# Original Article microRNA-497-5p targeted SOX9 to inhibit proliferation, migration and invasion of glioma cells

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**Abstract:** Increasing studies indicate that deregulation of microRNAs (miRNAs) contributes to the carcinogenesis and progression of glioma. The present study was aimed to investigate the roles of miR-497-5p in the progression of glioma and to elucidate underlying miR-497-5p-mediated molecular mechanisms in glioma. Our results showed that miR-497-5p was significantly down-regulated in glioma tissues and cell lines. Enforced miR-497-5p expression inhibited cell proliferation, migration and invasion of glioma cells. Moreover, bioinformatics analysis predicated that SRY-related high mobility group-Box gene 9 (SOX9) was a putative target of miR-497-5p. By using luciferase reporter assay, qRT-PCR and western blot, we demonstrated that SOX9 was a direct target gene of miR-497-5p in glioma. We also found that knockdown of SOX9 could simulate the suppressive functions of miR-497-5p in glioma. Taken together, these findings suggested that miR-497-5p functioned as a tumor suppressor in glioma by targeting SOX9. It might be a novel therapeutic target for patients with glioma.

Keywords: miR-497-5p, SOX9, proliferation, migration, invasion, glioma

#### Introduction

Human glioma, the most common form of brain malignancy, originates from astrocytes or astroglial precursors [1]. It accounts for about onethird of all intracranial tumors of the central nervous system among adults and children [2]. So far, the pathology of glioma remains elusive and is possible to be closely correlated with multiple factors, such as tumor origin, genetic factors, biochemical environment, ionizing radiation, nitroso compounds, air pollution, bad living habits and infection [3]. Depending on the degree of malignancy according to World Health Organization (WHO) classification, gliomas could be classified into four grades: low-grade astrocytomas (WHO grade I-II), anaplastic astrocytomas (WHO grade III), and glioblastoma (GBM, WHO grade IV) [4]. The overall survival rate of glioma patients with high-grade is 40% at one year, and the five-year survival rate is less than 10% [5]. In spite of the significant progress in the field of surgical resection, radiotherapy and chemotherapy treatment, the survival of patients is still quite short, especially when rapidly growth and metastasis occur [6]. Therefore, to fully understand the molecule mechanism involved in the glioma cell proliferation, invasion and metastasis is of particular important to the development of novel therapeutic targets for glioma patients.

Recently, microRNAs (miRNAs), a group of 19to 24-nucleotide short, endogenous, and noncoding RNAs, have emerged as key regulators of tumorigenesis and tumor development in various kinds of human cancers [7-10]. It has been firmly confirmed that miRNAs could trigger destruction of homologous mRNA or inhibition of cognate mRNA translation, and play a significant role in maintaining steady state of chromosome structure, thereby regulating the expression of protein-coding genes [11]. It is well established that miRNAs play important regulators in diverse biological processes of cancer, such as cell cycle, apoptosis, cell proliferation, migration, invasion, metastasis, differentiation and development [12, 13]. A great deal of studies showed that miRNAs were abnormal deregulated in human cancers, also including glioma, and specific miRNA may function as an oncogene or a tumor suppressor in different kinds of cancers [14-16]. These findings suggest the importance of miRNAs in carcinogenesis and cancer progression, and provide another glimmer of hope for glioma therapy.

In this study, we investigated the expression levels of miR-497-5p in glioma tissue and matched normal tissues, and found that miR-497-5p was significantly down-regulated in glioma tissues compared with that in the matched normal tissues. In addition, we also evaluated the functions of miR-497-5p in glioma cells. The results showed that miR-497-5p acted as a tumor suppressor in glioma cells, and restoration miR-497-5p expression inhibited glioma cells proliferation, migration and invasion. Moreover, SOX9 was demonstrated to be a direct target gene of miR-497-5p in glioma. All these findings suggested a novel target for targeted therapy of glioma patients.

