European Review for Medical and Pharmacological Sciences 2020; 24: 6211-6218

Circ_001680 stimulates glioma progression with the involvement of miR-186-5p

J. LI[1], D. LIU[2], Z. CUI[3]

[1]Department of Neurosurgery, Shanghai Fifth People's Hospital, Fudan University, Shanghai, China
[2]Department of Neurosurgery, Liaocheng Third People's Hospital, Liaocheng, China
[3]Department of Neurosurgery Ward 2, The First Hopital of Yulin, Yulin, China

Jun Li and De Liu contributed equally to this work

Abstract. – OBJECTIVE: This study aims to explore the influences of circ_001680 on glioma proliferation and metastasis, and the underlying mechanism.

PATIENTS AND METHODS: Circ_001680 levels in 40 pairs of glioma and normal tissues were detected by quantitative real-time polymerase chain reaction (qRT-PCR). The relationship between circ_001680 level and clinical indicators in glioma patients was analyzed. After knockdown of circ_001680 in U251 and U87 cells by transfection of sh-circ_001680, changes in proliferative, migratory and invasive abilities were assessed by cell counting kit-8 (CCK-8), transwell and wound healing assay, respectively. The binding relationship between circ_001680 and miR-186-5p was predicted by online bioinformatics tools and detected by Dual-Luciferase reporter assay. In addition, their synergistic regulations on glioma progression were explored by rescue experiments.

RESULTS: Circ_001680 was highly expressed in glioma tissues than normal ones. Incidences of lymphatic metastasis and distant metastasis were higher in glioma patients expressing higher level of circ_001680 than those with a lower level. Cell function experiments uncovered that knockdown of circ_001680 inhibited proliferative and metastatic abilities in U251 and U87 cells. MiR-186-5p was downregulated in glioma tissues and negatively correlated to that of circ_001680. Knockdown of miR-186-5p could abolish the inhibitory effects of silenced circ_001680 on glioma progression.

CONCLUSIONS: Circ_001680 stimulates proliferative and metastatic abilities in glioma by negatively regulating miR-186-5p level. High level of circ_001680 is closely linked to lymphatic metastasis, distant metastasis and poor prognosis in glioma patients.

Key Words:
Circ_001680, MiR-186-5p, Glioma.

Introduction

Glioma is a common primary intracranial tumor that originates from neuroepithelial ectoderm. Glioma accounts for more than 50% of primary intracranial tumors, which is featured by high recurrence rate, high mortality and strong invasiveness[1-3]. The currently applied surgery combined chemotherapy and/or radiotherapy cannot effectively improve the prognosis in glioma. Abnormally expressed proto-oncogenes and tumor suppressors enable glioma cells to evade normal regulatory mechanisms[4-7]. People have gradually realized that the occurrence and progression of glioma are complicated, involving multiple factors and pathways. It is necessary to seek for glioma biomarkers and thus improve the clinical outcomes[8-10].

Changes in molecular genetics and epigenetics are responsible for the pathogenesis of glioma, including the involvement of proteomics, genomics, DNAs, protein-encoding RNAs and non-coding RNAs[9,11,12]. Non-coding RNAs, including miRNAs, lncRNAs and circRNAs, have been identified to be vital regulators in glioma progression[12,13]. CircRNAs are abundantly expressed, highly stable and tissue specific. They are resistant to RNase owing to the covalently closed loop structure without 3' and 5' ends[14-16]. CircRNAs are mainly produced by reverse splicing of exons or intron lariat[16]. The role of circRNAs in tumor progression has been previously reported[14,17]. Our research group analyzed differentially circRNAs in glioma profiling by high-throughput sequencing and circ_001680 was selected to be the research object. Circ_001680 has been found to be closely linked to the malignant progression of colorec-

Corresponding Author: Zheng Cui, MM; e-mail: cuizheng007@163.com

tal cancer[18]. Its biological function in glioma progression remains unclear, which will be explored in this paper.

Patients and Methods

Glioma Patients and Tissue Samples

Tumor tissues and adjacent normal ones were collected from 40 patients treated by radical surgery of glioma. All patients did not receive any radiotherapy or chemotherapy before surgery. Tumor node metastasis (TNM) staging and histological subtypes were evaluated based on the criteria proposed by The Union for International Cancer Control (UICC). In addition, this study was in line with the declaration of Helsinki clinical practice guidelines. This was approved by the Ethics Committee of Shanghai Fifth People's Hospital, Fudan University and it was conducted after informed consent of each subject.

