

RESEARCH ARTICLE

Circular RNA hsa_circ_0000285 regulates the microRNA-599/G-protein subunit gamma 12 (miR-599/GNG12) axis to promote glioma progression

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Abstract

Objective: Glioma is the most common, rapidly progressing, lethal brain tumor. However, underlying mechanisms behind its abnormal progression remain largely unknown. This study aimed to investigate mechanism of action and effects of the hsa_circ_0000285 on glioma progression.

Methods: RT-qPCR was utilized to study RNA expression in glioma tissues and cell lines. The effects of hsa_circ_0000285 on glioma progression were studied by measuring cell proliferation and migration, apoptosis, tumor volume and weight in both glioma cells and xenograft glioma mice. The features of hsa_circ_0000285 were identified using chromatin fractionation and RNase digestion. Its mechanism of action was analyzed using bioinformatics, RNA-binding protein immunoprecipitation, and luciferase reporter assay.

Results: We found glioma tissues and cell lines were overexpressing hsa_circ_0000285. While hsa_circ_0000285 promoted cell proliferation and migration, it inhibited apoptosis in vitro. It also increased tumor volume and weight in vivo. Using bioinformatic analysis and verification experiments for studying its mechanisms, we confirmed that hsa_circ_0000285 sponged miR-599, which negatively regulated GNG12 by binding to its mRNA.

Conclusion: Hsa_circ_0000285 is overexpressed in the glioma and promotes its progression by directly regulating the miR-599/GNG12 axis. This novel mechanism, therefore, shows that the hsa_circ_0000285/miR-599/GNG12 axis may be a promising therapeutic target for glioma treatment.

KEYWORDS

0000285, apoptosis, circ, glioma, GNG12, hsa, migration, miR-599, progression, proliferation

1 | INTRODUCTION

Glioma is the most common among intracranial tumors, accounting for 40%–50% of all cases.¹ The morbidity rate of gliomas is approximately 80%, among malignant brain tumors.² Previous reports have

found that areas with a high socioeconomic status have a higher glioma incidence.³ Risk factors for glioma are linked to genetics, race, age, and gender,^{4,5} and considering the rapid progression and lethality of glioma, it is imperative to develop an effective treatment strategy.

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Circular RNAs (circRNAs) that are divided into non-coding and coding circRNAs, are endogenous small RNAs with wide distribution, different classes, and multiple regulatory functions, mainly existing in eukaryotic cells.^{6,7} The downstream splicing donor site of circRNA is covalently linked to the upstream splicing receptor site, forming a closed circular structure without a 5' end cap and 3' end polyadenylation tail, and no terminal structure.⁸ After biogenesis in the nucleus, most exonic circRNAs are transported to the cytoplasm to perform their regulatory functions. A common way of regulation happens when circRNAs acting as mRNA sponges, are degraded in an argonaute 2 (Ago2)-dependent manner, resulting in free mRNAs that translate into proteins affecting physiological and pathological processes. circRNAs are linked to a variety of diseases, especially cancer, and can act as both oncogenes and tumor suppressors, depending on the tumor type. Additionally, circRNAs being stable and resistant to exonuclease digestion, make them potential diagnostic and prognostic biomarkers for tumors.^{9,10} While upregulated circ_PVT1 and circ_0075930 are a diagnostic biomarker of osteosarcoma and a prognostic biomarker of lung cancer, respectively, downregulated circ_PLEKHM3 serves both as a diagnostic and prognostic biomarker of ovarian cancer.¹¹⁻¹³ In glioma, many abnormally expressed circRNAs play an important role in tumor progression in several ways, like sponging with miRNA, encoding proteins, interacting with RNA-binding proteins, and regulating blood vessel formation.^{14,15} Although hsa_circ_0000285 is reportedly involved in several tumors, its role in glioma remains unclear.

GNG12 is a G-protein subunit involved in multiple signaling pathways, including the PI3K/Akt pathway. Recent studies have found that overexpression of GNG12 can be a potential biomarker of different cancers like glioma, endometrial cancer, pancreatic ductal adenocarcinoma, and colorectal cancers.¹⁶⁻²⁰ However, the effect of GNG12 on tumor development and progression is unreported in vitro, and its upstream regulator is still unknown.

Therefore, in this study, we combined bioinformatics approaches based on glioma tissues along with confirmatory experiments in both glioma cell lines and xenograft glioma mice to investigate effects and mechanisms of action of hsa_circ_0000285 on glioma progression.

2 | METHODS

2.1 | Glioma tissues

Glioma and adjacent non-tumorous tissues were obtained with informed consent from 18 patients at Taikang Tongji Hospital (Wuhan) from 2018 to 2019. Histopathological examination confirmed the diagnosis of glioma after surgery. The inclusion criteria were: (1) the diagnosis matches with the diagnostic criteria of glioma; (2) newly diagnosed glioma; (3) patients who received surgical treatment but did not receive any anti-tumor treatment before surgery; (4) the expected patient survival time was over six months; and (5) patients who had complete case data. Exclusion criteria were: (1) patients with malignant tumors in other body parts; (2) patients with other intracranial diseases; (3) patients with heart, liver, kidney, and other

important organ dysfunction; and (4) pregnant or lactating women. Finally, 9 men and 9 women, aged 26-77 (55.62 ± 8.23) years were selected. All the specimens obtained from patients were stored in liquid nitrogen. The study was approved by the relevant ethics committee and conducted in accordance with the Declaration of Helsinki.

2.2 | Cell lines

Human glioma cell lines SHG-44, U251, and A172 were obtained from the National Collection of Authenticated Cell Cultures (Shanghai, China), and the normal human astrocyte (NHA) cell line was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). These cell lines were cultured in complete medium (Invitrogen, Carlsbad, CA, USA) comprising 90% Dulbecco's Modified Eagle Medium and 10% fetal bovine serum, and maintained at 37°C in an atmosphere of 5% CO₂ and air.

2.3 | Cell transfection

siRNAs of hsa_circ_0000285 (si-circ), siRNAs of GNG12 (si-GNG12), miR-599 agomir (represented as miR-599 mimic), miR-599 antagomir (represented as miR-599 inhibitor), and negative controls (NCs) were generated by GenePharma (Shanghai, China). Transfections (50 nM NC, miR-599 agomir or miR-599 antagomir, and 50 ng siRNA-NC or siRNA-hsa_circ_0000285) were performed using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4 | CCK-8 viability assay

Cell proliferation was detected using the Cell Counting Kit-8 (CCK-8 kit; Beyotime, Shanghai, China). Cells were plated in 96-well plates and incubated for 24 h, 48 h, 72 h, and 96 h. Then, 10 μ L CCK-8 was added to each well and incubated for 2 h at 37°C. The absorbance was detected at 450 nm using a microplate reader.

2.5 | Chromatin fractionation

Nucleoplasm and cytoplasmic fractionation were conducted using the PARIS™ kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The separated RNA was reverse-transcribed and amplified by PCR. The endogenous controls for the nucleus and cytoplasm were U6 and GAPDH, respectively.

2.6 | RNase digestion

Total RNA extraction was digested with RNase R (Epicentre Technologies, USA). Two micrograms of total RNA was digested with

0.2 μ L RNase R (20 U/ μ L) in diethyl pyrocarbonate-treated water at 37°C for 30 mins, and hsa_circ_0000285 and GAPDH mRNA were detected.

