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Circ-ABCB10 acts as an oncogene in glioma cells *via* regulation of the miR-620/FABP5 axis

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Abstract. – OBJECTIVE: This study aims to investigate the biological function of circular RNA ABCB10 (circ-ABCB10) in regulating the progression of glioma and to study the possible underlying mechanisms.

PATIENTS AND METHODS: The expression levels of circ-ABCB10, miR-620 and FABP5 mRNA in glioma tissues, normal surrounding tissues and glioma cell lines were measured by Real-time PCR (RT-PCR). Circ-ABCB10 was silenced by siRNA in glioma cell lines (U87, T98G). The proliferation, migration and invasion of glioma cells were measured by MTT, wound healing and transwell assays, respectively. The relationship between circ-ABCB10, miR-620 and FABP5 was tested by Dual-Luciferase assay. The expression of proteins was measured by Western blot. The cell cycle distribution and apoptosis were measured by flow cytometry.

RESULTS: The expression levels of circ-ABCB10 and FABP5 in glioma tissues and cells were significantly higher than those in their normal counterparts. Moreover, the expression of miR-620 was lower in glioma tissues. Silencing of circ-ABCB10 in glioma cells significantly inhibited the proliferation, migration and invasion of glioma cells. Moreover, downregulation of circ-ABCB10 induced cell cycle arrest and apoptosis in glioma cells. Furthermore, inhibition of miR-620 showed the opposite effects to silencing circ-ABCB10 on glioma cells. Dual-Luciferase reporter assays demonstrated that circ-ABCB10 could bind to miR-620 and that FABP5 was a direct target of miR-620. Western blot results showed that circ-ABCB10 could stabilize the expression of FABP5, while miR-620 decreased the expression of FABP5. Furthermore, overexpression of FABP5 abrogated the silencing effects of circ-ABCB10 in glioma cells.

CONCLUSIONS: These data suggest that circ-ABCB10 affects glioma progression by regulating the miR-620/FABP5 axis, and circ-ABCB10 might be used as a potential target for the treatment of glioma.

Key Words:
Glioma, CircRNA, MiR-620, FABP5.

Abbreviations

CNST = central nervous system tumours; FITC = fluorescein 5-isothiocyanate; PBS = phosphate-buffered saline; RIPA buffer = radioimmune precipitation buffer; PVDF membrane = polyvinylidene difluoride membrane; ECL = enhanced chemiluminescence; ANOVA = analysis of variance; UTR = untranslated regions; NHAs = normal human astrocytes; MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; PI = propidium iodide; FABP = fatty acid binding proteins.

Introduction

Glioma is the most common malignant tumour among central nervous system tumours (CNST) affecting adults and which causes high mortality and morbidity worldwide[1]. Although great efforts have been made in the past decades to treat glioma, the prognosis of glioma patients is still dismal. Therefore, there is an urgent need to investigate the molecular mechanisms underlying the initiation and development of glioma and to identify potential therapeutic targets for glioma treatment[2].

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Noncoding RNAs (ncRNAs) have received great attention in recent years due to their complex roles in the initiation and progression of various human diseases. Based on their size and structure, ncRNAs can be classified into long noncoding RNAs (lncRNAs), microRNAs (miRNAs) and the newly identified circular RNAs (circRNAs)[3]. CircRNAs are a group of single-stranded closed circular RNAs that are characterized by stable structure, evolutionary conservation and tissue specificity[4]. CircRNAs are predominantly localized in the cytoplasm of cells, where they can interact with miRNAs to thereby regulate the target genes of miRNAs[5]. CircRNAs play essential roles in the progression of glioma. Circ-SMAD7 promoted glioma cell proliferation and metastasis *via* upregulation of PCNA[6]. Circ-PTN also acted as an oncogene in glioma *via* interaction with miR-122 and further regulation of SOX6[7]. Although several studies have confirmed the important roles of circRNAs in glioma, the molecular mechanisms by which circRNAs affect the progression of glioma remain largely elusive.

Circ-ABCB10 is a newly identified circRNA that exerts oncogenic effects in various cancers. For example, circ-ABCB10 could enhance the proliferation of breast cancer cells by sponging miR-1271[8]. In addition, circ-ABCB10 was also found to be upregulated and to promote tumour progression of renal carcinoma cells[9]. However, whether circ-ABCB10 has any role in the regulation of glioma development is still elusive. In the present study, we investigated the biological function of circ-ABCB10 and are the first to propose an effect of the circ-ABCB10/microRNA-620/FABP5 regulatory network on glioma progression.

Patients and Methods

Clinical Samples

This study was approved by the Ethics Committee of Lishui People's Hospital. Forty glioma tumour tissue samples (WHO grades I-IV) and adjacent normal tissues were collected at the Lishui People's Hospital from July 2017 to August 2018 and stored at -80 degrees. All patients signed an informed consent form prior to the experiment.

