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Original Article

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Y-box protein-1 modulates circSPECC1 to promote glioma tumorigenesis via miR-615-5p/HIP1/AKT axis

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Abstract

Y-box binding protein-1 (YB-1) is upregulated in glioma and plays an important role in its occurrence and drug resistance. However, the involved regulatory processes and downstream pathways are still unclear. Since various circular RNAs (circRNAs) and microRNAs (miRNAs) also play roles in the pathogenesis of glioma, we hypothesize that YB-1 may exert its function through a circRNA-miRNA-protein interaction network. In this study, we use the RNA binding protein immunoprecipitation assay and quantitative reverse transcription polymerase chain reaction to determine the circRNAs involved in the regulation of YB-1 and further elucidate their biological functions. The level of circSPECC1 (hsa_circ_0000745) modulated by YB-1 is significantly upregulated in the U251 and U87 glioma cell lines. Downregulation of circSPECC1 markedly inhibits the proliferation and invasiveness of U251 and U87 cells by inducing apoptosis. Bioinformatics analysis reveals that miR-615-5p could interact with circSPECC1 and huntingtin-interacting protein-1 (HIP-1). Then we determine the interactions between miR-615-5p, circSPECC1, and HIP1 using dual luciferase reporter system and pull-down assays. Mechanistic analysis indicates that the downregulation of circSPECC1 results in a decreased HIP1 expression. This study demonstrates that circSPECC1 modulated by YB-1 is increased in glioma cell lines. In addition, circSPECC1 promotes glioma growth through the upregulation of HIP1 by sponging miR-615-5p and targeting the HIP1/AKT pathway. This indicates that YB-1 and circSPECC1 may both be promising targets for glioma treatment.

Key words glioma, YB-1, circSPECC1, miR-615-5p, HIP1

Introduction

Gliomas represent approximately 75% of malignant primary brain tumors in adults and have the highest mortality rate compared with other types of brain tumors [1,2]. Diffuse gliomas are diagnosed and classified according to histopathology and molecular biology [3,4]. Glioblastoma multiforme (GBM), a subtype of glioma, is characterized by its invasiveness and high rate of recurrence; it accounts for 57.3% of gliomas, with the lowest five-year relative survival of all brain tumors (6.8%)[1]. After surgery, radiotherapy, chemotherapy (temozolomide), and immunotherapy (bevacizumab), the overall survival rate of GBM and other subtypes is still very low [5–7]. Therefore, diagnosing and treating glioma is challenging, especially from the molecular point of view. To determine potential molecular therapeutic targets, a full understanding of the molecular mechanisms of glioma recurrence and invasiveness is crucial. In recent years, non-coding RNAs and their networks of interaction in glioma have attracted widespread attention.

Circular RNAs (circRNAs), a unique kind of non-coding RNA, are single-stranded, covalently closed circular transcripts with no 5′ cap or 3′ poly(A) tail. Because of their special structure and significant biological functions, circRNAs have been recognized as special tools in many biological fields, especially cancer diagnosis and therapy [8,9]. Various circRNAs were also confirmed to play a role in the pathogenesis of gliomas [10]. However, the specific role of circRNAs

in glioma remains largely unknown.

MicroRNAs (miRNAs), another kind of small non-coding RNAs, are transcripts 20–25 nucleotides long. miRNAs play critical roles in tumorigenesis and cancer biology by binding with target mRNAs and regulating their gene expression [11,12]. Abnormal miRNA expression profiles have been observed in glioma [13,14] and could be a potential biomarker for its diagnosis [15,16]. miR-615 has been found to inhibit cell proliferation, migration, and invasion in human glioblastoma [17]. However, the upstream and downstream molecular mechanisms remain unclear. Previous studies have shown that miRNA-mediated dysregulation of mRNA and relevant signaling pathways are closely associated with tumor progression and treatment resistance. circRNAs can act as competing endogenous RNAs (ceRNAs) or miRNA sponges, "isolate" miRNAs from target mRNAs and inhibit target mRNAs, thereby upregulating the expressions of target genes via miRNA response elements [18]. Therefore, we speculate that miR-615-5p may also function in this way in glioma.

Y-box binding protein-1 (YB-1) is a member of the cold shock protein superfamily. It contains highly conserved nucleic acid sequences and is a multifunctional protein. YB-1 participates in a series of DNA/RNA-dependent events, such as DNA repair, alternative splicing of mRNA, and regulation of mRNA stability and translation, and plays an important role in cell proliferation, differentiation, stress response, and transformation of malignant cells. Moreover, YB-1 may be a prognostic biomarker or target in cancer therapy [19,20]. Normal neural stem cells express YB-1 at high levels. However, *YB-1* is silenced during glial differentiation [21]. Previous studies showed that YB-1 is highly expressed in different glioma cell lines, such as U373 and U87 [22], and is also highly expressed in neural stem cells but not in terminally differentiated astrocytes [21], suggesting that YB-1 may be a potential marker of glioma and that it is possible to use YB-1 as a target for glioma treatment. YB-1 immunization combined with Treg depletion can induce specific T-cell responses against neuroblastoma and may be a potential strategy for the prevention and treatment of neuroblastoma in the early stage [23]. Silencing of *YB-1* can suppress neuroblastoma cell growth both *in vitro* and *in vivo* [24]. In our previous research, we found that YB-1 is overexpressed in glioma tissues and plays a key role in the occurrence and resistance of gliomas [25]. However, its regulatory processes, such as the transcription and translation of its target genes, are still unclear.

In this study, we aimed to explore the YB-1 signaling pathway and its downstream effects on glioma, especially the circRNA-miRNA-protein interaction network.

Materials and Methods

Cell culture

The U251 (astrocytoma) and U87 (glioblastoma) human glioma cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The present study was approved by the Institutional Review Board/Ethics of Medical Biology Research Ethics, School of Medicine, Xi'an Jiaotong University (Approval number: 2021-605). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Gibco, Grand Island, USA) and 100 U/mL penicillin-streptomycin (Gibco) and incubated at 37°C with 5% CO_2 and 95% air. Trypsin-EDTA (0.25%; Gibco) was used for cell digestion and passage.