Cell Lines and Reagents

Glioma cell lines (U87, A172, LN229, SHG-44, U251) and normal glia (HEB) were purchased from Life Technologies (Gaithersburg, MD, USA). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in a 5% CO_2 incubator at 37°C.

Transfection

Cells were implanted in 6-well plates and cultured to 30-50% density. They were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and harvested at 48 h. Transfection plasmids were constructed by GenePharma (Shanghai, China).

Cell Proliferation Assay

2×10^3 cells were implanted in each well of a 6-well plate, where 10 μL of cell counting kit-8 (CCK-8) solution was added (TaKaRa, Dalian, China). After 1-h culturing in the dark, 450 nm absorbance was measured using a microplate reader. Blank group was set by adding medium and experimental solution without cells.

Transwell Migration and Invasion Assay

Transwell chambers (Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate. 3×10^5 cells suspended in 200 μL of serum-free medium was applied in the upper layer of the chamber with 600 μL of medium in the bottom. After 48-h incubation, migratory cells in the bottom were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Migratory cells were counted in 10 random selected fields per sample. For transwell invasion assay, transwell chambers were pre-coated with Matrigel diluted in serum-free medium (1:100).

Wound Healing Assay

Cells were prepared into suspension with 5×10^5 cells/mL, and implanted in 6-well plates. Until 90% of cell attachment, an artificial wound was made using a sterilized pipette tip. Cells were washed in phosphate-buffered saline (PBS) for 2-3 times and cultured in the medium containing 1% FBS. 24 hours later, wound closure was captured for calculating the percentage of wound healing.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for isolating total cellular RNAs, which were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) (Primescript RT Reagent; TaKaRa, Dalian, China). Using the SYBR® Premix Ex Taq™ kit (TaKaRa, Dalian, China) and StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA), qRT-PCR was carried out. Relative level was calculated by $2^{-\Delta\Delta Ct}$ and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6. circ_001680: forward: 5'-ATCACTTTAGCAGGCACAC-3', reverse: 5'-TTTTAGAGGAGGGGATT-3'; GAPDH: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'; miR-186-5p: forward: 5'-GCGGATCCGAGCCATGCTTATGCTACTG-3', reverse: 5'-GCGCGGCCGCCCAGGTATATGGCA-3'; U6: forward: 5'-CGCAAGGATGACACGCAAATTC-3', reverse: 5'-TATATCACTCTTGCTTCA-3'.

Dual-Luciferase Reporter Assay

Seed sequences paired in the 3′ untranslated region (3′ UTR) of circ_001680 and miR-186-5p (wild-type) and mutant ones (mutant-type) were inserted into pmirGLO vectors for constructing Luciferase vectors. Cells were implanted in 24-well plates, and co-transfected with NC mimic/miR-186-5p mimic and pmirGLO-

circ_001680-WT/pmirGLO-circ_001680-MUT/pmirGLO for 48 h. Luciferase activity was finally measured (Promega, Madison, WI, USA).

Statistical Analysis

Data were expressed as mean ± standard deviation and processed by Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA). Measurement data were compared using the Student's *t*-test, and categorical variables were analyzed by χ^2-test or Fisher's exact test. Chi-square analysis was conducted for analyzing the influences of circ_001680 on clinical indicators in glioma patients. Pearson correlation test was applied for evaluating the correlation between relative expressions of two genes. Each experiment was conducted in triplicate. A significant difference was set at $p<0.05$.

Results

Circ_001680 Was Highly Expressed In Glioma

Circ_001680 was found to be upregulated in glioma tissues than normal ones (Figure 1A). Compared with the normal human glia, circ_001680 was highly expressed in glioma cell lines, especially U251 and U87 cell lines (Figure 1B). In the following experiments, U251 and U87 cells were utilized for establishing circ_001680 knockdown model.

Recruited glioma patients were divided into high circ_001680 expression group and low circ_001680 expression group based on the median level of circ_001680 in glioma tissues. It is shown that circ_001680 level was positively correlated to the incidences of lymphatic metastasis and distant metastasis in glioma patients, whereas it was unrelated to age, sex and tumor staging (Table I). Kaplan-Meier curves uncovered worse survival in glioma patients of high circ_001680 expression group (Figure 1C).

