Cancer Management and Research

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ORIGINAL RESEARCH

CircTTBK2 Contributes to the Progression of Glioma Through Regulating miR-145-5p/CPEB4 Axis

This article was published in the following Dove Press journal:
Cancer Management and Research

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Background: Currently, circular RNAs (circRNAs) have been demonstrated to play vital roles in malignant tumors, including glioma. Nevertheless, the functions of circTTBK2 in glioma are largely unclear.

Materials and Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) was applied for the expression levels of circTTBK2, TTBK2 mRNA, miR-145-5p and cytoplasmic polyadenylation element binding protein 4 (CPEB4) mRNA. Actinomycin D and RNase R digestion assays were utilized for the characteristics of circTTBK2. 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay, flow cytometry analysis and transwell assay were conducted for cell proliferation, apoptosis and metastasis, respectively. The glycolysis level was estimated with specific kits. Western blot assay was adopted for the protein levels of hexokinase2 (HK2) and CPEB4. The targeting relationship between miR-145-5p and circTTBK2 or CPEB4 was verified by Dual-luciferase reporter and RNA immunoprecipitation (RIP) assays. Murine xenograft assay was used for the role of circTTBK2 in tumorigenesis in vivo.

Results: CircTTBK2 was upregulated in glioma tissues and cells, and its level was associated with poor survival of glioma patients. CircTTBK2 knockdown suppressed glioma cell proliferation, migration, invasion and glycolysis and accelerated apoptosis in vitro and hampered tumor growth in vivo. CircTTBK2 functioned as a sponge of miR-145-5p, and miR-145-5p inhibition restored the effects of circTTBK2 knockdown on the malignant behaviors of glioma cells. Moreover, CPEB4 was the direct target gene of miR-145-5p, and miR-145-5p inhibition facilitated glioma cell progression by targeting CPEB4.

Conclusion: CircTTBK2 functioned as a tumor promoter in glioma by modulating miR-145-5p/CPEB4 axis, which might offer a new sight for glioma therapy.

Keywords: circTTBK2, miR-145-5p, CPEB4, glioma

Introduction

Glioma is a common intracranial brain tumor that is featured by refractory, local dissemination and recurrence.[1,2] In recent years, with the advancement of medical science, the treatment technology of glioma, a comprehensive treatment strategy based on surgical resection, combined with radiotherapy, chemotherapy, molecular targeting and other therapies has been gradually formed.[3–5] Even so, the prognosis of glioma patients remains unsatisfactory and the molecular mechanisms of gliomagenesis are still largely unknown. Accordingly, it is urgent to seek feasible and effective therapeutic targets for this fatal disease.

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Circular RNAs (circRNAs) are a family of non-coding RNAs (ncRNAs) that harbor covalently closed-loop structures.[6] Recently, the effects of circRNAs on tumorigenesis have attracted more attention. Mounting evidence has shown that circRNAs can alter the carcinogenesis of diverse human tumors, including glioma, via functioning as microRNA (miRNA) sponges to regulate gene expression at the post-transcriptional level.[7] For example, circ_0079593 impeded glioma cell proliferation, motility and facilitated apoptosis by sponging miR-499a-5p and regulating KPNA2.[8] Circ_0037251 served as a tumor promoter in glioma via modulation of miR-1229-3p expression.[9] Moreover, Zhang et al have disclosed that circTTBK2 plays an oncogenic role in glioma through modulating miR-217 and HNF1β.[10] Whereas, the exact roles and related regulatory mechanisms of circTTBK2 in glioma are largely unelucidated.

As a class of small ncRNAs, miRNAs have also been verified to be closely linked to the tumorigenesis of human cancers, including glioma. It has been documented that miR-145-5p takes part in regulating the malignant behaviors of multiple cancers, such as colorectal cancer,[11] bladder cancer,[12] gastric cancer[13] and thyroid papillary cancer.[14] Of note, Chen et al demonstrated that miR-145-5p could participate in regulating glioma cell growth and cell cycle process by targeting SOX9.[15] As a member of the cytoplasmic polyadenylation element binding protein (CPEB) family, CPEB4 level has been verified to be elevated in glioma and related to the malignancy and dismal prognosis of glioma.[16,17] Previous studies also suggested that CPEB4 could be targeted by miRNAs, such as miR-30c-5p,[18] miR-98-5p,[19] miR-29c-5p[20] and miR-1246.[21] Nevertheless, the association between miR-145-5p and CPEB4 in glioma progression remains unexplored.

In the research, we detected the abundance of circTTBK2, miR-145-5p and CPEB4 in glioma tissue specimens and cells. Moreover, the functional role and regulatory mechanism of circTTBK2 in glioma development were investigated through functional and mechanism analysis.

Materials and Methods

Tissues Acquisition

Forty glioma tissue specimens were received from glioma patients and 40 non-glioma tissue specimens were resected from non-glioma patients who required the resection of brain tissues for cerebral hemorrhage, laceration and cerebral contusion at General Hospital of Tianjin Medical University. The surgical resection was conducted following the approval of the Ethics Committee of General Hospital of Tianjin Medical University (No.2019TJ881). Written informed consents were also acquired from the participants. The samples were stored at −80°C until use.

Cell Culture

Glioma cell lines (T98G and LN229) were acquired from Procell (Wuhan, China) and normal human astrocytes (NHAs) were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Solarbio, Beijing, China) added with 10% fetal bovine serum (FBS; Solarbio) and 1% penicillin-streptomycin (Solarbio) at 37°C in an incubator consisting of 5% CO_2.

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

Total RNA was extracted from tissues and cells utilizing RNAiso Plus (Takara, Dalian, China). Avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA) or TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was utilized to reversely transcribe RNA into cDNA. After that, qRT-PCR reaction was executed on an ABI 7500 PCR system (Applied Biosystems) using AceQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) and specific primers (Sangon, Shanghai, China). The used primers were: circTTBK2: (F: 5′-AGTGCAA CATTTTCCCTGGTG-3′ and R: 5′-GCTTGATTTTGGC TTGGCT-3′); TTBK2: (F: 5′-TTCCATAGGGTCGGAAT CTG-3′ and R: 5′-AAGGTCCCTACCGGTTTGAT-3′); miR-145-5p: (F: 5′-CTCACGGTCCAGTTTTCCCA-3′ and R: 5′-ACCTCAAGAACAGTATTTCCAGG-3′); CPEB4: (F: 5′-ACAGTGACTTTGTGAGATGGATGG-3′ and R: 5′-TTATCATCGCAAGCTCCACA-3′); β-actin: (F: 5′-CCCGAGCCGTGTTTCC-3′ and R: 5′-GTCC CAGTTGGTGACGATGC-3′); U6: (F: 5′-AGAGCCT GTGGTGTCCG-3′ and R: 5′-CATCTTCAAA GCACTTCCCT-3′). The relative expression was quantified via the $2^{-\Delta\Delta Ct}$ strategy. β-actin or U6 was used as the internal reference.

Actinomycin D (Act D) and RNase R Digestion Assays

To prevent transcription, T98G cells were exposed to Act D (2 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) or

dimethyl sulfoxide (DMSO; Sigma-Aldrich) at indicated time points. For RNase R treatment, 1 μg RNA was treated with or without 2 U of RNase R (Epicentre, Madison, Wisconsin, USA) for 20 min at 37°C. After Act D or RNase R treatment, qRT-PCR assay was implemented for the determination of circTTBK2 and TTBK2 mRNA levels.

