CircRNA PIP5K1A promotes the progression of glioma through upregulation of TCF12/PI3K/AKT pathway by sponging miR-515-5p

Kebin Zheng
Affiliated Hospital of Hebei University
Haipeng Xie
Affiliated Hospital of Hebei University
Xiaosong Wu
Affiliated Hospital of Hebei University
Xichao Wen
Affiliated Hospital of Hebei University
Zhaomu Zeng
Affiliated Hospital of Hebei University
Yanfang Shi (bdsyy1206@163.com)
Affiliated Hospital of Hebei University https://orcid.org/0000-0002-6538-6202

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Abstract

Background

Increasing studies have revealed that circular RNAs (CircRNAs) make great contribution to regulating tumor progression. Therefore, we intended to explore the expression characteristics, function, and related mechanisms of a novel type of circRNA, PIP5K1A in glioma.

Methods

Firstly, RT-PCR was carried out to examine CircPIP5K1A expression in glioma tissues and adjacent normal tissues, and the correlation between CircPIP5K1A level and the clinical pathological indicators of glioma was analyzed. Then, the CircPIP5K1A expression in various glioma cell lines was detected, and a cell model of CircPIP5K1A overexpression and knockdown was constructed. Subsequently, cell proliferation and viability were detected by CCK8 method and BrdU staining, apoptosis was detected by flow cytometry, and cell invasion was examined by Transwell assay. The expression of TCF12, PI3K/AKT pathway apoptotic related proteins (including Caspase3, Bax and Bcl2) and epithelial-mesenchymal transition (EMT) markers (including E-cadherin, Vimentin and N-cadherin) by western blot or RT-PCR.

Results

The results manifested that CircPIP5K1A was obviously upregulated in glioma tissues (compared with that in normal adjacent tissues), and overexpressed CircPIP5K1A was distinctly related to glioma volume and histopathological grade. Functionally, overexpressing CircPIP5K1A notably elevated the proliferation, invasion, EMT of glioma cells, and inhibited apoptosis both in vivo and in vitro. Besides, CircPIP5K1A also upregulated TCF12 and PI3K/AKT pathway activation. Bioinformatics analysis testified that miR-515-5p was a common target of CircPIP5K1A and TCF12, while dual luciferase reporter assay and RNA immunocoprecipitation (RIP) experiment further confirmed that CircPIP5K1A targeted miR-515-5p, which bound the 3'-untranslated region (UTR) of TCF12.

Conclusions

Altogether, the study illustrated that CircPIP5K1A is a potential prognostic marker in glioma and regulates the development of glioma through the modulating miR-515-5p mediated TCF12/PI3K/AKT axis.

1 Background

Glioma is the most common tumors of the central nervous system with a high incidence and high malignancy [1]. Currently, surgery, chemoradiotherapy, immunotherapy and other comprehensive methods are mainly used for its treatment clinically [2]. However, the overall therapeutic effects remain unsatisfactory due to the unclear pathogenesis and lack of specific treatment targets. In recent years, gene therapy for glioma has been widely studied, but the specific target and mechanism have not yet

been well determined [3]. Hence, exploring new molecular mechanism of glioma development is of great value for its treatment and prognosis.

CircRNAs are newly discovered circular noncoding RNAs that are involved in the regulation of gene expression at transcriptional and post-transcriptional levels [4]. Growing evidence shows that circRNAs play prominent roles in tumorigenesis and cancer development by regulating the downstream targeted miRNAs [5]. For example, circ-0006948 directly binds to miR-490-3p which targets the 3'UTR of the oncogene high mobility group protein A2 (HMGA2), and enhances HMGA2 expression by sponging miR-490-3p, thus inducing EMT and promoting cancer progression in esophageal squamous cell carcinoma (ESCC) [6]. In addition, circ-0037251 affects glioma proliferation and metastasis by regulating the miR-1229-3p/mTOR axis [7]. CircPIP5K1A is a newly discovered cancer-related circRNA, which is a powerful regulator in the process of tumors. For example, Zhang Qu et al. demonstrated that increasing circPIP5K1A enhances AP-1 expression and dampens the expression of IRF-4, CDX-2 and Zic-1 by downregulating miR-1273a, thus aggravating the migration and invasion of colon cancer [8]. Interestingly, the mechanism of action of circPIP5K1A in other diseases, including glioma, is rarely reported.

MicroRNAs (miRNAs) belong to the same class of noncoding RNAs as circRNAs, which are only 18 to 25 nucleotides in length. However, multiple miRNAs are found to regulate the pathological processes of many diseases through post-transcriptional gene silencing [9]. For instance, as a tumor suppressor, miR-187 attenuates cell growth and metastasis in glioma by dampening SMAD1 expression [10]. In addition, miR-214-3p abates EMT and transfer of endometrial cancer (EC) cells by targeting TWIST1 [11]. As an important member of the miRNAs, miR-515-5p is located at 19q13.42 and its pre-miRNA is 83 bp in length. In addition, it has been proved to be a key molecular in a variety of tumors. For example, miR-515-5p directly targets the 3'-UTR end of TRIP13 and negatively regulates the expression of it, thereby acting as a tumor suppressor in prostate cancer [12]. Besides, miR-515-5p impedes the tumor cell by targeting CXCL6 in non-small cell lung cancer cells (NSCLC) [13]. Nevertheless, the role of miR-515-5p in glioma needs further investigation.

Transcription factor 12 (TCF12) is a member of the helix-loop-helix protein family and serves either as an oncogene or tumor suppressor in multiple human cancers [14]. For example, TCF12 is a direct target of miR-26a, which has been shown to inhibit the growth of epithelial ovarian cancer (OC) and induce apoptosis by inhibiting the TCF12 expression [15]. In addition, Yang Jing et al. confirmed that TCF12 promotes the occurrence and development of hepatocellular carcinoma (HCC) by upregulating CXCR4 [16]. What's more, PI3K is an intracellular phosphatidylinositol kinase and AKT is a serine/threonine-specific protein. Existing studies have manifested that the PI3K/AKT signaling pathway is activated in most cancers, including glioma [17]. For example, matrine has anti-tumor effects in glioma cells via inducing apoptosis and autophagy through inhibition of PI3K/AKT and Wnt-β-catenin pathways. [18]. Besides, some studies have found that overexpressing TCF12 markedly upregulates p-AKT and p-PI3K, and promotes the development of gastric cancer (GC) [19]. However, whether circPIP5K1A and TCF12 affects the progress of glioma by regulating the conduction of PI3K/AKT signaling pathway remains elusive.

Here, we discovered that there is a targeted regulatory relationship between circPIP5K1A and miR-515-5p, miR-515-5p and TCF12 through bioinformatics. By detecting the expression of circPIP5K1A, miR-515-5p, TCF12 and PI3K/AKT in glioma tissues and cells and exploring the relationship among these molecules, it was illustrated that circPIP5K1A inhibits miR-515-5p level, upregulates TCF12 by serving as a competitive endogenous RNA (ceRNA) of miR-515-5p, which in turn affects the proliferation and metastasis of glioma cells. In summary, this study aims to research the function of a novel network of circPIP5K1A/miR-515-5p/TCF12/PI3K/AKT axis in glioma progression, and further improve the study of its molecular mechanism, and provide referential molecular markers for clinical diagnosis and treatment.

2. Methods

2.1 Clinical specimen collection and processing

Forty-five cancerous and adjacent non-cancer tissues of patients with glioma who underwent resection in the Affiliated Hospital of Hebei University from March 2014 to March 2015 were selected. None of the patients received adjuvant treatment such as chemotherapy and radiotherapy before surgery. The control group specimens were obtained from the normal tissues of the same patient (at least 1 cm from the surgical margin), and no cancer cells were found through pathological examination. The glioma was diagnosed pathologically according to World Health Organization (WHO) criteria. Immediately after removal, all specimens were stored in -196℃ liquid nitrogen until used for RNA extraction. The Ethics Committee of Affiliated Hospital of Hebei University approved our study and all of the involved patients signed informed consent.

