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Reduced Expression of Jumonji AT-Rich Interactive Domain 2 (JARID2) in Glioma Inhibits Tumor Growth In Vitro and In Vivo

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Jumonji AT-rich interactive domain 2 (JARID2) is a member of the Jumonji family of proteins and has been proposed as an oncogene in several types of human cancer. However, the role of JARID2 in human glioma has not yet been understood. The present study was designed to determine the roles of JARID2 in the proliferation and migration in human glioma cells and the growth of glioma cells in nude mice. Our data indicate that JARID2 is upregulated in human glioma tissues and cell lines. Knockdown of JARID2 obviously inhibits the proliferation of U87MG cells and tumor growth in vivo. Furthermore, knockdown of JARID2 inhibits migration and invasion as well as the epithelial–mesenchymal transition (EMT) process in U87MG cells. Mechanistically, knockdown of JARID2 reduces the phosphorylation levels of PI3K and Akt in U87MG cells. In summary, our study is the first one in our knowledge to indicate that JARID2 plays an important role in glioma development and progression. Therefore, JARID2 may serve as a potential therapeutic target for the treatment of glioma.

Key words: Jumonji AT-rich interactive domain 2 (JARID2); Glioma; Proliferation; Invasion

INTRODUCTION

Glioma is the most devastating and aggressive tumor in the brain, accounting for 40%–50% of all intracranial tumors[1]. Despite considerable progress in treatment strategies, including surgery, radiotherapy, and chemotherapy, the 5-year survival of patients remains low due to high recurrence and metastasis of glioma[2–4]. Thus, making a thorough investigation into the mechanisms of tumor invasion and metastasis may facilitate the discovery of potential molecular targets for the treatment of glioma.

Jumonji AT-rich interactive domain 2 (JARID2) is a member of the Jumonji family of proteins and contains a DNA-binding domain, denoted as the AT-rich interaction domain (ARID); a zinc finger domain; a jumonji N (JmjN) domain; and a JmjC domain[5]. A growing body of evidence suggests that JARID2 plays an important role in regulating hematopoietic stem cell function and differentiation of embryonic stem cells[6–8]. It was reported that depletion of JARID2 enhances the in vitro expansion and in vivo reconstitution capacity of human hematopoietic stem and progenitor cells[9]. In addition, recent studies demonstrated that JARID2 has been proposed as an oncogene in several types of human cancer[10–12]. For example, Lei et al. confirmed that downregulation of JARID2 greatly suppressed hepatocellular carcinoma cell migration, invasion, proliferation in vitro, and metastasis in vivo[10]. However, the role of JARID2 in human glioma has not yet been understood. The present study was designed to determine the roles of JARID2 in the proliferation and migration in human glioma cells, and glioma cell growth in nude mice. Our results demonstrated that JARID2 plays an important role in glioma development and progression.

MATERIALS AND METHODS

Tissue Specimens

Fresh glioma and corresponding normal tissue samples were obtained from patients with glioma. No patients received chemotherapy or radiotherapy before surgery. The specimens were quickly frozen in liquid nitrogen and stored at −80°C until use. This study was approved by the Research Ethics Committee of Huaihe Hospital of Henan University (P.R. China). Written, informed consent was obtained from all patients.

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Cell Culture

Human glioma cell lines (U87MG, U251, and LN229) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Bio-Rad, Hercules, CA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher, Waltham, MA, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C under a humidified 5% CO_2/95% air atmosphere. Human microglia cells (Sciencell Research Laboratories, Carlsbad, CA, USA) were used as the control.

