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Circ_0005075 stimulates the proliferation and metastasis of glioma *via* downregulating SIRT1

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Abstract. – OBJECTIVE: The aim of this study was to uncover the potential influence of circ_0005075 on the malignant progression of glioma and the underlying mechanism.

PATIENTS AND METHODS: Circ_0005075 level in glioma tissues and cell lines was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relation between circ_0005075 expression and metastasis of glioma patients was analyzed. Prognostic potential of circ_0005075 in glioma was assessed by calculating overall survival (OS) and progression-free survival (PFS). After knockdown or overexpression of circ_0005075, changes in the viability, migration, and wound closure percentage of T98-G and U87 cells were examined, respectively. Subsequently, expression pattern and prognostic value of SIRT1 in glioma patients were determined. Furthermore, the involvement of SIRT1 in glioma progression affected by circ_0005075 was evaluated through rescue experiments.

RESULTS: Circ_0005075 was significantly up-regulated in glioma tissues and cell lines. Meanwhile, its expression level was significantly higher in glioma patients with lymphatic metastasis or distant metastasis when compared with those with negative metastasis. OS and PFS were both remarkably worse in glioma patients with high expression level of circ_0005075. Knockdown of circ_0005075 decreased the viability, migration, and wound closure percentage of T98-G cells. However, overexpression of circ_0005075 in U87 cells yielded the opposite trends. SIRT1 expression level was negatively regulated by circ_0005075 in glioma. QRT-PCR results demonstrated that SIRT1 was significantly down-regulated in glioma tissues and cell lines. High level of SIRT1 predicted better prognosis of glioma patients. Rescue experiments confirmed that SIRT1 was responsible for the regulatory role of circ_0005075 in the malignant progression of glioma.

CONCLUSIONS: Circ_0005075 is up-regulated in glioma tissues and correlated with distant metastasis and poor prognosis of glioma patients. Furthermore, it aggravates the malignant progression of glioma by down-regulating SIRT1.

Key Words:
Circ_0005075, SIRT1, Glioma, Metastasis.

Introduction

Glioma is one of the most common primary tumors in the central nervous system[1-3]. Current treatments for glioma include surgical resection, radiotherapy, and temozolomide-based chemotherapy. Unfortunately, the prognosis of high-grade glioma, especially glioblastoma, is extremely poor even after active treatment of surgery and postoperative chemotherapy or radiotherapy[2,4-7]. With the development of medicine, the pathology of glioma has been widely explored[5]. Researchers[8-10] have demonstrated that the occurrence and progression of glioma are very complex. Therefore, it is necessary to uncover the molecular hallmarks for early diagnosis, prognosis evaluation, and target therapy of glioma. Furthermore, some reports[11,12] have discovered many circRNAs that are closely linked to tumor progression.

CircRNAs are a type of single-stranded RNAs with a closed-loop structure. They do not have 5' end and 3' end poly(A) tail in structure[12]. Due to its special looped structure, circRNA is stable and cannot easily be degraded by RNase. Meanwhile, it is conservative in evolution[13,14]. CircRNAs serve as competitive endogenous RNA (ceRNA) to sponge miRNAs[15]. Based on these characteristics, circRNAs may be utilized for biomarkers for drug development[16]. Recently, plenty of differen-

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tially expressed circRNAs have been screened out in tumor tissues by high-throughput sequencing technology[17,18]. These certain circRNAs are of great significance during the occurrence and progression of tumors[19,20]. One study[21] has established a circRNA microarray profiling by analyzing differentially expressed circRNAs in glioma tissues/para-cancerous tissues. Circ_0005075 has been identified to be closely related to the pathological indexes of glioma patients. In addition, it is able to affect the proliferative and metastatic capacities of glioma cells[22,23].

In this paper, we first constructed *in vitro* overexpression and knockdown models of circ_0005075. Meanwhile, its regulatory effects on glioma progression were further investigated. Our findings might provide references for clinical monitor and treatment of glioma.

