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Circ-DONSON promotes malignant progression of glioma through modulating FOXO3

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Abstract. – OBJECTIVE: The aim of this study was to investigate the expression level of circ-DONSON in glioma and to explore its effect on glioma metastasis and the underlying mechanism.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine circ-DONSON expression in 40 paired glioma tumor tissues and adjacent tissues. Meanwhile, the relation between circ-DONSON level and clinical parameters of glioma and the prognosis of patients was analyzed. The expression of circ-DONSON in glioma cell lines was analyzed by qRT-PCR as well. In addition, circs-DONSON silencing model was constructed in glioma cell lines. Cell counting kit-8 (CCK-8), cell scratch, and transwell migration assays were performed to investigate the effect of circ-DONSON on biological functions of glioma cells. Finally, the interplay between FOXO3 and circ-DONSON was explored.

RESULTS: QRT-PCR results revealed that the expression level of circ-DONSON in glioma tumor tissues was remarkably higher than that of adjacent tissues, and the difference was statistically significant ($p<0.05$). Compared with patients with low expression of circ-DONSON, significantly higher prevalence of lymph node or distant metastasis and worse prognosis were observed in patients with high expression of circ-DONSON ($p<0.05$). The proliferation and migration abilities of glioma cells in circ-DONSON silenced group were remarkably suppressed when compared with NC group ($p<0.05$). Additionally, FOXO3 expression was remarkably down-regulated in glioma cell lines and tissues. FOXO3 expression was negatively correlated with circ-DONSON expression. In addition, cell reverse experiment demonstrated that circ-DONSON and FOXO3 can regulate each other, thereby together affecting the malignant progression of glioma.

CONCLUSIONS: Circ-DONSON was remarkably associated with lymph node or distant metastasis, as well as poor prognosis of patients with glioma. Furthermore, it promoted the metastasis of glioma cells *via* regulating FOXO3.

Key Words:
Circ-DONSON, FOXO3, Glioma, Metastasis.

Introduction

Glioma is the most common primary tumor of the central nervous system[1,2]. Currently, treatment methods for glioma mainly include surgical resection, radiotherapy, and chemotherapy with temozolomide. However, even after surgery and postoperative radiotherapy and chemotherapy, the prognosis of high-grade glioma, especially glioblastoma, is still very poor. This seriously threatens human life and health[3,4]. With the development and progress of medicine, people's understanding of glioma has gradually deepened[5,6]. It has been found that the occurrence and development of glioma is an extremely complex process. Therefore, it is of great significance to elucidate the molecular mechanism of glioma and to search for new molecular markers for early diagnosis, prognosis, and treatment targets[7,8].

Some studies[9,10] have found that circRNAs are closely related to the incidence and development of various tumors. CircRNAs (circRNAs) are a class of single-stranded closed circRNAs with neither 5'-terminal nor 3'-terminal poly A tails[11,12]. Due to its special stable structure, it may not be degraded by the RNA enzyme. Therefore, it is evolutionarily conservative[12]. Currently, researches[13,14] have demonstrated that circRNAs play an important role as competitive endogenous RNAs (ceRNAs). Based on these characteristics of circRNAs, researches[14,15] on circRNAs may provide new ideas for drug development and new directions for the study of life evolution as a biomarker. With the development of high-throughput sequencing technology,

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a great number of differentially expressed circRNAs in tumors have been screened out. The important roles of circRNAs in the incidence and development of tumors have been revealed in recent years[16]. These studies mainly focus on liver cancer, colon cancer, cervical cancer, etc. However, few reports[17] have explored the expression and function of circRNAs in glioma[17]. High throughput sequencing results have revealed that circ-DONSON is highly expressed in tumor tissues. All these findings indicate that circ-DONSON may play an important role in glioma progression. In addition, circ-DONSON is densely distributed with multiple binding sites of miRNA on its sequence. Therefore, we determined it as the research target here[18].

Numerous studies[13-15] have found that circRNAs have many specific gene binding sites. This may more effectively regulate the functions of downstream target genes through binding. In the present work, target gene prediction software (MiRanda and Targetscan) was used to predict the correlation between circRNA and mRNA for differentially expressed circRNAs. The interaction network between circRNA and mRNA can provide convenience for further study on the mechanism of circRNA in glioma[15]. According to bioinformatics analysis, circ-DONSON may bind to FOXO3. However, no research has elucidated the mechanism of circ-DONSON on FOXO3 in glioma cells. In this study, the possible roles of circ-DONSON and FOXO3 in the occurrence and development of glioma as well as their molecular regulatory mechanisms were elaborated, respectively. Our findings might help to bring new ideas for the diagnosis and treatment of glioma.