Short Hairpin RNA and Cell Transfection

Short hairpin RNA targeting JARID2 (sh-JARID2) or empty vector (vector) was purchased from Invitrogen (Carlsbad, CA, USA). U87MG cells were transfected with sh-JARID2 or vector using Lipofectamine 2000 (Invitrogen), respectively, according to the manufacturer's protocols. Transfection efficiency was confirmed using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from frozen tissues or cells using the TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using the PrimeScript® RT Reagent Kit (TaKaRa, Shiga, Japan). qRT-PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with FastStart Universal SYBR Green Master (Roche, Indianapolis, IN, USA). The primers used for JARID2 and β-actin were the following: JARID2, 5′-GACACCAAACCCAATCACC AC-3′ (sense) and 5′- GTTCAACCTGCCACTGACC TT-3′ (antisense); β-actin, 5′-TGGCACCCAGCACAAT GAA-3′ (sense) and 5′-CTAAGTCATAGTCCGCCTAG AAGCA-3′ (antisense). Data were analyzed using the formula[13]: $R = 2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$.

Western Blot

Total protein was extracted from frozen tissues or cells using RIPA lysis buffer (Beyotime, Nantong, P.R. China). A total of 30 μg of protein samples from each cell line was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% nonfat dry milk in 0.05% Tween 20 phosphate-buffered saline for 1 h at room temperature, membranes were incubated with the corresponding primary antibodies (mouse anti-JARID2, anti-E-cadherin, anti-N-cadherin, anti-phospho-PI3K, anti-PI3K, anti-phospho-Akt, anti-Akt, or anti-GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Subsequently, membranes were rinsed and incubated with goat anti-mouse horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology). Bound antibodies were detected using enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, IL, USA).

Cell Proliferation Assay

Cell proliferation was evaluated by the cell counting kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan). In brief, U87MG cells at a density of 1×10^4 cells/well were plated in 96-well plates and transfected with sh-JARID2 or vector. Cells were assayed 24 to 96 h posttransfection. The CCK-8 reagents were added and incubated with the cells for 1 h. The absorbance of each well was then determined with a microplate reader at a 450 nm wavelength.

Cell Migration and Invasion Assays

Cell migration assay was performed using Transwell™ chambers (Costar, Boston, MA, USA). In brief, infected U87MG cells were seeded into the upper chamber of the Transwell plate, and 500 μl of DMEM containing 10% FBS was added to the lower chamber as a chemotactic factor. After 24 h of incubation, cells that transferred to the lower surface of the base membrane were fixed, stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA), and counted under a microscope (magnification: 100×). Cell invasion migration assay was performed by the same procedure, except that the inserts were precoated with Matrigel (8-mm pore size; BD Biosciences, San Jose, CA, USA).

In Vivo Experiment

The animal study was approved by the Institutional Animal Care and Use Committee of Huaihe Hospital of Henan University. Female nude mice (4–6 weeks, 18–23 g) were purchased from the laboratory animal center of Henan Province. The animals were housed in a temperature- and humidity-controlled room with a 12-h on–off light cycle and given free access to food and water. For the transplantation tumor experiment, a suspension of stable transduced U87MG cells (5×10^6) was injected subcutaneously into the left flank of each group ($n = 6$ per group). Tumor volumes were measured every 7 days using a caliper. After 4 weeks, the mice were euthanized, and tumor weights were measured.

Statistical Analysis

All of the data were expressed as the mean ± SD. Statistical significance was analyzed with one-way factorial ANOVA or Student's two-tailed *t*-test. Values of $p < 0.05$ were considered significant.

RESULTS

JARID2 Is Upregulated in Human Glioma Tissues and Cell Lines

First, we investigated the expression level of JARID2 in human glioma tissues using qRT-PCR and Western