Cell Transfection

Small interfering RNA targeting circTTBK2 (si-circTTBK2), siRNA against CPEB4 (si-CPEB4) and scramble control si-NC, the overexpression plasmid of circTTBK2 (oe-circTTBK2) and its control (Vector), miR-145-5p mimic and miR-NC mimic, miR-145-5p inhibitor and miR-NC inhibitor, short hairpin RNA against circTTBK2 (sh-circTTBK2) and sh-NC were designed by GenePharma (Shanghai, China). Then the indicated synthetic plasmids or oligonucleotides were transfected into glioma cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in reference to the guidelines of the manufacturer.

3-(4, 5-Dimethyl-2-Thiazolyl)-2, 5-Diphenyl-2-H-Tetrazolium Bromide (MTT) Assay

After relevant transfection, the capacity of T98G and LN229 cell proliferation was assessed by MTT assay. In brief, transfected T98G and LN229 cells (2×10^4 cells/well) were implanted into 96-well plates and cultivated for 24 h. Then, 20 μL MTT (Sigma-Aldrich) was supplemented into each well at indicated time points and maintained for another 4 h. Afterward, cell supernatant was abandoned and 150 μL DMSO (Sigma-Aldrich) was used for the formazan crystals dissolving. Finally, the OD value was examined with a microplate reader (Potenov, Beijing, China) at 490 nm.

Flow Cytometry Analysis

The Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Vazyme) was utilized to assess the apoptosis rate of glioma cells according to the manufacturers' instructions. Briefly, transfected T98G and LN229 cells were collected, resuspended, washed and then mixed with 5 μL Annexin V-FITC and 5 μL propidium iodide (PI); 15 min later, the apoptotic cells were determined with a FACScan® flow cytometry (Beckman Coulter, Atlanta, GA, USA).

Transwell Assay

To test cell migration and invasion, about 1×10^4 cells were suspended in serum-free DMEM (Solarbio) and then 100 μL cell suspension was added into the top compartment of transwell inserts (BD Bioscience, San Jose, CA, USA) pre-coated with (invasion) or without (migration) Matrigel (BD Biosciences). Meanwhile, 600 μL culture medium containing 10% FBS was plated into the lower compartment; 24 h later, cells migrated or invaded to the bottom surface were fixed with methanol, dyed with 0.1% crystal violet (Solarbio) and counted with a microscope under the magnification of 100×.

Detection of Glucose Consumption and Lactate Production

The levels of glucose consumption and lactate production in T98G and LN229 cells were examined using glucose assay kit (Sigma-Aldrich) and lactate assay kit (Sigma-Aldrich), respectively. The procedures were based on the instructions of manufacturers.

Western Blot Assay

Protein extraction was finished using RIPA buffer (CWBio, Beijing, China). The protein concentrations were examined via a BCA Protein Quantification Kit (Vazyme). Then, an equal amount of proteins was separated through sodium dodecyl sulfonate-polyacrylamide gel (Solarbio) electrophoresis and blotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat milk for 1 h at indoor temperature and immunoblotted with primary antibodies against hexokinase2 (HK2; bs-9455R; Bioss, Beijing, China), CPEB4 (bs-14020R; Bioss) and β-actin (bs-0061R; Bioss) overnight at 4°C followed by interaction with indicated horseradish peroxidase-conjugated secondary antibody (bs-0295M-HRP; Bioss) for 1 h. The immunoreactive bands were developed using ECL kit (Beyotime, Shanghai, China) and analyzed using Image J software.

Dual-Luciferase Reporter Assay

The sequences of circTTBK2 containing the wild-type (WT) or mutant (MUT) binding sites of miR-145-5p were cloned and inserted into psiCHECK-2 plasmid (GenePharma), constructing WT-circTTBK2 and MUT-circTTBK2, respectively. Similarly, luciferase reporter plasmids WT-CPEB4 and MUT-CPEB4 were generated.

Then, T98G and LN229 cells were plated into 6-well plates and co-transfected with miR-145-5p mimic or miR-NC mimic and the luciferase reporter plasmids; 48 h post-transfection, Dual-Luciferase Reporter Assay System (Promega) was exploited for the measurement of firefly and renilla luciferase activities.

RNA Immunoprecipitation (RIP) Assay

RIP analysis was executed with Magna RNA-binding protein immunoprecipitation kit (Millipore). After being transfected with miR-145-5p mimic or miR-NC mimic, T98G and LN229 cells were disrupted in RIP buffer and then cell extraction was interacted with magnetic beads bound with antibodies immunoglobulin G (IgG; Abcam, Cambridge, MA, USA) or Argonaute-2 (Ago2; Abcam). Then the aforementioned qRT-PCR analysis was executed for the abundance of circTTBK2 and CPEB4 in the immunoprecipitant complexes.

Murine Xenograft Model

Following the animal test was approved by the Ethics Committee of Animal Research of General Hospital of Tianjin Medical University, sh-circTTBK2 or sh-NC was introduced into T98G cells and then the cells were subcutaneously implanted into the BALB/c nude mice (4–6 weeks old; Vital River Laboratory, Beijing, China). The xenograft tumors were monitored every week and computed with the equation: $(\text{Length}\times\text{Width}^2)\times0.5$. After 4 weeks, the mice were sacrificed and neoplasm weights were measured. Moreover, the xenograft tumor tissues were stored at −80°C for other tests. Animal studies were performed in compliance with the ARRIVE guidelines and the Basel Declaration. All animals received humane care according to the National Institutes of Health (USA) guidelines.

Statistical Analysis

The experiments were conducted in triplicate. The results were analyzed using GraphPad Prism 7 and presented as mean ± standard deviation. The comparisons were evaluated through Student's *t*-test and one-way analysis of variance. The survival curve of glioma patients was generated by Kaplan-Meier plot and analyzed by Log rank test. Spearman correlation coefficient analysis was utilized to estimate the correlation among circTTBK2, miR-145-5p and CPEB4 levels in glioma tissues. It was considered as significant when $P<0.05$.

Results

CircTTBK2 Was Highly Expressed in Glioma Tissues and Cells

To investigate the function of circTTBK2 in glioma, we firstly determined the expression of circTTBK2 in glioma tissues and normal tissues using qRT-PCR assay. The results showed that circTTBK2 level was obviously increased in glioma tissues compared to that in normal tissues (Figure 1A). In 50% (20/40) glioma tissues, circTTBK2 level had a fold-change of more than 2 compared with normal tissues (Figure 1B). Then we divided the glioma tissues into 2 groups based on circTTBK2 expression: High circTTBK2 expression group (n=20) and Low circTTBK2 expression group (n=20). As exhibited in Figure 1C, the survival time of glioma patients with high circTTBK2 expression was shorter than that of glioma patients with low circTTBK2 expression. We also determined the expression level of circTTBK2 in glioma cells, and the results showed that circTTBK2 level was markedly elevated in T98G and LN229 cells in comparison with NHA cells (Figure 1D). In addition, we evaluated the characteristics of circTTBK2 using Act D assay and RNase R digestion assay. Act D assay showed that circTTBK2 had a longer half-life than linear TTBK2 in T98G cells (Figure 1E). RNase R digestion assay displayed that circTTBK2 was resistant to RNase R treatment, while TTBK2 mRNA was evidently digested following the treatment of RNase R in T98G cells (Figure 1F). Collectively, circTTBK2 had a loop structure and might be involved in the development of glioma.

