miR-128-3p inhibits glioma cell proliferation and differentiation by targeting NPTX1 through IRS-1/PI3K/AKT signaling pathway

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Abstract. It has been reported that glioma has a higher morbidity and mortality than other types of malignant brain tumor. While glioma has been extensively researched, the exact molecular mechanisms of its genesis and progression have remained to be fully elucidated. In order to explore a novel glioma-associated pathway which may represent a therapeutic target, 61 pairs of tumor tissues and adjacent normal tissues of glioma patients were collected and subjected to reverse-transcription quantitative polymerase chain reaction analysis, indicating that the relative expression of microRNA (miR)-128-3p was significantly decreased in the tumor tissues. However, the expression of neuronal pentraxin 1 (NPTX1) was obviously elevated. Through a bioinformatics analysis using Targetscan and transfection experiments, it was confirmed that NPTX1 was targeted by miR-128-3p. In the U251 human glioma cell line, transfection with miR-128-3p mimics increased the levels of phosphorylated insulin receptor substrate 1 (p-IRS-1), phosphoinositide-3 kinase (PI3K) and p-AKT, as demonstrated by western blot analysis. In addition, the proliferation rate of the cells was notably decreased following transfection with miR-128-3p mimics. Conversely, transfection with miR-128-3p inhibitor significantly increased the levels of p-IRS-1, PI3K and p-AKT, accompanied by an elevated proliferation rate of the cells. Therefore, it was indicated that miR-128-3p could reversely regulate NPTX1 expression. After the expression of NPTX1 was inhibited with specific small interfering RNA, the levels of p-IRS-1, PI3K and p-AKT were obviously decreased, while the expression of miR-128-3p was not significantly changed. Overall, it was concluded that miR-128-3p suppresses glioma through the NPTX1/IRS-1/PI3K/AKT signaling pathway.

Introduction

Gliomas are the most prevalent type of malignant brain tumor, accounting for 46-70% of all central nervous system (CNS) tumors, and have a devastating impact on the human health and life (1). The major characteristics of gliomas are their invasive growth and high rate of recurrence after intracranial operation, resulting in a poor prognosis of affected patients (2,3). Gliomas have been classified by the World Health Organization into four grades: Grades I and II are for low-grade tumors, with Grade I tumors being benign tumors and Grade II tumors having the ability to recur as high-grade gliomas. Grade III and IV gliomas are high-grade tumors, of which Grade III gliomas tend to metastasize and recur, and Grade IV tumors are the most malignant gliomas (4). In spite of significant advances in surgical techniques and chemotherapeutic treatments, the median survival rate of glioma patients remains poor (5-7). It has been reported that the 5-year survival rate of patients with Grade I and II glioma is 30-70%, while it is only 9-12 months for patients with Grade III and IV glioma (8). Therefore, it is of great importance to explore the molecular mechanisms underlying the development of gliomas and to identify novel biomarkers for predicting the progression of gliomas and identifying approaches for targeted therapies.

MicroRNAs (miRNAs/miRs) are a class of non-coding, single-stranded, endogenous small RNA molecules of 21-23 nucleotides in length, which regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of an mRNA target to promote mRNA degradation and/or translational repression (9,10). It has been reported that miRNAs are involved in the modulation of a variety of biological processes, including cell proliferation, differentiation, apoptosis, migration and tumorigenesis (11,12). miR-128-3p was identified to speed up cell cycle arrest and chromosomal instability in mitomycin C-treated lung cancer cells by suppressing spectrin alpha, non-erythrocytic 1 (13). It was also indicated that miR-128-3p is intimately associated with hepatocellular carcinoma (14,15) and acute lymphoblastic leukemia (16). However, to date, the association between miR-128-3p and gliomas has remained to be determined in combination with a clinical and fundamental study.

