGGAGG-3'
TWIST1 F	5'-TCTACCAGGTCCTCCAGAGC-3'
TWIST1 R	5'-CTCCATCCTCCAGACCGAGA-3'
GAPDH F	5'-GTCAAGGCTGAGAACGGGAA-3'
GAPDH R	5'-AAATGAGCCCCAGCCTTCTC-3'

prepared 75% ethanol and dissolved in an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water (Beyotime, Shanghai, China), and the concentration was measured using a NanoDrop spectrophotometer. After that, the primers were designed by means of Primer Premier 6.0 software and synthesized by Sangon Biotech (Shanghai, China) Co., Ltd. The primer sequences of miR-489, TWIST1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in Table 1. MiR-489 was synthesized into first-strand complementary DNA (cDNA) using the qScript microRNA cDNA kit, and the total RNA was synthesized into cDNA through the random primers from RT Master Mix kit for TWIST1 and GAPDH. Next, qRT-PCR was performed using the SYBR Green Real-Time PCR Master Mix kit and ABI 7500 sequence detection system in accordance with the manufacturer's protocol. The transcription level was evaluated *via* cycle threshold (Ct) value. The target amount of standardized internal reference was calculated through $2^{-\Delta\Delta Ct}$ method.

Cell counting kit-8 (CCK-8) assay

The cells in good growth status were inoculated into a 96-well plates at 4×10^3 cells/well, with 6 replicate wells for each group, and then cultured in the incubator with 5% CO_2 at 37°C. After adherence, the cells were cultured for another 24 h and 48 h. After discarding the medium, another freshly prepared medium supplemented with 10 μL of CCK-8 assay solution (Dojindo, Kumamoto, Japan) was added into each well, and the cells were cultured again in the incubator for 4 h, and the optical density (OD) value at the wavelength of 450 nm was measured using a microplate reader. The experiment was repeated for 3 times, and the average value of the experimental results was taken as the final result.

Flow cytometry

The cells in each group were digested into single-cell suspension using 0.25% trypsin + 0.02% EDTA (ethylenediaminetetraacetic acid), washed with PBS for 3 times and counted. After that, the cell density was adjusted to 5×10^5 cells/per well, and the cells were incubated with Annexin V and Propidium Iodide (PI) at 4°C in the dark for 30 min. After washing with PBS for 3 times, the cell density was adjusted to 10^6 cells/mL, followed by detection on a flow cytometer (C6) and data analysis using CFlow Plus software.

Luciferase assay

The cells in each group were transfected with miR-489 mimics or miR-NC and wild-type TWIST1 (TWIST1-WT) or mutant-type TWIST1 (TWIST1-MUT) for 48 h. After that, DMEM was discarded, and the cells in each group were washed with PBS for 3 times and lysed by 50 μL of freshly prepared RIPA lysis buffer for 30 min. Then, 10 μL of RIPA lysis buffer was added with 100 μL of luciferase assay reagent prepared in advance, and the luciferase activity was determined by means of the luciferase reporter gene assay system combined with the bioluminescent plate reader. Each experiment was set with 3 parallel controls and repeated for three times.

Western blotting (WB)

Each group of cells was added with a proper amount of cell lysate, then the lysate suspension was collected using a cell scraper, and the cells were lysed at 4°C overnight, followed by centrifugation at 13,000 rpm to extract the total protein and detection of protein concentration through bicinchoninic acid (BCA) method. Next, the proteins were separated *via* 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. Subsequently, the membrane was sealed in 5% skim milk powder and 0.1% Tris-buffered saline-Tween 20, and incubated with primary antibodies against TWIST1, Caspase-3 and β-actin separately by gently shaking at 4°C overnight. After that, horse radish peroxidase (HRP)-labeled secondary antibodies were added for incubation, and the proteins to be detected were exposed using electrochemiluminescence (ECL) reagent.

Statistics

SPSS 20.0 software (IBM, Armonk, NY, USA) was used for data recording and processing. The data in each group were presented as mean ± standard deviation. Differences between two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using One-way ANOVA test followed by *post hoc* test (least significant difference). $P<0.05$ suggested that the difference was statistically significant.

Results

Expression level of miR-489 in glioma tissues and cells

The GBM tissues and para-tumor tissues were isolated. The results of qRT-PCR indicated that the expression level of miR-489 in tumor tissues was remarkably lower than that in normal para-tumor tissues ($p<0.01$) (Figure 1A). Moreover, the GBM cells were isolated and cultured *in vitro*, and the normal cells isolated from para-tumor tissues and cultured were set as the controls, so as to detect the expression level of miR-489. It was manifested that the expression level of miR-489 in GBM was also evidently lower than that in normal cells ($p<0.01$) (Figure 1B).