Knockdown of Circ_001680 Inhibited Proliferative and Metastatic Potentials In Glioma

Transfection of sh-circ_001680 could effectively downregulate circ_001680 level in U251 and U87 cells (Figure 2A). Knockdown of circ_001680 markedly decreased viability in

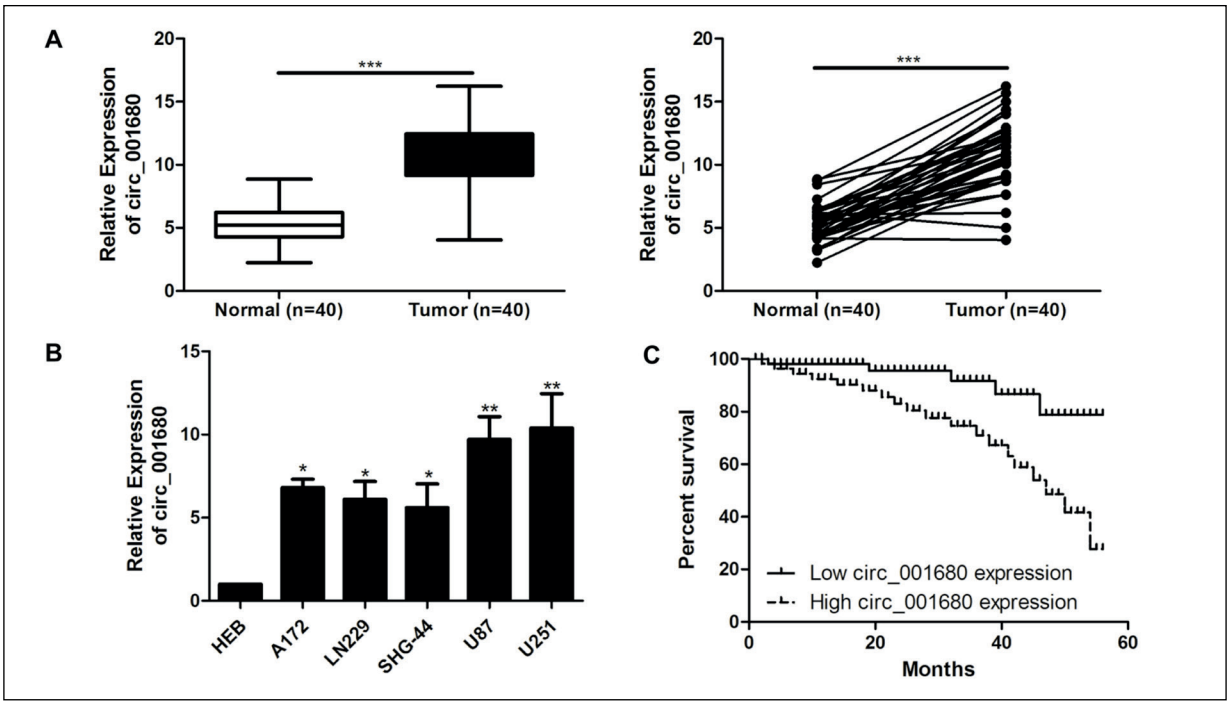

Figure 1. Circ_001680 was highly expressed in glioma. **A,** Circ_001680 levels in glioma tissues (n=40) and normal tissues (n=40). **B,** Circ_001680 levels in glioma cell lines. **C,** Overall survival in glioma patients expressing high or low level of circ_001680. Data were expressed as mean±SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table I. Association of circ_001680 expression with clinicopathologic characteristics of glioma.

Parameters	Number of cases	circ_001680 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.536
< 60	21	12	9	
≥ 60	19	9	10	
Gender				0.536
Male	19	9	10	
Female	21	12	9	
T stage				0.935
T1-T2	25	13	12	
T3-T4	15	8	7	
Lymph node metastasis				0.010
No	27	18	9	
Yes	13	3	10	
Distance metastasis				0.026
No	26	17	9	
Yes	14	4	10	

U251 and U87 cells, indicating that the proliferative ability in glioma was inhibited (Figure 2B). Transwell assay uncovered that transfection of sh-circ_001680 in glioma cells reduced numbers of migratory and invasive cells (Figure 2C). Similarly, the percentage of wound closure was lower in U251 and U87 cells transfected with sh-circ_001680 than those of controls (Figure 2D). The above data demonstrated that circ_001680 was able to stimulate proliferative and metastatic abilities in glioma.

