microRNA-101 inhibits proliferation, migration and invasion of human glioblastoma by targeting SOX9

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common primary malignant tumors originating in the brain parenchyma. At present, GBM patients have a poor prognosis despite the continuous progress in therapeutic technologies including surgery, radiotherapy, photodynamic therapy, and chemotherapy. Recent studies revealed that miR-101 was remarkably down-regulated in kinds of human cancers and was associated with aggressive tumor cell proliferation and stem cell self-renewal. Data also showed that miR-101 was down-regulated in primary glioma samples and cell lines, but the underlying molecular mechanism of the deregulation of miR-101 in glioma remained largely unknown. In this study, we found that miR-101 could inhibit the proliferation and invasion of glioma cells both in vitro and in vivo by directly targeting SOX9 [sex-determining region Y (SRY)-box9 protein]. Silencing of SOX9 exerted similar effects with miR-101 overexpression on glioma cells proliferation and invasion. Quantitative reverse transcription PCR and Western blotting analysis revealed a negative relationship between miR-101 and SOX9 in human glioma U251MG and U87MG cells, and the luciferase assay indicated that miR-101 altered SOX9 expression by directly targeting on 3’UTR. Taken together, our findings suggest that miR-101 regulates glioma proliferation, migration and invasion via directly down-regulating SOX9 both in vitro and in vivo, and miR-101 may be a potential therapeutic target for future glioma treatment.

INTRODUCTION

Glioma is the most frequent and malignant brain tumor, which usually originates from neural mesenchymal cells and can be classified into astrocytoma, glioblastoma, medulloblastoma, ependymoma, and oligodendroglioma [1]. Glioma is also divided into four grades according to the WHO classification system, each grade includes varieties of pathological subtypes [2]. Nowadays, the prognosis of traditional treatments such as surgery and radiation therapy is poor [3–7], resulting in low cure rate and short lifetime. The 5-year survival rate of glioblastoma is less than 5%, and the average survival period is only 14 months [8].

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Glioblastoma is the highest grade of glioma. It shows low sensitivity to radiation therapy and chemotherapy, with poor prognosis and high recurrence rate [9–11]. At present, the international standard treatment of Glioblastoma is temozolomide (TMZ) synchronous chemotherapy after surgical resection, following by cycle specific chemotherapy by TMZ [12, 13]. However, because of the strong radiotherapy and chemotherapy resistance, only 30% patients’ median survival period could reach 2 years, among whom only 9.8% could reach 5 years [14]. Theoretically the microRNA (miRNA) exhibits biological function of glioma occurrence inhibition at the molecular level and improve the survival rate. Studies have also shown that the knockout of miR-21 could inhibit glioblastoma (GBM) proliferation and induce glioma cells apoptosis [15, 16]. These findings provide a potential new strategy for clinical treatment of glioma.

MiRNA is a small endogenous single-stranded RNA (19-25nt) [17] without open reading frame. As a non-coding nucleotide, it plays a vital role in the process of many important life activities and diseases [18]. MiRNA regulates the expression of target genes in transcription level through partial-complementary with its 3′-untranslated region [19]. MiRNA participates in individual development, cell proliferation, differentiation and apoptosis, and other important life activities. All those activities mentioned above have great significance in the occurrence and development of glioma [20]. For example, miR-21, miR-7, miR-128 and miR-221/22 are involved in the progression of gliomas [21, 22]. Several researches have shown that miR-101 was remarkably downregulated in samples from patients and cell lines of human cancers such as lung cancer [28], breast cancer [29], laryngeal squamous cell carcinoma [30], embryonal rhabdomyosarcoma [31] and glioblastoma [32]. In this study, we performed functionally investigation of miR-101 in GBM. Our data suggested that miR-101 regulated glioma cells proliferation and invasion both in vitro and in vivo by directly targeted SOX9. Simultaneously, SOX9 was proved to be essential for glioma progression. These findings make miR-101 as a new target for glioma therapy and verify the importance of SOX9 in glioma tumorigenesis.