Influence of miR-489 on proliferation of GBM cells

The GBM cells were cultured *in vitro* and transfected with miR-NC, miR-489 inhibitor and miR-489 mimics, with the cells cultured normally as the controls. The results of qRT-PCR showed that the expression level of miR-489 was markedly down-regulated after transfection with miR-489 inhibitor ($p<0.01$) but up-regulated notably after transfection with miR-489 mimics ($p<0.01$), while the transfection with miR-NC had no significant impact on the miR-489 expression level ($p>0.05$) (Figure 2A). According to the cell proliferation detected through CCK-8 assay, the proliferative capacity of the cells transfected with miR-489 inhibitor for 24 h ($p<0.01$)

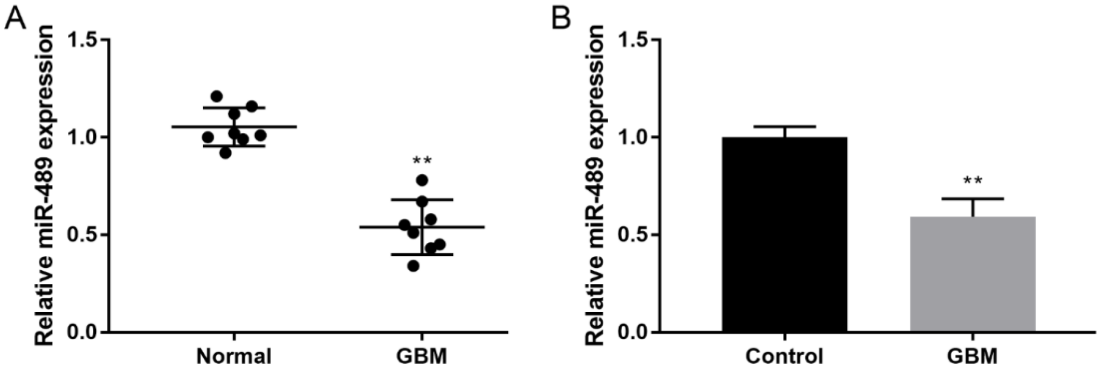

Figure 1. Expression level of miR-489 detected via qRT-PCR. **A:** Expression level of miR-489 in GBM tissues and paratumor tissues. **B:** Expression level of miR-489 in GBM cells and normal cells isolated and cultured *in vitro* (**p<0.01).

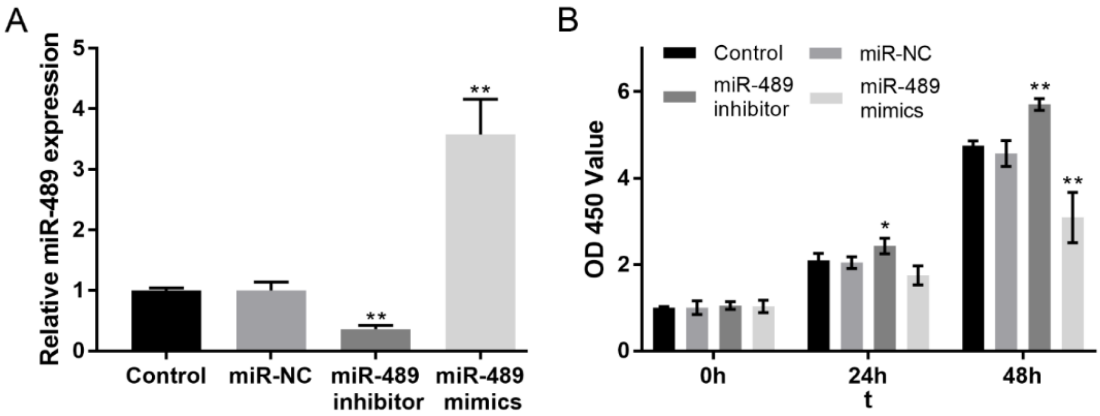

Figure 2. Influence of miR-489 on the proliferation of GBM cells. **A:** Expression level of miR-489 after transfection of GBM cells with miR-NC, miR-489 inhibitor and miR-489 mimics detected through qRT-PCR. **B:** Proliferative capacity of cells in each group at 0, 24 and 48 h determined via CCK-8 assay (*p<0.05 & **p<0.01).

