**MicroRNA-423-3p promotes glioma growth by targeting PANX2**

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**Abstract.** Previously, a number of microRNAs (miRs) have been identified to participate in the development and progression of glioma via the regulation of their target genes. However, the molecular mechanisms underlying the effect of miR-423-3p in glioma growth remain unclear. In the present study, the reverse transcription-quantitative polymerase chain reaction and western blotting were used to assess the mRNA and protein expression levels of miR-423-3p, respectively. An MTT assay and flow cytometry were performed to determine cell proliferation and apoptosis, respectively. A luciferase reporter gene assay was performed to determine the target association between pannexin 2 (PANX2) and miR-423-3p. It was revealed that miR-423-3p was significantly upregulated in glioma tissues compared with normal brain tissues, and the increased expression of miR-423-3p was significantly associated with an advanced grade as well as a poorer prognosis of patients with glioma. Inhibition of miR-423-3p using an miR-423-3p inhibitor resulted in the decreased proliferation of glioma U251 and U87MG Uppsala cells, and the induction of apoptosis. PANX2 was identified as a novel target gene of miR-423-3p. Knockdown of PANX2 attenuated the effects of miR-423-3p inhibition on glioma cell proliferation and apoptosis. Furthermore, miR-423-3p was significantly downregulated in glioma tissues compared with normal brain tissues, and its levels were markedly lower in World Health Organization (WHO) stage III-IV gliomas compared with WHO stage I-II gliomas. Additionally, the expression levels of PANX2 were inversely correlated with miR-423-3p expression levels in glioma tissues. Consequently, targeting miR-423-3p may inhibit glioma growth via the upregulation of PANX2.

**Introduction**

Malignant glioma is the most common type of cancer in the brain, accounting for 80% of all malignant brain tumors worldwide (1,2). In previous years, the deregulation of numerous oncogenes and tumor suppressors including microRNAs (miRs) has been observed in glioma, and investigating the underlying molecular mechanism may be beneficial for developing effective therapeutic strategies for this disease (2-4). miRs are a class of non-coding RNAs that are 18-25 nucleotides in length, and have been demonstrated to act as key regulators of gene expression by directly binding to the complementary regions of their target mRNA, leading to mRNA degradation or translational inhibition (5). Through the inhibition of the expression of their target mRNA, a multitude of miRs participate in various physiological and pathological biological processes, including differentiation, development, angiogenesis and tumorigenesis (5-7). Glioma, like other types of cancer, possesses a distinct miR expression signature, and previous studies have identified that a number of miRs are involved in the regulation of glioma cell proliferation, survival, cell cycle progression, migration and invasion (8-10). Furthermore, a number of miRs have been suggested as potential therapeutic targets in the treatment of glioma (8,10,11).

miR-423 is involved in a number of physiological and pathological progresses, including skeletal muscle development (12), childhood obesity (13), heart failure (14), idiopathic pulmonary fibrosis (15) and acute graft-versus-host disease (16). Furthermore, the deregulation of miR-423 has been identified to be involved in several types of human cancer (17,18). For instance, miR-423 was upregulated in head and neck squamous cell carcinoma tissues compared with normal tissues (17). It may also promote the proliferation of hepatocellular carcinoma cells through regulating the G/S transition by targeting cyclin-dependent kinase inhibitor 1 (p21Cip1/Waf1) (18). Additionally, miR-423 promotes cell proliferation in breast cancer cell lines through its miR-423-3p strand as opposed to its miR-423-5p strand (19). Previously, miR-423-5p was demonstrated to be significantly upregulated in glioma, and promoted the malignant phenotypes of glioma cells as well as their temozolomide resistance (20), suggesting that miR-423-5p serves an oncogenic function in glioma. However, the regulatory function of miR-423-3p in glioma remains unclear.