Effects of circTTBK2 Silencing on Glioma Cell Proliferation, Apoptosis, Migration, Invasion and Glycolysis

To validate the functional roles of circTTBK2 in glioma cell progression, si-circTTBK2 was transfected into T98G and LN229 cells to knock down the expression of circTTBK2. As shown in Figure 2A, si-circTTBK2 transfection led to a remarkable downregulation of circTTBK2 expression level in both T98G and LN229 cells, indicating that si-circTTBK2 was successfully transfected into 98G and LN229 cells. MTT assay indicated that circTTBK2 silencing obviously suppressed the proliferation capacity of T98G and LN229 cells

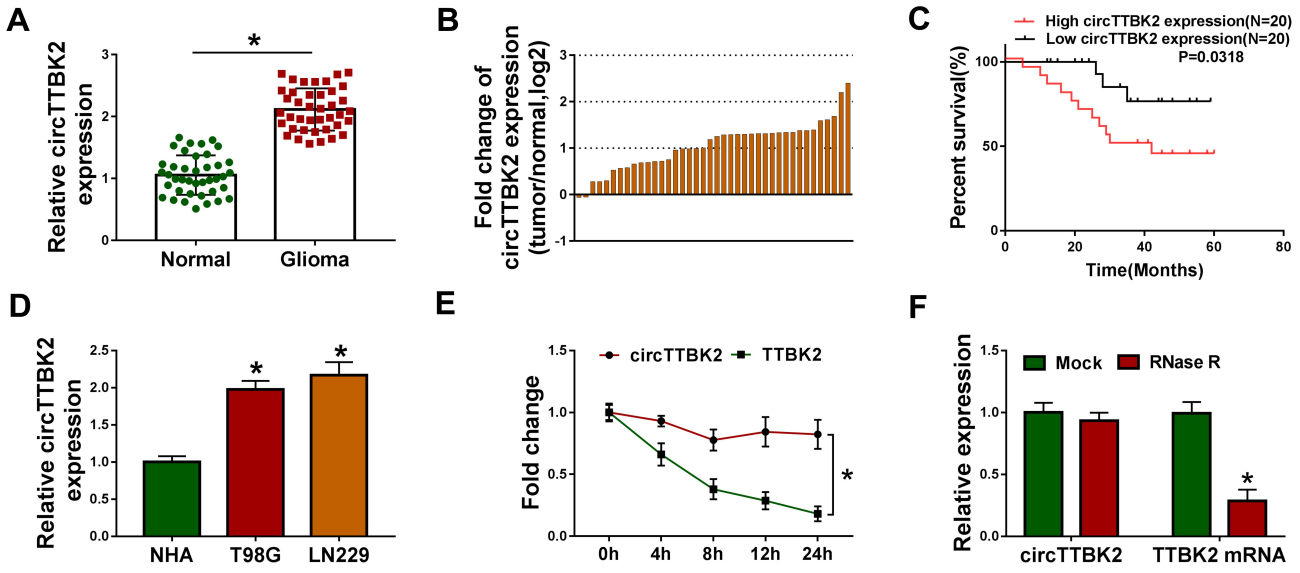

Figure 1 CircTTBK2 level was increased in glioma tissues and cells. (**A**) The expression level of circTTBK2 in glioma tissues and normal tissues was detected through qRT-PCR assay. (**B**) Fold change of circTTBK2 expression level was presented as log2 (tumor/normal). (**C**) The overall survival of glioma patients with high/low circTTBK2 expression level was analyzed by Kaplan-Meier survival analysis. (**D**) The expression level of circTTBK2 in T98G, LN229 and NHA cells was determined by qRT-PCR assay. (**E**) After T98G cells were treated with Act D at indicated time points, the abundance of circTTBK2 and TTBK2 was examined via qRT-PCR assay. (**F**) The expression levels of circTTBK2 and TTBK2 mRNA in T98G cells treated with or without RNase R were examined by qRT-PCR assay. *$P<0.05$.

compared to si-NC control groups (Figure 2B). Flow cytometry analysis demonstrated that the apoptosis of T98G and LN229 cells was conspicuously induced following the downregulation of circTTBK2 compared to control groups (Figure 2C). As illustrated by transwell assay, the migration and invasion of T98G and LN229 cells were all drastically repressed by decreasing circTTBK2 expression in comparison with si-NC groups (Figure 2D and E). In addition, we determined the effect of circTTBK2 knockdown on glycolysis in T98G and LN229 cells by detecting the levels of glucose consumption, lactate production and HK2. Our results indicated that circTTBK2 deficiency markedly reduced the levels of glucose consumption, lactate production and HK2 protein in both T98G and LN229 cells (Figure 2F–H). To sum up, circTTBK2 knockdown suppressed cell proliferation, metastasis and glycolysis and induced apoptosis in glioma cells.

CircTTBK2 Sponged miR-145-5p and Negatively Regulated miR-145-5p Expression in Glioma Cells

To investigate the underlying mechanism of circTTBK2 in regulating glioma cell progression, online tools StarBase (http://starbase.sysu.edu.cn/agoClipRNA.php?source=circRNA) and circinteractome (https://circinteractome.nia.nih.gov/?tdsourcetag=s_pcqq_aiomsg) were used for searching the potential target miRNAs of circTTBK2. The results showed that 5 miRNAs (miR-197, miR-526b, miR-145-5p, miR-140-3p and miR-217) were simultaneously predicted to be the targets of circTTBK2 (Figure S1A). Then, we designed 3′-biotinylated circTTBK2 probe to analyze the interaction between these miRNAs and circTTBK2. The results showed that circTTBK2 could pull down circTTBK2 and circTTBK2 overexpression elevated the pull-down efficiency in T98G and LN229 cells (Figure S1B). Moreover, we found that miR-145-5p was conspicuously pulled down by circTTBK2 probe in SK-N-SH and SK-N-AS cells (Figure S1C). Thus, we selected miR-145-5p as the object of our study. The potential binding sites between circTTBK2 and miR-145-5p were presented in Figure 3A. To confirm this prediction, we conducted dual-luciferase reporter assay. The results showed that miR-145-5p mimic transfection caused an obvious suppression in the luciferase activity of WT-circTTBK2 in T98G and LN229 cells when compared to miR-NC groups, whereas no change was observed in MUT-circTTBK2 groups (Figure 3B). Then, we further performed RIP assay to verify the combination between circTTBK2 and miR-145-5p. RIP assay results showed that circTTBK2 enrichment was markedly enhanced in Ago2 immunoprecipitation complex in both T98G and