2.2 Cell culture

Human normal glial cell HEB and glioma cell lines (U87, TJ861, TJ905, U251, H4 and A172) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were incubated with RPMI1640 (Thermo Fisher Scientific, MA, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific,MA, USA) and 1% penicillin/streptomycin (Invitrogen, CA, USA)) at 37℃ and 5% CO_2. Cells during the logarithmic growth phase were treated with 0.25% trypsin (Thermo Fisher, HyClone, Utah, USA) for trypsinization and passage. It was found that CircPIP5K1A was the lowest expressed in U87 cells and the highest expressed in A172. Therefore, U87 and A172 cells were chosen as the research object in subsequent studies.

2.3 Cell transfection

CircPIP5K1A overexpression plasmids (pcDNA3.1-CircRNA PIP5K1A) and its small inference RNA (Si-CircPIP5K1A), miR-515-5p mimics, TCF12 overexpression plasmids (pcDNA3.1-TCF12) and its small inference RNA (Si-TCF12) and the corresponding negative controls were obtained from GenePharma (Shanghai, China). U87 and A172 cells were taken and seeded in 24-well culture plates at 3×10^5 cells/well, then incubated at 37 ℃ with 5% CO_2 for 24 hours before transfection using Lipofectamine® 3000 (Invitrogen; ThermoFisherScientific, Inc.) according to the supplier's instructions. RT-PCR was used

to determine the transfection efficiency, and the cells were incubated at 37˚C with 5% CO_2 for 24 hours for further analysis.

2.4 RT-PCR

Firstly, total RNA from tissues or cells was extracted using TRIzol reagent (Invitrogen, Waltham, MA, USA). Then, Nanodrop-spectrophotometer was employed to measure RNA concentration and purity. Subsequently, we used a PrimeScript-RT Kit (Madison, WI, USA) to reverse-transcribe 1 μg of total RNA to synthesize its complementary DNA (cDNA), and then adopted SYBR® Premix-Ex-Taq ™ (Takara, TX, USA) and ABI7300 system for real-time polymerase chain reaction (RT-PCR) of cDNA according to the manufacturer's protocol. The total volume of the PCR system was 30 μl, and each sample contained 300 μg of cDNA. The amplification was initially performed at 95℃ for 10 minutes for 45 cycles. Namely, 95 ℃ for 10 s, 60℃ for 30 s, and 85℃ for 20 s. We converted all fluorescence data to relative quantification, β-actin was the internal reference of CircRNA PIP5K1A and TCF12, while U6 was the internal reference of miR-515-5p. The RT-PCR experiment was repeated three times. The primers were designed and synthesized by Guangzhou Ruibo Company. CircPIP5K1A: forward primer 5'-AGATTCCCTAACCTCAACCAGA-3', reverse primer 5'-CGAATGTTCTTGCCACCTGC-3' ; TCF12: forward primer 5'-TCTGCCCCTAGATGAGACCT-3', reverse primer 5'-GGCAATCATTCGGTCCTGTC-3'; miR-515-5p, forward primer: 5'-TTCTCCAAAAGAAAGCACTTTCTG-3, reverse primer 5'-CTCGCTTCGGCAGCACA-3'; GAPDH: forward primer 5'-TGATCTTCATGGTCGACGGT-3, reverse primer 5'-CCACGAGACCACCACCTACAACT-3'; U6, forward primer 5'-CTCGCTTCGGCAGCACA-3 ', reverse primer 5'-AACGCTTCACGAATTTGCGT-3'.

2.5 CCK 8 assay

CCK 8 assay was used to detect cell viability using a CCK8 kit (Beyotime, Shanghai, China). Firstly, 100 μL cell suspension at 2×10^5/ml of glioma cells U87 and A172 were inoculated into 96-well plates per well. On the next day, the original culture medium was removed and cell culture medium was supplemented. Subsequently, the medium was taken out after cultured in an incubator with 5% CO_2 for 3 hours, 6 hours, 12 hours, and 24 hours, respectively. Finally, CCK8 reagent at 10 μL/well was added and incubated for 2 hours, and the OD value at 450 nm wavelength was obtained by microplate.

2.6 BrdU assay

Firstly, cells at the logarithmic phase were added with 10 μmol/L BrdU (Sigma, Shanghai, China). After DNA denaturation, the cells were incubated with BrdU primary antibody (Abcam, ab6326,1:1000, CA, USA) at room temperature for 2 hours. Then, fluorescent secondary antibodies were added and incubated for 2 hours at room temperature. Later on, the nucleus was labeled by 10 μmol/L Hoechst33342. Finally, fluorescence inverted microscope was used for imaging and statistical analysis.

2.7 Transwell assay

Transwell chambers (Corting, NY, USA) were coated with 200 mg/mL Matrigel (BD, SanJose, USA) and incubated overnight. Then, U87 and A172 cells (5×10^5/ml) was suspended by serum-free RPMI1640 medium and 200 μL cell suspension was added into the upper chambers. Subsequently, RPMI1640 (500 μL) containing 10% FBS was placed in the lower chambers as a chemotactic agent. After incubation for 24 hours, all uninvaded cells were removed. Then, matrigel membranes were fixed with paraformaldehyde and then dyed with crystal violet buffer. At last, the number of invaded cells was counted by a phase contrast microscope (Olympus, Tokyo, Japan). The experiment was repeated three times.

2.8 RIP experiment

Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Merck Millipore, USA) was used to conduct RIP assay. 2×10^7 U87 cells transfected with miR-505-5p or its negative control were collected and added with 200 μL of RIP Lysis Buffer, then they were cleaved on the ice for 5 min and centrifuged at 1500 rpm for 15 min to obtain the supernatant. Then, the extract was incubated with anti-Ago2 or anti-IgG (Sigma) overnight. Subsequently, the supernatant was discarded after magnetic beads were washed with Wash Buffer for 5 times, and then protease K lysate was added to the magnetic beads for lysis at 55℃ for 30 min. Finally, the supernatant was placed in a new centrifuge tube, total RNA was extracted by phenol-chloroform-isoamyl alcohol extraction and purified by isopropanol centrifugation. The level of CircPIP5K1A and TCF12 were tested by RT-PCR.

2.9 Dual luciferase reporter assay

All luciferase reporter vectors (CircPIP5K1A-WT, CircPIP5K1A-MUT, TCF12-WT, TCT12-MUT) were obtained from Promega (Promega, Madison, WI, USA). U87 cells (4.5×10^4) were seeded in 48-well plates to 70% confluence. Then, the U87 cells were co-transfected with miR-515-5p or negative control with CircPIP5K1A-WT, CircPIP5K1A-MUT, TCF12-WT and TCF12-MUT using liposome 2000. Forty-eight hours after transfection, luciferase activity was determined according to manufacturer's guidelines. All experiments were made in triplicate and repeated three times.

2.10 Western blot

The cells were collected and then washed with cold PBS for 3 times, and 100 ~ 200 μL RIPA lysate (Beyotime Biotcchnology, Shanghai,China) was added to lyse the cells on ice. Then, the protein in the lysates were isolated by centrifugation and the protein concentration was determined by Bradford method. Equal protein in each group was isolated on 10% SDS-PAGE, and then the proteins in the gel were transferred to PVDF membranes (Millipore, Bedford, MA, USA). Subsequently, the membranes were blocked at 4℃ for 1 hour by 5% BSA, and incubated with primary antibodies anti-TCF12 antibody (ab70746, 1:1000, abcam, MA, USA), anti-PI3K antibody (ab191606,1:1000, abcam, MA, USA), anti-PI3k (phosphoY607) antibody (ab182651, 1:1000, abcam, MA, USA), anti-pan-AKT antibody (ab18785, 1:1000, abcam, MA, USA), anti-AKT (phospho T308) antibody (ab38449, 1:1000, abcam,MA,USA), anti-Bax antibody (ab32503,1: 1000, Abcam,MA,USA), Anti-Bcl-2 antibody (ab59348,1:1000, abcam,MA,USA), Anti-Caspase 3 antibody (ab13847, 1:1000, abcam, MA, USA), Anti-E-cadherin antibody (ab16505, 1:1000, abcam, MA, USA), Anti-Vimentin-antibody (ab92547, 1:1000, abcam, MA, USA) and Anti-N-cadherin

antibody (ab18203,1:1000,abcam,MA,USA) at 4℃ overnight. After being washed with TBST for 2 times, the membranes were incubated with HRP labeled goat-anti-rabbit secondary antibody (ab205718, 1:2500, abcam) at room temperature for 1 hour. Finally, the membranes were washed for 3 times, exposed with ECL color reagent (Millipore,Bedford,MA,USA), and imaged with a scanner.

