MicroRNA-599 suppresses glioma progression by targeting RAB27B

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Abstract. MicroRNAs (miRNAs/miRs) serve tumor promoting or suppressive roles in different human cancer types, including glioma; however, the regulatory underlying mechanism by which miR-599 affects glioma progression remains largely unknown. The aim of the present study was to investigate the expression of miR-599 in glioma, as well as the underlying regulatory mechanism. Reverse transcription-quantitative polymerase chain reaction and western blot analysis were used to examine mRNA and protein expression, respectively. MTT, wound healing and transwell assays were conducted to study cell proliferation, migration and invasion, respectively. A dual-luciferase reporter gene assay was used to confirm the targeting association between miR-599 and Ras-related protein Rab-27B (hereafter RAB27B). In the present study, miR-599 expression was observed to be significantly downregulated in human glioma tissues and cell lines, when compared with normal brain tissues and normal human astrocyte cells, respectively. Low miR-599 expression was significantly associated with glioma progression. Ectopic expression of miR-599 caused a significant reduction in the proliferation, migration and invasion of U-87MG Uppsala and U251 cells. Bioinformatics analysis and dual-luciferase reporter gene assay data identified that RAB27B was a direct target gene of miR-599. The mRNA and protein expression of RAB27B was significantly downregulated following miR-599 overexpression in U-87MG Uppsala and U251 cells. Rescue experiments demonstrated that RAB27B rescued the miR-599-induced inhibition of glioma cell growth, migration and invasion. In addition, RAB27B expression was significantly upregulated in glioma tissues and cell lines, and the expression levels of RAB27B were inversely correlated with miR-599 levels in glioma tissues; therefore, the present study demonstrated that miR-599 exerts a tumor-suppressive role in glioma progression by targeting RAB27B, indicating that miR-599 may be used as a potential candidate for glioma treatment.

Introduction

Glioma is the most common type of primary brain tumor, and one of the most aggressive and lethal human malignancies (1). The majority of patients are diagnosed at an advanced stage of the disease, despite great efforts towards the early detection of glioma (1,2). The overall prognosis of patients with advanced glioma is poor (1,2). In recent years, a number of studies have focused on investigating the pathogenesis of glioma (3-5). However, the molecular mechanisms underlying glioma progression remain largely unknown; therefore, there is an urgent requirement to identify novel and effective diagnostic and therapeutic targets for this disease.

MicroRNAs (miRNAs/miRs) are a class of short, endogenous, single-stranded RNAs that have been demonstrated to function as key regulators for gene expression by directly binding to the 3'-untranslational region (3'-UTR) of target mRNAs, causing mRNA degradation or translation repression (6,7). Previous studies have demonstrated that miRNAs are involved in a variety of cellular biological processes, including development and differentiation, cell proliferation, apoptosis and migration, in addition to tumorigenesis (8,9). Complete deregulation of miRNAs has been observed in various human cancer types, indicating that an altered miRNA expression profile may contribute to the development and malignant progression of human cancer (10,11). Recently, Zhang et al (12) reported that miR-599 inhibited the proliferation and invasion of glioma by targeting periostin. As one miRNA can have numerous target genes, other target genes of miR-599 may also serve important roles in glioma.

Ras-related protein Rab-27B (hereafter RAB27B) is a member of the Rab protein family, which are prenylated, membrane-bound proteins involved in vesicular fusion and
trafficking (13). Deregulation of RAB27B has been observed in a number of common cancer types (14,15). For instance, RAB27B was determined to be significantly upregulated in ovarian cancer tissues, where it was associated with distant metastasis and poor prognosis (15). Furthermore, patients with high-grade glioma harboring RAB27B hypomethylation or overexpression exhibited a reduced survival time, indicating that the upregulation of RAB27B contributes to glioma progression and poor patient prognosis (16); however, whether miR-599 regulates RAB27B expression in glioma remains unclear.

Therefore, the present study aimed to investigate the clinical significance of miR-599 and RAB27B expression in glioma. The regulatory mechanism of miR-599 and RAB27B underlying glioma progression was studied.

