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Circular RNA TTBK2 promotes the development of human glioma cells via miR-520b/EZH2 axis

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Abstract. – OBJECTIVE: Glioma is a highly malignant human disease characterized by limited response to clinical therapy. Evidence indicated that circular RNA Tau tubulin kinase 2 circular RNAs (circ-TTBK2) participated in glioma pathogenesis. However, the precise effect of circ-TTBK2 on glioma progression is needed to be highlighted.

MATERIALS AND METHODS: The levels of circ-TTBK2, microRNA-520b (miR-520b), and enhancer of zeste homologue 2 (EZH2) were detected via quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) or Western blot. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was performed to determine cell proliferation *in vitro*. Besides, a flow cytometry assay was conducted to examine apoptosis of A172 and U251 cells. Cell invasion was identified using the transwell assay. Moreover, Dual-Luciferase reporter assay was used to confirm the interaction between miR-520b and circ-TTBK2 or EZH2. The role of circ-TTBK2 in glioma progression was exposed using xenograft tumor experiments.

RESULTS: The levels of circ-TTBK2 and EZH2 were markedly augmented, whereas miR-520b expression level was notably reduced in glioma tissues and cell lines. Either circ-TTBK2 or EZH2 detection could clearly facilitate cell apoptosis and block proliferation and invasion in A172 and U251 cells, while the effect of circ-TTBK2 or EZH2 deficiency was reverted by co-transfecting with miR-520 inhibitor. Moreover, circ-TTBK2 exerted its roles via miR-520b/EZH2 axis in glioma cells, and the knockdown of circ-TTBK2 could hinder the progression of glioma.

CONCLUSIONS: Circ-TTBK2/miR-520b/EZH2 axis modulated cell proliferation, apoptosis, and invasion in glioma cell lines, and might serve as potential targets for glioma diagnosis and therapy.

Key Words:
Circ-TTBK2, MiR-520b, EZH2, Glioma, Cell proliferation, Apoptosis, Invasion.

Introduction

Glioma is a malignant tumor characterized by the higher morbidity and mortality rate, and its highly aggressive nature leads to poor prognoses in patients. Recently, the prevalence of glioma is increased year by year[1,2]. Despite ongoing studies, the modulator mechanisms of gliomagenesis and progression are still not clearly understood.

Circular RNAs (circRNAs) are members of non-coding RNAs (ncRNAs)[3], formed by a covalently closed loop. Over the past decades, circRNAs are discovered to widely exist in eukaryotes[4], and they derive from nonrandom back-splice events[5,6]. The biogenesis of circRNAs is related to several distinct mechanisms. With regard to their function, increasing reports[7] have discovered that circRNAs mainly act as a sponge of microRNAs (miRNAs) to modulate the targeted gene expression. For example, circRNA mitochondrial translation optimization 1 homologue (circMTO1) modulates the expression of miR-9 to curb the progression of hepatocellular carcinoma[8]. Besides, Tau tubulin kinase 2 circular RNAs (circ-TTBK2) is deemed as a kinase[9]. The linear TTBK2 is connected with diverse diseases, and its upregulation can improve the progression and initiation of amyotrophic lateral sclerosis[10]. Interestingly, TTBK2 overexpression can weaken the apoptosis of kidney carcinoma, which is stimulated by sunitinib[11]. Nevertheless, the circular RNA TTBK2 (circ-TTBK2) has been little investigated before this study. Zheng et al[12] find that circ-TTBK2 contributes to the malignancy of glioma *via* targeting microRNA-217. Thereby, elucidating the biological role of circ-TTBK2 is still a continuous process in glioma researches.

Currently, miRNAs are a family of endogenous ncRNAs[13]. They modify the targeted gene

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expression at the transcriptional and post-transcriptional level, thereby downregulating the levels of mRNAs[14]. Besides, researchers suggest that miRNAs are the target genes of long-non-coding RNAs (lncRNAs)[15] or circRNAs[16]. For example, circRNA 0001564 regulates cell proliferation and apoptosis in osteosarcoma by sponging miRNAs[17]. In this study, we focus on the biological function of miR-520b in glioma pathogenesis. Previous works discover that miR-520b participates in multiple cancers. For instance, miR-520b modulates cell proliferation and migration of gastric cancer *via* targeting epidermal growth factor receptor (EGFR)[18]. MiR-520b represses hepatoma cell proliferation by regulating the expression of ten-eleven translocation 1 (TET1)[19]. Herein, we focus on the role of miR-520b in glioma cell proliferation, apoptosis, and invasion *in vitro*. The interaction between miR-520b and circ-TTBK2 is also the purpose of this study.

Recently, the enhancer of zeste homologue 2 (EZH2) belongs to the polycomb-group family. Generally, EZH2 is expressed at low levels in normal cells but is increased in various stem cells[20,21]. What's more, EZH2 has been reported to act as an oncogenic gene and contribute to neoplastic transformation in breast epithelial cells[22]. In this study, we speculate that EZH2 is a target gene of miR-520b to regulate cell behaviors in glioma.

Furthermore, we detected the expression levels of circ-TTBK2, miR-520b, and EZH2 in tumor tissues and cell lines (A172 and U251). The association between circ-TTBK2 expression and glioma cell behaviors (proliferation, apoptosis, and invasion) was also researched to expose the role of circ-TTBK2 in glioma. Moreover, this study explored whether circ-TTBK2 exerted its functions *via* miR-520b/EZH2 axis in glioma cells.

Materials and Methods

Patient Samples and Cell Culture

With the approval of the Ethics Committee of Zhuji People's Hospital of Zhejiang Province, we received the glioma tissues (n=30, marked as T) and peritumoral brain edema tissues (n=30, marked as N). These specimens were donated by glioma patients who underwent surgical resection at the Zhuji People's Hospital of Zhejiang Province. Written informed consent was also signed from each participant. The samples were transported with dry ice, and then stored in -80°C.

Glioma cell lines of A172 and U251 and Normal Human Astrocytes (NHA) were purchased from BeNa Culture Collection Co., Ltd. (Beijing, China). Besides, 293T cells used for lentivirus packing were also purchased from BeNa Culture Collection Co., Ltd. (Beijing, China). Then, the total cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South-Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1′ Penicillin-Streptomycin (Gibco, 100 U/mL of penicillin and 100 μg/mL of streptomycin) at 37°C with 5% CO_2.