2.7 | RNA-binding protein immunoprecipitation (RIP) assay

EZ-Magna RIP™ RNA Binding Protein Immunoprecipitation Kit (Millipore,

Billerica, MA, USA) was used to pull down proteins (Ago2 or IgG) from RNAs using magnetic beads. The expression of hsa_circ_0000285 and miR-599 was measured according to the manufacturer's instructions.

2.8 | Wound healing assays

SHG-44 and U251 cell lines were transfected with siRNA-NC/siRNA-hsa_circ_0000285/siRNA-GNG12, with or without inhibitor, depending on the experimental design, and then plated on 6-well plates (6×10^5 cells/well). When ~80% cell confluence was observed, wounds of the same size were scraped on monolayer cells using a micropipette tip. Cells were then rinsed with phosphate-buffered saline to remove cell debris and subsequently cultured in fresh medium. The distance between gaps was recorded and analyzed after 24 h.

2.9 | Xenograft glioma mice models

All animal experimental protocols were approved by the Ethical Committee of the Taikang Tongji Hospital (Wuhan) and conformed to the National Institutes of Health Laboratory Animal Care and Use Guidelines. Male athymic BALB/c nude mice (aged 4–5 weeks) were obtained. Mice were housed in a sterile pathogen-free animal room, which was maintained at a temperature of ~22°C and 12-h light/12-h dark cycle, and they were provided ad libitum with sterile food and water. The pLVX-IRES-Puro vector was used to construct plasmids of shRNA-hsa_circ_0000285 or shRNA-NC (GenePharma, Shanghai, China). After transformation and amplification in *Escherichia coli* DH5 α -competent bacteria, pLVX-IRES-Puro-shRNA-hsa_circ_0000285/shRNA-NC were extracted from the bacteria. 3 μ g of pLVX-IRES-Puro-shRNA-hsa_circ_0000285/shRNA-NC and 1.5 μ g helper packaging vectors (pLP/VSVG, pLP1, pLP2; Invitrogen, Carlsbad, CA, USA) were transfected into 293T cells (5×10^6 cells/well) and incubated at 37°C for 48 h. Then, the 293T lentiviral supernatant was harvested and used to transfect SHG-44 cells (5×10^6 cells/well) and incubated at 37°C for 48 h. Transfected SHG-44 cells were selected using puromycin. Total RNA was extracted from transfected SHG-44 cells and RT-qPCR was used to verify the efficiency of knockdown. Subsequently, SHG-44 cells containing shRNA-hsa_circ_0000285 or shRNA-NC were inoculated subcutaneously into

the right flank of nude mice. Tumor volume and weight were measured weekly for five weeks.

2.10 | Bioinformatics analysis

With adj. $p < 0.05$, log FC > 2 , mRNA microarray GSE104291 and mRNA expression database Gene Expression Profiling Interactive Analysis (GEPIA) were used to screen for upregulated genes in glioma samples. Metascape was used to analyze key processes, and the WebGestalt (WEB-based GENE Set Analysis Toolkit) was used to examine the signaling pathways involved. We used starBase to predict the miRNAs sponged by hsa_circ_0000285 and TargetScan to predict the miRNAs targeting GNG12.

2.11 | Western blotting

Protein extraction of different groups of cells was performed using a lysis kit (Beyotime, Shanghai, China), and lysed cells were centrifuged at 12,000 \times g for 10 mins. After determination of concentration using a BCA kit (Beyotime, Shanghai, China), proteins were separated by 10% SDS-PAGE. Separated proteins on the gel were subsequently transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). After incubation in blocking buffer for 1 h, target proteins on membranes were mixed with primary antibodies and incubated overnight at 4°C. The primary antibodies included anti-Bax (1:2000, Abcam, Cat# ab32503), anti-Bcl-2 (1:2000, Abcam, Cat# ab32124), anti-GNG12 (1:2000, Abcam, Cat# ab154698), and anti-GAPDH (1:5000, Abcam, Cat# ab8245) antibodies. After incubation with secondary antibodies, an enhanced ECL kit (Millipore, Billerica, MA, USA) was used to visualize protein bands.

2.12 | RT-qPCR

For total RNA extraction from human glioma tissues and SHG-44, U251, A172 and NHA cells, collected tissues and cells were lysed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A Reverse Transcription Kit (Takara, Otsu, Japan) and specific primers (Sangon Biotech, Shanghai, China) were used for reverse transcription of the separated total RNA. The individual primers sequences are mentioned in Table 1.

2.13 | Luciferase Reporter Assay

The target genes of miR-599 were predicted using starBase and TargetScan. The wild-type (wt) and mutated target genes (mut) were inserted into the pMIR-Reporter luciferase reporter plasmid using the GeneArt™ platform (Invitrogen, Carlsbad, CA, USA) to obtain Luc-hsa_circ_0000285-WT/GNG12-3'UTR-WT and Luc-hsa_circ_0000285 Mut/GNG12-3'UTR-Mut1/2/3. SHG-44 and

TABLE 1 Primers for RT-qPCR

Gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
Has_circ_0000285	GCTCAGTTTGGTTGTGGTGA	TCACATGAATTTAGGTGGGACTT
miR – 599	GTTGTGTCAGTTTA	CAGTGCCTGTCGTGGAGT
miR – 2467-3p	GCCGAGGGACAGGCACCTGA	CTCAACTGGTGTCTGTGA
GNG12	GCAAAACAGCAAGCACCAAC	CTATCAGCAAAGGGTCACTCC
GAPDH	CTGGGCTACTGAGCACC	AAGTGGTCGTTGAGGGCAATG
U6	CTCGCTTCGGCAGCACATATACT	ACGCTTACGAATTTGCGTGTGTC

Note: miR-599, microRNA-599; miR-2467-3p, microRNA-2467-3p; GNG12, G-protein subunit gamma 12; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; U6, U6 small nuclear RNA.