A Negative Interaction Between Circ_001680 and MiR-186-5p

A Venn diagram was depicted to illustrate the potential miRNAs binding circ_001680 predicted in miRBase, StarBase, TargetScan and microRA.org (Figure 3A). MiR-186-5p was selected as the candidate binding circ_001680, which was downregulated in glioma tissues we collected (Figure 3B). As expected, miR-186-5p was lowly expressed in glioma cells as well (Figure 3C). A negative correlation was identified between expressions of miR-186-5p and circ_001680 in glioma tissues (Figure 3D). In addition, the luciferase activity markedly decreased in the pmirGLO-circ_001680-WT vector by overexpression of miR-186-5p in U251 and U87 cells (Figure 3E). However, miR-186-5p could not influence the luciferase activity in the pmirGLO-circ_001680-MUT vector. We therefore demonstrated the binding relationship between miR-186-5p and circ_001680.

Circ_001680 Exerted Biological Functions in Glioma Via the Involvement of MiR-186-5p

To uncover the role of both circ_001680 and miR-186-5p in glioma progression, co-transfection of sh-circ_001680 and miR-186-5p inhibitor was conducted in U251 and U87 cells. Decreased level of circ_001680 in glioma cells transfected with sh-circ_001680 was upregulated by knockdown of miR-186-5p (Figure 4A). Meanwhile, transfection efficacy of miR-186-5p inhibitor was confirmed as well (Figure 4B). Knockdown of circ_001680 was proven to be able to decrease the viability in glioma cells, and such an inhibited trend was abolished by silence of miR-186-5p (Figure 4C). Similar results were identified in transwell assay that knockdown of miR-186-5p in glioma cells abolished the reduced number of migratory cells induced by transfection of sh-circ_001680 (Figure 4D).

Discussion

The pathogenesis of glioma remains largely unclear[1-3]. Currently, genetic factors (i.e., neurofibromatosis or other hereditary diseases), environmental stimuli (i.e., ionizing radiation, carcinogens and teratogens) and unhealthy lifestyle (i.e., long-term smoking, drinking and stay up late) are considered as risk factors for glioma. However, no evidence has suggested that there is a direct relationship between certain factors and the tumorigenesis of glioma[2-5]. Multiple can-

