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MiR-186 promotes the apoptosis of glioma U87 cells by down-regulating the expression of Smad6

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Abstract. – OBJECTIVE: MiRNA family gene is an evolutionarily conserved non-coding small RNA that directly participates in a variety of physiological processes and cancer development via regulating gene expression in the biological level of transcription. To research the specific mechanism by which miR-186 regulates apoptosis within gliomas.

PATIENTS AND METHODS: RT-qPCR was performed to verify the transcriptional level of miR-186 within glioma tissues and glioma cells. miRanda and Dual-Luciferase assay were performed to predict and confirm that Smad6 gene is an effective target of miR-186 within glioma. The expression of Smad6 protein was tested by Western blot following cell effective transfection. Apoptosis of gliomas was analyzed by inverted fluorescence microscopy and flow cytometry.

RESULTS: The mRNA level of miR-186 was suppressed within glioma tissues and glioma U87 cells. MiR-186 is associated with apoptosis in glioma. Overexpression of miR-186 promoted U87 cell apoptosis, whereas suppression of miR-186 had the opposite effect. Besides, miR-186 directly targeted Smad6 and suppress its expression in glioma. The expression of Smad6 affected the regulation of miR-186 on glioma cell apoptosis, restoration of Smad6 rescued apoptosis of glioma U87 cells induced by miR-186 mimics, whereas inhibition of Smad6 promoted apoptosis.

CONCLUSIONS: As noted above, miR-186 exerts a tumor-suppressing effect by targeting Smad6. We propose that miR-186 can be used as a novel biomarker for glioma diagnosis in the future, or as a new pharmacy target in the cure of gliomas.

Key Words:
MiR-186, Smad6, Glioma, Apoptosis.

Introduction

Tumors derived from neuroepithelial cells are collectively referred to as gliomas, which account for 40% to 50% of brain tumors. Currently, gliomas have developed into the most common intracranial malignancy, and are associated with the survival rates of patients[1,2]. Currently, chemotherapy and radiotherapy are the main treatments. However, despite the advancement of glioma treatment, there is still an inferior survival rate of glioma sufferers and no definitive therapeutic measures were found[3,4]. Therefore, there is an urgent need to elucidate the molecular mechanisms and regulatory pathways of glioma malignancies. Further investigation may provide a more effective or potential therapeutic measure for gliomas.

MicroRNAs are a cluster of non-coding RNA sequences of approximately 18-25 bp that interacts with the target gene RNA (mRNA, messenger RNA) 3' untranslated region to inhibit expression of the target gene[5,6]. MiRNAs are vital regulators of a variety of metabolic processes, and more importantly, they are directly referred to tumor progression, which serves as an irreplaceable effect on the infinite proliferation and multi-directional transfer of various tumor cells[7,8]. MiRNA levels are imbalanced in a variety of malignant cells, including gliomas[9,10]. Up to now, studies have shown that miR-186 is a critical regulator in tumorigenesis[11,12]. MiR-186 inhibits tumor development by inhibiting tumor cell proliferation and diffusions, such as Non-small cell lung cancer (NSCLC), bladder can-

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cer, and squamous cell carcinoma (SCC)[13,14]. Previous studies have shown that miR-186 regulates the biological behavior of gliomas[15]. However, the particular mechanism and physiological and biochemical functions of miR-186 are still unclear. To investigate the role of miR-186 in glioma progression and its specific mechanism, a timed quantitative reverse transcription PCR system was used to monitor miR-186 mRNA levels. Further, the mechanism of miR-186 in glioma cell apoptosis was explored. Our results showed that Smad6 was a direct target of miR-186 in glioma, which was negatively regulated by miR-186. In conclusion, we found that miR-186 expression decreased rapidly in gliomas. It is hypothesized that the increase in the expression of miR-186 is associated with apoptosis in glioma cells. MiR-186 may promote glioma cells apoptosis by targeting Smad6. Our data demonstrate the effect of miR-186 on tumor progression and provide a theoretical basis for clinical treatment of glioma.

Patients and Methods

Tissue Samples

The glioma specimen of human was obtained by surgical resection. The non-tumor brain tissues, which was derived from internal decompression, were obtained from patients with traumatic brain injury. The participating patients would continue to track for 55 months, and the duration of their survival would be recorded after the operation. All samples were obtained from the Second People's Hospital of Liaocheng. The study was approved by the Second People's Hospital of Liaocheng Ethical Committee and by the participants who signed the informed consent.

Cell Lines

Human U87 cell lines and astrocyte HA1800 cell lines were obtained from West China Hospital of Sichuan University (Chengdu, Sichuan, China). Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) was used to culture all cell lines. 10% fetal bovine serum was added to the medium, and cells were cultured at 37°C in a humid incubator containing 5% CO_2. Cultured cells are used for RNA extraction or total intracellular protein extraction.