Cell Culture and Transfection

The glioma cell lines U251, A172, U87, and T98G were purchased from Chinese Type Culture Collection (Chinese Academy of Sciences, Shanghai, China). Cells were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 1% penicillin and 1% streptomycin (Life Technologies, Carlsbad, CA, USA). Cells were cultured in a 37°C, 5% CO_2 incubator. Cells in the logarithmic growth phase were transfected with negative control siRNA (si-NC), circ-ABCB10 siRNA (si-circ-ABCB10), FABP5 siRNA (si-FABP5), negative control miRNA inhibitor (miR-NC inhibitor), miRNA-620 inhibitor (miR-620 inhibitor), empty vector (pcDNA3.1), or FABP5 overexpression vector (pcDNA3.1 FABP5; GenePharma, Suzhou, China). Transfections were carried out using Lipofectamine 2000 according to the manufacturer's guidelines (Life Technologies, Carlsbad, CA, USA).

RNA Purification and RT-PCR

TRIzol reagent (Life Technologies, Carlsbad, CA, USA) was used to extract total RNA from tissues/cells according to the manufacturer's instructions. RNA was reverse transcribed using a PrimeScript™ RT Master Mix Kit (Takara, Dalian, China). RT-PCR was performed using SYBR Green qPCR Mix (Takara, Dalian, China) and a QuantStudio™ Dx system (Applied Biosystems, Singapore). Levels of circRNA and mRNA were normalized to GAPDH levels, and miRNA levels were normalized to U6 RNA levels. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

MTT Assay

To measure cell viability, we performed the MTT assay as described before[10]. Briefly, cells were plated into 96-well plates at a density of 1×10^6 cells/well. After cell culture for 24 h, the cells were subjected to different treatments for another 24 h, and 20 µl of MTT solution (Solarbio Biotechnology, Beijing, China) was added to each well and incubated at 37°C for 4 h. Then, stop solution (200 µl) was added into each well, and the absorbance was read at 450 nm.

Cell Cycle and Apoptosis Assay

After treatment, the cells were harvested and fixed with 70% ethanol for another 4 h. Then, cells were stained with 25 mg/ml PI (propidium iodide) and 50 mg/ml RNase A (Solarbio Biotechnology, Beijing, China) for another 30 min. For the apoptosis assay, the cells were stained with an Annexin V-FITC-PI staining kit (Roche,

Mannheim, Germany) according to the manufacturer's instructions. Cell cycle distribution and apoptosis were determined using flow cytometry (BD Bioscience, San Jose, USA), and the results were analysed by FlowJo (ThreeStar, San Carlos, USA).

Cell Migration and Invasion Assays

The wound healing assay was performed using 6-well plates (Corning, San Diego, USA). A total of 1×10^6 cells were seeded in each well and cultured overnight until confluent. A 200 µl pipette tip was used to make a straight scratch in the cell monolayer. The detached cells were washed off gently using PBS, and fresh medium was added. The initial gap length (0 h) and the gap length at 24 h after wounding were calculated from photomicrographs. For the cell invasion assay, a transwell chamber (Corning, San Diego, USA) was used. The upper chamber was coated with Matrigel (BD Bioscience, San Jose, USA), and the culture medium was added to the lower chamber. Glioma cells were resuspended in serum-free medium and placed in the upper chamber. Forty-eight hours later, the non-invading cells were removed, and invasive cells were fixed and stained with crystal violet (Solarbio Biotechnology, Beijing, China). The number of cells migrating to the lower chamber was counted under an inverted microscope (Olympus, Tokyo, Japan). Five fields were randomly selected for each sample. This experiment was repeated three times.

Caspase-3 Activity Assay

Caspase-3 activity was assayed using a colorimetric assay kit purchased from Abcam (Cambridge, MA, USA) according to the manufacturer's instructions.

Luciferase Activity Assay

Bioinformatics methods were used to analyse the relationship between circ-ABCB10-WT, miR-620 and FABP5. Predicted binding sites for miR-620 in circ-ABCB10 and predicted binding sites for miR-620 in the 3'UTR of FABP5 were cloned into a Dual-Luciferase reporter vector, psi-check2, by Synbio Technologies (Suzhou, China). The constructed vectors were then transfected with miR-650 mimics using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). Forty-eight hours after transfection, the cells were harvested, and Luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega, Madison, USA) according to the manufacturer's instructions.

Western Blot Assay

Total protein was extracted from cells using RIPA lysis buffer (Cell Signaling Technologies, Beverly, USA). The protein was quantified by a BCA protein assay kit (Solarbio, Beijing, USA), and 20 µg of total protein was separated by 12% SDS-PAGE gels and transferred onto PVDF membranes. After blocking with skimmed milk for 1 h at room temperature, the membrane was incubated with primary antibody overnight at 4°C. Then, the membrane was washed and incubated with secondary antibody at room temperature for 1 h. The protein bands were visualized using an ECL Prime Western Blotting System (Sigma-Aldrich, St. Louis, USA). All antibodies were purchased from CST (Cell Signaling Technologies, Beverly, USA).

