

Over-expression of miR-187 inhibited cell proliferation and metastasis of glioma *via* down-regulating SMAD1

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) have been identified to participate in the tumorigenesis and progression of glioma. However, the expression and function of miR-187 have not been fully elucidated in glioma so far. Therefore, the aim of this study was to investigate the role of miR-187 in glioma and to explore the possible underlying mechanism.

PATIENTS AND METHODS: The expression levels of miR-187 in 67 glioma tissues and 21 normal brain tissues, as well as 4 glioma-derived cell lines were measured using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). MiR-187 was overexpressed or inhibited in U251 or U87MG cells using miR-187 mimics or inhibitor transfection, respectively. Colony formation assay and Cell Counting Kit-8 (CCK-8) assay were employed to detect the proliferation ability of cells. Meanwhile, transwell assay and wound-healing assay were applied to evaluate the invasion and migration capacities of cells. Furthermore, Dual-Luciferase assay and Western blot analysis were used to verify the downstream target gene of miR-187 in glioma.

RESULTS: MiR-187 expression was significantly lower in glioma tissues and cells when compared with normal brain tissues and cell lines. Up-regulation of miR-187 markedly reduced the proliferation, migration and invasion of U251 cells compared with the negative control group. However, down-regulation of miR-187 remarkably accelerated U87MG cell growth and metastasis compared with inhibitor negative control group. Furthermore, SMAD1 was identified as a direct target for miR-187 in glioma, which could be repressed by miR-187. In addition, over-expression of SMAD1 restored the influence of miR-187 mimics in glioma cells.

CONCLUSIONS: MiR-187 was lowly expressed in glioma tissues and cell lines. Acting as a tumor suppressor, miR-187 inhibited cell growth, invasion, and migration in glioma *via* repressing SMAD1 expression. Our findings might provide a novel insight into the biological diagnosis and treatment in glioma.

Key Words:

MiR-187, Proliferation, Metastasis, Glioma, SMAD1.

Introduction

Glioma accounts for about 80% of primary malignant tumors of the central nervous system. The vast majority of gliomas are anaplastic gliomas and glioblastomas¹. Currently, surgical resection and radiotherapy combined with temozolomide (TMZ) adjuvant chemotherapy are the standard treatment strategy for glioma. However, due to its low differentiation, rapid proliferation, invasiveness and invasive growth, glioma cannot be completely removed by current surgical methods. Furthermore, tumor tissues produce resistance to radiotherapy and chemotherapy, thereby increasing the recurrence rate of gliomas. It is reported that the median survival of patients with anaplastic glioma is 2 to 5 years. However, the median survival of patients with glioblastoma is only 12 to 15 months^{2,3}. Therefore, an in-depth study of the pathological mechanism of gliomas and malignant tumors from the perspective of molecular biology is of great significance to provide clinical diagnostic markers and specific therapeutic targets for glioma⁴.

MicroRNAs (miRNAs) are a class of non-coding small RNAs (ncRNAs) that can regulate the expression of genes by targeting the corresponding messenger RNAs (mRNAs)^{5,6}. Numerous experiments have found that miRNAs are involved in almost all biological processes in tumors, including proliferation, apoptosis, metastasis, angiogenesis, and immune responses. Meanwhile, they play vital roles in promoting or suppressing malignant tumors by inhibiting the expression of

specific molecules in signaling networks⁷. Using genome-wide detection methods, researchers⁸ have found that miRNAs are abnormally expressed in a variety of human tumor tissues. Due to high sensitivity and specificity, miRNAs are likely to become novel markers for tumor diagnosis, treatment and prognosis prediction. In recent years, there have been more and more studies on glioma-associated miRNAs. For example, miR-181b-5p regulates chemosensitivity of glioma cells to temozolomide by targeting Bcl-2. MiR-16-5p is lowly expressed in astrocytic gliomas, which inhibits cell proliferation, increases cell apoptosis and induces cell response to cytotoxic medicine. Meanwhile, miR-6807-3p accelerates the development of glioma *via* inhibiting its downstream DACH1. In addition, down-regulation of miR-204 promotes glioma stem cell-like phenotype and migration. Loss of miRNA-637 promotes cell proliferation, invasion, and migration through direct targeting Akt1⁹⁻¹³. However, the expression and function of miR-187 in glioma have not been fully elucidated.

In our study, we first collected 67 glioma tissues and 21 normal brain tissues and detected the relative expression of miR-187 in tissues. The expression of miR-187 in glioma cells was detected as well. Next, we interfered and overexpressed miR-187 expression in U87MG and U251 cells, respectively. Changes in the proliferation and metastasis abilities of glioma cells were evaluated using functional experiments. The underlying mechanism of miR-187 in glioma was further explored. In this work, we investigated the exact function of miR-187 in glioma, which might provide a new target for glioma diagnosis and treatment.