Transfection of Cells

MiR-186 mimics, inhibitors purchased from RiboBio (Huangpu, Guangzhou, Guangdong, China). Sangon Biotech (Songjiang, Shanghai, China) synthesized small interfering RNAs targeting Smad6 and negative control. Smad6 was amplified from the astrocyte HA1800 cell line, and its gene was cloned into the pcDNA3.1 vector, while Smad6 was expressed in the expression host cell, and the empty vector as a negative control. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect all plasmids according to the manufacturer's instruction. Cells were cultured into 6-well plates before the experiment. Cultured cells were collected for the experiment analysis following 72 hours of transfection.

Luciferase Activity Assay

Wt 3′-UTR or mut 3′-UTR of Smad6 was amplified into psi-CHECK2 reporter vector. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for co-transfection miR-186 mimics and scrambling with wt 3'-UTR or mut 3'-UTR of the Smad6 psi-CHECK2 vector, which was used for Luciferase activity assays. After 72 hours, Luciferase activity was detected following cell harvesting and lysing. Luciferase activity was measured by the Dual-Luciferase Reporter Assay System.

RNA Extraction and RT-qPCR

TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract the total RNA from tissue samples, and reversely transcribed into complementary DNA (cDNA) or cultured cells. Monitoring miR-186 or Smad6, primers purchased from RiboBio (Huangpu, Guangzhou, Guangdong, China). Purchased from Invitrogen, SYBR Green PCR Master Mix was used to carry RT-qPCR. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were controlled for miR-186 and Smad6, respectively. Primers were displayed in Table I. The $2^{-\Delta\Delta Ct}$ methods were used to calculate the relative expression. This experiment was repeated three more times.

The Extraction of Total Intracellular

Table I. Primer sequences used in this study.

Primer name	(5′-3′) Primer sequences
F-miR-186	5′-CCCGATAAAGCTAGATAACC-3′
R-miR-186	5′-CAGTGCGTGTCGTGGAGT-3′
F-U6	5′-CTTCGGCAGCA CATATACT-3′
R-U6	5 ′-TGGAACGCTT CACGAATTT- 3′
F-GAPDH	5′-CTTCTT TTGCGTCGCC-3′
R-GAPDH	5′-AAAGCAGCC CTGGTG-3′

Protein

Washing the cultured cells twice with 1×PBS and suspended in 1×PBS. The suspended cells were added to a 1.5 mL Eppendorf (EP) tube, centrifuged for 5 min at 1500 rpm in a refrigerated centrifuge. 50-120 μl cell lysate (requires protease inhibitor 1:100 before cell lysate) was used to suspend the cells and lysed on ice for 20 min. The suspension was centrifuged at 4°C, 13000 rpm for 13 min, and the supernatant was taken out. The concentration of cell extracts was evaluated using the bicinchoninic acid assay (BCA; Invitrogen, Carlsbad, CA) kit. The corresponding volume of 5×Loading buffer was added, the mixture was boiled at 91°C for 12 min and stored at –81°C.

Western Blot Analysis

The protein was treated with 10% sodium dodecyl sulphate (SDS)-page, and the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (PVDF; Millipore, Billerica, MA, USA). The PVDF membrane was placed in 6% milk and incubated with the primary antibody overnight at 4°C for blocking. At the same time, GAPDH was used as a control. On the next day, the transfected membrane was washed and incubated with a secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1.5 hours at room temperature. In the end, Pierce® enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA) Western blot Substrate's A and B solutions were mixed in a 1:1 ratio and evenly dropped onto the PVDF membrane. The signals were exposed to the image in the Tanon 5200.

Cell Apoptosis Analysis

Annexin V-FITC/PI Apoptosis Detection Kit was purchased from Beyotime (Songjiang, Shanghai, China) for apoptosis analysis following the manufacturer's instructions. In detail, centrifuging the 12-well cell culture plate at 1000 × g for 5 min after the induction of apoptosis. Aspirate the medium and wash it twice with 1 phosphate-buffered saline (PBS) for 5 min each time. 500 μl binding buffer were added to suspend cells. Then, 5 μL FITC-conjugated antibody and 5 μL propidium iodide (PI) were added followed by incubation for 15 min on ice in the dark. Finally, based on cell binding to Annexin V apoptosis was assessed by flow cytometry.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 (San Diego, CA, USA). All data are expressed as the mean ± SEM of three independent experiments. A comparison of the mean between the control and treatment groups was performed using the paired *t*-test or one-way ANOVA. A *p*-value <0.05 was considered statistically significant.

