GGNBP2 Suppresses the Proliferation, Invasion, and Migration of Human Glioma Cells

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Gliomas are the most common and aggressive type of primary adult brain tumors. Although GGNBP2 has previously been considered to be a tumor suppressor gene, little is known about the association between GGNBP2 and glioma. In this study, we clearly demonstrated that GGNBP2 was downexpressed in glioma tissues, and its downexpression is related to the pathological grade and overall survival of patients with gliomas. Overexpression of GGNBP2 suppressed the proliferation, migration, and invasion of glioma cells. Mechanistically, we demonstrated that the PI3K/Akt and Wnt/β-catenin signaling pathways were suppressed by GGNBP2 overexpression. In contrast, knockdown of GGNBP2 has precisely the opposite effect. Collectively, these data indicate that GGNBP2 shows tumor suppressive activity in human glioma cells and may stand out as a potential therapeutic target for glioma.

Key words: GGNBP2; Glioma; Proliferation; Invasion; Migration

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor, accounting for 45% of all primary brain tumors[1,2]. GBM originates from neural mesenchymal and parenchymal cells, and GBMs have high migratory and proliferative abilities and are characterized by a high incidence, recurrence, and mortality as well as a low cure rate[3,4]. The current standard of care consists of surgical resection followed by radiotherapy and chemotherapy[5]. However, the prognosis of GBM remains poor, with an average survival time of less than 1 year[6]. Advances in glioma therapy have been restricted because the pathophysiological mechanisms underlying the development of this disease are not fully known. Therefore, elucidating the molecular mechanism for glioma development will help in the identification of novel therapeutic targets and the development of strategies for glioma treatment.

Gametogenetin-binding protein 2 (GGNBP2; also known as ZFP403, ZNF403, DIF3, LCRG1, and LZK1) is encoded in human chromosome 17q12–q23, a region known as a breast and ovarian cancer susceptibility locus[7]. The full-length murine GGNBP2 protein is composed of 740 amino acids. Over the past several years, accumulating studies have shown that GGNBP2 was expressed in a number of tissues such as testis, heart, brain, lung, liver, kidney, pancreas, placenta, and skeletal muscle[8–11]. Previous research showed that GGNBP2 was highly expressed in the adult testis, and its expression was tightly associated with the start of spermatogenesis[8]. One recent study has shown that GGNBP2 is a breast cancer tumor suppressor, functioning as a nuclear receptor corepressor to inhibit Era activity and tumorigenesis[7]. Furthermore, loss of GGNBP2 in trophoblast giant cells caused aberrant overactivation of c-Met–Stat3 signaling, resulting in a dysregulation of trophoblast proliferation and differentiation[12]. However, the role of GGNBP2 in the carcinogenesis of glioma is still poorly understood.

In the present study, we showed that GGNBP2 in human gliomas is correlated with tumor progression and overall survival. Our results demonstrated that GGNBP2 inhibited glioma proliferation, migration, and invasion through inactivation of the PI3K/Akt and Wnt/β-catenin signaling pathways.

MATERIALS AND METHODS

Cell Culture and Reagents

U87 and U251 glioma cell lines were obtained from Shanghai Life Academy of Sciences Cell Library. The

two glioma cell lines were maintained in a 5% CO_2 atmosphere at 37°C in DMEM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin (HyClone, Logan UT, USA), and 10% FBS. Antibodies against GGNBP2 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Akt, phospho-Akt, phospho-PI3K, PI3K, PCNA, Wnt, MMP2, and MMP9 were obtained from Cell Signaling Technology (Danvers, MA, USA). GAPDH was purchased from KangCheng Biotech (Shanghai, P.R. China).

Patients and Tissue Preparation

In total, 80 glioma and 15 nontumor brain samples were obtained between 2012 and 2015 in The First Affiliated Hospitals of Chongqing Medical University. None of the patients providing these samples had received chemotherapy or radiotherapy prior to resection. These tissue specimens were formalin fixed and paraffin embedded. Additionally, four gliomas and the corresponding adjacent noncancerous brain tissues were collected in 2016 for Western blot analysis and quantitative real-time polymerase chain reaction (qRT-PCR) and stored in liquid nitrogen until use. For the use of these clinical materials for research purposes, prior consent and approval were obtained from patients. This investigation was approved by the Institutional Research Ethics Committee of Chongqing Medical University and the People's Hospital of Leshan and was conducted in accordance with the ethical standards of the Declaration of Helsinki and national and international guidelines.

Immunohistochemistry

Formalin-fixed GBM tissues were embedded in paraffin, and 4-μm-thick sections were cut and mounted on slides. After deparaffinization and antigen recovery, the slides were washed three times in peroxidase blocking solution. The slides were then incubated with rabbit anti-human GGNBP2 polyclonal antibodies (1:100; Sigma-Aldrich) overnight at 4°C, followed by incubation with a secondary antibody at 25°C for 30 min. The immuno-labeled slides were visualized by diaminobenzidine for 5 min, counterstained with hematoxylin, and observed under a microscope (DM6000 B; Leica, Wetzlar, Germany).

Immunohistochemical staining of GGNBP2 was calculated as both percentage of positive cells and color intensity. The percentage of the positivity was classified as "0" (negative), "1" (<10%), "2" (10%–50%), and "3" (>50%). The intensity was graded as "0" (absent), "1" (light yellow), "2" (yellowish brown), and "3" (brown). The expression of GGNBP2 was evaluated by staining index (SI), which was calculated using the following formula: SI=proportion score×intensity score. SI of 0 was categorized as negative (−), 1–2 as low expression (1+), 3–4 as moderate expression (2+), and 6 or 9 as high expression (3+).

Cell Transfections

GGNBP2 lentivirus [(overGGNBP2) used for GGNBP2 overexpression and corresponding negative control lentivirus (overCON)] as well as lentiviral constructs expressing GGNBP2 shRNA (shGGNBP2) and matched empty lentivirus (shCON) were purchased from GeneChem Co., Ltd (Montreal, Quebec, Canada). Glioma cells stably expressing the GGNBP2 shRNA were used for targeting the sequence ACAGTCATTTATGGCTAATAA. One day before transfection, 5×10^4 cells per well (reaching about 30% confluency at the time of transfection) were cultured in six-well plates. These lentiviruses were introduced into glioma cells treated with 8 μg/ml polybrene and complete medium. Transfection effects were observed by a fluorescence microscope after 48 h. Puromycin was used to purify these infected cells. Effective knockdown or forced expression of GGNBP2 was monitored by RT-PCR and Western blot analysis after 72 h.