Patients and Methods

Clinical Glioma Samples

67 glioma specimens were collected from patients in The First Affiliated Hospital of Xinjiang Medical University from September 2013 to October 2015. Meanwhile, 21 normal brain tissues were removed and decompressed during the same period as the control group. The collected specimen was temporarily stored in liquid nitrogen, followed by storage in a refrigerator at -80°C until use. This study was approved by the Ethics Committee of The First Affiliated Hospital of Xinjiang Medical University. Informed consents were obtained from all participants before the study.

Cell Culture

Four different glioma cell lines (U87MG, U373, SW1783 and U251) and one human glial cell line (HBE) was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and maintained in an incubator with 5% CO₂ at 37°C. After adherent growth, the cells were sub-cultured and selected for subsequent experiments.

Cell Transfection

MiR-187 mimics (Mimics) and negative control (NC), as well as miR-187 inhibitor (Inhibitor) and miR-187 inhibitor negative control (Inhibitor NC) were synthesized by GeneWiz Technology Co., Ltd. (Suzhou, China). When cell density reached 40%-50%, miR-187 mimics, Inhibitor, NC, and Inhibitor NC were transfected into cells according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), respectively. The pcDNA for SAMD1 was bought from GenepWiz (Suzhou, China) and transfected into U251 cells using Lipofectamine 3000. Transfection efficiency was confirmed by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RNA Isolation and Real Time-Quantitative Polymerase Chain Reaction

Total RNA in tissues and cells was extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using Fast Quant RT Kit (TaKaRa, Tokyo, Japan). Quantitative Real Time-Polymerase Chain Reaction (QRT-PCR) analysis was performed using SYBR Green (TaKaRa, Tokyo, Japan) with cDNA as a template. The expression level of miR-187 was calculated by the $2^{-\Delta\Delta C_t}$ method. U6 was used as an internal reference. Primer sequences used in this study were as follows: miR-187, F: 5'-GCAGGAACATCTCCGGCTC-3', R: 5'-GCTAGGAGCTGTCCTTTAGGA-3'; SMAD1, F: 5'-CGATTTGTCCACATCACGACTG-3', R: 5'-GATTGCCCGTCGTGAGTCAAG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCTGTTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Cell Counting Kit-8 (CCK-8) Assay

MiR-187 mimics or inhibitor transfected U251 or U87MG cells and control cells were first harvested and adjusted to a concentration of 3×10^5 /mL. Then, the cells were seeded into 96-well-plates at 3000/well, followed by culture for 0, 24, 48 and 72 h, respectively. Briefly, 10 μ L of Cell Counting Kit-8 solution (CCK-8; Dojindo, Kumamoto, Japan) was added in each well and incubated in the dark for 2 h. The absorbance of each well at the wavelength of 470 nm was detected by a microplate reader. This experiment was repeated at least 3 times.

Colony Formation Assay

After transfected U251 or U87MG cells were treated into single cell suspension, the cells were seeded into 6-well plates with 500 cells per well. After culturing in complete medium containing 10% FBS for 3 weeks, formed colonies were fixed with methanol and stained with crystal violet. Finally, the number of colonies containing more than 40 cells was counted and recorded.

Wound Healing Assay

Transfected U251 or U87MG cells were cultured in 6-well plates until the cells covered the entire plate. Three spikes were drawn vertically on the surface of the plate using a 200 μ L tip. Subsequently, the cells were maintained for 48 h in a serum-free medium. The healing condition of wounds was photographed under a microscope. Healing rate was calculated based on five randomly selected fields of view.

Transwell Assay

Transwell assay was employed to detect the migration and invasion abilities of U251 or U87MG cells. 8- μ m transwell insert (Millipore, Billerica, MA, USA) and Matrigel gel (BD Biosciences, Franklin Lakes, NJ, USA) were purchased and prepared. For migration assay, a total of 5×10^5 /mL cells suspended in 200 μ L FBS-free DMEM medium was inoculated into the upper chamber. Meanwhile, 800 μ L of DMEM medium containing 15% FBS were added into the lower chamber. After 36 h of incubation, the insert was harvested and washed 3 times with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA). The cells were fixed with methanol and stained with crystal violet. Using cotton swabs, upper chamber cells were cleaned. Migrating cells were observed under an inverted microscope, and the number of

cells was counted. 6 fields of view were randomly selected for each sample.

For invasion assay, Matrigel gel was first added to the upper chamber of 8- μ m transwell inserts. The other steps were the same as the migration assay.

Luciferase Reporter Gene Assay

A total of 1×10^6 U251 cells were first uniformly placed in 6-well plates. Then, cells were transfected with negative control, miRNA-187 mimics, or co-transfected with miRNA-87 mimics and SMAD1 3'-untranslated region (3'-UTR) wild-type/mutant plasmids, respectively. After 48 h of culture, the cells were collected. Luciferase activity was detected according to the manufacturer's instructions of the Dual-Luciferase[®] Reporter Assay System (Promega, Madison, WI, USA).