2.11 Flow cytometry

After being treated with different factors, the cells were trypsinized, and then collected by centrifugation (1500r/min, 3 min). The harvested cells were handled according to cell apoptosis detection kit(Shanghai Aladdin Bio-Chem Technology Co., LTD): after the cells were washed with PBS twice, 400 μL precooling PBS was added, then 10 μL of AnnexinV-FITC and 5 μL of PI were supplemented respectively and incubated at 4℃ in the darkness for 30 min. Immediately after that, flow cytometry instrument was adopted to measure cell apoptosis, and the percentage of apoptotic cells was calculated after computer processing.

2.12 Tumor formation assay in nude mice

Firstly, 4-6-week-old BALB/c-nu nude mice were selected to construct tumor formation model. U87 and A172 cells in the logarithmic phase were chosen, and the cell concentration was adjusted to $2 \times 10^{8/}$ml. Subsequently, 0.1 ml cell suspension was injected subcutaneously into the armpit of the left forelimb of each nude mouse. Each group had a total of 10 mice. The survival rate, weight and status of the mice were monitored, and the size and weight of the tumor in the newly dead mice were measured within 25 days after the injection.

2.13 Statistical Methods

SPSS22.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis, and the results were expressed as mean ± SD ($\bar{x} \pm s$). The measurement data between the two groups were compared by t test, and $P < 0.05$ was considered statistically significant.

3 Results

3.1 CircPIP5K1A was highly expressed in glioma tissues and cells

Firstly, we carried out RT-PCR to investigate the circPIP5K1A level in glioma tissues. It turned out that circPIP5K1A was distinctly upregulated compared with that in normal adjacent tissues ($P < 0.05$, Fig. 1A). In addition, the circPIP5K1A expression in different glioma cell lines was compared by RT-PCR, and the results manifested that it was obviously upregulated in glioma cell lines (U87, TJ861, TJ905, U251, H4 and A172) compared with that in normal human glial cells (HEB) ($P < 0.05$, Fig. 1B). Moreover, the survival time of glioma patients with high circPIP5K1A expression was shorter than that of with low CircPIP5K1A level, with more larger tumor volume, higher tumor stage and Ki-67 rate (Fig. 1C and Table 1). These

results suggested that CircPIP5K1A is associated with the malignant phenotypes of glioma cells and is carcinogenic.

Table 1
Relationship between the CircPIP5K1A level and clinical characteristics in tissue samples from glioma patients

Characteristics	Patients	Expression of CircPIP5K1A		P-value
		Low-CircPIP5K1A	High-CircPIP5K1A	
Total	45	20	25	
Age(years)				
45	20	8	12	0.592
≥ 45	25	12	13	
Gender				
Male	24	11	13	0.841
Female	21	9	12	
IDH1 mutation				
no mutation	24	10	14	0.8241
mutation	19	9	11	
MGMT promoter methylation				
unmethylation	26	13	12	0.5549
methylation	23	10	13	
Tumor stage (WHO)				
I-II	27	11	16	0.014*
III-IV	18	4	14	
Ki-67 rate level				
Low	19	13	6	0.0057*
High	26	7	19	
Tumor volume				
< 5 cm	28	15	11	0.036*
≥ 5 cm	17	5	14	
Note: $P < 0.05$ was considered to be statically significant				

3.2 CircPIP5K1A effects proliferation, invasion, apoptosis and EMT of glioma cells

We constructed an overexpression and knockdown model of circPIP5K1A respectively in glioma cell lines U87 and A172 to explore the effect of circPIP5K1A in glioma progression ($P < 0.05$, Fig. 2A). CCK8 and BrdU experiments testified that the cell proliferation and viability were significantly strengthened after circPIP5K1A overexpression, while the reverse effect was observed after circPIP5K1A knockdown ($P < 0.05$, Fig. 2B-D). Similarly, we employed flow cytometry and western blot to detect cell apoptosis. The results revealed that the apoptosis rate was markedly dampened after CircPIP5K1A overexpression but elevated with circPIP5K1A knockdown ($P < 0.05$, Fig. 2E and F). Besides, Transwell assay showed that overexpressing circPIP5K1A enhanced the invasion of cells was obviously, while knocking down circPIP5K1A weakened cell invasion ($P < 0.05$, Fig. 2G). Furthermore, we conducted western blot to detect the expression of EMT related markers E-cadherin, Vimentin and N-cadherin. As shown in the figure, E-cadherin protein expression was notably attenuated after CircPIP5K1A overexpression, while Vimentin and N-cadherin protein expression was significantly elevated. In contrast, E-cadherin protein was markedly upregulated, while Vimentin and N-cadherin protein were obviously downregulated after circPIP5K1A knockdown ($P < 0.05$, Fig. 2H). These results demonstrated that circPIP5K1A is involved in the development of glioma cells through elevating the growth, invasion and EMT of tumor cells, and decreasing their apoptosis.

3.3 CircPIP5K1A promoted glioma growth and EMT in vivo

We constructed overexpressed and knocked down circPIP5K1A cell lines in U87 and A172 respectively, and in vivo tumor formation assay in nude mice was conducted to verify the effect of circPIP5K1A on glioma growth *in vivo*. We found that the tumor volume and mass were elevated by overexpressing circPIP5K1A, while were dramatically weakened by knocking down circPIP5K1A ($P < 0.05$, Fig. 3A-D). In addition, western blot suggested that E-cadherin protein was significantly downregulated, while Vimentin and N-cadherin protein was obviously upregulated after circPIP5K1A overexpression. However, circPIP5K1A knockdown had the opposite effects (Fig. 3E). Hence, these results further confirmed that circPIP5K1A promoted the growth and EMT of glioma cells.

3.4 CircPIP5K1A promoted the expression of the TCF12 and PI3K/AKT signaling pathway activation

We conducted western blot to preliminarily explore the expressive characteristics of TCF12 in tumors, and found that TCF12 was notably upregulated in glioma tissues (compared adjacent normal tissues (Fig. 4A and B). Besides, we discovered that TCF12 was upregulated both in GBM (glioblastoma) and LGG (low-grade glioma) as shown in GEPIA (http://gepia.cancer-pku.cn/) (Fig. 4C). On the other hand, TCF12 was found to be overexpressed in glioma tissues, which mainly distributed in the nucleus (Fig. 4D, data from The Human Protein Atlas (https://www.proteinatlas.org/)). Interestingly, there was a positive relationship

between circPIP5K1A and TCF12 in glioma tissues (R^2 = 0.499, P < 0.0001, Fig. 4E). In the cell model, upregulating circPIP5K1A increased TCF12 expression (Fig. 4F and G). Moreover, we found that TCF12 was positively related to AKT1 in LGG through GEPIA database analysis (http://gepia.cancer-pku.cn/) (Fig. 4H). Furthermore, western blot was employed to detect the effect of CircPIP5K1A on the PI3K/AKT signaling pathway. The results testified that overexpressing CircPIP5K1A elevated the expression of p-PI3K and p-AKT, while knocking down CircPIP5K1A resulted in the opposite effect (P < 0.05, Figure. 4I). These results indicated that TCF12 and PI3K/AKT signaling pathways were positively regulated by circPIP5K1A.