Cell Transfection

MiR-520b mimic (miR-520b), miR-520b inhibitor (anti-miR-520b), and their controls (miR-NC for mimic and anti-miR-NC for inhibitor) were purchased from GenePharma Co., Ltd. (Suzhou, China). Short hairpin (shRNA) against circ-TTBK2 (sh-circ-TTBK2) and EZH2 (sh-EZH2) and their negative control (sh-NC), overexpression vector of circ-TTBK2 (circ-TTBK2) and the empty overexpression plasmid (pcDNA) were obtained from HanBio Biotechnology Co., Ltd (Shanghai, China). These vectors and oligonucleotides were transfected into A172 and U251 cells utilizing Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with producer's specifications. Besides, we also used lentivirus-mediated sh-circ-TTBK2 or sh-circ-TTBK2 to stably establish the transfected A172 cells.

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

The total RNA was isolated and extracted from patient samples and cells (A172 and U251) by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) based on manufacturer's instructions. The isolated RNA worked as a template to reversely transcribe cDNA using First Strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). The relative gene expression level of circ-TTBK2 was quantified utilizing TaqMan probe. The primer sequences and probe of circ-TTBK2 were as following: circ-TTBK2: (forward 5'-AGTGCAACATTTTCCCTGGTG-3', reverse 5'-GCTTGATTTTGGCTTGGCTC-3') and probe: (FAM+CCCCAATCTTTCTCAATGGTCTGACG+BHQ1).

Besides, TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) were used for miRNA and

mRNA reverse transcription, respectively. The primers of miR-520b, U6, EZH2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized from GenePharma Co., Ltd (Suzhou, China) and the sequences as listed: miR-520b: (forward 5'-AAAGTGCTTCCTTTTAGAGGG-3', reverse 5'-GCGAGCACAGAATTAATACGACTCACTATAGG-3'); EZH2: (forward 5'-TACTTGTGGAGCCGCTGAC-3', reverse 5'-CTGCCACGTCAGATGGTG-3'); GAPDH: (forward 5'-CAATGACCCCTTCATTGACC-3', reverse 5'-GACAAGCTTCCCGTTCTCAG-3'); U6: (forward 5'-CTCGCTTCGGCAGCACA-3', reverse 5'-AACGCTTCACGAATTTGCGT-3'). The expression levels were standardized to endogenous controls (GAPDH and U6), and the relative levels were calculated *via* $2^{-\Delta\Delta Ct}$ method.

Western Blot Assay

Briefly, RIPA lysis buffer (Absin Bioscience, Shanghai, China) supplemented with 1% Phenylmethylsulfonyl Fluoride (PMSF; Beyotime, Shanghai, China) was used to homogenize the tissues and cells. The mixtures were loaded onto sodium dodecyl sulfate-polyacrylamide gel by electrophoresis (SDS-PAGE) and then, transfected onto the polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) membrane. Subsequently, the blots were incubated with 1:1500 diluted rabbit polyclonal EZH2 (ab186006, Abcam, Cambridge, MA, USA) at 4°C overnight. Next, the membranes were covered with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) after Tris-Buffered Saline Tween-20 (TBST; Beyotime, Shanghai, China) wash for thrice. Unique protein was appeared by enhanced chemiluminescence substrates (Millipore, Billerica, MA, USA). After that, the EZH2 expression level was normalized to internal GAPDH (1:10000; ab181602, Abcam, Cambridge, MA, USA).

Cell Proliferation Assay

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) was employed to assay the capacity of cell proliferation. A172 and U251 cells were plated (2×10^4 cells/well) in a 96-well plate with three replicate wells. After transfection with vectors or oligonucleotides for 24 h, 48 h, or 72 h, 20 μL of MTT was supplemented into each well at the same time, and the cells were incubated for 4 h. Next, the supernatant was abandoned, and 200 μL dimethyl sulfoxide (DMSO) was added to dissolve formazan. Lastly, the optical density (OD) value could be used to determine the level of cell proliferation, and it was detected using a microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 490 nm.

Flow Cytometry Assay for Cell Apoptosis

Apoptotic cells were measured using Annexin V-fluorescein isothiocyanate/propidium iodide (PI) (Annexin V-FITC/PI) Apoptosis Detection Kit (Yeasen Biotechnology, Shanghai, China) following the manuals of the manufacturer at 48 h post-transfection. Briefly, the transfected cells were re-suspended with 1×binding buffer. Then, Annexin V-FITC (5 μL) and PI (5 μL) were mildly mixed with the cell suspension. After incubation for 15 min at the temperature, apoptotic cells were distinguished by FACScan flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Transwell Assay

For cell invasion assay, the upper chamber was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Then, the transfected A172 and U251 cells (5×10^4) were plated in the upper part of the transwell chamber (Corning Inc., Corning, NY, USA) with serum-free medium. The lower chamber was filled with complete medium. After 24 h incubation, the migrated cells were stained by crystal violet (Sigma-Aldrich, St. Louis, MO, USA), the photographs of 10 randomly picked fields were taken, and the stained cell numbers were calculated by an inverted microscope (Olympus, Tokyo, Japan).

Dual-Luciferase Reporter Assay

The assay was referenced as previously described[23]. In brief, the common sequences of miR-520b and circ-TTBK2 or EZH2 were cloned by qRT-PCR and inserted into the downstream of the Firefly Luciferase gene, thereby constructed Luciferase reporter (WT-circ-TTBK2 and EZH2 3'UTR-MUT). Similarly, the mutant types of the Luciferase reporter vectors were also constructed (MUT-circ-TTBK2 and EZH2 3'UTR-MUT). For the Dual-Luciferase reporter assay, the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was carried out to evaluate the activities of Renilla and firefly. Renilla activity functioned as the reference to normalize firefly activity.

Murine Xenograft Assay

Six-week nude mice (18-20 g, n=7/group) were purchased from Shanghai Laboratory Animal

Center Co., Ltd. (Shanghai, China). They were fed in specific-pathogen-free (SPF) condition with free rodent chow and water. The animal experiments were authorized by the Institutional Animal Care and Use Committee of Zhuji People's Hospital of Zhejiang Province. In this study, the mice were randomly assigned into two groups (control or treatment). Then, the stably transfected (lentivirus-mediated sh-NC or sh-circ-TTBK2) A172 cells were subcutaneously injected into the right flank of the relative mice. Subsequently, the tumor volumes were monitored every 3 days for a total of 23 days post-injection. The mice were sacrificed at day 23, and the tumor weights of each nude mouse were measured. Moreover, the qRT-PCR assay was carried out to examine circ-TTBK2, miR-520b, and EZH2 levels in xenograft tumors, and Western blot assay was employed to assess the protein level of EZH2 in tumors.