Materials and methods

Clinical tissue samples. The present study was approved by the Ethics Committee of Sun Yat-sen University Cancer Center (Guangzhou, China). A total of 50 glioma tissues were collected from patients with primary glioma at Sun Yat-sen University Cancer Center between April 2014 and March 2016, as were 13 matched normal brain tissues. The age of patients with normal brain tissue ranged between 34-58 years, with 8 male and 5 female. The 50 patients with glioma included 29 men and 21 women, aged 14-69 years old, with a mean age of 43.3 years old. Written informed consent was obtained from all patients. All tissues were pathologically confirmed at Sun Yat-sen University Cancer Center, and necrosis in glioma tissues was also evaluated pathologically. The patients with glioma were classified according to the World Health Organization criteria and staged according to the Tumor-Node-Metastasis classification (17). Following surgical resection, tissues were immediately snap-frozen in liquid nitrogen. The clinicopathological characteristics of these patients with glioma are summarized in Table I.

Cell culture. Normal human astrocyte (NHA) cells were purchased from the American Type Cell Culture Collection (Manassas, VA, USA). Human glioma cell lines (U-373MG Uppsala, U-87MG Uppsala, U251 and T98G) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.), according to the manufacturer's instruction. U6 was used as internal reference. GAPDH was used as internal reference. The primer sequences were as follows: RAB27B, forward, TAGACTTTCCGGGAAACGCTGTG, and reverse, AGAAGCTCTGTGACTGTTGTA; GAPDH, forward, GGAGCGGATCCCCTCAAAAT, and reverse, GGCTGTTGTCATATTCTCATGG. The thermocycling conditions were as follows: Denaturation at 95˚C for 5 min followed by 35 cycles of denaturation at 95˚C for 15 sec and annealing/elongation at 60˚C for 30 sec. The relative expression was analyzed using the 2^ΔΔCq method (18).

Western blot analysis. Cells (NHA, U-373MG Uppsala, U-87MG Uppsala, U251 and T98G) were solubilized in cold radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). The protein concentration was determined using a BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer’s instruction. Proteins (60 µg/lane) were separated by 12% SDS-PAGE and were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Inc.). The PVDF membrane was then incubated with PBS (Thermo Fisher Scientific, Inc.) containing 5% non-fat milk at room temperature for 3 h. Following three washes with PBS, the PVDF membrane was incubated with rabbit polyclonal anti-RAB27B antibody (dilution, 1:50; cat. no. ab103418; Abcam, Cambridge, MA, USA) or rabbit polyclonal anti-GAPDH antibody (dilution, 1:50; cat. no. ab9485; Abcam) at room temperature for 3 h. After being washed with PBS three times, the PVDF membrane was then incubated with the horseradish peroxidase-conjugated goat anti-rabbit monoclonal secondary antibody (dilution, 1:5,000; cat. no. ab6721; Abcam) at room temperature for 40 min. An Enhanced Chemiluminescence Western Blotting kit (Pierce; Thermo Fisher Scientific, Inc.) was used to detect immune complex on the PVDF membrane. Protein expression was analyzed using Image-Pro Plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). GAPDH was used as an internal control.

MTT assay. To analyze cellular proliferation, 5x10^4 U-87MG Uppsala and U251 cells were cultured in a 96-well plate with 100 µl of DMEM containing 0.5 g/l MTT (Thermo Fisher Scientific, Inc.). Following this, U251 cells were cultured at 37˚C for 12, 24, 48 or 72 h, after which the medium was

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues and cells (NHA, U-373MG Uppsala, U-87MG Uppsala, U251 and T98G) using TRIzol® reagent (Thermo Fisher Scientific, Inc.). RevertAid RT Reverse Transcription kit (Thermo Fisher Scientific, Inc.) was used to convert 1 µg RNA into cDNA. To detect the expression of miR-599, qPCR was conducted using the All-in-one™ miRNA qRT-PCR detection kit (GeneCopeia, Inc., Rockville, MD, USA), according to the manufacturer's instruction. GAPDH was used as internal reference. The primers for miR-599 and U6 were directly purchased from Fulengen, Guangzhou, China. For detecting mRNA expression, qPCR was conducted using the SYBR Green PCR Master mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's instruction. GAPDH was used as internal reference. The primer sequences were as follows: RAB27B, forward, TAGACTTTCCGGGAAACGCTGTG, and reverse, AGAAGCTCTGTGACTGTTGTA; GAPDH, forward, GGAGCGGATCCCCTCAAAAT, and reverse, GGCTGTTGTCATATTCTCATGG. The thermocycling conditions were as follows: Denaturation at 95˚C for 5 min followed by 35 cycles of denaturation at 95˚C for 15 sec and annealing/elongation at 60˚C for 30 sec. The relative expression was analyzed using the 2^ΔΔCq method (18).