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 7.0 (GraphPad Software, 2007, La Jolla, CA, USA). Data are expressed as the mean ± SD. One-way ANOVA was used to determine the significant differences between multiple groups. A post-hoc test was used to calculate the significant differences between two groups. A p value<0.05 (two-tailed) was considered statistically significant.

Results

Circ-ABCB10 Was Upregulated in Glioma Tissues and Cell Lines

We first measured the levels of circ-ABCB10 in 40 pairs of glioma and corresponding normal tissues by qRT-PCR. It was shown that circ-ABCB10 was highly expressed in glioma tissues (Figure 1A). Compared with that in normal human astrocytes (NHAs), the expression of circ-ABCB10 in glioma cell lines (U261, A172, U87, T98G) was much higher (Figure 1B). In particular, U87 and T98G cells showed the most pronounced expression of circ-ABCB10 among the four glioma cell lines and were chosen for subsequent experiments. Three siRNAs against circ-ABCB10 were tested, and it was found that si-circ-ABCB10#1 was the most effective siRNA to knockdown circ-ABCB10 (Figure 1C). Then, we tested the proliferative change in glioma cells after the downregulation of circ-ABCB10.

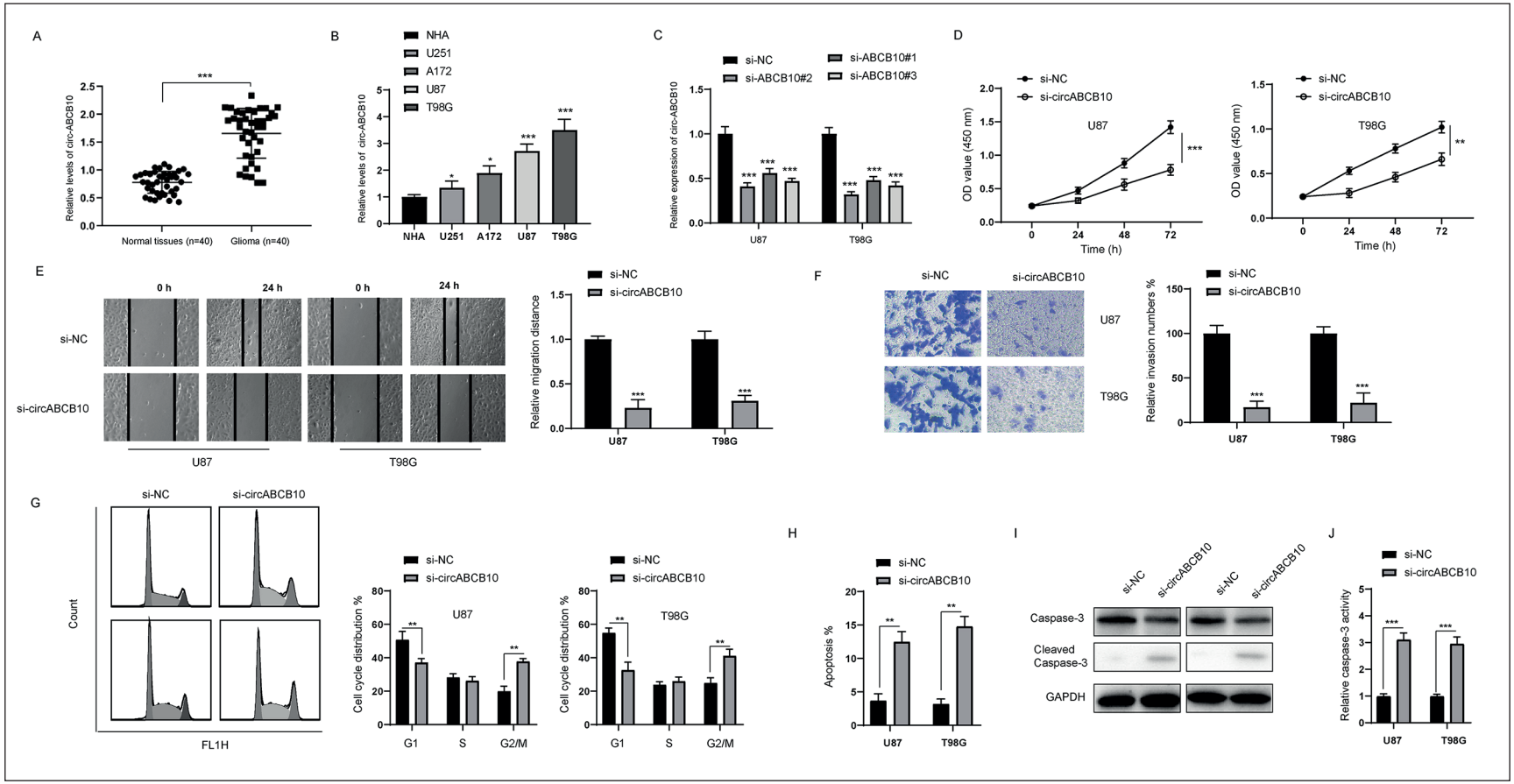

Figure 1. Circ-ABCB10 knockdown inhibited the proliferation, migration, and invasion of glioma cells. **A,** The expression of circ-ABCB10 was evaluated in 40 pairs of glioma and adjacent normal tissues. **B,** The expression of circ-ABCB10 was tested in NHAs and four glioma cell lines. **C,** Glioma cell lines were transfected with three different siRNAs against circ-ABCB10 for 24 h, and then, the expression of circ-ABCB10 was evaluated. **D,** Glioma cells were transfected with si-NC or si-circ-ABCB10, and cell viability was assayed at different time points. **E,** Glioma cells were transfected with si-NC or si-circ-ABCB10, and cell migration was assayed (magnification, 50 ×). **F,** Glioma cells were transfected with si-NC or si-circ-ABCB10, and cell invasion was assayed (magnification, 50 ×). **G,** Glioma cells were transfected with si-NC or si-circ-ABCB10, and the cell cycle distribution was assayed. **H,** Glioma cells were transfected with si-NC or si-circ-ABCB10, and apoptosis was assayed. **I,** Glioma cells were transfected with si-NC or si-circ-ABCB10, and the protein levels of caspase-3 were measured by Western blot. (**J**) Glioma cells were transfected with si-NC or si-circ-ABCB10, and caspase-3 activity was measured. Data are presented as the mean ± SD; **p<0.01, ***p<0.001.