3.5 Overexpressing TCF12 aggregated the malignant phenotypes of glioma cells

To verify the role of TCF12 in glioma, we conducted gain- and loss-of functions assay of TCF12 in U87 and A172 glioma cell lines respectively to further explore the TCF12 effect in glioma (P < 0.05, Fig. 5A). In addition, CCK8 and BrdU experiments were used to detect the cell growth and viability. It was proved that overexpressing TCF12 notably enhanced cell proliferation and viability, while knocking down TCF12 led to the opposite effects (P < 0.05, Fig. 5B-D). Besides, flow cytometry and western blot certified that the apoptosis rate was notably dampened after TCF12 overexpression, while was dramatically increased after TCF12 knockdown compared with NC group (P < 0.05, Fig. 5E and F). Further, Transwell assay was carried out to examine the effect of TCF12 regulation on cell invasion. As shown in the figure, the cell invasion was significantly enhanced after TCF12 overexpression, while was obviously inhibited after TCF12 knockdown (P < 0.05, Fig. 5G). Furthermore, Transwell assay was employed to investigate the TCF12 regulation on EMT markers E-cadherin, Vimentin and N-cadherin in glioma cells. The results revealed that TCF12 overexpression repressed E-cadherin expression but upregulated Vimentin and N-cadherin level. Conversely, knocking down TCF12 increased E-cadherin level but inhibited the expression of Vimentin and N-cadherin (Fig. 5H). Similarly, western blot was conducted to detect the activation state of PI3K/AKT signaling pathway. The results manifested that overexpressing TCF12 promoted the expression of p-PI3K and p-AKT compared with NC, while knocking down TCF12 showed the opposite result (P < 0.05, Fig. 5I). The above results indicated that overexpressing TCF12 elevated the proliferation, invasion and EMT and weakened apoptosis of glioma cells, and promoted the expression of PI3K/AKT signaling pathway.

3.6 MiR-515-5p shared the target with CircPIP5K1A and TCF12

Inspired by the circRNA-miRNA-mRNA regulatory network, we conducted bioinformatics analysis to explore the miRNA target of circPIP5K1A and TCF12 through Starbase (http://starbase.sysu.edu.cn/). The results showed that a total of 22 miRNAs were common targets of CircPIP5K1A and TCF12 (Fig. 6A). Next, we used RT-PCR to determine the levels of the 22 miRNAs circPIP5K1A overexpressed cell. It was found that miR-515-5p was most significantly downregulated (Fig. 6B). Next, the miR-515-5p level in

glioma tissues was detected by RT-PCR, and it was found to be obviously reduced in glioma tissues compared with that in the adjacent normal tissues ($P < 0.05$, Fig. 6C). In addition, Person correlation analysis revealed that circPIP5K1A and miR-515-5p was negatively correlated in glioma cells ($R^2 = 0.571$, $P < 0.001$, Fig. 6D), which was the same of TCF12 and miR-515-5p ($R^2 = 0.463$, $P < 0.001$, Fig. 6E). The binding relationship of miR-515-5p with both circPIP5K1A and TCF12 are shown in Fig. 6F. In order to clarify the targeting relationship among the three molecules, we performed RIP assay and dual luciferase reporter gene assay. The results revealed that the amount of precipitated circPIP5K1A and TCF12 in the Ago2 antibody group was significantly higher than that in the IgG group after miR-515-5p transfection, suggesting that circPIP5K1A and TCF12 bound to Ago2 protein through miR-515-5p ($P < 0.05$, Fig. 6G and H). Furthermore, miR-515-5p markedly inhibited the luciferase activity of circPIP5K1A-WT and TCF12-WT, while had no marked effect on circPIP5K1A-MUT and TCF12-MUT ($P < 0.05$, Fig. 6I and J). These results illustrated that miR-515-5p was a downstream target of circPIP5K1A and an upstream target of TCF12.

3.7 CircPIP5K1A regulated the TCF12/PI3K/Akt expression by sponging miR-515-5p

For the purpose of verifying whether there was a regulatory axis of circPIP5K1A/miR-515-5p/TCF12/PI3K/Akt in glioma, rescue experiments were conducted. It turned out that circPIP5K1A was notably downregulated, while miR-515-5p was obviously upregulated in miR-515-5p mimic group. On the other hand, circPIP5K1A was upregulated, while miR-515-5p was markedly downregulated after CircPIP5K1A overexpression compared with the miR-515-5p group ($P < 0.05$, Fig. 7A-B). Next, we conducted RT-PCR and Western blot to compare alteration in TCF12 level. The results manifested that TCF12 mRNA and protein were significantly downregulated with miR-515-5p overexpression while supplementing circPIP5K1A enhanced TCF level ($P < 0.05$, Figs. 7C-D). Further, we used western blot to test the activation of PI3K/AKT signaling pathway. The results proved that upregulating miR-515-5p inhibited the levels of p-PI3K and p-AKT compared with NC, while adding circPIP5K1A in miR-515-5p group increased p-PI3K and p-AKT expression ($P < 0.05$, Fig. 7E). The above results suggested that circPIP5K1A regulated TCF12 expression by sponging miR-515-5p, thereby promoting the activation of PI3K/AKT signaling pathway

4 Discussion

Here, we explored a novel circPIP5K1A in the development of glioma. Our data suggested that circPIP5K1A is upregulated in glioma and predicts worse survival of glioma patients. Further, circPIP5K1A targeted miR-515-5p, thus upregulating the TCF12-PI3K/AKT axis.

CircRNAs are involved in modulating the occurrence and development of multiple tumors, including glioma [20]. For example, circSMO742 promotes glioma growth by sponging miR-338-3p to regulate SMO expression [21]. In addition, Ding Chenyu et al. showed that circNFIX inhibited glioma proliferation and metastasis by upregulating miR-378e and inhibiting RPN2 expression [22]. Meanwhile, accumulating

evidence has confirmed that circPIP5K1A is a powerful regulator in different types of cancers. For instance, circPIP5K1A is upregulated in ovarian cancer and inhibits the migration, proliferation, and invasion of ovarian cancer cells [23]. Additionally, it has also been demonstrated that circPIP5K1A promotes NSCLC proliferation and metastasis by upregulating HIF-1α [24]. In view of the above studies, we speculated that circPIP5K1A also plays a vital regulatory role in glioma. Interestingly, we found that circPIP5K1A is overexpressed in glioma tissues and cell lines, and our experiments both *in vivo* and *in vitro* proves that circPIP5K1A promotes the proliferation, invasion and EMT while attenuated the apoptosis of glioma cells, suggesting that circPIP5K1A also functions as a prognostic factor and a therapy target in glioma.

Previously, abundant studies have reported that miR-515-5p is a powerful tumor suppressor. For example, miR-515-5p, negatively regulated by LINC00673, is downregulated in breast cancer, and it exerts an anti-tumor effect by downregulating the MAPK4/Hippo signaling pathway [25]. Similarly, miR-515-5p dampens the proliferation and metastasis of prostate cancer by targeting TRIP13 [26]. The results of this study also revealed that miR-515-5p was downregulated in glioma tissues and cell lines, and negatively correlated with circPIP5K1A expression, which was consistent with the above reports on the anti-tumor effect of miR-515-5p in tumors.

Growing studies have found that circRNAs act as the ceRNAs of miRNA. For example, circZNF609 sponges miR-134-5p to promote BTG-2 expression as a ceRNA, thus weakening proliferation and migration of glioma cells [27]. In addition, overexpressing circPCMTD1 downregulates miR-224-5p and upregulates mTOR, thus aggregating the glioma development [28]. Here, we found a binding site between circPIP5K1A and miR-515-5p through StarBase. Combined with the above studies, we hypothesized that miR-515-5p might act as a downstream molecule of circPIP5K1A in glioma. Nest, we verified the targeting relationship between the two by RIP and dual luciferase reporter gene assay. Furthermore, overexpressing circPIP5K1A was proved to dampen the inhibitory effects of miR-515-5p. Therefore, circPIP5K1A could exert its biofunctions via sponging miR-515-5p.