Cell transfection

Transfection was performed using X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) according to manufacturer's protocol. Briefly, U251 cells were seeded and incubated for 24 h and then transfected with a YB-1-Flag plasmid (GenePharma, Shanghai, China) when they reached 80%–90% confluence. After 4 to 6 h of transfection, the Opti-MEM medium (Gibco) was changed, and the transfection was continued for 48 h. Transfection efficiency was evaluated and then screened with 500 μg/mL G418 for 14 days to obtain a stable expression strain, which was then expanded for subsequent experiments. Nontransfected cells and cells transfected with a control flag plasmid were used as blank and negative controls, respectively. The small interfering RNAs (siRNAs; GenePharma) were as follows: si-YB-1, sense: 5′-GGUCCUCCACG CAAUUACCAGCAAA-3′; si-circSPECC1, sense: 5′-GCCAAGGGGCCT TTACAACAA-3′; si-HIP1, sense: 5′-GTTGTGGCCTCAACCATT-3′; and nonspecific siRNA (si-NC), sense: 5′-GACTCTCCACACAGGG CTGTATG-3′.

RNA binding protein immunoprecipitation (RIP) assay

Glioma U251 cells were collected and processed using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, USA) according to the manufacturer's protocol. Rabbit anti-human HuR antibody or normal rabbit IgG antibody was added at 5 μg, and the cell lysate was incubated with the antibody at 4°C overnight. After specific intracellular proteins were captured by specific antibodies, the protein-RNA complexes were obtained. Proteins were then digested with proteinase K, and RNAs were extracted. During the experiment, the magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible, and the target RNAs were then subjected to high-throughput sequencing according to the needs of the experiment. The bound RNAs were eluted and reverse transcribed to cDNA and subsequently detected by qPCR.

CCK-8 assay

Cells from different groups were suspended at a concentration of 1×10^6 cells/mL. Each group was seeded in a 96-well plate at 4×10^3 cells/well. Four duplicate wells were seeded for each group. After 24 h, 48 h, 72 h, and 96 h of culture, 10 μL of Cell Counting Kit-8 solution (Beyotime Institute of Biotechnology, Shanghai, China) was added to each well and incubated at 37°C for 2 h in the dark. A microplate reader was used to detect absorbance at 450 nm, and the cell survival rates were calculated. The experiment was repeated three times.

Wound-healing assay

In six-well plates, five straight parallel lines were drawn at the bottom of each well. Cells from different groups were suspended, seeded at 2.5×10^5 cells/well, and cultured in an incubator until their confluence reached 90%–95%. The cells were washed three times with PBS, a vertical line was drawn with a 10-μL pipette tip, and serum-free medium was added. Cells were then placed in an incubator at 37 °C and 5% CO_2. Finally, the phase was determined under an inverted microscope (100 ×) at 0 h, 24 h and 48 h, and the distance between the wound margins was calculated using ImageJ software (version 1.51; National Institutes of Health, Bethesda, USA) (http://rsb.info.nih.gov/ij/). The relative migration rate was defined as the decreasing distance normalized to the 0 h control.

The experiment was repeated three times.

Transwell invasion assay

The migration ability of cells was measured using 24-well Transwell chambers (Corning, Kenneburg, USA). The chambers were coated with 20 μL Matrigel (Shanghai Yisheng Biotechnology, Shanghai, China) to assess the invasive ability of glioma cells. Cells from different groups were suspended at a concentration of 2.5×10^5 cells/mL. In the upper compartments of the transwell chambers, 1×10^5 cells and 200 μL of serum-free culture medium were added. In each lower compartment, 600 μL of medium was added. After 36 h of incubation, the cells on the upper surface of the filter were removed with a cotton swab, while those on the lower surface were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Cells were rinsed with PBS until the background was clear. The cells were then observed and counted under a microscope in 5 fixed fields (at a magnification of 100 ×), and the migration ability of each group was evaluated. The experiment was repeated three times.

Dual-luciferase reporter assay

circSPECC1 and huntingtin-interacting protein-1 (HIP1) wild-type (WT) and mutant sequences, with or without the miR-615-5p binding site, were cloned and inserted into pGL3 luciferase plasmids. Each recombined plasmid (1.5 μg) was cotransfected into HEK293 cells with a miR-615-5p mimic (100 nM; GenePharma) and a pRL-TK plasmid (10 ng). Twenty-four hours after transfection, the cells were collected and lysed. Luciferase activities were evaluated using a dual-luciferase reporter assay system (Promega, Madison, USA). The intensities of the firefly and Renilla luciferases were recorded and measured.

RT-qPCR

Total RNA was extracted from cells by using TRIzol reagent (Invitrogen, Carlsbad, USA). Reverse transcription was performed using the PrimeScriptTM RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instructions. Complementary DNA amplification was subsequently performed by quantitative reverse transcription PCR (RT-PCR), using GoTaq qPCR Master Mix (Promega) on an ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, USA). Forty amplification cycles took place with the following parameters: pre-denaturation at 95°C for 2 min, denaturation at 94°C for 15 s, and annealing and extension at 58°C for 30 s. Three duplicate wells were set up. The dissolution curve was used to determine the specificity of the product, and *U6* was used as an internal reference; the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ quantification method. The primer sequences for RT-qPCR were as follows: circSPECC1 forward, 5′-ATGTTGAAAGTAGCCCGAGCAG-3′, and reverse, 5′-TGGGAGTGTTGGAAGAAGTTGG-3′; miR-615-5p forward, 5′-GGGGGTCCCCGGTGCT-3′, and reverse, 5′-AGTGCAGGGTCCGAGGTATT-3′.

Flow cytometer analysis

Apoptotic cells were assayed by flow cytometer analysis using an Annexin V apoptosis detection kit (BD Bioscience, San Jose, USA) following the manufacturer's instructions. U251 and U87 cells were suspended separately and seeded at 1×10^5 cells/well in 6-well plates. Cells from different treatment groups were collected and labeled with reagents. The labeled cells were analyzed on a FACS Aria flow cytometer (BD Biosciences). Different cells and their ratios were calculated with BD Cell QuestPro software (version 5.1; BD Biosciences).

Western blot analysis

Cells from different groups were harvested and extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Cell extracts were mixed with loading buffer (CWBio, Shanghai, China), and equal amounts of proteins were separated by 10% SDS-PAGE, followed by transfer onto polyvinylidene fluoride (PVDF) membranes (Solarbio Science & Technology, Beijing, China). After being blocked with 5% BSA, the PVDF membranes were incubated overnight at 4°C with the following primary antibodies: anti-HIP1 antibody (1:2000; Proteintech, Chicago, USA), anti-P-AKT antibody (1:2000; Proteintech), anti-caspase-9 antibody (1:2000; Proteintech), or anti-β-actin antibody (1:2000; Proteintech) and then washed with phosphate buffer solution supplemented with 0.05% Tween 20 (PBST). The membranes were then incubated with the secondary antibody (Proteintech) at 25°C for 1 h and then washed three times with PBST. Protein bands were detected using a chromogenic kit (Millipore) and quantified using ImageJ software. β-Actin was used as the loading control.