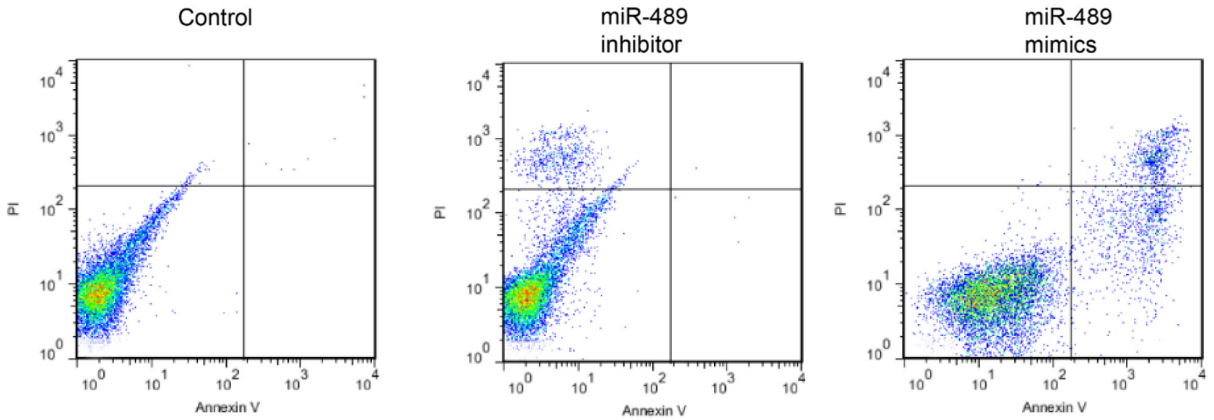

Figure 3. Apoptosis rate detected via flow cytometry showing miR-489 transfected mimics was distinctly higher than that in the other two groups.

and 48 h (p<0.01) was significantly enhanced, while that of cells transfected with miR-489 mimics for 48 h was clearly weakened (p<0.01). However, no apparent influence of miR-NC on the proliferative capacity of the cells was observed (p>0.05) (Figure 2B).

Influence of miR-489 on apoptosis of GBM cells

The analysis of cell apoptosis by means of flow cytometry revealed that the apoptosis rate of normally cultured cells was 2.3±0.4%, the apoptosis rate of cells transfected with miR-489 inhibitor was 1.8±0.5%, and the apoptosis rate of cells transfected with miR-489 mimics was 19.6±1.2%. In short, the apoptosis rate of the cells transfected with miR-489 mimics was distinctly higher than that in the other two groups (p<0.01) (Figure 3).

Relationship between miR-489 and TWIST1

The targeted regulating correlation between miR-489 and TWIST1 was predicted, and the

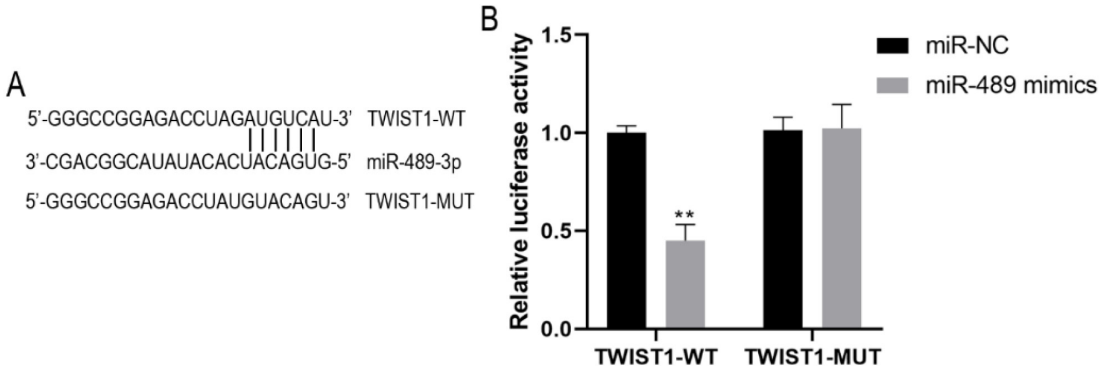

Figure 4. Interrelation between TWIST1-WT and TWIST1-MUT under the actions of miR-489 mimics and miR-NC verified via luciferase reporter gene assay system. **A:** Comparison of sequences of miR-489 and TWIST1 target gene. **B:** Relative luciferase activity (**p<0.01).