Patients and Methods

Patients and Glioma Samples

Glioma tissue samples and para-cancerous tissues were collected from 40 patients undergoing glioma radical resection. All patients did not receive any radiotherapy or chemotherapy before surgery. The pathological classification and staging criteria of glioma were performed according to the international collateral cancer staging criteria (international union against cancer, UICC). Informed consent was obtained from patients and their families before the study. This investigation was approved by the Ethics Oversight Committee of our hospital.

Cell Lines and Reagents

Human glioma cell lines (U251, U87, T98-G, A172) and human brain normal glial cell line (HEB) were purchased from ATCC (American Type Culture Collection; Manassas, VA, USA). High glucose Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). All cells were cultured in DMEM high glucose medium containing 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) in a 37°C incubator with 5% CO_2.

Cell Transfection

Negative control (NC) and siRNA containing circ-DONSON interference sequence (circ-DONSON-S) were purchased from Shanghai Jima Company (Shanghai, China). A172 and U251 cells were first seeded into 6-well plates and cultured to a cell density of 50% to 70%. Cell transfection was performed according to the manufacturer's instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 h later, transfected cells were collected for quantitative Real Time Polymerase Chain Reaction (qRT-PCR) analysis and cell function experiments.

Cell Proliferation Assay

Cell proliferation was examined using Cell Counting Kit-8 kit (CCK-8; Dojindo Laboratories, Kumamoto, Japan). 48 h after transfection, the cells were harvested and plated into 96-well plates at a density of 2000 cells per well. After culture for 24 h, 48 h, 72 h, and 96 h, respectively, CCK-8 reagent was added to each well, followed by incubation for 2 h in the dark. Optical density (OD) value of each well at the absorption wavelength of 490 nm was measured by a micro-plate reader.

Cell Wound Healing Assay

Transfected cells for 48 h were digested, centrifuged, and re-suspended in serum-medium. The density of cells was adjusted to 5 x 10^5 cells/mL. The density of plated cells was determined according to the size of cells (the majority of the number of cells plated was set to 50000 cells/well). On the next day, the confluency of cells could reach 90% or more. After the stroke, the cells were rinsed gently with phosphate-buffered saline (PBS) for 2-3 times, and added with low-concentration serum medium. Next, the cells were observed again after 24 h. Finally, the dif-

ference in cell healing ability was judged according to the migration area.

Transwell Assay

Cells in each treatment group were separately trypsinized, and plated into 24-well plates of transwell chamber. 100 μL (cell density: 1×10^5 cells/mL) of cell suspension was added to the upper chamber. Meanwhile, 250 μL medium supplemented with 10% FBS was added to the lower chamber. Subsequently, the cells were cultured for 48 h at 37°C, and the chamber was taken out. Next, the cells in the upper chamber of the microporous membrane were wiped off with a cotton swab, followed by washing carefully with PBS for 2 times. Then, the cells adhered to the microporous membrane of the chamber were fixed with 4% paraformaldehyde for 15 min and stained with crystal violet for 15 min. Afterwards, the chamber was washed with PBS and dried. Migrating cells were finally observed under a 100-fold inverted microscope.

QRT-PCR

Quantitative Real Time-Polymerase Chain Reaction was used to examine the mRNA expressions of FOXO3, β-actin, and circ-DONSON in glioma tissues and cells. Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan) reverse transcription kit. Primers were designed using Primer 5.0 software. Specific qRT-PCR reaction was performed using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Three replicate wells were repeated for each sample, and each experiment was repeated three times. Bio-Rad (Hercules, CA, USA) PCR instrument was used to analyze and process the data. β-actin and U6 were used as internal parameters. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: circ-DONSON, F: 5'-CCACATCGCGCTGGTTACGTC-3', R: 5'-GACTACGATCGTCGTCAAGGCA-3'; FOXO3, F: 5'-GAAGTCAACAGTCGTCGACG-3', R: 5'-GAGTGAGTATCGAGAGCCG-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'; β-actin: F: 5'-CCTGGCACCCAGCACAAT-3', R: 5'-GCTGATCCACATCTGCTGGAA-3'.