In the present study, a total of 61 pairs of tumor tissues and paratumor tissues were collected from glioma patients.
and it was identified that the relative expression of miR-218-5p was downregulated, while neuronal pentraxin 1 (NPTX1) was upregulated in the tumor tissues. Through a bioinformatics analysis using Targetscan, NPTX1 was identified as a potential target of miR-218-5p, which was confirmed using a luciferase reporter assay. After the human glioma cells were transfected with miR-218-5p mimics or inhibitor, the insulin receptor substrate 1 (IRS-1)/phosphoinositide-3 kinase (PI3K)/AKT signaling pathway was suppressed or promoted, respectively. Furthermore, the IRS-1/PI3K/AKT signaling pathway was inhibited after NPTX1 was suppressed. Thus, miR-128-3p regulates glioma development via the IRS-1/PI3K/AKT signaling pathway by targeting NPTX1.

**Materials and methods**

**Patients and tissue samples.** Between March 2009 and April 2016, a total of 61 pairs of primary glioma and matched paratumor tissue samples were collected at the Department of Neurosurgery of the First Hospital of Lanzhou University (Lanzhou, China). The tumor and adjacent normal tissues were rapidly frozen in liquid nitrogen and preserved at -70˚C until use. None of the patients had received any blood transfusion, chemotherapy or radiotherapy prior to the surgery. Written informed consent was obtained from each patient and approval of the Ethics Committee of the First Hospital of Lanzhou University was obtained for using their tissue samples in the present study.

**U251 cell culture and transfection.** The U251 human glioma cell line was purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China) and was cultured in minimum essential medium/Earle's balanced salt solution (HyClone; GE Healthcare, Little Chalfont, UK) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare), 50 U/ml penicillin and 50 mg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO₂. The cells were seeded in a 6-well plate at 5x10⁴ cells/well and the medium was replaced every 3 days until the confluency reached ~70%. miR-128-3p mimics (100 nM) or negative control (NC, nonsense miR); Sangon Biotech Co., Ltd., Shanghai, China) were transfected into U251 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), 1 µg total RNA was reverse transcribed using a PrimeScript RT Reagent kit with a gDNA Eraser (cat. nos. RR047A and RR047B; each, Takara Bio, Inc., Dalian, China) according to the manufacturer's protocol. Subsequently, PCR amplification was performed in a total reaction volume of 20 µl comprising 1 µl NPTX1 primers (0.2 µmol/l, Sangon Biotech Co., Ltd.), 10 µl SYBR green (Takara Bio, Inc.), 6 µl diethyl pyrocarbonate-treated water (Sangon Biotech Co., Ltd.) and 2 µl complementary DNA product, in a Real-Time LightCycler® 96 System (Roche Diagnostics, Basel, Switzerland). The sequences of the primers were as follows: miR-128-3p forward, 5'-GACTGC CGAGGAGGCG-3' and reverse, 5'-GGACGCGGCCACTCT TCTCTCT-3'; U6 forward, 5'-CCATCGGAATTCGCTATA CAAGATT-3' and reverse, 5'-GGGCTTCTCAACTTGGGT GTCAG-3'; NPTX1 forward, 5'-TTGACAGTTCGATCA CAAAG-3' and reverse, 5'-CCAGCTATTGCGCTGGA CG-3'; β-actin forward, GCTGTCCCTGTATGCCTT-3' and reverse, TGTACGCAGATTCC-3'. The following thermocycling conditions were applied: 5 sec at 95˚C, followed by 40 cycles of 1 sec at 95˚C and 20 sec at 65˚C, followed by 5 sec at 72˚C. The comparative 2^{-ΔΔCt} method was used for relative quantification of gene expression (17). Each sample utilized in reverse transcription and the PCR amplification of cDNA were analyzed three times.