RESULTS

Overexpression of miR-101 inhibits glioma cell invasion, migration, and proliferation in vitro

In order to test the expression level of miR-101 in glioma, we collected 20 clinical specimens, including 10 grade IV cases, 10 grade III cases, 10 grade II cases, 10 grade I cases and 4 cases of brain injury brain tissues. Our data suggested that miR-101 level in glioma tissue is much lower compared to normal brain tissue (Figure 1A), and the expression level of miR-101 in U87MG, U251MG, A172 and T98 glioma cells are lower than that of HEB cells (Figure 1B). To test miR-101 function in glioma cell lines, U87MG and U251MG were infected with a lentivirus encoding the mature sequence of miR-101. The infected cells expressed high level of miR-101 both in U87MG and U251MG glioma cells (Figure 1C). Wound-healing assay was performed to detect the effect of miR-101 on cell migration, and the miR-101-overexpressing cells showed considerably slower migration compared with the miR-control cells either in U87MG and U251MG glioma cells lines (Figure 1D and 1E). Furthermore, Transwell migration assays illustrated that overexpression of miR-101 significantly suppressed glioma cell migration and invasion (Figure 1F-I). The invasion ability of miR-101-U87MG cells was only approximately one-eighth of miR-control-U87MG cells, and the migration ability was reduced to two-fifth by miR-101-U87MG compared with that of miR-control cells (Figure 1F and 1I). The invasion ability of miR-101-U251MG cell was only approximately one-ninth of miR-control-U251MG cells, and the migration ability was reduced to two-fifth by miR-101-U251MG compared with that of miR-control cells (Figure 1H and 1I). MTT assays were taken to examine the effect of miR-101 on cell proliferation. As shown in Figure 1J and 1K, the cell proliferation rate was decreased approximately one-ninth by miR-101-U251MG compared with that of miR-control cells (Figure 1J and 1K). The miR-101-U87MG cells and their respective control cells were implanted into the left and right flanks (3.0×10^6 cells per flank) of nude mice by subcutaneous injection, respectively. At 30 days post-injection, data showed that the mean volumes of xenograft tumors of miR-101-U87 cells were significantly smaller than that of miR-control-U87 cells (n = 5 animals per group, P = 2.89×10^{-2}; Figure 2A, 2B and 2C). Immunohistochemical staining results showed that the number of Ki67 positive cells in miR-101-U87MG tumors was less than that in miR-control-U87MG tumors (Figure 2D and Supplementary Figure 1). Thus, miR-101 overexpression significantly inhibited the glioma proliferation both in vitro and in vivo.

MiR-101 directly targets SOX9 in GBM

Bioinformatics methods were adopted to predict the potential targets of miR-101 in human GBM. The TargetScan Program suggested that the 3′UTR region
of the SOX9 gene containing the binding sites of miR-
101 (Figure 3A), and the expression level of SOX9 in
glioma (II-III) tissue was higher than that of the normal
brains tissue (Figure 3B and Supplementary Figure 5).
Furthermore, qRT-PCR analysis showed that SOX9
was obviously down-regulated in miR-101-U87 tumor
compared with the miR-NC-U87 tumor in tumor xenograft
model (Supplementary Figure 3), indicating that SOX9
might be a potential target gene of miR-101. In order to
test the regulating manner between miR-101 and SOX9,
we used qRT-PCR and Western blotting to compare the
expression level of SOX9 in the two glioma cell lines
transfected with miR-101 or miR-control as shown in
Figure 3C. Both mRNA level and protein level of SOX9
was obviously decreased upon miR-101 overexpression
(Figure 3E and 3D). Then we constructed a luciferase
reporter plasmid containing the 3’UTR of SOX9. We
found that the luciferase activity in the Luc-SOX9-UTR-
transfected cells was prominent decreased compared with
the luciferase activity in the miR-101 target site mutant
SOX9 3’UTR and negative control cells (Figure 3C). All
these results suggested that SOX9 was a direct target of
miR-101 in glioma. We further used immunofluorescence
to compare the SOX9 expression between miR-control
U87MG and miR-101-U87MG cells (Supplementary
Figure 2). The results showed that overexpression of miR-
101 only reduced the SOX9 expression level but did not
change the SOX9 expression pattern (Figure 3E).

**The tumor suppressing function of SOX9 in vitro and in vivo**

To acknowledge the targeting relationship between
SOX9 and miR-101 in GBM, we also studied the SOX9
function in glioma. SOX9 was silenced in U87MG and
U251MG by sh-SOX9-1 and sh-SOX9-2. The result
showed that sh-hSOX9-1 showed a better knockdown
efficiency (Figure 4A and 4B). MTT assay, wound healing
assays and trans-well assay were used to test the effect of
SOX9 on glioma cells. U87MG and U251MG glioma cell
lines were infected with a lentivirus encoding the mature
sequence of sh-hSOX9-1. First, MTT assay was used to
test the effect of SOX9 on U251 MG and U87MG
cells proliferation. As shown in Figure 4C and 4D, the cell
proliferation rate was significantly decreased in the miR-
101 group compared with that of control group at 24 to

