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Original Article

Down-regulation of AQP8 suppresses glioma cells growth and invasion/migration via cell cycle pathway

Rui Dong, Shengzhong Tao, Zhan Liu, Wei Zheng, Dan Yu

Department of Neurosurgery, The Second Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China

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Abstract: Aquaporin8 (AQP8), has a pivotal role in keeping the fluid and electrolyte balance, which is weakly distributed in mammalian brains. Overexpression of AQP8 has been reported in several types of human cancer but roles in human glioma have yet to be clearly defined. Here, we investigated the expression changes of AQP8 in 35 cases of human brain glioma tumors using Real-time PCR, and then down-regulated expression of AQP8 gene in human glioma U373 and T98G cells using specific targeted short hairpin RNA (shRNA). Its specific functions and mechanisms in human glioma cells were investigated as a potential therapeutic target for glioma. In this study, we found that AQP8 expression level was up-regulated in gliomas than normal brain tissues. Down-regulation of AQP8 in U373 and T98G cells showed a significant inhibitory effect on cell proliferation and invasion/migration, which accompanied with cell cycle S arrest as well as down-regulating the cell cycle protein expression. In summary, this study suggested that AQP8 suppresses glioma cells growth and invasion/migration via cell cycle pathway, and may be useful for developing a new therapeutic strategy for glioma.

Keywords: Aquaporin8, glioma cells, proliferation, invasion, cell cycle

Introduction

Glioma is the most common primary brain tumors in central nervous system, with a survival rate of 20% ~ 30% only in 2 years, posing a serious threat to human health [1]. Although the treatment has been improvements due to the progress of the gene therapy, the mechanisms of glioma growth and invasion are yet to emerge. Previous studies have demonstrated aquaporins (AQPs) involved in cell migration, angiogenesis, and tumor growth directly as a key factor [2-5], and may accelerate glioma cell migration by facilitating rapid changes in cell volume that accompany the changes in cell shape [6].

The AQP8 gene, situated in chromosome 16 p12 [7], encodes a 261 amino acid protein that participates in water metabolism, may contribute to the proliferation of astrocytomas, and tumor cells overexpressed AQP8 with an intracellular distribution, especially glioma. AQP8 may be a biomarker and candidate therapy target for glioma patients, as its protein and mRNA expression are strongly elevated in glioblastoma tissues [8], However, the molecular mechanism of AQP8 during the growth and invasion of glioma have not been fully established yet.

Therefore, in this study, we compared the expression level of AQP8 in glioma and matched adjacent nontumorous tissues, and then we explored the functions of AQP8 that involves cell proliferation, invasion and cell cycle in glioma cells, verify it at the histological and cytological level. AQP8 expression is strongly elevated in glioma tissues, and hence suggesting its vital role in the growth of glioma cell.

Materials and methods

Patients and cell culture

35 pairs of human glioma and matched adjacent nontumorous tissue samples were collected from 2012 to 2014 at Second Affiliated Hospital of Zhengzhou University. The ages of the patients ranged from 35 to 75 years old. The pathological grades of the 35 tumor specimens were defined as follows: 7 cases of grade I, 9 cases of grade II, 10 cases of grade III, and 8 cases of grade IV. All human tissues were acquired with informed consent and the pathological grades of tumor were determined by

neuropathologist according to the WHO criteria for gliomas. Human glioma tissue samples were stored at -80°C.

Glioma cell lines (U373 and T98G) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin, which were obtained from the Cell Bank of the Chinese Academy of Sciences. Cells were maintained in a humidified incubator at 37°C and supplemented with 5% CO_2.

RNA extraction and real-time PCR

Total RNA from 35 of glioma tissue samples and glioma cell lines were extracted with TRIzol reagent (Invitrogen, USA). Complementary DNA synthesis was performed by PrimeScript RT reagent kit (Takara, China). Real-time PCR (RT-PCR) was carried out using SYBR-Green PCR Kit. The primers for AQP8 were as followed: forward 5'-GAGGAGGCTCTAGGTTCTTG-3', and reverse 5'-GCGGGAAATGAGCTGATG-3'. The primers for GAPDH which acted as an internal control were as followed: forward 5'-CACCCACTCCTCCACCTTTG-3', and reverse 5'-CCACCACCCTGTTGCTGTAG-3'. The expression level of AQP8 was determined by TaqMan miRNA assays (Fermentas, USA) according to the provided protocol, and U6 small nuclear RNA was used to normalize the expression.