**Western blot analysis.** Total protein was extracted from collected tissues and cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with 100X proteinase inhibitor and phosphatase inhibitor, and then the proteins (25 µg) were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA), which was then blocked with 5% non-fat milk for 1 h at room temperature. The primary
antibodies were anti-NPTX1 (cat. no. 3725; 1:1,000 dilution), anti-IRS-1 (cat. no. 2382; 1:500 dilution), anti-phosphorylated (p)-IRS-1 ( Tyr895) (cat. no. 3070; 1:500 dilution), anti-P13K (cat. no. 4292; 1:1,000 dilution) anti-p-AKT (ser473; cat. no. 12694; 1:1,000 dilution), anti-AKT (cat. no. 2938; 1:1,000 dilution) and anti-β-actin (cat. no. 4970; 1:1,000 dilution) and were all rabbit monoclonal antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) in 4°C overnight. The secondary antibody (cat. no. A0208; Beyotime Institute of Biotechnology) was used at the dilution of 1:5,000 and incubated at room temperature for 90 min. Blots were visualized with an enhanced chemiluminescence kit (cat. no. 15159; Invitrogen; Thermo Fisher Scientific, Inc.). The gray bands were analyzed with Image J software 10.8 (National Institutes of Health, Bethesda, MD, USA) to compare the expression between targeted proteins and internal controls.

**U251 proliferation assay.** To detect the proliferation activity of transfected U251 cells, the MTT assay was performed using a Cell Proliferation Kit I (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a 96-well plate at a density of 4x10^3 per well according to the manufacturer's instructions. After 24, 48 and 72 h of incubation, MTT-formazan production was estimated by a VersaMax (Molecular Devices, LLC, Sunnyvale, CA, USA) at 570 nm to evaluate the proliferation rate. The index was determined at 48 and 72 h and normalized to that at 24 h.

**Statistical analysis.** Values are expressed as the mean ± standard error of the mean. All statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The differences between two groups were compared using a paired Student's t-test and the differences between three or more groups using one-way analysis of variance followed by a Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**miR-128-3p and NPTX1 are differentially expressed in glioma tissues.** The Targetscan search indicated that NPTX1 was a target gene of miR-128-3p. Thus, miR-128-3p and NPTX1 was assessed in glioma tissues. Between March 2009 and April 2016, a total of 61 pairs of glioma tumor tissues and the adjacent normal tissues were collected. RT-qPCR analysis indicated that the relative expression of miR-128-3p was significantly decreased, while NPTX1 was elevated in the tumor tissues compared with that in the normal tissues (Fig. 1A and B). In addition, the relative expression of miR-128-3p was also lower in metastatic tissues than that in non-metastatic tissues, but the NPTX1 expression was higher in metastatic tissues (Fig. 1C and D). According to the diagnostic criteria of the world health organization, the glioma patients were stratified according to their grade [grade I (n=22), II (n=20) and III+IV (n=19)]. The miR-128-3p expression was lowest in the grade III+IV group but highest in the grade I group. Conversely, NPTX1 expression was the highest in the grade III+IV group, while it was lowest in the grade I group (Fig. 1E and F). These results illustrated that miR-128-3p and NPTX1 are closely associated with gliomas.

**miR-128-3p is targeted by miR-128-3p.** In order to further clarify the association between miR-128-3p and NPTX1, a Targetscan software analysis was performed to identify the miR-128-3p binding sequence in the 3'-UTR of NPTX1 and the core of the binding sequence was deleted to generate a mutant sequence (Fig. 2A and B). Subsequently, U251 cells were co-transfected with miR-128-3p mimics and luciferase reporter vectors containing the WT or mutant miR-128-3p binding sequence of the 3'-UTR of NPTX1. It was revealed that the luciferase activity was significantly decreased in the WT group (NPTX1 WT + miR-128-3p mimic group) in comparison with the mutant (NPTX1 mutant + miR-128-3p mimic group) and blank group (Blank + miR-128-3p mimic group), while no difference was observed between the mutant and blank groups (Fig. 2C). Of note, the luciferase activity was upregulated after the reporter vector driven by the binding sequence in the 3'-UTR of NPTX1 and miR-128-3p inhibitor (NPTX1 3'-UTR + miR-128-3p inhibitor group) were co-transfected into the U251 cells compared with the blank group (NPTX1 3'-UTR + Blank). Furthermore, the opposite result was obtained after the reporter vector driven by the binding sequence in the 3'-UTR of NPTX1 and miR-128-3p mimics (NPTX1 3'-UTR + miR-128-3p mimic group) were transfected into the cells in comparison with the blank group (Fig. 2D). In U251 cells transfected with miR-128-3p mimics, the expression of NPTX1 was decreased at the protein level (Fig. 2E), while the opposite effect was obtained after miR-128-3p inhibitor was transfected into the cells (Fig. 2F).