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**Figure 1: Overexpression of miR-101 inhibits glioma cell invasion, migration, and proliferation in vitro.**

A. miR-101 expression levels in normal brain tissue and glioma tumor tissues were detected by qRT-PCR analysis. B. The expression level of miR-101 in U87MG, U251MG, A172 and T98 glioma cells were detected by qRT-PCR analysis. C. The efficiency of lentivirus in U87MG and U251MG glioma cell lines were detected by qRT-PCR analysis. D, and E. Representative images of wound healing assay detecting cell migration in miR-101-U87MG, NC-U87MG, miR-101-U251MG and NC-U251MG cells. F. Cell invasion and migration of miR-101-U87MG and NC-U87MG cells were detected by trans-well assay. G. Quantitative analysis of the cell number of U87MG cell trans-well invasion and migration assay. H. Cell invasion and migration of miR-101-U251MG and NC-U251MG cells were detected by trans-well assay. I. Quantitative analysis of the cell number of U251MG cell trans-well invasion and migration assay. J. and K. Growth curves of miR-101-U251MG and NC-U251MG, miR-101-U87MG and NC-U87MG cell lines.
Figure 2: Overexpression of miR-101 suppresses the tumor growth in vivo. A. and B. Determination of the tumor growth, tumor volume was calculated every five days after injection (n = 5). ***p < 0.001. C. Representative image for tumor growth is shown. Nude mice were subcutaneously injected with 3.0×10^6 cells per flank miR-101 or miR-NC stable transfected U87MG cells. D. Immunohistochemistry assay detected the level of Ki67 in overexpression-miR-101 and miR-NC xenograft tumor tissues, 200 ×. Scale bar = 100 μm.

Figure 3: SOX9 is a direct target of miR-101. A. Predicted miR-101 target sequences in 3'UTR of SOX9 and mutant containing eight mutated nucleotides in 3'UTR of SOX9 (SOX9-mut). B. Immunohistochemistry assay detected the level of SOX9 in glioma (II-III) tissue and the normal brains tissue. C. U87MG and U251MG cells were co-transfected with miR-101 and luciferase reporters containing either the predicted miRNA target site in SOX9 3'UTR or its corresponding mutant form, the values obtained from the Has-miR-101 vector and PGL3 were set as 100%. D. Western blot analysis for the SOX9 expression in U87MG and U251MG cells. E. qRT-PCR analysis of SOX9 expression in U87MG and U251MG cells transfected with miR-101 or negative control.
96 hours after transfection (*P < 0.05). Subsequently, trans-well assay illustrated that the invasion ability and migration ability was suppressed prominently by SOX9 silencing both in U87MG and U251MG cells (Figure 4E-4H). Wound-healing assay was used to detect the effect of SOX9 on cell migration (Figure 4I and 4J), and the SOX9-KD-U251MG and SOX9-KD-U87MG exhibited slower migration compared to the SOX9-control cells, respectively. Furthermore, SOX9 silencing also markedly inhibited mice xenograft tumor growth (Supplementary Figure 4), indicating that SOX9 was essential for glioma cell proliferation both in vitro and in vivo.

DISCUSSION

Glioma is one of the most common primary malignant tumors in the brain parenchyma. Malignant glioma has poor prognosis despite the continuous progress in therapeutic technologies, including surgery, radiotherapy, photodynamic therapy, and chemotherapy. The high incidence and mortality of glioma prompt us to search for new therapeutic strategies. The miRNAs are kind of endogenous non - coding RNA, they can negatively regulate the target genes expression on post - transcriptional level through binding to the 3'UTR of their mRNA [33]. The deregulation of miRNAs had been found in many human cancers, such as ovarian carcinoma [34], lung cancer [35,36], liver cancer [37], colon cancer [38, 39] and GBM [40]. MiRNA deregulation has become a new characteristic of malignant tumor, so some specific miRNAs are potentially novel biomarkers of cancer diagnosis and prognosis [41–45].

MiR-101 has been reported to be down-regulated in several human cancers. Study indicated that miR-101 could repress lung cancer invasion and proliferation by inhibiting interaction of fibroblasts and cancer cells by directly targeting CXCL12 [27]. MiR-101 exercises its biological function in multiple cancer types by interating with CXCR7 [29], CDK8 [30], EZH2 level [31, 47] and CPEB1 [32]. In GBM, research showed that miR-101...
could act as a tumor suppressor by targeting Kruppel-like Factor 6 in glioblastoma stem cells [46]. Furthermore, miR-101 could reverse the hypomethylation of the LMO3 promoter in glioma cells [48]. In a word, miR-101 was an important regulator in different cancers including malignant glioma.