Statistical analysis

Each experiment was repeated at least three times. Data are shown as the mean ± SD. Groups were compared using either Student's *t* test or one-way analysis of variance. All statistical analyses were performed using SPSS 18.0 software (IBM-SPSS, Chicago, USA). $P < 0.05$ was considered statistically significant.

Results

circSPECC1 is abundantly expressed in glioma cells

RIP was performed in U251 cells to identify YB-1-RNA complexes. We found 21 circRNAs that can bind with YB-1, five of which (circSPECC1, circHPK3, circHPK2, circFOXO3, and circZNF286B) have the largest changes in expression, as quantitatively determined by RT-qPCR (Figure 1A). RT-qPCR in *YB-1*-knockdown U251 cells demonstrated that circSPECC1 is the circRNA most affected by the YB-1 protein (Figure 1B). Furthermore, RT-qPCR assays showed that overexpression of YB-1 facilitated the expression of circSPECC1 (Figure 1C), while knockdown of *YB-1* inhibited the level of circSPECC1 in U251 and U87 cells (Figure 1D). Therefore, YB-1 might promote the expression of circSPECC1.

circSPECC1 downregulation suppresses the proliferation, migration, and invasion of glioma cells and promotes their apoptosis

To further determine the potential function of circSPECC1 in glioma, siRNAs were used to interfere with the expression level of circSPECC1 in U251 and U87 cell lines. RT-qPCR results indicated that circSPECC1 siRNAs could effectively suppress the expression of circSPECC1 (Figure 2A). Then, we explored the biological function of circSPECC1 in cell proliferation, migration, and invasion in glioma cell lines. CCK-8 assay showed that downregulation of circSPECC1 expression could markedly suppress glioma cell proliferation (Figure 2B). In addition, wound-healing assay revealed that *circSPECC1* knockdown repressed the migration of glioma cells

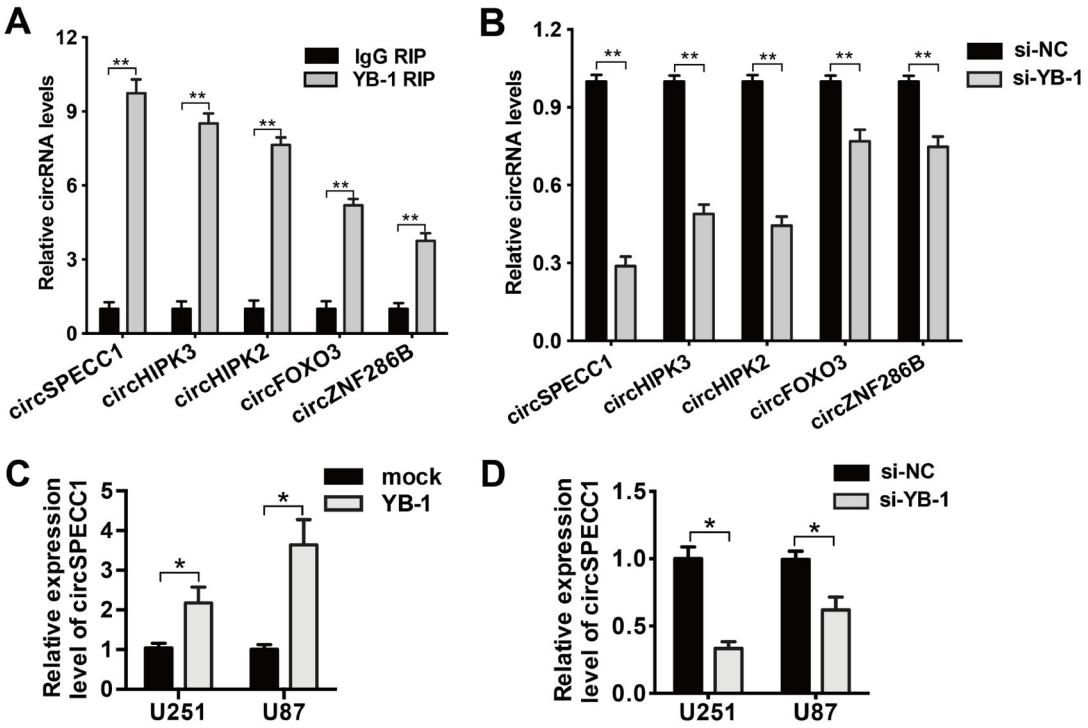

Figure 1. circSPECC1 is abundantly expressed in glioma cells (A) The top five upregulated circRNAs interacting with YB-1 in U251 cells measured by the RNA binding protein immunoprecipitation assay. $^{**}P < 0.01$. (B) The expression level of the top five circRNAs in U251 cells after YB-1 downregulation measured by RT-qPCR. $^{**}P < 0.01$. (C) RT-qPCR was used to detect circSPECC1 expression in U251 and U87 cells upon YB-1 upregulation treatment. $^{*}P < 0.05$. (D) RT-qPCR results of the circSPECC1 expression in U251 and U87 cells upon YB-1 downregulation treatment. $^{*}P < 0.05$.

(Figure 2C,D). Moreover, transwell assay revealed that the inhibition of circSPECC1 could suppress the invasiveness of glioma cells (Figure 2E,F). Finally, FACS results showed that downregulation of circSPECC1 induced apoptosis in U251 and U87 cells (Figure 2G,H). All of the above results suggested that circSPECC1 could promote cell proliferation, migration and invasion and inhibit apoptosis in glioma cells, which is shown to behave as an oncogenic factor for cancer genesis and progression of glioma cells.

circSPECC1 acts as an efficient miR-615-5p sponge in glioma cells

To understand how circSPECC1 modulates the genesis and progression of glioma, we identified potential downstream miRNA targets through the circular RNA interactome database (https://circinteractome.nia.nih.gov/index.html) and found that miR-615-5p could be a candidate. The binding sites for the miR-615-5p and circSPECC1 interaction were hypothesized (Figure 3A). Then, several assays were performed to clarify the binding relationship between the two. First, RT-qPCR revealed that downregulation of circSPECC1 promoted miR-615-5p expression (Figure 3B). Moreover, the RNA pull-down assay showed that in U251 and U87 cells, circSPECC1 was significantly enriched in circSPECC1–miR-615-5p complexes (Figure 3C). Furthermore, the dual-luciferase reporter assay revealed that transfection with a miR-615-5p mimic visibly attenuated the luciferase activity of WT circSPECC1 compared with the control (Figure 3D). Meanwhile, the miR-615-5p mimics did not affect the luciferase activity in the circSPECC1 mutant type (Mut) (Figure 3E). The above results revealed that circSPECC1 might function as a sponge of miR-615-5p in glioma cells.