Western Blot

Tissue or cells were first added with pre-cooled tissue lysate on ice, followed by lysis on ice for 30 min. Protein concentration was determined by the Bradford method. Subsequently, protein samples were denatured in a water bath at 100°C for 5 min, and an appropriate amount of the loading buffer was applied for electrophoresis. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Roche, Basel, Switzerland). After blocking with 5% skimmed milk for 2 h, the membranes were incubated with primary antibodies (FOXO3, PTEN, AKT, Erk) on a 4°C shaker overnight. On the next day, the membranes were incubated with corresponding secondary antibody (concentration: 1:1000) for 2 h at room temperature. Immunoreactive bands were exposed by enhanced chemiluminescence reagent (ECL) method. Gray value was finally scanned and used for further analysis.

Statistically Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. The *t*-test was used to compare measurement data. Categorical variables were analyzed by χ^2-test or Fisher's exact probability method. Survival analysis was performed using the Kaplan-Meier method, and survival curves were plotted. Experimental data were expressed as mean ± standard deviation. $p<0.05$ was considered statistically significant.

Results

Circ-DONSON Was Highly Expressed in Glioma Tissues and Cell Lines

To clarify the role of circ-DONSON in glioma, we first collected 40 pairs of glioma tissues and adjacent tissues. Circ-DONSON level in tissues was detected using qRT-PCR. Compared with para-cancerous tissues, the expression level of circ-DONSON in glioma tissues was remarkably up-regulated, and the difference was statistically significant ($p<0.05$) (Figures 1A and 1B). In addition, circ-DONSON level in glioma cell lines was remarkably higher than that of normal cells, especially in A172 and U251 cells ($p<0.05$). These results suggested that circ-DONSON might serve as an oncogene in glioma (Figure 1C).