Real-Time-PCR

Total RNA in glioma cells was extracted using the TRIzol reagent and precipitated with buffer containing 2.5 volumes of ethanol. The RNA was dissolved in TE solution (10 mM Tris-HCl 7.5, 0.1 mM EDTA) and further treated with RNase-free DNase I (Takara, Japan). The RNA was recovered using phenol/chloroform extraction followed by ethanol precipitation. The RNA concentration was estimated by measuring the absorbance at 260 nm using a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was synthesized using RT primer and SuperScript III reverse transcriptase following the manufacturer's instructions (Takara). qRT-PCR was performed using iQ5™ Multicolor Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA) with Real-Time PCR Master Mix (SYBR Green; Toyobo, Osaka, Japan). GAPDH was chosen as the endogenous control in the assay. The primer sequence and product size for GGBNP2 was 5′-TTATGAAGGCTTGCGGT GCT-3′ (forward) and 5′-ATGCCTTTCTCTTCGCCCT C-3′ (reverse), 123 bp, and for GAPDH, which was used as a standard, was 5′-ACATCAAGAAGGTGGTGAA G-3′ (forward) and 5′-ATACCAGGAAATGAGCTTG A-3′ (reverse), 173 bp.

Western Blot

Total protein from tissue and cells was harvested in RIPA lysis buffer. After centrifugation at 12,000 rpm for 10 min, the protein concentration was measured by BCA protein assay kit (Beyotime Institute of Biotechnology, Beijing, P.R. China). Total protein (30 μg) was then

loaded onto an SDS-PAGE gel. After electrophoresis, proteins were transferred to a PVDF membrane (Millipore, Boston, MA, USA) and incubated with blocking buffer for 60 min at room temperature and then incubated with primary antibody at 4°C overnight. Finally, the PVDF membranes were washed three times with TBST buffer and incubated with secondary antibody (1:5,000) for 1 h at 37°C. The primary antibodies used were as follows: GGNBP2 (1:1,000), total AKT1 (1:1,000), phospho-Akt (1:250), total PI3K (1:1,000), phospho-PI3K (1:500), GAPDH (1:1,000), Wnt (1:1,000), PCNA (1:1,000), MMP2 (1:800), and MMP9 (1:800). The membranes were then washed three times in TBST buffer, and the amount of protein in each band was quantified using the Quantity One 4.6 computer software (Bio-Rad).

Cell Proliferation Assay

Cells were seeded into 96-well plates at a density of 2,000 cells/well. After 24, 48, 72, and 96 h, 10 µl of cell counting kit-8 (Beyotime, Beijing, P.R. China) was added to each well, and the cells were incubated for another 1 h. The absorbance values were read at 450 nm using an enzyme-labeled instrument.

Migration and Invasion Assay

For Transwell migration assays, 10^5 cells were suspended in medium without serum and placed on the top side of polycarbonate Transwell filters without Matrigel in the upper chamber of the QCM™ 24-Well Cell Invasion Assay (Cell Biolabs, Inc., USA/Canada), and the medium without serum was used in the lower chamber. For the invasion assays, the experimental procedures were similar to those for migration assays except that the filters with Matrigel were used instead of the ones without Matrigel. The cells were incubated at 37°C for 8 h for migration assays or 48 h for invasion assays. The cells in the top chambers were removed with cotton swabs. The migrated and invaded cells on the lower membrane surface were washed with PBS buffer twice and fixed in 4% paraformaldehyde for 30 min. The well was stained with 0.1% crystal violet for 20 min after another repeat washing using PBS buffer. Following washing in ddH$_2$O more than three times, the membrane was dried and observed under a light microscope. The assays were performed in triplets, and the data were presented as the means±standard error of mean (SEM).

Statistical Analysis

Statistical analyses were performed using SPSS 17.0 (Chicago, IL, USA). Statistical differences among groups were analyzed by *t*-test or chi-square test. The prognostic significance analysis was performed using Kaplan–Meier method and log-rank tests. A value of $p < 0.05$ was considered to be statistically significant. All data are presented as mean±standard deviation (SD).

RESULTS

Expression of GGNBP2 in Glioma

First, the expression of GGNBP2 was detected in 80 glioma specimens and 15 normal brain tissues by immunohistochemistry (data not shown). The results showed that GGNBP2 expression in normal brain tissues was significantly higher than the expression in low-grade glioma tissues (WHO I and WHO II) and high-grade glioma tissues (WHO III and WHO IV) and that the low-grade tissues presented a higher expression level in GGBNP2 than in high-grade tissues (Fig. 1A and B). There were no significant statistical differences between gender or age according to the staining results (data not shown). We found that the mRNA and protein levels of GGNBP2 were significantly lower in glioma tissues compared with the matched normal brain tissues by qRT-PCR (Fig. 1C) and Western blot analysis (Fig. 1D). Kaplan–Meier analysis showed that GGNBP2 downregulation was significantly associated with poor overall survival in 80 glioma cases (Fig. 1E).

GGNBP2 Inhibits the Proliferation of Glioma Cells

To investigate the function of the GGNBP2 gene in glioma cells, we generated GGNBP2-overexpressing stable cells and matching control cells (designated as overGGBNP2 cells and overCON cells, respectively) as well as GGNBP2 knockdown stable cells and the corresponding control cells (designated as shGGNBP2 cells and shCON, respectively), as described in the Materials and Methods section. qRT-PCR and Western blot results confirmed a remarkable upregulation of GGNBP2 expression in overGGNBP2 U87 and U251 cells (Fig. 2A–F) and a significant downregulation of GGNBP2 expression in shGGNBP2 U87 and U251 cells (Fig. 3A–F). CCK-8 cell proliferation assay was utilized to determine cell proliferation. Overexpression of GGNBP2 inhibited cell proliferation in both U87 and U251 cells compared with the control group (Fig. 2G and H). Meanwhile, our data showed that knockdown of GGNBP2 promoted cell proliferation in both glioma cell lines (Fig. 3G and H).