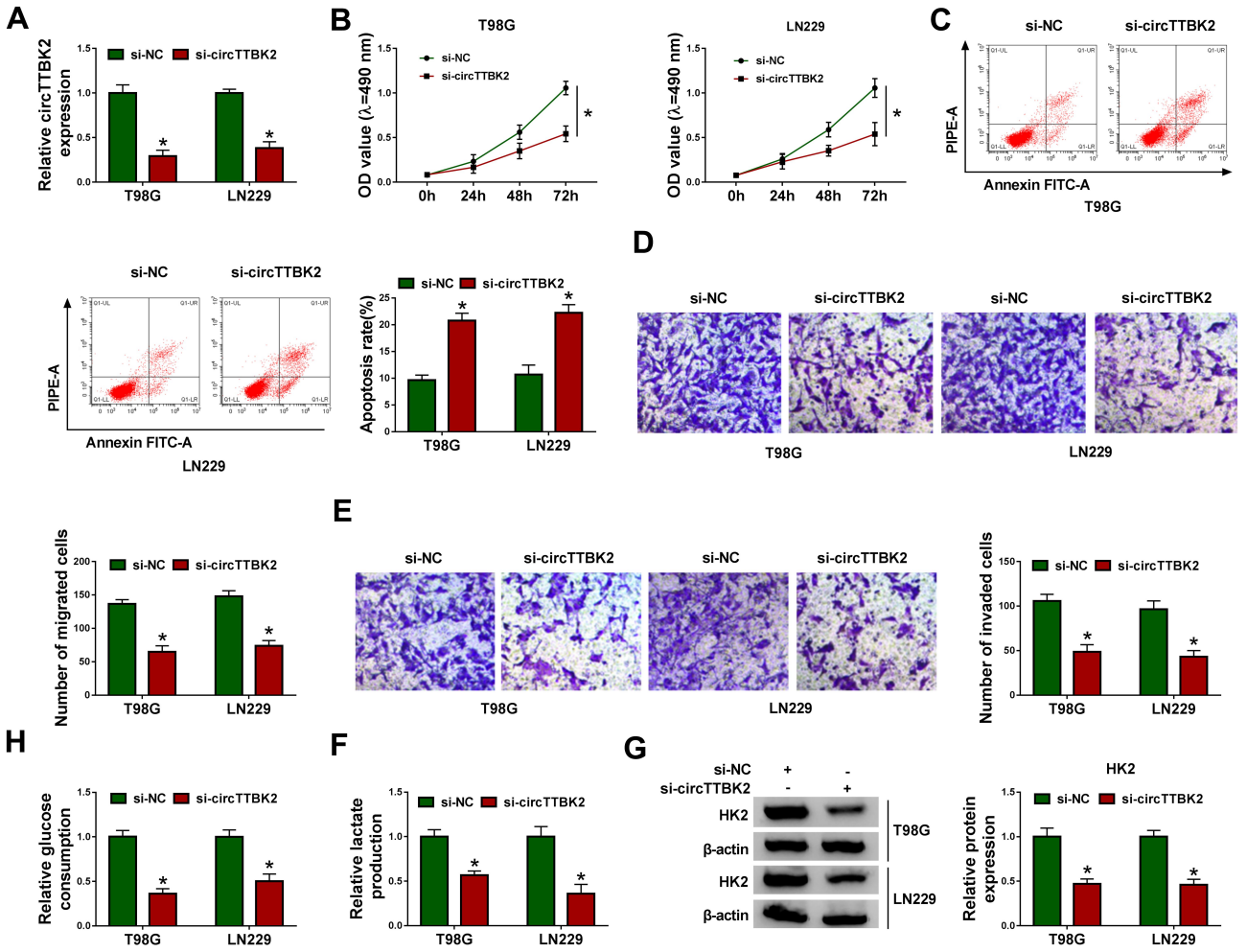

Figure 2 CircTTBK2 knockdown relieved the malignant behaviors of glioma cells. T98G and LN229 cells were transfected with si-NC or si-circTTBK2. (**A**) QRT-PCR assay was performed for circTTBK2 expression level in T98G and LN229 cells. (**B**) The proliferation, (**C**) apoptosis, (**D** and **E**) migration and invasion of T98G and LN229 cells were assessed through MTT assay, flow cytometry analysis and transwell assay, respectively. (**F** and **G**) The levels of glucose uptake and lactate production in T98G and LN229 cells were measured with specific kits. (**H**) The protein level of HK2 in T98G and LN229 cells was measured via Western blot assay. *$P<0.05$.

LN229 cells following the transfection of miR-145-5p mimic compared to IgG control groups (Figure 3C). After that, we successfully transfected oe-circTTBK2 into T98G and LN229 cells to elevate circTTBK2 expression, and the transfection efficiency was demonstrated by qRT-PCT analysis (Figure 3D). Next, the effect of circTTBK2 on miR-145-5p expression was determined using qRT-PCR assay. As a result, circTTBK2 silencing apparently increased miR-145-5p expression level in T98G and LN229 cells, while circTTBK2 overexpression exhibited the opposite results (Figure 3E). Expectedly, miR-145-5p was lowly expressed in glioma tissues and cells relative to normal tissues and cells (Figure 3F and G). As estimated by Spearman correlation coefficient analysis, miR-145-5p expression level was negatively correlated with circTTBK2 expression level in glioma tissues (Figure 3H). These results suggested that circTTBK2 negatively modulated miR-145-5p expression by direct targeting miR-145-5p in glioma cells.

MiR-145-5p Inhibition Ameliorated the Effects of circTTBK2 Knockdown on Glioma Cell Proliferation, Apoptosis, Migration, Invasion and Glycolysis

As exhibited in Figure 4A, miR-145-5p inhibitor transfection effectively reduced the level of miR-145-5p in T98G and LN229 cells. To explore the effects of circTTBK2/miR-145-5p axis on the malignant phenotypes of glioma cells, rescue experiments were conducted through transfecting miR-NC inhibitor,

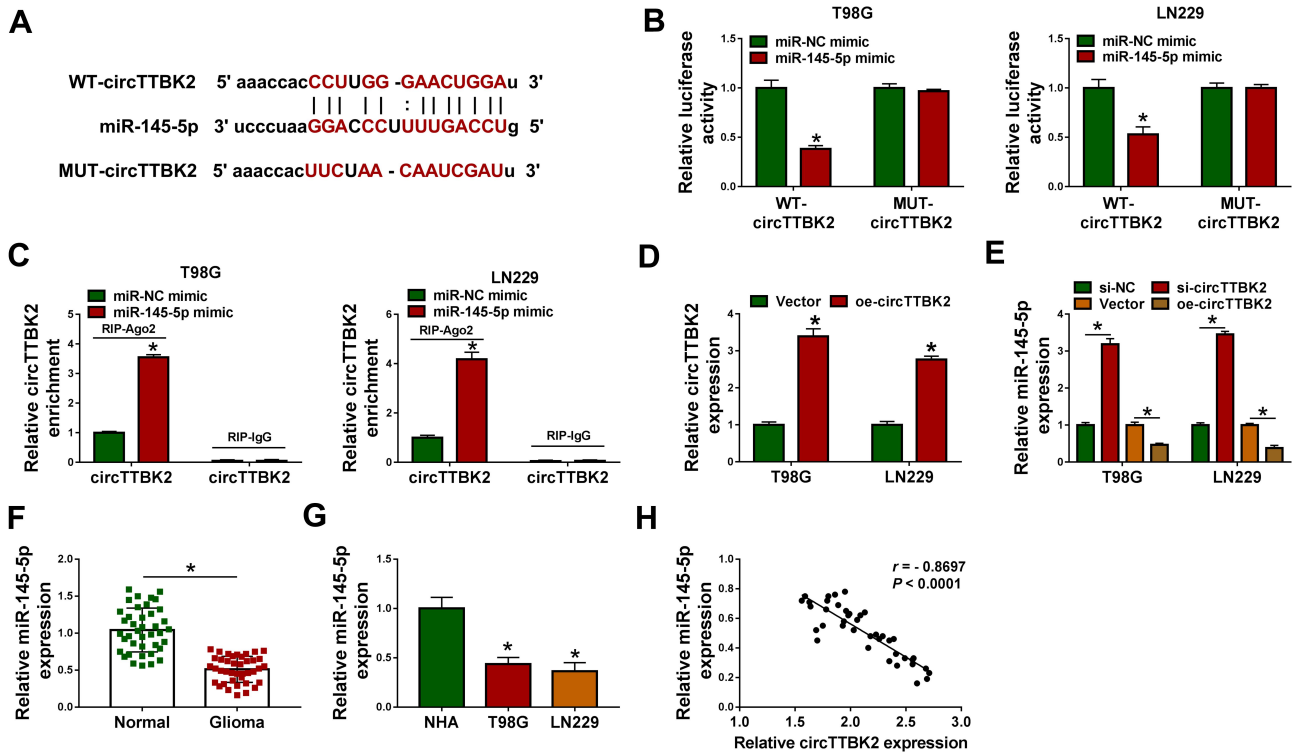

Figure 3 CircTTBK2 functioned as a sponge of miR-145-5p in glioma cells. (**A**) The predicted binding sites between circTTBK2 and miR-145-5p. (**B**) The luciferase activity of WT-circTTBK2/MUT-circTTBK2 in T98G and LN229 cells transfected with miR-145-5p mimic/miR-NC mimic was measured through dual-luciferase reporter assay. (**C**) The enrichment of circTTBK2 in Ago2/IgG immunoprecipitates in T98G and LN229 cells transfected with miR-145-5p mimic or miR-NC mimic was determined by qRT-PCR following RIP assay. (**D**) The expression level of circTTBK2 in T98G and LN229 cells transfected with oe-circTTBK2 or Vector was determined by qRT-PCR analysis. (**E**) The expression level of miR-145-5p in T98G and LN229 cells transfected with si-NC, si-circTTBK2, Vector or oe-circTTBK2 was measured by qRT-PCR. (**F** and **G**) QRT-PCR assay was performed for miR-145-5p expression level in glioma tissues, cells and corresponding normal tissues and cells. (**H**) The correlation between the levels of miR-145-5p and circTTBK2 was analyzed by Spearman correlation coefficient analysis. *$P<0.05$.