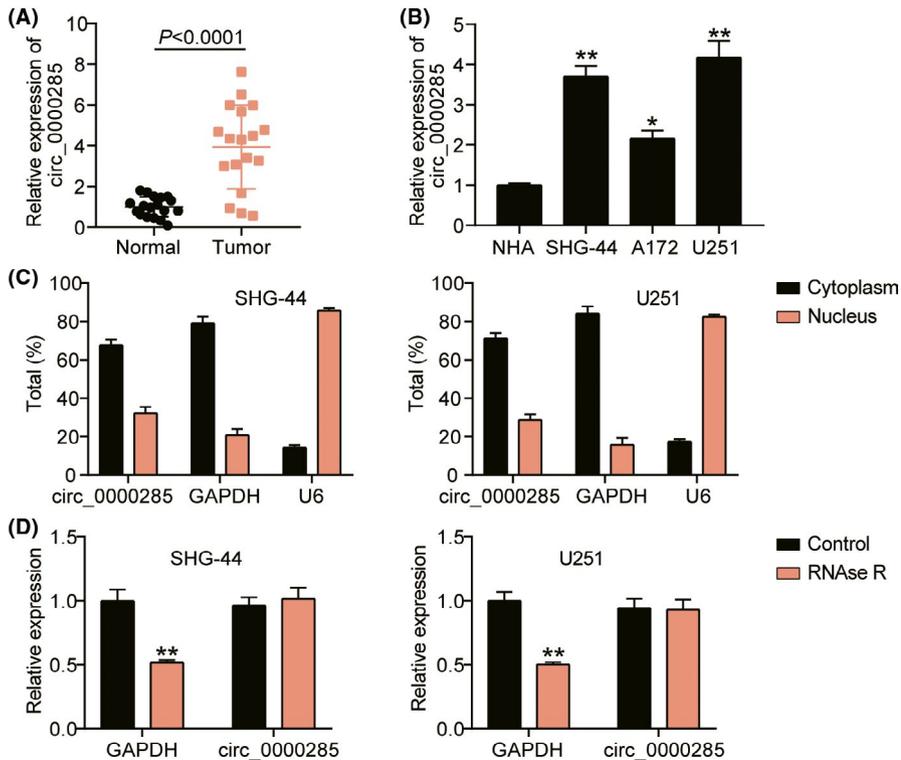


FIGURE 1 The characteristics of hsa_circ_0000285 in glioma by detection with RT-qPCR. (A) The expression of hsa_circ_0000285 in the glioma tissues (n = 18) and the matched adjacent normal tissues (n = 18) from patients. (B) The expression of hsa_circ_0000285 in normal human astrocytes (NHA) cells and glioma cell lines (SHG-44, A172, U251). * $p < 0.05$, ** $p < 0.001$, compared with the NHA group. (C) The subcellular enrichment of hsa_circ_0000285, GAPDH, and U6 in SHG-44 and U251. (D) The expression of hsa_circ_0000285 and GAPDH digested by RNase R in SHG-44 and U251. ** $p < 0.001$, compared with the control group

U251 cells were seeded into 6-well plates and transfected with plasmids containing wild-type (Luc-hsa_circ_0000285-WT/GNG12-3'UTR-WT) or mutation (Luc-hsa_circ_0000285-Mut/GNG12-3'UTR-Mut1/2/3) and miR-599 mimics using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 48 h, a dual-luciferase reporter gene assay (Promega, Madison, WI, USA) was performed to measure luciferase activity.

2.14 | Statistical Analyses

SPSS 22.0 was used for statistical analysis. All data are presented as mean \pm standard deviation. Two-tailed Student's t-test and one-way ANOVA were used for comparisons between two groups and multiple, respectively. Statistical significance was set at $p < 0.05$. Correlation analysis was performed using the Pearson's correlation test.

3 | RESULTS

3.1 | The expression, distribution and circular characteristics of hsa_circ_0000285 in glioma

To assess whether hsa_circ_0000285 is a discriminative gene of glioma, we first measured the expression level of hsa_circ_0000285 in glioma tissues and matched adjacent normal tissues. As shown in Figure 1A, hsa_circ_0000285 was significantly overexpressed in glioma tissues as compared to normal tissues. Meanwhile, compared with NHA, hsa_circ_0000285 was also highly expressed in glioma cell lines, including SHG-44, A172, and U251 (Figure 1B). Subsequently, chromatin fractionation and RNase digestion were used to characterize hsa_circ_0000285 in SHG-44 and U251 cells. As shown in Figure 1C, when compared with controls for cytoplasmic and nuclear localization (GAPDH and U6), hsa_circ_0000285 was mostly localized in the cytoplasm. Additionally, hsa_circ_0000285

was also left undigested by RNase R (Figure 1D). Therefore, these results indicate that hsa_circ_0000285 not only had circular characteristics, but it may also serve as a post-transcriptional regulator through cytosolic enrichment.

3.2 | Hsa_circ_0000285 promoted cell proliferation, migration, inhibited apoptosis in vitro, and also contributed to tumor progression in vivo

To investigate the effect of hsa_circ_0000285 overexpression on tumor progression, we used siRNA to knock down hsa_circ_0000285 in SHG-44 and U251 cells. The results in Figure 2A show that siRNA-hsa_circ_0000285 significantly reduced the level of hsa_circ_0000285. Compared with siRNA-NC, siRNA-hsa_circ_0000285 significantly inhibited cell proliferation (Figure 2B). siRNA-hsa_circ_0000285 reduced the distance of migration 24 h post scraping (Figure 2C). In addition, siRNA-hsa_circ_0000285 elevated Bax/Bcl-2 levels to promote apoptosis in glioma cell lines (Figure 2D). For further verification, we used SHG-44 carrying shRNA-hsa_circ_0000285 to generate xenograft gliomas in nude mice. As shown in Figure 2E, tumor volume and weight of the xenograft glioma containing the shRNA-hsa_circ_0000285 decreased significantly as compared to shRNA-NC. Therefore, these results demonstrate that hsa_circ_0000285 promotes glioma progression.

3.3 | miR-599/GNG12 axis might lie downstream of the hsa_circ_0000285 in glioma

Using the mRNA microarray GSE104291 and mRNA expression database GEPIA, we screened 297 common upregulated genes in glioma samples (Figure 3A). Using Metascape for biological process enrichment of 297 genes, we showed that 20 key genes out of 297 were important for pathways in cancer (Figure 3B). On uploading these 20 key genes to the WebGestalt (WEB-based GENE Set Analysis Toolkit), the PI3K-Akt signaling pathway, including CDK2, COL4A2, F2R, FN1, GNG12, ITGB1, LAMC1, LPAR6, MYC, and VEGFA attracted our attention (Figure 3C). Since multiple genes, like CDK2, COL4A2, F2R, FN1, ITGB1, LAMC1, MYC, and VEGFA were previously investigated in glioma,²¹⁻²⁸ we selected our genes of interest from GNG12 and LPAR6. In our clinical samples, as GNG12 expression was significantly upregulated in glioma tissues as compared with LPAR6; we, therefore, selected GNG12 as our gene of interest (Figure 3D). We then used starBase to predict miRNAs sponged by hsa_circ_0000285 and TargetScan to predict the miRNAs targeting GNG12, and found that miR-599 and miR-2467-3p overlapped (Figure 3E). In our clinical samples, since miR-599 expression was significantly downregulated in glioma tissues as compared to miR-2467-3p; we then selected it as our miRNA of interest (Figure 3F).

3.4 | Hsa_circ_0000285 acted as a miR-599 sponge

To verify the bioinformatics analysis results of the relationship between hsa_circ_0000285 and miR-599, we conducted experiments on glioma cells and tissues. The putative complementary binding sites are shown in Figure 4A. Based on the complementary sequence, pMIR-wild-type-hsa_circ_0000285 (circ-wt) or pMIR-mutant type-hsa_circ_0000285 (circ-mut) plasmids were constructed and co-transfected with miR-599 mimics or mimic-NC in SHG-44 or U251 for luciferase reporter assay. The results in Figure 4B demonstrated that hsa_circ_0000285 directly bond to miR-599. We observed greater enrichment of hsa_circ_0000285 and miR-599 after pulling down Ago2, in the Ago2 immunoprecipitation assay (Figure 4C). These results thus confirm that hsa_circ_0000285 is a sponge of miR-599. In contrast to the high expression of hsa_circ_0000285, the expression of miR-599 was lower in glioma cells than in NHA cells (Figure 4D). In addition, correlation analysis in tumor tissues verified the relationship of negative regulation between hsa_circ_0000285 and miR-599 (Figure 4E).