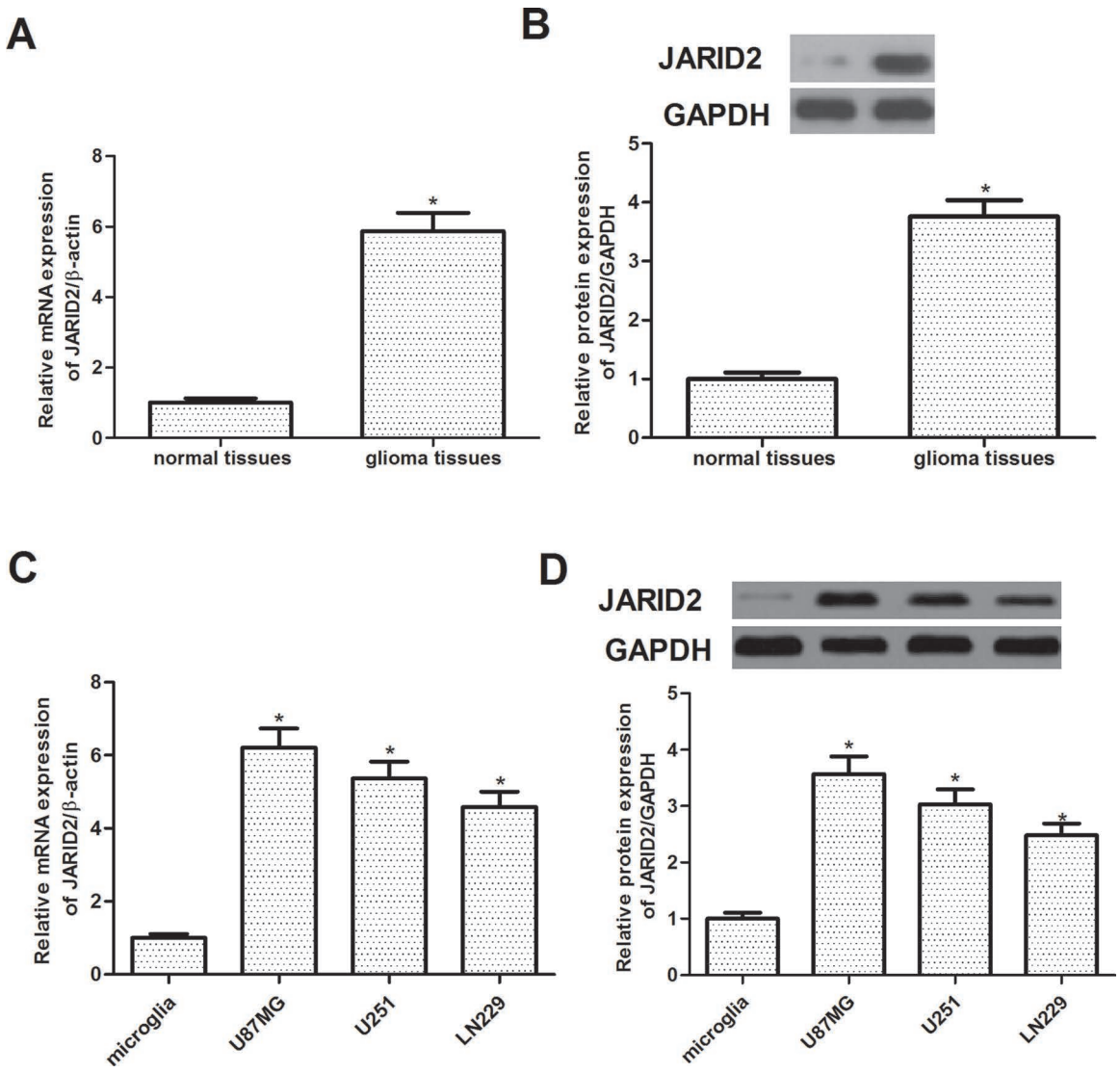

Figure 1. JARID2 is upregulated in human glioma tissues and cell lines. (A) The mRNA expression of JARID2 was measured by qRT-PCR in human glioma tissues. (B) Representative Western image of JARID2 protein in human glioma tissues. (C) The mRNA expression of JARID2 in human glioma lines. (D) Representative Western image of JARID2 protein in human glioma lines. Results presented are mean ± SD. $*p < 0.05$ versus control group.

blotting analysis. As shown in Figure 1A, compared to the corresponding normal tissue samples, the mRNA expression of JARID2 was markedly elevated in human glioma tissues. The results of the Western blotting analysis demonstrated that the JARID2 protein level was significantly higher in human glioma tissues than in control tissues (Fig. 1B). Then we examined the expression of JARID2 in human glioma cell lines. All cell lines examined showed higher JARID2 expression at both mRNA and protein levels compared with the microglia cells (Fig. 1C and D).