Results

MiR-186 Was Downregulated in Glioma Tissue Samples and Glioma U87 Cells

Studies have shown that miR-186 was downregulated in a variety of tumors[16,17]. In this study, to survey the expression of miR-186 in glioma,

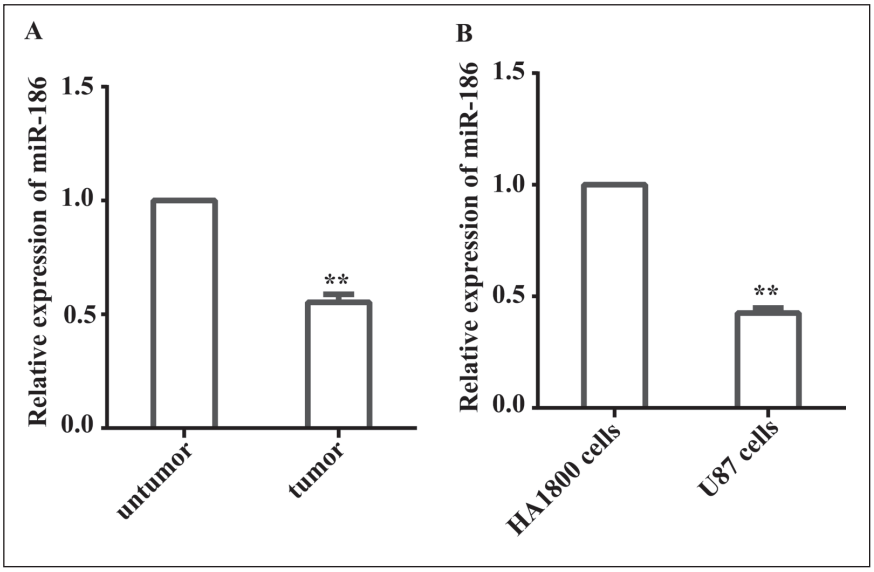

Figure 1. MiR-186 level was decreased in glioma tissue samples and cell lines. **A**, Expression of miR-186 in glioma tissues (tumor) and the corresponding normal tissues (un-tumor) as determined by RT-qPCR. **B**, Expression of miR-186 in U87 cells and normal astrocyte HA1800. Comparisons were performed using *t*-test. **indicates $p<0.01$. Error bars represent SEM. Data represent three independent experiments.

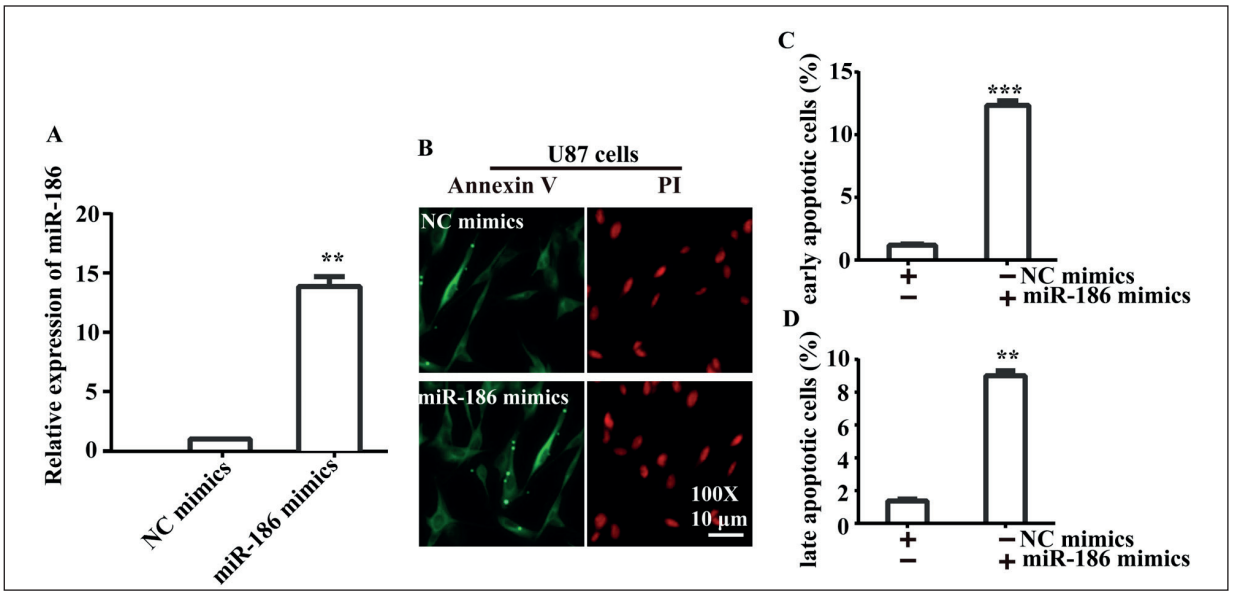

Figure 2. MiR-186 promoted U87 cells apoptosis. **A**, U87 cells were first transfected with miR-186 mimic, and then the expression of miR-186 was detected by RT-qPCR after transfection. **B**, Inverted fluorescence microscopy was performed to observe the apoptosis of U87 cells following effective transfection (100×). **C-D**, Percentage of apoptotic U87 cells was analyzed by flow cytometry following miR-186 overexpressing. Comparisons were performed using *t*-test. **indicates $p<0.01$, ***indicates $p<0.001$. Error bars represent SEM. Data represent three independent experiments.