**miR-128-3p mimics suppress IRS-1/P13K/AKT signaling in U251 cells.** Cultured U251 cells were transfected with miR-128-3p mimics or NC. Through visual observation, it was determined that the proliferation rate of the cells was reduced in the miR-128-3p mimics group (Fig. 3A) in comparison with that in the NC group (Fig. 3B). An MTT assay confirmed that the proliferation rate of the cells in the miR-128-3p mimics group was significantly lower than that in the NC group (Fig. 3C). In addition, western blot analysis clearly proved that compared with those in the NC group, the protein levels of p-IRS-1, P13K and p-AKT were significantly decreased in the miR-128-3p mimics group, while the expression of IRS-1 and AKT was significantly increased (Fig. 3D and E). This result indicated that the IRS-1/P13K/AKT signaling pathway was suppressed by the miR-128-3p mimics.

**miR-128-3p inhibitor increases IRS-1/P13K/AKT signaling in U251 cells.** miR-128-3p inhibitor and its NC were transfected into the cultured U251 cells, and visual observation revealed that the proliferation rate of the cells was increased in the miR-128-3p inhibitor group (Fig. 4A) in comparison with that in the NC group (Fig. 4B). An MTT assay confirmed that the proliferation rate of the cells in the miR-128-3p inhibitor group was higher than that in the NC group (Fig. 4C). Furthermore, compared with those in the NC group, the levels of p-IRS-1, P13K and p-AKT were significantly increased in the miR-128-3p inhibitor group, while the expression of IRS-1 and AKT was not significantly different (Fig. 4D and E). This result indicated that IRS-1/P13K/AKT signaling was enhanced after miR-128-3p was inhibited.
Suppression of NPTX1 inhibits IRS-1/PI3K/AKT signaling. In order to further demonstrate the association between NPTX1 and the IRS-1/PI3K/AKT signaling pathway, NPTX1 siRNA was transfected into the cultured U251 cells, which caused a significant decrease in the levels of p-IRS-1, PI3K and p-AKT compared with that in the NC group (Fig. 5A and B). However, the expression of total IRS-1 and AKT was increased in the NPTX1 siRNA group. This meant that compared with the protein levels normalized to β-actin, the p-IRS-1 and p-AKT levels relative to IRS-1 and AKT, respectively, were even higher. However, after the expression of NPTX1 was inhibited with siRNA, the expression of miR-128-3p was not significantly different (Fig. 5C).

Discussion

Glioma is the most prevalent type of malignant carcinoma of the CNS and has a yearly incidence of almost 6 per 100,000 individuals in the US (18). Gliomas represent a severe treat to human health and life, and are therefore intensively studied. During glioma cell migration and invasion, transforming growth factor-β receptor type 2 exerts a key role and it may...
serve as a prognostic predictor and a therapeutic target for glioma (19). It has been indicated that sea buckthorn leaf extract suppresses the proliferation of rat glioma cells via inducing early events of apoptosis and it may therefore serve as a potential therapeutic for the treatment of glioma (20). It has been reported that atorvastatin has cytotoxic effects and

\[ \text{NFTX-1 is a direct target of miR-128-3p.} \]