Effects of JARID2 on Glioma Cell Proliferation

To investigate the significance of JARID2 in the tumorigenesis of glioma, we generated U87MG cells expressing a lentiviral shRNA targeting of JARID2. qRT-PCR and Western blotting confirmed the downregulation of JARID2 expressions at both the mRNA (Fig. 2A) and protein levels (Fig. 2B) in U87MG cells. Furthermore, the effect of JARID2 on glioma cell proliferation was detected using the CCK-8 assay. As shown in Figure 2C, a significant decrease in cell proliferation was observed in U87MG cells after transfection with sh-JARID2 when compared to the vector group.

Effects of JARID2 on Tumor Growth in a Xenograft Model

To further evaluate the possible function of JARID2 in the regulation of tumor growth in vivo, we established a

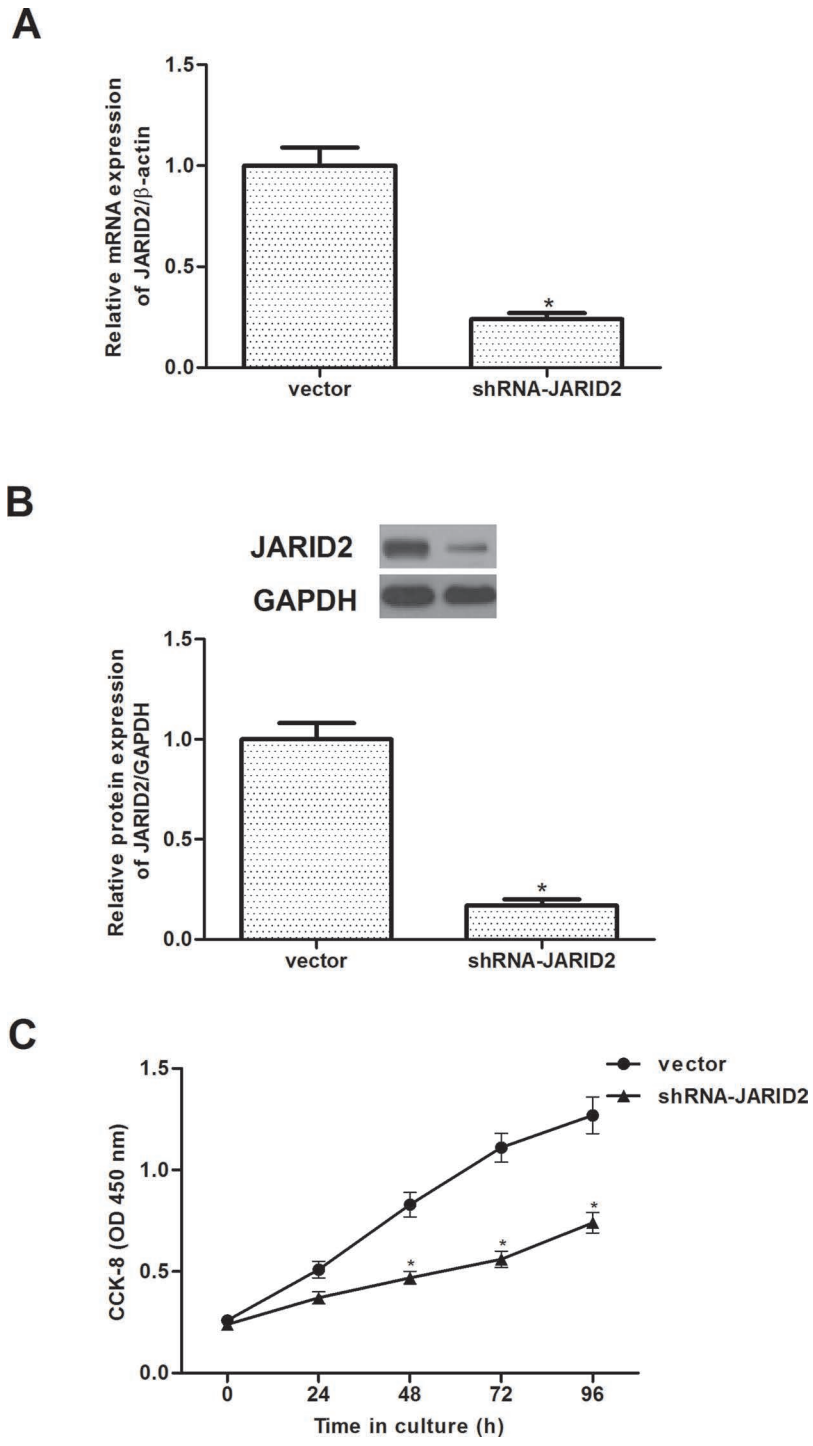

Figure 2. Effects of JARID2 on glioma cell proliferation. U87MG cells were transfected with sh-JARID2 or vector for 24 h. (A) The mRNA expression level of JARID2 was detected with qRT-PCR. (B) The protein expression level of JARID2 was detected with Western blotting. (C) Cell proliferation was detected using the CCK-8 assay. Results presented are mean ± SD. *$p < 0.05$ versus vector group.