3.5 | Hsa_circ_0000285 promoted glioma cells progression via miR-599

To further confirm how the hsa_circ_0000285 sponging of miR-599 regulates tumor progression, we applied a model related to miR-599 inhibitor and si-hsa_circ_0000285 in SHG-44 and U251 cells. The results in Figure 5A revealed that miR-599 inhibitor significantly reduced the expression of miR-599 as compared with inhibitor-NC; hsa_circ_0000285 knockdown significantly increased levels of miR-599 as compared to siRNA-NC; and miR-599 inhibitor reversed the siRNA-hsa_circ_0000285 induced elevation. Since these indicated that hsa_circ_0000285 and miR-599 were regulatorily related, we investigated the effect of this regulatory relationship on cell proliferation, migration, and apoptosis. As shown in Figure 5B, siRNA-hsa_circ_0000285 significantly inhibited the cell viability as compared to the siRNA-NC treated group, and miR-599 inhibitor significantly promoted cell viability as compared to inhibitor-NC, and miR-599 inhibitor + si-hsa_circ_0000285 restored the silencing of hsa_circ_0000285-induced inhibition of cell viability. As shown in Figure 5C, while siRNA-hsa_circ_0000285 inhibited the migration as compared to the siRNA-NC treated group, miR-599 inhibitor instead stimulated migration. Thus, we saw cells co-transfected with miR-599 inhibitor migrating farther than the siRNA-hsa_circ_0000285 group, thereby indicating that miR-599 inhibitor reversed the siRNA-hsa_circ_0000285-induced inhibition of migration. Similarly, in Figure 5D, we see that siRNA-hsa_circ_0000285 increased the Bax expression and decreased the Bcl-2 expression; while miR-599 inhibitor decreased the expression of Bax /Bcl-2, and miR-599 inhibitor reversed the effect of si-hsa_circ_0000285 induced apoptosis. Therefore, all these results confirm that hsa_circ_0000285 not only regulates the expression of miR-599, but also regulated glioma progression, including cell proliferation, migration, and apoptosis, via negative regulation of miR-599.

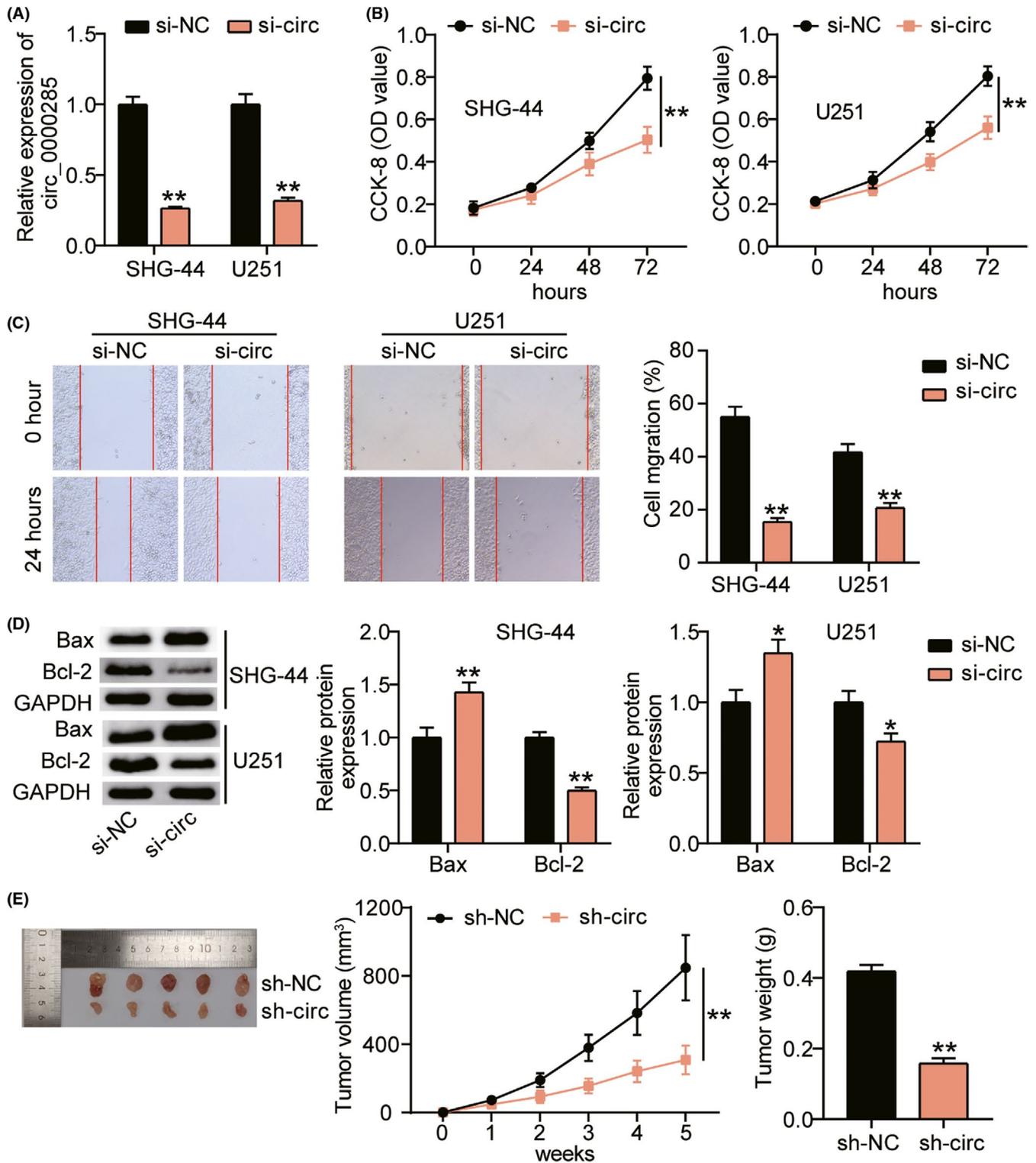


FIGURE 2 Hsa_circ_0000285 promoted cell proliferation, migration and inhibited apoptosis in vitro, and hsa_circ_0000285 contributed to tumor progression in vivo. (A) The expression of hsa_circ_0000285 in glioma cells transfected with siRNA-hsa_circ_0000285 or siRNA-NC as control. (B) The proliferation of glioma cells (SHG-44 and U251) transfected with siRNA-hsa_circ_0000285 or siRNA-NC at 24h, 48h, 72h by measuring with CCK-8 assay. (C) The migration of cells (SHG-44 and U251) transfected with siRNA-hsa_circ_0000285 or siRNA-NC at 0h and 24h in wound healing assay. (D) The expression of Bcl-2 and Bax in glioma cells (SHG-44 and U251) transfected with siRNA-hsa_circ_0000285 or siRNA-NC by detection with western blotting. (E) The tumor volume and weights of xenograft nude mice, which were inoculated with shRNA-hsa_circ_0000285 or shRNA-NC transfected SHG-44 cells for 5 weeks. ** $p < 0.001$, * $p < 0.05$, compared with si-NC group