AQP8 shRNA design and cell transfection

The primers were shown as follow: AQP8-1 shRNA forward: 5'-CCGGGAGCCTGAATTTGGCAATGACTCGAGAGTCATTGCCAAATTCAGGCTCTTTTTC-3', reverse: 5'-AATTGAAAAAGAGCCTGAATTTGGCAATGACTCGAGAGTCATTGCCAAATTCAGGCTC-3'; AQP8-2 shRNA forward: 5'-CCGGGAGAGGTTCTGGAATGCATCTTCGAGAAGATGCATTCCAGAACCTCTCTTTTTC-3', reverse: 5'-AATTGAAAAAGAGAGGTTCTGGAATGCATCTTCGAGAAGATGCATTCCAGAACCTCTC-3'; AQP8-3 shRNA forward: 5'-CCGGGAGGCTCTAGGTTCTTGGAATTCGAGAATTCCAAGAACCTAGAGCCTCTTTTTC-3', reverse: 5'-AATTGAAAAAGAGGCTCTAGGTTCTTGGAATTCGAGAATTCCAAGAACCTAGAGCCTC -3'.

U373 and T98G Cells were seeded into 6 well plates and transfected in Opti-mem (Gibco, USA), Control, NC or AQP8 shRNA, using Lipofectamin 2000 (Invitrogen, USA).

Cell proliferation

Forty-eight hours after transfection, monolayers were scratched with a plastic pipette tip and washed twice with PBS to move away dead cells and hatched in serum-free media with a concentration of 3×10^4 cell/ml. At the designated time (0, 24, 48 and 72 h after scratching), mixed in 1:10 volume ratio with Cell Counting Kit -8 (CCK-8) and serum-free media, then incubation 1 h. The cell viability was assayed according to the manufacturer's protocol by enzyme micro-plate reader (Perlong, Beijing, China) and repeat in three times. The mean number of cells of the control group was described as 100% and the mean number of other groups was normalized by means of the control group, respectively.

Flow cytometry

For cell cycle assay, 48 h of post-transfection, the glioma cells were harvested and fixed in 70% ice-ethanol at 4°C overnight. Then the cells were subsequently in 300 ml PBS with 25 ml propidium iodide (PI) and 10 ml RNase at 37°C for 30 min. The DNA content was quantified by FACS Calibur flow cytometer (BD Biosciences, USA). The results were modified using FLOWJO software.

Transwell invasion assay

To assess cell invasion in vitro, cells (104 in serum-free medium) were placed into the top chamber of transwell coated with 150 mg Matrigel (BD Biosciences, USA). After 48 h of incubation at 37°C, cells adhering to the lower membrane were fixed and stained with 0.1% crystal violet and imaged using microscope (Olympus).

Western blotting

Total protein from tissue samples and cell lysis were prepared by RIPA buffer. Western blot analysis was performed with standard procedure. Proteins were separated by 10% SDS-PAGE and transferred to membranes. After incubating with primary antibodies, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG as secondary antibodies and using ECL methods to blotting. The primary antibodies were as followed: anti-AQP8, anti-CCNB1, anti-CCNB2, anti-CDK1, anti-KIF4A, anti-PLK4, anti-FEN1 (1:1000; Abcam, UK), anti-GAPDH (1:2000; Abcam, UK).