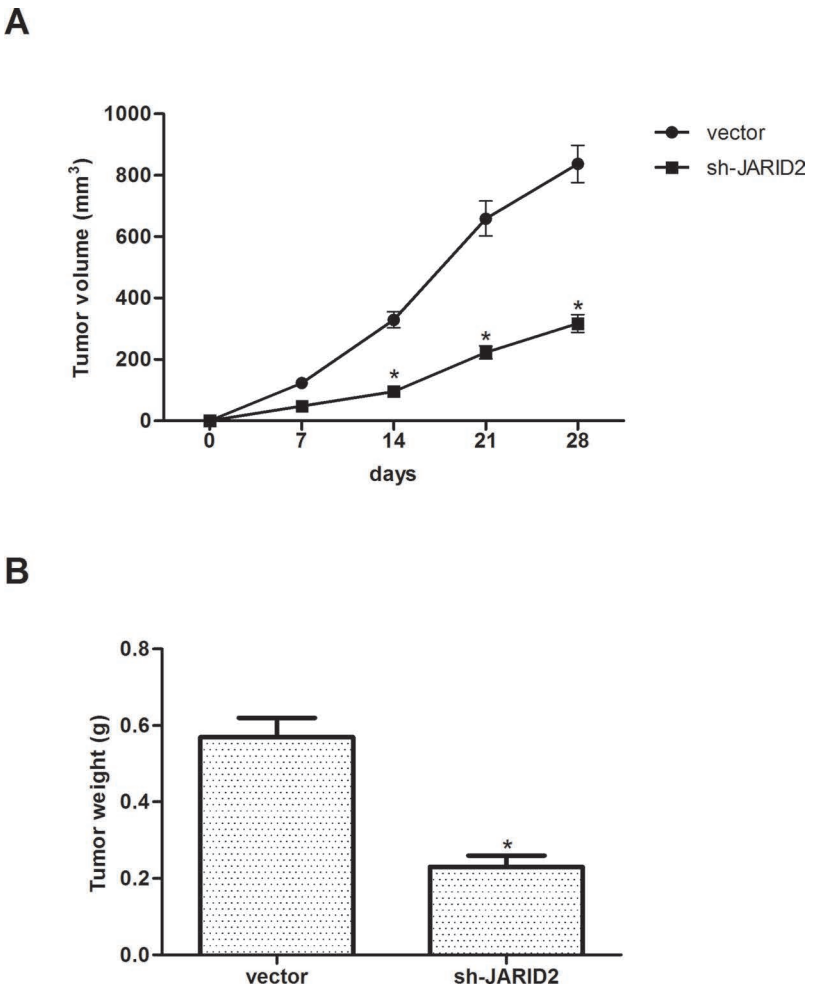

Figure 3. Effects of JARID2 on tumor growth in a xenograft model. JARID2-depleted or control U87MG cells were injected subcutaneously into the left flank of nude mice, and then the tumor growth was monitored. (A) Growth curves of tumor size. (B) The tumor weights of the two groups. Results presented are mean ± SD. $*p < 0.05$ versus vector group.

xenograft model. We found that knockdown of JARID2 remarkably attenuated the subcutaneous tumor volume (Fig. 3A). In addition, mean tumor weight in the sh-JARID2 group was also significantly lower than those of the vector group (Fig. 3B).

Effects of JARID2 on Glioma Cell Migration and Invasion

We performed Transwell migration and Matrigel® invasion assays to investigate the effects of JARID2 on glioma cell migration and invasion. As shown in Figure 4A and B, the average migrating or invading cell count of sh-JARID2-infected U87MG cells was much less than that of vector-infected cells. To better elucidate the effects of JARID2 on glioma cell migration and invasion, we examined the expression of epithelial–mesenchymal transition (EMT)-related markers using Western blotting. As indicated in Figure 4C, knockdown of JARID2 caused a 2.7-fold increase in E-cadherin expression and a significant decrease in N-cadherin expression in U87MG cells.

Effects of JARID2 on the PI3K/Akt Pathway in Glioma Cells

The PI3K/Akt signaling pathway plays an essential role in the development and progression of glioma[14]. Thus, in order to investigate the potential mechanism by which JARID2 affected glioma cell proliferation and invasion, we examined whether JARID2 suppressed activation of the PI3K/Akt signaling pathway in U87MG cells. As indicated in Figure 5, knockdown of JARID2 significantly reduced phosphorylation levels of PI3K and Akt, compared with the vector group.

DISCUSSION

To our knowledge, the present study was the first to reveal the role of JARID2 in glioma. Our data indicated that JARID2 is upregulated in human glioma tissues and