Patients and Methods

Patients and Glioma Samples

A total of 40 matched glioma tissues and para-cancerous tissues were surgically resected from glioma patients. Tumor staging of glioma was assessed based on the criteria proposed by UICC. Clinical indexes and follow-up data were collected. Informed consent was obtained from patients and their families. This investigation was approved by the Ethics Committee of Huai'an First Hospital Affiliated to Nanjing Medical University. The Declaration of Helsinki has been respected.

Cell Culture

Human glioma cell lines (U251, U87, T98-G, A172) and human brain normal glial cell line (HEB) were provided by ATCC (American Type Culture Collection; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 5% CO_2 incubator at 37°C.

Cell Transfection

Cells were first inoculated into 6-well plates and cultured until 70% of confluence. Cell transfection was conducted according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells for 48 h were harvested for functional experiments. Transfection plasmids were constructed by GenePharma (Shanghai, China).

Cell Counting Kit (CCK-8) Assay

Transfected cells were seeded into 96-well plates at a density of 2×10^3 cells per well. At day 1, 2, 3, and 4, respectively, CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) was added to each well, followed by incubation for 2 h in the dark. Absorbance value at 450 nm was recorded by a micro-plate reader. Finally, the viability curve was plotted.

Transwell Assay

Transfected cells were inoculated into 24-well plate with 3.0×10^5 cells per well. 200 μL of cell suspension was applied in the upper side of transwell chambers (Millipore, Billerica, MA, USA) inserted in a 24-well plate. Meanwhile, 600 μL of complete medium containing 10% FBS was applied to the lower side. After 48 h of incubation, cells penetrated to the bottom side were fixed in methanol for 15 min and dyed with crystal violet for 20 min. Migrating cells were observed under a microscope. Numbers of invasive and migratory cells were counted in 10 randomly selected fields per sample (magnification 200×).

Wound Healing Assay

Cells were seeded into 24-well plates with 5.0×10^5 cells/well. After cell adherence, an artificial wound was created in the confluent cell monolayer using a 200 μL pipette tip. Wound closure images were taken at 0 h (cell adherence) and 24 h using an inverted microscope, respectively. Finally, the percentage of wound closure was calculated.

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

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After purified by DNase I treatment, extracted RNA was reverse transcribed into cDNA using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). Obtained cDNA was subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Ostu, Shiga, Japan). β-actin and U6 were used as internal references. Each sample was performed in triplicate. Relative expression level of genes was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer 5.0 was used for designing qRT-PCR primers. Primer sequences used in this study were as follows: circ_0005075, F: 5'-GCCTAACACGTCGCT-

CAACTCG-3', R: 5'-GACAACATACGTAGAG-CCCG-3'; SIRT1, F: 5'-ATGCTAGTTCGGCAT-GCGACACA-3', R: 5'-CGGTGTGAATGCGT-CAGATTCT-3'; U6: F: 5'- CTCGCTTCGGCAG-CACA-3', R: 5'-AACGCTTCACGAATTTG-CGT-3'; β-actin: F: 5'-CCTGGCACCCAGCA-CAAT-3', R: 5'-GCTGATCCACATCTGCTG-GAA-3'.

Western Blot

Total protein in tissues and cells was extracted, and protein concentration was determined. Subsequently, protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% skimmed milk for 1 h, the membranes were incubated with primary antibodies overnight at 4°C. On the next day, the membranes were incubated with corresponding secondary antibody for 2 h at room temperature, followed by washing with 1×Tris-Buffered Saline and Tween-20 (TBST) for 1 min. Immunoreactive bands were finally exposed by the enhanced chemiluminescent (ECL) substrate kit.

Statistical Analysis

Statistical Product and Service Solution SPSS 22.0 (SPSS IBM Corp., Armonk, NY USA) was used for all statistical analyses. Experimental data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the *t*-test. Kaplan-Meier curves were introduced for survival analysis. $p < 0.05$ was considered statistically significant.