Protein Extraction and Western Blot

Transfected U251 or U87MG cells were isolated using radioimmunoprecipitation assay reagent (RIPA; Beyotime, Shanghai, China) on ice after washing twice with pre-cooled PBS. The concentration of extracted protein was measured by the bicinchoninic acid Kit (BCA; Beyotime, Shanghai, China). A total of 20 μ L protein sample was separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% bovine serum albumin (BSA) solution, the membranes were washed with PBS solution 3 times and incubated with primary antibodies of SAMD1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000, Abcam, Cambridge, MA, USA) overnight at 4°C. On the next day, the membranes were incubated with corresponding secondary antibody (Beyotime, Shanghai, China) for 2 h. Electrochemiluminescence Kit (ECL; Millipore, Billerica, MA, USA) was applied to detect the relative protein expression of SMAD1. GAPDH was used as an internal reference. This experiment was repeated three times.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Measurement data were shown as mean \pm SD (standard deviation). Independent *t*-test was used to compare the difference between the two groups. $p < 0.05$ was considered statistically significant.

Results

MiR-187 Was Lowly Expressed in Glioma Tissues and Cell Lines

In this study, we first collected 67 glioma tissues and 21 normal brain tissues. The expression level of miR-187 in tissues was detected using qRT-PCR. As shown in Figure 1A, miR-187 expression was markedly lower in glioma tissues than that of normal brain tissues. The expression of miR-187 in glioma cell lines decreased significantly compared with human glial cell line HBE (Figure 1B). These results indicated that miR-187 served as a potential tumor suppressor in glioma.

To further investigate the function of miR-187, we up-regulated miR-187 level using miR-187 mimics transfection in U251 cells, whereas down-regulated miR-187 level using miR-187 inhibitor in U87MG cells. The expression level of

miR-187 in transfected U251 (Mimics) cells increased by about 4.88 fold than the negative control (NC) group. However, miR-187 expression in transfected U87MG (Inhibitor) cells decreased by 70.5% than the inhibitor control group (INC; Figure 1C, 1D).

MiR-187 Inhibited the Proliferation of Glioma Cells

To study the effect of miR-187 on cell growth, colony formation and CCK-8 assays were performed. U251 cells transfected with miR-187 mimics formed significant fewer colonies than the NC group, while U87MG cells transfected with miR-187 inhibitor formed markedly more colonies than the INC group. The results indicated miR-187 inhibited colony formation ability of glioma cells (Figure 2A, 2B). Similarly, CCK-8 assay showed that over-expression of miR-187