GGNBP2 Suppresses Migration and Invasion of Glioma Cells

To determine whether GGNBP2 could affect the migratory and invasive abilities of glioma cells, we first performed Transwell assays. GGNBP2 overexpression in U87 and U251 cells elicited a significant reduction of cell invasion (Fig. 4A and B) compared with the control groups. Similarly, cell migration was significantly blocked in overGGNBP2 U87 and U251 cells compared with control cells (Fig. 5A and B).
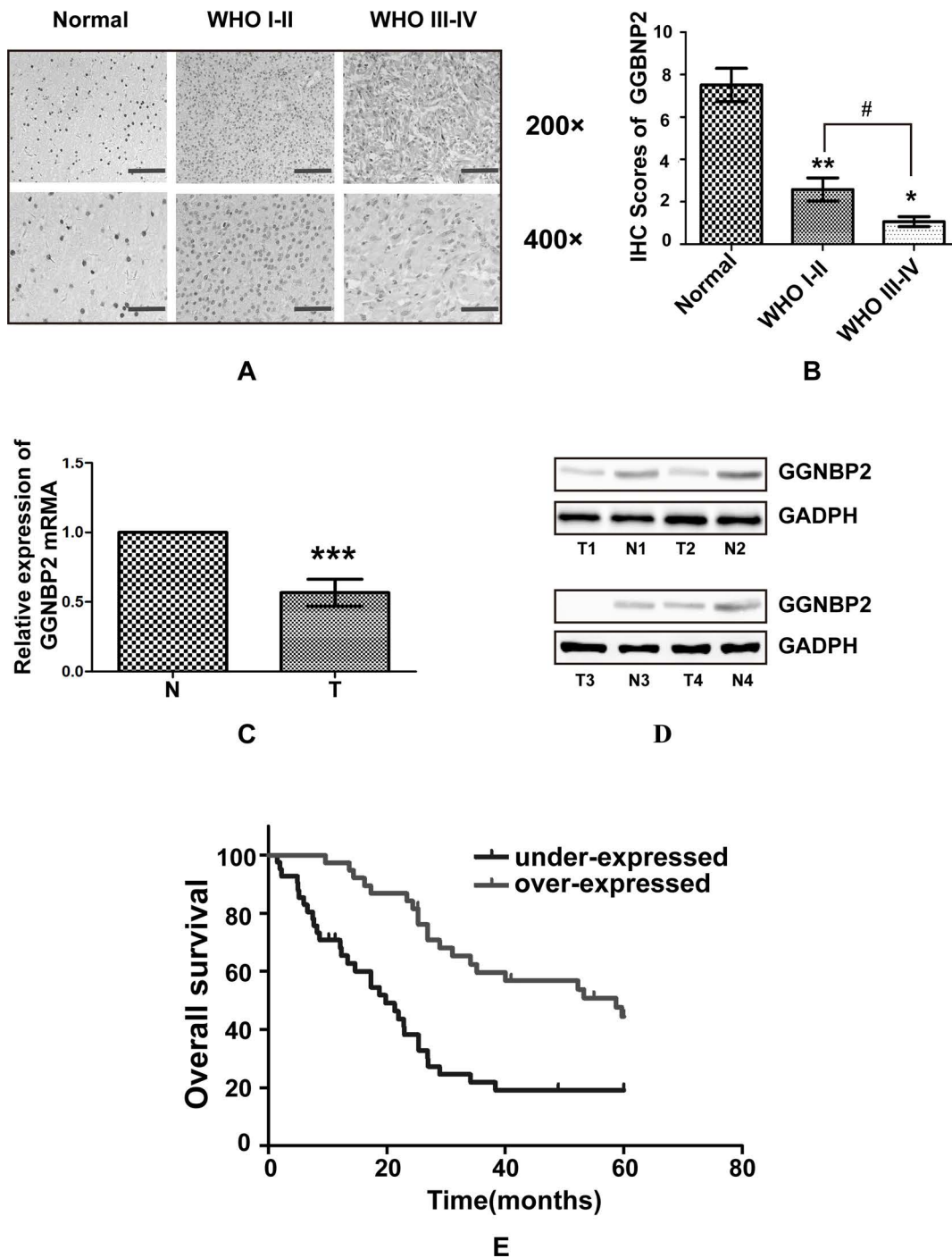
Figure 1. Low GGNBP2 expression predicted a worse prognosis for patients with glioma. (A) GGNBP2 expression in normal brain tissues and glioma tissues was determined by IHC. Scale bars: 100 μm (low power, 200×), 50 μm (high power, 400×). (B) Statistical quantification of the mean optical density in normal brain tissues, low-grade glioma tissues, and high-grade glioma tissues (*$p < 0.05$, **$p < 0.01$ vs. normal brain tissues; #$p < 0.05$ between the high-grade group and low-grade group). (C) qRT-PCR analysis of GGNBP2 mRNA in four glioma tissues and paired adjacent