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MicroRNA-199a-5p regulates glioma progression *via* targeting MARCH8

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Abstract. – OBJECTIVE: MicroRNA-199a-5p (miR-199a-5p) was reported to play crucial roles in cancer progression. However, its role in glioma remains largely unknown.

MATERIALS AND METHODS: RT-qPCR was employed to analyze miR-199a-5p expression level in glioma cell lines. The effects of miR-199a-5p on cell proliferation and invasion were investigated *in vitro*.

RESULTS: We showed that miR-199a-5p expression level was significantly downregulated in glioma cell lines compared with a normal cell. In addition, miR-199a-5p overexpression suppresses glioma cell proliferation and invasion *in vitro*. Bioinformatic analysis and Luciferase assay suggested that the membrane-associated ring-CH-type finger 8 (MARCH8) was a target of miR-199a-5p. Furthermore, MARCH8 overexpression could partially reverse the effects of miR-199a-5p on glioma cells.

CONCLUSIONS: These findings suggested that miR-199a-5p may function as a tumor suppressor in glioma by targeting MARCH8.

Key Words:
MiR-199a-5p, MARCH8, Glioma, Growth, Invasion.

Introduction

Glioma is a nervous system tumor with its 5-year overall survival rate of around 33%[1,2]. Surgical resection combined with chemotherapy remains the first-line treatment option for glioma, although recent improvements in targeted therapy or immunotherapy have been achieved[3]. Hence, a deep understanding of the mechanisms underlying glioma progression is necessary to improve the survival quality of cancer patients.

MicroRNAs (miRNAs) are often abnormally expressed in human cancers by binding with the 3'-untranslated region (3'-UTR) of the target message RNAs (mRNAs)[4,5]. Lu et al[6] indicated that miRNAs were crucial regulators for multiplies cellular processes. To date, several investigations[7-9] have validated that miRNAs can regulate glioma cell behaviors, including cell metastasis, apoptosis, autophagy, and angiogenesis. Hence, targeting miRNAs represents a strong therapeutic measure in glioma.

Several analyses[10-12] revealed that miR-199a-5p expression was dysregulated in various cancers. MiR-199a-5p was found to be downregulated in colorectal cancer, and its overexpression could inhibit cancer metastasis *via* regulating Rho-associated coiled coil-containing protein kinase 1 expression[10]. In hemangioma, miR-199a-5p was shown to reduce the expression and inhibited cell growth, but promoted cell apoptosis by targeting HIF1A[11]. In glioma, recently Wang et al[12] revealed that miR-199a-5p reduced its expression and regulated cancer cell metastasis *via* targeting magnesium transporter 1.

Here, the miR-199a-5p expression level in glioma cell lines was examined by RT-qPCR method. Subsequently, we investigated the biological roles of miR-199a-5p in regulating glioma cell malignant phenotypes and the associated mechanisms.

Materials and Methods

Cell Culture

Glioma cell lines U87 and U251 purchased from Cell Bank of the Chinese Academy of Science

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(Shanghai, China) were maintained at Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). The normal human astrocytes (NHAs) purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) were incubated at RPMI-1640 medium (Thermo Fisher Scientific Inc., Waltham, MA, USA). The cell medium was supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Waltham, MA, USA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Thermo Fisher Scientific Inc., Waltham, MA, USA). The cells were incubated at 37°C humidified atmosphere containing 5% of CO_2.

Cell Transfection

MiR-199a-5p mimic, corresponding negative control (miR-NC), and pcDNA3.1 containing the coding sequence of the membrane-associated ring-CH-type finger 8 (pMARCH8) were purchased from GenScript (Nanjing, Jiangsu, China). Lipofectamine 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for cell transfection. The cells were collected for further analyses after 48 h of transfection.