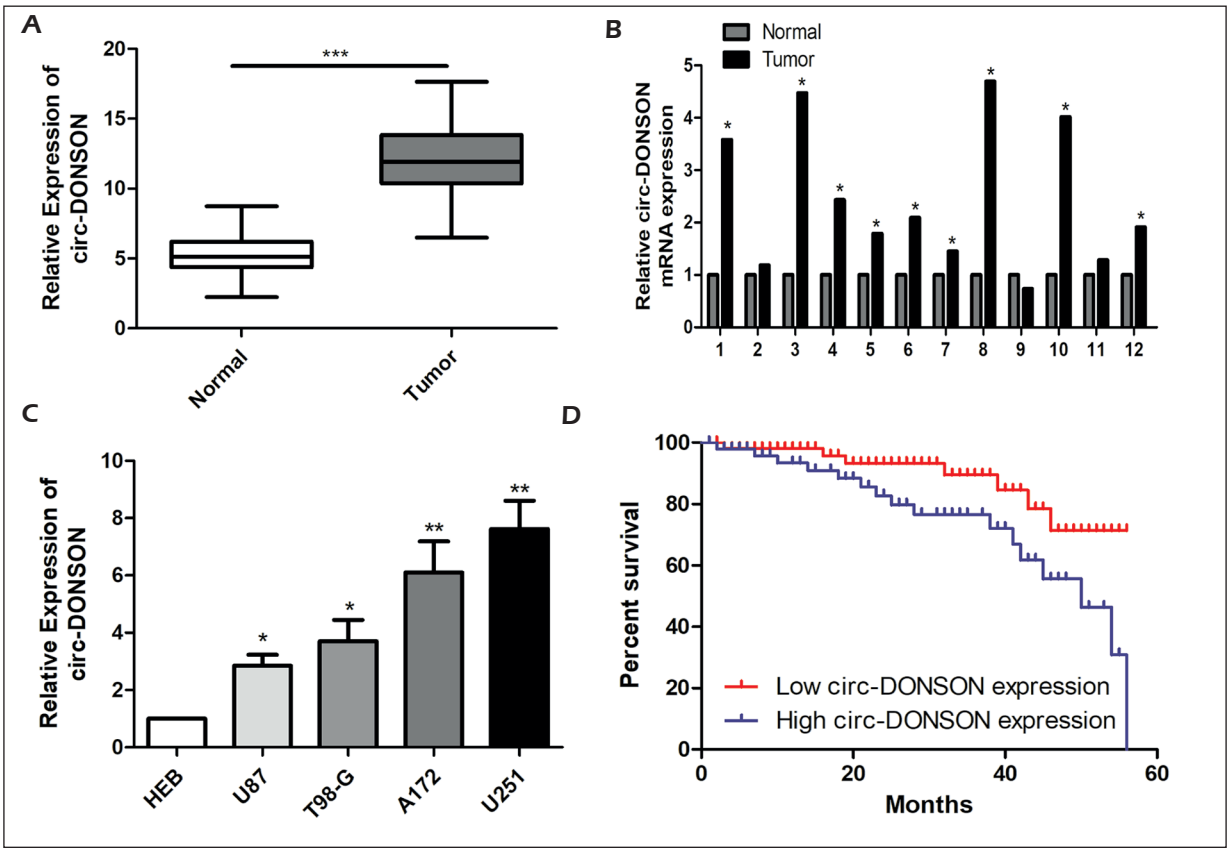

Figure 1. Circ-DONSON was highly expressed in glioma tissues and cell lines. **A,** QRT-PCR was used to detect the expression of circ-DONSON in glioma tissues and adjacent tissues. **B,** QRT-PCR was used to detect the expression of circ-DONSON in 12 glioma tissues and adjacent tissues. **C,** QRT-PCR was used to detect the expression level of circ-DONSON in glioma cell lines. **D,** Kaplan Meier survival curve of glioma patients based on circ-DONSON expression; the prognosis of patients with high expression was significantly worse than that of patients with low expression. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Circ-DONSON Expression Was Correlated with Lymph Node, Distance Metastasis, and Poor Prognosis of Glioma Patients

According to the mRNA level of circ-DONSON, glioma patients were divided into two groups, including high circ-DONSON expression group and low circ-DONSON expression group. The relation between the expression of circ-DONSON and age, sex, pathological stage, lymph node metastasis, and distant metastasis of glioma patients was analyzed. As shown in Table I, low expression of circ-DONSON was positively correlated with glioma lymph node metastasis and distant metastasis, whereas it was not associated with age, gender, and pathological stage. In addition, to explore the relation between the expression of circ-DONSON and the prognosis of patients with glioma, relevant follow-up data were collected. Kaplan-Meier survival curves revealed that high expression of circ-DONSON was remarkably associated with poor prognosis (p<0.05; Figure 1D). These results demonstrated that circ-DONSON level was correlated with lymph node, distance metastasis, and overall survival of glioma patients.

Knockdown of Circ-DONSON Inhibited Cell Proliferation and Migration

To explore the effect of circ-DONSON on glioma *in vitro,* circ-DONSON knockout expression model was successfully constructed and verified by qRT-PCR (Figure 2A). Cell proliferation, migration, and crawling experiments were performed in A172 and U251 cell lines, respectively. CCK-8 results showed that the proliferation ability of cells in the circ-DONSON silenced group was remarkably lower than that of NC group, and the difference was statistically significant (p<0.05, Figure 2B). In addition, the effects of

Table I. Association of circ-DONSON expression with clinicopathologic characteristics of glioma.

Parameters	No. of cases	Circ-DONSON 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	