Results

Upregulation of Circ_0005075 in Glioma Tissues and Cell Lines

Compared with para-cancerous tissues, circ_0005075 was significantly up-regulated in glioma tissues (Figure 1A). Similarly, the expression level of circ_0005075 was significantly higher in glioma cells relative to normal glial cells (Figure 1B). Among the four glioma cell lines, T98-G and U87 cells expressed the highest and lowest abundance of circ_0005075, respectively. Therefore, these two cell lines were used for the following experiments.

Circ_0005075 Expression Was Correlated with Metastasis and Prognosis of Glioma Patients

By analyzing the clinical data of enrolled 40 glioma patients, we found that circ_0005075 level was correlated with lymphatic and distant metastasis, rather than age, gender, and tumor staging of glioma patients (Table I). Glioma patients with lymphatic or distant metastasis expressed significantly higher level of circ_0005075 than those with negative metastasis (Figure 1C, 1D). Follow-up data were collected from glioma patients, and the prognostic potential of circ_0005075 was evaluated. Kaplan-Meier curves indicated

Table I. Association of circ_0005075 expression with clinicopathologic characteristics of glioma.

Parameters	No. of cases	Circ_0005075 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.746
< 60	18	10	8	
≥ 60	16	8	8	
Gender				0.746
Male	16	8	8	
Female	18	10	8	
T stage				0.800
T1-T2	22	12	10	
T3-T4	12	6	6	
Lymph node metastasis				**0.038**
No	23	15	8	
Yes	11	3	8	
Distance metastasis				**0.016**
No	22	15	7	
Yes	12	3	9	

that OS and PFS were both remarkably worse in glioma patients with high level of circ_0005075 (Figure 1E, 1F).

Circ_0005075 Influenced Proliferative and Migratory Capacities of Glioma

In vitro circ_0005075 knockdown and overexpression models were constructed in T98-G and U87 cells, respectively (Figure 2A). Transfection of si-circ_0005075 remarkably reduced the viability, migration, and wound closure percentage of T98-G cells. Conversely, transfection of pcDNA-circ_0005075 in U87 cells yielded the opposite results (Figure 2B-2D). These findings suggested that circ_0005075 promoted the proliferation and migration of glioma.