miR-145-5p inhibitor, si-circTTBK2+miR-NC inhibitor or si-circTTBK2+miR-145-5p inhibitor into T98G and LN229 cells. As demonstrated by MTT assay, flow cytometry analysis and transwell assay, miR-145-5p inhibition distinctly promoted the proliferation, migration and invasion abilities of T98G and LN229 cells and apparently impeded apoptosis; moreover, si-circTTBK2-mediated cell phenotypes were all restored by decreasing miR-145-5p (Figure 4B–E). Furthermore, we demonstrated that miR-145-5p inhibition elevated the levels of glucose consumption, lactate production and HK2 protein in T98G and LN229 cells; moreover, miR-145-5p suppression overturned the inhibitory effects of circTTBK2 knockdown on cell glucose consumption, lactate production and HK2 protein (Figure 4F–H). Taken together, circTTBK2 knockdown relieved the malignant behaviors of glioma cells by targeting miR-145-5p.

CPEB4 Was a Direct Target Gene of miR-145-5p in Glioma Cells

Through using bioinformatics software TargetScanHuman (http://www.targetscan.org/vert_72/), ZEB2, CPEB4, SOX9, CDK6, TLR4 and CCND2 were predicted to be the target genes of miR-145-5p. Moreover, we demonstrated that miR-145-5p mimic transfection led to a marked elevation in CPEB4 mRNA level in T98G and LN229 cells (Figure S2D). Thus, we selected CPEB4 as our research object. The potential complementary sequences between miR-145-5p and CPEB4 are shown in Figure 5A. Then, dual-luciferase reporter assay and RIP assay were carried out to verify this prediction. As suggested by dual-luciferase reporter assay, compared with WT-CPEB4 and miR-NC mimic co-transfected groups, the luciferase activity in WT-CPEB4 and miR-145-5p mimic co-transfected T98G and LN229 cells was notably repressed, but the luciferase activity was not affected in

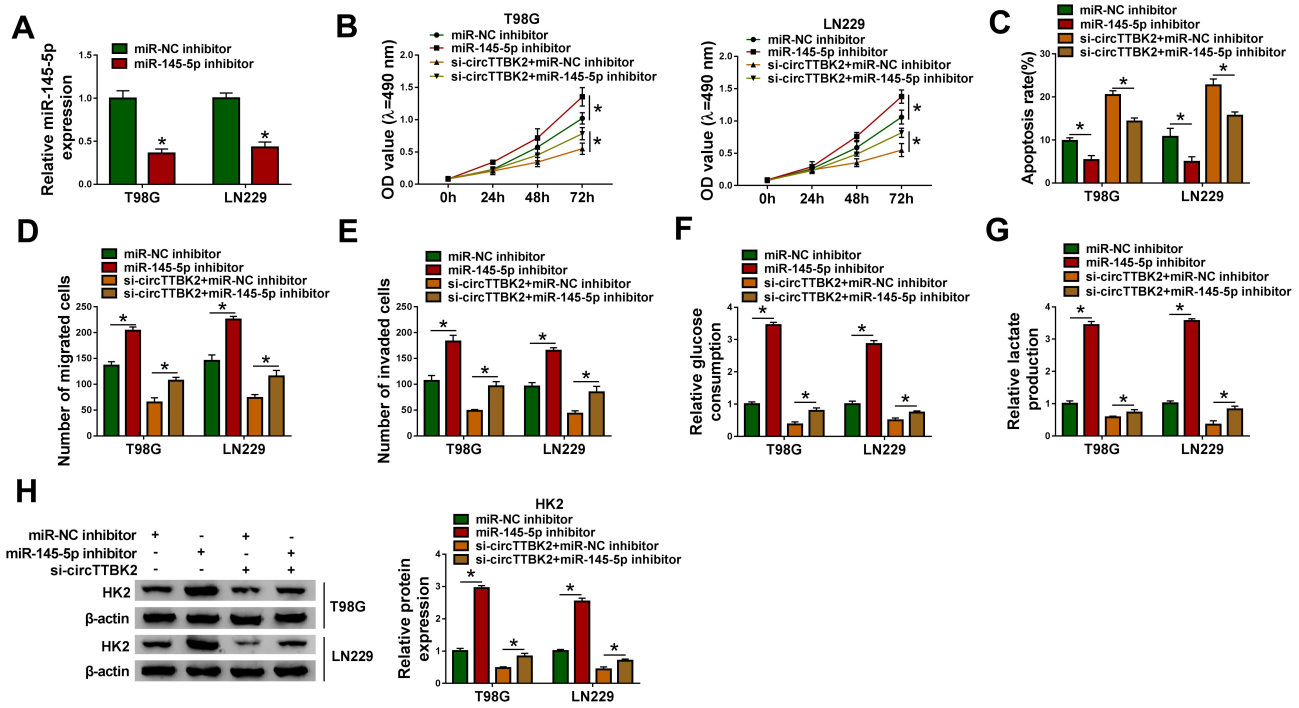

Figure 4 Inhibition of miR-145-5p abrogated the effects of circTTBK2 silencing on glioma cell behaviors. (**A**) T98G and LN229 cells were transfected with miR-145-5p inhibitor or miR-NC inhibitor and then the expression of miR-145-5p was measured by qRT-PCR assay. (**B-H**) T98G and LN229 cells were transfected with miR-NC inhibitor, miR-145-5p inhibitor, si-circTTBK2+miR-NC inhibitor or si-circTTBK2+miR-145-5p inhibitor. (**B**) Cell proliferation, (**C**) apoptosis, (**D** and **E**) migration and invasion in T98G and LN229 cells were examined by MTT assay, flow cytometry analysis and transwell assay, respectively. (**F** and **G**) The levels of glucose consumption and lactate production were detected using relevant kits. (**H**) Western blot assay was used for HK2 protein level in T98G and LN229 cells. *$P<0.05$.

MUT-CPEB4 groups (Figure 5B). The results of RIP assay showed that CPEB4 was markedly enriched in Ago2 immunoprecipitation complex in T98G and LN229 cells transfected with miR-145-5p mimic compared to IgG groups (Figure 5C). As presented in Figure 5D, miR-145-5p mimic transfection led to a distinct elevation in miR-145-5p level in T98G and LN229 cells. Next, the impact of miR-145-5p on CPEB4 expression was measured by qRT-PCR assay and Western blot assay. The data exhibited that miR-145-5p inhibition notably elevated the mRNA and protein levels of CPEB4 in T98G and LN229 cells, while miR-145-5p overexpression showed the opposite results (Figure 5E and F). After that, we determined the mRNA and protein levels of CPEB4 in glioma tissues and cells, showing that CPEB4 mRNA and protein levels were obviously increased in glioma tissues and cells when compared to normal tissues and cells (Figure 5G–J). Additionally, we observed that there was an inverse correlation between the expression levels of miR-145-5p and CPEB4 mRNA in glioma tissues (Figure 5K). These outcomes demonstrated that miR-145-5p negatively regulated CPEB4 expression by direct interaction.