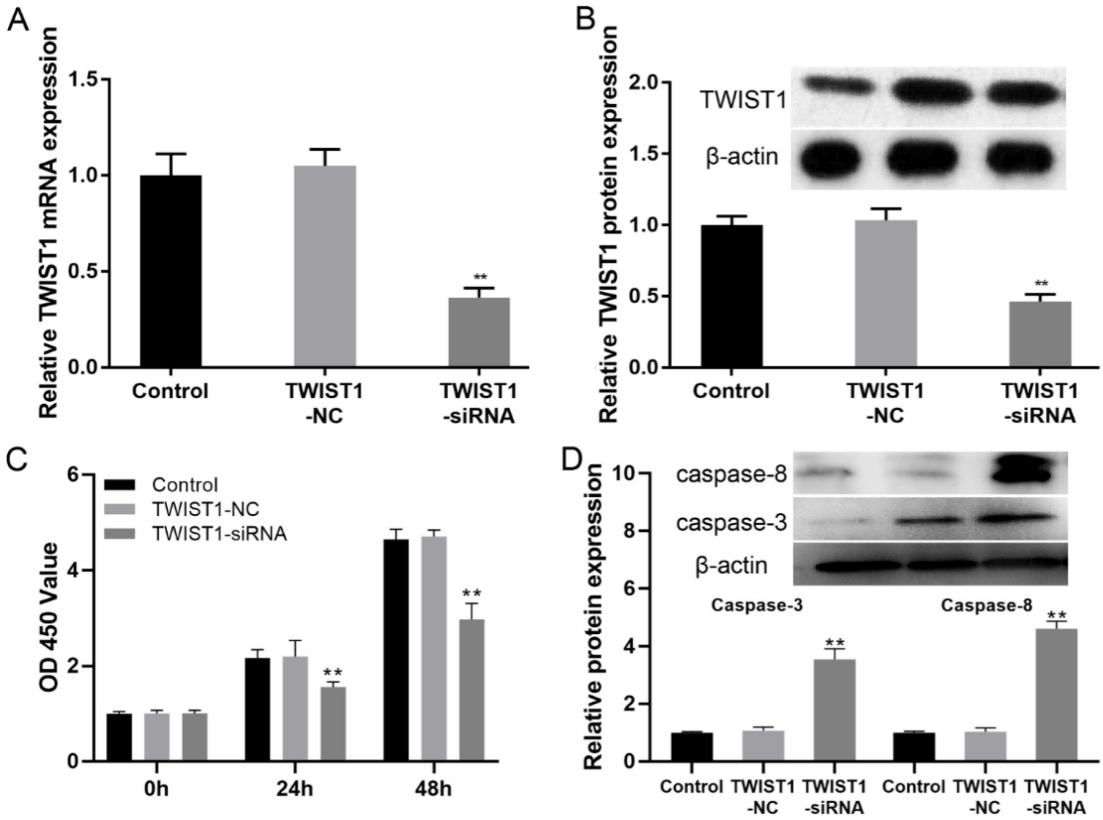

Figure 5. Impacts of TWIST1 expression on cell proliferation and apoptosis. **A:** Expression of TWIST1 mRNA in cells measured by qRT-PCR. **B:** Protein expression of TWIST1 detected via western blotting (WB) assay and relative quantitative analysis. **C:** Proliferative ability of cells cultured *in vitro* detected via CCK-8 assay. **D:** Expressions of apoptotic proteins Caspase-3 and Caspase-8 in cells determined via WB assay and relative quantitative analysis (**p<0.01).

target gene sequences are shown in Figure 4A. Besides, the luciferase reporter gene vectors of TWIST1 containing the predicted miR-489 target sites were constructed. The GBM cells were transfected with TWIST1-WT plasmid, TWIST1-MUT plasmid, miR-489 mimics and miR-NC separately. The detection results of luciferase activity manifested that the luciferase activity of TWIST1-WT was remarkably suppressed by miR-489 mimics ($p<0.01$), but there was no clear change in the luciferase activity of TWIST1-MUT ($p>0.05$) (Figure 4B).