In the present study, the molecular mechanisms underlying the effect of miR-423-3p on glioma growth were investigated.
Materials and methods

Tissue collection. The present study was approved by the Ethics Committee of Xiangya Hospital, Central South University (Changsha, China). A total of 58 cases of glioma specimens and 10 cases of normal brain tissues were obtained from Xiangya Hospital, Central South University (Changsha, China) between January 2010 and March 2012. Written informed consent was obtained from all patients prior to the study. Tissues were snap-frozen in liquid nitrogen following surgical resection, and stored in liquid nitrogen until use. The clinicopathological information of the patients with glioma included in the present study is summarized in Table I.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the tissues and cells using TRIzol® reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. The MirVana™ RT-PCR microRNA detection kit (Thermo Fisher Scientific, Inc.) was used to examine miR expression, according to the manufacturer's protocol. U6 was used as an internal reference. The standard SYBR Green RT-PCR Kit (Takara Bio, Inc., Otsu, Japan) was used to examine mRNA expression, according to the manufacturer's protocol. GAPDH was used as an internal reference. The primers used were as follows: miR-423 forward, 5'-ATGTTTCGTGGGTAGGGGCAGAGAGCAGGAGGCTCCCCAGGTATTCG-3' and reverse, 5'-GTGCAGGTCCGGTGGGTA-3'; U6 forward, 5'-CTCGCTTCGGCAGCAC-3' and reverse, 5'-AACGCTTCCAGATTTTCGT-3'; PANX2 forward, 5'-CCAAGACCTTCGCAAGGAA-3' and reverse, 5'-GGCCGCGAACATGGTGC-3'; GAPDH forward, 5'-GGAGGAGGATCCTCCAAATAT-3' and reverse, 5'-GGCTTGTGTCATACCTTCTCATGG-3'. The reaction mixture included cDNA solution (1 µl), PCR master mix (10 µl), primers (2 µl) and water (7 µl). The reaction conditions were 9˚C for 3 min, followed by 40 cycles of 15 s at 95˚C, 1 min at 55˚C and 1 min at 72˚C. The relative expression level was quantified using the 2^ΔΔCq method (21).

Cell culture. Human glioma U251 and U87MG Uppsala cell lines were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) was used to perform transfection, according to the manufacturer's protocol. Briefly, U251 and U87MG Uppsala cells were transfected with a negative control (NC) inhibitor, miR-423-3p inhibitor, non-specific small interfering RNA (siRNA) or PANX2-specific siRNA, respectively were purchased from Guangzhou FulenGen Co. Ltd. (Guangzhou, China). Following transfection at 37˚C for 48 h, the expression assay was performed.

Western blotting. U251 and U87MG Uppsala cells were lysed in RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China). The protein concentration was quantified using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Protein (50 µg) was separated by SDS-PAGE (12% gel), transferred onto a polyvinylidene fluoride membrane (Thermo Fisher Scientific, Inc.), and then blocked using 5% non-fat dried milk (Yili Group, Beijing, China) in Tris-buffered saline with Tween-20 (TBST; Beyotime Institute of Biotechnology) at room temperature for 3 h. The membrane was incubated with rabbit polyclonal anti-PANX2 primary antibody (1:100; cat no. ab55917; Abcam, Cambridge, MA, USA) or rabbit polyclonal anti-GAPDH primary antibody (1:100; cat no. ab9485; Abcam) at room temperature for 3 h, and then washed three times using TBST. Subsequently, the membrane was incubated with goat monoclonal anti-rabbit secondary antibody (1:5,000; cat no. ab190492; Abcam) for 1 h at room temperature, and washed three times using TBST. The immune complexes were detected using an enhanced chemiluminescence western blotting kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Image J software (version 1.0, National Institutes of Health, Bethesda, MD, USA) was used to analyze the relative protein expression, represented as the density ratio relative to GAPDH.

Cell proliferation analysis. U251 and U87MG Uppsala cells (2x10^4 cells per well) were seeded in 96-well plates, and 100 µl fresh serum-free DMEM with 0.5 g/l MTT solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added. Following incubation at 37˚C for 0, 24, 48 and 72 h, the medium containing MTT solution was removed, and 50 µl dimethylsulfoxide (Sigma-Aldrich; Merck KGaA) was added. Following incubation at 37˚C for 10 min, the absorbance at 570 nm of each sample was determined using a plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell apoptosis analysis. A flow cytometer was used to determine the cell apoptosis with an Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection kit (Sigma-Aldrich; Merck KGaA). Cells were harvested and washed with ice-cold PBS twice, and 10^6 cells were resuspended in 200 µl binding buffer with 10 µl annexin-V-FITC and 5 µl propidium iodide-phycocerythrin, prior to incubation in the dark at 4˚C for 30 min. Subsequently, 300 µl binding buffer was added, followed by analysis using flow cytometry (BD Accuri C6 software, 1.0, C6; BD Biosciences, Franklin Lakes, NJ, USA).