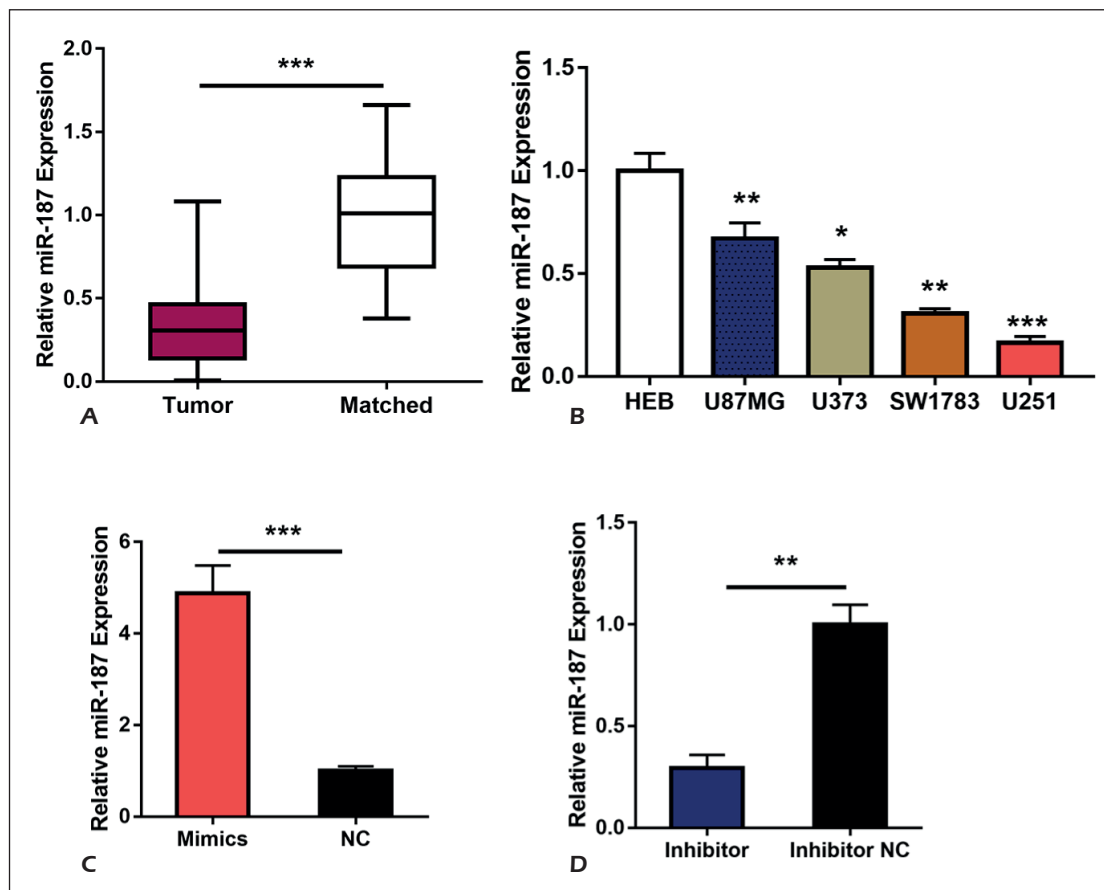


Figure 1. MiR-187 was lowly expressed in glioma tissues and cell lines. **A**, Analysis of the expression level of miR-187 in 67 glioma tissues and 21 normal brain tissues. **B**, Analysis of miR-187 expression level in glioma cell lines (U251, U373, SW1783, U87MG) and human normal brain cell line (HEB). **C**, Expression of miR-187 in U251 cells transfected with miR-187 mimics. **D**, Expression of miR-187 in U87MG cells transfected with miR-187 inhibitor. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

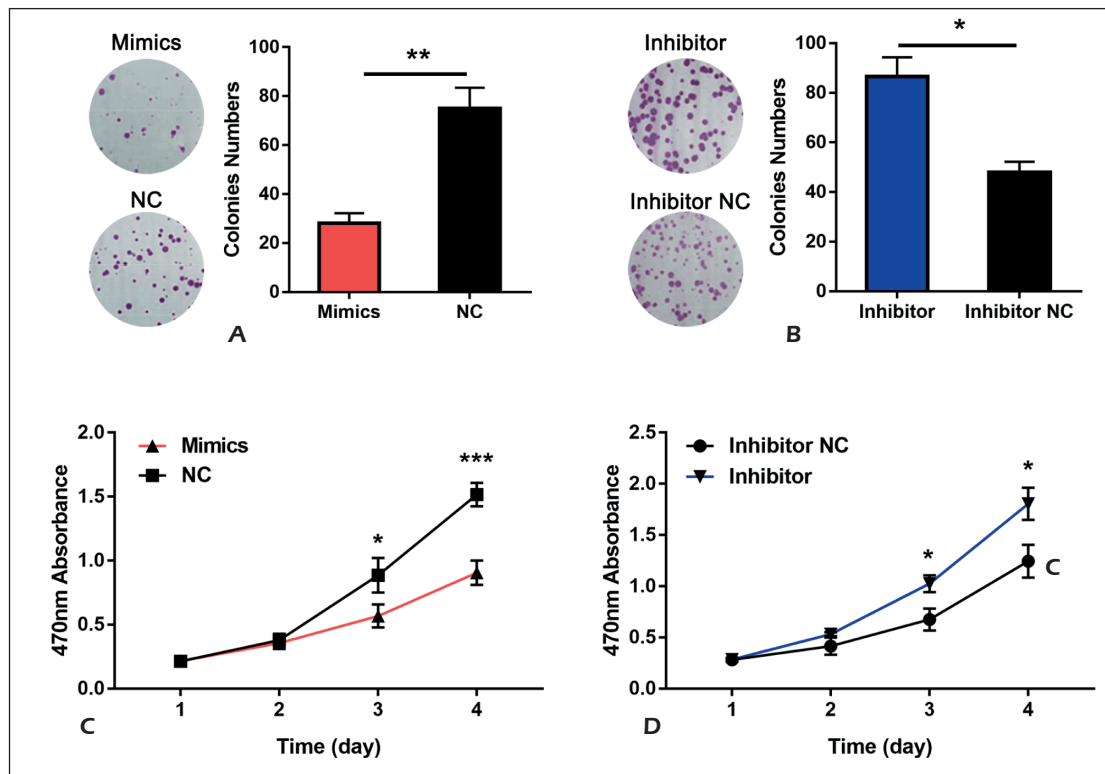


Figure 2. MiR-187 inhibited the proliferation of glioma cells. **A-B**, Colony formation assay was performed to determine the growth of U251 **A**, or U87MG **B**, cells transfected with mimics or inhibitor, respectively (Magnification $\times 20$). **C-D**, CCK-8 assay was performed to determine the proliferation of U251 **C**, or U87MG **D**, cells treated with miR-187 mimics or inhibitors compared with the negative control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

remarkably decreased the proliferation of U251 cells compared with the NC group. However, the interference of miR-187 remarkably promoted the growth of U87MG cells (Figure 2C, 2D). These results confirmed that miR-187 could inhibit the proliferation of glioma cells.