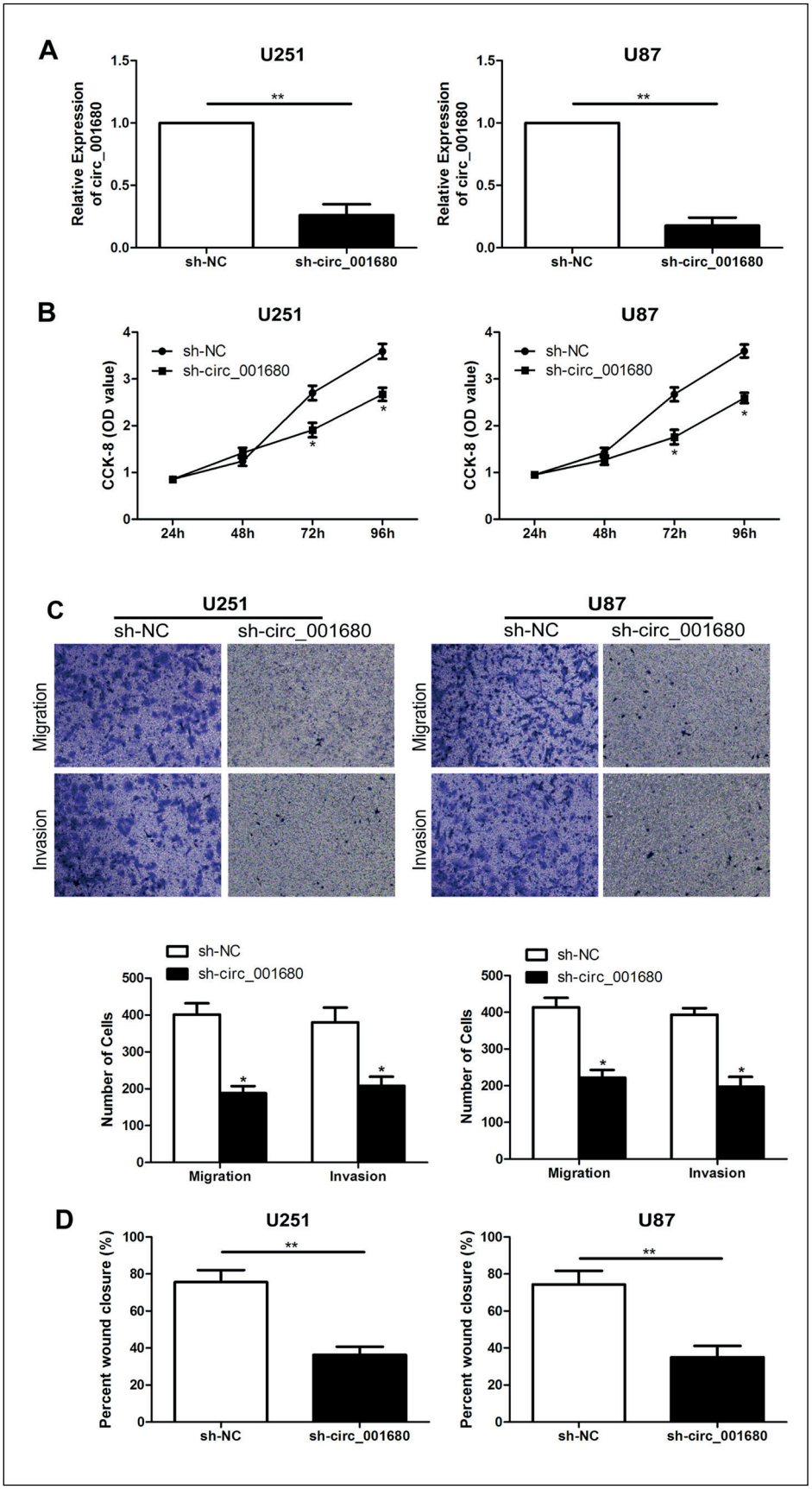

Figure 2. Knockdown of circ_001680 inhibited proliferative and metastatic potentials in glioma. **A,** Transfection efficacy of sh-circ_001680 in U251 and U87 cells. **B,** Viability in U251 and U87 cells transfected with sh-NC or sh-circ_001680. **C,** Migration and invasion in U251 and U87 cells transfected with sh-NC or sh-circ_001680 (magnification: 40×). **D,** Wound closure in U251 and U87 cells transfected with sh-NC or sh-circ_001680. Data were expressed as mean±SD. *p<0.05, **p<0.01.