nontumor tissues (***$p < 0.001$ vs. paired adjacent nontumor tissues). (D) GGNBP2 protein level was determined in four pairs of glioma tissues compared to paired adjacent nontumor tissues by Western blot. (E) Kaplan–Meier analysis of overall survival based on GGNBP2 expression in 80 patients with glioma ($p < 0.001$).
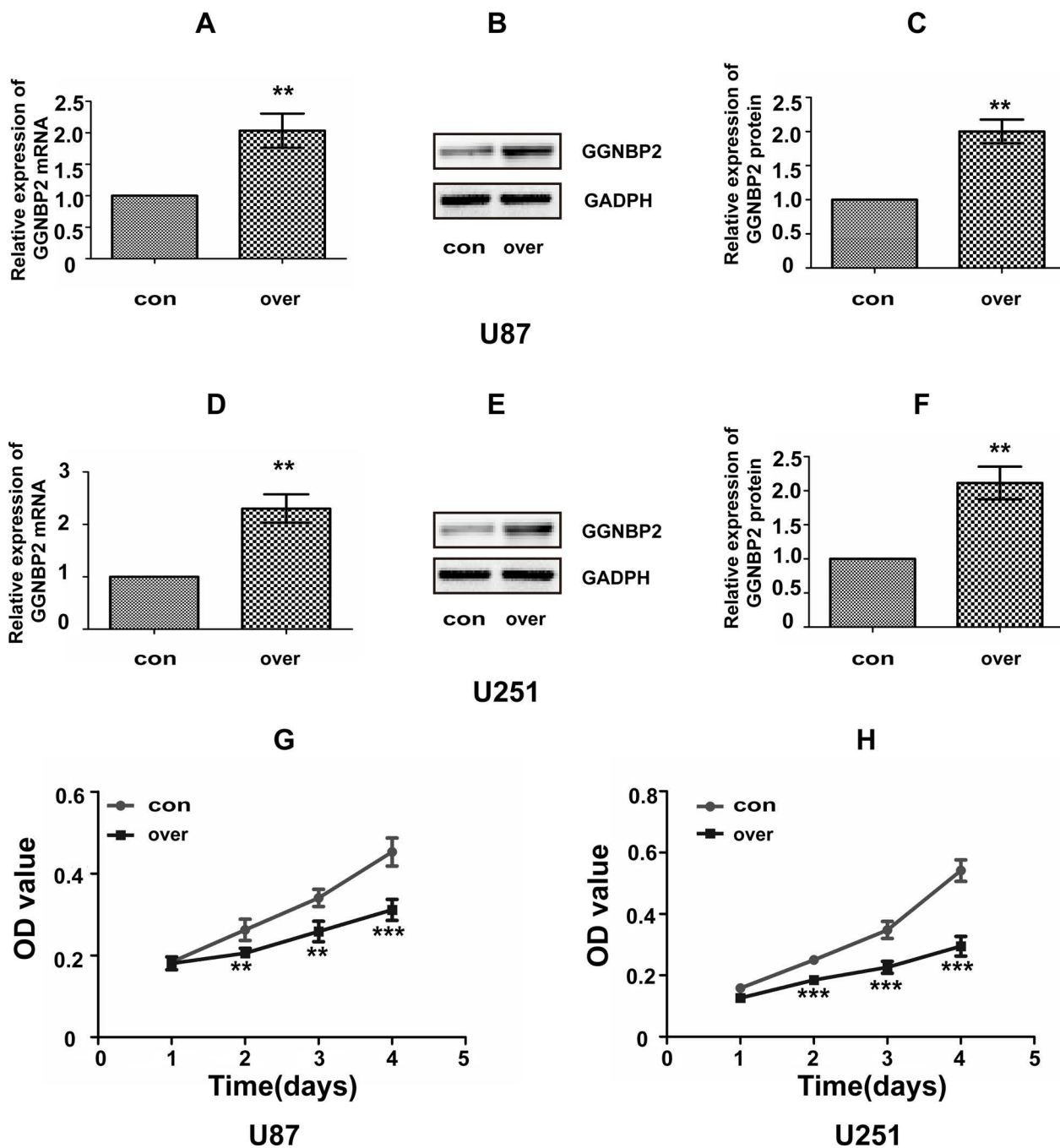
Figure 2. Upregulation of GGNBP2 inhibited proliferation of glioma cells. (A) qRT-PCR was conducted to determine the mRNA levels of GGNBP2 in U87 cells. (B, C) Expression of GGNBP2 in U87 cells was analyzed by Western blot. (D) qRT-PCR was conducted to determine the mRNA levels of GGBNP2 in U87 cells. (E, F) Expression of GGNBP2 in U87 cells was analyzed by Western blot. (G, H) Cell proliferation was detected in U87 and U251 cells by CCK-8 assay. con, transfected with empty vectors; over, transfected with GGNBP2 vectors. Data were based on at least three independent experiments and shown as the mean±SD ($**p<0.01$, $***p<0.001$, compared with con).
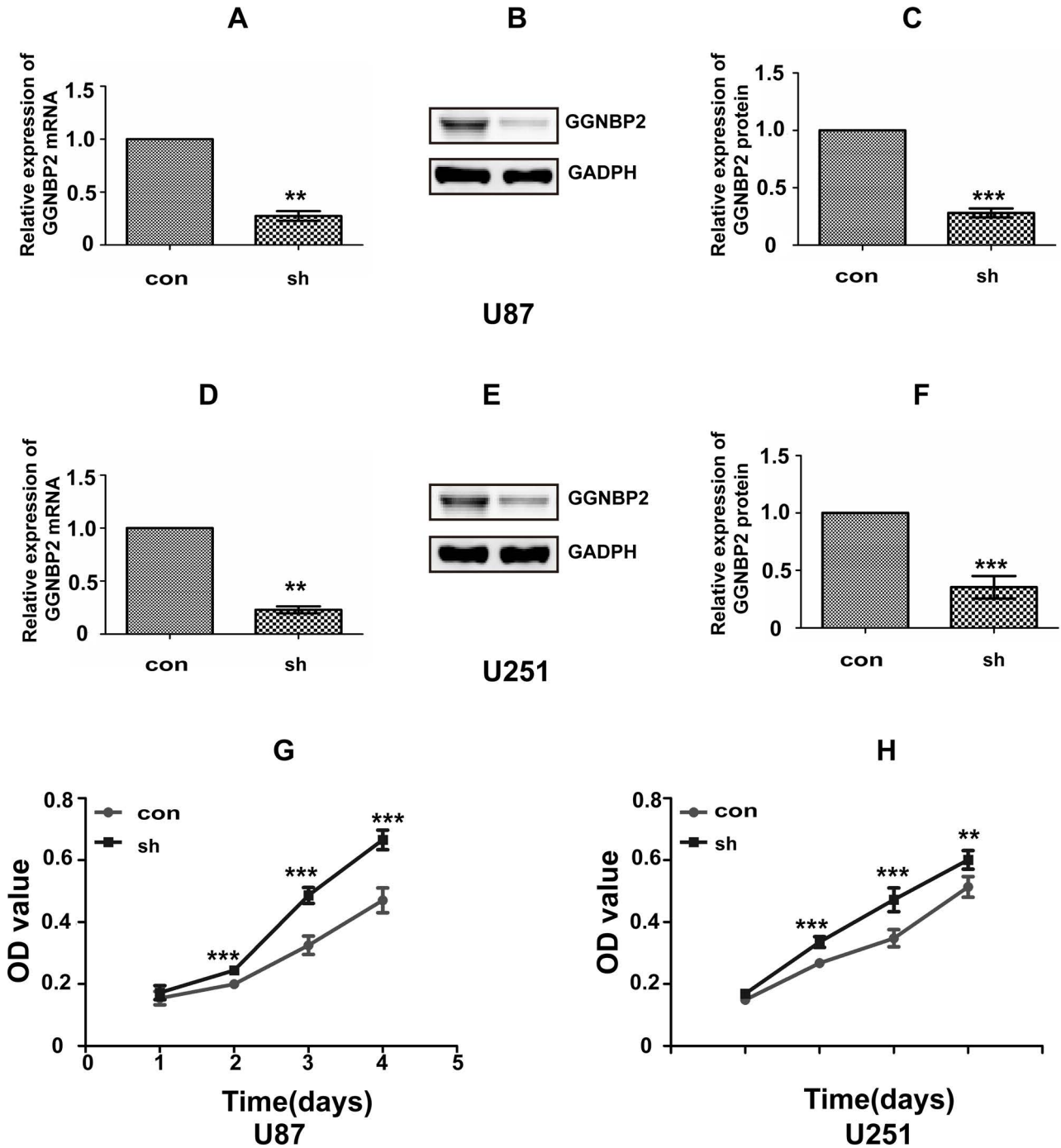