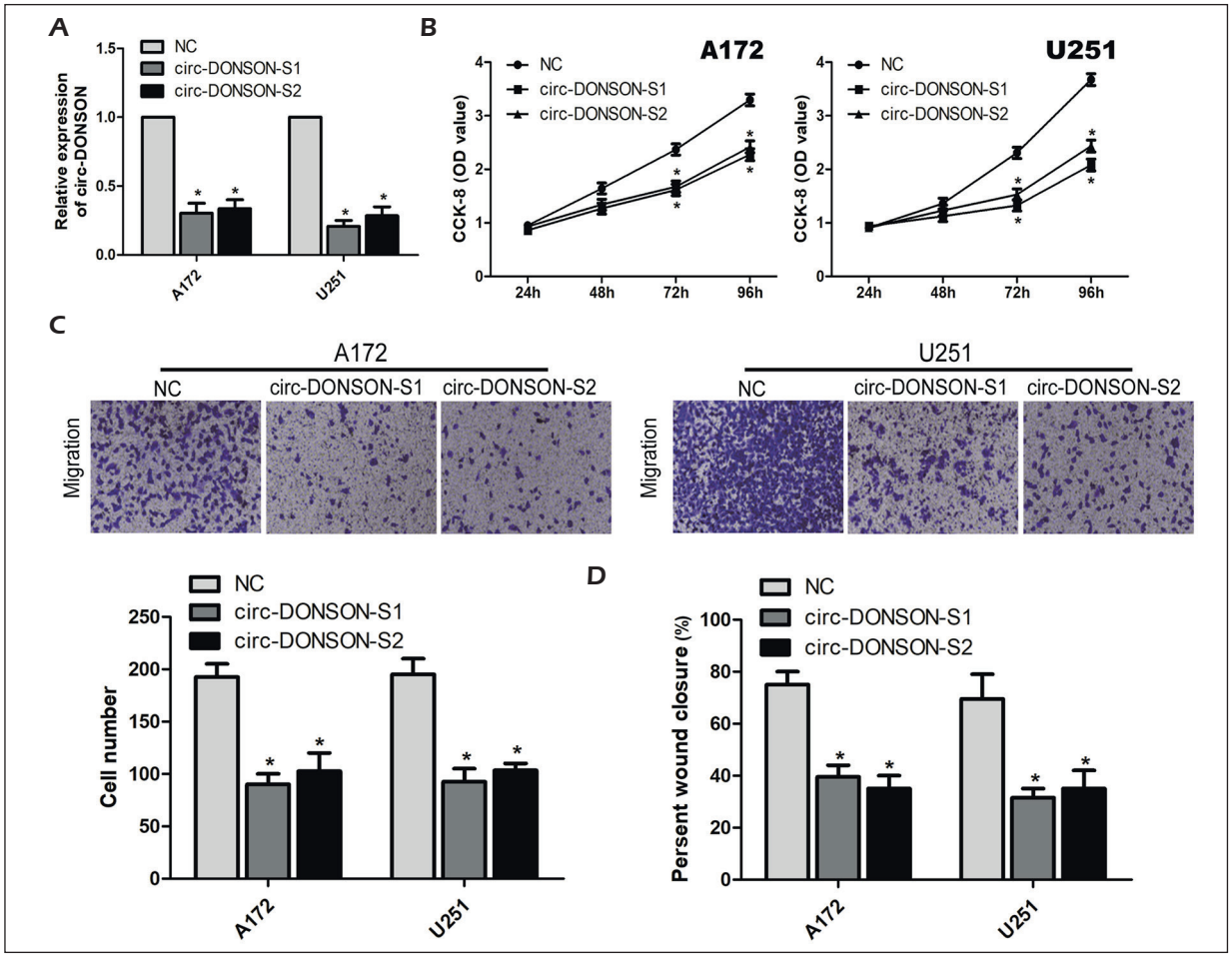

Figure 2. Silencing circ-DONSON inhibited glioma cell proliferation and metastasis. **A,** QRT-PCR verified the interference efficiency of circ-DONSON after transfection of circ-DONSON knockdown vector in A172 and U251 cell lines. **B,** CCK-8 assay detected the role of circ-DONSON knockdown vectors in promoting the proliferation of A172 and U251 cell lines. **C,** Transwell migration assay detected the ability of circ-DONSON knockdown vector in promoting the migration of A172 and U251 cell lines (magnification: 200×). **D,** Cell scratch assay detected the effect of circ-DONSON knockdown vectors on promoting the crawling ability of CYC and U251 cell lines. Data were expressed as mean ± SD, *$p<0.05$.

circ-DONSON on the metastatic ability of glioma cells were explored using transwell migration and cell scratch experiments. The results revealed that compared with NC group, the number of transmembrane glioma cells was remarkably reduced in circ-DONSON silenced group (p<0.05). This suggested that the migration ability was inhibited after knockdown of circ-DONSON (Figure 2C). In addition, cell scratch test reflected significantly decreased crawling ability of circ-DONSON silenced glioma cells (p<0.05, Figure 2D). These results suggested that knockdown of circ-DONSON inhibited proliferation and migration of glioma cells.

FOXO3 Was Lowly Expressed in Glioma Tissues and Cell Lines

Bioinformatics revealed that there might be some association between circ-DONSON and FOXO3 in glioma. QRT-PCR results demonstrated that silencing circ-DONSON remarkably up-regulated FOXO3 expression (Figure 3A). Similarly, Western Blot showed that silencing of circ-DONSON remarkably increased FOXO3 expression, while decreased PTEN, AKT, and Erk protein expressions in A172 and U251 cell lines. These findings indicated that circ-DONSON was closely correlated with FOXO3-related proteins (Figure 3B). Subsequently, we found that FOXO3 expression was remarkably reduced in glioma tissues when compared with para-cancerous tissues (Figure 3C). In addition, FOXO3 was remarkably down-regulated in glioma cells than HEB cells, and the difference was statistically significant (p<0.05, Figure 3D). Therefore, the expressions of circ-DONSON and FOXO3 in 40 glioma tissues and adjacent tissues were detected by qRT-PCR. The results demonstrated that circ-DONSON expression was negatively correlated with FOXO3 in glioma tissues (Figure 3E).