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

To detect the levels of miR-199a-5p, the complementary DNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, Liaoning, China) from the RNA extracted from cell line by TRIzol (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). RT-qPCR was conducted at ABI 7900 system (Applied Biosystems, Foster City, CA, USA) using SYBR Green Mix (TaKaRa, Dalian, Liaoning, China) with the following parameters: 95°C for 3 min, 40 cycles of 95°C for 10 s, and 58°C for 30 s. The primers used were as follows: miR-199a-5p forward: 5'-TCAAGAGCAATAACGAAAAATGT-3', reverse: 5'-GCTGTCAACGATACGCTACGT-3'; U6 snRNA forward: 5'-ATTGGAACGATACAGAGAAGATT-3', reverse: 5'-GTCCTTGGTGCCCGAGTG-3'. The relative expression level was normalized to U6 snRNA and was calculated with the $2^{-\Delta\Delta Ct}$ method.

Western Blot

The total protein was isolated with RIPA buffer supplemented with protease inhibitor (Beyotime, Haimen, Jiangsu, China), and quantified with BCA kit (Beyotime, Haimen, Jiangsu, China). After that, an equal amount of protein sample was separated using 10% SDS-PAGE and transferred to polyethylene difluoride (PVDF) membranes. After being blocked with 5% non-fat milk, the membranes were incubated with primary antibodies (anti-MARCH8: ab109690, anti-GAPDH: ab181602; Abcam, Cambridge, MA, USA) for overnight at 4°C, followed by incubation with secondary antibody (ab6721, Abcam) for 1 h at 37°C. Finally, BeyoECL kit was utilized to visualize the band signals.

CCK-8 Assay

The cells were seeded into 96-well plate and incubated for the indicated time. At these time points, CCK-8 reagent (Beyotime, Haimen, Jiangsu, China) was added to the well and incubated for other 4 h. The optical density was measured at 450 nm using a microplate reader.

Transwell Invasion Assay

The transwell invasion assay was performed using Matrigel (BD Biosciences, San José, CA, USA) pre-coated 8-μm pore polycarbonate membrane chamber. 5×10^4 cells in FBS-free medium were plated to the upper chamber; while the medium contains FBS was placed in the lower chamber. After incubation for 48 h, the invasive cells were fixed with methanol and stained with crystal violet. Finally, the invasive cell numbers were counted under the microscope.

Luciferase Reporter Assay

TargetScan predicted MARCH8 was a putative target for miR-199a-5p. Wild-type and mutant 3'-UTR of MARCH8 were inserted into pGL3-control vector (Promega, Madison, WI, USA) and designated as WT-MARCH8 or MUT-MARCH8, respectively. The cells were transfected with miRNAs and the Luciferase vectors using Lipofectamine 2000. After transfection for 48 h, the cells were collected for relative Luciferase activity analysis using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

The data were presented as mean ± standard error after analyzing at GraphPad Prism 5 (San Diego, CA, USA). The two-tailed Student's *t*-test or One-way analysis of variance and Tukey post-hoc test were used to analyze the differences in the groups. A *p*-value less than 0.05 represents statistical significance in the groups.

Results

MiR-199a-5p Expression Was Reduced in Glioma Cell Lines

RT-qPCR analysis results showed that miR-199a-5p expression level was significantly decreased in glioma cells compared with normal cell (Figure 1).

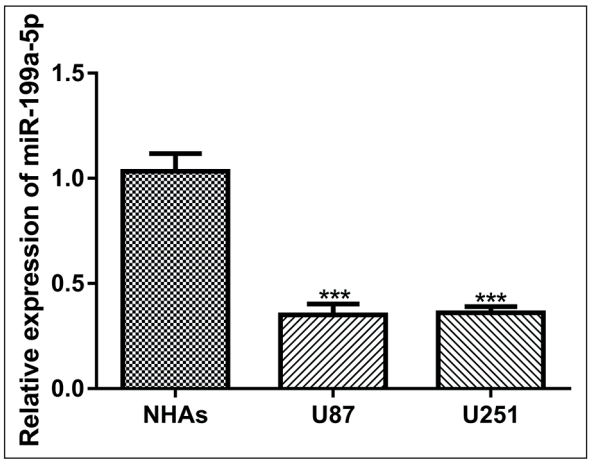

Figure 1. Lower expression level of miR-199a-5p in glioma cell lines compared with the normal cell line. MiR-199a-5p: microRNA-199a-5p.