MiR-187 Inhibited the Migration and Invasion of Glioma Cells

Next, we investigated the function of miR-187 in cell metastasis using wound-healing assay and transwell assay. As shown in Figure 3A, the up-regulation of miR-187 significantly decreased the wound-healing rate of U251 cells compared with NC cells. However, down-regulation of miR-187 accelerated the healing rate of U87MG cells compared with the INC group (Figure 3B). Transwell migration assay indicated that the migration ability of U251 cells decreased markedly after transfection of miR-187 mimics. However, the migration of U87MG cells increased remarkably after interfering with miR-187 inhibitor. This confirmed the results of the wound-healing assay

(Figure 3C). In addition, the transwell invasion assay showed that over-expressed miR-187 significantly inhibited the invasion ability of U251, while down-expressed miR-187 promoted the invasion ability of U87MG cells (Figure 3D). These findings suggested that miR-187 inhibited migration and invasion of glioma cells.

SMAD1 Was a Direct Target for MiR-187 in Glioma

Numerous studies have confirmed that miRNAs play their roles in diseases *via* direct binding to the 3'-untranslated region of target genes and repressing their protein expressions. Therefore, we searched several databases in this work, including TargetScan, miRWalk, PiTar and miRBase. The results found that miR-187 could bind to the 3'-UTR of SMAD1 (Figure 4A). To verify the assumption, Dual-Luciferase reporter gene assay was performed. The results found that the Luciferase activity decreased markedly in wild-type SMAD1 3'-UTR group. However, no significant difference was observed in the Luciferase activity of the mu-