RT-qPCR was performed on glioma tissues and corresponding non-tumor tissues. From Figure 1A, the result indicated that the transcription level of miR-186 was significantly down-regulated in glioma tissues. Further, the intracellular level of miR-186 in glioma U87 cells and astrocyte HA1800 cells were studied. The RT-qPCR result showed that the expression of miR-186 in glioma U87 cells was relatively lower than the expression in astrocyte HA1800 cells (Figure 1B).

MiR-186 Promoted Glioma U87 Cells Apoptosis

In recent years, a series of research have shown that miR-186 promotes tumor cell apoptosis and inhibits tumor cell invasion and migration[17,18]. To investigate the effect of miR-186 on the biological functions of glioma cells, the miR-186 mimetic was transfected into glioma U87 cells to express miR-186 (Figure 2A). Meanwhile, Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime, Shanghai, China) was used to analyze the apoptosis following miR-186 overexpression. Inverted fluorescence microscopy analysis results showed that there was a large number of glioma U87 apoptotic cells following miR-186 overexpression (Figure 2B). Finally, Annexin V apoptosis was assessed by flow cytometry, based on cell binding to Annexin V. Results showed that overexpression of miR-186 significantly promoted the apoptosis of U87 cells (Figure 2C, D).

Suppression of MiR-186 Inhibited Glioma U87 Cells Apoptosis

To further investigate the role of miR-186 in the biological function of glioma cells, the miR-186 inhibitor was transfected into glioma U87 cells to suppress miR-186 by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA; Figure 3A). Similarly, cell apoptosis was analyzed by Annexin V-FITC/PI Apoptosis Detection Kit following miR-186 suppression. A small amount of apoptotic glioma U87 cells was observed under inverted fluorescence microscopy following miR-186 suppressed (Figure 3B). Besides, flow cytometry results indicated that suppression of miR-186 inhibited the apoptosis of U87 cells (Figure 3C, D). Based on the above findings, we suggest that miR-186 is involved in the regulation of U87 cell apoptosis.

Smad6 Was a Direct Target of MiR-186 in Glioma U87 Cells

MiRNAs regulate a series of biological processes and tumor progression by targeting some protein synthesis. For example, miR-186 affects cervical cancer cells proliferation and invasion by targeting EGFR gene[18]. In this study, to determine miR-186 in gliomas, the potential possible target

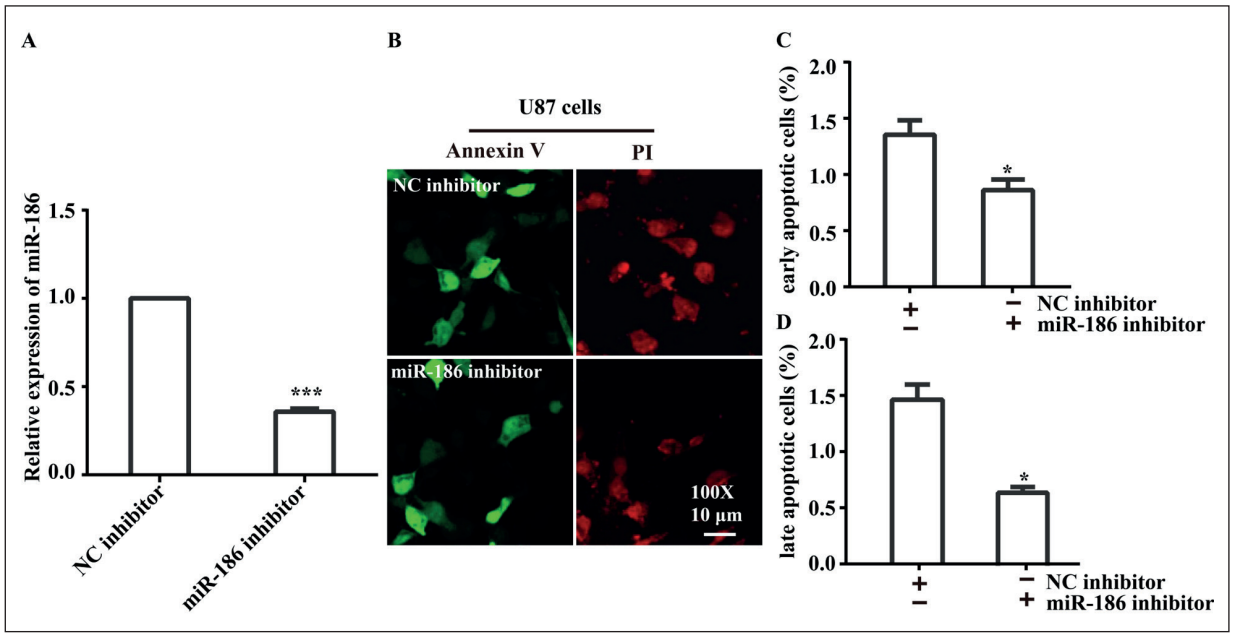

Figure 3. Suppression of miR-186 inhibited U87 cells apoptosis. **A**, MiR-186 inhibitor was first transfected into U87 cells, and the expression of miR-186 was detected by RT-qPCR after transfection. **B**, Inverted fluorescence microscopy was performed to observe the apoptosis of U87 cells following effective transfection (100×). **C-D**, Percentage of apoptotic U87 cells was analyzed by flow cytometry following miR-186 suppressing. Comparisons were performed using *t*-test. *indicates $p<0.05$, *** indicates $p<0.001$. Error bars represent SEM. Data represent three independent experiments.