MiR-199a-5p Overexpression Inhibits Glioma Cell Proliferation and Invasion

To explore the roles of miR-199a-5p in glioma, the synthetic miRNAs were transfected into glioma cells. As presented in Figure 2A, the introduction of miR-199a-5p mimic significantly elevated miR-199a-5p levels in glioma. Next, we found that miR-199a-5p overexpression significantly inhibited cell proliferation ability (Figure 2B). The transwell invasion assay showed that force miR-199a-5p expression inhibits cell invasion ability (Figure 2C). These results indicated that miR-199a-5p overexpression inhibits glioma cell growth and invasion.

MARCH8 Was a Direct Target of MiR-199a-5p

TargetScan predicted MARCH8 was a putative target of miR-199a-5p (Figure 3A). The Luciferase activity reporter assay showed that

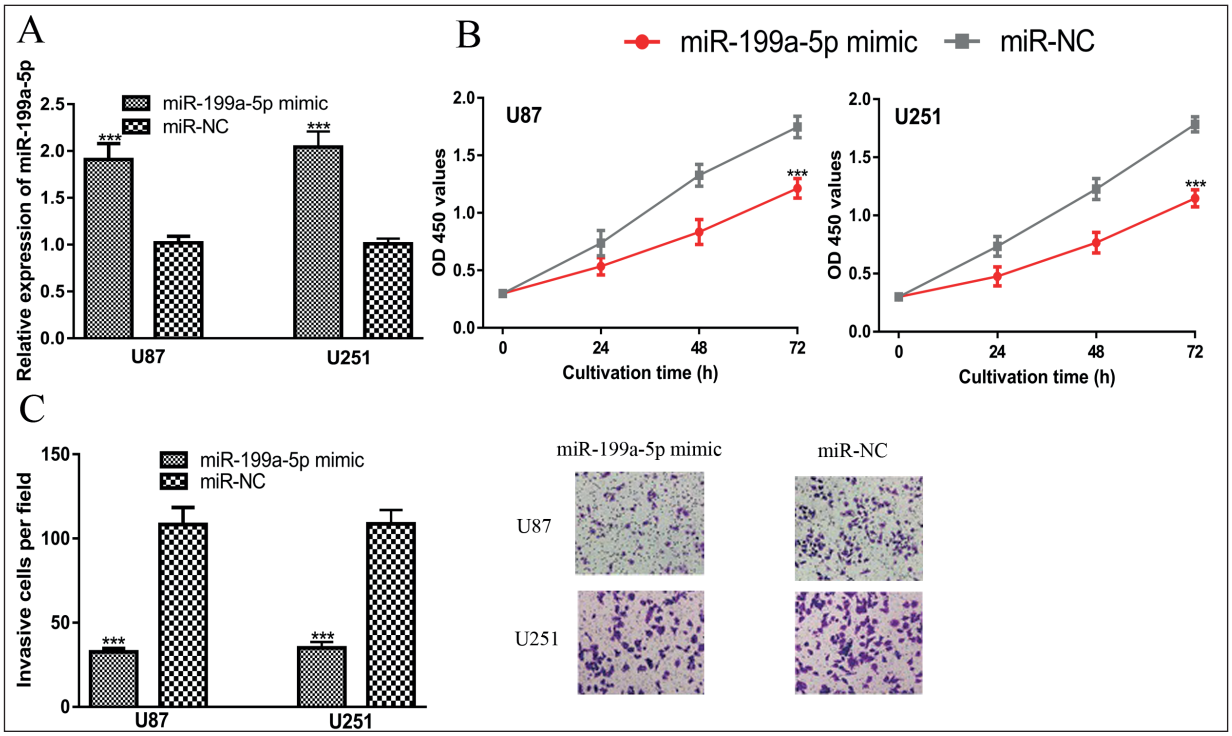

Figure 2. Overexpression of miR-199a-5p inhibits glioma cell proliferation and invasion. ***(A)*** MiR-199a-5p expression, ***(B)*** Cell proliferation, and ***(C)*** Cell invasion in glioma cells transfected with synthetic miRNAs as examined by RT-qPCR, CCK-8 assay, and transwell invasion assay, respectively (200×). MiR-199a-5p: microRNA-199a-5p; RT-qPCR: quantitative Real-Time Polymerase Chain Reaction; CCK-8: cell counting kit-8.