## Material and methods

## Human samples and cell lines

This study was approved by the Ethic committee of Hospital. Twenty-five paired human glioma tissues and matched normal tissues were collected at the time of surgical resection. The matched normal tissue was taken from 3 cm away from the tumor. None of these glioma patients had received chemotherapy, radiotherapy before surgery and other treatments. Glioma tissues and matched normal tissues were immediately snap-frozen and stored at liquid nitrogen.

Human glioma cell lines (U87, U251, U373, A172) were obtained from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Normal human glial cell line (HEB), used as a control, was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were maintained in Dulbecco's modified eagle's medium (DMEM) (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Gibco), at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

## miRNA mimics and siRNAs transfection

Mature miR-497-5p mimics, miRNA mimics negative control (NC), SOX9 siRNA and negative control siRNA (NC siRNA) were purchased from GenePharma corporation (Shanghai, China). Transfection of miRNA mimics or siRNAs was performed using HiPerFect Transfection Reagent (Qiagen, Germany), according to the manufacturer's instructions. Forty-eight hours after transfection, the expression levels of miR-497-5p were measured by quantitative real-time reverse transcription-PCR (qRT-PCR).

#### RNA isolation and qRT-PCR

Total RNA was extracted from tissues and cells using Trizol solution (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The concentration and purity of total RNA was evaluated with a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Houston, TX, USA). SYBR PrimeScript miRNA RT-PCR Kit (TaKaRa, Ohtsu, Japan) was used to detect miR-497-5p expression with U6 small nuclear RNA as an internal control. For the detection of SOX9 mRNA expression, cDNA synthesis was performed using a PrimeScript RT Reagent kit (TaKaRa) following to the manufacturer's instructions. Quantitative PCR was conducted using SYBR Green PCR master mix (TaKaRa), with GADPH as an internal control, Each sample was run in triplicate.

## Cell counting kit-8 (CCK8) assay

To investigate the effects of miR-497-5p on proliferation of glioma cells, CCK8 assay (Dojindo, Kumamoto, Japan) was performed. Cells were plated into 96-well plates at a density of 3,000 cells/well. After incubation overnight, cells were trasnfected with miRNA mimics or siRNA. Cell proliferation was determined at 24 h, 48 h, 72 h, and 96 h after transfection. In briefly, 10  $\mu$ l of CCK8 solution was added to each well of the 96-well plates and incubated for 2 h at 37°C. The absorbance was detected at 450 nm using a microplate reader.

## Migration and invasion assays

To determine the effects of miR-497-5p on metastasis of glioma cells, migration and invasion assays were performed using transwell chambers with 8 mm pores (Corning, Cambridge, USA). The transwell membranes were coated with or without Matrigel (BD Biosciences, San Jose, CA) for migration assay and invasion assay, respectively. At 48 h after transfection, 5×10<sup>4</sup> transfected cells in serum-free culture medium were plated into the upper chamber and complete culture medium containing 20% FBS was added into the lower chamber. After incubation at 37°C for 24 h, the chambers were washed with PBS, fixed with 100% methanol, stained with 0.5% crystal violet and cell number was counted in five random areas of each transwell chamber.



**Figure 1.** Depressed expression of miR-497-5p in glioma. A. miR-497-5p expression levels in glioma tissues and matched normal tissues were measured by qRT-PCR. B. The relative expression of miR-497-5p in glioma cell lines and normal human glial cell line. \*P < 0.05.

#### Protein extraction and western blot

Total proteins were extracted from transfected cells using RIPA buffer (SolarBio, Beijing, China), supplemented with protease inhibitor (Sigma-Aldrich, St. Louis, Missouri, USA) and phosphorylated proteinase inhibitor (Sigma-Aldrich). Total proteins were quantified and a total of 40 µg of total proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto a polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, the membranes were blocked in 5% non-fat skimmed milk in phosphate-buffered saline with Tween-20 (PBST), and probed with specific primary antibodies overnight at 4°C. The following primary antibodies were used: mouse anti-human monoclonal SOX9 antibody (sc-166505; Santa Cruz Biotechnology, CA, USA) and antihuman monoclonal GADPH antibody (sc-166574; Santa Cruz Biotechnology, CA, USA). After washing with TBST, corresponding Horseradish peroxidase (HRP)-conjugated secondary antibody and enhanced chemiluminescence regents (Pierce, Rockford, IL, USA) was used to visual the signals on the membranes. The relative integrated density of SOX9 was determined based on the GAPDH protein expression level as a control.