Figure 3. Knockdown GGNBP2 promoted proliferation of glioma cells. (A) qRT-PCR was conducted to determine the mRNA levels of GGNBP2 in U87 cells. (B, C) Expression of GGNBP2 in U87 cells was analyzed by Western blot. (D) qRT-PCR was conducted to determine the mRNA levels of GGNBP2 in U87 cells. (E, F) Expression of GGNBP2 in U87 cells was analyzed by Western blot. (G, H) Cell proliferation was detected in U87 and U251 cells by CCK-8 assay. con, transfected with empty vectors; over, transfected with shGGNBP2 vectors. Data were based on at least three independent experiments and shown as the mean±SD (**$p < 0.01$, ***$p < 0.001$, compared with con).
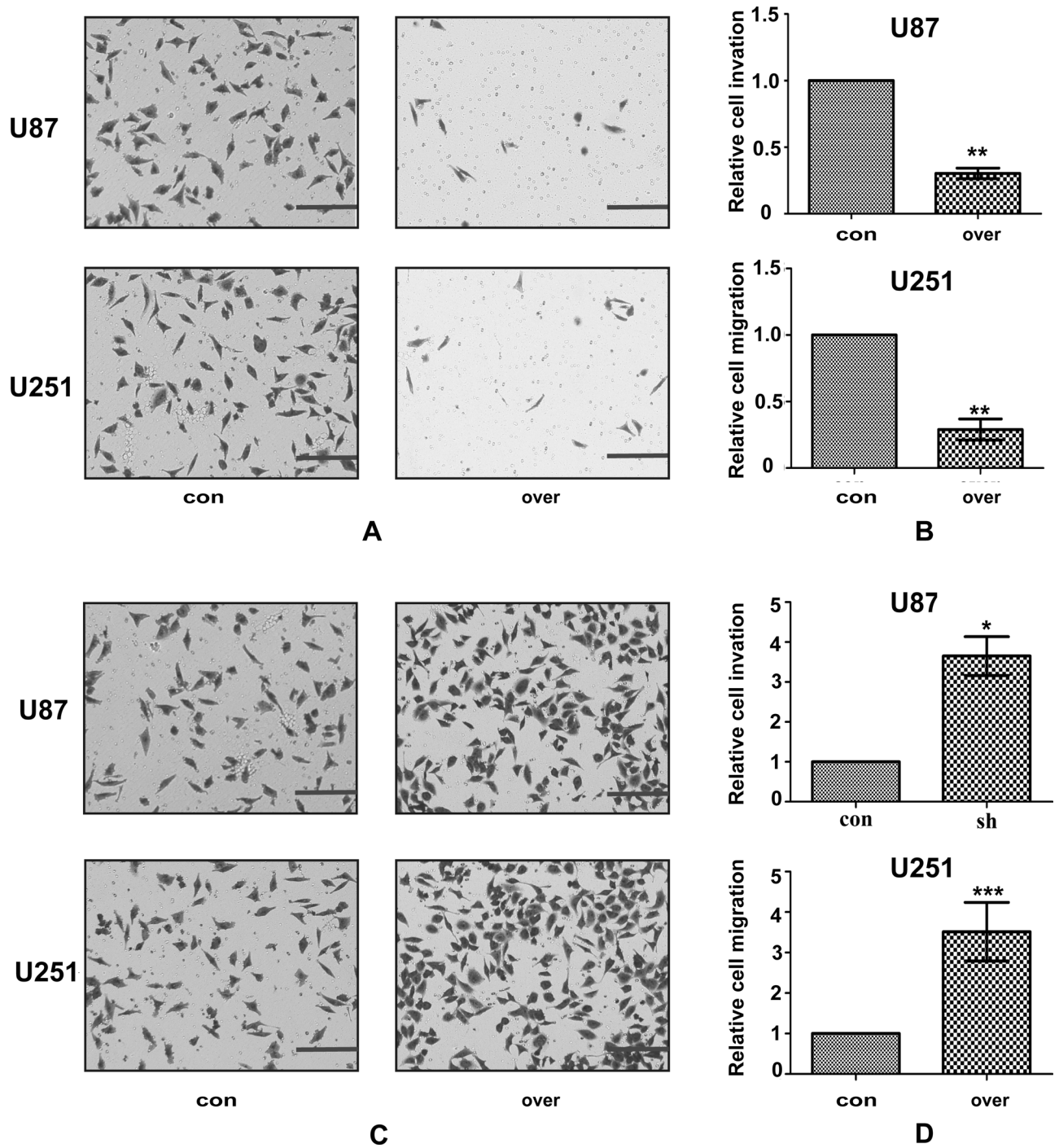
Figure 4. GGNBP2 inhibited invasion of glioma cells. (A, B) Upregulation of GGNBP2 inhibited cell invasion in the U87 and U251 cell lines (100×). (C, D) Downregulation of GGNBP2 after shRNA treatment promoted cell invasion in the U87 and U251 cell lines (100×). Data were based on at least three independent experiments and shown as the mean±SD (*$p<0.05$, **$p<0.01$, ***$p<0.001$, compared with con).