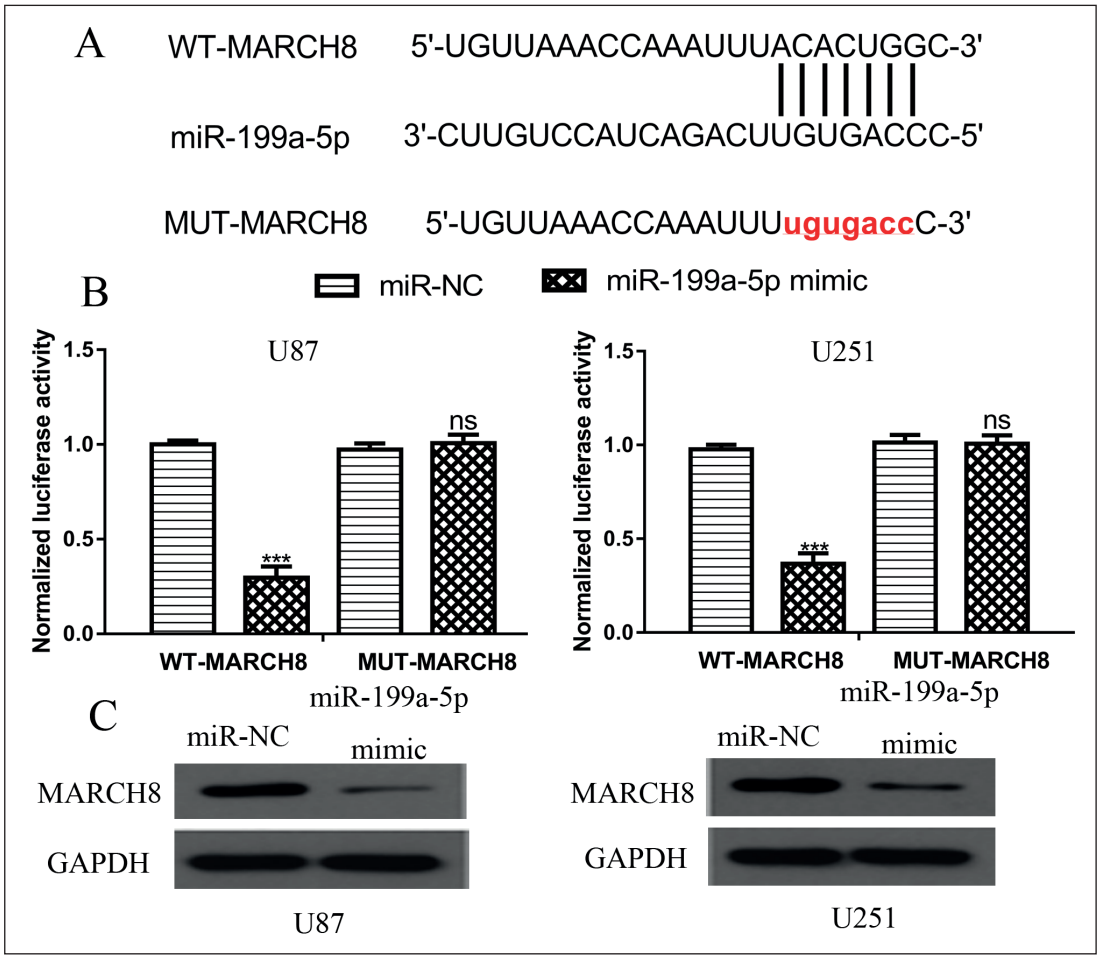

Figure 3. MARCH8 was a direct target of miR-199a-5p. ***A,*** Sequence alignment of miR-199a-5p and the MARCH8 3'-UTR. ***B,*** Luciferase assay in glioma cells cotransfected with miR-199a-5p mimic and Luciferase reporter containing the WT-MARCH8 orMUT-MARCH8. ***C,*** The miR-199a-5p transfection affects MARCH8 protein levels. MiR-199a-5p: microRNA-199a-5p; MARCH8: membrane associated ring-CH-type finger 8; 3'-UTR: 3'-untranslated region; WT: wild type; MUT: mutant.

miR-199a-5p overexpression inhibited the relative Luciferase activity in the cells transfected with WT-MARCH8 (Figure 3B). Western blot showed that introduction of miR-199a-5p mimic significantly decreased the protein level of MARCH8 in glioma cells (Figure 3C).

MARCH8 Overexpression Promoted Cell Proliferation and Invasion

Then, we performed gain-of-function experiments to analyze the role of MARCH8 in glioma. We found that the pMARCH8 transfection increased the protein levels of MARCH8 in glioma cells (Figure 4A). CCK-8 assay indicated that the MARCH8 overexpression significantly promoted glioma cell proliferation (Figure 4B). The transwell invasion assay demonstrated that MARCH8 overexpression notably elevated cell invasion ability in glioma cells (Figure 4C). We also showed that MARCH8 overexpression partially reversed the effects of miR-199a-5p mimic on glioma cell proliferation and invasion (Figures 4A-4C).

Discussion

In the past decades, numerous molecular biomarkers have been identified for glioma[13-18]. LncRNA LINC00909 was reported[13] to be highly expressed in glioma and correlated with poorer overall survival of cancer patients. LINC00909 overexpression was shown to promote glioma progression *via* regulating miR-194/MUC1-C axis[13]. Another work[14] showed that ras suppressor-1 was able to promote glioma invasion. In addition, high phospholipase A2 group V was re-