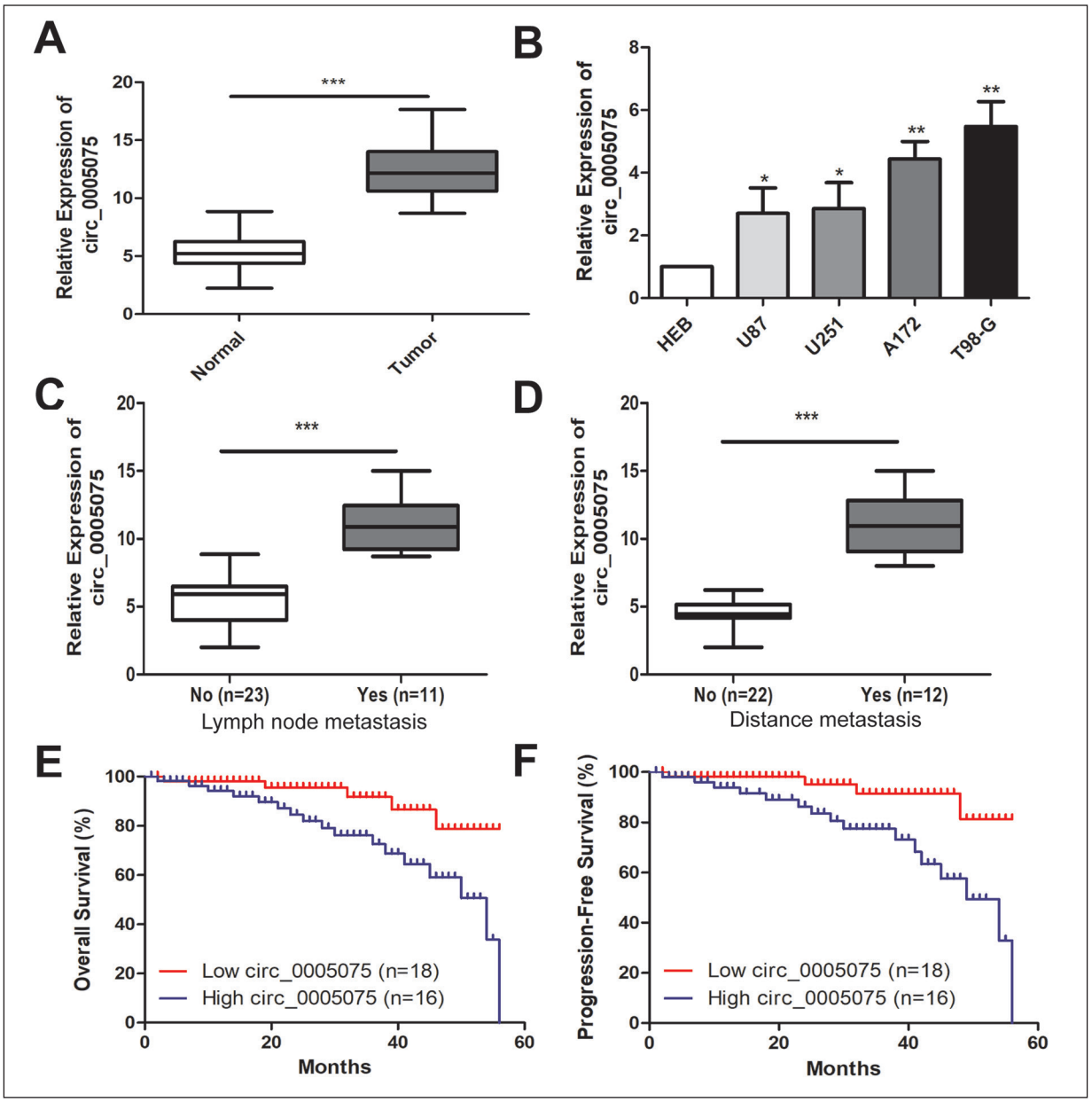

Figure 1. Upregulation of circ_0005075 in glioma tissues and cell lines. **A**, Circ_0005075 level in para-cancerous tissues and glioma tissues. **B,** Circ_0005075 level in normal glial cells and glioma cells. **C,** Circ_0005075 level in glioma patients either with lymphatic metastasis or not. **D,** Circ_0005075 level in glioma patients either with distant metastasis or not. **E,** Overall survival of glioma patients with high and low level of circ_0005075. **F,** Progression-free survival of glioma patients with high and low level of circ_0005075.