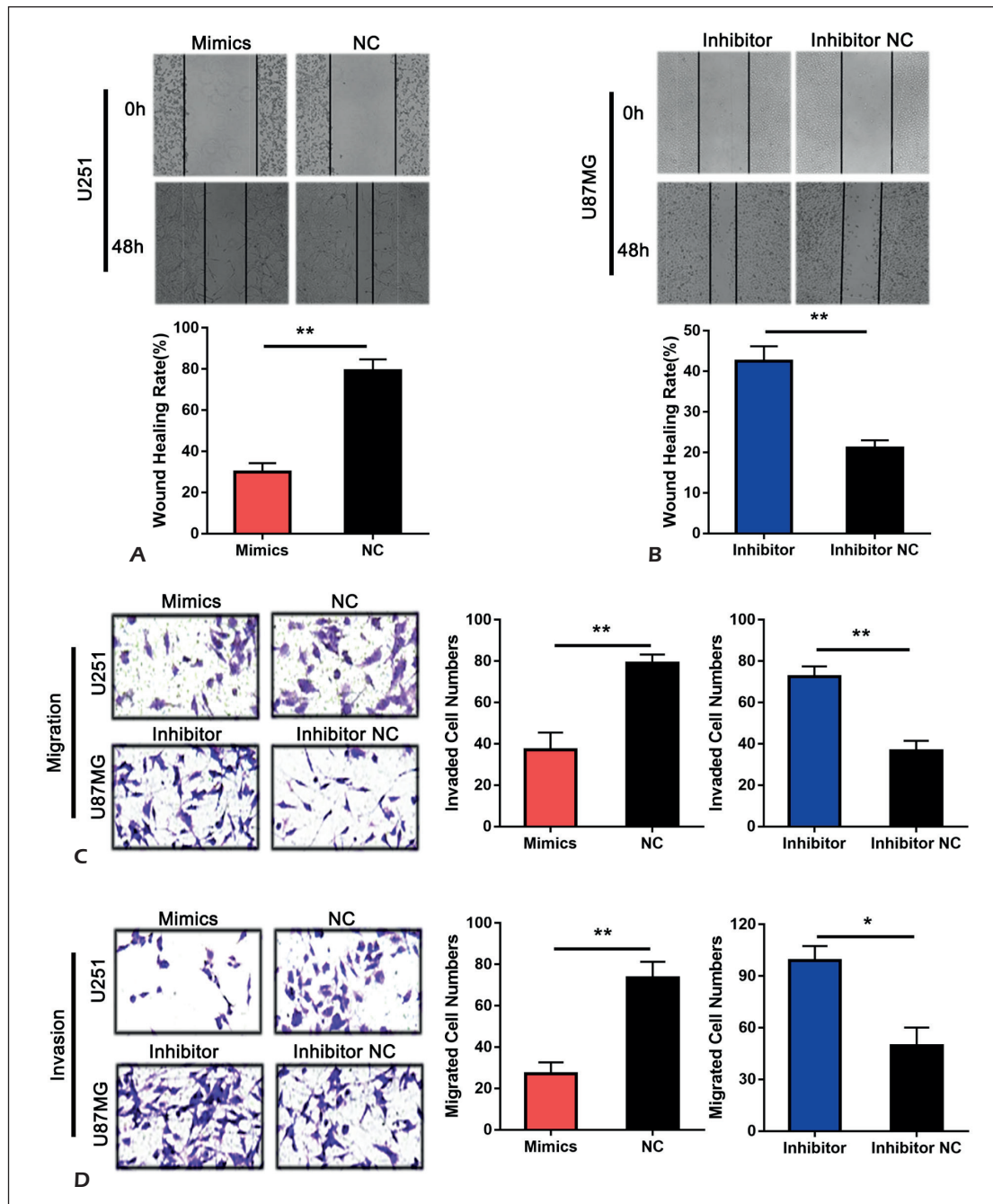


Figure 3. MiR-187 inhibited the invasion and migration of glioma cells. **A-B**, Wound-healing as-say was used to detect the invasion ability of miR-187 mimics treated U251 cells **A**, or miR-187 in-hibitors treated U87MG cells **B**. **C-D**, Transwell migration assay was used to detect the migration **C**, and invasion **D**, abilities of miR-187 mimics treated U251 cells or miR-187 inhibitor treated U87MG cells (Magnification $\times 40$). Data were presented as mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

tant group (Figure 4B). This confirmed that miR-187 could bind to the 3'-UTR of SMAD1. Next, the protein level of SMAD1 in glioma cells was measured using Western blot. U251 cells transfected with miR-187 mimics expressed a remarkably low-

er level of SMAD1. However, the protein expression of SMAD1 was significantly up-regulated in U87MG cells treated with miR-187 inhibitor (Figure 4C). These findings suggested that SMAD1 was a direct target for miR-187 in glioma.