genes were predicted by the miRanda method. In Figure 4A, bioinformatics analysis revealed miR-186 was placed in the 3'-UTR of Smad6. To further confirm the prediction, Luciferase activity assay was continued used to in U87 cells. In detail, Lipofectamine 2000 was used to transfect the miR-186 mimics and scramble the wt 3′-UTR or mut 3′-UTR of Smad6 psi-CHECK2 vector into U87 cells. The Luciferase activity was measured employing the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). From Figure 4B, wt 3'-UTR Luciferase activity was significantly inhibited by the miR-186 mimetic, but mut 3'-UTR Luciferase activity in U87 cells was not significantly inhibited. Inversely, the miR-186 inhibitor significantly promoted the wt 3′-UTR Luciferase activity in U87 cells. There was no significant difference in mut 3'-UTR experimental group (Figure 4C). Based on the above results, we propose that Smad6 is a direct target of miR-186 in gliomas.

Smad6 Was Negatively Regulated by MiR-186 in Gliomas

To further verify the negative regulation of miR-186 on Smad6, on the one hand, we overexpressed miR-186 by transfecting the miR-186 mimetic into glioma U87 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Subsequently, total intracellular proteins were extracted for Western blot detection. The results indicated that Smad6 expression in glioma U87 cells was significantly inhibited with the overexpression of miR-186 (Figure 5A). On the other hand, we transfected the miR-186 inhibitor into glioma U87 cells by the use of the same method to inhibit miR-186. Western blot results indicated that Smad6 protein expression was markedly promoted after inhibition of miR-186 (Figure 5B). All the results implied that Smad6 was negatively regulated miR-186 in glioma.

Smad6 Overexpression Rescued Glioma U87 Cells Apoptosis Induced by MiR-186

Prior studies[19] suggest that Smad6 is associated with tumorigenesis and metastasis. At the same time, we have demonstrated that Smad6 was negatively regulated by miR-186 in glioma. To further investigate the interaction between miR-186 and Smad6 in glioma, we used Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) to transfect pcDNA3.1-Smad6 plasmid (Smad6) to rescue the suppression of Smad6 induced by miR-186 mimics in U87 cells, empty plasmid or NC mimics as a negative control. The expression of Smad6 in glioma