The MTT assay results showed that silencing circ-ABCB10 significantly inhibited the proliferation of glioma cells (Figure 1D). Subsequently, we examined the effects of circ-ABCB10 on migration and invasion changes by wound healing and transwell assays, respectively. As shown in Figure 1E-H, knockdown of circ-ABCB10 in glioma cells significantly inhibited the migration and invasion of glioma cells. Next, we investigated whether circ-ABCB10 had any effects on cell cycle progression. Silencing of circ-ABCB10 led to cell cycle arrest at the G2/M phase in glioma cells (Figure 1G). Moreover, we also observed that silencing of circ-ABCB10 led to apoptosis in glioma cells (Figure 1H). Furthermore, cleavage of caspase-3 and an increase in caspase-3 activity were observed after silencing circ-ABCB10 in glioma cells (Figure 1I, J). Taken together, these data suggested that circ-ABCB10 had a potential carcinogenic effect in glioma.

Circ-ABCB10 Regulated the Proliferation and Migration of Glioma Cells by Binding to MiR-620

The subcellular localization of lncRNAs is directly related to their biological function. To determine the subcellular localization of circ-ABCB10 in glioma cells, cytoplasmic and nuclear extraction assays were performed. It was found that U6 was mainly present in the nucleus, while GAPDH was mainly present in the cytoplasm, and the results further indicated that circ-ABCB10 was predominantly located in the cytoplasm of glioma cells (Figure 2A). Therefore, circ-ABCB10 might be involved in the development and progression of glioma at the posttranscriptional level. Then, we examined whether there are any miRNAs that bind to circ-ABCB10 through bioinformatics analysis. Ultimately, miR-620 was identified (Figure 2B). To verify the interaction between miR-620 and circ-ABCB10, glioma cells were co-transfected with miR-620/miR-NC mimics and wild-type pGL3-circ-ABCB10/mutant pGL3-circ-ABCB10. The Dual-Luciferase reporter assay showed decreased Luciferase activity in the wild-type circ-ABCB10 group. However, no significant change in Luciferase activity was observed in the circ-ABCB10 mut group (Figure 2C). These findings suggest that miR-620 binds to circ-ABCB10. Furthermore, we found that silencing of circ-ABCB10 led to the upregulation of miR-620 in glioma cells (Figure 2D). Subsequently, we evaluated the expression of miR-620 in glioma and adjacent normal tissues by RT-PCR. It was shown that miR-620 was downregulated in glioma tissues (Figure 2E). By analysing the expression of circ-ABCB10 and miR-620 in glioma tissues, a negative correlation was found between them (Figure 2F). Subsequently, we investigated the effects of miR-620 on glioma cells. The inhibited proliferation, migration and invasion of glioma cells induced by silencing circ-ABCB10 were partially reversed by inhibition of miR-620 (Figure 2G, H, I). Furthermore, cell cycle arrest at the G2/M phase, apoptosis, cleavage of caspase-3 and increased activities induced by silencing circ-ABCB10 were all partially abrogated by inhibition of miR-620 (Figure 2J, K, L, M). These data indicated that circ-ABCB10 may regulate the oncogenic properties of glioma cells by binding to miR-620.