Bioinformatics analysis and luciferase reporter assay. TargetScan Human 5.1 software (22) (www.targetscan.org) was used to determine the putative target of miR-423-3p. The wild-type (WT) PANX2 3'-untranslated region (UTR) was constructed by PCR, which was performed by Yeartbio (Changsha, China), and inserted into the pMIR-REPORT miRNA Expression Reporter vector (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The mutant type (MT) of PANX2 3'-UTR was constructed using the Easy Mutagenesis System kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol, and then inserted into the pMIR-REPORT miRNA Expression Reporter vector (Thermo Fisher Scientific, Inc.). U251 and U87MG Uppsala cells were co-transfected with WT PANX2-3'UTR
plasmid or MT PANX2-3’UTR plasmid, and miR-NC or miR-423-3p mimics, using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Inc.). Following transfection at 37˚C for 48 h, the activity of Renilla luciferase and firefly luciferase were determined using a dual-luciferase reporter assay system (Promega Corporation), 48 h after transfection. The activity of Renilla luciferase was normalized to that of the firefly luciferase.

Statistical analysis. Data are expressed as the mean ± standard deviation. The differences between two groups were analyzed using two-tailed Student’s t-test. Statistical analysis was performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). Pearson’s correlation analysis was performed to examine the correlation between the miR-423-3p and PANX2 expression in glioma tissues. Kaplan Meier analysis with log rank tests were used for the survival analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Upregulation of miR-423-3p is associated with glioma progression. In the present study, qPCR was used to determine the expression of miR-423-3p in glioma tissues. Normal brain tissues were used as controls. As indicated in Fig. 1A, miR-423-3p expression levels were significantly increased in glioma tissues compared with normal brain tissues. Furthermore, the expression of miR-423-3p was increased in World Health Organization (WHO) III-IV grade glioma compared with WHO I-II grade glioma (Fig. 1B). These glioma tissues were further divided into two groups, a high miR-423-3p group and a low miR-423-3p group, on the basis of mean expression value. It was observed that high expression of miR-423-3p was significantly associated with an advanced grade of glioma as well as a low Karnofsky performance score (KPS), but not with age and sex (Table I). Furthermore, as presented in Fig. 1C, the patients with glioma with high
Figure 2. Comparisons between miR-423-3p expression levels, cell proliferation and cell apoptosis in cells with a miR-423-3p inhibitor and an NC inhibitor. The reverse transcription-quantitative polymerase chain reaction was performed to examine the miR-423-3p expression levels in (A) U251 and (B) U87MG Uppsala cells transfected with either miR-423-3p inhibitor or NC inhibitor. An MTT assay was used to determine proliferation in (C) U251 and (D) U87MG Uppsala cells. Flow cytometry was used to detect apoptosis in (E) U251 cells and (F) U87MG Uppsala cells. "P<0.01 vs. NC inhibitor. miR, microRNA; NC, negative control."
miR-423-3p expression levels had a shorter survival time, compared with those with low miR-423-3p expression levels. Therefore, miR-423-3p upregulation may contribute to the malignant progression of glioma as well as poor prognosis in patients with glioma.

**Knockdown of miR-423-3p decreases U251 and U87MG Uppsala cell proliferation and induces cell apoptosis.** As miR-423-3p was upregulated in glioma, glioma U251 and U87MG Uppsala cells were transfected with an miR-423-3p inhibitor in order to knock down miR-423-3p expression. Transfection with an NC inhibitor was used as the control group. Following transfection with miR-423-3p inhibitor, miR-423-3p expression levels were significantly decreased compared with the NC inhibitor group (Fig. 2A and B). An MTT assay further indicated that the inhibition of miR-423-3p
led to the decreased proliferation of U251 and U87MG Uppsala cells (Fig. 2C and D). Cell apoptosis was further examined and it was revealed that the knockdown of miR-423-3p resulted in a significant increase in U251 and U87MG Uppsala cell apoptosis (Fig. 2E and F). Therefore, the results of the present study demonstrated that the knockdown of miR-423-3p decreases U251 and U87MG Uppsala cell proliferation and induces cell apoptosis.