Circ-DONSON Modulated FOXO3 Expression in Human Glioma Cells

To further explore the underlying mechanism in which circ-DONSON promoted the

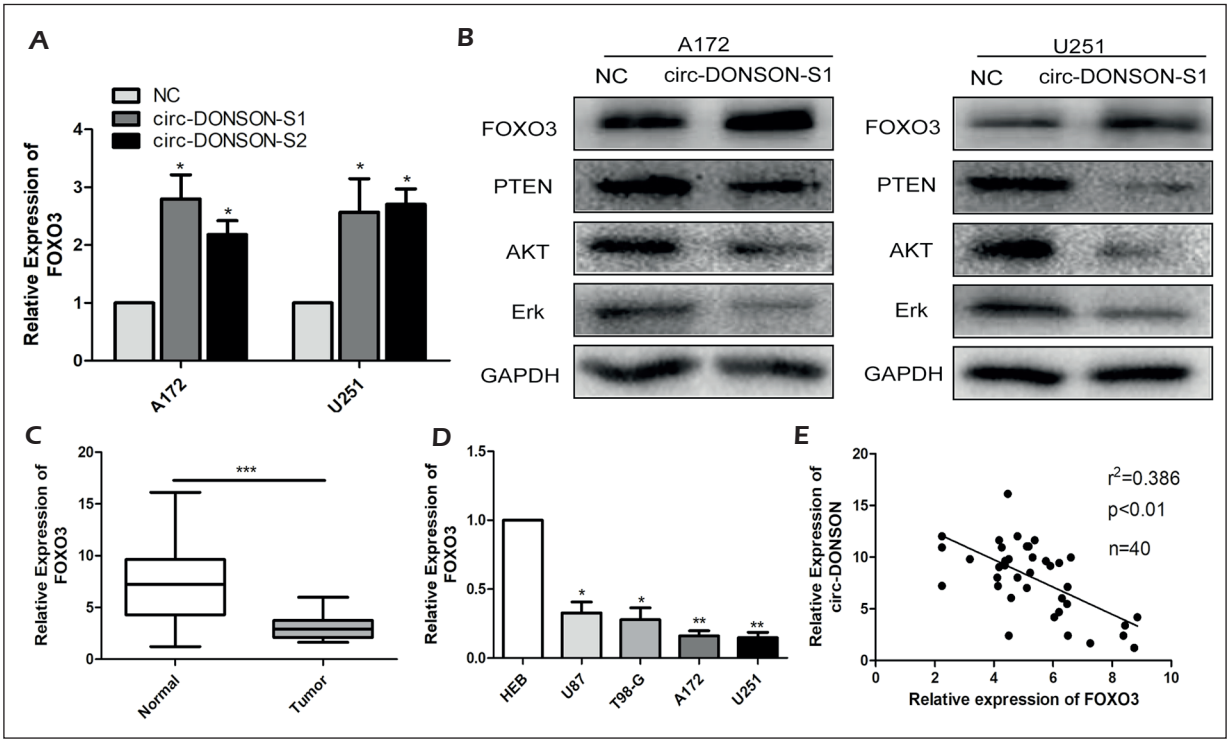

Figure 3. FOXO3 was lowly expressed in glioma tissues and cell lines. **A,** QRT-PCR was used to detect the expression level of FOXO3 after transfection of circ-DONSON knockdown vector. **B,** Western blot verified the changes in the protein expression levels of FOXO3, PTEN, AKT, Erk after transfection of circ-DONSON knockdown vector in A172 and U251 cell lines. **C,** QRT-PCR was used to detect the expression of FOXO3 in glioma tissues and adjacent tissues. **D,** QRT-PCR was used to detect the expression level of FOXO3 in glioma cell lines. **E,** There was a significant negative correlation between the expression levels of circ-DONSON and FOXO3 in glioma tissues. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

malignant progression of glioma, FOXO3 was silenced in circ-DONSON-silenced A172 and U251 cell lines. QRT-PCR was performed to verify the transfection efficiency (Figure 4A). Our results confirmed that silencing of FOXO3 remarkably reversed the effect of circ-DONSON silencing on cell proliferation, migration, and crawling ability. All these findings revealed that circ-DONSON regulated glioma progression by modulating FOXO3 level (Figure 4B-4D).