TCF12 is reported to be involved in the regulation of cell growth and differentiation and is carcinogenic in multiple malignant tumors [16]. For example, downregulation of TCF12 dampens the growth, transfer and invasion of ovarian cancer and promotes apoptosis [29]. In addition, Shu Longwen et al. showed that overexpressing TCF12 attenuated the inhibitory effect of miR-204 on cervical cancer cell metastasis [30]. Surprisingly, TCF12 was confirmed to activate the PI3K/AKT signaling pathway to affect tumor progression. For example, TCF12 promotes the progression of hepatocellular carcinoma (HCC) by upregulating CXCR4 and its ligand CXCL12 and activating MAPK/ERK and PI3K/AKT pathway [31]. Besides, Wang Xuekui et al. found that TCF12 accelerates gastric cancer development by targeting miR-183 and activating PI3K/AKT signaling [32]. Here, we have confirmed through *in vitro* experiments that overexpressing TCF12 promotes the proliferation, invasion and EMT, weakens apoptosis, and activates PI3K/AKT signaling pathway in glioma. Through the bioinformatics database, the targeted binding relationship between miR-515-5p and TCF12 was analyzed, which prompted us to further explore whether circPIP5K1A plays a carcinogenic role by indirectly upregulating TCF12 expression through miR-515-5p.

Moreover, miR-515-5p negatively regulated TCF12 expression. Meanwhile, circPIP5K1A positively modulated TCF12 and PI3K/AKT pathway. These results suggested that circPIP5K1A plays a biological role by modulating the TCF12-PI3K/AKT, which is consistent with our previous hypothesis that circPIP5K1A promotes glioma progression through the miR-515-5p-TCF12-PI3K/AKT axis.

Overall, circPIP5K1A promotes glioma proliferation, metastasis and EMT, and inhibits apoptosis by targeting the miR-515-5p-TCF12-PI3K/AKT axis. The result provides a better understanding for gene targeted therapy and prognosis of glioma, but more molecular mechanisms need to be further explored in terms of the circPIP5K1A- miR-515-5p-TCF12-PI3K/AKT network.

Declarations

Ethics approval and consent to participate

Our study was approved by the Ethics Review Board of Affiliated Hospital of Hebei University.

Consent for publication

Not applicable.

Data Availability Statement

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

YFS conceived and designed the experiments. KBZ performed the experiments. HPX and XSW analyzed the statistics. XCW analyzed the formal. ZMZ investigated the patient data and resourcs. KBZ was the major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Figures

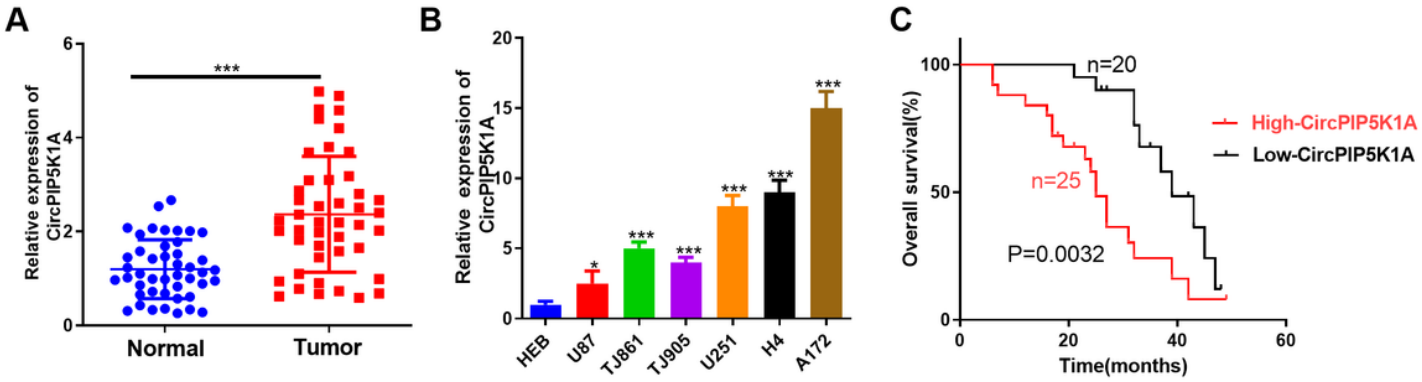

Figure 1

CircPIP5K1A expression in normal and cancerous tissues of glioma A: RT-PCR was used to verify the CircPIP5K1A expression in the tumorous and adjacent normal tissues. ***P < 0.0001. B. RT-PCR was adopted to determine the circPIP5K1A expression in normal glioma cells HEB and tumor cell lines (U87, TJ861, TJ905, U251,H4 and A172), *P< 0.05,***P<0.001 vs. HEB group. C. KM plotter was used for analyzing the relationship between circPIP5K1A expression with glioma prognosis.