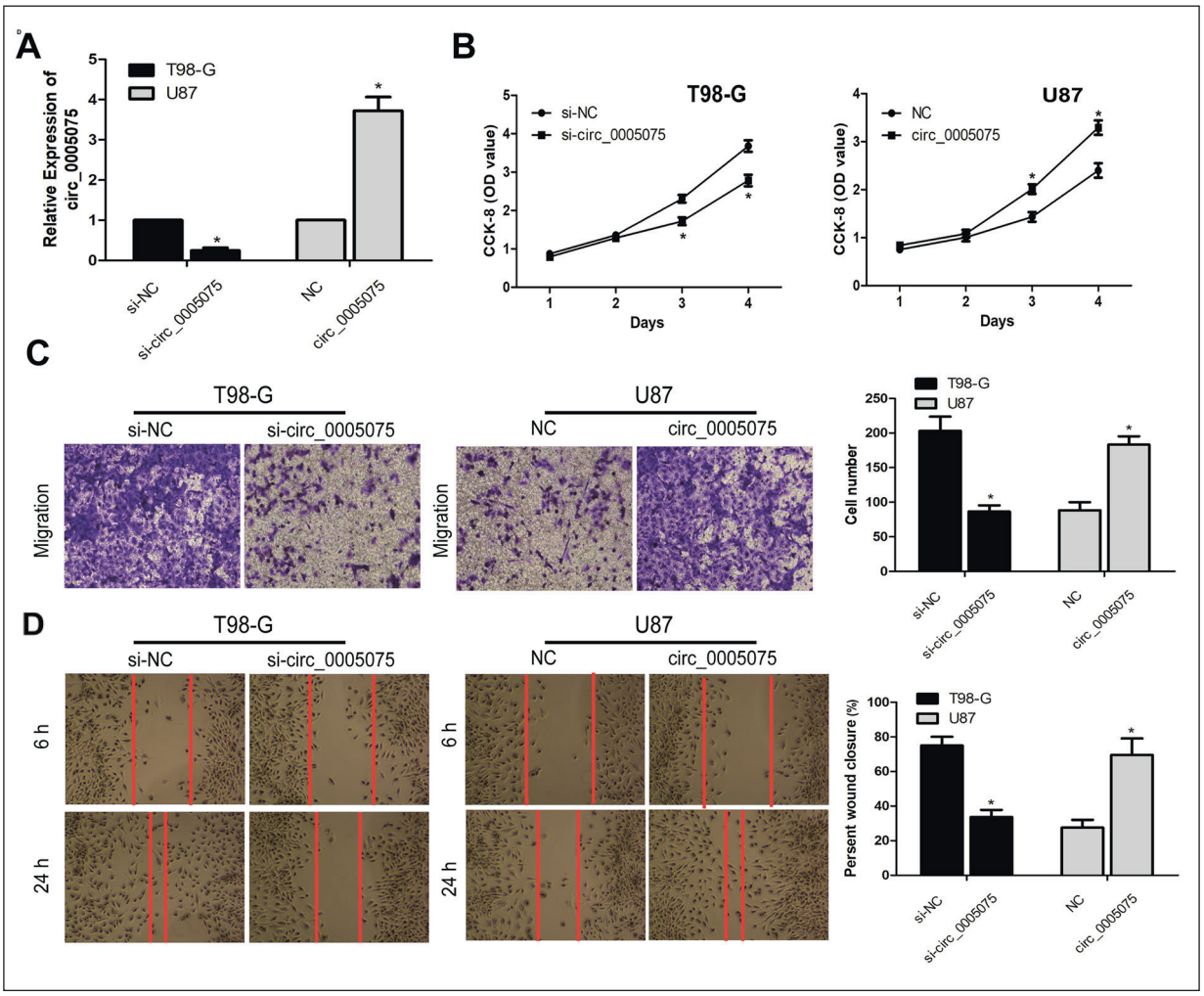

Figure 2. Circ_0005075 influenced proliferative and migratory capacities of glioma cells. T98-G cells were transfected with si-NC or si-circ_0005075. U87 cells were transfected with NC or pcDNA-circ_0005075. **A,** Relative level of circ_0005075. **B,** Viability at day 1, 2, 3 and 4. **C,** Migratory cell number (magnification: 100×). **D,** Wound closure percentage (magnification: 40×).

Downregulation of SIRT1 in Glioma Tissues and Cell Lines

Bioinformatics predicted a potential interaction between circ_0005075 and SIRT1 (data not shown). Both the mRNA and protein levels of SIRT1 were identified significantly upregulated in T98-G cells with circ_0005075 knockdown. However, they were significantly downregulated in U87 cells overexpressing circ_0005075 (Figures 3A, 3B). Subsequently, the expression pattern and prognostic potential of SIRT1 in glioma were explored. The results demonstrated that SIRT1 was markedly downregulated in glioma tissues and cell lines (Figures 3C, 3D). A negative correlation was identified between SIRT1 level and circ_0005075 level in glioma tissues (Figure 3E). Besides, the worse prognosis was observed in glioma patients with low expression level of SIRT1 (Figure 3F).