Statistical Analysis

All the experiments were run in triplicate. The data were exhibited as mean ± standard deviation (SD), and the comparisons between the two-group data were examined by the Student's *t*-test, and One-way analysis of variance (ANOVA) was conducted to compare the differences in the multiple groups. The significant difference was considered when *p*-value was less than 0.05.

Results

Circ-TTBK2 and EZH2 Levels are Upregulated in Glioma Tissues and Cell Lines

To investigate the potential role of circ-TTBK2 in glioma, the level of circ-TTBK2 was evaluated using qRT-PCR, and the results indicated that circ-TTBK2 level was notably increased in glioma tissues and cell lines (Figure 1A and 1B). Subsequently, the mRNA and protein levels of EZH2 were also determined by qRT-PCR and Western blot assays, respectively. The results showed that mRNA and the protein levels of EZH2 were both improved in tumor tissues compared with matched control (Figure 1C and 1D). What's more, the level of EZH2 was also highly expressed in glioma cell lines at the aspects of mRNA and protein (Figure 1E and 1F). These data meant that circ-TTBK2 and EZH2 played a vital role in glioma pathogenesis and progression.

Circ-TTBK2 Detection Accelerates Cell Apoptosis, Suppresses Cell Proliferation and Invasion In Vitro

To identify the biological role of circ-TTBK2 in glioma cells, sh-circ-TTBK2 or sh-NC was transfected into A172 and U251 cells. As shown in Figure 2A, the expression level of circ-TTBK2

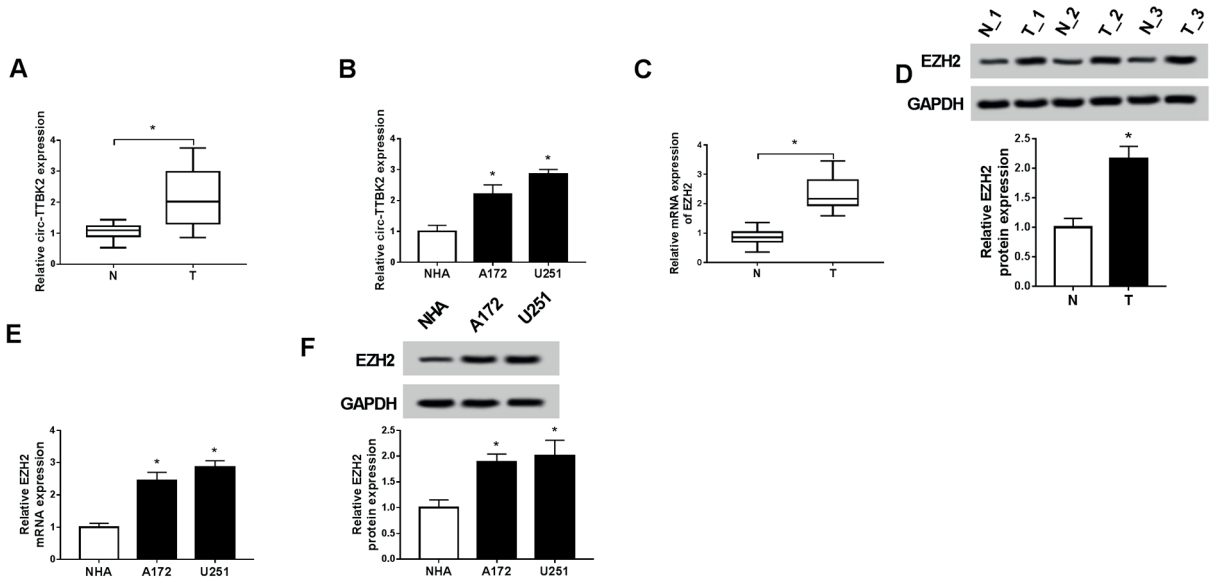

Figure 1. Circ-TTBK2 and EZH2 levels were upregulated in glioma tissues and cell lines. **A,** and **B,** QRT-PCR based on probe was employed to analyze the expression level of circ-TTBK2 in glioma tissues and cell lines compared with relative controls. **C-F,** The mRNA and protein expression levels of EZH2 were determined by **C,** and **E,** qRT-PCR and **D,** and **F,** Western blot assays, respectively. * $p<0.05$.

was remarkably impeded in both A172 and U251 cells transfected with sh-circ-TTBK2. Hence, we used sh-circ-TTBK2 to further investigate the role of circ-TTBK2 in cell behaviors. MTT analysis displayed that the proliferation of A172 and U251 cells was significantly hampered after transfection with sh-circ-TTBK2 (Figure 2B and 2C). Conversely, the knockdown of circ-TTBK2 could clearly boost cell apoptosis *in vitro* (Figure 2D). Simultaneously, the transwell assay was employed to detect the invasiveness of A172 and U251 cells, and the results suggested that circ-TTBK2 detection effectively constrained cell invasion in glioma cell lines (Figure 2E). All the data demonstrated that circ-TTBK2 detection could suppress the glioma malignancy *in vitro*.

Circ-TTBK2 is a Sponge of MiR-520b

As mentioned above, circ-TTBK2 acted as an oncogenic regulator, and we used starBase to find the potential targets of circ-TTBK2. As the results, miR-520b was discovered to exist in some complementary bases with circ-TTBK2 (Figure 3A). After which, WT-circ-TTBK2 or MUT-circ-TTBK2 reporter plasmid was co-transfected with miR-NC or miR-520b into A172 and U251 cells. After 48 h of transfection, miR-520b notably impeded the Luciferase activity of WT-circ-TTBK2 reporter group, whereas it had no distinct role in the Luciferase activity of MUT-circ-TTBK2 plasmid (Figure 3B and 3C). Then, sh-circ-TTBK2 or circ-TTBK2 was introduced into glioma cells. qRT-PCR analysis revealed that miR-520b level was significantly decreased in A172 and U251 cells after transfection with circ-TTBK2, but it was increased in glioma cells with circ-TTBK2 deficiency (Figure 3D). Besides, the level of miR-520b was apparently curbed in glioma tissues and cell lines (Figure 3E and 3F), and its level was negatively correlated with circ-TTBK2 level (Figure 3G). Subsequently, the regulatory mechanism between miR-520b and circ-TTBK2 was researched; next, we manifested that miR-520b inhibitor could abolish the promoting effect of circ-TTBK2 detection on miR-520b level in A172 and U251 cells (Figure 3H). Moreover, the inhibitory effect of circ-TTBK2 silencing on cell proliferation was rescued by suppression of miR-520b (Figure 3I and 3J). Furthermore, the reintroduction of miR-520b inhibitor reversed the effect of sh-circ-TTBK2 on apoptosis in A172 and U251 cells (Figure 3K). Meanwhile, the transwell analysis exhibited that the inhibiting role of circ-TTBK2 deficiency on cell invasion was relieved by miR-140-5p inhibitor *in vitro* (Figure 3L). All the evidence might suggest that circ-TTBK2 exerted its carcinogenic role partially *via* sponging miR-520b in glioma cells.