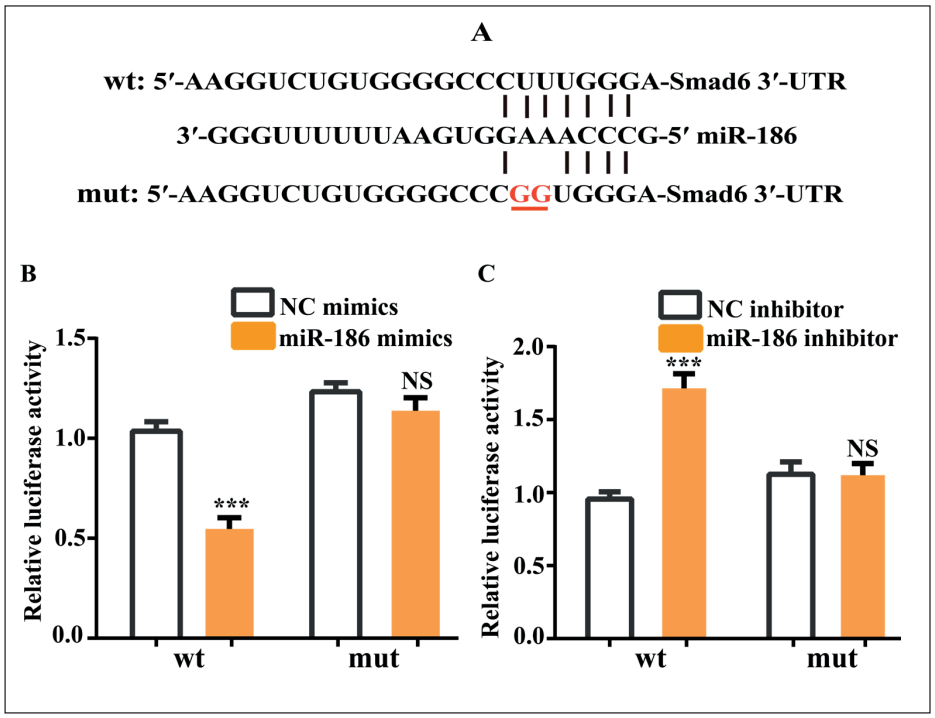

Figure 4. Smad6 was a direct target of miR-186 in U87 cells. **A**, Sequences of miR-186 against the WT or mut 3′-UTR of Smad6 predicted by miRanda. **B**, MiR-186 mimics or the NC mimics and WT or mut 3′-UTR of Smad6 reporter vector were co-transfected into U87 cells. **C**, MiR-186 inhibitor or the NC inhibitor and WT or mut 3′-UTR of Smad6 reporter vector were cotrans-fected into U87 cells. Comparisons were performed using t-test. ***indicates $p<0.001$, NS indicates no significant difference. Error bars represent SEM. Data represent three independent experiments.

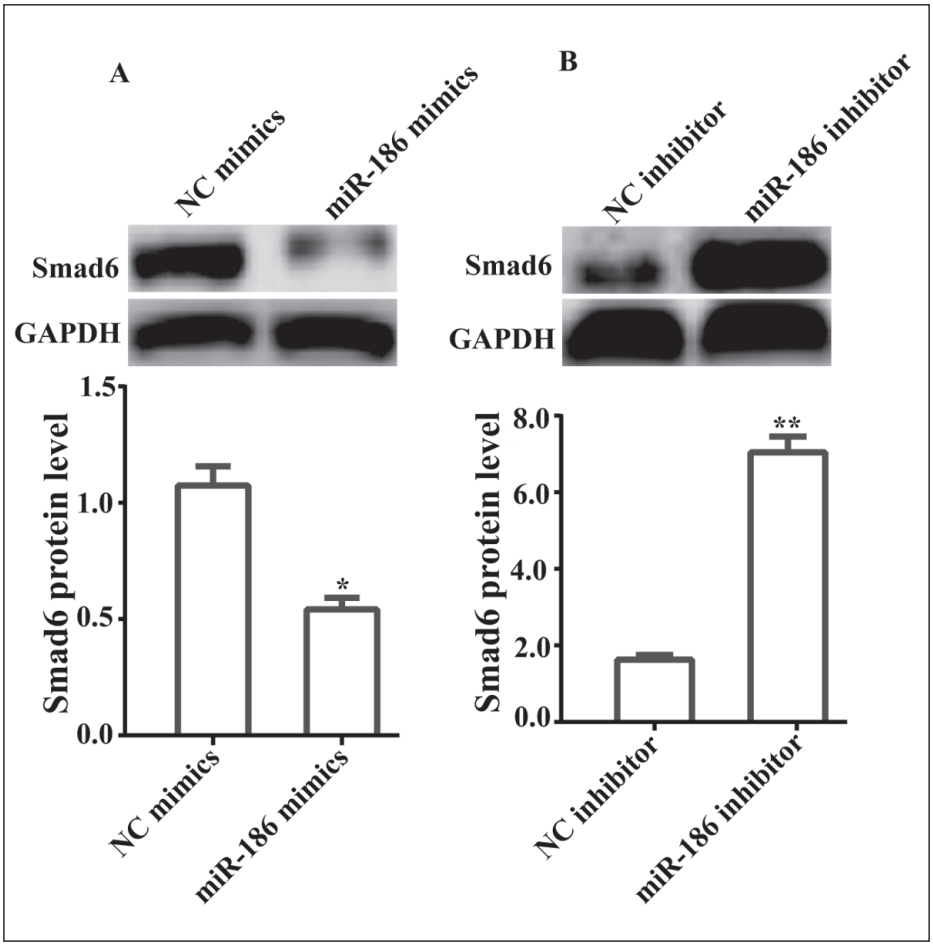

Figure 5. Smad6 was negatively regulated miR-186. A, Results of Smad6 protein expression in U87 cells were examined by Western blot analysis after overexpression of miR-186. B, Results of Smad6 protein expression in U87 cells were examined by Western blot analysis after the suppression of miR-186. Comparisons were performed using t-test. *indicates $p<0.05$, ** indicates $p<0.01$. Error bars represent SEM. Data represent three independent experiments.