FABP5 Is a Direct Target of MiR-620

Through similar methods, FABP5 was identified as the target gene of miR-620 (Figure 3A). The Dual-Luciferase reporter assay showed decreased Luciferase activity in the FABP5-WT 3'-UTR group (Figure 3B). However, no significant change in Luciferase activity was observed in the FABP5-Mut 3'-UTR group (Figure 3B). Overexpression of miR-620 led to the downregulation of both the mRNA and protein levels of FABP5 in glioma cells (Figure 3C, D). Subsequently, we examined the expression of FABP5 in glioma and adjacent normal tissues by RT-PCR. The data showed that FABP5 was highly expressed in glioma tissues (Figure 3E). By analysing the expression of miR-620 and FABP5 in glioma tissues, a negative correlation was found between the two genes (Figure 3F). Then, we tested the effects of FABP5 on glioma cells. We used siRNA to successfully downregulate FABP5 (Figure 3G). As shown in Figure 3H, I, J, knockdown of FABP5 led to the inhibition of proliferation, migration and invasion of glioma cells. Furthermore, downregulation of FABP5 also led to cell cycle arrest at G2/M phase, apoptosis, cleavage of caspase-3 and increase in caspase-3 activity in glioma cells (Figure 3K, L, M, N). These data indicated that FABP5 is a target of miR-620.

Upregulation of FABP5 Interfered with the Antitumor Effects of Silencing circ-ABCB10

Next, we further evaluated the role of FABP5 by forced expression of FABP5 in glioma cells (Figure 4A). Interestingly, the inhibition of proliferation and cell cycle arrest at G2/M phase