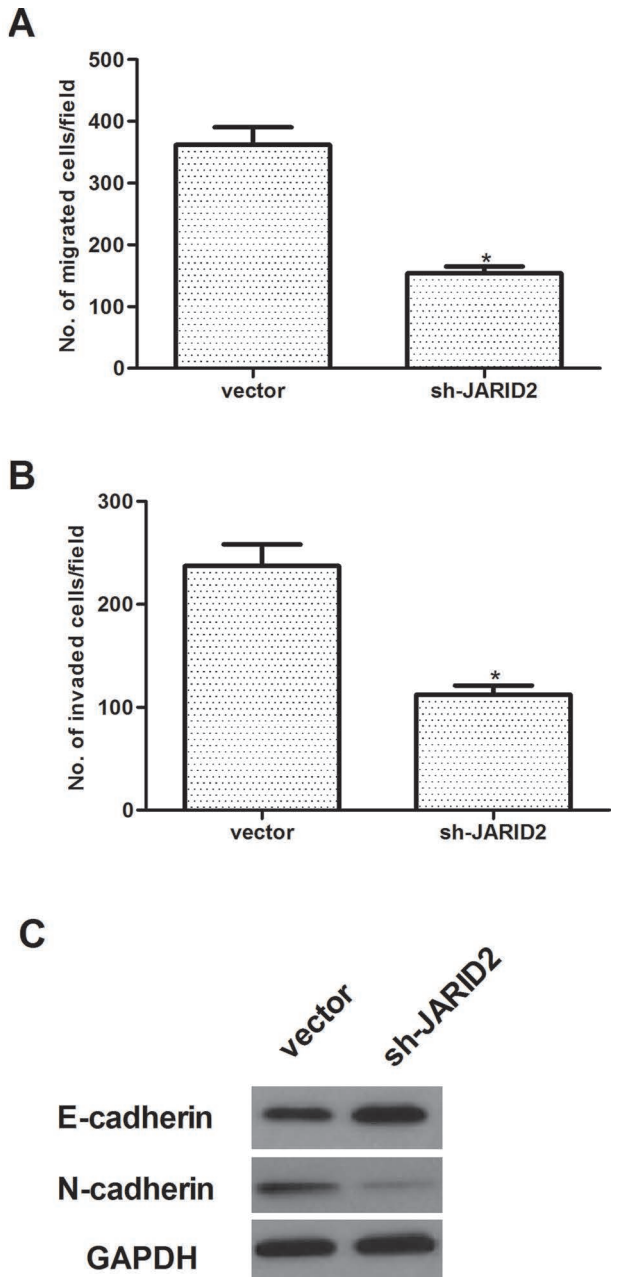

Figure 4. Effects of JARID2 on glioma cell migration and invasion. U87MG cells were transfected with sh-JARID2 or vector for 24 h. (A) Cell migration was evaluated by Transwell migration assay. (B) Cell invasion was determined using the Matrigel® invasion assay. (C) The protein levels of E-cadherin and N-cadherin were detected with Western blotting, and the relative protein expression levels of E-cadherin and N-cadherin were quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. Results presented are mean ± SD. *$p < 0.05$ versus vector group.