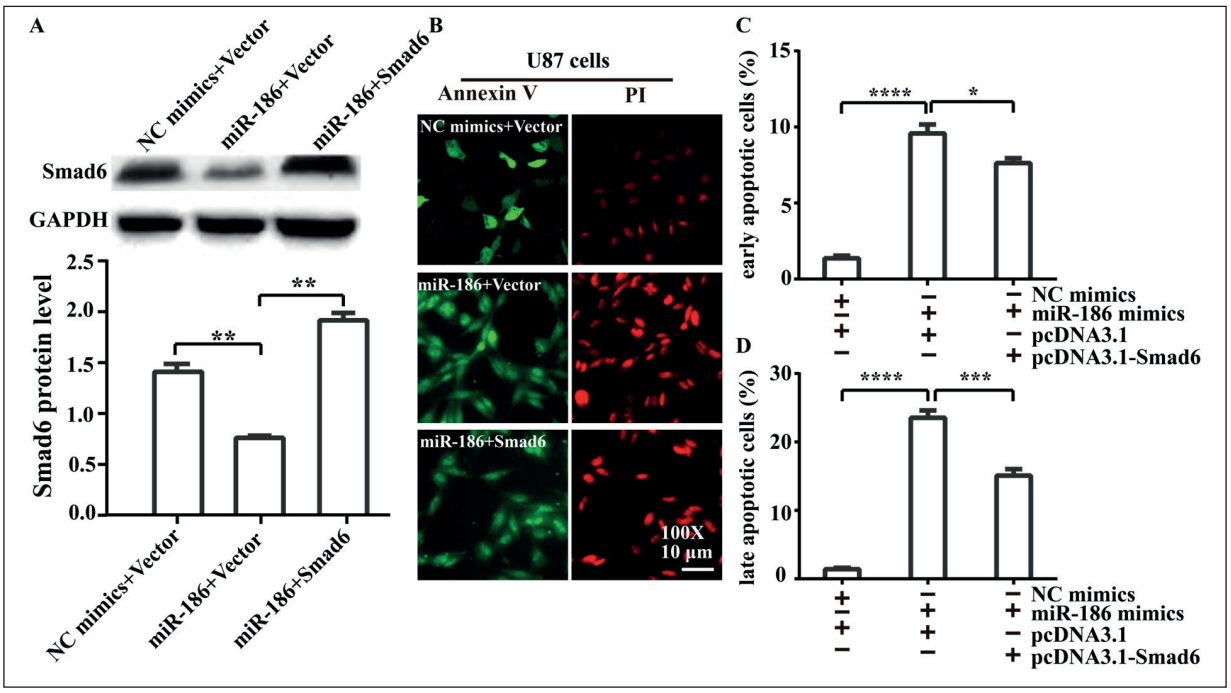

Figure 6. Smad6 overexpression rescued U87 cells apoptosis induced by miR-186. **A**, Results of Smad6 protein expression were detected by Western blot in U87 cells overexpressing miR-186 with or without Smad6 restoration. **B**, Inverted fluorescence microscopy was performed to observe the apoptosis of U87 cells with or without Smad6 restoration following miR-186 overexpression (100×). **C-D**, Percentage of apoptotic U87 cells with or without Smad6 restoration was analyzed by flow cytometry following miR-186 overexpressing. Comparisons were performed using one-way ANOVA. *indicates $p<0.05$, ** indicates $p<0.01$. Error bars represent SEM. Data represent three independent experiments.

U87 cells was observed by Western blot following transfection (Figure 6A). Meanwhile, Annexin V-FITC/PI Apoptosis Detection Kit was used to analyze cell apoptosis following transfection. Under inverted fluorescence microscopy, we observed a small amount of apoptotic glioma U87 cells following overexpressing Smad6 (Figure 6B). Consistently, flow cytometry analysis results showed that apoptotic glioma U87 cells were significantly reduced following overexpression of Smad6 compared with control (Figure 6C and 6D).

Smad6 Suppression Promoted Glioma U87 Cells Apoptosis Induced by MiR-186

We have demonstrated that exogenous overexpression of Smad6 rescued the apoptosis of glioma U87 cells induced by miR-186 mimics. Further, to investigate the effect of Smad6 inhibition on miR-186-induced apoptosis in glioma U87 cells, we transfected si-Smad6 to suppress the expression of Smad6 in U87 cells, si-Ctrl or NC mimics as a negative control. Western blot analysis indicated that the si-Smad6 and miR-186 mimics effectively inhibited the expression of Smad6 protein in glioma U87 cells following transfection (Figure 7A). Similarly, Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime, Shanghai, China) was used to analyze cell apoptosis following transfection. Under inverted fluorescence microscopy, we observed a large number of apoptotic glioma U87 cells following suppressing Smad6 compared to control (Figure 7B). Meanwhile, the results of flow cytometry analysis in keeping with the above results (Figure 7C and 7D).

According to the above results, we suggest that miR-186 may regulate apoptosis by targeting Smad6 in glioma.