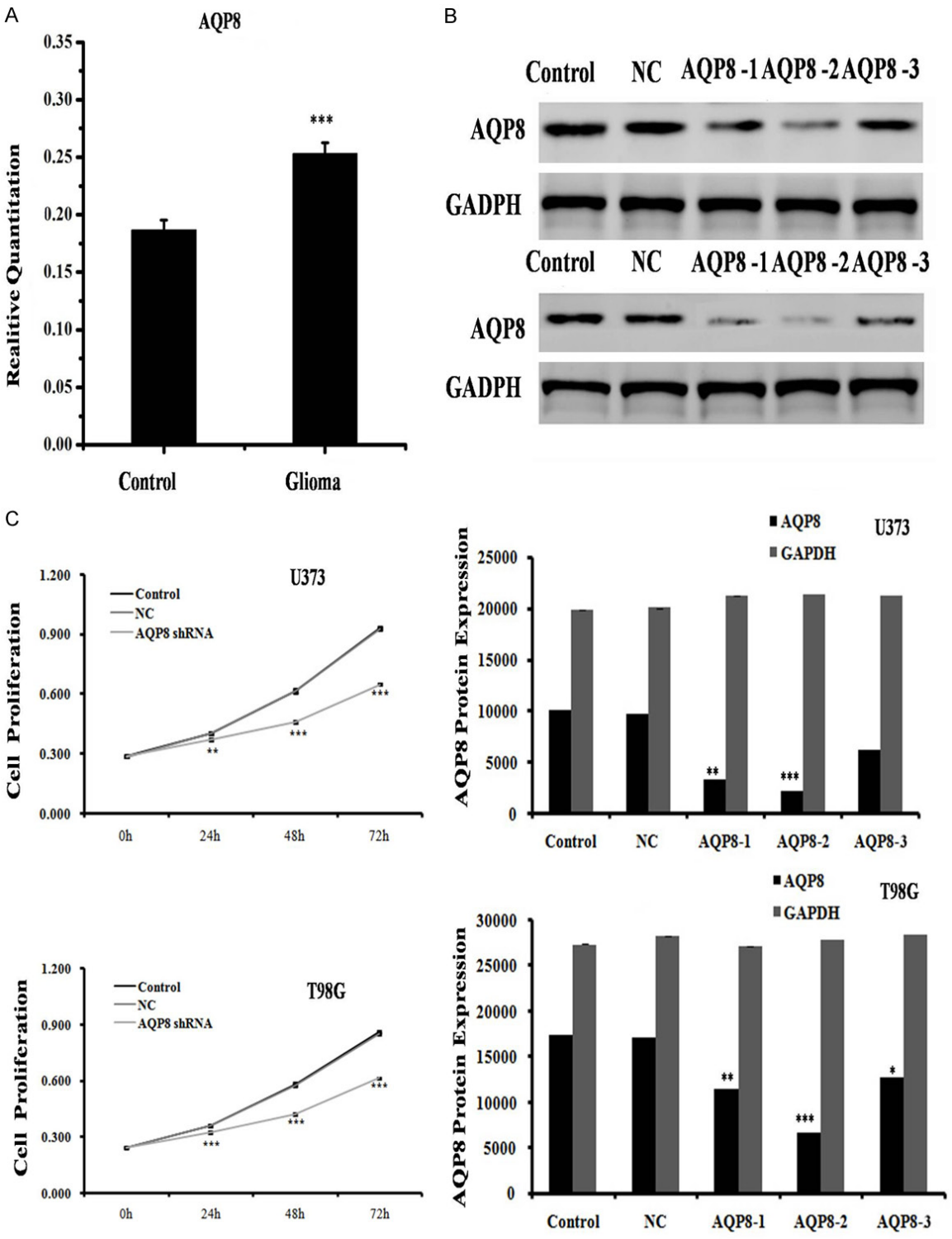


Figure 1. Level of AQP8 is up-regulated in human Glioma tissues and cell proliferation after transfected with AQP8-1 shRNA. A. The AQP8 mRNA levels in 35 paired samples of glioma tissues and adjacent nontumorous tissues were evaluated by RT-PCR. Each bar represents the mean ± SD from three samples (***P < 0.001 vs. the control). B. Three interference targets of AQP8 gene shRNA were used to transfect U373 and T98G cells, we found that the best sits of interference effectiveness was AQP8-2. C. The cells viability was measured at 24, 48 and 72 h after transfection. Data are either representative of three similar experiments or are shown as mean ± SD of five experiments (***P < 0.001 vs. NC).