# Bioinformatics analysis and luciferase report assay

The target genes of miR-497-5p were analyzed using miRanda (www.microrna.org) and Targets can (www.targetscan.org).

The pmirGLO-SOX9-3'UTR Wt and pmirGLO-SOX9-3'UTR Mut luciferase report vectors were

obtained from GenePharma. For the luciferase reporter assay, cells were plated into 24-well plates. After incubation overnight, cells were co-transfected with luciferase report vectors and miRNA mimics. Following transfection 48 h, the Firefly luciferase and Renilla luciferase activities were detected using the Dual-Luciferase Reporter Assay System (Promega, Manheim, Germany). The experiment was performed in triplicate.

## Statistical analysis

All values were expressed as mean  $\pm$  S.D. Statistical differences among groups were determined using a Student's t test. P < 0.05 was considered to be statistically significant.

## Results

# Decreased expression of miR-497-5p in glioma tissues and cell lines

To investigate whether miR-497-5p was downregulated in glioma, we firstly measured its levels in glioma tissues and matched normal tissues by using qRT-PCR. As shown in **Figure 1A**, miR-497-5p expression was lower in glioma tissues than in matched normal tissues. We also evaluated miR-497-5p expression levels in 4 glioma cell lines (U87, U373, U251, A172) and normal human glial cell line (HEB). The results showed that miR-497-5p was significantly down-regulated in all 4 glioma cell lines compared with that in HEB (**Figure 1B**). In all the four glioma cell lines, U87 and U373 cells showed lower miR-497-5p expression levels and were selected for the following studies. All



**Figure 2.** Ectopic expression of miR-497-5p inhibited proliferation of glioma cells. A. The expression of miR-497-5p was up-regulated in U87 and U373 cells by qRT-PCR after transfection with miR-497-5p mimic. B. Cell proliferation was measured at posttransfection by CCK-8 assay. \*P < 0.05.



these results indicated that miR-497-5p was frequently down-regulated in glioma.

## Over-expression of miR-497-5p inhibited proliferation of glioma cells

Firstly, we transfected either miR-497-5p mimics or NC into U87 and U373 cells, and measured miR-497-5p expression levels to assess its transfection efficiency. The data showed that miR-497-5p expression was remarkably increased in the U87 and U373 cells after transfection with miR-497-5p mimics (**Figure 2A**).

To explore whether miR-497-5p affected the proliferation of glioma cells, CCK8 assay was performed. As shown in **Figure 2B**, miR-497-5p reduced proliferation of U87 and U373 cells at 72 and 96 h following transfection with miR-497-5p mimics. The results demonstrated that miR-497-5p inhibited the ability to proliferate in glioma cells.

## miR-497-5p decreased the migration and invasion capacities of glioma cells

Cell migration and invasion abilities are two essential processes of metastasis in human

cancers. Therefore, the effects of miR-497-5p on migration and invasion capacities of glioma cells were also evaluated by using transwell migration and invasion assays. As shown in **Figure 3**, restored expression of miR-497-5p decreased migration and invasion abilities of U87 and U373 cells. The results indicated that miR-497-5p inhibited the ability to metastasis in glioma cells.

## SOX9 was a direct target of miR-497-5p

To investigate the molecular mechanisms by which miR-497-5p inhibited growth and metastasis of glioma cells, putative targets of miR-497-5p were retrieved from miRanda and TargetScan. Among these putative targets, SOX9 attracted our attention as it involved in growth and metastasis of glioma [17, 18]. To verify whether SOX9 was a direct target gene of miR-497-5p in glioma, a human SOX9-3'UTR fragment containing the binding sites of miR-497-5p or the mutant sites (Figure 4A) was together with miR-497-5p or NC were co-transfected into U87 and U373 cells. After transfection 48 h, luciferase report assay was performed, and showed that luciferase activities were significantly reduced in U87 and U373