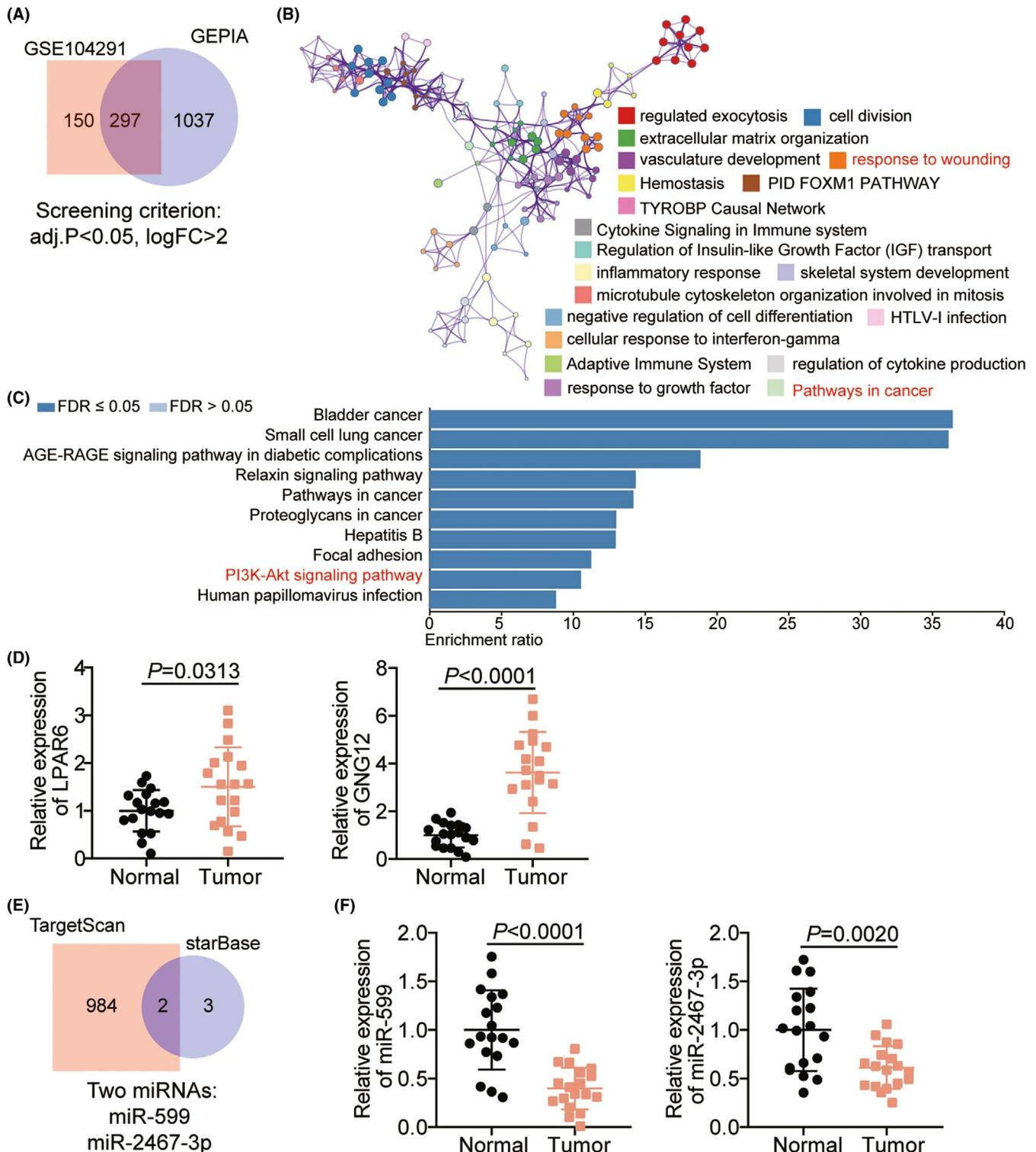


FIGURE 3 miR-599/GNG12 axis might be sponged by hsa_circ_0000285 in glioma (A) 297 upregulated genes in glioma samples were screened from GSE104291 (an mRNA microarray) and GEPIA (a mRNA expression database) with adj. $p < 0.05$ and log FC > 2. (B) The key biological processes for 297 upregulated genes were analyzed by Metascape. (C) WEB-based Gene SeT AnaLysis Toolkit was used to analyze the 20 genes of the Pathways in cancer. (D) The expression of GNG12 and LPAR6 in our clinical samples (normal: $n = 18$; tumor: $n = 18$) were detected by RT-qPCR. (E) miR-599 and miR-2467-3p was overlapped from TargetScan and starBase. TargetScan was used to predict the miRNAs targeting GNG12, and starBase was used to predict the miRNAs sponged by hsa_circ_0000285. (F) The expression of miR-599 and miR-2467-3p in our clinical samples (normal: $n = 18$; tumor: $n = 18$) was detected by RT-qPCR