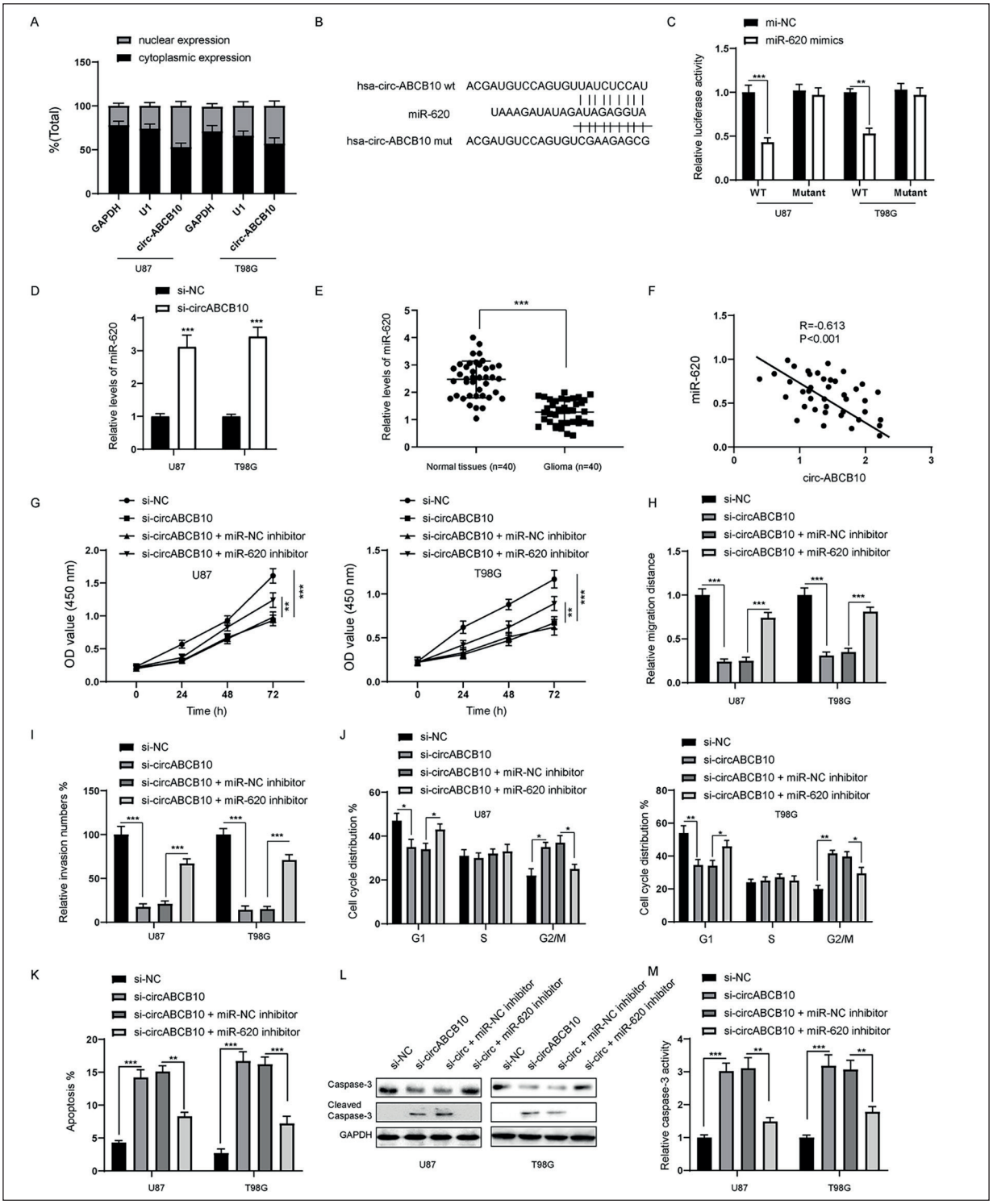

Figure 2. The relationship between circ-ABCB10 and miR-620. **A,** The location of circ-ABCB10 was assayed in glioma cells. **B,** The predicted binding sites between miR-620 and circ-ABCB10. **C,** Dual-Luciferase reporter assay indicated that miR-620 could bind to circ-ABCB10. **D,** After silencing circ-ABCB10, the levels of miR-620 were assayed by RT-PCR. **E,** The levels of miR-620 were measured in 40 pairs of glioma and adjacent normal tissues. **F,** The relationship between miR-620 and circ-ABCB10 was assayed in glioma tissues. **G,** The inhibited proliferation. **H,** The inhibited migration. **I,** The inhibited invasion. **J,** The induced cell cycle arrest. **K,** The induced apoptosis. **L,** The induced cleavage of caspase-3. **M,** The increased activities of caspase-3 in glioma cells caused by circ-ABCB10 knockdown were partially reversed by inhibition of miR-620. Data are presented as the mean ± SD; *p<0.05, **p<0.01, ***p<0.001.

caused by silencing circ-ABCB10 in glioma cells could be partially rescued by overexpression of FABP5 (Figure 4B, C). Furthermore, the inhibition of the migration and invasion of glioma cells by silencing circ-ABCB10 could also be partially rescued by overexpression of FABP5 (Figure 4D,