miR-615-5p inhibits the proliferation, migration, and invasion of glioma cells

To further understand the function of miR-615-5p in glioma, we explored its expression in glioma cells. RT-qPCR showed that the relative expression level of miR-615-5p was lower in U251 and U87 cells than in normal gliocytes (Figure 4A). RNA pull-down results suggested that the downregulation of circSPECC1 reduced the relative miR-615-5p level in the pulled-down complexes (Figure 4B), indicating that circSPECC1 interacts with miR-615-5p. Several experiments clarified the function of miR-615-5p as follows. First, CCK-8 assay demonstrated that overexpression of miR-615-5p inhibited the proliferation of U251 and U87 cells (Figure 4C). Furthermore, wound-healing assay revealed that overexpression of miR-615-5p could suppress the migration of glioma cells (Figure 4D,E). Moreover, transwell assay showed that upregulation of miR-615-5p could suppress glioma cell invasiveness (Figure 4F, G). Finally, FACS results showed that upregulation of miR-615-5p induced cell apoptosis in U251 and U87 cells (Figure 4H,I). Therefore, miR-615-5p served as a suppressor in the proliferation, migration and invasion of glioma cells and induced cell apoptosis.

HIP1 is a target of miR-615-5p

Next, we explored the potential target genes of miR-615-5p with TargetScan, where HIP1 was predicted as a potential downstream target of miR-615-5p. The putative interactive site is shown in Figure 5A. Western blot analysis showed that overexpression of miR-615-5p significantly suppressed HIP1 expression in U251 and U87 cells compared to the mimic control groups (Figure 5B,C). In addition, HIP1 wild-type plasmid (HIP1-WT) and mutant plasmid

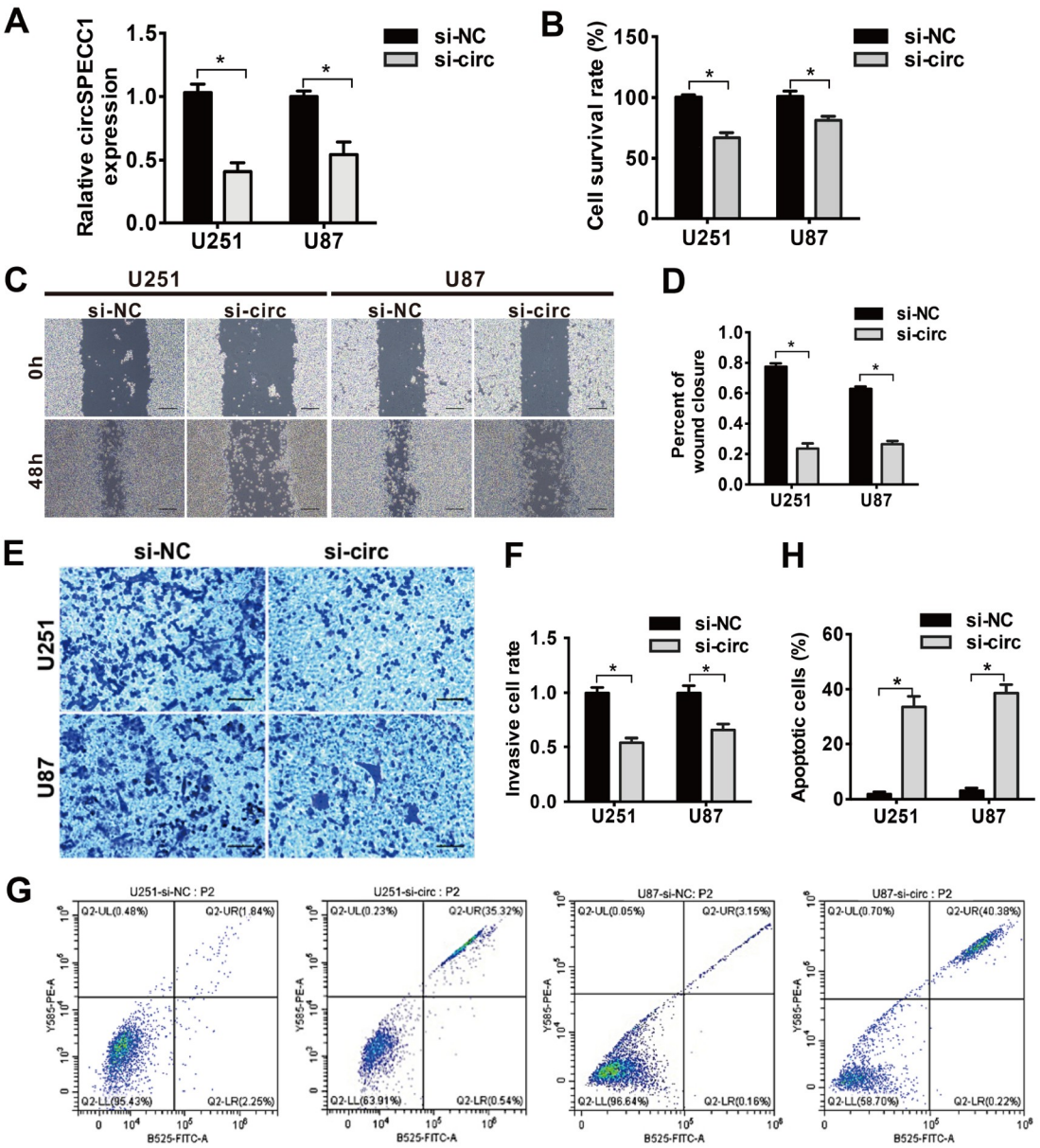

Figure 2. circSPECC1 downregulation suppresses the proliferation, migration, and invasion and promotes the apoptosis of glioma cells (A) RT-qPCR was used to detect circSPECC1 expression in U251 and U87 cells upon circSPECC1 downregulation. *$P < 0.05$. (B) CCK8 assay of U251 and U87 cells upon siRNA transfection treatment. *$P < 0.05$. (C,D) Wound-healing assay for the detection of migration ability of U251 and U87 cells upon circSPECC1 downregulation. *$P < 0.05$. Scale bar: 200 μm. (E,F) Transwell assay for the detection of invasion ability of U251 and U87 cells upon siRNA transfection. *$P < 0.05$. Scale bar: 200 μm. (G,H) Flow cytometric analysis of apoptosis in U251 and U87 cells upon circSPECC1 downregulation. *$P < 0.05$.