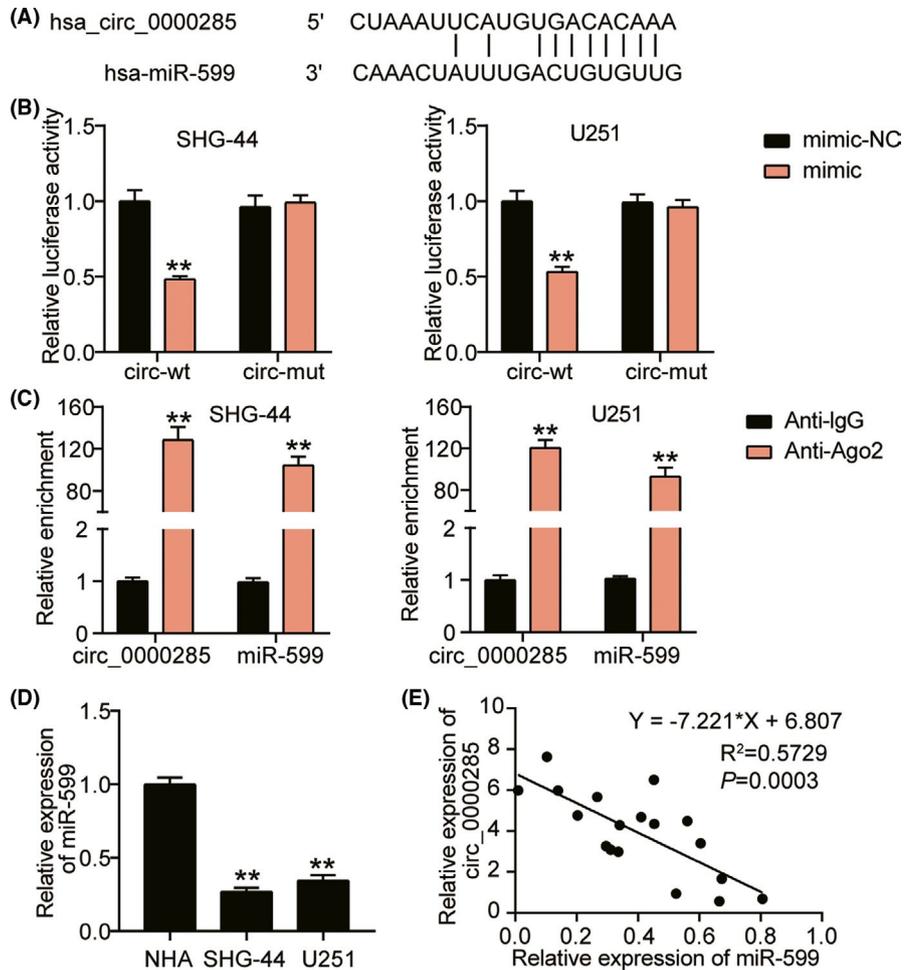


FIGURE 4 Direct interaction and negative regulation between hsa_circ_0000285 and miR-599. (A) Complementary binding sites within hsa_circ_0000285 and miR-599 were predicted using online bioinformatic prediction of starBase. (B) Luciferase reporter assay in glioma cells (SHG-44 and U251) co-transfected with miR-599 mimics NC or miR-599 mimics and pMIR wild-type hsa_circ_0000285 (circ-Wt) or pMIR-mutant type hsa_circ_0000285 (circ-mut) plasmids. $**p < 0.001$, compared with mimic-NC. (C) The dissociated hsa_circ_0000285 and miR-599 from Ago 2 after Ago 2 immunoprecipitation. $**p < 0.001$, compared with Anti-IgG. (D) The expression of miR-599 in NHA, SHG-44 and U251 cells. $**p < 0.001$, compared with NHA. (E) The correlation analysis between hsa_circ_0000285 and miR-599 in tumor tissues

3.6 | miR-599 directly negatively regulates GNG12

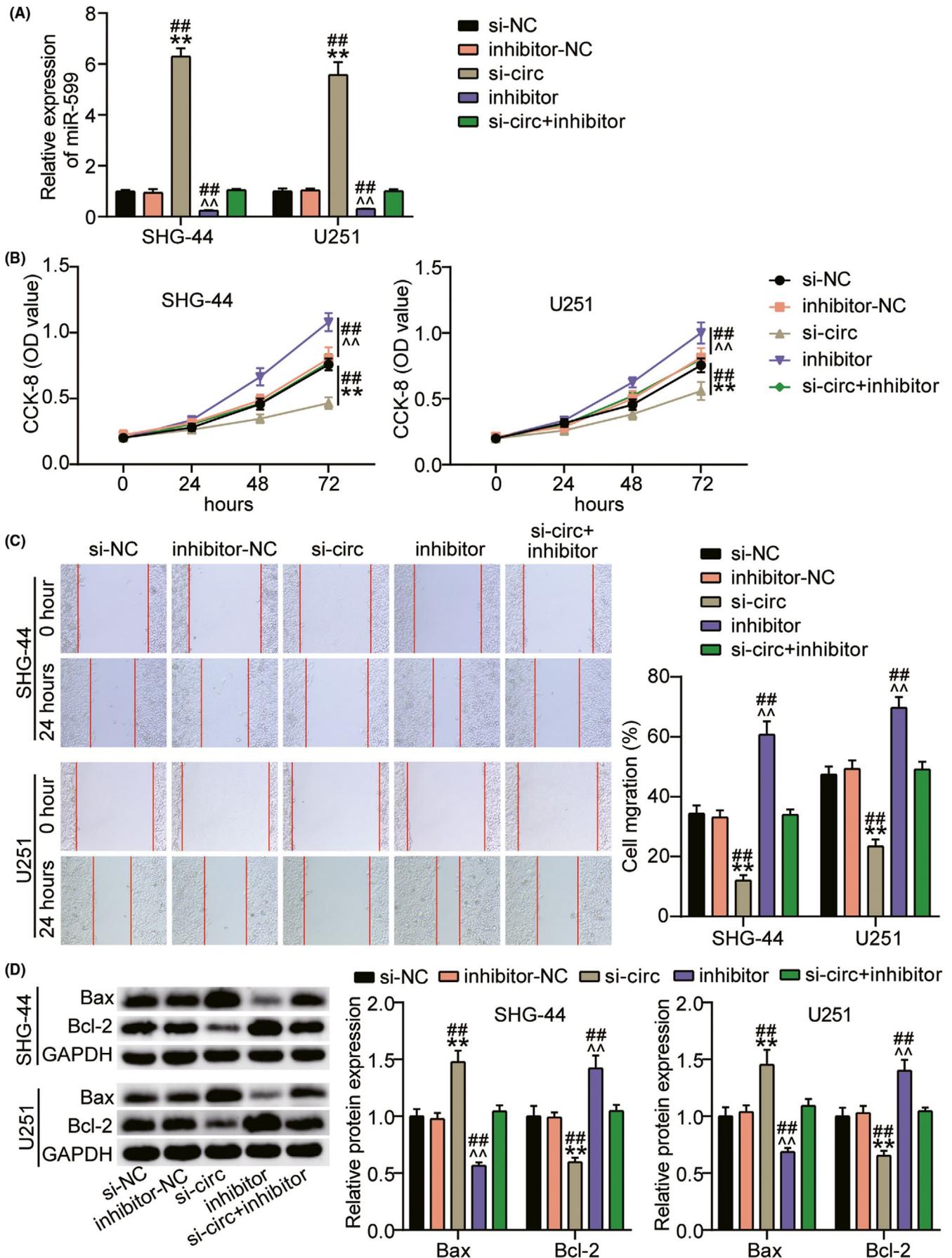
To identify the binding and regulatory relationship between *GNG12* and miR-599, we predicted and obtained three binding sites of miR-599 in the 3'UTR of *GNG12* using TargetScan (Figure 6A) and performed a luciferase activity assay. We transfected the SHG-44 and U251 cells with the luciferase reporters constructed with three types of mutations in the binding sites of 3'UTR of *GNG12* and miR-599 mimics. In the luciferase activity assay, if a miRNA interacted with the targeting 3'UTR, it would inhibit the luciferase. As shown in Figure 6B, wt + miR-599 mimics had reduced luciferase activity as compared to WT + mimic-NC. The mut1, mut2, mut3 elevated the luciferase activity to different degrees as compared to the WT + miR-599 mimics, and Co-mut showed the strongest reversal when compared with the individual mutation-treated group.

This result demonstrates the direct binding between miR-599 and the 3'UTR of *GNG12*. We then explored *GNG12* expression in cell lines. Compared with NHA, we observed high *GNG12* expression in the SHG-44 and U251 cells (Figure 6C), which was in stark contrast to the low miR-599 expression (Figure 4D). As expected, a correlation analysis revealed a negative correlation between miR-599 and *GNG12* in tumor tissues (Figure 6D). These results thus revealed that miR-599 directly binds *GNG12* mRNA and negatively regulates it.