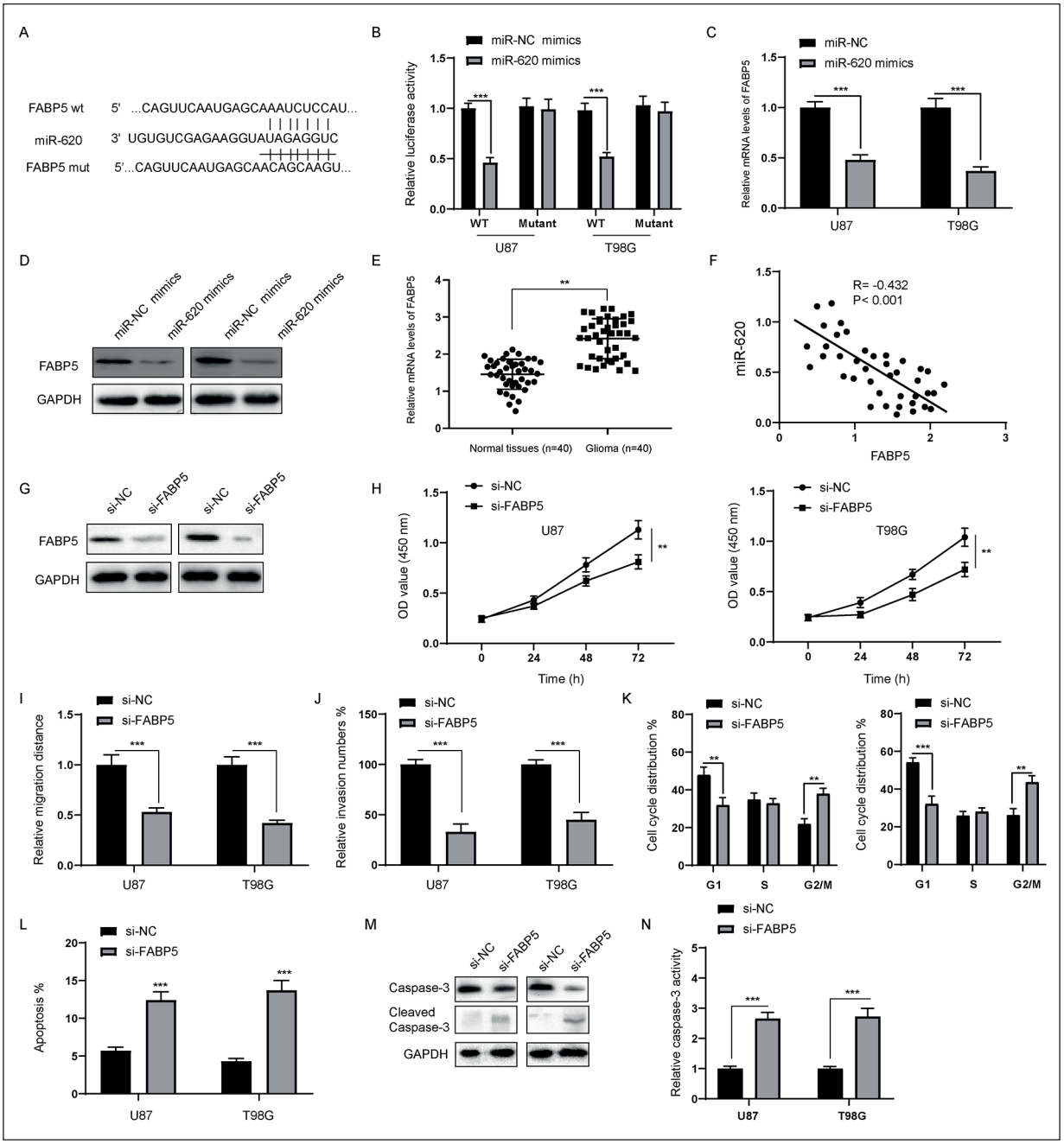

Figure 3. FABP5 is a direct target of miR-620. **A,** The predicted binding sites between FABP5 and miR-620. **B,** Dual-Luciferase reporter assay verified that miR-620 could bind to the FABP5 3'-UTR. **C,** Glioma cells were transfected with miR-620 mimics, and the mRNA levels of FABP5 were assayed by RT-PCR. **D,** Overexpression of miR-620 in glioma cells was achieved, and the protein levels of FABP5 were assayed by Western blot. **E,** The mRNA levels of FABP5 were evaluated in 40 pairs of glioma and normal tissues. **F,** The correlation between the mRNA levels of FABP5 and miR-620 in glioma tissues. **G,** Glioma cells were transfected with si-NC or si-FABP5, and then the protein levels of FABP5 were assayed by Western blot. **H,** Glioma cells were transfected with si-NC or si-FABP5 for the indicated times, and then the proliferation of glioma cells was examined. After silencing FABP5 in glioma cells, the migration (**I**), invasion (**J**), cell cycle distribution (**K**), apoptosis (**L**), cleavage of caspase-3 (M) and activity of caspase-3 were determined (N). Data are presented as the mean ± SD; **p<0.01, ***p<0.001.

E). Finally, the apoptosis, cleavage of caspase-3 and increased activity of caspase-3 caused by silencing of circ-ABCB10 could also be blocked by overexpression of FABP5 (Figure 4F, G, H). Taken together, these data suggested that the antitumor effects of silencing circ-ABCB10 were at least partially mediated by the regulation of FABP5.

Discussion

Glioma is a CNST solid tumour characterized by high proliferative potential and aggressive angiogenesis, leading to poor prognosis worldwide[11]. Currently, the main treatments for glioma include cancer resection, radiotherapy and chemotherapy[12]. Although great efforts have been made to improve the treatment, the prognosis of glioma patients is still unsatisfactory. Therefore, insights into the molecular mechanisms underlying tumorigenesis in glioma might allow for earlier detection and improved outcomes.

CircRNAs are abundantly present in eukaryotic cells and involved in the regulation of various human diseases. Due to the lack of 3' and 5' ends, circRNAs are structurally stable and resistant to degradation by RNase. Although many circRNAs are derived from protein-coding genes, most circRNAs are unable to encode proteins. In the present study, we revealed that circ-ABCB10 was highly expressed in glioma tissues and cell lines. Subsequent experiments showed that silencing circ-ABCB10 could inhibit the proliferation, migration and invasion of glioma cells. In addition, downregulation of circ-ABCB10 also led to cell