Inhibition of miR-145-5p Contributed to Glioma Cell Progression by Binding to CPEB4

As displayed in Figure 6A and B, si-CPEB4 transfection evidently decreased the mRNA and protein levels of CPEB4 in T98G and LN229 cells, indicating that si-CPEB4 was successfully transfected into T98G and LN229 cells. To clarify the relationship between miR-145-5p and CPEB4 in regulating the malignant phenotypes of glioma cells, T98G and LN229 cells were divided into 4 groups: si-NC, si-CPEB4, miR-145-5p inhibitor+si-NC and miR-145-5p+si-CPEB4. The deficiency of CPEB4 markedly hampered cell proliferation, migration and invasion and facilitated apoptosis in T98G and LN229 cells compared to si-NC groups (Figure 6C–F). Furthermore, CPEB4 deficiency rescued the impacts of miR-145-5p inhibition on the malignant biological behaviors (Figure 6C–F). In the meanwhile, we confirmed that CPEB4 interference decreased the levels of glucose consumption and lactate production, as well as HK2 protein in both T98G and LN229 cells; moreover, miR-145-5p inhibitor-mediated effects on glucose consumption, lactate production and HK2 protein could be abated by

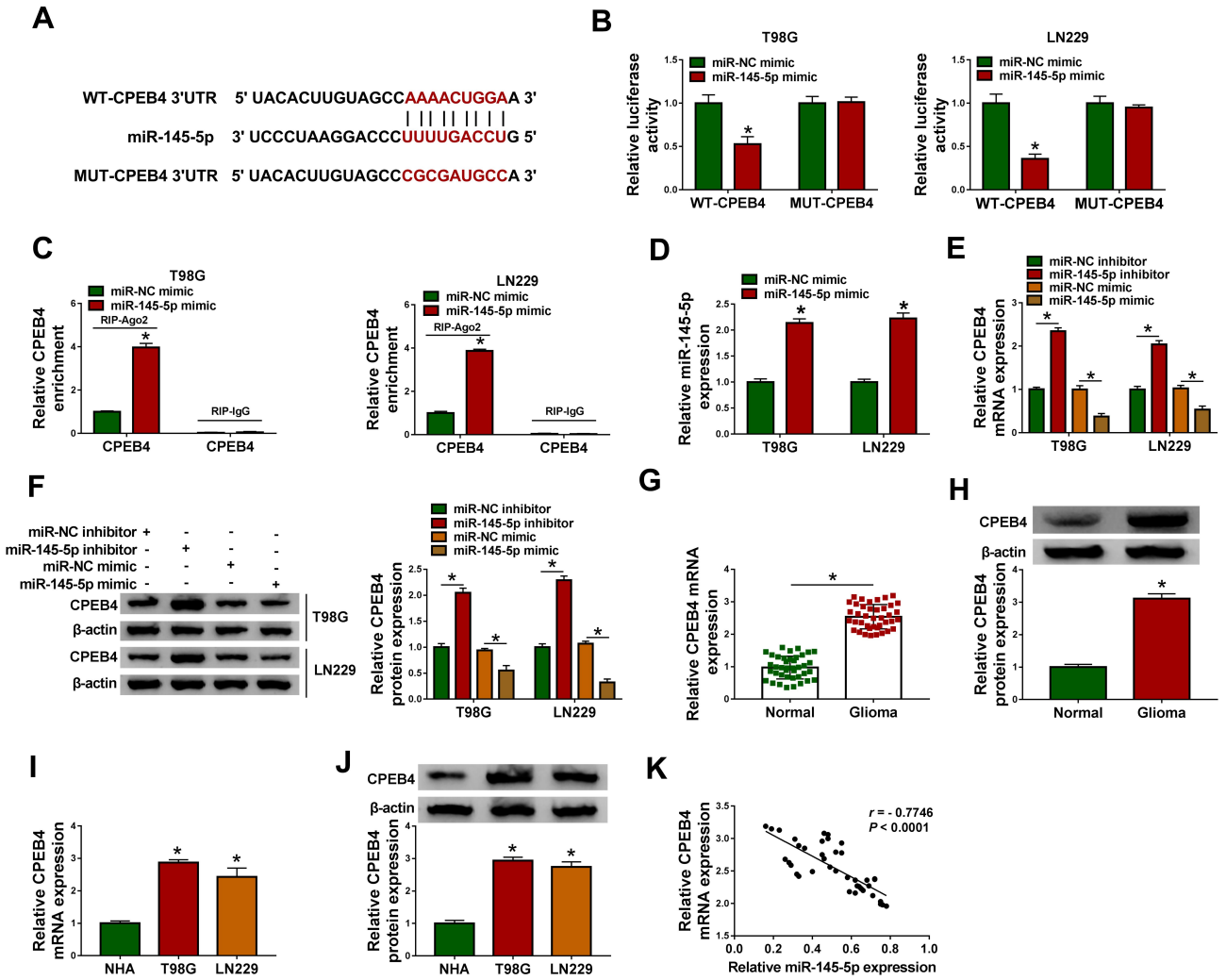

Figure 5 CPEB4 was targeted by miR-145-5p in glioma cells. (**A**) The potential binding sites between CPEB4 and miR-145-5p were predicted by TargetScan. (**B**) The luciferase activity in T98G and LN229 cells co-transfected with miR-NC mimic/miR-145-5p mimic and WT-CPEB4/MUT-CPEB4 was measured by dual-luciferase reporter assay. (**C**) After RIP assay, CPEB4 level in Ago2/IgG immunoprecipitates in T98G and LN229 cells transfected with miR-145-5p mimic or miR-NC mimic was detected through qRT-PCR assay. (**D**) QRT-PCR assay was conducted for miR-145-5p expression level in T98G and LN229 cells transfected with miR-145-5p mimic or miR-NC mimic. (**E** and **F**) T98G and LN229 cells were transfected with miR-NC inhibitor, miR-145-5p inhibitor, miR-NC mimic or miR-145-5p mimic, and then CPEB4 mRNA and protein levels were measured by qRT-PCR assay and Western blot assay, respectively. (**G-J**) The mRNA and protein levels of CPEB4 in glioma tissues, cells and corresponding normal tissues and cells were determined by qRT-PCR analysis and Western blot analysis, respectively. (**K**) The correlation between miR-145-5p expression level and CPEB4 mRNA expression level was analyzed by Spearman correlation coefficient analysis. *P<0.05.

CPEB4 interference (Figure 6G–I). These results suggested that miR-145-5p inhibition aggravated the progression of glioma cells via interacting with CPEB4.

CircTTBK2 Positively Regulated CPEB4 Expression by Targeting miR-145-5p in Glioma Cells

As estimated by Spearman correlation coefficient analysis, CPEB4 mRNA expression level was positively correlated with circTTBK2 expression level in glioma tissues (Figure 7A). To further explore the mechanism among circTTBK2, miR-145-5p and CPEB4, T98G and LN229 cells were transfected with si-NC, si-circTTBK2, si-circTTBK2+miR-NC inhibitor or si-circTTBK2+miR-145-5p inhibitor. Then the mRNA and protein levels of CPEB4 were measured using qRT-PCR assay and Western blot assay, respectively. The results showed that circTTBK2 silencing notably decreased the mRNA and protein levels of CPEB4 in T98G and LN229 cells, while the impacts were partially abolished by the inhibition of miR-145-5p (Figure 7B and C). These data suggested that circTTBK2 knockdown suppressed CPEB4 expression by sponging miR-145-5p in glioma cells.