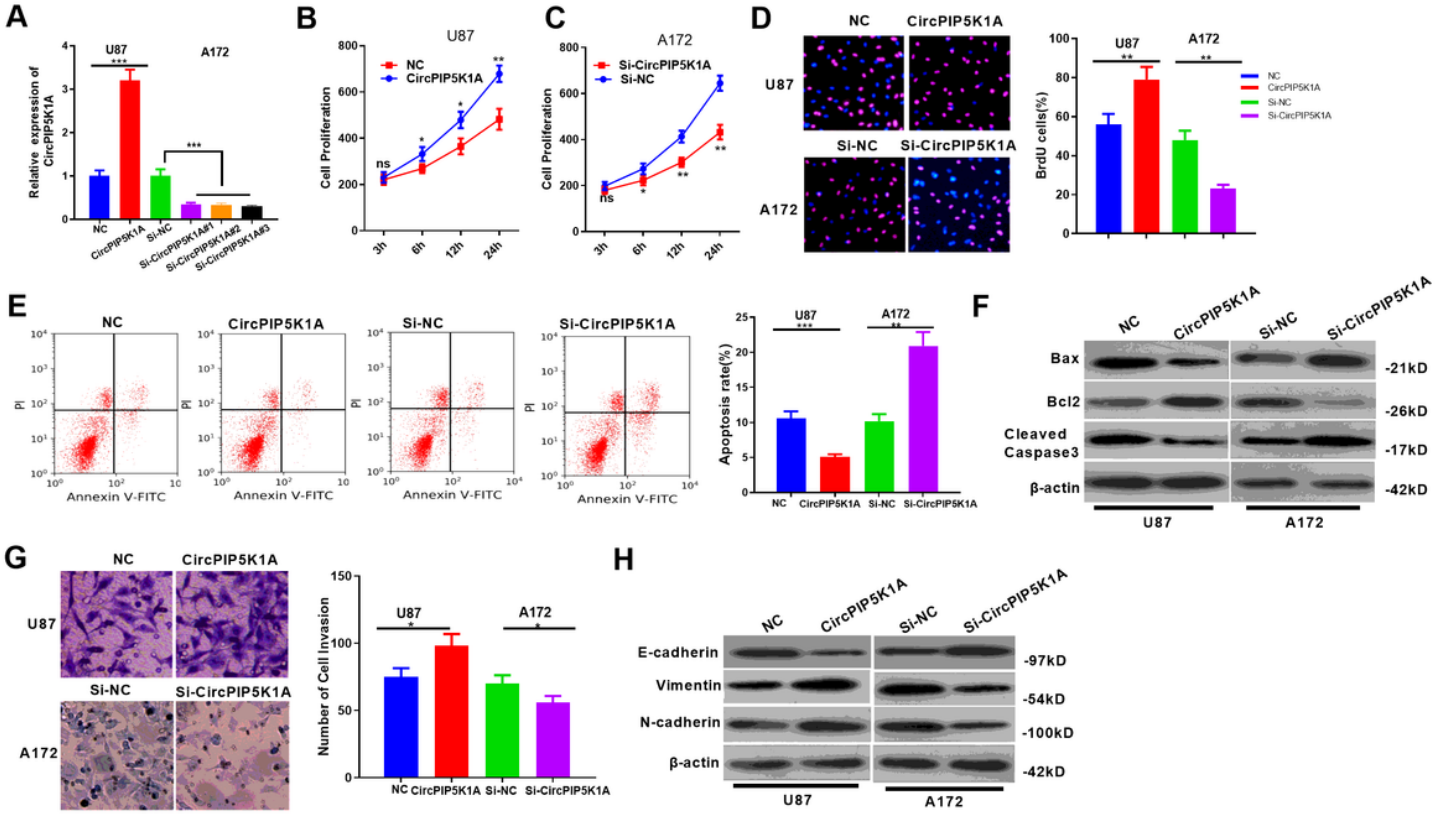

Figure 2

CircPIP5K1A's effects on glioma cell proliferation, invasion, apoptosis and EMT A: CircPIP5K1A overexpression and knockdown models were constructed in glioma cell lines U87 and A172, respectively. B-D: CCK-8 method (B and C) and BrdU assay (D) were adopted to determine cell proliferation. E-F: flow cytometry and western blot were conducted to detect the apoptosis of U87 and A172 cells. G: Transwell assay was employed to determine the invasive ability of U87 and A172 cells. H: Western blot was employed to test EMT markers of E-cadherin, Vimentin and N-cadherin. * P < 0.05, **P < 0.01, ***P < 0.001.

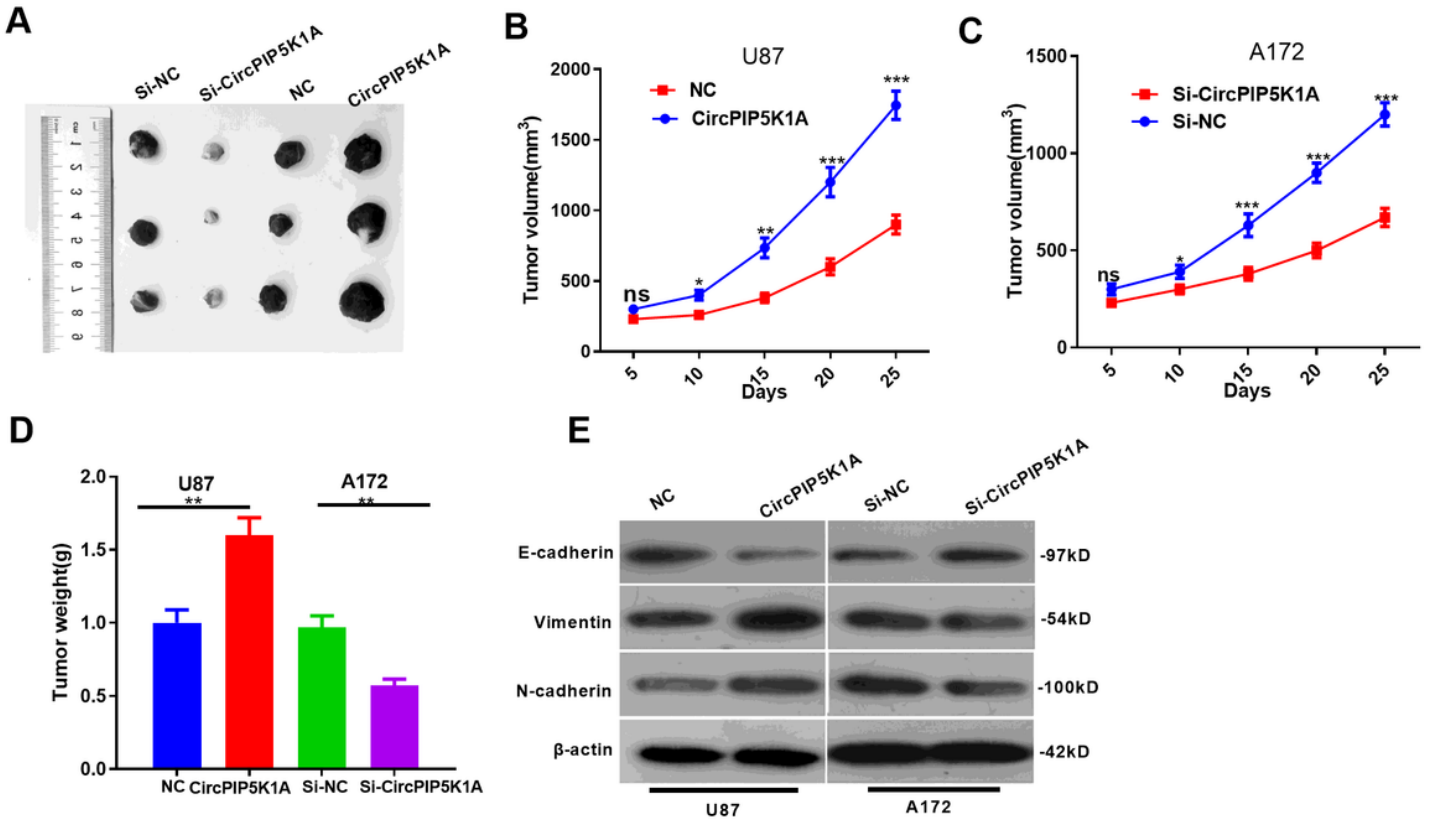

Figure 3

CircPIP5K1A inhibited glioma growth and EMT transformation in vivo A: The nude mice were sacrificed 25 days later, the subcutaneous nodules were taken out and the image of tumors in each group was showen. B-D. The volume and weight of the tumor nodules were calculated. E: Western blot was employed to test EMT markers of E-cadherin, Vimentin and N-cadherin. NS P >0.05, *P<0.05, **P<0.01,***P<0.001 vs.NC group or si-NC group.