PANX2 is a novel target of miR-423-3p in U251 and U87MG Uppsala cells. Bioinformatics analysis indicated that PANX2 was a putative target gene of miR-423-3p (Fig. 3A). To the best of our knowledge, this targeting association has never previously been reported. In the present study, luciferase reporter plasmids were constructed containing WT or MT PANX2 3'-UTR (Fig. 3B and C). The luciferase reporter gene assay was then performed in U251 and U87MG Uppsala cells. The results of the present study indicated that luciferase activity was significantly decreased in U251 and U87MG Uppsala cells co-transfected with the WT PANX2 3'-UTR plasmid and miR-423-3p mimic compared with the control group, which was eliminated during transfection with the MT PANX2 3'-UTR plasmid (Fig. 3D and E). Subsequently, it was revealed that the inhibition of miR-423-3p significantly increased the expression of PANX2 protein in U251 and U87MG Uppsala cells (Fig. 3F and G). Therefore, PANX2 is a potential novel target gene of miR-423-3p in U251 and U87MG Uppsala cells.

Knockdown of PANX2 attenuates the effects of miR-423-3p inhibition on U251 and U87MG Uppsala cells. On the basis of the aforementioned results, it was hypothesized that PANX2 may be involved in miR-423-3p-mediated glioma growth. To investigate this hypothesis, U251 and U87MG Uppsala cells were co-transfected with miR-423-3p inhibitor and PANX2 siRNA, or miR-423-3p inhibitor and NC siRNA. Following transfection, the mRNA and protein levels of PANX2 were significantly decreased in the miR-423-3p inhibitor + PANX2 siRNA group, compared with the miR-423-3p inhibitor + NC siRNA group (Fig. 4A-D). An MTT assay further demonstrated that the proliferation of U251 and U87MG Uppsala cells was significantly increased in the
miR-423-3p inhibitor + PANX2 siRNA group, compared with the miR-423-3p inhibitor + NC siRNA group (Fig. 5A and B), indicating that knockdown of PANX2 attenuates the suppressive effects of miR-423-3p inhibition on glioma cell proliferation. Cell apoptosis was then assessed. It was revealed that the apoptosis of U251 and U87MG Uppsala cells was significantly decreased in the miR-423-3p inhibitor + PANX2 siRNA group, compared with the miR-423-3p inhibitor + NC siRNA group (Fig. 5C and D), indicating that the knockdown of PANX2 attenuates the effect of miR-423-3p inhibition on glioma cell apoptosis. As a result, it may be suggested that the knockdown of miR-423-3p at least partially inhibits proliferation and induces the apoptosis of glioma cells, via the direct targeting of PANX2.

Figure 5. U251 and U87MG Uppsala cells were co-transfected with miR-423-3p inhibitor and PANX2 siRNA, or miR-423-3p inhibitor and NC siRNA. An MTT assay was used to determine proliferation in (A) U251 and (B) U87MG Uppsala cells. Flow cytometry was used to detect apoptosis in (C) U251 and (D) U87MG Uppsala cells. *P<0.01 vs. miR-423-3p inhibitor + NC siRNA. miR, microRNA; siRNA, small interfering RNA; NC, negative control.

PANX2, which is downregulated in glioma, is inversely correlated with the miR-423-3p expression. Finally, RT-qPCR was performed to examine the mRNA expression levels of PANX2 in glioma. The expression of PANX2 mRNA was demonstrated to be significantly decreased in glioma tissues compared with normal brain tissues (Fig. 6A). Furthermore, PANX2 mRNA expression levels in WHO III-IV grade glioma were decreased compared with in WHO I-II grade glioma (Fig. 6B). Notably, the PANX2 mRNA expression levels were inversely correlated with the miR-423-3p expression levels in glioma tissues (Fig. 6C). The decreased expression of PANX2 may be due to the upregulation of miR-423-3p in glioma.