Impacts of TWIST1 expression on cell proliferation and apoptosis

In order to testify the impacts of TWIST1 on the proliferation and apoptosis of GBM cells, the mRNA expression of TWIST1 in GBM cells was interfered. After interference with TWIST1-siRNA, the mRNA and protein expression levels of TWIST1 declined remarkably ($p<0.01$) (Figure 5A & 5B). The results of CCK-8 assay showed that TWIST1-siRNA evidently attenuated the proliferative capacity of GBM cells cultured *in vitro* for 24 and 48 h ($p<0.01$) (Figure 5C). Furthermore, the expressions of apoptotic proteins Caspase-3 and Caspase-8 in cells were determined *via* western blotting (WB) assay, which demonstrated that the protein expression levels of Caspase-3 and Caspase-8 were markedly elevated in TWIST1-siRNA group compared with those in control group and TWIST1-NC group ($p<0.01$) (Figure 5D).

Discussion

As the most common brain malignancy, GBM has a morbidity rate of 5.6/100,000 in males and 7.8/100,000 in females [20]. Based on the location and grade of tumor, patients may manifest various acute symptoms such as epileptic seizure, movement disorder and loss of consciousness [21], and other progressive symptoms including headache, personality change and cognitive problems [22]. MiRs, a category of important non-coding regulatory genes, have vital effects on multiple biological functions [23-25]. Among them, miR-489 serves as a tumor-suppressor by targeting a variety of carcinogenic cascades in different types of cancers. In addition, it can act on the LINC01446/miR-489-3p/TPT1 axis through targeting p21-activated kinase 5 (PAK5), thus modulating the progression of GBM [26]. As one of the effectors of lncRNA ENST01108, miR-489 is able to target SIK1 and inhibit GBM progression [26]. Moreover, it can induce cell apoptosis and arrest cell cycle progression by targeting the SPIN1-mediated PI3K/AKT pathway [27]. A latest study demonstrated that miR-489 is capable of reducing Bcl-2 expression and raising Bax expression through the PAK5/RAF1/MMP2 pathway, thereby increasing cell apoptosis and decreasing the migratory and invasive ability of cells [8]. Similar to the results of previous studies, the results in this research indicated that the expression level of miR-489 was down-regulated in glioma tissues and cells, while the overexpression of miR-489 in GBM cells could obviously weaken the proliferative capacity of cells and facilitate cell apoptosis. Another study revealed that most miRs regulate hundreds of targets, and many genes are controlled by several miRs at the same time [28]. In this research, it was predicted online that miR-489 might repress TWIST1 gene expression, and the targeting relationship between miR-489 and TWIST1 was verified using the luciferase reporter gene assay system. The results indicated that miR-489 expression was negatively correlated with TWIST1 expression, suggesting that the elevation of TWIST1 expression in GBM is probably related to the down-regulation of miR-489 expression.

TWIST, a bHLH transcription factor, can regulate mesodermal development, and it is expressed in neurons instead of glial cells in the brain and mature brain of embryo and fetus, becoming an essential player in central nervous system development or normal neuronal physiology. Meanwhile, TWIST can promote the migration of tumor cells, respond to cytotoxic stress and improve cell survival rate [29]. Besides, the anti-apoptotic function of TWIST implies that TWIST can work as an oncogene [30]. It has been observed that the mRNA expression of TWIST is raised in mesenchymal tumors, rhabdomyosarcomas and osteosarcomas [31,32]. The transcription factor TWIST1 can facilitate the formation and maintenance of breast cancer stem cells [33], promote metastasis of breast cancer and induce epithelial-mesenchymal transition simultaneously. Additionally, TWIST1 can further activate Src by inducing the expression of platelet-derived growth factor receptor alpha (PDGFRα), thus accelerating the generation of invadopodia in cells. The co-expression of TWIST1 and PDGFRα signifies the poor survival of breast cancer patients [33]. Elias et al [29] researched and discovered that TWIST expression can be detected in the majority of human neuroglioma-derived cell lines, and the increased mRNA expression level of TWIST is associated with glioma in the highest grade. Furthermore, TWIST1 exhibits up-regulated expression level in GBM and promotes the invasion of GBM *in vitro*. It was revealed in this

research that the expression level of TWIST was elevated in GBM cells, while the inhibited TWIST expression decreased the proliferative capacity of cells distinctly but up-regulated the expression level of apoptotic proteins in cells. In summary, the expression of miR-489 in GBM cells suggests that the proliferation and apoptosis of GBM cells are closely correlated with the miR-489/TWIST1 axis.

Conclusions

The results of this research elucidate that miR-489 may repress the proliferation and promote the apoptosis of glioma cells by targeting TWIST1.

Conflict of interests

The authors declare no conflict of interests.

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