EZH2 Is Directly Targeted by MiR-520b

Similarly, we also searched the target genes of miR-520b using starBase software, and its results showed that EZH2 was a probable target of miR-520b (Figure 4A). Then, the Dual-Luciferase reporter assay indicated that the Luciferase activity was decreased in the wild type group, but the Luciferase activity was not evidently different in the mutant group. The data jointly proved that EZH2 was directly targeted by miR-520b in glioma cells (Figure 4B and 4C). Additionally, we also implied that miR-520b level was passively correlated with EZH2 (Figure 4D). Furthermore, the level of EZH2 was regulated by miR-520b, and the introduction of miR-520 mimic could conspicuously reduce EZH2 mRNA and the protein levels in A172 and U251 cells, while the role of miR-520 inhibitor was contrary to miR-520b mimic in regulating EZH2 level (Figure 4E-4H). These data might elucidate that miR-520b modified the development of glioma *via* targeting EZH2.

MiR-520b Inhibitor Can Abrogate the Effect of EZH2 Silencing on Cell Behaviors In Vitro

Based on the above descriptions, we declared that EZH2 was negatively regulated by miR-520b. Subsequently, the regulatory mechanism between miR-520b and EZH2 was explored at the aspects of cell behaviors. Firstly, the efficiency of sh-EZH2 was confirmed by measuring the mRNA and protein levels of EZH2 (Figure 5A and 5B). Then, sh-NC, sh-EZH2, sh-EZH2+anti-miR-NC, or sh-EZH2+anti-miR-520b were transfected into A172 and U251 cells, respectively. MTT results indicated that EZH2 knockdown could dramatically hinder cell proliferation, while the effect of EZH2 knockdown on cell proliferation was overturned *via* co-transfection with miR-520b inhibitor (Figure 5C and 5D). At the same time, the reintroduction of miR-520b inhibitor could abrogate the enhanced effect of EZH2 detection on cell apoptotic rate in A172 and U251 cells (Figure 5E). What's more, miR-520b inhibitor also abolished the repressive role of EZH2 silencing in the capacity of invasion in glioma cells (Figure 5F). In brief, we revealed that miR-520b inhibitor reversed the effect of EZH2 deficiency on cell behaviors in A172 and U251 cells.

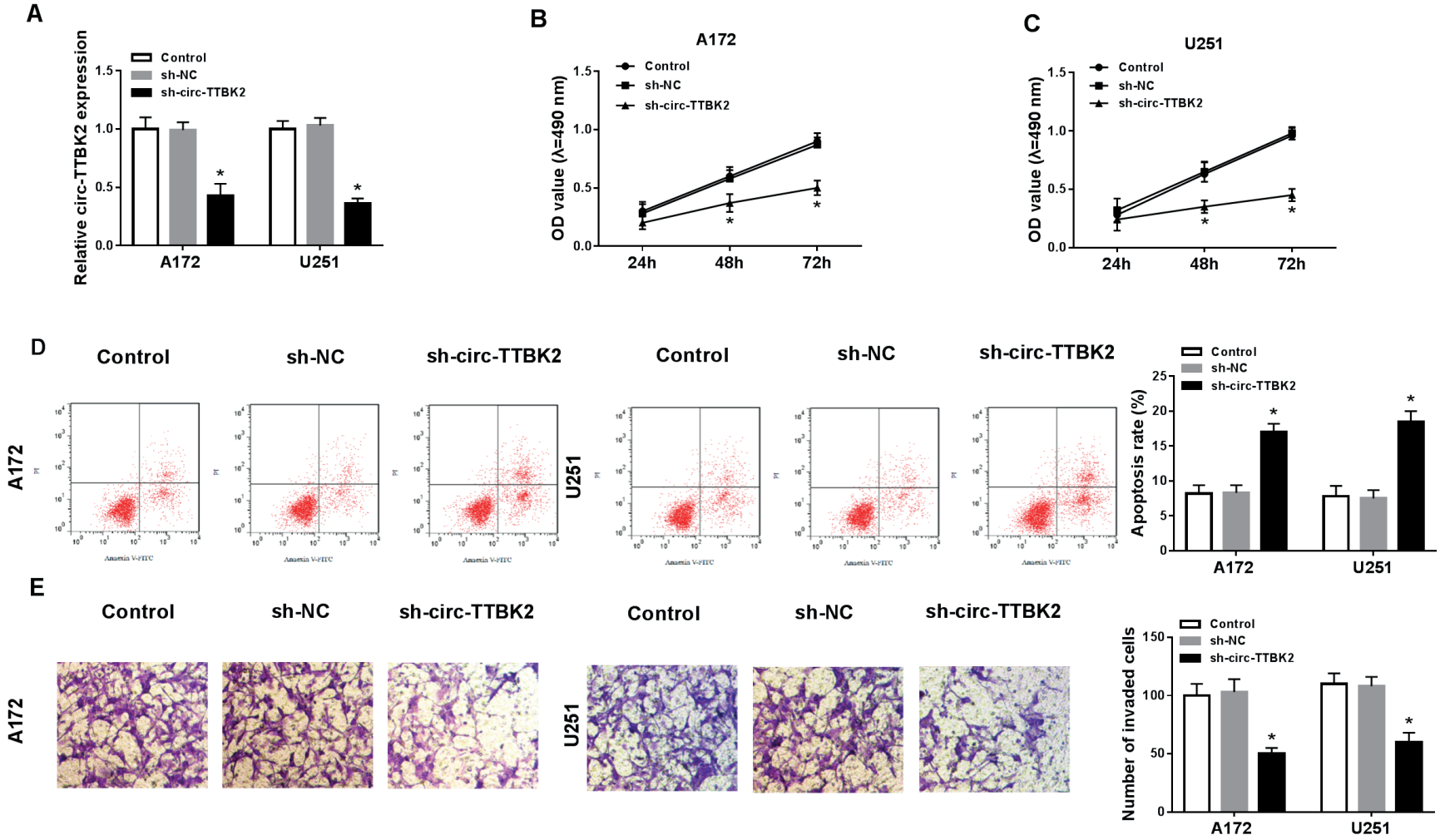

Figure 2. Circ-TTBK2 detection accelerated cell apoptosis, suppresses cell proliferation and invasion *in vitro*. Sh-NC or sh-circ-TTBK2 was transfected into A172 and U251 cells, (**A**) and the knockdown efficiency was measured by qRT-PCR. **B,** and **C,** MTT assay was applied to evaluate the proliferation effect of circ-TTBK2 silencing on A172 and U251 cells. **D,** The impact of circ-TTBK2 detection on cell apoptosis was assessed by flow cytometry assay. **E,** Transwell assay was conducted to assay the capacity of invasion in A172 and U251 cells (100×). **p*<0.05.