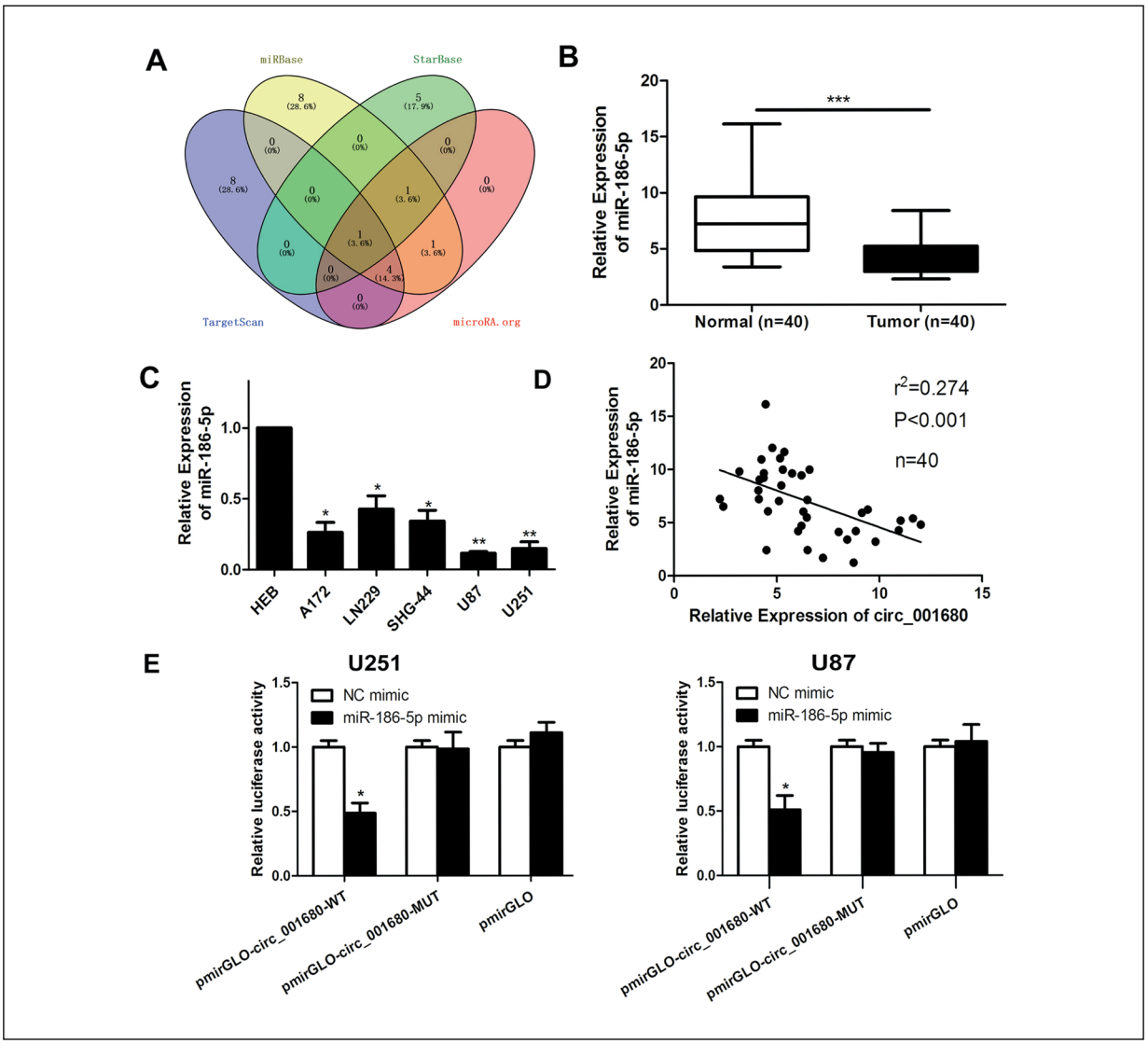

Figure 3. A negative interaction between circ_001680 and miR-186-5p. **A,** A Venn diagram depicted for illustrating the potential miRNAs binding circ_001680 predicted in miRBase, StarBase, TargetScan and microRA.org. **B,** MiR-186-5p levels in glioma tissues (n=40) and normal tissues (n=40). **C,** MiR-186-5p levels in glioma cell lines. **D,** A negative interaction between circ_001680 and miR-186-5p in glioma tissues. **E,** Luciferase activity in U251 and U87 cells co-transfected with NC mimic/miR-186-5p mimic and pmirGLO-circ_001680-WT/pmirGLO-circ_001680-MUT/pmirGLO. Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

cer-associated genes and pathways are involved in the progression of glioma[6-8]. At present, glioma is mainly treated by surgery, chemotherapy, radiotherapy or adjuvant treatment[4,5,9]. For advanced glioma or drug-resistant cases, their prognosis is extremely poor. Molecule-targeted intervention may be a novel idea for glioma treatment[8-10].

A growing number of studies have highlighted the vital functions of circRNAs in tumor growth, metastasis and recurrence[14,17]. Jian et al[18] suggested that circ_001680 serves as a tumor-promotive gene. However, the mechanism of circ_001680 in glioma is not clear. Therefore, the objective of this study was firstly to elucidate the oncogenic role of circ_001680 in the occurrence and development of glioma. In this paper, circ_001680 was detected to be upregulated in glioma tissues and positively linked to incidences of lymphatic metastasis and distant metastasis in glioma. We thereafter speculated that circ_001680 may drive the malignant progression of glioma. Subsequently, circ_001680 knockdown model was established in U251 and U87 cells by transfection