(A) The predicted binding sequences of the 3’-UTR of NPTX1 and miR-128-3p. (B) The sequences on the top refer to the complete binding sequences of the 3’-UTR of NPTX1 and miR-128-3p, while the ones at the bottom refer to the mutation sequences without core sequences. (C) After the binding sequences of 3’-UTR of NPTX1 and miR-128-3p mimics were transfected into U251 cells, the luciferase activity was obviously decreased; however, no statistically significant difference was identified after their mutated sequences were transfected into the cells. (D) The luciferase activity was significantly promoted after the binding sequences of NPTX1 3’-UTR and miR-128-3p inhibitor were transfected into U251 cells, but the results were opposite after the binding sequences of the NPTX1 3’-UTR and miR-128-3p mimics were transfected into the cells. (E and F) After U251 cells were transfected with miR-128-3p mimics, the protein expression of NPTX1 was decreased compared with that in the NC group, while it was increased after transfection with miR-128-3p inhibitor. *P<0.05, **P<0.01, ***P<0.001. NPTX1, neuronal pentraxin 1; miR, microRNA; UTR, untranslated region; WT, wild-type; NC, negative control; hsa, Homo sapiens.

serve as a prognostic predictor and a therapeutic target for glioma (19). It has been indicated that sea buckthorn leaf extract suppresses the proliferation of rat glioma cells via inducing early events of apoptosis and it may therefore serve as a potential therapeutic for the treatment of glioma (20). It has been reported that atorvastatin has cytotoxic effects and
induces autophagy, and simultaneously reduces the migration and proliferation of human A172 glioma cells (21). However, in spite of the abundance of studies on gliomas, the exact molecular mechanisms associated with gliomas and therapeutic targets leading to an efficient treatment approach remain to be elucidated, and additional study is therefore required.

Certain miRNAs are known to be associated with tumor development and progression through regulating the translation or causing degradation of their target mRNAs (22, 23). Regarding the elucidation of the exact mechanisms underlying the pathological processes of various diseases, particularly cancers, miRNAs may provide a novel approach for their diagnosis and treatment (24). A variety of studies revealed that miRNAs are involved in the development and progression of gliomas. A previous study suggested that low expression of miR‑508‑5p was associated with glioma patient survival; it may therefore serve as a novel prognostic factor and also lead to novel treatment strategies for glioma (25). It has been proved that overexpression of miR‑497 is associated with resistance to temozolomide in human glioma cells by targeting mammalian target of rapamycin/B‑cell lymphoma 2 (26). It has also been reported that miR‑302a is an important tumor suppressor of glioma progression by directly targeting GRB2-associated binding protein, thus providing novel insight into the molecular mechanisms underlying the genesis, development and progression of glioma (27). Furthermore, miR‑128‑3p was reported

Figure 3. miR-128-3p mimics suppress the IRS-1/PI3K/AKT pathway. (A and B) The amount of U251 cells was after transfection with miR-128-3p mimics as compared with that in the NC group (magnification, x200 magnification). (C) An MTT assay revealed that the proliferation rate of the cells was statistically decreased in the miR-128-3p mimics group in comparison with that in the NC group. (D and E) Western blot analysis indicated that the levels of p-IRS-1, PI3K and p-AKT in the miR-128-3p mimics group were significantly decreased compared with those in the NC group. Furthermore, the expression of IRS-1 and AKT in the miR-128-3p mimics group was higher than that in the NC group. The ratio of (F) p-IRS-1/IRS-1 and (G) p-AKT/AKT. *P<0.05, **P<0.01, ***P<0.001. p-IRS-1, phosphorylated insulin receptor substrate 1; PI3K, phosphoinositide-3 kinase; miR, microRNA; NC, negative control.
to be closely associated with lung cancer (13), hepatocellular carcinoma (14,15) and gastric cancer (28). However, the association between miR-128-3p and glioma has remained to be determined.