SIRT1 Was Responsible for Glioma Progression Regulated by Circ_0005075

To further uncover the biological function of SIRT1 in glioma progression, si-SIRT1 and pcDNA-SIRT1 were constructed. Transfection efficacy of si-SIRT1 and pcDNA-SIRT1 in T98-G and U87 cells, respectively, was verified by qRT-PCR (Figures 4A, 4B). Silence of circ_0005075 downregulated circ_0005075 level in T-98G cells, which could be upregulated by co-transfection of si-SIRT1. Meanwhile, overexpression of SIRT1 downregulated the up-regu-

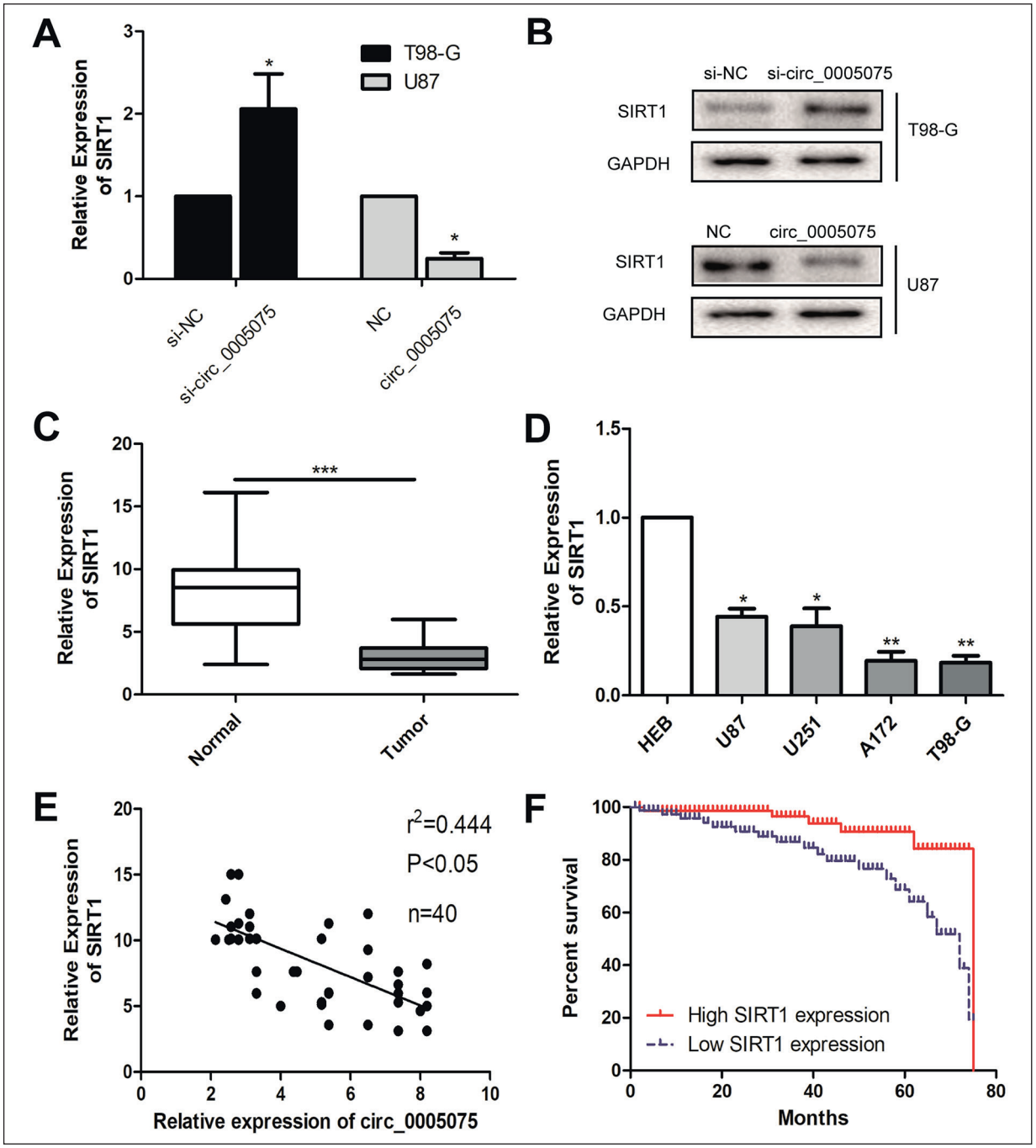

Figure 3. Downregulation of SIRT1 in glioma tissues and cell lines. T98-G cells were transfected with si-NC or si-circ_0005075. U87 cells were transfected with NC or pcDNA-circ_0005075. The mRNA **A,** and protein **B,** levels of SIRT1. **C,** SIRT1 level in para-cancerous tissues and glioma tissues. **D,** SIRT1 level in normal glial cells and glioma cells. **E,** A negative correlation between expression levels of circ_0005075 and SIRT1 in glioma tissues. **F,** Overall survival of glioma patients with high and low level of SIRT1.