EZH2 is Co-Regulated by MiR-520b and Circ-TTBK2

After the above investigations, we needed to explore the mechanism between EZH2 and miR-520b or circ-TTBK2. Firstly, vector of miR-NC, miR-520b, miR-520b+pcDNA, or miR-520b+-circ-TTBK2 was introduced into A172 and U251 cells. The mRNA and protein levels of EZH2 were measured using qRT-PCR and Western blot assays, respectively. The results presented that the inhibitory role of miR-520b mimic in EZH2 mRNA and the protein levels were restored after co-transfection with circ-TTBK2 in both A172 and U251 cells (Figure 6A and 6B). The data suggested that EZH2 was co-modulated by miR-520b and circ-TTBK2 in glioma cell lines.

Knockdown of Circ-TTBK2 Suppresses the Progression of Tumor In Vivo

The above findings proved the segmental function of circ-TTBK2 *in vitro*. Herein, the A172 cells mediated by lentivirus (sh-circ-TTBK2 or sh-NC) were injected into nude mice. The tumor volumes were evidently smaller in circ-TTBK2 knockdown group compared with control (Figure 7A). In addition, after circ-TTBK2 detection, tumor weight was also decreased *in vivo* (Figure 7B). Synchronously, we selected three samples from treatment and control groups; qRT-PCR analysis pointed out that the levels of circ-TTBK2 and EZH2 were prominently reduced, but miR-520b level was clearly enhanced in tumor samples of treatment group (Figure 7C). Finally, the protein level of EZH2 was also constrained after circ-TTBK2 knockdown (Figure 7D). In short, circ-TTBK2 deficiency could retard the progression of glioma *in vivo*.

Discussion

The study proved that mRNA levels of circ-TTBK2 and EZH2 were augmented in tumor tissues and cell lines (A172 and U251). The knockdown of circ-TTBK2 impeded the malignant process of glioma. On the contrary, miR-520b was evidently blocked in glioma tissues and two types of cell lines, and its restoration constrained the malignant progression of glioma cells. In addition, miR-520b was directly targeted by circ-TTBK2, and EZH2 bound to miR-520b in a sequence-dependent manner. Furthermore, miR-520b mimic significantly repressed the level of EZH2, while the effect was regained by circ-TTBK2 overexpression. Besides, EZH2 silencing remarkably boosted cell apoptosis, restrained proliferation, and invasion in glioma cells. Above all, the tumor-suppressive role of circ-TTBK2 deficiency was also verified *via in vivo* studies.

Recently, circRNAs have been found in the past decades, while their precise functions remained hugely incomprehensible. Emerging reports[24,25] showed that the dysregulation of circRNAs existed in numerous tumors and was related to the various cellular progression of tumor cells. Although the effect of circRNAs on the regulatory mechanism of tumors was complex, earlier works revealed that circRNA Forkhead box O3 (circ-FOXO3) level was downregulated in some tumors, and its upregulation could hamper cell proliferation *via* targeting cyclin-dependent kinase 2 (CDK2) and p21[26]. In addition, many reports have indicated circRNAs as major regulators of the progression and initiation of diseases, including Alzheimer's disease[27] and multiple cancers[28,29]. Previous studies[12,30] have uncovered that circ-TTBK2 was independent with linear TTBK2. In our study, we only investigated the expression of circ-TTBK2 in glioma tissues and cells, and the results proved that circ-TTBK2 expression was significantly improved. Based on our preliminary results, we showed that decreased circ-TTBK2 retarded malignant progression of glioma *in vitro* and *in vivo*. Thus, circ-TTBK2 might exert its critical role in the modulation of glioma pathogenesis and progression.

To date, circRNAs are regarded as the sponges of miRNAs, and the alteration of circRNAs regulates the levels of the targeted miRNAs[31]. For example, circGFRA1 exerted the oncogenic role *via* targeting miR-449a in ovarian cancer cells 31417634. Furthermore, starBase exhibited that the putative binding sites between miR-520b and circ-TTBK2. More researches elucidated that miR-520b regulated the Luciferase activity of WT-circ-TTBK2, and the results meant that circ-TTBK2 was a sponge of miR-520b. About miR-520b, it has been proved[32-34] to mediate epigenetic alterations in multiple cancers. Thus, we have ample reasons to speculate that unusual expression of miR-520b influences several cellular pathways, ultimately regulating cancer malignancy. In the present study, we showed that miR-520b inhibitor could reverse the effect of circ-TTBK2 detection on cell behaviors in A172 and U251 cells. Furthermore, the target gene of miR-520b was required to be found.

According to starBase analysis, we discovered that miR-520b might target numerous genes. In