Discussion

Glioblastoma is the most malignant type of brain glioma, with an average survival of only 14.6 months[1-3]. Surgery, adjuvant chemo-radiotherapy, and other treatments for glioma have made considerable progress in recent years. However, the prognosis of glioma patients has not been remarkably improved[4-6]. Strong migration ability of glioma cells is an important reason for the poor prognosis of glioma patients[7]. Multiple studies[8-10]

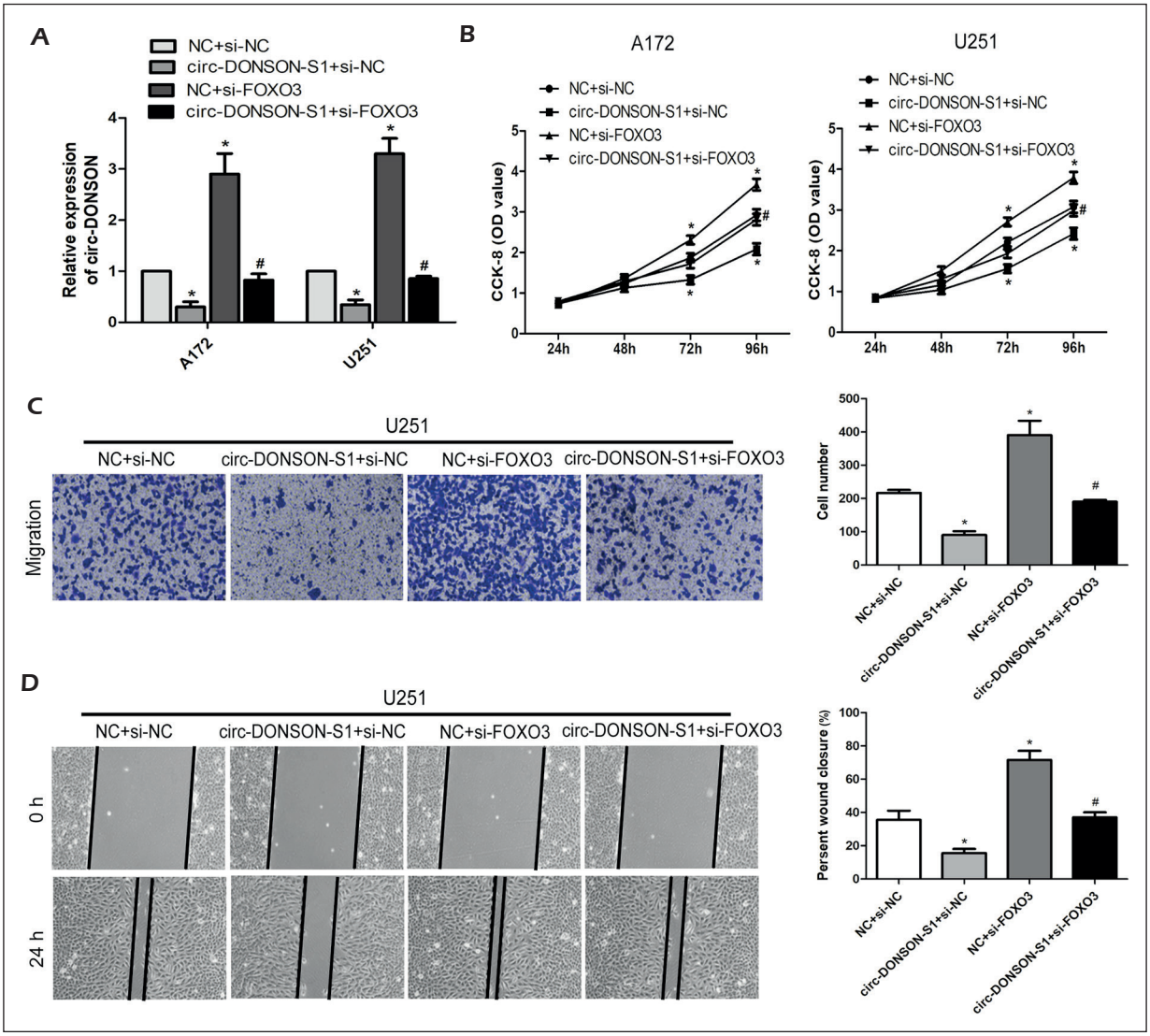

Figure 4. Circ-DONSON could regulate the expression of FOXO3 in glioma tissues and cell lines. **A,** Circ-DONSON expression in circ-DONSON and FOXO3 co-transfected cell lines was detected by qRT-PCR. **B,** CCK-8 assay was used to detect the proliferation of glioma cells after co-transfection of circ-DONSON and FOXO3. **C,** Transwell migration assay was used to detect the migration of glioma cells after co-transfection of circ-DONSON and FOXO3 (magnification: 200×). **D,** Cell scratch assay was used to detect the crawling ability of glioma cells after co-transfection of circ-DONSON and FOXO3 (magnification: 200×). Data were expressed as mean ± SD, $*^{\#}p<0.05$.

on non-coding RNAs have shown that abnormal expression of microRNA and/or lncRNA can regulate the migration ability of glioma cells. However, studies on circRNA regulating glioma cell migration have not been reported.

Rapid development of high-throughput sequencing technology and bioinformatics analysis has brought great convenience to the basic research of tumors[9,10]. Currently, a large number of abnormally expressed genes in tumors have been found. Combined with bioinformatics analysis, researchers are able to conduct further studies on targeted genes with more directional orientation[10]. In recent years, increasing molecules, including mRNA, microRNA and DNA methylation, have been proposed as diagnostic markers, drug targets, and prognostic factors for glioma[8,10]. Abnormally expressed RNA plays a crucial role in the occurrence and development of tumors[9]. CircRNA is a single-chain circular closed structure formed by connecting the head and tail of precursor RNA after splicing, with structural stability and evolutionary conservatism[11,12]. In recent years, with the rapid development of deep RNA sequencing and bioinformatics technology, more and more circRNAs have been discovered. Researchers have reported[13-15] that circRNAs play an important role in the development and progression of various malignant tumors. CircRNAs mainly function as microRNA sponges, which can directly regulate other RNAs through base complementary pairing. In addition, a small number of circRNAs can be used as templates for protein synthesis[13-16]. Therefore, understanding the role and mechanism of circRNAs in malignant tumors is especially significant.