(HIP1-Mut) were synthesized according to the predicted binding site of miR-615-5p and HIP1. The results of the luciferase reporter assay showed that miR-615-5p mimics reduced the luciferase activity of the HIP1-WT reporter but not the HIP1-Mut reporter (Figure 5D). Therefore, HIP1 is a target of miR-615-5p, and circSPECC1 may participate in the progression of glioma by sponging miR-615-5p to promote the expression of HIP1.

circSPECC1 regulates the HIP1/AKT pathway through miR-615-5p

We then explored the function of HIP1 in glioma cells. CCK-8, wound-healing, and transwell assays showed that decreased expression of HIP1 clearly inhibited the proliferation, migration, and invasion of glioma cells (Figure 6A–E) and promoted cell apoptosis (Figure 6F,G). Since HIP1 might participate in tumorigenesis through AKT or apoptosis, the phosphorylated AKT (p-AKT) and cleaved caspase-9 pathways were both evaluated in glioma cells via western blot analysis. The results revealed that miR-615-5p overexpression inhibited HIP1 and p-AKT expression and promoted the production of cleaved caspase-9, implying that cell proliferation was inhibited while apoptosis was promoted. This phenomenon was partly reversed by circSPEEC1 (Figure 7A,B), but this was not observed in total AKT. To explore whether circSPECC1 serves its biological function through the circSPECC1/miR-615-5p/HIP1 axis,

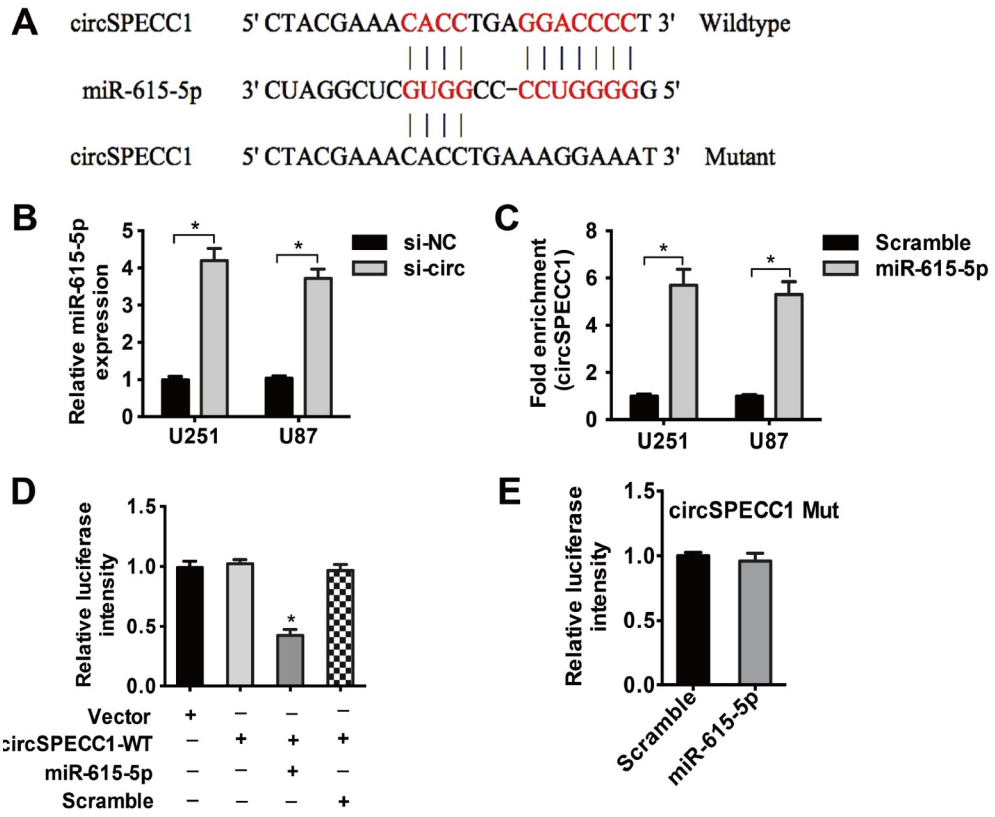

Figure 3. circSPECC1 functions as an efficient miR-615-5p sponge in glioma cells (A) The putative binding sites of miR-615-5p with the circSPECC1 wild-type (WT) or mutant sequence are shown. (B) RT-qPCR results of the expression level of miR-615-5p in U251 and U87 cells upon circSPECC1 downregulation. *P < 0.05. (C) RNA pulldown assay was used to confirm the interaction of circSPECC1 with miR-615-5p. *P < 0.05. (D,E) Dual-luciferase reporter assay for the detection of the relative luciferase activities of U251 cells cotransfected with miR-615-5p or scramble and circSPECC1 or vector or circSPECC1 mutant cotransfected with miR-615-5p or scramble. *P < 0.05, compared with the circSPECC1-WT group.

rescue experiments were designed using miR-615-5p mimics and inhibitors. Western blot analysis demonstrated that circSPCCC1 overexpression increased HIP1 expression and p-AKT expression, and the effect could be counteracted by miR-615-5p mimics, while downregulation of circSPECC1 reduced the levels of HIP1 and p-AKT, and the effect could be abolished by miR-615-5p inhibitors (Figure 7A–C). Therefore, the rescue experiments demonstrated that circSPECC1 might serve as a ceRNA to sponge miR-615-5p and regulate its role in glioma through the HIP1/AKT signaling pathway.

Discussion

YB-1 is a 42-kDa multifunctional protein belonging to the cold shock protein superfamily. It participates in the tumorigenesis, development, and treatment resistance of various tumors [19,20,26–28] and could be a diagnostic or prognostic biomarker of certain types of tumors. Previous studies have revealed that YB-1 may be a key factor in glioma progression, proliferation, prognosis, and drug resistance [25,29–31]. Consistently, our previous study demonstrated that *YB-1* knockdown inhibits glioma cell proliferation and migration and induces cell apoptosis both *in vivo* and *in vitro* [32]. However, its regulatory processes, such as the transcription and translation of target genes, are still unclear. In addition, several noncoding RNAs, such as circRNAs and miRNAs, which are associated with the occurrence and development of gliomas have been identified. The pattern and characteristics of circRNA and miRNA expression in gliomas have attracted increasing attention [16,18,33–35]. Therefore, in the present study, we explored the possible pathway through which YB-1 promotes glioma progression, especially the downstream circRNA-miRNA-protein interaction network.