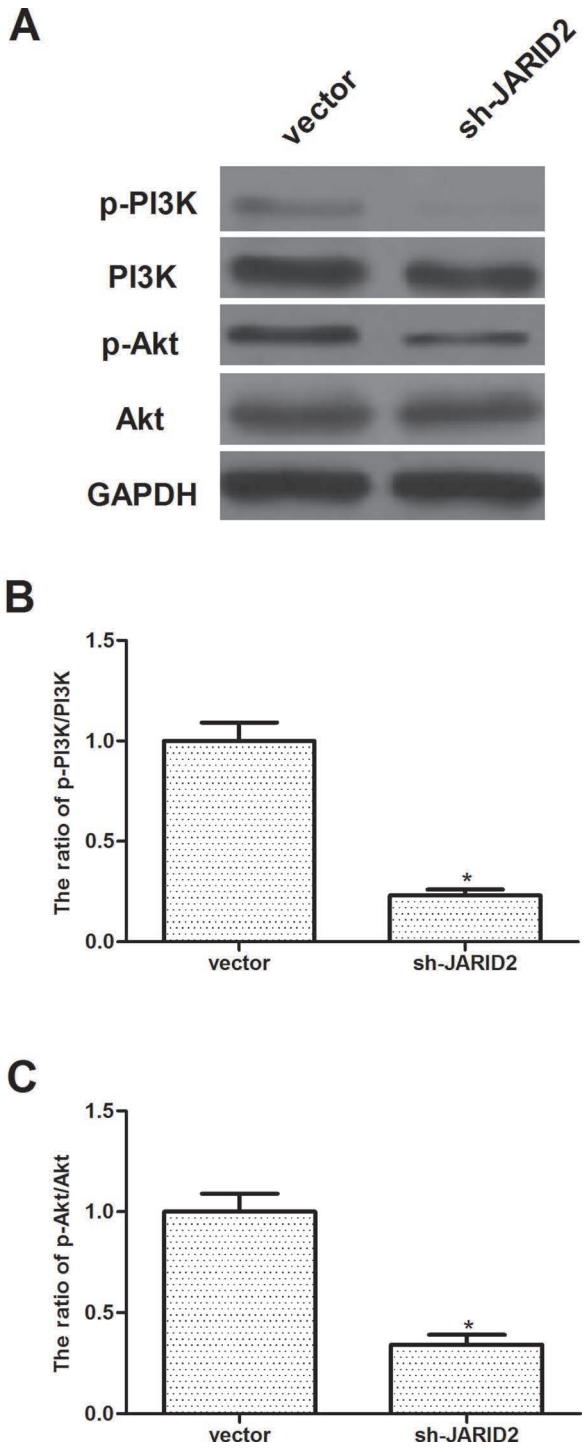

Figure 5. Effects of JARID2 on the PI3K/Akt pathway in glioma cells. (A) U87MG cells were transfected with sh-JARID2 or vector for 24 h. The PI3K and Akt phosphorylation was determined by Western blotting using phospho-PI3K- and phospho-Akt-specific antibody, respectively. (B, C) Quantification analysis of p-PI3K/PI3K and p-Akt/Akt was performed using the Gel-Pro Analyzer version 4.0 software. Results presented are mean ± SD. *$p < 0.05$ versus vector group.

cell lines. Knockdown of JARID2 obviously inhibits the proliferation of U87MG cells and the tumor growth in vivo. Furthermore, knockdown of JARID2 inhibits migration and invasion, as well as the EMT process in U87MG cells. Mechanistically, knockdown of JARID2 reduces phosphorylation levels of PI3K and Akt in U87MG cells.

JARID2 has been reported to be overexpressed in several human carcinomas, and the expression levels were associated with tumor progression[15–17]. Walters et al. confirmed that JARID2 is highly expressed in rhabdomyosarcoma tissues; silencing JARID2 reduced the proliferation of rhabdomyosarcoma cells by inducing myogenic differentiation[12]. However, as far as we know, there are no studies in the literature regarding the role of JARID2 in glioma. In agreement with the above findings, we found that JARID2 is upregulated in human glioma tissues and cell lines, and in vitro cell experiments confirmed that knockdown of JARID2 obviously inhibits the proliferation of U87MG cells. In xenograft experiments, tumor cells silencing JARID2 showed a decreased growth rate and tumorigenic capacity. Taken together, these results suggest that JARID2 may function as an oncogene in the development and progression of glioma.