Discussion

As an important regulator of various biological processes and tumor progression, miRNAs regulate the proliferation and metastasis of a variety of tumor cells[20]. As one of the miRNAs, miR-186 has been reported to inhibit multiple myeloma cells and prostate cancer cell proliferation[21]. In addition, miR-186 has been reported to regulate cell metastasis in a variety of cancers, including SCC and NSCLC[18,22]. In this research, we aimed to

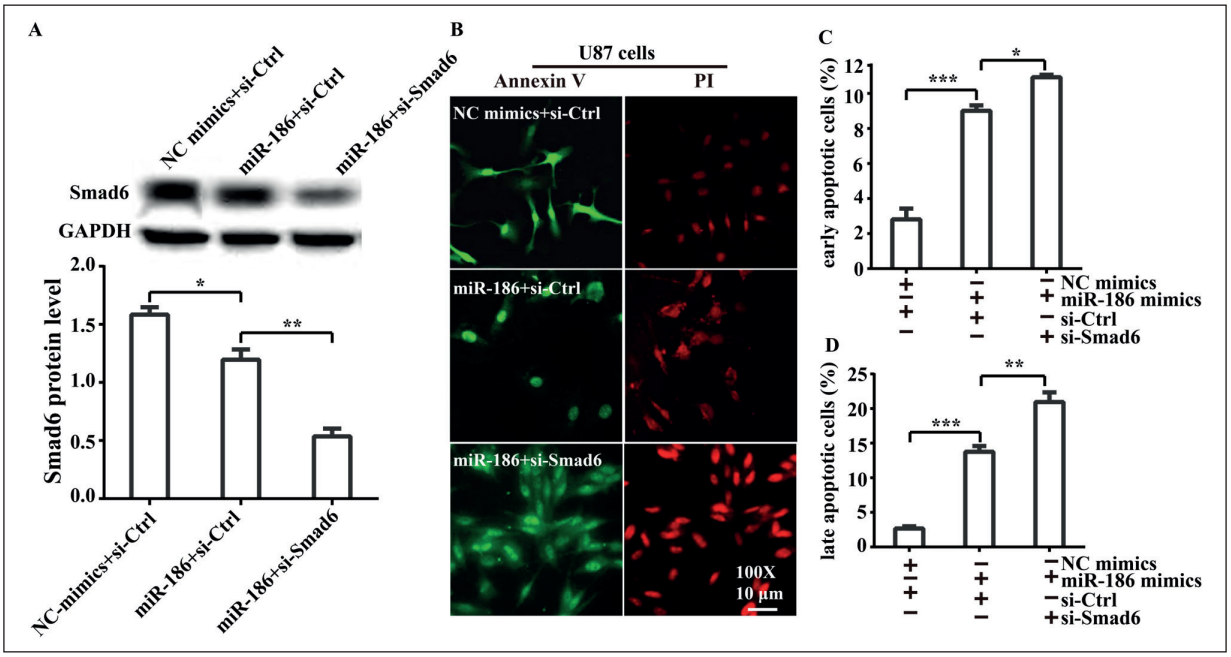

Figure 7. Smad6 suppression promoted U87 cells apoptosis induced by miR-186. **A**, Expression of Smad6 protein in U87 cells with or without Smad6 silence following miR-186 suppression. **B**, Inverted fluorescence microscopy was performed to observe the apoptosis of U87 cells with or without Smad6 silence following miR-186 suppression (100×). **C-D**, Percentage of apoptotic U87 cells with or without Smad6 silence was analyzed by flow cytometry following miR-186 suppressing. Comparisons were performed using one-way ANOVA. *indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. Error bars represent SEM. Data represent three independent experiments.

reveal the molecular mechanisms and regulatory pathways of glioma malignancies, figuring out the specific mechanism by which miR-186 regulates the progression of glioma. This study found that the expression of miR-186 in gliomas decreased rapidly, but the apoptosis of glioma cells was associated with increased expression of miR-186.

SMADs, the critical mediator of the canonical TGF-β signaling pathway, are proteins responsible for transducing the TGF-β signal into the nucleus[23]. Among the Smad6 family, Smad7 is demonstrated to be involved in TGFβ signaling, whereas Smad6 generally mediates bone morphogenetic protein signals. Furthermore, previous studies[19] have demonstrated the critical role of Smad6 in tumorigenesis. In this research, we mainly investigated the role of Smad6 in the progression of glioma, and its interaction with miR-186 in gliomas. The results indicated that Smad6 was a direct target of miR-186, and its expression in glioma was regulated by miR-186. In addition, recovery or inhibition of Smad6 significantly reduced the effects of miR-186 mimics and inhibitors on apoptosis in glioma U87 cells. Therefore, it can be shown that miR-186 plays an antitumor effect by targeting Smad6 in gliomas.

As described above, miR-186 expression in gliomas decreased, and the increase in the expression of miR-186 was associated with apoptosis of glioma cells. In addition, miR-186 can promote glioma cell apoptosis by targeting Smad6. We propose that miR-186 is a promising new biomarker for the diagnosis of glioma or a new therapeutic target for the treatment of glioma.

Conclusions

The aforementioned results showed that miR-186 expression in gliomas decreased, and the increase in the expression of miR-186 was associated with apoptosis of glioma cells. In addition, miR-186 can promote glioma cell apoptosis by targeting Smad6. We suggest that miR-186 is a promising new biomarker for the diagnosis of glioma or a new therapeutic target for the treatment of glioma.

Conflict of Interests

The authors declare that they have no conflict of interests.

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