vealed to be associated with unfavorable prognosis, epithelial-mesenchymal transition, and isocitrate dehydrogenase 1 mutation status[15]. Through these studies, we believe it is useful to explore the mechanisms underlying glioma progression to provide novel treatment options.

In this investigation, we detected miR-199a-5p expression levels in glioma cell lines. We found that miR-199a-5p expression was significantly reduced in glioma cells compared with a normal cell. Moreover, we showed that miR-199a-5p overexpression was able to inhibit glioma cell proliferation and invasion *in vitro*. In combination with previous report[12], we suggested that miR-199a-5p functioned as a tumor suppressor in glioma. MiRNAs functioned by modulating various genes expression during carcinogenesis. Recently, multiple targets[10-12] for miR-199a-5p have been identified and helped us to understand the role of miR-199a-5p in cancer. By utilizing TargetScan, we showed that MARCH8 was a putative target of miR-199a-5p. MARCH8 contains a N-terminal RING finger domain and is able to interact with E2 enzyme and two trans-membrane domains[19]. Massive reports[20,21] have indicated that MARCH8 functioned as an oncogene in human cancers. For example, the knockdown of MARCH8 reduced gastric cancer cell viability and induced apoptosis[20]. Moreover, MARCH8 was found highly expressed in esophageal tumor, and its knockdown could inhibit cancer proliferation, migration, invasion, but promote apoptosis[21]. Here, we showed that MARCH8 overexpression promoted glioma cell growth and invasion, and partially reversed the effects of miR-199a-5p mimic on glioma cell behaviors.

Conclusions

MiR-199a-5p expression was downregulated in glioma, and it was able to regulate glioma cell proliferation and invasion *via* targeting MARCH8. It may be a useful target for glioma patients.