3.7 | The effects of *GNG12* on glioma cells progression were regulated by miR-599

To verify the relationship of negative regulation between miR-599 and *GNG12*, we determined *GNG12* protein levels in cells transfected

FIGURE 5 Hsa_circ_0000285 promoted glioma cells progression via miR-599. (A) The expression of miR-599 in SHG-44 or U251 transfected with siRNA-NC, siRNA-hsa_circ_0000285, miR-599 inhibitor-NC, miR-599 inhibitor, or siRNA-hsa_circ_0000285 + miR-599 inhibitor, respectively. (B) The viability of SHG-44 or U251 transfected with siRNA-NC, siRNA-hsa_circ_0000285, miR-599 inhibitor-NC, miR-599 inhibitor, or siRNA-hsa_circ_0000285 + miR-599 inhibitor, respectively, at 0, 24, 48, 72 h. (C) In wounding healing assay, the migration of SHG-44 or U251 transfected with siRNA-NC, siRNA-hsa_circ_0000285, miR-599 inhibitor-NC, miR-599 inhibitor, or siRNA-hsa_circ_0000285 + miR-599 inhibitor, respectively, at 0, 24 h. (D) The expression of transfused Bcl-2/Bax in SHG-44 or U251 transfected with siRNA-NC, siRNA-hsa_circ_0000285, miR-599 inhibitor-NC, miR-599 inhibitor, or siRNA-hsa_circ_0000285 + miR-599 inhibitor, respectively. $**p < 0.001$, compared with si-NC; $^{\wedge}p < 0.001$, compared with inhibitor-NC; $^{\#\#}p < 0.001$, compared with si-circ + inhibitor



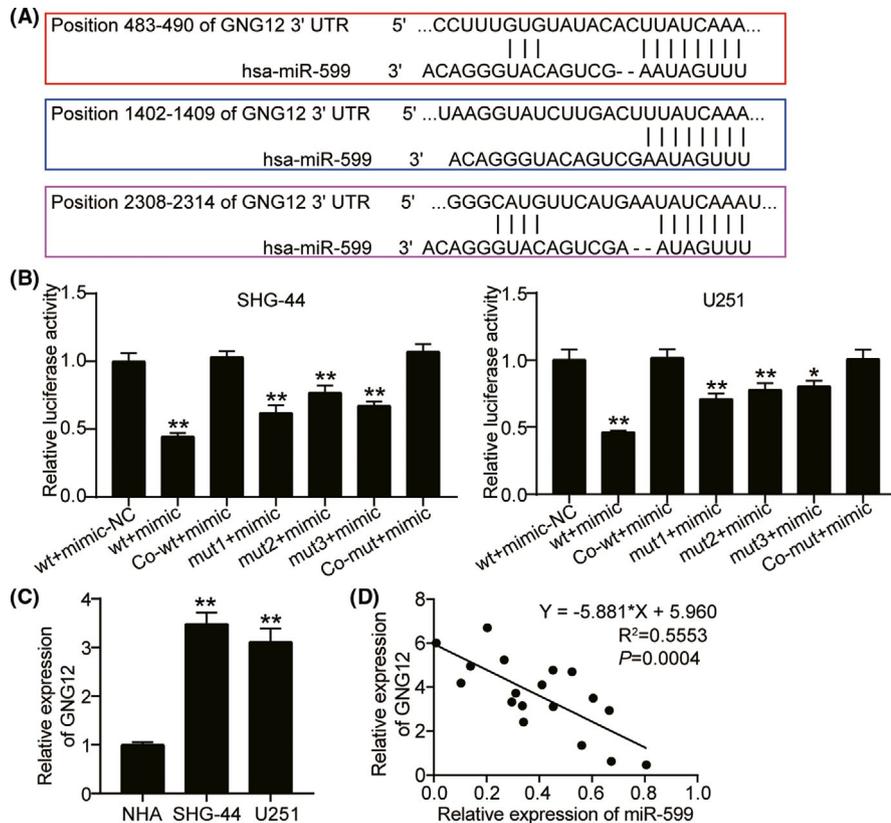


FIGURE 6 Relationship between miR-599 and GNG12. (A) The 3 binding sites between miR-599 and 3' UTR of GNG12 predicted by TargetScan and the luciferase reporters were constructed with the wild-type or mutant GNG12 3'UTR. (B) The luciferase plasmids of WT-GNG12 3'UTR or mut- GNG12 3'UTR (mut 1, mut 2, mut 3 or three mutants together presented as co-mut) was co-transfected with miR-599 mimics in SHG-44 and U251, and NC was the negative controls of miR-599 mimics. * $p < 0.05$, ** $p < 0.001$, compared with wt + mimic-NC. (C) The expression level of GNG12 in NHA, SHG-44 and U251 cells. ** $p < 0.001$, compared with NHA. (D) The correlation analysis between miR-599 and GNG12 in patients' tumor tissues

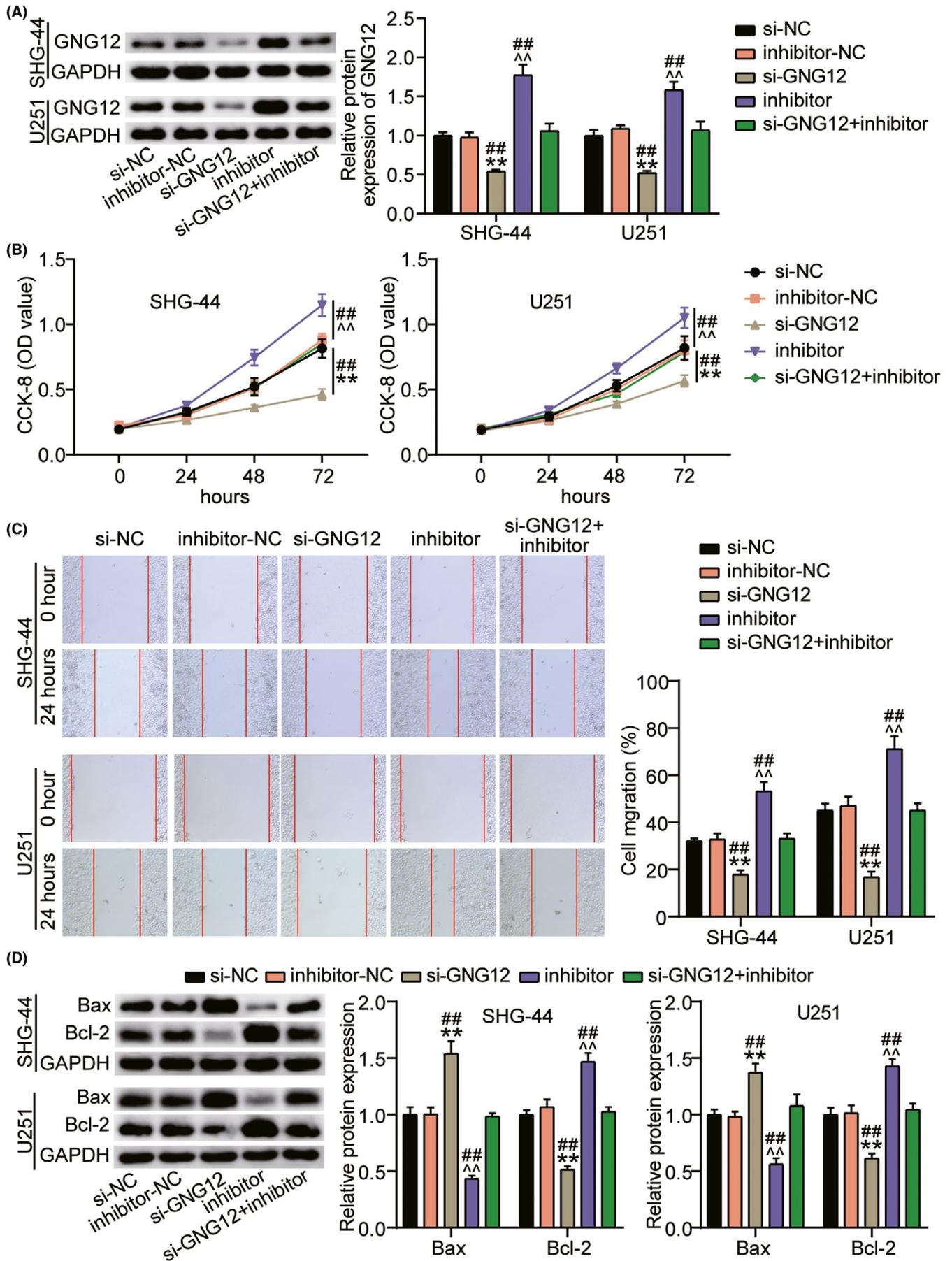
with the miR-599 inhibitor, siRNA-GNG12, and the corresponding control. The results in Figure 7A show that siRNA-GNG12 silenced about half of the GNG12 protein expression as compared to siRNA-NC. However, miR-599 inhibitor enhanced GNG12 protein expression approximately two-fold as compared to inhibitor-NC. The miR-599 inhibitor rescued the reduction in GNG12 expression, induced by siRNA-GNG12 to the level of control. This result thereby confirms that miR-599 negatively regulates the GNG12 protein expression. In addition, while siRNA-GNG12 reduced the proliferation, miR-599 inhibitor instead increased cell viability and rescued the siRNA-GNG12-induced reduction of cell proliferation (Figure 7B). Similarly, the miR-599 inhibitor also reversed the siRNA-GNG12-induced reduction of migration (Figure 7C). As shown in Figure 7D, miR-599 inhibitor also reversed the siRNA-GNG12-induced increase in Bax/Bcl-2 expression. These above results thus not only indicate that GNG12 contributes to glioma cell proliferation, migration, and inhibition of apoptosis, but also show that miR-599 negatively regulates all these biological processes.