It is noteworthy that a study illustrated that miR-101 directly targets SOX9 in human hepatocellular carcinoma (HCC), and miR-101 could suppress SOX9-dependent tumorigenicity and promotes favorable prognosis of HCC [49]. While, SOX9 is a high mobility group box transcription factor which plays critical roles during embryogenesis, differentiation, tumor initiation, invasion and stem cell self-renewal [50, 51]. These studies prompt that SOX9 might involves in miR-101 tumor suppressing process. Therefore, we investigated the relationship between SOX9 and miR-101 in glioma in this study. Our data confirmed that miR-101 could inhibit proliferation, migration and invasion of human glioblastoma by directly targeting SOX9. Results also showed that SOX9 was essential for glioma tumorigenesis both \textit{in vitro} and \textit{in vivo}. SOX9 is reported to be regulated by EGFR [52], Notch [53], SHH [54], and in turn regulate Akt [55], Wnt [56], BMI1 [57] pathways. So we conclude that miR-101 and SOX9 regulation axis regulates proliferation, migration and invasion of human GBM by regulating Akt, Wnt, BMI1 signal pathway (Figure 5). This study suggests us that miR-101 and SOX9 are key regulators in human glioblastoma and provides new therapeutic targets for glioma therapy.

**MATERIALS AND METHODS**

**Cell cultures and human tissue Samples**

Human glioma cell lines U251MG and U87MG were purchased from the Chinese Academy of Sciences Cell Bank in 2012, A172, T98 and HEB Normal glial cell lines were purchased from Beijing ChuangLian biotechnology company (Beijing, China). The authenticity of cancer cell lines was tested by short tandem repeat profiling. All cancer cells were maintained in high glucose DMEM (Invitrogen, Grand Island, NY, USA) supplemented with 10% FBS (GIBCO, USA), 100 units/mL penicillin (NCPC, Shijiazhuang, China) and 100 μg/mL streptomycin (NCPC, Shijiazhuang, China), and incubated in 5% CO\textsubscript{2} at 37°C. Specimens of human glioma tissues were collected from 25 patients treated in Tangdu Hospital of Fourth Military Medical University, P. R. China. The normal brain tissue specimens were taken from...
isolated from frozen samples and cells using Trizol reagent (Invitrogen, CA, USA) under the guidance of manufacturer’s protocol. RNA was treated with RNase-free DNase I (Roche, Switzerland). Then, BcaBest RNA PCR kit (TaKaRa, Dalian, China) was used to synthesize the cDNA according to the manufacturer’s protocol. All primers were synthesized by Shanghai Sangon Technology co., LTD. Quantitative RT-PCR was carried out by the q5 Multicolor Real-Time PCR Detection System (Bio-Rad) with Real-time PCR Master Mix (SYBR Green).

**Vector construction and transfection**

To construct the miR-101 overexpression vector, the primers of miR-101 were synthesized by Shanghai Generay Biotech Co., Ltd. Primer sets (5’-3’) used for amplifying the miR-101 were as follows, (Forward primer: CCTGAAATTCTCTTGAATTTAATTCAGAAG; Reverse primer: TATGGATCTCCAGCACAACATGGGCTGCA), containing EcoR I and BamH I respectively. Amplified miR-101 was then subcloned into pCDH1 vector between EcoR I and BamH I sites (Promega, Madison, WI, USA). The predicted miR-101 target site GUACUGU was mutated into GAUGACA by site-directed mutagenesis. The SOX9 Wild type primers used were GAATTCTCAGTGCGCCAGGCAAACCTTV-5’ and CATATGGAACTGATCACATAACACAA-3’, mutagenic primers used were ATATTTTTTAGATGAGCATGAGATATGTCATGAAT-5’ and ATGAAATCATACGTGTCATACTAATACCAATTATAT-3’.

**MTT assay**

The MTT assays were performed as described before [23]. In brief, 1 × 10⁵ cells/well was seeded in 96-well plates with 200 μl culture medium. After treatments, the medium was replaced with 200 μL DMEM/FBS containing 0.5 mg/mL MTT and incubated at 37°C for 4 h. The supernatant was then discarded, and the cells were lysed in 200 μL DMSO for 10 min at 37°C. The optical density (OD) values were measured at 490 nm (SpectraMax 190; Molecular Devices Sunnyvale, CA, USA).