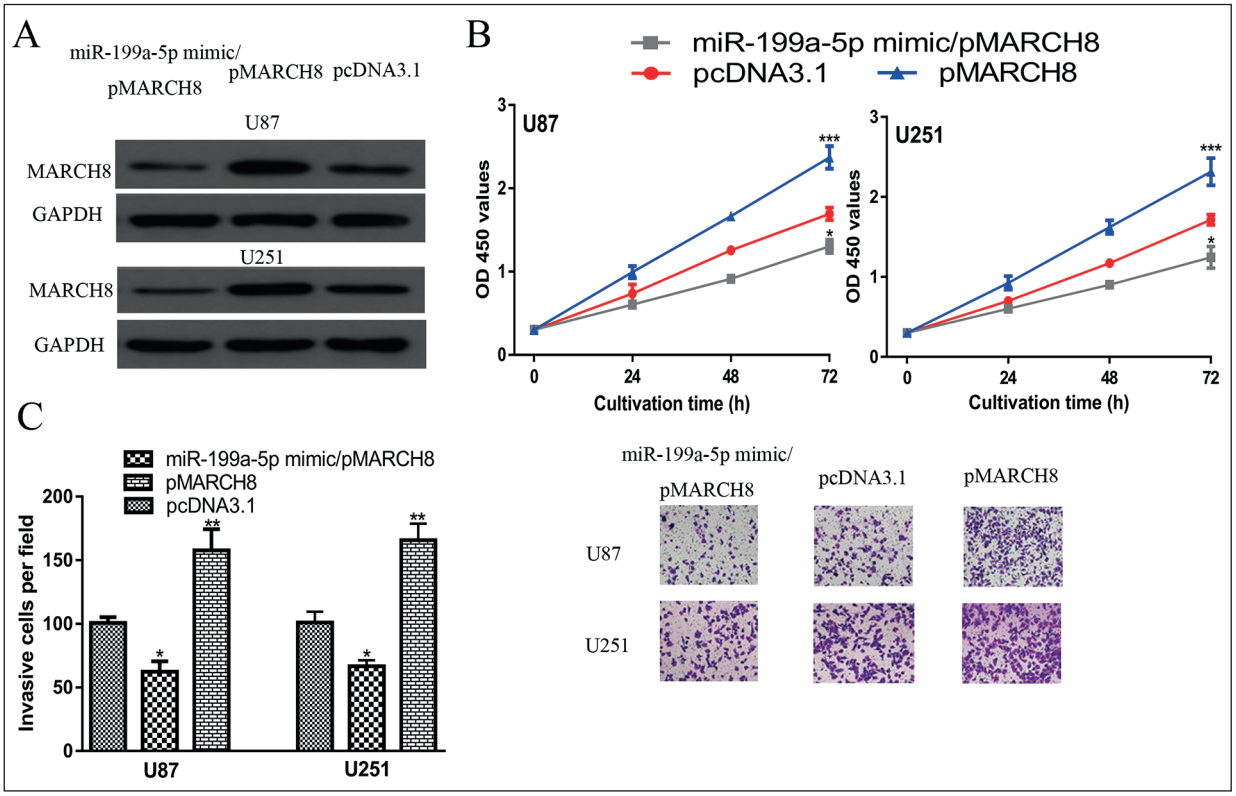

Figure 4. Overexpression of MARCH8 promotes glioma cell proliferation and invasion. ***(A)*** MARCH8 expression, ***(B)*** Cell proliferation, and ***(C)*** Cell invasion in glioma cells transfected with pMARCH8, pcDNA3.1 or pMARCH8, and miR-199a-5p mimic as examined by Western blot, CCK-8 assay, and transwell invasion assay, respectively. MiR-199a-5p: microRNA-199a-5p; MARCH8: membrane associated ring-CH-type finger 8; CCK-8: cell counting kit-8 (200x).

Conflict of Interests

The Authors declare that they have no conflict of interests.

Funding

Not applicable.

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