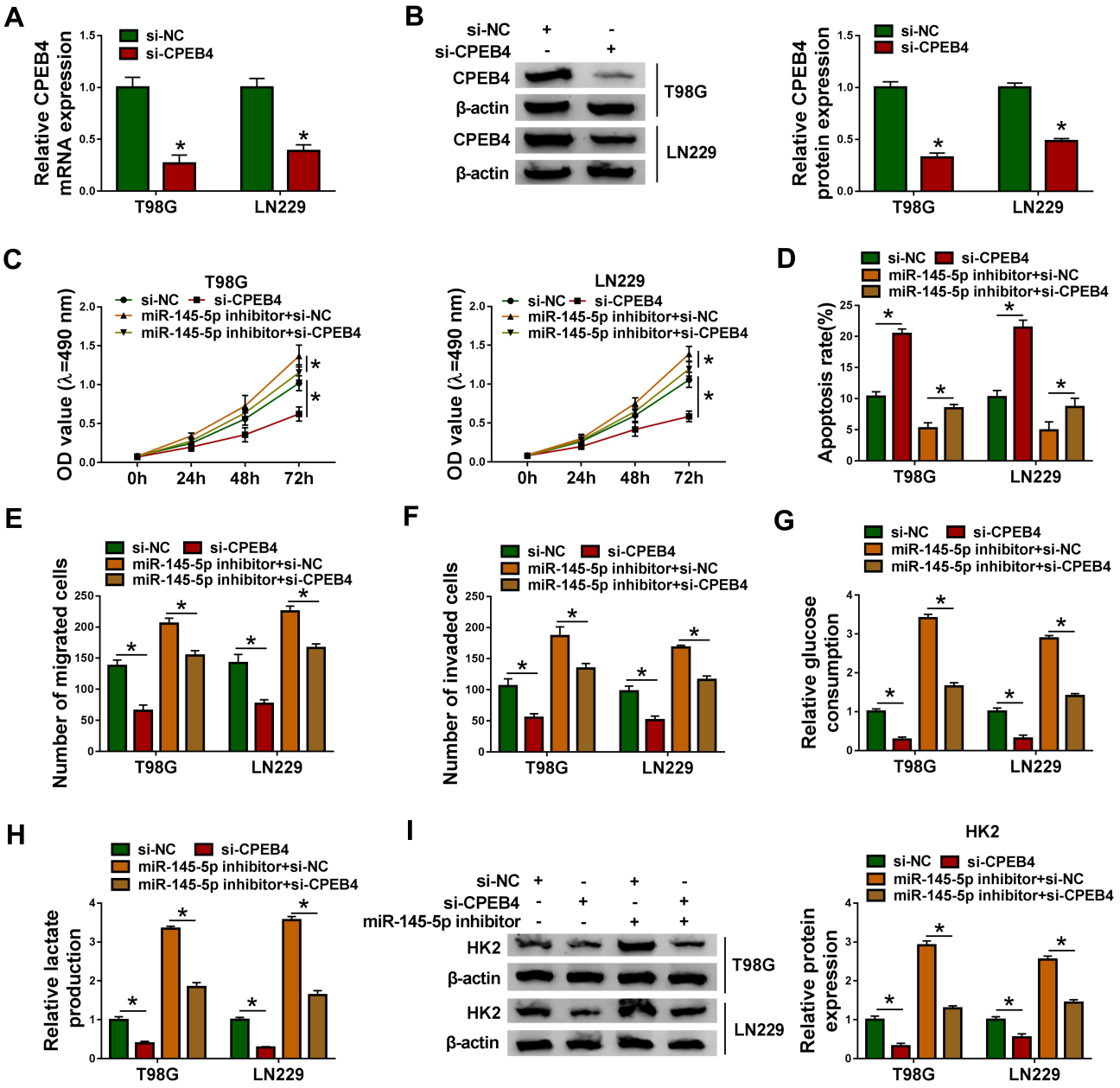

Figure 6 CPEB4 knockdown reversed miR-145-5p inhibitor-mediated glioma cell proliferation, apoptosis, migration, invasion and glycolysis. (**A** and **B**) The mRNA and protein levels of CPEB4 in T98G and LN229 cells transfected with si-CPEB4 or si-NC were determined by qRT-PCR assay and Western blot assay, respectively. (**C-I**) T98G and LN229 cells were transfected with si-NC, si-CPEB4, miR-145-5p inhibitor+si-NC or miR-145-5p+si-CPEB4. (**C**) Cell proliferation, (**D**) apoptosis, (**E** and **F**) migration and invasion in T98G and LN229 cells were evaluated by MTT assay, flow cytometry analysis and transwell assay, respectively. (**G** and **H**) The levels of glucose uptake and lactate production in T98G and LN229 cells were examined using specific kits. (**I**) The protein level of HK2 in T98G and LN229 cells was measured by Western blot assay. *$P<0.05$.

CircTTBK2 Knockdown Suppressed Tumorigenesis of Glioma in vivo

To elucidate the tumorigenic function of circTTBK2 in vivo, T98G cells transfected with sh-circTTBK2 or sh-NC were implanted into nude mice. As exhibited in Figure 8A and B, mice treated with sh-circTTBK2 showed reduced tumor volume and weight when compared to mice treated with sh-NC. Subsequently, our results of qRT-PCR assay and Western blot assay showed that circTTBK2, CPEB4 mRNA and CPEB4 protein levels were prominently reduced, while miR-145-5p level was enhanced in tumor tissues collected

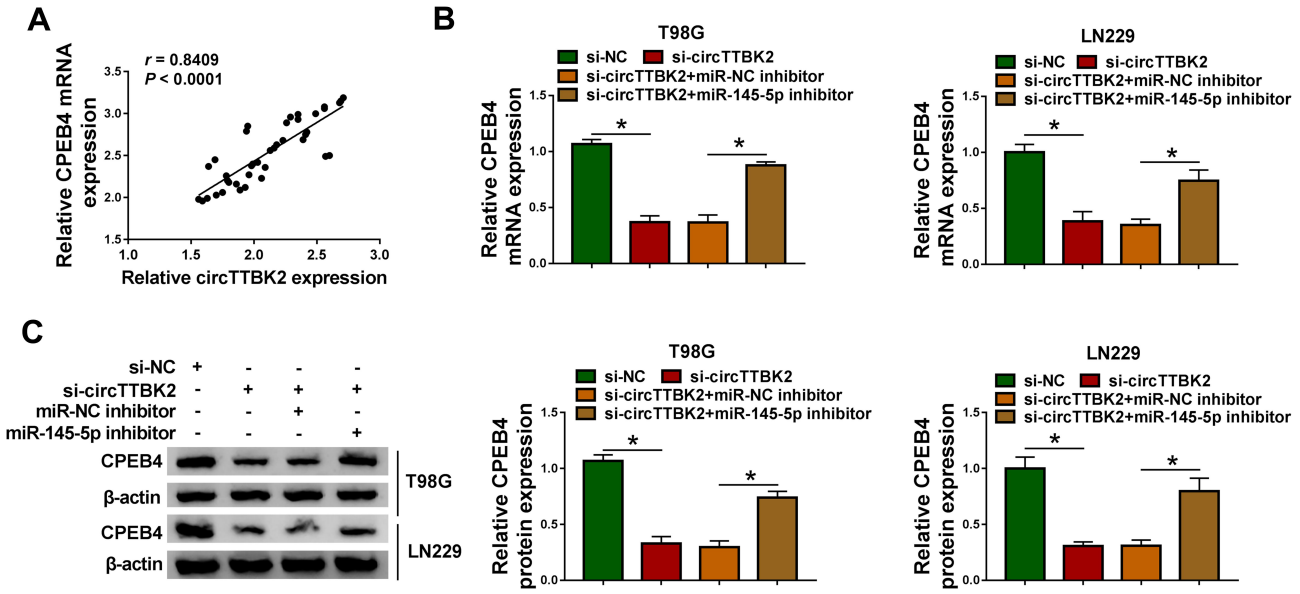

Figure 7 CircTTBK2 positively modulated CPEB4 expression through targeting miR-145-5p in glioma cells. (**A**) The correlation between the levels of CPEB4 mRNA and circTTBK2 in glioma tissues was analyzed by Spearman correlation coefficient analysis. (**B** and **C**) Si-NC, si-circTTBK2, si-circTTBK2+miR-NC inhibitor or si-circTTBK2 +miR-145-5p inhibitor was transfected into T98G and LN229 cells, and then qRT-PCR and Western blot assays were conducted for CPEB4 mRNA and protein levels. *P<0.05.