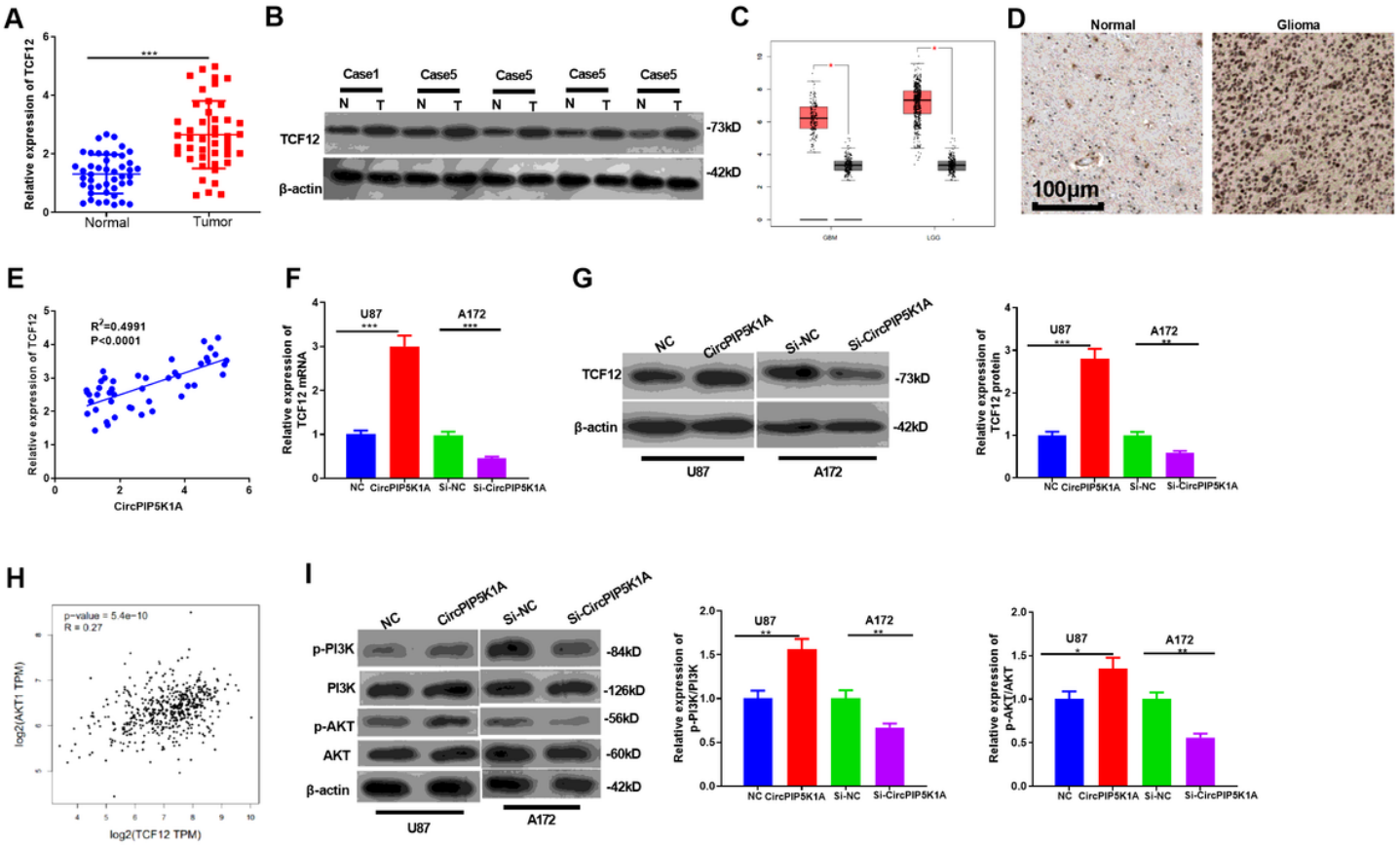

Figure 4

CircPIP5K1A promoted the expression of the TCF12 and PI3K/AKT signaling pathways A. TCF12 expression in glioma and adjacent normal tissues was detected by RT-PCR. B. Protein expression of TCF12 in five cases of glioma and adjacent normal tissues was detected by western blot. C: GEPIA (http://gepia.cancer-pku.cn/) was used for analyzing TCF12 expression in GBM and LGG. D. TCF12 in glioma tissues and normal cerebral tissues was detected by IHC (data from The Human Protein Atlas (https://www.proteinatlas.org/). E. Person correlation analysis detected the correlation between circPIP5K1A and TCF12 in the glioma tissues. F and G: RT-PCR was performed to determine the expression of TCF12 mRNA (F) and protein (G). H: GEPIA database showed a positive correlation between TCF12 and AKT1 in LGG. I: Western blot was conducted to detect the activation of PI3K/AKT signaling pathway. *P< 0.05, **P<0.01, ***P<0.001.

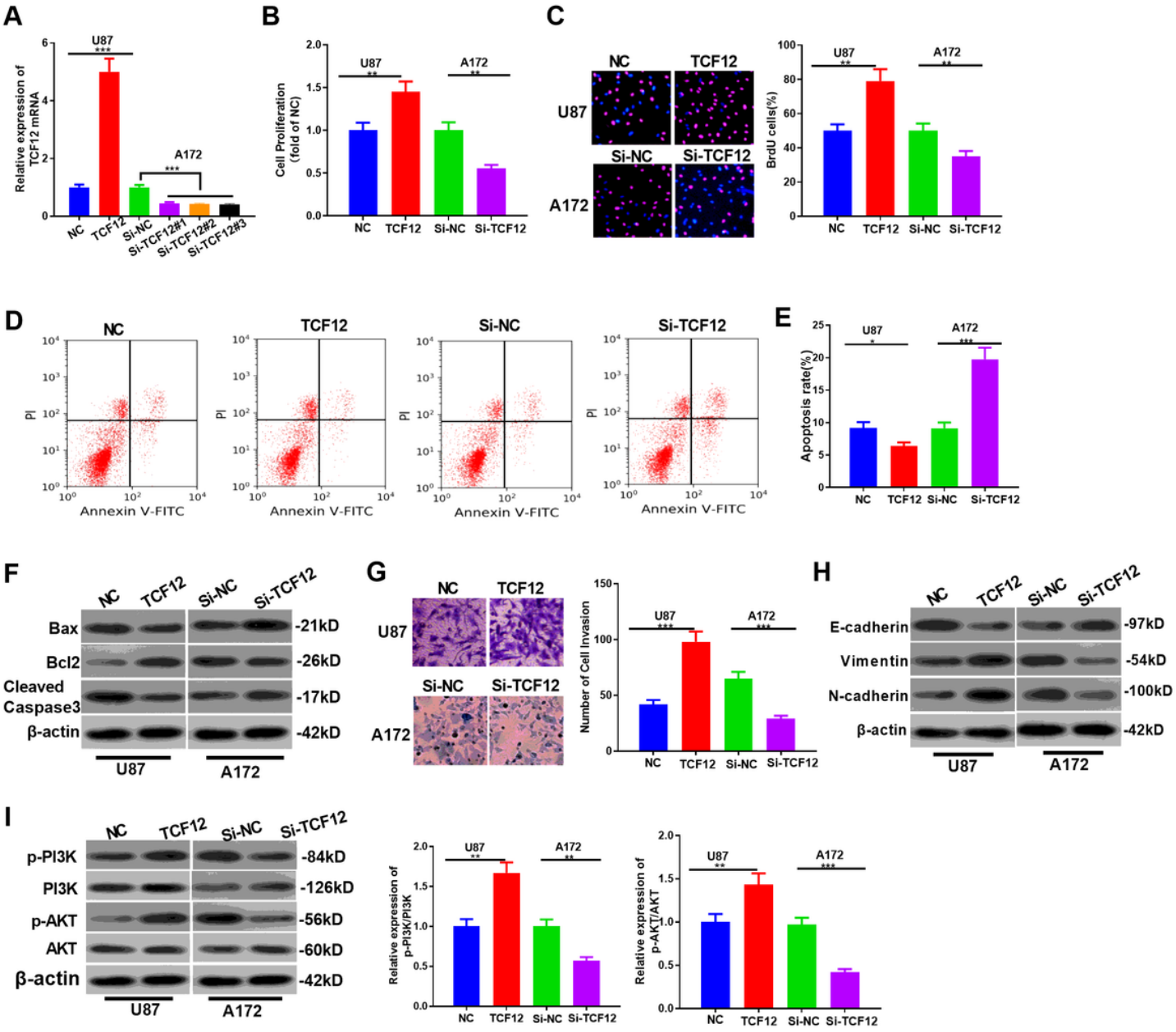

Figure 5

Overexpressing TCF12 promoted the malignant phenotypes of glioma cells A: Overexpression and knockdown models of TCF12 were constructed in U87 and A172 glioma cell lines, respectively, and protein expression of TCF12 was detected by western blot. B-C: CCK8 (B) and BrdU assay (C) were employed to detect cell proliferation. E-G: Flow cytometry and western blot were used to detect and verify the effect of TCF12 regulation on apoptosis. G: Transwell assay was conducted to examine the effect of TCF12 regulation on cell invasion. H: Western blot was carried out to measure the effect of TCF12 regulation on EMT. I: the effect of TCF12 on PI3K/AKT signaling was determined by Western blot. *P< 0.05, **P<0.01, ***P<0.001.