Figure 3. Ectopic expression of miR-497-5p decreased migration and invasion of glioma cells. Cell migration (A) and invasion (B) was determined in glioma cells by migration and invasion assay after transfection with miR-497-5p mimics or NC. \*P < 0.05.

cells transfected with miR-497-5p and pmir-GLO-SOX9-3'UTR Wt, but not in the cells with the miR-497-5p and pmirGLO-SOX9-3'UTR Mut (**Figure 3B**). Subsequent, qRT-PCR and western blot was adopted to explore whether miR-497-5p had regulation effect on SOX9 expression. The results showed that over-expression of miR-497-5p reduced SOX9 expression at mRNA (**Figure 4C**) and protein levels (**Figure 4D**) in U87 and U373 cells. Taken together, these results indicated that miR-497-5p decreased the expression levels of SOX9 by directly binding to its 3'UTR.

Knockdown of SOX9 could simulate the suppressive functions of miR-497-5p in glioma

The above results demonstrated that miR-497-5p was a direct target gene of miR-497-5p in glioma. Hence, we wondered whether knock-



**Figure 4.** SOX9 was a direct target of miR-497-5p. A. The predicted binding sites for miR-497-5p in the 3'UTR of SOX9 and the mutations in the binding sites were shown. B. The miR-497-5p mimics reduced the luciferase activities of pmirGLO-SOX9-3'UTR Wt, while the pmirGLO-SOX9-3'UTR Mut failed to alter the luciferase activities. C. SOX9 mRNA expression levels in U87 and U373 cells were determined by qRT-PCR after transfection with the miR-497-5p mimics or NC. D. SOX9 protein expression levels in U87 and U373 cells were measured by western blot after transfection with the miR-497-5p mimics or NC. \*P < 0.05.

down of SOX9 could simulate the suppressive functions of miR-497-5p in glioma cells. In addressing this issue, SOX9 siRNA or NC siRNA was introduced into U87 cells. At 48 h after transfection, western blot was performed to determine the expression levels of SOX9 in U87 cells. As shown in **Figure 5A**, SOX9 was significantly down-regulated by SOX9 siRNA in U87 cells. Meanwhile, CCK8 assay, migration and invasion assays showed that the inhibitory effects of SOX9 were similar with those induced by miR-497-5p in U87 cells (**Figure 5B, 5C**). These findings indicated that SOX9 contributed to the suppressive functions of miR-497-5p in glioma.

#### Discussion

Gliomas are the most common and incurable primary brain tumors with high morbidity and mortality [19, 20]. Tremendous progress has been developed in glioma diagnosis and treatment. However, the prognosis of patients with glioma remains poor. It is thus necessary to develop alternative targets suitable for novel therapies. Several studies have demonstrated that miRNAs play an important role in diverse biological processes, such as proliferation, apoptosis cellular differentiation, metastasis and carcinogenesis [12, 13]. So any deregulated expression of miRNAs may cause disorder in gene regulating networks and cellular process-



**Figure 5.** Knockdown of SOX9 could simulate the suppressive functions of miR-497-5p in glioma. A. The protein levels of SOX9 in U87 and U373 cells were detected by western blot after transfection with the SOX9 siRNA or NC siRNA. B. Cell proliferation was analyzed using CCK-8 assay after transfection with SOX9 siRNA or NC siRNA. C. Cell migration and invasion was determined in glioma cells by migration and invasion assay after transfection with SOX9 siRNA or NC siRNA or NC siRNA or NC siRNA. \*P < 0.05.

es, especially in tumorigenesis and metastasis [21, 22]. Therefore, fully understanding the expression, and functions of miRNAs in glioma may provide potential clinical therapeutic targets for patients with glioma. In this study, we found that miR-497-5p was down-regulated in glioma tissues and cell lines. Enforced miR-497-5p expression significantly inhibited proliferation, migration and invasion of glioma cells. To the best of our knowledge, the present study was the first to identify a molecular level that miR-497-5p regulated SOX9 expression by targeting its 3'UTR. Taken together, these findings indicated that the low expression of miR497-5p may enhance the carcinogenesis and progression of glioma.