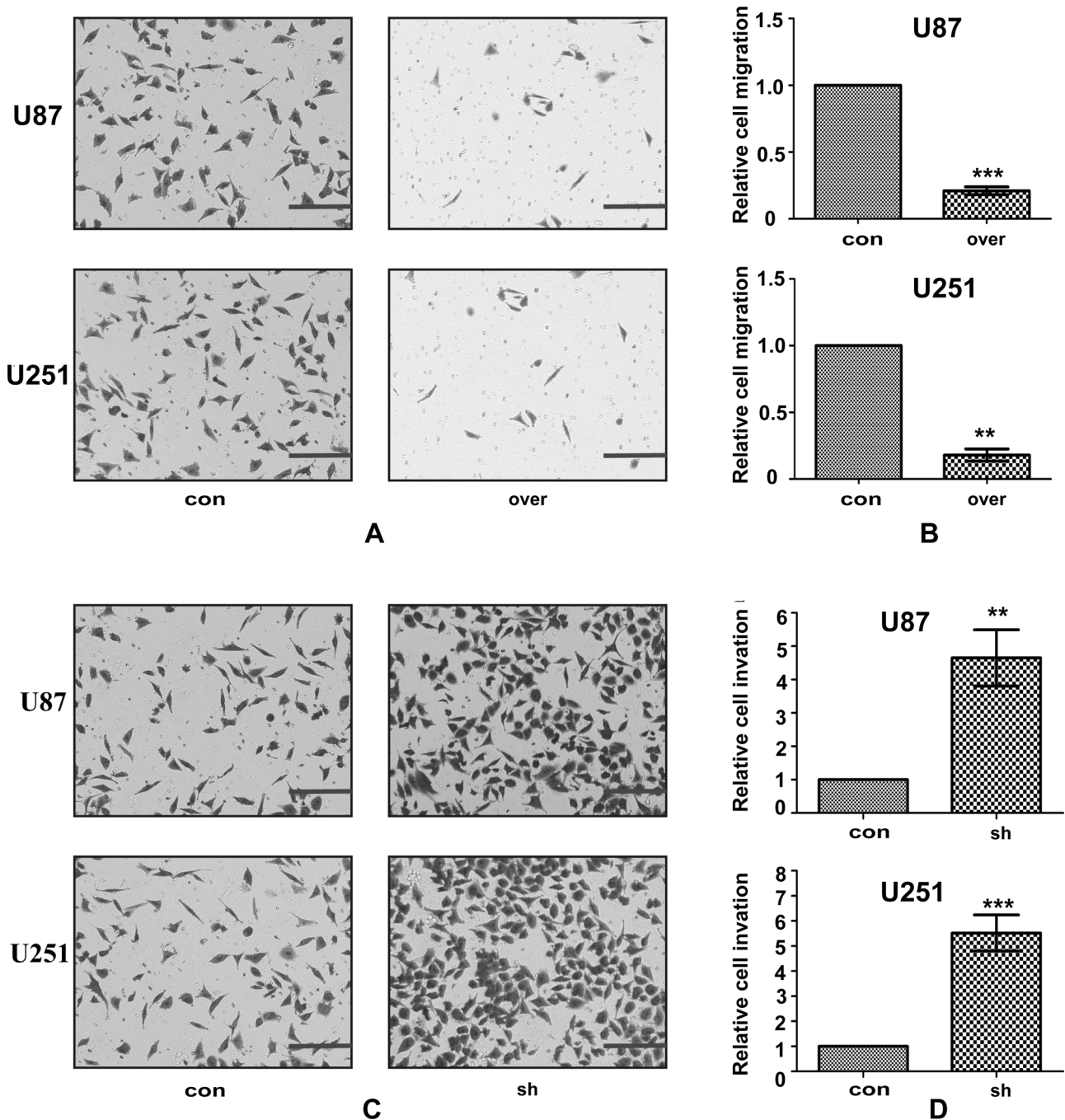**Figure 5.** GGNBP2 inhibited migration of glioma cells. (A, B) Upregulation of GGBNP2 inhibited cell migration in the U87 and U251 cell lines (100×). (C, D) Downregulation of GGNBP2 after shRNA treatment promoted cell migration in the U87 and U251 cell lines (100×). Data were based on at least three independent experiments and shown as the mean±SD (**$p < 0.01$, ***$p < 0.001$, compared with con).