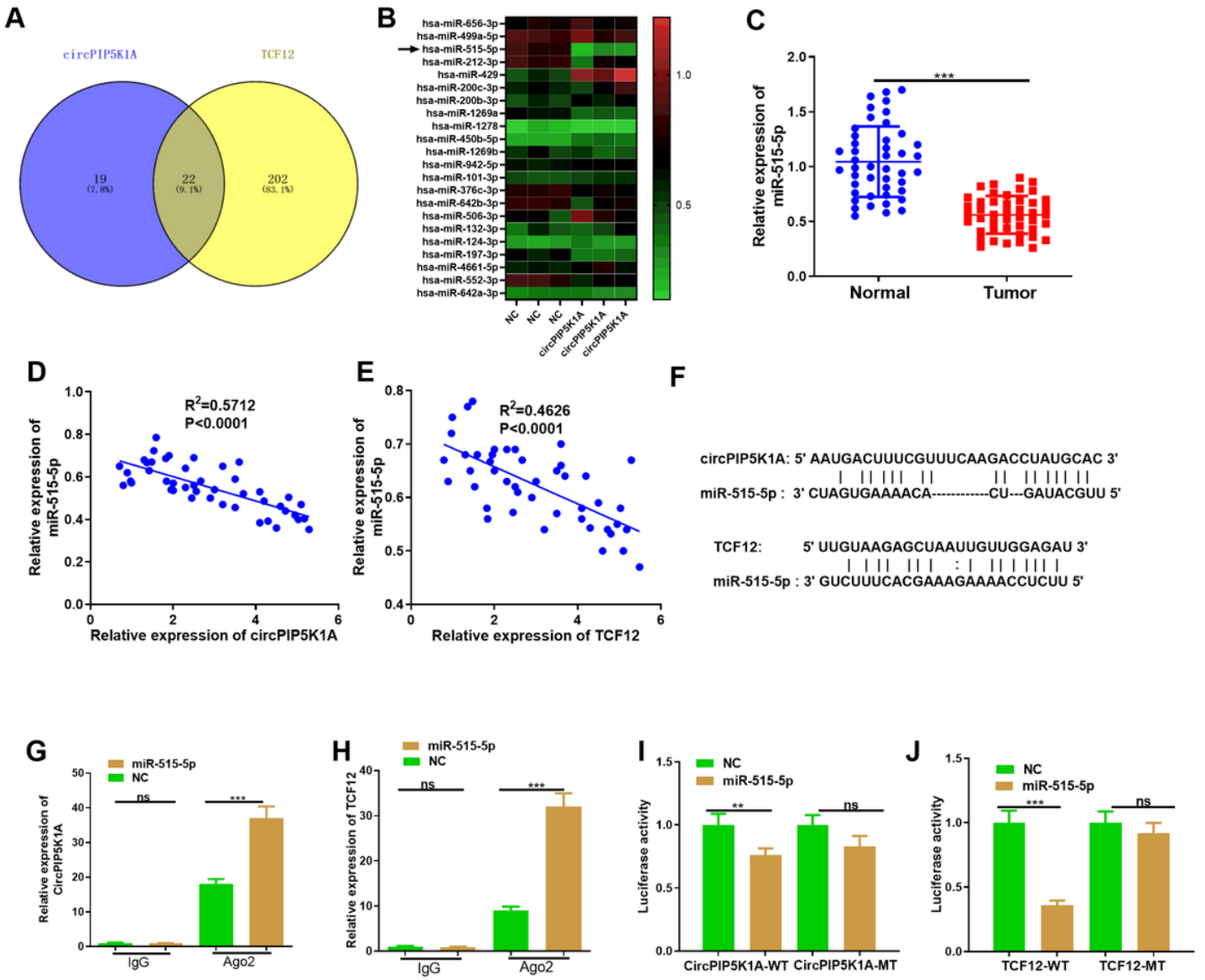

Figure 6

The targeted relationships among circPIP5K1A, TCF12 and miR-515-5p. A. The miRNA target of circPIP5K1A and TCF12 was analyzed through Starbase (http://starbase.sysu.edu.cn/), and a total of 22 miRNAs were common targets of CircPIP5K1A and TCF12. B. RT-PCR was used to determine the levels of the 22 miRNAs circPIP5K1A overexpressed cell. C. The miR-515-5p expression in glioma tissues and adjacent normal tissues was detected by RT-PCR. D-E: Person correlation analysis was adopted to detect the correlation between miR-515-5p and circPIP5K1A, miR-515-5p and TCF12 in the tissues of glioma patients. F: The binding relationship of miR-515-5p with both circPIP5K1A and TCF12 are shown. G and H. RIP assay was employed to examine the bindings of CircPIP5K1A and TCF12 with ant-Ago2 antibody in U87 cells. I and J: Dual luciferase reporter assay was analyzed to make clear the targeting binding relationships between miR-515-5p and circPIP5K1A, miR-515-5p and TCF12 in U87 cells. Ns P>0.05, **P<0.01, ***P<0.001.

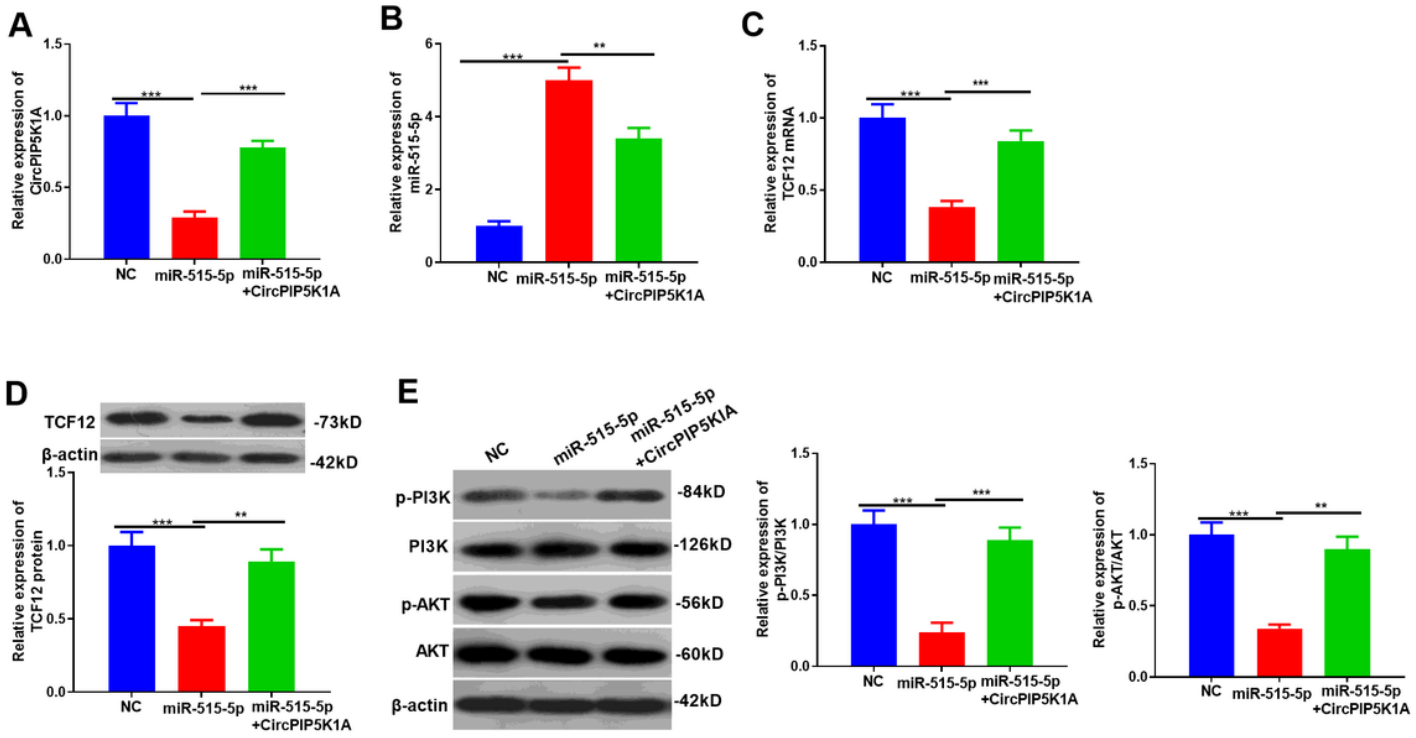

Figure 7

CircPIP5K1A regulated TCF12/PI3K/AKT expression by sponging miR-515-5p A-C: RT-PCR was carried out to detect the CircPIP5K1A miR-515-5p and TCF12 mRNA expressions in U87 cells after transfecting circPIP5K1A overexpression plasmids or miR-515-5p mimics. D-E: Western blot was constructed to detect the expression of TCF12 protein and PI3K/AKT signaling pathway. **P<0.01, ***P<0.001.