**Cell invasion and migration assays**

Cells (2.5 × 10⁵) were suspended in 250 μL serum-free DMEM and seeded in the top chambers of 24-well transwell plates (Corning Inc., Corning, NY, USA) coated with 30 μL Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The bottom chambers of the trans-well plates were filled with 600 μL DMEM containing 10% FBS. Cells were allowed to migrate for 48 hours (invasion assay) at 37°C [24]. After migration, cells in the top chambers were removed using a cotton swab, and the cells which migrated to the bottom chambers were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich) and stained with Hoechst staining. The fixed and stained cells were counted in five independent fields under a light microscope. At least three chambers were counted for each experiment. For the migration assay, a similar protocol was followed except for the replacement of the top chamber of the trans-well plate with an uncoated chamber. The culture medium in the bottom chamber was replaced with DMEM containing 2.5% FBS, and cells were allowed to migrate for 8 hours.

**Wound healing assays**

Cells were seeded in 6-well plates and cultured until they reached confluence, then a wound was created by manually scraping the cell monolayer with a sterile 200 μL pipette tip. Cells were washed twice with PBS to remove the floating cells, and then incubated in DMEM supplemented with 1% FBS. Cell migration was observed at three preselected time points (0, 12, and 24 hours). Images were acquired with a Nikon DS-5M Camera System mounted on a phase-contrast Leitz microscope.
**Immunohistochemistry assay**

For immunohistochemistry (IHC), 8 μm sections of formalin-fixed and paraffin embedded brain tissues were first de-waxed and rehydrated before antigen retrieval. The SOX9-antibody (1:100 dilution; Abcam, Cambridge, USA) and Ki67-antibody (1:100; Roche, Basel, Switzerland) were used for this study. After incubation with the primary antibodies, the cells were rinsed and incubated for one hour with Biotin-labeled secondary antibodies at room temperature (Molecular Probes 1:800). Nuclei were stained by Hematoxylin. Stained sections were examined under a light microscope and the positive cells in five high power fields (1×200) were counted for statistic study.

**Western blotting**

The total cell lysates were dissolved in high KCl lysis buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 300 mM KCl, 0.5% Triton X-100, 1 mM EDTA, and 0.5% sodium deoxycholate) with complete protease inhibitor cocktail (Roche). The protein concentrations were determined by a protein assay kit (Bio-Rad, Hercules, CA). The Western blot assay has been previously described [25]. SOX9-antibody was used as primary antibodies. Immunoreactivity was visualized by enhanced chemiluminescence amplification according to the manufacturer’s protocol (GE healthcare, Buckinghamshire, UK).

**Luciferase assay**

The Hsa-miR-101 vector (GenePharma Co.) and PGL3, PGL3-SOX9 3’-UTR, PGL3-SOX9 3’-UTR-mut were cotransfected into HEK293T cells. Twenty four hours after transfection, cell lysates were harvested. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA).

**Animal experiments**

All animal experiments were approved by the Research Ethics Committee of Tangdu Hospital of Fourth Military Medical University, P. R. China. Athymic/nude immunocompromised mice were purchased from Fourth Military Medical University (Shanxi, China) and breeding colonies were maintained in our animal facility under standard conditions. Xenografted transplantation of glioma cells into athymic/nude immunocompromised mice was performed as previously described [26]. There are miR-NC group and miR-101 group for the U87MG cell lines. After pre-transplant preparation of the recipient mice and anesthesia with 10% chloral hydrate, isolated miR-101-U87MG cells (3.0×10⁶ in 5 mL PBS) and their respective control cells were implanted into the left and right flanks (cells per flank) of nude mice by subcutaneous injection to establish the xenograft model. The weight change of each animal was measured daily. Tumor volumes were determined by measuring the length (a) and the width (b). The tumor volume (V) was calculated according to the formula $V = \frac{ab^2}{2} [27]$.

**Statistical analysis**

Independent samples were analyzed by using one-sided unpaired Student’s t tests with SPSS17.0 for windows (SPSS Inc., Chicago, IL, USA). All statistical results from the quantitative analysis of the in vitro experiments are presented as means ± SEM, as specified in the figure legends. $p$ values < 0.05 were considered statistically significant.

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**CONFLICTS OF INTEREST**

There is no conflicts of interest.

**Author contributions**

YYT, YSZ, WLJ, WHW, HWY conceived and designed most experiments. WLJ, YDC, XW and YYT developed the methodology. NL, LZ and ZW performed most experiments. PXZ and HL did the histological analysis of mice. NL, LZ and ZW did the analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis). NL and ZW wrote, reviewed, and revised the manuscript. YYT supported study supervision.

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