With the in-depth research of circRNA, the role of circRNAs in the incidence and development of malignant tumors has been widely reported[11,12]. In this study, we focused on the effect of circ-DONSON on glioma cell biology. Our results found that circ-DONSON was remarkably up-regulated in glioma, suggesting that circ-DONSON exhibited a cancer-promoting effect in glioma. In addition, circ-DONSON expression was positively correlated with lymph node metastasis and distant metastasis. Tumor metastasis is the process, in which tumor cells fall off in situ and spread to the remote target organs and adapt to the new tissue microenvironment. The two necessary conditions for the smooth realization of the process include movement and survival[19,20]. To further explore the effect of circ-DONSON on the biological function of glioma, a circ-DONSON knockout model was established using lentivirus transfection. CCK-8, cell scratch assay, and transwell migration assay revealed that circ-DONSON could promote the metastasis of glioma cells, thereby playing an important role in glioma. However, the specific molecular mechanism was not clear.

To clarify the biological function of circRNA, we further looked up for its target genes and explored the effect of its interaction with mRNA on the development of glioma[13-15]. The expression of FOXO3 was detected by qRT-PCR, and the results revealed that FOXO3 was markedly down-regulated in glioma tissues compared with adjacent tissues. In addition, the expression level of FOXO3 decreased remarkably in glioma cell lines. Circ-DONSON silencing significantly up-regulated the mRNA and protein levels of FOXO3. Rescue experiment verified that silencing FOXO3 could remarkably reverse the proliferation and crawling ability of cells in circ-DONSON silencing group, thereby counteracting the role of circ-DONSON in glioma progression. Therefore, we believed that circ-DONSON might promote the malignant progression of glioma by regulating FOXO3.

Conclusions

Altogether, circ-DONSON may promote the progression of glioma by regulating FOXO3. Meanwhile, circ-DONSON was remarkably associated with lymph node metastasis and distant metastasis and poor prognosis of glioma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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