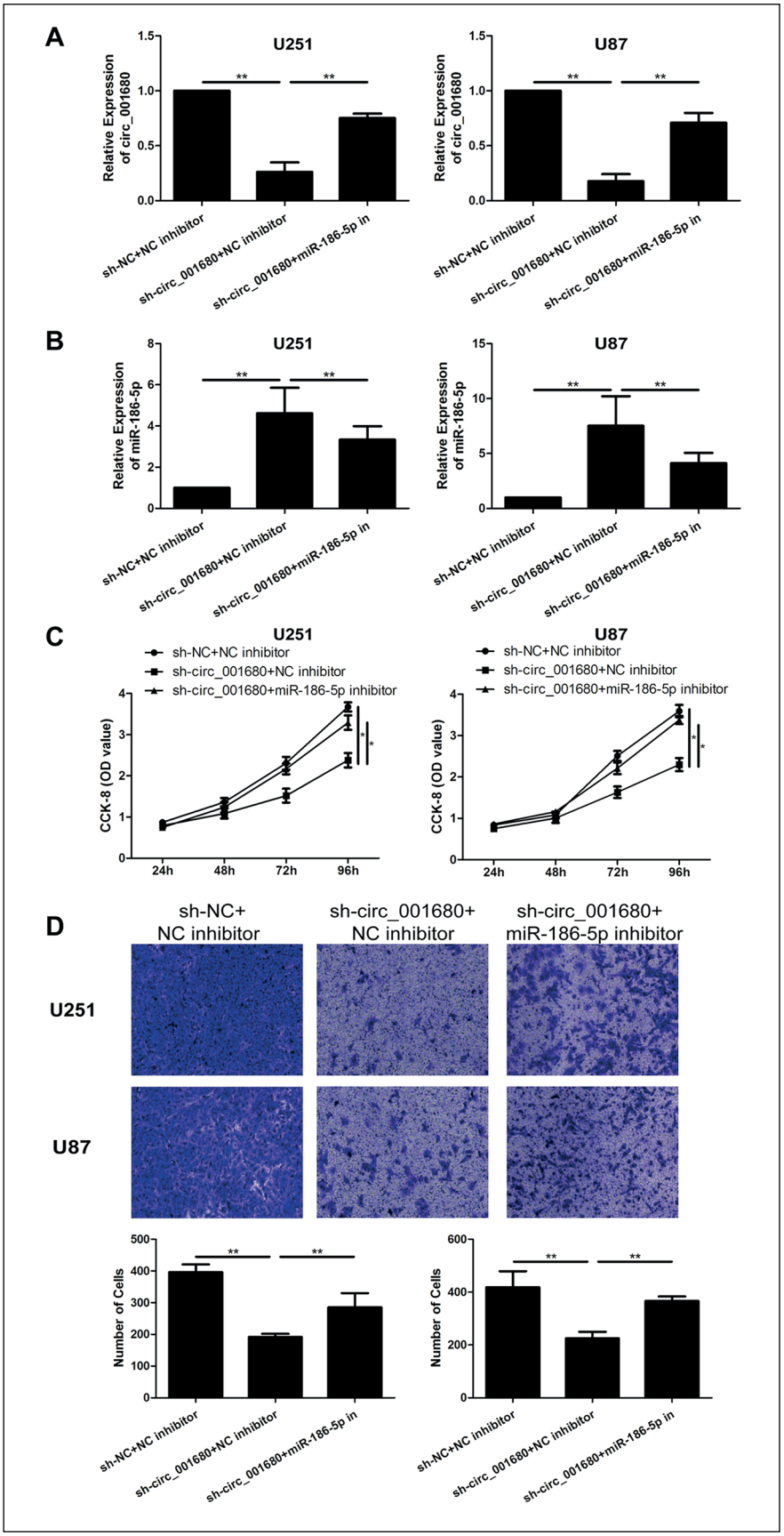

Figure 4. Circ_001680 exerted biological functions in glioma *via* the involvement of miR-186-5p. **A,** Circ_001680 level in U251 and U87 cells co-transfected with sh-NC+NC inhibitor, sh-circ_001680+NC inhibitor or sh-circ_001680+miR-186-5p inhibitor. **B,** MiR-186-5p level in U251 and U87 cells co-transfected with sh-NC+NC inhibitor, sh-circ_001680+NC inhibitor or sh-circ_001680+miR-186-5p inhibitor. **C,** Viability in U251 and U87 cells co-transfected with sh-NC+NC inhibitor, sh-circ_001680+NC inhibitor or sh-circ_001680+miR-186-5p inhibitor. **D,** Migration in U251 and U87 cells co-transfected with sh-NC+NC inhibitor, sh-circ_001680+NC inhibitor or sh-circ_001680+miR-186-5p inhibitor (magnification: 40×). Data were expressed as mean±SD. *$p<0.05$, **$p<0.01$.

of sh-circ_001680. A series of functional experiments have demonstrated that knockdown of circ_001680 inhibited proliferative and metastatic abilities in glioma cells.

Recent attention has been focused on circRNA functions as follows: 1. circRNA's sponge effect on a miRNA; 2. Binding to RNA-binding proteins to inhibit protein activities; 3. mRNA regulations by limited base pairing; 4. Translation template for guiding protein synthesis[15,16,19]. Here, we focused on the sponge effect of circ_001680 and predicted its miRNA targets in online databases. MiR-186-5p was indicated to be the sponged miRNA by circ_001680, which was downregulated in glioma tissues and negatively linked to circ_001680 level. Importantly, miR-186-5p could abolish the regulatory effects of silenced circ_001680 on viability and migratory ability in glioma. Collectively, circ_001680 stimulated the malignant progression of glioma requiring the involvement of miR-186-5p. Our findings proposed a novel idea in the malignant progress of glioma, which might be conducive to improve the diagnosis and treatment of glioma by detecting circ_001680 and miR-186-5p levels.