Conversely, cell invasion was significantly enhanced upon GGNBP2 knockdown in U87 and U251 cells (Fig. 4C and D). Consistently, GGNBP2 knockdown in U87 and U251 cells evidently promoted tumor cell migration ($p < 0.05$) (Fig. 5C and D).

GGNBP2 Regulates Activation of the PI3K/Akt and Wnt/β-Catenin Signaling Pathways in Glioma Cells

We then tested the signaling pathways associated with the proliferation, invasion, and migration of glioma cells. The effects of GGNBP2 on PI3K/Akt and

Wnt/β-catenin signaling pathways were evaluated. We found that overexpression of GGNBP2 in U87 and U251 cells obviously inhibited the protein levels of p-PI3K, p-Akt, Wnt, and β-catenin in U87 and U251 cells (Fig. 6A–C). In addition, we found that PCNA, MMP2, and MMP9, the downstream targets of the PI3K/Akt and Wnt/β-catenin signaling pathways, were suppressed in over GGNBP2 cells (Fig. 6A–C). Conversely, down-regulation of GGNBP2 promoted the expression of those molecules (Fig. 7A–C).
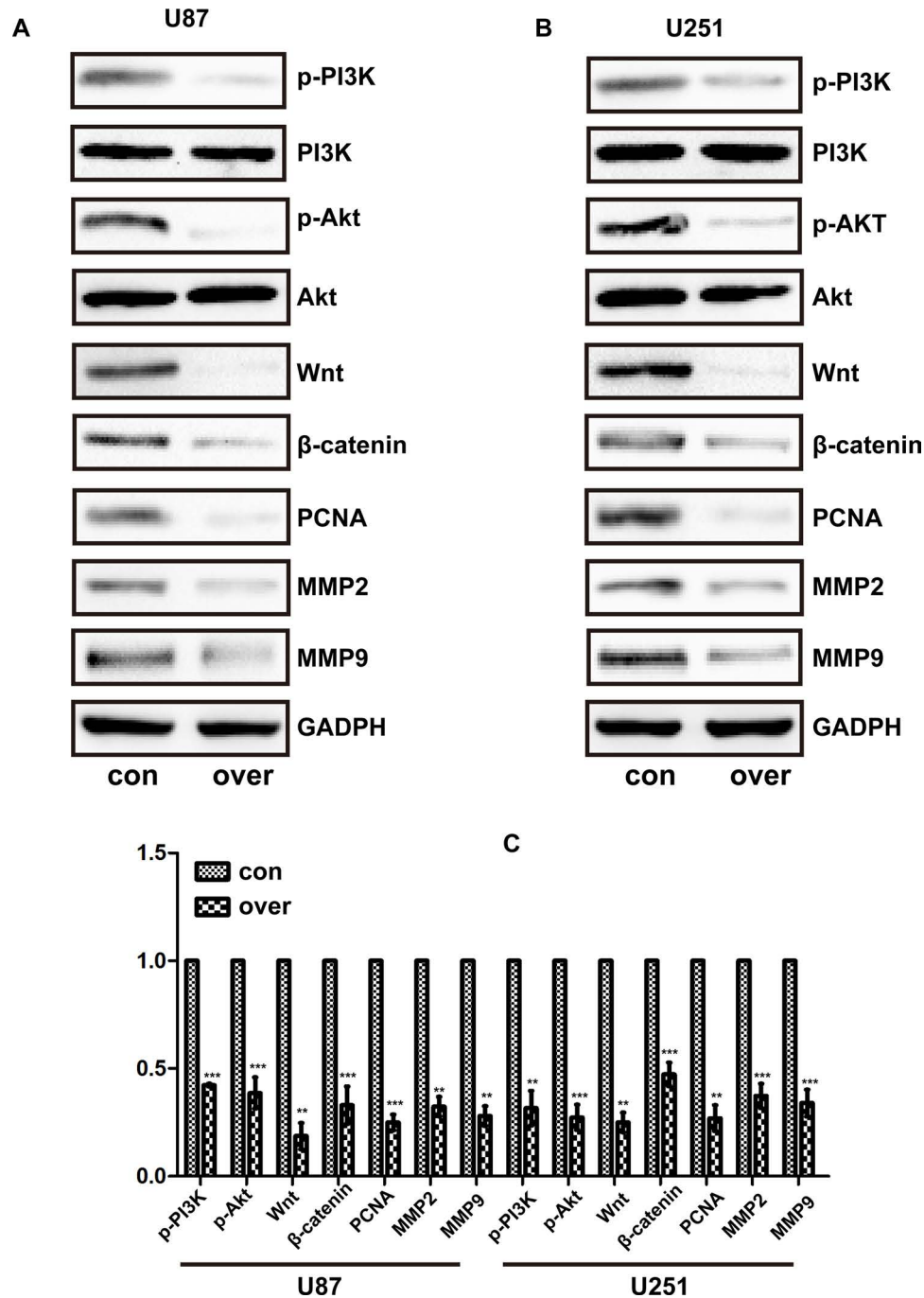

Figure 6. Upregulation of GGNBP2 suppressed PI3K/Akt signaling and Wnt/β-catenin signaling in glioma cells. (A–C) GGNBP2 overexpression inhibits the PI3K/Akt and Wnt/β-catenin signaling pathways and the expression of PCNA, MMP2, and MMP9 in the U87 and U251 cells. Data were based on at least three independent experiments and shown as the mean±SD (**$p < 0.01$, ***$p < 0.001$, compared with con).
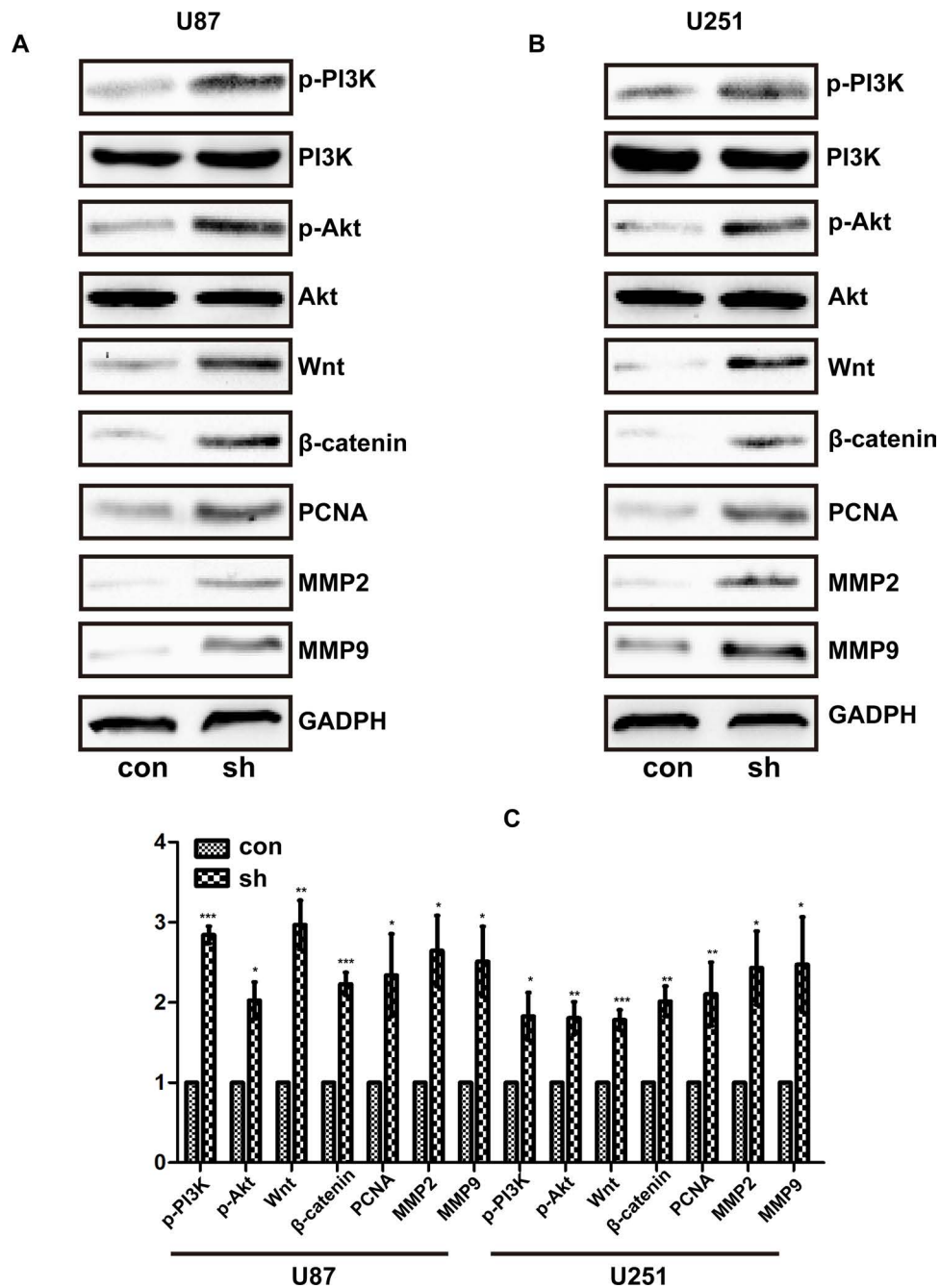
Figure 7. Knockdown of GGNBP2 promoted PI3K/Akt signaling and Wnt/β-catenin signaling in glioma cells. (A–C) GGNBP2 downexpression promotes the PI3K/Akt and Wnt/β-catenin signaling pathways and the expression of PCNA, MMP2, and MMP9 in the U87 and U251 cells. Data were based on at least three independent experiments and shown as the mean±SD ($*p<0.05$, $**p<0.01$, $***p<0.001$, compared with con).