In our study, circSPECC1 (hsa_circ_0000745) was found to be regulated by YB-1 and abundantly expressed in glioma cell lines. The potential regulatory mechanisms of circSPECC1 in glioma were determined through several experiments. We showed that downregulation of circSPECC1 can inhibit cell proliferation, migration, and invasion and promote cell apoptosis, suggesting that circSPECC1 may promote malignant progression of glioma cells and inhibit their apoptosis. Furthermore, miR-615-5p was downregulated in glioma cells compared to normal cells. Inhibition of circSPECC1 was correlated with the upregulated expression of miR-615-5p in glioma cells. We also found that miR-615-5p expression is related to the activation of the HIP1/AKT pathway. Thus, this study is the first to reveal the role of circSPECC1 in glioma and how it promotes the malignant behavior of glioma cells.

In recent years, an increasing number of circRNAs have been demonstrated to participate in tumor pathogenesis, serving as promoters or suppressors in the biological progression of cancer. For instance, hsa_circ_0014359, circDENND2A, circMAN2B2, circPTN, circ-MAPK4 and circNT5E were found to be related to the pathogenesis and malignancy of glioma [36–41]. We found that circSPECC1, which is associated with the prognosis of glioma patients, is regulated by YB-1 and significantly upregulated in

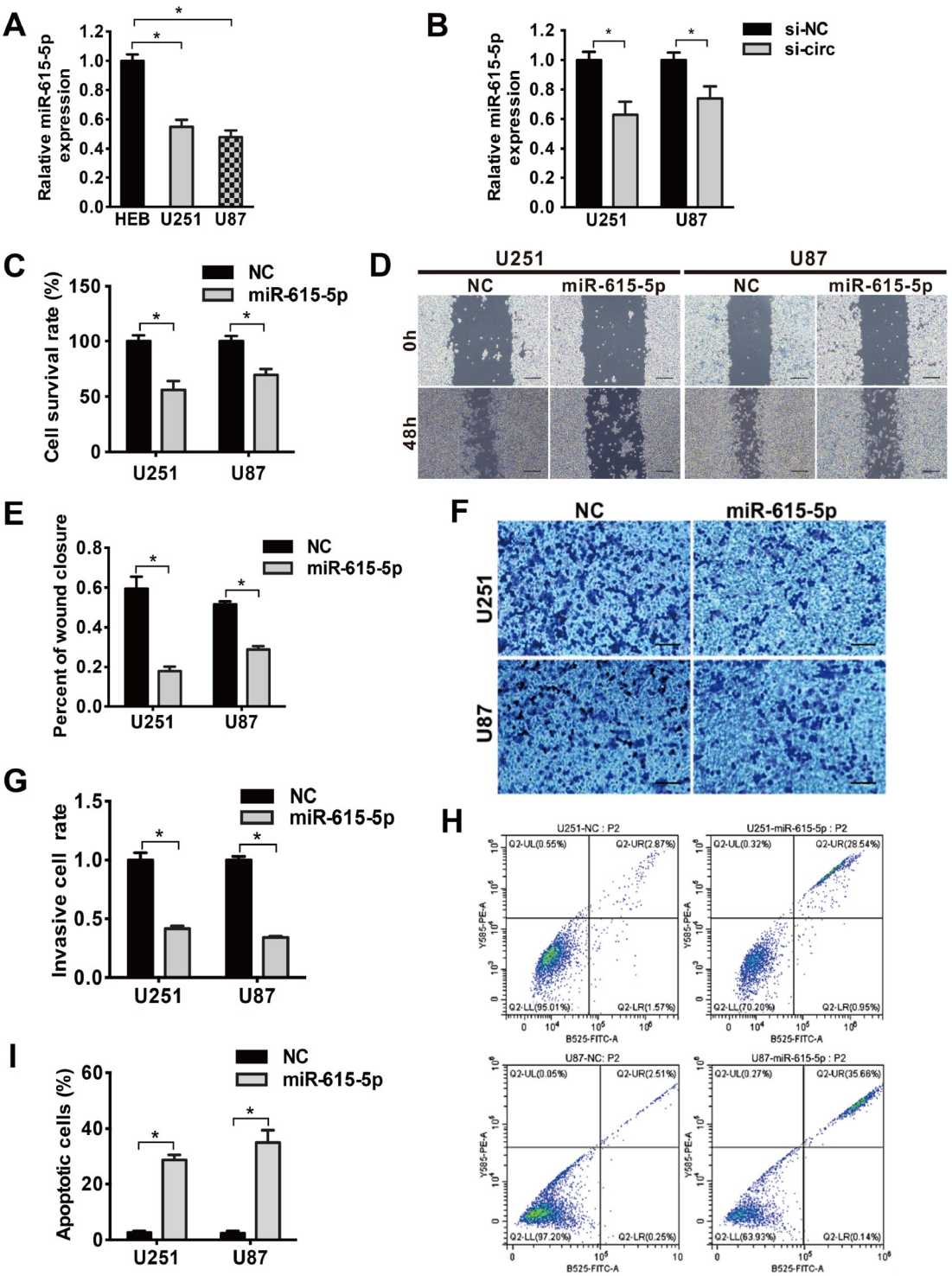

Figure 4. Overexpression of miR-615-5p inhibits the proliferation, migration, and invasion of glioma cells (A) RT-qPCR results of the miR-615-5p expression in U251 and U87 cells and compared to normal control cells. *$P < 0.01$. (B) RT-qPCR results of the miR-615-5p levels in the pulled-down complexes in U251 and U87 cells upon *circSPECC1* knockdown. *$P < 0.01$. (C) CCK-8 assay of U251 and U87 cells upon miR-615-5p overexpression. *$P < 0.01$. (D,E) Wound-healing assay for the detection of the migration ability of U251 and U87 cells upon miR-615-5p overexpression. *$P < 0.01$. Scale bar: 200 μm. (F,G) Transwell assay was used to measure the invasion ability of U251 and U87 cells upon miR-615-5p overexpression. *$P < 0.01$. Scale bar: 200 μm. (H,I) Flow cytometric analysis for the detection of apoptosis of U251 and U87 cells in the miR-615-5p mimic groups. *$P < 0.05$.

glioma cells. circSPECC1 is known to participate in the tumorigenesis and progression of gastric and cervical cancers [42–44]. In this study, we revealed that circSPECC1 also plays a key role in glioma by promoting the proliferation, migration, and invasion of glioma cells.