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removed. Next, 50 µl dimethyl sulfoxide (Thermo Fisher Scientific, Inc.) was added. Following incubation at 37˚C for 10 min, the A570 of each sample was measured at a wavelength of 570 nm using an enzyme immunoassay analyzer (Tecan Infinite® M200; Tecan Group Ltd., Männedorf, Switzerland).

**Wound-healing assay.** U-87MG Uppsala and U251 cells were cultured to full confluence and a wound of ~1 mm width was created using a plastic scriber. Next, cells were washed in DMEM and incubated in serum-free DMEM at 37˚C for 24 h. For the negative control, cells were fixed using 90% ethanol at room temperature for 10 min and observed under an inverted microscope (magnification, x40; Olympus Corporation, Tokyo, Japan). Following this, cells were incubated at 37˚C in DMEM supplemented with 10% FBS for 48 h. Next, cells were fixed using 90% ethanol at room temperature for 10 min and observed under an inverted microscope (magnification, x40; Olympus Corporation). This experiment was repeated 3 times.

**Invasion assay.** Transwell chambers pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used to conduct cell invasion analysis. The U-87MG Uppsala and U251 cell suspension (3x10^5 cells/ml) was prepared in DMEM. Next, 300 µl cell suspension was added into the upper chamber, and 300 µl DMEM supplemented with 10% FBS was added into the lower chamber. Following incubation at 37˚C for 24 h, the cells that did not invade through the membrane were removed using a cotton-tipped swab. Cells that invaded the membrane were fixed in 90% ethyl alcohol at room temperature for 10 min, and then stained with 0.1% crystal violet (Beyotime Institute of Biotechnology) at room temperature for 10 min. The invaded cells were counted under an inverted microscope (magnification, x400).

**Bioinformatics prediction.** Targetscan 7.1 software (http://www.targetscan.org/vert_71/) was used to predict the potential target genes of miR-599. The search terms ‘Human’ and ‘miR-599’ were used.

**Dual-luciferase reporter assay.** The wild type (WT) RAB27B 3'-UTR containing the predicted binding sequences of miR-599 was constructed using PCR, which was then subcloned downstream of the Renilla luciferase gene in the psiCHECK-2 vector (Promega Corporation, Madison, WI, USA). The mutant type (MT) RAB27B 3'-UTR lacking the binding sequences of miR-599 was constructed using the Directed Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA), which was also subcloned downstream of the Renilla luciferase gene in the psiCHECK-2 vector. U-87MG Uppsala and U251 cells were then co-transfected with WT or MT RAB27B 3'-UTR plasmid, and miR-599 mimic or miR-negative control (NC), using Lipofectamine 2000 according to the manufacturer's instruction. Following transfection for 48 h, the luciferase activity was determined using the Dual-Luciferase Reporter Assay system (Promega Corporation) on an Lmax multwell luminometer (Molecular Devices, LLC, Sunnyvale, CA, USA), according to the manufacturer's instruction. The Renilla luciferase activity was normalized to firefly luciferase activity.

**Statistical analysis.** The data are presented as the mean ± standard deviation of three independent experiments. Statistical analysis was conducted using the SPSS Graduate Pack, version 19.0 (IBM Corp., Armonk, NY, USA). Differences were analyzed using a Student's t-test for two-group comparison or a one-way analysis of variance for multiple-group comparison followed by Tukey's post hoc test. The correlation between the expression of miR-599 and

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*P<0.05. miR-599, microRNA-599.

Table I. Association between miR-599 expression and clinicopathological characteristics of patients with glioma.
RAB27B was analyzed using Pearson's correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of miR‑599 expression is associated with glioma progression. RT-qPCR analysis was conducted to determine the expression of miR‑599 in glioma tissues, which was then compared to that in normal brain tissues. These data indicated that glioma tissues exhibited significantly lower expression levels of miR‑599, than normal brain tissues (Fig. 1A). Furthermore, miR‑599 expression was also significantly downregulated in glioma cell lines, compared with NHA cells (Fig. 1B).