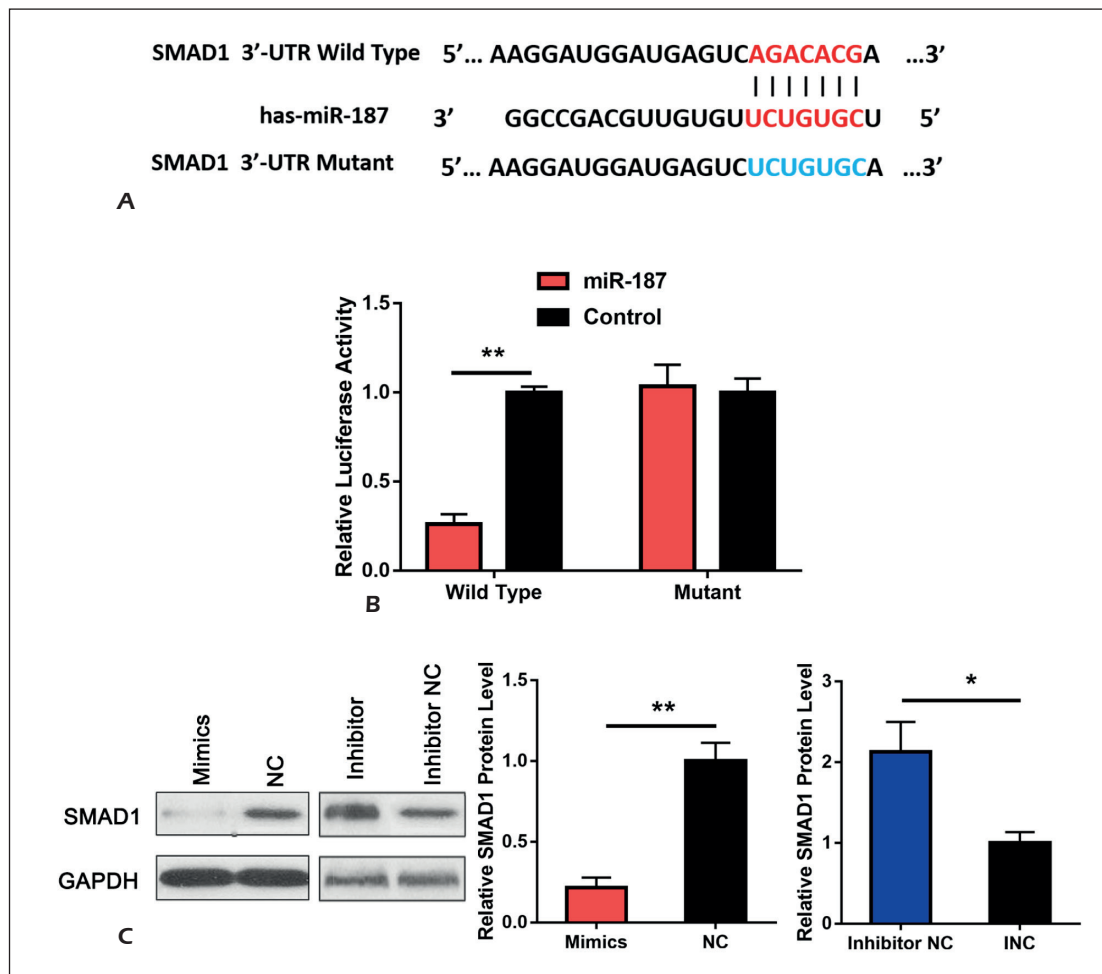


Figure 4. SMAD1 was a direct target of miR-187. **A**, The predicted binding sites of miR-187 in the 3'-UTR of SMAD1. **B**, TDual-Luciferase reporter assay was used to determine the binding site. **C**, TProtein levels of SMAD1 and GAPDH measured by Western blot in miR-187 over-expressed U251 cells and miR-187 down-expressed U87MG cells. The relative protein level of SMAD1 was normal-ized to GAPDH. Data were presented as mean \pm SD of three independent experiments. * p <0.05, ** p <0.01.

MiR-187 Inhibited Glioma Cell Proliferation and Metastasis via Repressing SMAD1

The above findings demonstrated that SMAD1 was a direct target for miR-187 in glioma. We then restored SMAD1 expression in miR-187 up-regulated U251 cells using pcDNA-SMAD1. The protein level of SMAD1 in co-treated (miR-187 mimics + pcDNA-SMAD1) group was significantly higher than miR-187 mimics group (Figure 5A). Next, we explored cell proliferation and metastasis abilities *via* CCK-8 and transwell assays, respectively. Restoration of SMAD1 rescued cell growth inhibited by miR-187 mimics (Figure 5B). Meanwhile, the metastasis of U251 cells was promoted by SMAD1 over-expression compared with miR-187 mimics group (Figure 5C). These results suggested that the inhibition of cell

growth and metastasis by miR-187 could be rescued by SMAD1 up-regulation. All our findings confirmed that miR-187 inhibited glioma cell proliferation and metastasis *via* repressing SMAD1.

Discussion

Glioma is a common central nervous system tumor, accounting for about 35% to 60% of intracranial tumors. Statistics^{3,14} have shown that the 2-year survival rate of patients with high-grade glioma is only 5%, while the 10-year survival rate of patients with low-grade glioma remains only 20%. Therefore, searching for new ways to effectively treat gliomas has become an urgent need for clinicians and patients.

Nearly 100 new miRNAs have been reported to participate in the development of glioma. Based on this, specific changes in miRNA targets and related functions have been explored^{7,8}. For instance, miR-218-5p regulates the proliferation, migration and EMT of human glioma cells *via* targeting LHFPL3. Downregulation of miR-200a leads to over-expression of *Gai1*, thereby activating Akt to promote the proliferation of human glioma cells. In addition, down-regulation of miRNA-637 indicates poor prognosis of glioma, which promotes cell proliferation, invasion and migration by repressing Akt^{13,15,16}.

MiR-187 has been identified as a tumor suppressor in several tumors, such as cervical cancer, hepatocellular carcinoma, non-small cell lung cancer, osteosarcoma and colorectal cancer. It can moderate different target gene expression, including CD276, CYP1B1, S100A4, ZEB2, PTRF, IGF-1R, FGF9, and HPV16 E6¹⁷⁻²⁵. However, the expression and function of miR-187 in glioma has not been fully elucidated.

Here, we first detected the relative expression of miR-187 in 67 glioma tissues and 21 normal brain tissues. The results found miR-187 was lowly expressed in glioma tissues. Meanwhile, glioma-derived cell lines showed significantly de-