lation of circ_0005075 in U87 cells transfected with pcDNA-circ_0005075 (Figure 4C). Notably, attenuated viability and migration of T-98G cells with circ_0005075 knockdown were reversed by co-transfection of si-SIRT1. Co-overexpression of SIRT1 reversed the stimulated viability and migration of U87 cells overexpressing circ_0005075 (Figures 4D, 4E). As a result, SIRT1 was responsible for circ_0005075 in regulating cellular behaviors of glioma.

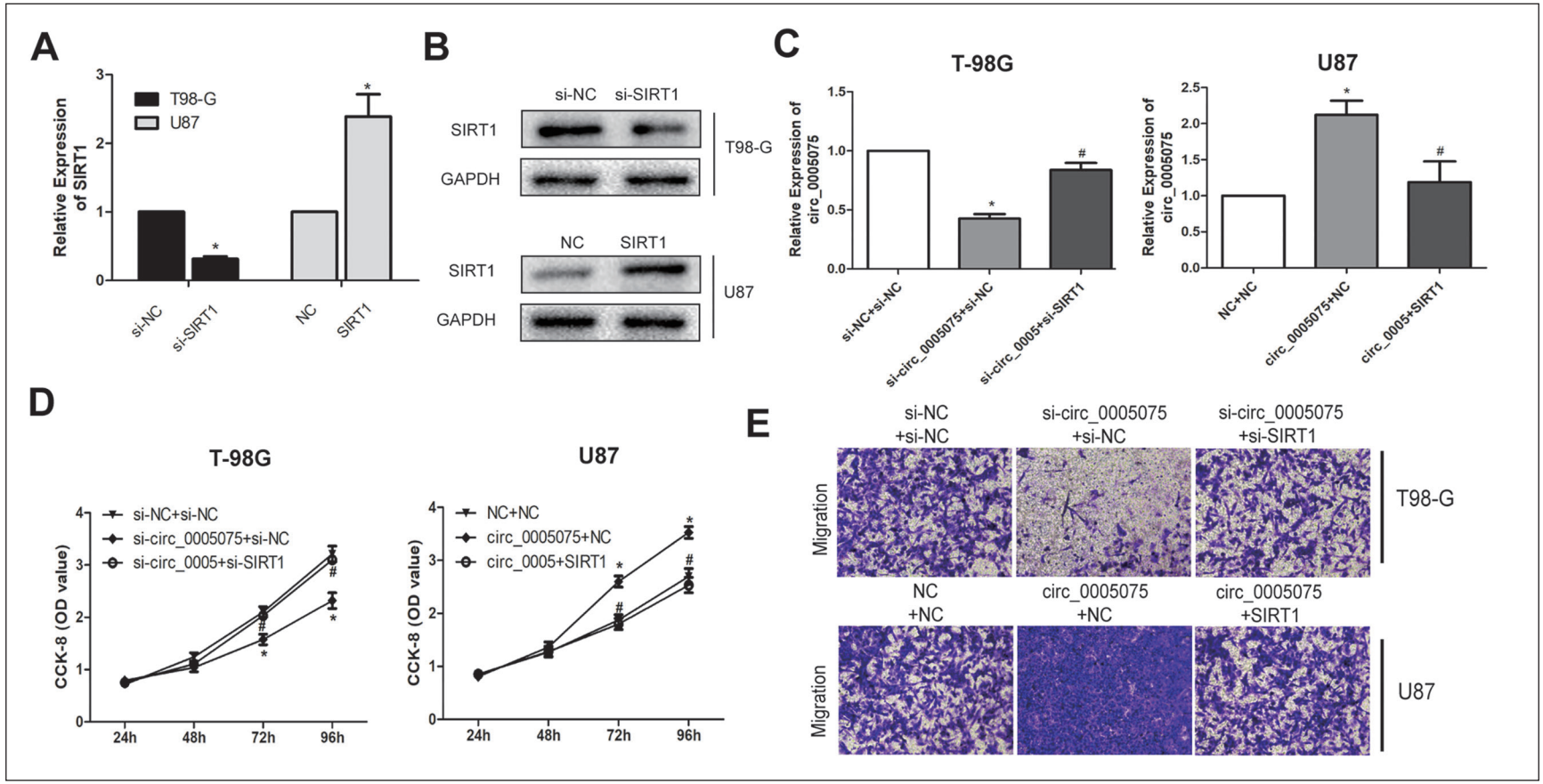

Figure 4. SIRT1 was responsible for glioma progression regulated by circ_0005075. T98-G cells were transfected with si-NC or si-SIRT1. U87 cells were transfected with NC or pcDNA-SIRT1. The mRNA **A,** and protein **B,** levels of SIRT1. T98-G cells were transfected with si-NC, si-circ_0005075 + si-NC or si-circ_0005075 + si-SIRT1. U87 cells were transfected with NC, pcDNA-circ_0005075 + NC or pcDNA-circ_0005075 + pcDNA-SIRT1. **C,** Relative level of circ_0005075. **D,** Viability at day 1, 2, 3 and 4. **E,** Migratory cell number (magnification: 100×).

Discussion

Glioblastoma is the most malignant subtype of glioma, with an average survival of only 14.6 months[1,2]. Even great strides have been achieved in the treatment of glioma, the prognosis of glioma has not been significantly improved[4-6]. Strong invasiveness and metastasis are vital reasons for the poor prognosis of glioma patients[6,7]. With the rapid development of high-throughput sequencing technology and bioinformatics analysis, fundamental researches on tumors have been advanced[8]. Abundant abnormally expressed genes have been searched from tumor tissues for further exploration of their potential functions[8-10]. Recently, mRNAs, miRNAs, and DNA methylation are proposed to be diagnostic markers and therapeutic targets for glioma[10,12]. Cellular behaviors of glioma can also be affected by abnormally expressed genes, thus influencing the progression and prognosis of the disease[11-13].