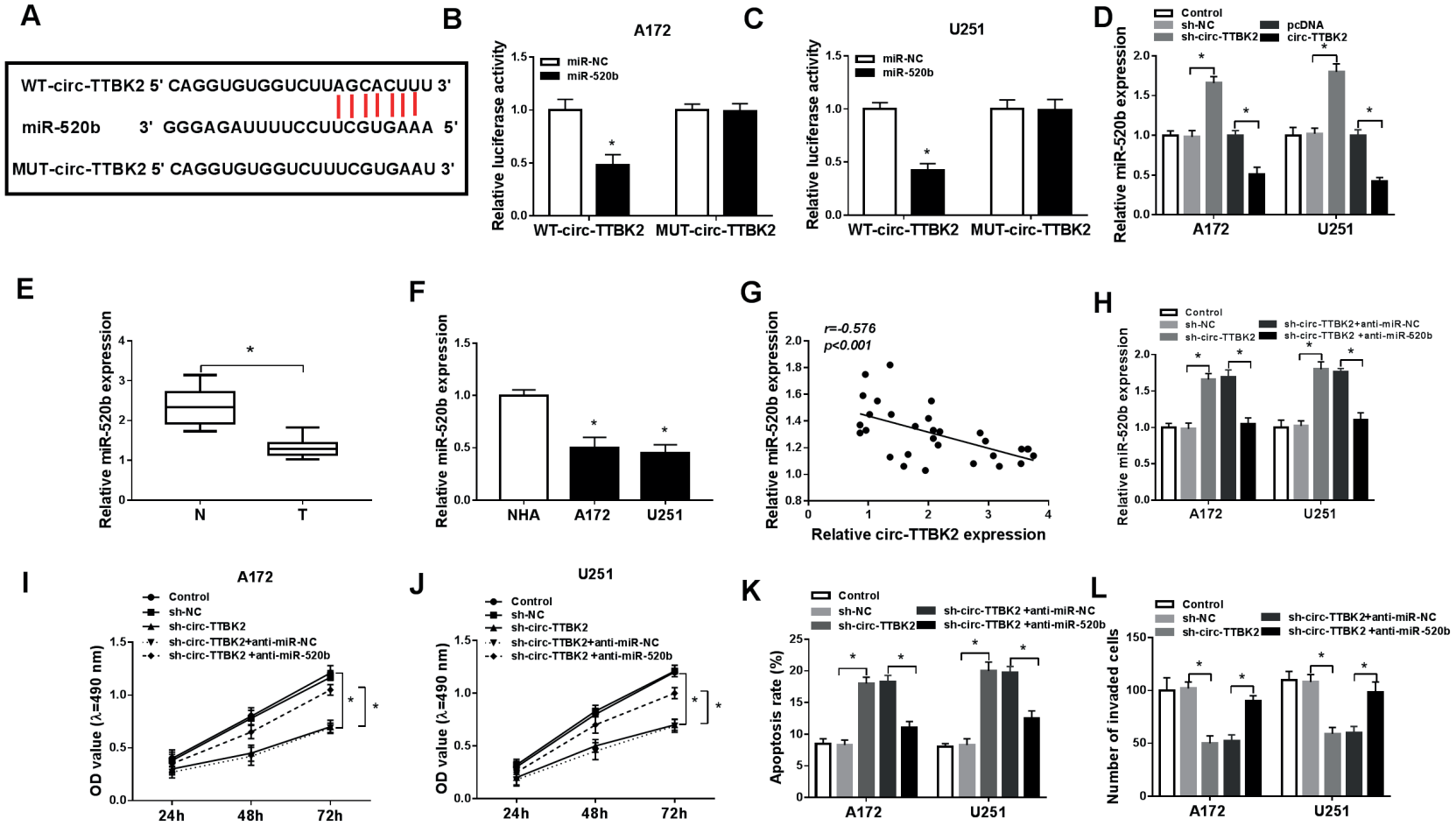

Figure 3. Circ-TTBK2 was a sponge of miR-520b. **A,** The predicted miR-520b binding sites in circ-TTBK2 or designed mutant sequence were shown. **B,** and **C,** A172 and U251 cells co-transfected with WT-circ-TTBK2 or MUT-circ-TTBK2 and miR-520b or miR-NC, and Dual-Luciferase reporter assay was used to detect the relative firefly activity. **D,** The effect of circ-TTBK2 or sh-circ-TTBK2 on miR-520 level was identified by qRT-PCR. **E,** and **F,** The level of miR-520b in (**E**) tissues and (**F**) cell lines was examined using qRT-PCR. **G,** QRT-PCR was carried out to analyze the correlation between miR-520b level and circ-TTBK2 level. A172 and U251 cells were transfected with sh-circ-TTBK2 alone, or along with anti-miR-520b, (**H**) and level of miR-520b was estimated utilizing qRT-PCR. **I,** and **J,** MTT assay was performed to assess cell proliferation in A172 and U251 cells. **K,** Flow cytometry assay was conducted to detect the role of sh-circ-TTBK2 and anti-miR-520b in cell apoptosis. **L,** The ability of invasiveness was evaluated using transwell assay. *p<0.05.