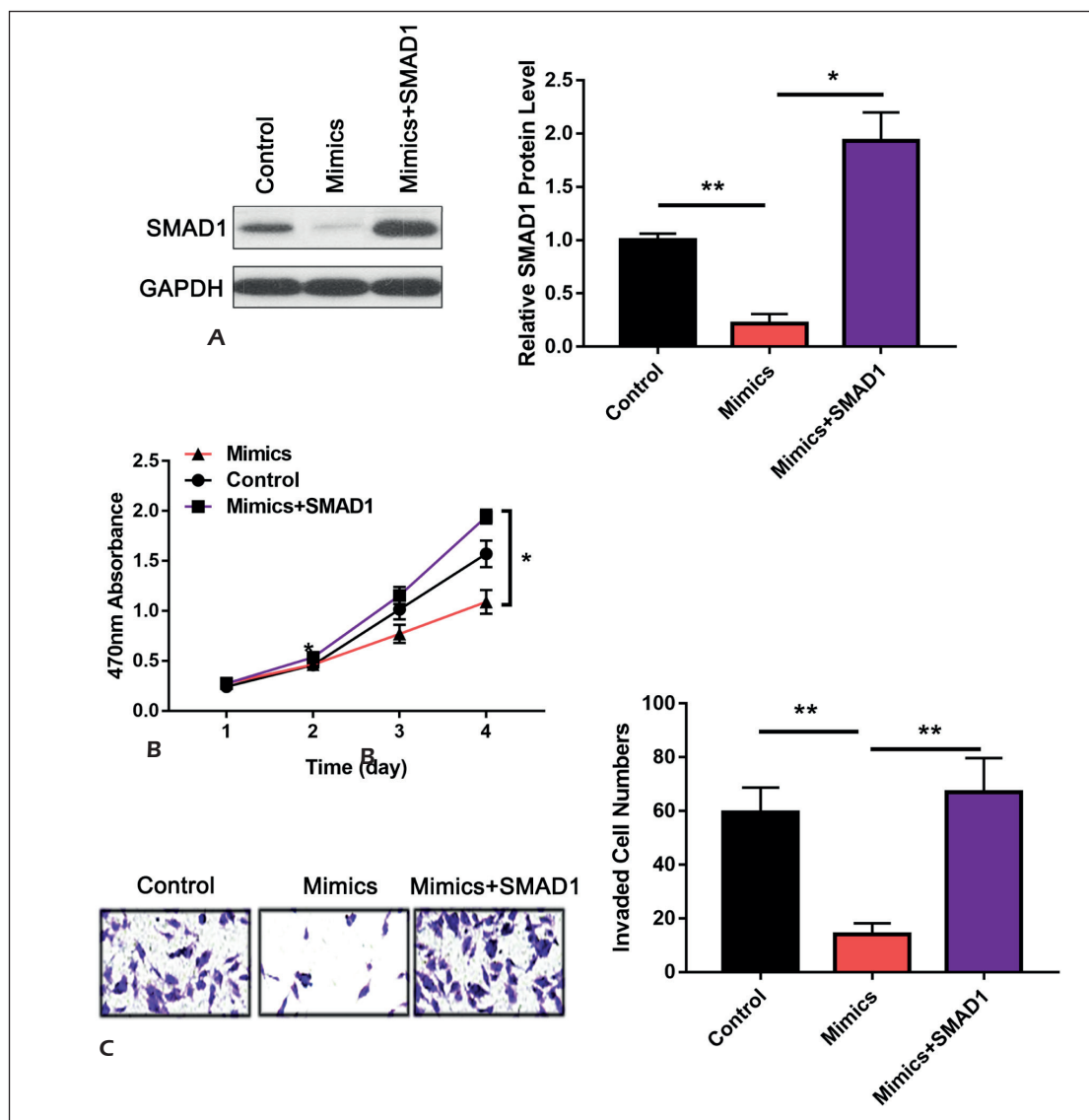


Figure 5. SMAD1 rescued the effects of miR-187 mimics in U251 cells. **A**, Western blot analyses of SMAD1. GAPDH was used as an internal control. **B**, Analysis of the proliferation ability by CCK-8 assay in control, mimics, or mimics+SMAD1 treated U251 cells. **C**, Cell invasion ability was measured by transwell assay (Magnification $\times 40$). Data were represented as mean \pm SD of three in-dependent experiments. * $p < 0.05$, ** $p < 0.01$.

creased miR-187 expression than normal human brain cells. These results were similar to previous studies that miR-187 functioned as a tumor suppressor in malignancies. Furthermore, using miR-187 mimics and inhibitor, several functional experiments were applied to study the influence of miR-187 on cell proliferation and metastasis. Our results indicated that over-expression of miR-187 markedly inhibited the growth, invasion, and migration of U251 cells, while inhibition of miR-187 promoted proliferation and metastasis of U87MG cells. All these findings detected that miR-187 dysregulation affected glioma development.

Next, SMAD1 was verified as a direct target for miR-187 in glioma cells. Western blot and luciferase reporter gene assay revealed that SMAD1 was regulated by miR-187 *via* binding to its 3'-UTR. SMAD1 has previously been verified as a tumor-promoting gene belonging to the SMAD family. SMAD1 can influence tumor progression together with the other SMAD factors including SMAD5 and SMAD8²⁶⁻³⁰. In glioma, SMAD1 and BMPR-IB promote cell growth and progression. Differential regulation of SMAD1/5/8 versus SMAD2/3 signaling regulates glioblastoma progression as well³¹. Here, we found miR-187 could reduce the development and progression of glioma *via* repressing SMAD1 expression. Restoration of SMAD1 could markedly reverse the inhibitory effect of miR-187 over-expression, which confirmed SAMD1 as a direct downstream molecule for miR-187.

Conclusions

We unraveled the expression of miR-187 in glioma for the first time. Our results found that miR-187 significantly inhibited the proliferation, invasion and migration of glioma cells *via* repressing SMAD1. However, an in-depth study of the underlying mechanism of miR-187 *in vivo* are still needed. Our work partially explained the function of miR-187 in glioma, which might provide a novel target for the biological treatment of glioma.

Conflict of Interests

The authors declare that they have no conflict of interest.

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