Next, the clinical significance of miR‑599 expression in glioma was studied. Patients with glioma were divided into high-miR-599-expression and low-miR-599-expression groups according to the mean value of miR-599 expression. Low expression of miR‑599 was significantly associated with necrosis and advanced clinical stage in glioma (Table I).

miR-599 could inhibit glioma cell proliferation, migration and invasion. As miR-599 expression was significantly downregulated in glioma, U-87MG Uppsala and U251 cells were transfected with miR-599 mimic to upregulate its expression. Following transfection, the miR-599 levels were significantly increased compared with the control group; however, transfection with scramble miRNA mimic did not affect the miR-599 expression in U-87MG Uppsala and U251 cells (Fig. 2A). The proliferative, migratory and invasive capacities of the transfected cells were examined using MTT, wound healing and transwell assays, respectively. As depicted in Fig. 2B-D, the overexpression of miR-599 caused a significant reduction in U-87MG Uppsala and U251 cell proliferation, migration and invasion. These data demonstrated that miR-599 could inhibit glioma cell proliferation, migration and invasion.

RAB27B is a target gene of miR-599 in glioma cells. As miRNAs function by mediating the expression of their target genes, Targetscan software was used to predict the potential target genes of miR-599. The 3'-UTR of RAB27B mRNA contained the binding sequences of miR-599 (Fig. 3A). To confirm this prediction, the WT RAB27B-3'-UTR and MT RAB27B 3'-UTR luciferase reporter plasmids were generated (Fig. 3A). A dual-luciferase reporter gene assay was then conducted in U-87MG Uppsala and U251 cells. As depicted in Fig. 3B, the luciferase activity was significantly decreased in cells co-transfected with miR-599 mimics and WT RAB27B 3'-UTR luciferase reporter plasmid, which was eliminated by transfection with the WT RAB27B 3'-UTR luciferase reporter plasmid. These results indicated that miR-599 could directly bind to the 3'-UTR of RAB27B mRNA in U-87MG Uppsala and U251 cells. Following this, the regulatory effect of miR-599 on the expression of RAB27B in U-87MG Uppsala and U251 cells was assessed. As depicted in Fig. 3C and D, the mRNA and protein levels of RAB27B were significantly downregulated following overexpression of miR-599. To confirm these findings, U-87MG Uppsala and U251 cells were transfected with miR-599 inhibitor or NC inhibitor. Following transfection, the miR-599 levels were significantly downregulated in the miR-599 inhibitor group, compared with the control group; however, transfection with the NC inhibitor did not affect its expression (Fig. 3E). It was determined that transfection with miR-599 inhibitor caused an upregulation of RAB27B expression, when compared with the NC inhibitor group (Fig. 3F and G). Therefore, the expression of RAB27B was negatively mediated by miR-599 in U-87MG Uppsala and U251 cells.

RAB27B rescues the inhibitory effects of miR-599 on the malignant phenotypes of glioma cells. As it was determined that overexpression of miR-599 resulted in inhibitory effects on the proliferation, migration and invasion of U-87MG Uppsala and U251 cells, accompanied by the significant downregulation of RAB27B, a rescue experiment was conducted to assess whether RAB27B was involved in the miR-599-mediated malignant phenotypes of U-87MG Uppsala and U251 cells. miR-599-overexpressing U-87MG Uppsala and U251 cells were transfected with a RAB27B expression plasmid to upregulate its expression levels. Following transfection, the mRNA and
protein levels of RAB27B were significantly increased in the miR-599+RAB27B group, compared with the miR-599+blank group (Fig. 4A and B). Next, the cell proliferation, migration and invasion capacities was examined using MTT, wound-healing
and transwell assays, respectively. As depicted in Fig. 4C-E, the proliferation, migration and invasion of U-87MG Uppsala and U251 cells were significantly upregulated in the miR-599+RAB27B group, compared with the miR-599+blank group, indicating that RAB27B rescued the inhibition effects of miR-599 on the malignant phenotypes of glioma cells. These data indicated that RAB27B acted as a downstream effector of miR-599 in U-87MG Uppsala and U251 cells.

**RAB27B is upregulated in glioma.** The expression of RAB27B in glioma tissues and cell lines was studied. As depicted in Fig. 5A, RT-qPCR data demonstrated that RAB27B was significantly upregulated in glioma tissues, compared with normal brain tissues. Similarly, the mRNA and protein expression of RAB27B was also increased in glioma cell lines, compared with NHA cells (Fig. 5B and C). Notably, an inverse correlation was observed between the expression of RAB27B and
Figure 4. RAB27B rescues the inhibitory effects of miR-599 on the malignant phenotypes of glioma cells. miR-599-overexpressing U-87MG Uppsala and U251 cells were transfected with RAB27B expression plasmid or a blank vector. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis were used to determine the mRNA and protein levels of RAB27B. (C) MTT, (D) wound healing and (E) transwell assays were conducted to examine cellular proliferation, migration and invasion, respectively. Magnification for wound healing assay, x40; magnification for transwell assay, x400. **P<0.01 vs. miR-599+blank. miR-599, microRNA-599; OD, optical density.
miR-599 in glioma tissues (Fig. 5D), which indicated that the reduced miR-599 expression may contribute to the increased expression of RAB27B in glioma tissues.