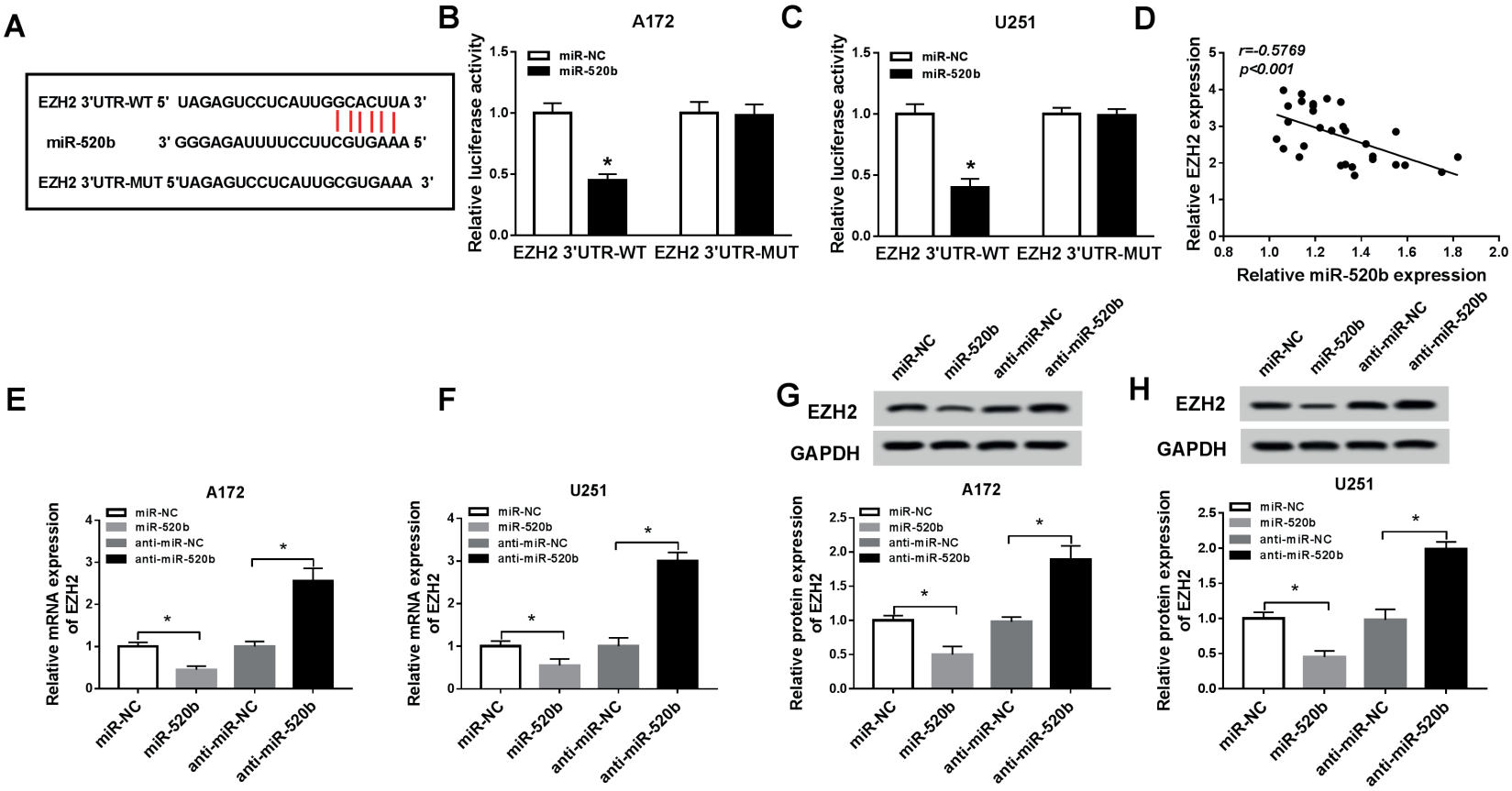

Figure 4. EZH2 was directly targeted by miR-520b. (**A**) The predicted miR-520b binding sites in EZH2 or designed mutant sequence were exhibited. **B,** and **C,** Dual-Luciferase reporter assay was performed to verify the interaction between miR-520b and EZH2 in A172 and U251 cells. **D,** The correlation between miR-520b and EZH2 was analyzed by qRT-PCR. **E-H,** The effect of miR-520b or anti-miR-520b on (**E**) and (**F**) mRNA and (**G**) and (**H**) protein expression levels was measured via QRT-PCR and Western blot assays, respectively. *p<0.05

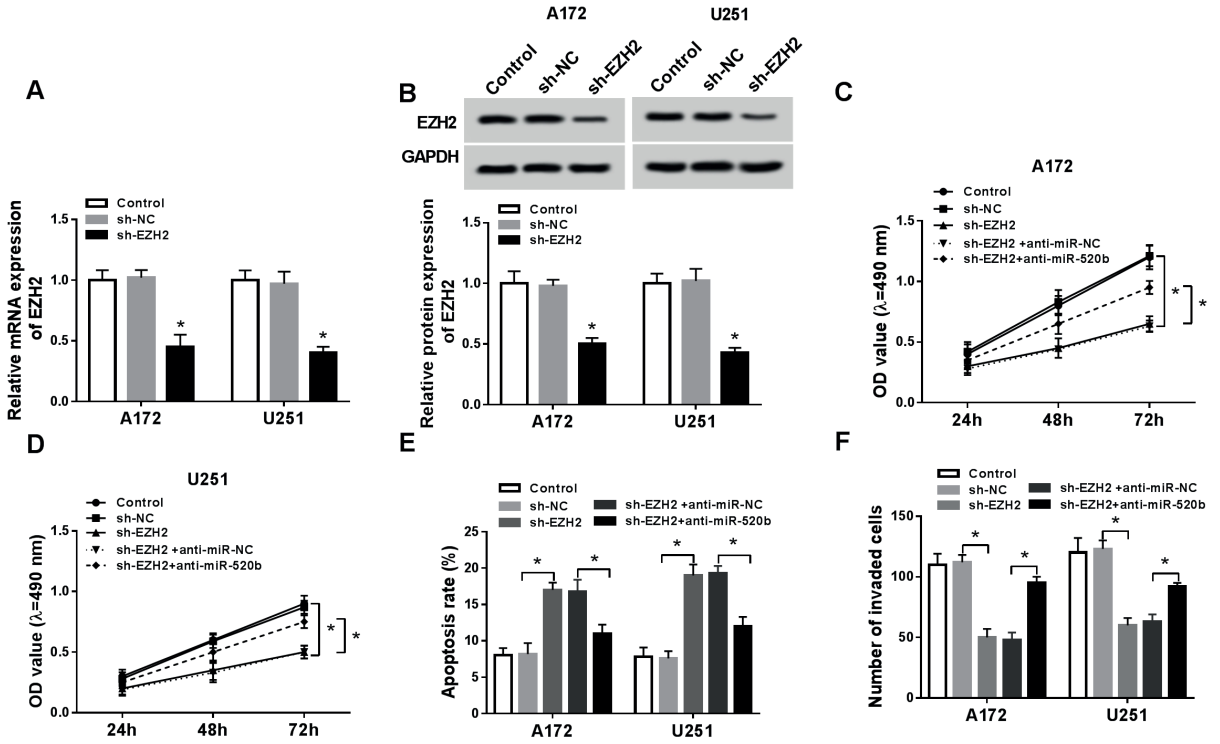

Figure 5. MiR-520b inhibitor could abrogate the effect of EZH2 silencing on cell behaviors *in vitro*. **A,** and **B,** The knockdown efficiency of sh-EZH2 was determined utilizing qRT-PCR and Western blot assays. Sh-NC, sh-EZH2, sh-EZH2+anti-miR-NC or sh-EZH2+anti-miR-520b was introduced into A172 and U251 cells, (**C** and **D**) and cell proliferation was detected by MTT assay at 24 h, 48 h, or 72 h post-transfection. **E,** Flow cytometry assay was carried out to evaluate cell apoptosis in A172 and U251 cells. **F,** The number of invaded cells was counted via transwell assay. *p<0.05.