miR-497-5p, a member of miR-15/16/195/ 424/497 family, is located on chromosomes 17p13.1 [23]. In breast cancer, miR-497-5p expression was lower in tumor samples and low miR-497 expression was associated with higher differentiation grade, positive HER-2 expression, higher incidence of lymph node metastasis and advanced clinical stage. Furthermore, breast cancer patients with high miR-497-5p expression had better 5-year disease-free and overall survival compared with the low miR-497-5p group. Univariate and multivariate analyses also demonstrated that low miR-497-5p expression was an independent poor prognostic factor for breast cancer patients [24]. Functional studies revealed that miR-497-5p

acted as a tumor suppressor in glioma, and contributed to proliferation, invasion, apoptosis, angiogenesis and epithelial mesenchymal transition [25-29]. Zhao et al. reported that miR-497-5p expression levels were reduced in non-small-cell lung cancer tissues, and ectopic of miR-497-5p expression inhibited cell proliferation, angiogenesis and colony formation [30]. miR-497-5p was also validated as a tumor suppressor in various kinds of human cancers, including pancreatic cancer [31], prostate cancer [32], endometrial cancer [33], renal cancer[34], nasopharyngeal carcinoma [35], hepatocellular carcinoma [36], colorectal cancer [23], and gastric cancer [37].

miRNAs exert biological functions through regulating target gene expression by mRNA cleavage or translational repression in a variety of model systems [38]. In this study, miR-497-5p, a new regulator of glioma, was found to inhibit proliferation, migration and invasion of glioma cells. Previous studies reported that miR-497-5p could regulate oncogenic transcripts in human cells, such as HIF-1a [28], Slug [27], VEGF [39], KSR1 [40], ANLN [41], HSPA4L [41], BCL-2 [29], YAP1 [42], and so on. In this study, a molecular link between miR-497-5p and SOX9 was validated. To explore the mechanism by which miR-497-5p exerts its function, bioinformatics analysis was used to search for the putative targets of miR-497-5p. The analysis showed that SOX9 was a potential target gene of miR-497-5p. Luciferase report assay demonstrated that miR-497-5p directly targeted the 3'UTR of SOX9. gRT-PCR and western blot showed that restoration miR-497-5p expression decreased SOX9 expression at both mRNA and protein levels. In addition, knockdown of SOX9 could simulate the suppressive functions of miR-497-5p in glioma. These findings suggested that miR-497-5p/SOX9 axis based targeted therapy could be a novel therapeutic target for glioma.

SOX9, a member of the SOX family of transcription factors, is a key regulator of developmental biological processes, including male sex determination, chondrogenesis, neurogenesis, and neural crest development [43, 44]. Recent studies also demonstrated that SOX9 is involved in carcinogenesis and progression in a variety of human cancers [45]. SOX9 has been found up-regulated in many human cancers, such as lung cancer [46], prostate cancer [47], breast cancer [48], colorectal cancer [49]. In human glioma, SOX9 expression levels were also higher in tumor tissues, and a high level of SOX9 expression was significantly correlated with advanced WHO grad and low Karnofsky performance score. In addition, the diseasefree and overall survival rates of patients with high SOX9 expression were obviously lower than those of patients with low SOX9 expression. Furthermore, multivariate analysis showed that high SOX9 expression was an independent prognostic factor for glioma patient [50]. SOX9 was also reported to involve in cell growth, cell cycle, apoptosis and metastasis of glioma cells [17, 18, 50]. Hence, SOX9 acts as an oncogene in glioma, and contributed to glioma carcinogenesis and progression.

In conclusion, our study showed that miR-497-5p was significantly down-regulated in glioma tissues and cell lines, and restoration of miR-497-5p expression inhibited proliferation, migration and invasion of glioma cells through directly binding to the 3'UTR of SOX9. Our findings provided new insight into the molecular mechanism of glioma carcinogenesis and progression, and identified miR-497-5p/SOX9 axis provided a potential therapeutic target to treatment of glioma.

# Disclosure of conflict of interest

## None.

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