CircRNA is formed by the cleavage of precursor RNA after shearing. It has structural stability and evolutionary conservation[12-14]. CircRNAs have been found extensively involved in tumor progression[15,16]. Biological functions of differentially expressed circRNAs in tumors are well-studied in recent years[17-20]. In the present research, our findings uncovered that circ_0005075 was significantly up-regulated in glioma. Its level was positively correlated with higher rates of metastasis and worse prognosis of glioma patients. Hence, circ_0005075 might exert a carcinogenic role in the progression of glioma. *In vitro* experiments disclosed that knockdown of circ_0005075 decreased the viability, migration, and wound closure percentage of glioma cells.

Regulatory mechanisms of circRNAs in tumors mainly include (1) inhibition of miRNA functions as miRNA sponges; (2) inhibition of protein activities by binding to proteins; (3) direct regulation on RNAs through complementary base pairing; and (4) guidance of protein synthesis as translation template[15-18]. Our results discovered that SIRT1 was responsible for circ_0005075-induced glioma progression. SIRT1 expression level was negatively regulated by circ_0005075 in glioma tissues. Importantly, SIRT1 could reverse the regulatory role of circ_0005075 in the malignant progression of glioma. As a result, we verified that circ_0005075 aggravated the malignant progression of glioma through negatively regulating SIRT1.

Conclusions

In brief, circ_0005075 was upregulated in glioma tissues and correlated with distant metastasis and poor prognosis of glioma patients. Furthermore, it aggravates the malignant progression of glioma by inhibiting SIRT1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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