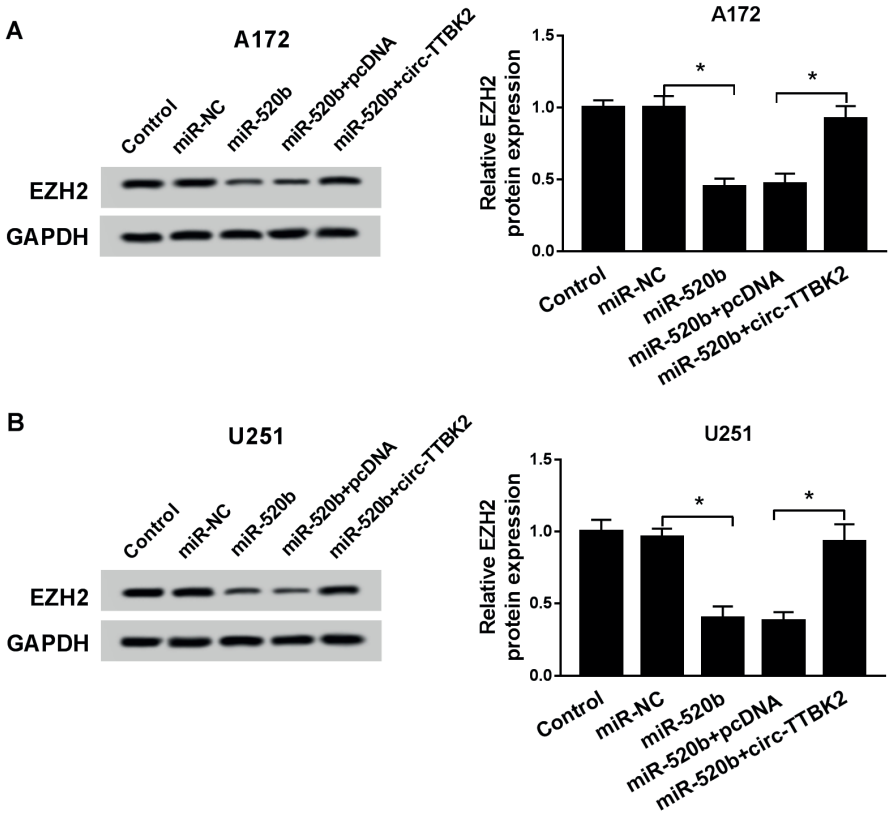

Figure 6. EZH2 was co-regulated by miR-520b and circ-TTBK2. (**A** and **B**) MiR-NC, miR-520b, miR-520b+pcDNA, or miR-520b+circ-TTBK2 were transfected into A172 and U251 cells, respectively. (**A**) The mRNA level of EZH2 was examined *via* qRT-PCR, (**B**) and protein expression level of EZH2 was identified using Western blot assay. *p<0.05.

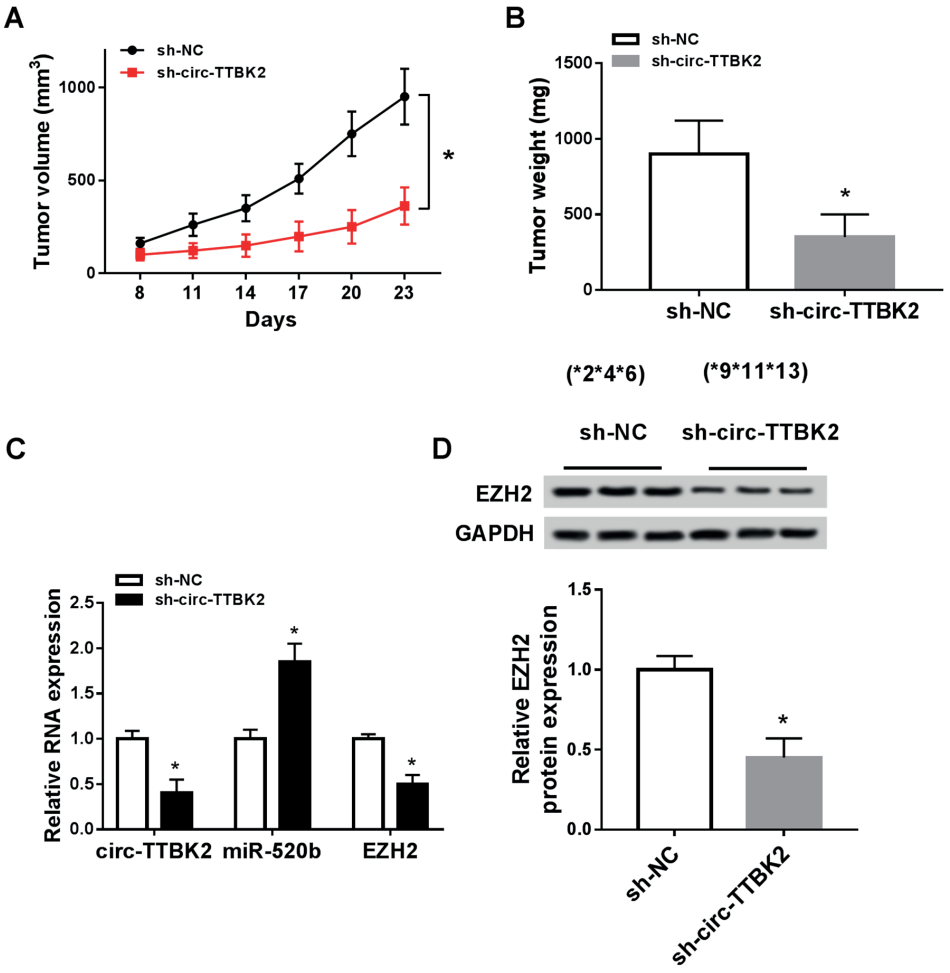

Figure 7. Knockdown of circ-TTBK2 suppressed the progression of tumor *in vivo*. The stably transfected A172 cells were used for the *in vivo* study. **A**, The volume of tumors carried by nude mice from respective groups were measured every 3 days at day 8 post-injection. **B**, Tumor weight was measured after mice were sacrificed. The tumor tissues were selected from three samples from both control and treatment groups. **C,** The levels of circ-TTBK2, miR-520b, and EZH2 were estimated *via* qRT-PCR assay. **D**, Western blot assay was performed to assay the protein expression level of EZH2 in tumor tissues. **p*<0.05.

view of the aberrant expression of EZH2 in glioma tissues and cell lines, we chose EZH2 as the potential targets of miR-520b. Scholars[35-37] report that EZH2 was generally upregulated in various cancers, and the association between EZH2 and tumorigenesis was also proved. Besides, EZH2 has been recognized as an oncogenic gene in several cancers, such as ovarian cancer[38], squamous cell carcinoma[39], and gastric cancer[40]. In our study, we demonstrated that EZH2 was co-regulated by circ-TTBK2 and miR-520b in glioma cells. The downregulation of EZH2 could significantly enhance cell apoptosis and block proliferation and invasion in A172 and U251 cells.

Conclusions

In summary, circ-TTBK2 and EZH2 were expressed at high levels, but miR-520b was expressed at a lower level in glioma tissues and cell lines. Circ-TTBK2 deficiency could trigger cell apoptosis, and constrain proliferation and

invasion in A172 and U251 cells. MiR-520b acted as the target gene of circ-TTBK2, and its inhibitor rescued the impact of circ-TTBK2 detection on cell behaviors *in vitro*. Besides, EZH2 was targeted by miR-520b, and the role of EZH2 knockdown in cell behaviors was abolished *via* co-transfecting with miR-520b inhibitor. We also found that the downregulation of circ-TTBK2 could suppress the development of glioma *via* miR-520b/EZH2 axis. However, the functions of circTTBK2 in the pathogenesis and progression need to be further explored.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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