Conclusions

Circ_001680 stimulates proliferative and metastatic abilities in glioma by negatively regulating miR-186-5p level. High level of circ_001680 is closely linked to lymphatic metastasis, distant metastasis and poor prognosis in glioma patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Schwartzbaum JA, Fisher JL, Aldape KD, Wrensch M. Epidemiology and molecular pathology of glioma. Nat Clin Pract Neurol 2006;2: 494-503.
2) Ostrom QT, Gittleman H, Stetson L, Virk SM, Barnholtz-Sloan JS. Epidemiology of gliomas. Cancer Treat Res 2015; 163: 1-14.
3) Ostrom QT, Bauchet L, Davis FG, Deltour I, Fisher JL, Langer CE, Pekmezci M, Schwartzbaum JA, Turner MC, Walsh KM, Wrensch MR, Barnholtz-Sloan JS. The epidemiology of glioma in adults: a "state of the science" review. Neuro Oncol 2014; 16: 896-913.
4) Bush NA, Chang SM, Berger MS. Current and future strategies for treatment of glioma. Neurosurg Rev 2017; 40: 1-14.
5) Woolf EC, Scheck AC. The ketogenic diet for the treatment of malignant glioma. J Lipid Res 2015; 56: 5-10.
6) Flavahan WA, Drier Y, Liau BB, Gillespie SM, Venteicher AS, Stemmer-Rachamimov AO, Suva ML, Bernstein BE. Insulator dysfunction and oncogene activation in IDH mutant gliomas. Nature 2016; 529: 110-114.
7) Miller JJ, Shih HA, Andronesi OC, Cahill DP. Isocitrate dehydrogenase-mutant glioma: Evolving clinical and therapeutic implications. Cancer-Am Cancer Soc 2017; 123: 4535-4546.
8) Wick W, Weller M, van den Bent M, Sanson M, Weiler M, von Deimling A, Plass C, Hegi M, Platten M, Reifenberger G. MGMT testing--the challenges for biomarker-based glioma treatment. Nat Rev Neurol 2014; 10: 372-385.
9) Gusyatiner O, Hegi ME. Glioma epigenetics: from subclassification to novel treatment options. Semin Cancer Biol 2018; 51: 50-58.
10) Ludwig K, Kornblum HI. Molecular markers in glioma. J Neurooncol 2017; 134: 505-512.
11) Peng Z, Liu C, Wu M. New insights into long noncoding RNAs and their roles in glioma. Mol Cancer 2018; 17: 61.
12) Shi J, Dong B, Cao J, Mao Y, Guan W, Peng Y, Wang S. Long non-coding RNA in glioma: signaling pathways. Oncotarget 2017; 8: 27582-27592.
13) Zhou Q, Liu J, Quan J, Liu W, Tan H, Li W. MicroRNAs as potential biomarkers for the diagnosis of glioma: a systematic review and meta-analysis. Cancer Sci 2018; 109: 2651-2659.
14) Patop IL, Kadener S. circRNAs in cancer. Curr Opin Genet Dev 2018; 48: 121-127.
15) Patop IL, Wust S, Kadener S. Past, present, and future of circRNAs. EMBO J 2019; 38: e100836.
16) Fischer JW, Leung AK. CircRNAs: a regulator of cellular stress. Crit Rev Biochem Mol Biol 2017; 52: 220-233.
17) Zhou R, Wu Y, Wang W, Su W, Liu Y, Wang Y, Fan C, Li X, Li G, Li Y, Xiong W, Zeng Z. Circular RNAs (circRNAs) in cancer. Cancer Lett 2018; 425: 134-142.
18) Jian X, He H, Zhu J, Zhang Q, Zheng Z, Liang X, Chen L, Yang M, Peng K, Zhang Z, Liu T, Ye Y, Jiao H, Wang S, Zhou W, Ding Y, Li T. Hsa_circ_001680 affects the proliferation and migration of CRC and mediates its chemoresistance by regulating BMI1 through miR-340. Mol Cancer 2020; 19: 20.
19) Xiao MS, Ai Y, Wilusz JE. Biogenesis and functions of circular RNAs come into focus. Trends Cell Biol 2020; 30: 226-240.