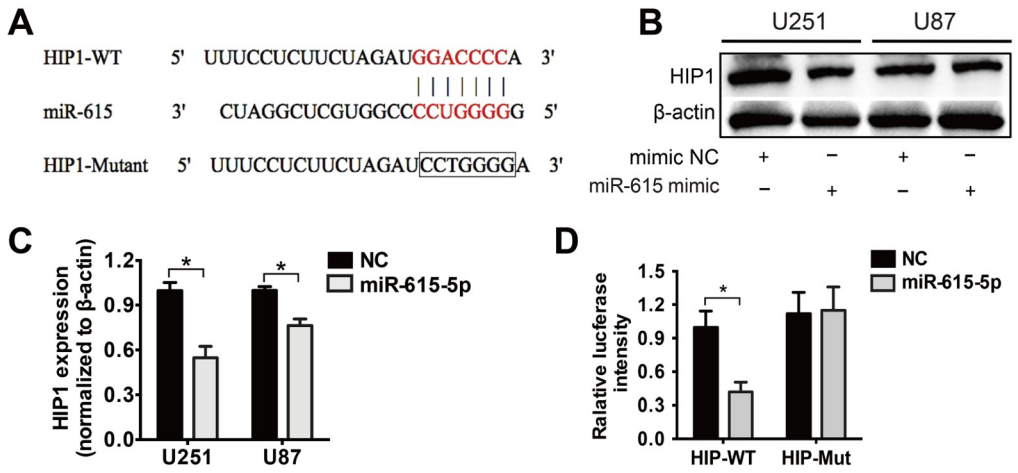

Figure 5. miR-615-5p targets the HIP1 gene and inhibits HIP1 expression (A) Putative complementary binding site between miR-615-5p and HIP1. (B,C) Western blot analysis of HIP1 levels upon miR-615-5p overexpression. *$P < 0.01$. (D) Dual-luciferase reporter assay of the relative luciferase activities of U251 cells co-transfected with HIP1-WT or HIP1-Mut and miR-615-5p mimics or miR-NC. *$P < 0.05$.

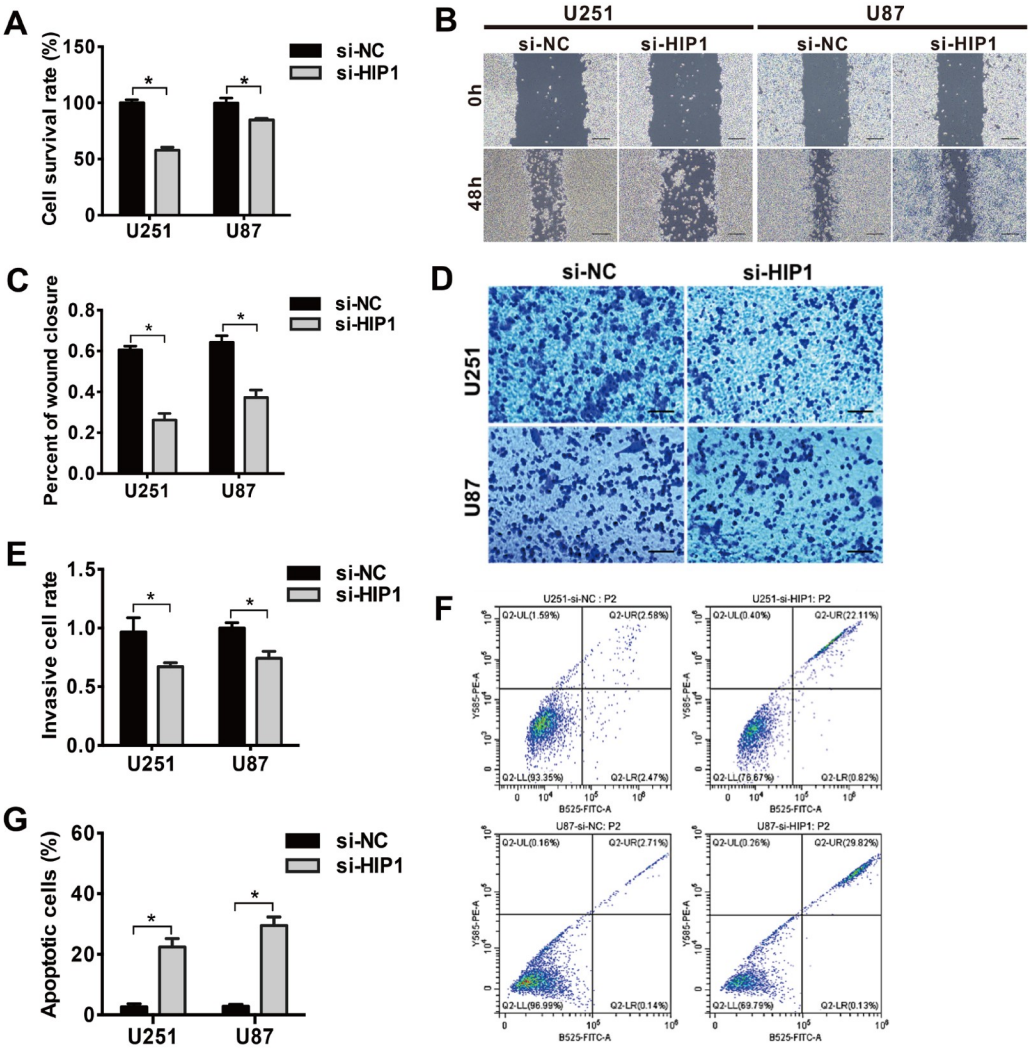

Figure 6. Downregulation of HIP1 inhibits the proliferation, migration, and invasion of glioma cells (A) CCK-8 assay of U251 and U87 cells upon *HIP1* knockdown treatment. *$P < 0.01$. (B,C) Wound-healing assay was used to detect the migration ability of U251 and U87 cells upon *HIP1* knockdown treatment. *$P < 0.01$. Scale bar: 200 μm. (D,E) Transwell assay was used to analyze the invasion ability of U251 and U87 cells upon HIP1 downregulation. *$P < 0.01$. Scale bar: 200 μm. (F,G) Apoptosis analysis of U251 and U87 cells upon *HIP1* knockdown treatment. *$P < 0.01$.