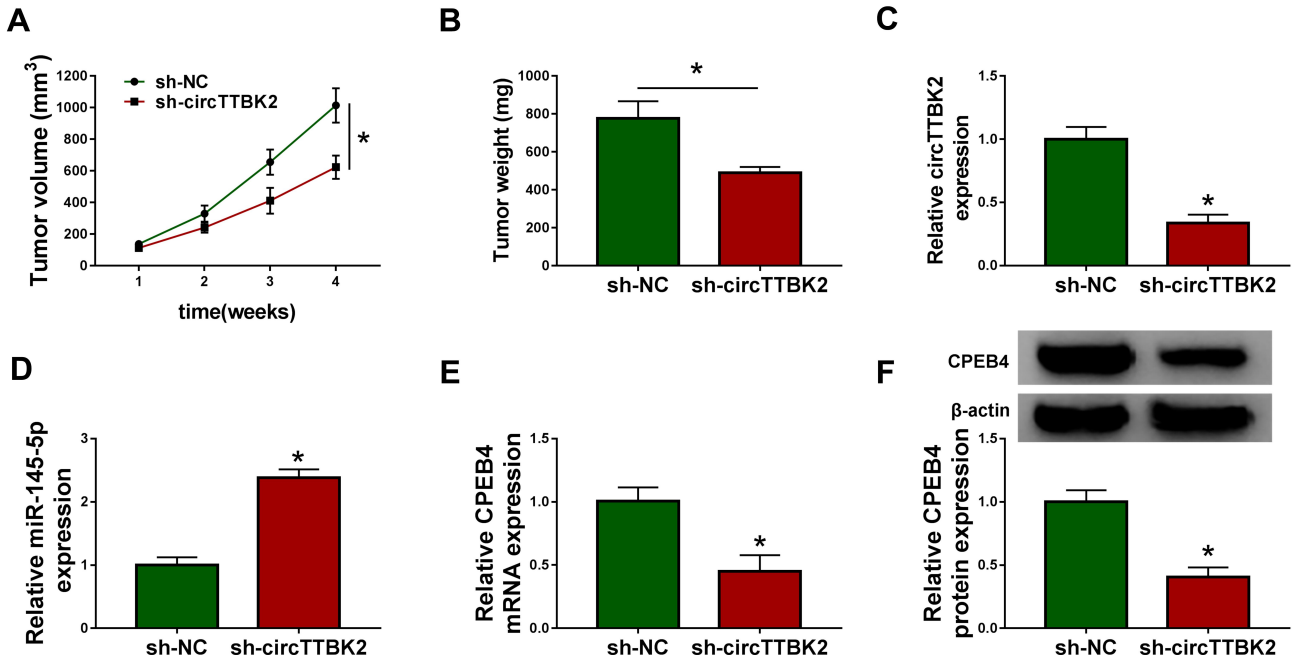

Figure 8 Silencing of circTTBK2 blocked tumor growth in vivo. Sh-circTTBK2 or sh-NC transfected T98G cells were injected into nude mice. (**A**) Tumor volume was monitored weekly. (**B**) Tumor weight was examined after 4 weeks. (**C-E**) The levels of circTTBK2, miR-145-5p and CPEB4 mRNA in the harvested tumors were determined by qRT-PCR assay. (**F**) The protein level of CPEB4 in the harvested tumors was measured by Western blot assay. *P<0.05.

from sh-circTTBK2 groups compared to those from sh-NC groups (Figure 8C–F). In a word, circTTBK2 knockdown restrained tumor progression in vivo.

Discussion

Exploring the innovative diagnosis and treatment technologies of glioma has always been a research hotspot in

neurosurgery. Growing publications have confirmed the crucial roles of circRNAs in glioma. In this paper, we mainly explored the precise functions and potential mechanisms of circTTBK2 in glioma. As a result, we demonstrated that circTTBK2 functioned as a tumor promoter in glioma through facilitating cell growth, motility and glycolysis and restraining apoptosis. Strikingly, a novel regulatory network circTTBK2/miR-145-5p/CPEB4 was discovered in glioma.

Zhang et al declared that circTTBK2 level was raised in glioma tissues and cells, and played a positive role in tumor cell growth and invasion by downregulating miR-761 and elevating ITGB8.[22] Yuan et al unraveled that circTTBK2 functioned as a positive regulator in glioma malignancy through inhibiting apoptosis and accelerating growth and invasion by modulating miR-520b/EZH2 axis.[23] In line with these previous researches, circTTBK2 was also observed to be highly expressed in glioma tissues and cells and linked to poor outcomes of patients. Loss-of-function experiments showed that circTTBK2 deficiency restrained the capacities of tumor cell growth and metastasis and induced the capacity of apoptosis. Metabolic change is a typical characteristic of cancers, and tumor cells tend to produce more energy through aerobic glycolysis to meet the needs for rapid growth.[24] Hence, we explored the effect of circTTBK2 on glycolysis. Our results showed that the levels of glucose consumption, lactate production and HK2 protein were all reduced in glioma cells with circTTBK2 knockdown, suggesting that glycolysis was inhibited in glioma cells by reducing circTTBK2. Moreover, circTTBK2 knockdown blocked tumorigenesis of glioma in vivo. These outcomes illustrated the tumor-promoting effect of circTTBK2 on glioma.

Subsequently, the potential mechanism of circTTBK2 in promoting glioma progression was further validated. We demonstrated that circTTBK2 sponged miR-145-5p and then enhanced CPEB4 expression. MiR-145-5p was observed to be underexpressed in glioma tissues and cells. Suppression of miR-145-5p caused a noteworthy promotion in glioma cell growth, metastasis and glycolysis and a distinct repression in apoptosis, indicating the anti-tumor role of miR-145-5p in glioma. Furthermore, circTTBK2 knockdown-mediated malignant glioma cell phenotypes were ameliorated by decreasing miR-145-5p, suggesting that circTTBK2 modulated glioma cell progression via sponging miR-145-5p. In support of our findings, circPTN aggravated the tumorigenicity of glioma through interacting with miR-145-5p.[15] CircCEP128 knockdown impeded the growth of glioma cells by inhibiting miR-145-5p.[25] Additionally, CPEB4 was demonstrated to be targeted by miR-145-5p. Wang et al and Zhi et al manifested that CPEB4 level was enhanced in glioma and associated with worse prognosis.[16,17] Gu et al proved that CPEB4 level could be restrained by miR-98-5p and then participated in regulating cell migration, growth and apoptosis in glioma cells.[19] In line with these reports, in this project, CPEB4 level was raised in tumor tissues and cell lines. Silencing of CPEB4 repressed the malignant biological behaviors of glioma cells and the promotional effects on glioma cell phenotypes mediated by miR-145-5p inhibition could be overturned by decreasing CPEB4.

Taken together, circTTBK2 aggravated the malignant phenotypes of glioma cells in vitro and facilitated tumor progression in vivo by modulation of miR-145-5p/CPEB4 axis. Our results, for the first time, demonstrated the interactions among circTTBK2, miR-145-5p and CPEB4. This research deepened our knowledge of the pathogenesis of glioma, which might offer a novel diagnostic biomarker and therapeutic strategy for glioma.

Funding

There is no funding to report.

Disclosure

The authors declare that they have no financial or non-financial conflicts of interest for this work.

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