Tumor metastasis is a complex process involving a complicated succession of invasion–metastasis steps[18]. EMT is one of the major molecular mechanisms involved in oncogenesis to promote tumor metastasis. It is characterized by the loss of adhesion, epithelial E-cadherin expression, and the acquisition of mesenchymal characteristics, such as expression of N-cadherin and vimentin and invasive motility[19,20]. Furthermore, one study report revealed that JARID2 knockdown inhibited TGF-β-induced changes of EMT-related genes, such as E-cadherin and fibronectin in human colon cancer cells[11]. In the present study, we found that knockdown of JARID2 inhibits the migration and invasion, as well as the EMT process, in U87MG cells. These results suggest that knockdown of JARID2 blocks the migration and invasion of U87MG cells by repressing the EMT process.

The PI3K/Akt signaling pathway is essential to the development and/or progression of most cancer types, including glioma[21–23]. Akt, a serine/threonine protein kinase, was activated through the PI3K pathway, which has been involved in cancer cell proliferation, resistance to cell death, invasiveness, angiogenesis, and metastasis[24–26]. Thus, inhibition of PI3K/Akt signaling may represent a potential strategy for glioma[22]. It was reported that activation of all three PI3K pathway members was significantly more frequent in glioblastoma and was closely related with reduced survival times[27]. Most interestingly, Lei et al. reported that overexpression of JARID2 significantly promoted proliferation, migration, and invasion through the activation of Akt in hepatocellular carcinoma cells[10]. Consistent with the previous studies, we observed that knockdown of JARID2 reduced phosphorylation levels of PI3K and Akt in U87MG cells. These results suggest that knockdown of JARID2 suppressed proliferation and tumorigenesis in glioma cells via inactivation of the PI3K/Akt signaling pathway.

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