DISCUSSION

GGNBP2, a potential tumor suppressor gene, is a highly conserved gene localized to the tumor suppressor locus D17S800–D17S930 but is also known to be involved in spermatogenesis[11]. The GGNBP2 protein has been

shown to interact with the testicular germ-specific protein gametogenetin 1 (GGN1)[10], whose mRNA is enriched in testes, expressed in late pachytene spermatocytes, and upregulated in round spermatids, a time window concomitant with the occurrence of meiosis. It has been

reported that GGNBP2 was an essential factor for pregnancy success through the maintenance of a balance of trophoblast stem cell proliferation and differentiation during placental development[12]. Moreover, forced expression of GGNBP2 suppressed the proliferation of Hela cells[10]. In addition to cell differentiation and proliferation, overexpression of GGNBP2 results in a decrease in migration and invasion of human breast cancer cells[7]. A recent study reported that downregulation of GGNBP2 resulted in a repression of cell proliferation of Hela cells[13]. However, the role of GGNBP2 in glioma has not been clearly studied. In this study, we investigated the clinicopathological and biological significance of GGNBP2 in glioma. Our study demonstrated that GGNBP2 was downexpressed in glioma. Moreover, we found that downexpression of GGNBP2 was correlated with shorter overall survival of patients, suggesting that GGNBP2 may function as a tumor suppressor in glioma. Additionally, we showed that GGNBP2 overexpression repressed glioma cell proliferation, while knockdown of GGNBP2 expression promoted cell proliferation. Furthermore, we found that overexpression of GGNBP2 could suppress the invasive and migratory capacities of glioma cells. Conversely, silencing GGNBP2 could enhance cell migration and invasion of glioma cells. Therefore, these data implied that GGNBP2 might be involved in the aggressiveness of glioma because it was shown to play an important role in cell proliferation, migration, and invasion.

Previous studies have demonstrated an overactivation of the Wnt/β-catenin pathway resulting from multiple oncogenes, maintaining the aggressive malignant phenotype of gliomas[14–16]. The Wnt/β-catenin pathway has a diversity of biological functions in regulating cell proliferation, migration, and cell invasion[17–22]. As reported, the PI3K/Akt signaling pathway is of great importance in the malignant development of various tumors[23,24]. It is well known that PI3K/Akt is a classic signaling pathway[25], and its activation induces cell proliferation, invasion, and migration[26–30]. In view of the crucial role of the PI3K/Akt and Wnt/β-catenin signaling pathway in tumor progression, we investigated whether GGNBP2 exerted any influence on these signaling pathways in glioma. Therefore, we examined whether GGNBP2 can regulate p-PI3K, p-Akt, Wnt, and β-catenin expression. We found that the protein levels of p-PI3K, p-Akt, Wnt, and β-catenin were downregulated by overexpression of GGNBP2, while knockdown of GGNBP2 could upregulate the protein levels of p-PI3K, p-Akt, Wnt, and β-catenin. In addition, PCNA, MMP2, and MMP9, downstream factors in the PI3K/Akt and Wnt/β-catenin signaling pathways that are related to tumor proliferation, migration, and invasion in glioma cells, were suppressed by the overexpression of GGNBP2. However, knockdown of GGNBP2 promoted the expression levels of PCNA, MMP2, and MMP9. These findings suggested that GGNBP2 might function as a tumor suppressor in glioma cells via inhibition of the PI3K/Akt and Wnt/β-catenin signaling pathways.

In conclusion, our study demonstrated that the mRNA and protein expression levels of GGNBP2 were significantly lower in glioma tissues. Low expression of GGNBP2 was significantly associated with shorter overall survival of glioma patients. GGNBP2 could suppress the proliferation, invasion, and migration of glioma cells via inactivating the PI3K/Akt and Wnt/β-catenin signaling pathways. Our study indicates that GGNBP2 may be a potential prognostic biomarker and a new therapeutic target for patients with gliomas.

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