The present study indicated that the expression of miR-128-3p was significantly lower in glioma tissues than in paratumor tissues. In addition, in metastatic tissues, the relative expression of miR-128-3p was also decreased in comparison with that in non-metastatic tumor tissues. Furthermore, the relative expression of miR-128-3p was lowest in grade III+IV glioma, followed by that in grade II and grade I glioma. These results demonstrated that miR-128-3p was intimately associated with gliomas. It has been demonstrated that a synergistic action of metformin and aspirin regulates the transcriptional profile of pancreatic cancer cells and NPTX1 was upregulated by >10-fold (29). Specific gene expression signatures have been identified in individuals and NPTX1 expression was decreased in mouse strains susceptible to pulmonary adenomas (30). NPTX1 was reported to be intimately associated with colorectal cancer (31), high-grade cervical intraepithelial neoplasia and cervical cancer (32) as biomarkers involved in methylation. These previous studies demonstrated that NPTX1 is closely associated with a variety of tumor types, but the association between NPTX1 and glioma has remained to be elucidated. In the present study, the expression of NPTX1 was significantly elevated in tumor tissues compared with that in normal tissues. In metastatic tissues, the relative expression of NPTX1 was also elevated in comparison with that in non-metastatic tumor tissues. In addition, the relative expression of NPTX1 was the highest in grade III+IV glioma. These results clearly illustrated that NPTX1 was
positively associated with glioma and inversely associated with miR-128-3p expression. A Targetscan analysis, which initially confirmed that miR-128-3p could target NPTX1, followed by a luciferase reporter assay and transfection experiments further confirmed this result. It has been demonstrated that leptin stimulates the migration of human prostate cancer cells, and that one of the underlying mechanisms was transcriptional upregulation of αvβ3 integrin expression through the [long form of the obese gene (leptin) receptor] OBR1/IRS-1/PI3K/Akt/nuclear factor (NF)-κB signal transduction pathway (33). In addition, leptin promotes the migration of chondrosarcoma cells by increasing αvβ3 integrin expression through the OBR1/IRS-1/PI3K/Akt/NF-κB signal transduction pathway (34). A previous study suggested that the IRS-1/PI3K/AKT signaling pathway was positively correlated with tumor growth, proliferation and migration. In the present study, after the glioma cells were transfected with miR-128-3p mimics, the IRS-1/PI3K/AKT signaling pathway was inhibited, while it was activated by transfection with miR-128-3p inhibitor. In addition, after NPTX1 was suppressed with specific siRNA, the IRS-1/PI3K/AKT signaling pathway was inhibited; however, the expression of miR-128-3p was not affected. These results demonstrated that in the growth and differentiation of glioma cells, miR-128-3p regulates the activity of the NPTX1/IRS-1/PI3K/AKT signaling pathway. Furthermore, the IRS-1/PI3K/AKT signaling pathway was situated downstream of NPTX1, which was able to modulate IRS-1/PI3K/AKT signaling pathway activity. However, miR-128-3p could not be reversely regulated by NPTX1 as expected, which was directly targeted by miR-128-3p.

The present study provided novel biomarkers, miR-128-3p and NPTX1, for the diagnosis of glioma, which may also
serve as therapeutic targets in the treatment of glioma. miR-128-3p was revealed to have a vital role in the regulation of glioma growth, proliferation through activating the IRS-1/P13K/AKT signaling pathway by targeting NPTX1.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QLG was fully responsible for the study design, experimental adjustment and drafting and finalizing the manuscript. LMH and BW performed the majority of the experiments. MHZ and YHZ performed the transfection assays and certain protein measurements by western blotting and statistical analysis. YHZ performed the western blotting and statistical analysis. MHZ and BW performed the majority of the experiments. MHZ and YHZ performed the western blotting and statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the First Hospital of Lanzhou University (Lanzhou, China) and written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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