Discussion

The underlying mechanism by which miR-599 regulates glioma progression remains largely unclear. The present study determined that low miR-599 expression was significantly associated with glioma progression. Ectopic expression of miR-599 reduced glioma cell proliferation, migration and invasion. Bioinformatic analysis identified that RAB27B was a direct target gene of miR-599, and its expression was suppressed by miR-599 in glioma cells. Overexpression of RAB27B rescued the miR-599-induced inhibition of glioma cell proliferation, migration and invasion. Additionally, RAB27B expression was significantly upregulated in glioma, and inversely correlated to the miR-599 expression in glioma tissues.

A previous study indicated that miR-599 inhibits the proliferation and migration of vascular smooth muscle cells by targeting transforming growth factor β2 (19); however, there are a limited number of studies regarding the function of miR-599 in human cancer. Tian et al (20) reported that miR-599 was upregulated in patients with non-small cell lung cancer, and promoted cancer cell proliferation and invasion by directly targeting special AT-rich sequence-binding protein 2. Chi et al (21) determined that miR-599 could directly inhibit the expression of inositol polyphosphate-4-phosphatase type II B, and thus served a tumor-suppressive role in melanoma. Recently, Zhang et al (12) reported that the expression of miR-599 was reduced in 33 glioma tissues, compared with adjacent normal brain tissues. The present study also demonstrated that miR‑599 was significantly downregulated in 67 glioma tissues, compared with normal brain tissues. Furthermore, the present study and that by Zhang et al (12) indicated that the increased expression of miR-599 was significantly associated with the malignant progression of glioma. In addition, Zhang et al (12) demonstrated that miR-599 could inhibit glioma cell proliferation and invasion by targeting
peristin expression. The present study also indicated that overexpression of miR-599 could inhibit glioma U251 cellular proliferation, migration and invasion.

Further investigation identified that RAB27B was a novel target gene of miR-599, and the expression levels of RAB27B was negatively affected by miR-599 in U-87MG Uppsala and U251 cells. RAB27B, a member of the Rab protein family, was previously reported to act as an oncogene in several common types of cancer (22,23). High expression of RAB27B is associated with poor prognosis of patients with breast cancer, and it promotes the invasive growth and metastasis in estrogen receptor-positive breast cancer cells (22). Pu et al (23) demonstrated that miR-193a-3p and miR-193a-5p suppressed the metastasis of osteosarcoma cells via inhibition of RAB27B expression. Wang et al (16) reported that high expression of RAB27B was associated with the poor prognosis of patients with glioma, and knockdown of RAB27B significantly inhibited glioma U-87MG Uppsala and LN229 cell invasion, possibly owing to a reduction in matrix metallopeptidase 9 expression and activation. Additionally, Wang et al (16) demonstrated that inhibition of RAB27B expression also reduced the tumor growth in vivo; their data also indicated that RAB27B acts as an oncogene in glioma. The present study determined that overexpression of miR-599 caused a reduction in the expression of RAB27B in U251 cells, which further confirmed that miR-599 could directly bind to RAB27B mRNA and repress its translation. RAB27B overexpression rescued the inhibitory effects of miR-599 on the proliferation, migration and invasion of U251 cells, indicating that RAB27B may be involved in the miR-599-mediated malignant phenotypes of glioma cells. Additionally, it was also determined that RAB27B was significantly upregulated in glioma, consistent with the previous report (16). The upregulation of RAB27B may be partly due to the reduced expression of miR-599; an inverse correlation was observed between miR-599 and RAB27B expression in glioma tissues.

In summary, to the best of our knowledge the present study is the first to have demonstrated that miR-599 served a suppressive role in regulating the proliferation, migration and invasion of glioma cells, at least in part, by directly targeting RAB27B. The present study indicated that miR-599 may represent a potential therapeutic candidate for glioma treatment.

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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

RL designed this study and revised this manuscript. YJ collected clinical tissues, performed statistical analysis and wrote the manuscript. XW and JZ performed the experiments.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Sun Yat-sen University Cancer Center (Guangzhou, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References