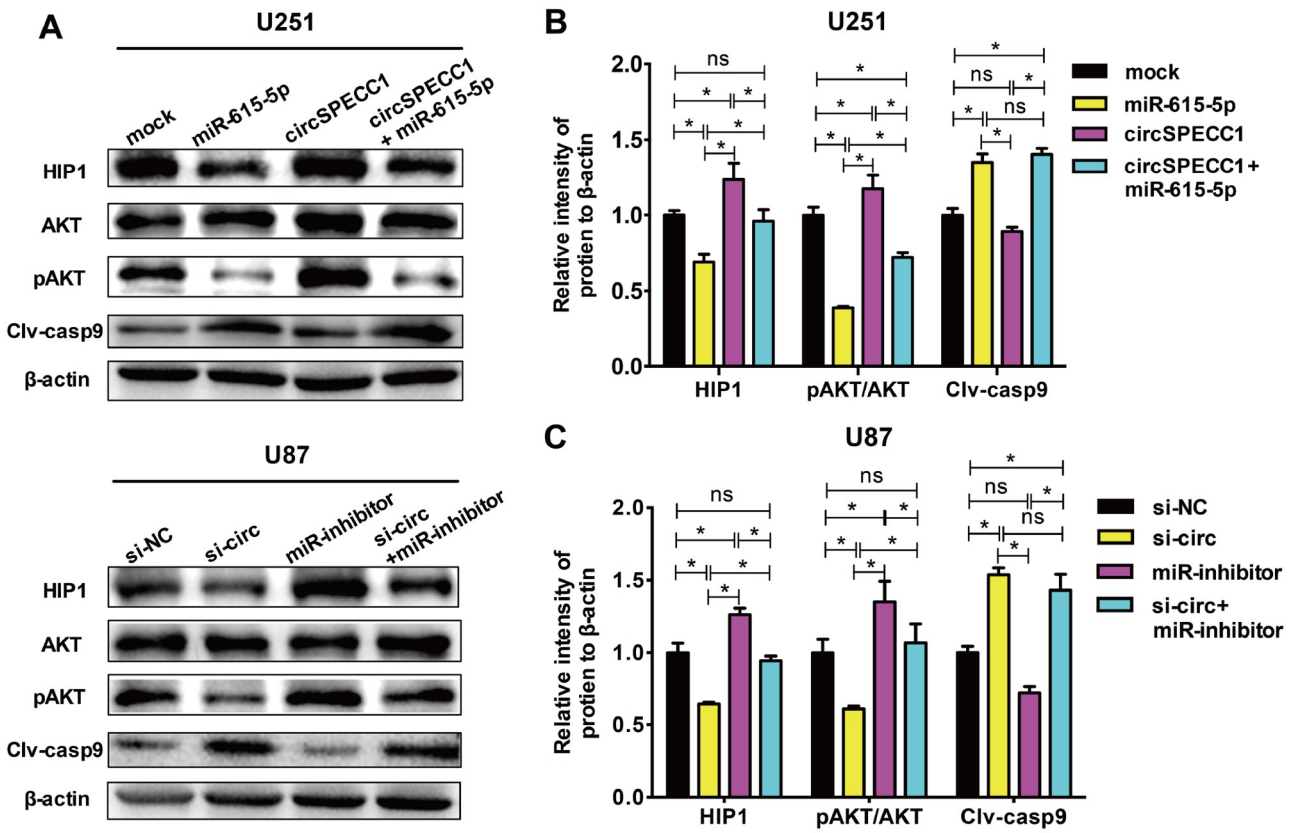

Figure 7. circSPECC1 may function through the HIP1/AKT pathway by sponging miR-615-5p (A–C) Western blot analysis of the protein expressions of HIP1, AKT, p-AKT, and cleaved caspase-9 in U251 cells upon miR-615-5p overexpression and in U87 cells upon miR-615-5p inhibitor treatment. $*P < 0.05$.

circRNAs are known to act as miRNA sponges owing to their ability to directly bind with miRNAs to regulate their functions [9]. Our study revealed that circSPECC1 plays a role through downstream miR-615-5p by targeting miR-615-5p and acting as a miR-615-5p sponge. It has been reported that miR-615-5p functions as a suppressor in various cancers, including glioma [17]. Herein, we also demonstrated that miR-615-5p is a suppressor of glioma, but its suppressive function is inhibited by circSPECC1.

HIP1 was identified as a protein interacting with the mutated gene of Huntington's disease [45]. HIP1 is a 116-kDa protein involved in clathrin-mediated endocytosis-related processes [46]. Many studies have suggested that HIP1 plays a role in tumorigenesis owing to its overexpression in several human tumors, including prostate cancer [47], breast cancer, brain tumors [48], Merkel cell carcinomas [49], and hematological malignancies [50]. HIP1 was also reported as a novel brain tumor marker associated with the epidermal growth factor receptor and an early-stage prognostic biomarker and metastatic suppressor of lung adenocarcinoma [51]. Herein, HIP1 downregulation in glioma cells significantly inhibited the viability, migration, and invasion capacity of cancer cells and promoted cell apoptosis, further indicating the oncogenic effect of HIP1 on glioma. Moreover, the effects of miR-615-5p overexpression on HIP1 expression, p-AKT, and cleaved caspase-9 were dramatically reversed by circSPECC1 via the inhibition of miR-615-5p, indicating that circSPECC1 binds to miR-615-5p to affect HIP1 expression.

Taken together, our study suggests that circSPECC1, which is modulated by YB-1, serves as a miR-615-5p sponge, thus promoting human glioma progression. This may occur through the HIP1/AKT pathway, implying that YB-1 may be involved in glioma behavior at the transcriptional and translational levels. Nevertheless, there are some limitations in our study. Since this study revealed that YB-1 could regulate circSPECC1 to play its regulatory role in downstream pathways, the possible regulatory mechanisms and related effects will be the direction of our future research. As we verified circSPECC1 and its function in cell lines, we will further add experimental contents of clinical samples and animal models in future experimental designs.

In summary, we focused on the expression of circRNAs regulated by YB-1 in human glioma. circSPECC1 was confirmed to be upregulated in glioma cells. We demonstrated that circSPECC1 upregulation promotes the malignant behavior of glioma cells by sponging miR-615-5p through the HIP1/AKT signaling pathway, suggesting its role as a promoter in glioma progression. Therefore, YB-1 and circSPECC1 could be promising targets for glioma treatment.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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