Discussion

The exact function of miR-423-3p in glioma growth as well as the underlying molecular mechanism remain unclear. In the present study, it was demonstrated that miR-423-3p was significantly upregulated in glioma tissues compared with normal brain tissues, and the increased expression of miR-423-3p was significantly associated with an advanced grade of glioma in addition to a poorer prognosis in patients with glioma. Further investigation suggested that miR-423-3p serves a promoting function in glioma growth via the direct targeting of PANX2. Furthermore, PANX2...
was significantly downregulated in glioma tissues compared with normal brain tissues, and PANX2 expression levels were inversely correlated with miR-423-3p expression levels in glioma tissues.

miR-423-3p has been demonstrated to serve a promoting function in several types of human cancer (23,24). For example, Guan et al (23) identified that miR-423-3p was upregulated in laryngeal carcinoma cells, and that the inhibition of miR-423-3p resulted in a significant decrease in cell proliferation, clonogenicity, cell migration and invasion. Additionally, miR-423-3p was upregulated in colorectal cancer (CRC), and promoted CRC cell proliferation via enhancing the G1/S transition by targeting p21Cip1/Waf1(24). Previously, miR-423-5p was demonstrated to be significantly upregulated in glioma, and the overexpression of miR-423-5p promoted glioma cell proliferation, angiogenesis and invasion by increasing the activities of protein kinase B and mitogen-activated protein kinase signaling pathways and suppressing the expression of inhibitor of growth family member 4 (20). In addition, miR-423-5p upregulation enhanced glioblastoma neurosphere formation and rendered glioma cells resistant to temozolomide (20). However, the exact function of miR-423-3p in glioma has not been uncovered. In the present study, it was identified that miR-423-3p was downregulated in glioma tissues compared with normal brain tissues, and its downregulation was associated with an advanced pathological grade and lower KPS in glioma. Furthermore, the patients with glioma with high miR-423-3p levels had a shorter survival time compared with patients with low miR-423-3p levels. As a result, it may be suggested that the upregulation of miR-423-3p contributes to glioma progression and a poorer prognosis in patients with glioma.

PANX2 was also identified as a novel target gene of miR-423-3p using bioinformatics analysis and a luciferase reporter assay, and knockdown of miR-423-3p increased the protein expression levels of PANX2 in U251 and U87MG Uppsala cells. PANX2 encodes the protein pannexin 2, belonging to the innexin family, the members of which are the structural components of gap junctions (25). Previous studies have demonstrated that PANX2 is abundantly expressed in the central neuronal system, and participates in neuronal development and adult neurogenesis (26,27). Furthermore, the upregulation and downregulation of PANX2 are associated with the development and progression of certain diseases, including neoplasms, multiple sclerosis, migraines and hypertension (28). Previously, gene array analysis indicated that there was a significant decrease in PANX2 expression in glioma, and that the decreased expression of PANX2 was associated with
a poorer prognosis in patients with glioma (29). Furthermore, the expression of PANX2 was also lower in human glioma cell lines compared with normal brain tissues and astrocytes (29). Consistent with this previous study, the results of the present study identified that PANX2 was downregulated in glioma tissues compared with normal brain tissues, and its expression levels in WHO III-IV grade glioma were decreased compared with those in WHO I-II grade glioma. Furthermore, Lai et al (29) identified that the restoration of PANX2 expression significantly decreased monolayer saturation density and anchorage-independent growth of rat C6 glioma cells in vitro, as well as tumor growth in vivo. However, the regulatory mechanism of PANX2 in glioma remains unknown. In the present study, it was demonstrated that the knockdown of PANX2 attenuated the effects of miR-423-3p inhibition on the proliferation and apoptosis of U251 and U87MG Uppsala cells, suggesting that miR-423-3p promotes glioma cell proliferation by directly targeting PANX2. Furthermore, the expression levels of PANX2 were inversely correlated with the miR-423-3p expression levels in glioma tissues, suggesting that the decreased expression of PANX2 may be caused by the upregulation of miR-423-3p.

In summary, the results of the present study demonstrated that miR-423-3p serves an oncogenic function in glioma cell proliferation by directly targeting PANX2, suggesting that miR-423-3p may be a potential therapeutic target for glioma.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
JX and JH collected clinical tissues. JXU, HH and RP performed the in vitro experiments. JX wrote the manuscript. JXU designed the study and revised the manuscript.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of Xiangya Hospital, Central South University (Changsha, China). Written informed consent was obtained from all patients prior to the study.

Consent for publication
Written informed consents for the publication of this data were obtained from all patients in the present study.

Competing interests
The authors declare that they have no competing interests.

References


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