4 | DISCUSSION

Through the course of this study, we observed for the first time that hsa_circ_0000285 (a) has the characteristics of circular RNA, (b) is overexpressed in glioma cell lines and tissues, (c) induces glioma cell proliferation and migration, and (d) inhibits apoptosis by releasing GNG12 via sponging miR-599. In brief, we identified a potential competing endogenous RNA (ceRNA) network in gliomas.

Although the effect of hsa_circ_0000285 in glioma is not reported yet, the literature survey shows its regulatory effect on several other cancers. In osteosarcoma, overexpression of hsa_circ_0000285 promotes the proliferation, migration, and invasion of osteosarcoma.²⁹ Upregulation of hsa_circ_0000285 is associated with cervical cancer development.³⁰ Our results were consistent with those from these studies, thus showing that hsa_circ_0000285 knockdown not only suppressed glioma cell proliferation and migration, but also promoted apoptosis. Therefore, it indicates that hsa_circ_0000285 plays a role as an oncogenic factor in gliomas.

FIGURE 7 Effects of GNG12 on glioma cells progression were regulated by miR-599. (A) By western blotting, the protein expression of GNG12 of SHG-44 or U251 cells, which were transfected with siRNA-GNG12, inhibitor of miR-599, siRNA-GNG12 + inhibitor of miR-599 and the corresponding control si-NC and inhibitor-NC. (B) The viability of SHG-44 or U251 transfected with siRNA-GNG12, inhibitor of miR-599, siRNA-GNG12 + inhibitor of miR-599 and the corresponding control si-NC and inhibitor-NC at 0, 24, 48,72 h. (C) In wounding healing assay, the migration of SHG-44 or U251 transfected with siRNA-GNG12, inhibitor of miR-599, siRNA-GNG12 + inhibitor of miR-599 and the corresponding control si-NC and inhibitor-NC at 0, 24 h. (D) The expression of Bcl-2/Bax in SHG-44 or U251 transfected with siRNA-GNG12, inhibitor of miR-599, siRNA-GNG12 + inhibitor of miR-599 and the corresponding control si-NC and inhibitor-NC. ** $p < 0.001$, compared with si-NC; ^ $p < 0.001$, compared with inhibitor-NC; # $p < 0.001$, compared with si-GNG12 + inhibitor



Additionally, we observed that hsa_circ_0000285 mostly localized in the cytoplasm of glioma cell lines, which was similar to finding from previous reports.^{29,30} In addition, studies have shown that hsa_circ_0000285 acts as a miRNA sponge or a ceRNA, by interacting with miR-409-3p and miR197-3p, respectively.^{29,30} Accordingly, we screened miR-599 to investigate the mechanism of action of hsa_circ_0000285 in glioma. MicroRNA is a small non-coding RNA that regulates gene expression by inhibiting translation or promoting mRNA degradation.³¹ Deregulation of miRNA expression is related with various cancers, which either involve tumor suppressor genes or oncogenes.³² A previous study has indicated that miR-599 is downregulated in glioma tissues and can be a prognostic biomarker.³³ This is further confirmed by another study, which has found that miR-599 inhibits the proliferation and invasion of glioma cells both in vitro and in vivo.³⁴ All these were in agreement with our results. First, we obtained predicted binding targets of miR-599 and hsa_circ_0000285 through bioinformatics analysis. Further verification experiments verified that hsa_circ_0000285 serves as a ceRNA and directly binds to miR-599 with the participation of Ago2, thereby promoting cell proliferation, migration, and reduction of apoptosis via negative regulation of miR-599. These results matched with a previous study that had demonstrated that hsa_circ_0000285 contributes to the progression of osteosarcoma by sponging miR-599.³⁵

A previous study reported that *GNG12* ranks sixth among the ten key genes as glioma biomarkers, identified using several advanced computational methods in three subtypes of glioma.²⁰ This is consistent with the results of our present study. Therefore, we confirm that overexpression of the key gene *GNG12*, not only promotes cell proliferation and migration, but also inhibits apoptosis of glioma cells. Through the target prediction of miR-599 and experimental validation, we thereby demonstrated that *GNG12* expression is negatively regulated directly by miR-599.

GNG12 regulates a variety of transmembrane signaling pathways, including the PI3K/Akt signaling pathway and other pathways in cancers, as shown in our bioinformatics analysis results. So, we will like to explore the related signaling pathways and illustrate the downstream network of *GNG12*, in our future investigations. In addition, we will also focus on the development of hsa_circ_0000285 for future clinical applications.

We can, therefore, conclude that this study is the first to demonstrate that hsa_circ_0000285 is overexpressed in gliomas and also contributes to glioma progression by directly regulating the miR-599/*GNG12* axis. Finally, this suggests that hsa_circ_0000285 can be used both as a biomarker for the predicting glioma progression and as a potential target for glioma treatment.

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CONSENT FOR PUBLICATION

Consent for publication was obtained from the participants.

CONSENT TO PARTICIPATE

All patients signed written informed consent.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interests.

AUTHOR CONTRIBUTIONS

FL and CD performed the experiments and data analysis. YH and FL conceived and designed the study. FL and YH made the acquisition of data. YH did the analysis and interpretation of data. All authors read and approved the manuscript.

CODE AVAILABILITY

Not available.

ETHICS APPROVAL

The present study was approved by the Ethics Committee of the Taikang Tongji (Wuhan) Hospital (Wuhan, China). The processing of clinical tissue samples is in strict compliance with the ethical standards of the Declaration of Helsinki. All patients signed written informed consent.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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