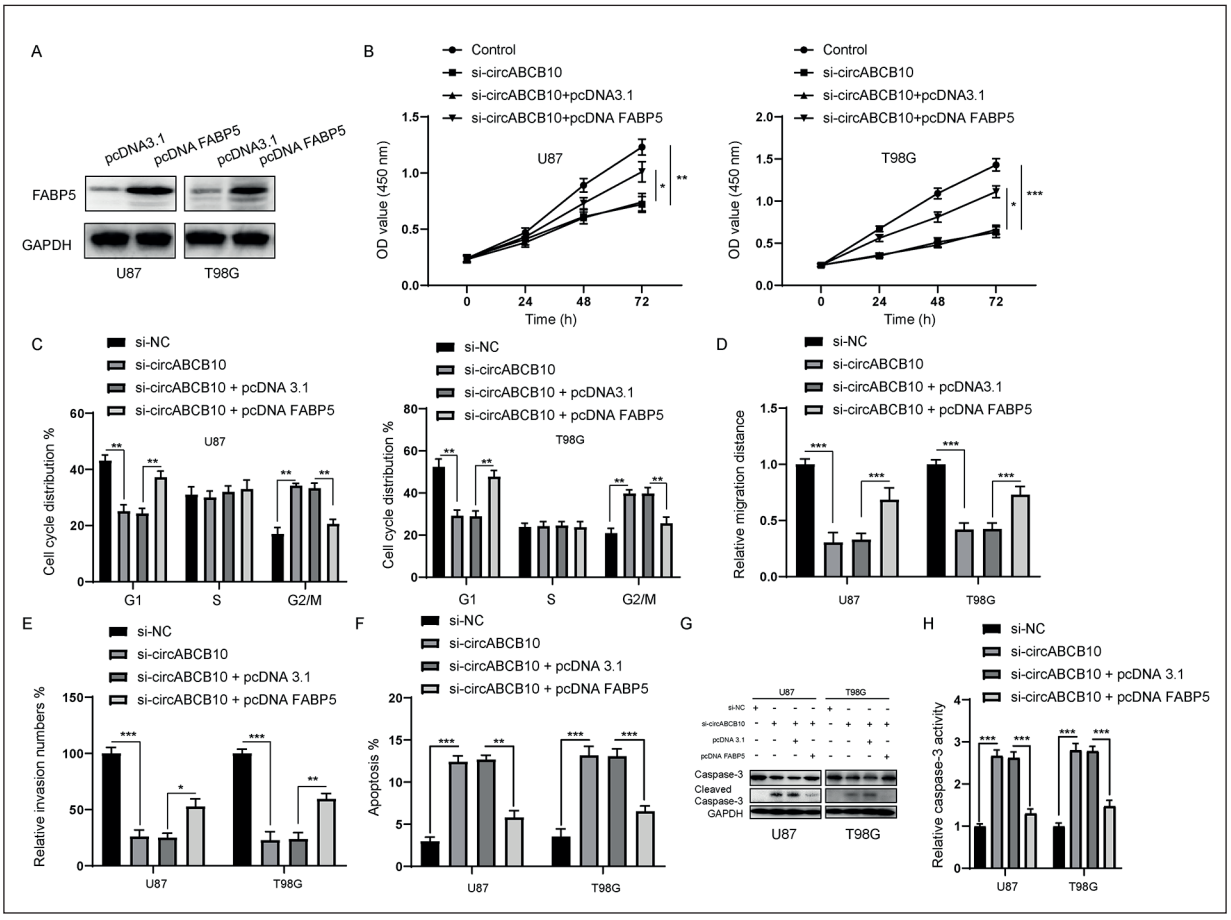

Figure 4. Forced expression of FABP5 abrogated the antitumour effects of silencing circ-ABCB10. **A,** Glioma cells were transfected with pcDNA3.1 (empty vector) or pcDNA FABP5, and then the protein levels of FABP5 were measured. Glioma cells were transfected as indicated. **B,** Cell viability was assayed. **C,** The cell cycle distribution was assayed. **D,** Cell migration was measured. **E,** Cell invasion was measured. **F,** Apoptosis was measured. **G,** The cleavage of caspase-3 was measured. **H,** The activity of caspase-3 was assayed. Data are presented as the mean ± SD; **p*<0.05; ***p*<0.01; ****p*<0.001.

cycle arrest at the G2/M phase and apoptosis of glioma cells. Our findings are in line with a previous study[13] in which circ-ABCB10 was reported to act as an oncogene in non-small cell lung cancer cells. To further investigate the mechanisms by which circ-ABCB10 affects the oncogenic properties of glioma cells, we searched for its target genes and identified FABP5. FABP5 belongs to the FABP (fatty acid binding protein) family of proteins, which are essential for the transport of fatty acids. FABP5 is a small protein (15 kD) that binds to different fatty acids that are recently transported into cells for storage and cell biofilm synthesis[14]. FABP5 is strongly involved in the occurrence, development and metastasis of various tumours. It promoted cervical tumour cell growth and metastasis and correlate with poor prognosis[15]. In addition, FABP5 also regulated the proliferation of clear cell renal carcinoma cells, prostate cancer and breast cancer[16-18]. In line with previous reports, our study further confirmed that FABP5 acted as an oncogene in glioma. We found that FABP5 was highly expressed in glioma tissues. Knockdown of FABP5 markedly decreased the proliferation, migration and invasion of glioma cells. Notably, forced expression of FABP5 partially abrogated the effects of silencing circ-ABCB10.

To reveal the mechanisms by which circ-ABCB10 regulates FABP5, we also searched for the link between circ-ABCB10 and FABP5. CircRNAs exert biological functions by binding to miRNAs and acting as a "sponge" to regulate miRNA expression[19]. Hence, we hypothesized that circ-ABCB10 may regulate FABP5 by sponging and regulating the expression of certain miRNAs. Through bioinformatic analysis, miR-620 was found to be able to interact with both circ-ABCB10 and the FABP5 3'UTR. We speculated that circ-ABCB10 might indirectly affect the expression of FABP5 by regulating miR-620. We also found that miR-620 was downregulated in glioma tissues and negatively correlated with the expression of circ-ABCB10.

Our study indicated that miR-620 might act as an anti-tumour gene in glioma. Interestingly, our findings are contradictory to previous studies[20,21] that showed that miR-620 acted as an oncogene in cervical cancer and lung cancer. This discrepancy might be due to the different cancer types, and further investigation about the role of miR-620 in carcinogenesis in more cancer types is needed. Based on these findings, we postulated that circ-ABCB10 indirectly promoted the expression of the oncogene FABP5 *via* inhibition of miR-620 expression, thereby promoting the occurrence and development of glioma.

Conclusions

We found that increased circ-ABCB10 acted as an oncogene in glioma by regulating the miR-620/FABP5 axis, suggesting that circ-ABCB10 may be used as a potential therapeutic target for glioma. There are some limitations to our study. First, our clinical samples are relatively small, and more clinical information related to the expression of circ-ABCB10 is needed. Second, investigation into the function of circ-ABCB10 in a mouse model is lacking. Therefore, further study of circ-ABCB10 may have clinical implications for future diagnosis and treatment of glioma and other diseases.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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