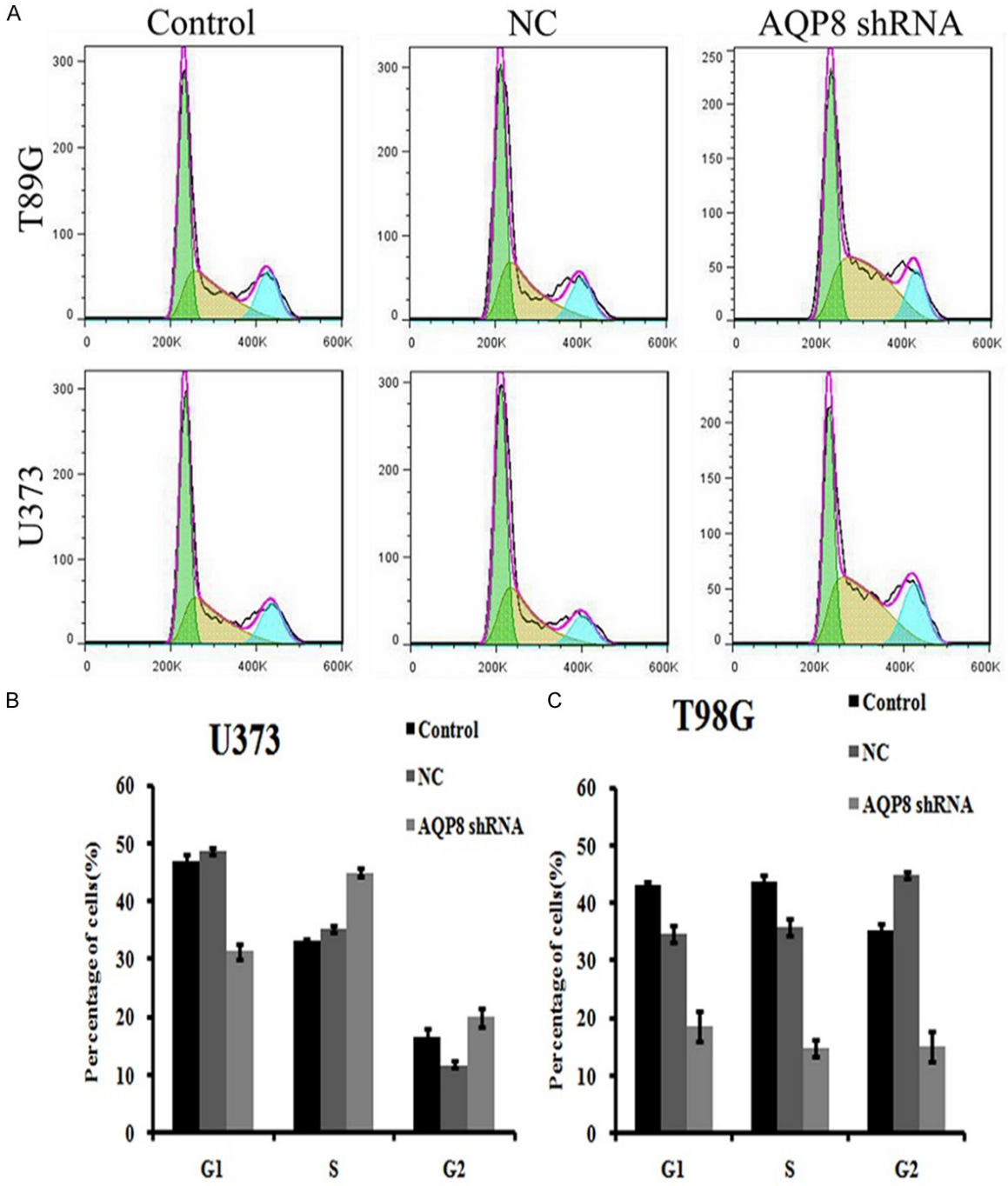


Figure 2. The influence on cell cycle progression of glioma cells by transfected with AQP8 shRNA. The cell cycle distribution of U373 and T98G cells transfected with AQP8 shRNA or NC were measured using propidium iodide staining and flow cytometry analyses.

Statistical analysis

Experiments were carried out at least in triplicate and results were expressed as mean ± S.D. Statistical analysis was performed with SPSS 21.0 (SPSS Inc., Chicago, IL). A value of $P < 0.05$ was considered to be statistically significant.

Results

AQP8 is overexpressed in glioma cells

AQP8 within the AQP family, is widely expressed in the epithelia of digestive tract, liver, Pancreas [9] and reproductive systems [10]. However, the expression of AQP8 in glioma cells is rarely

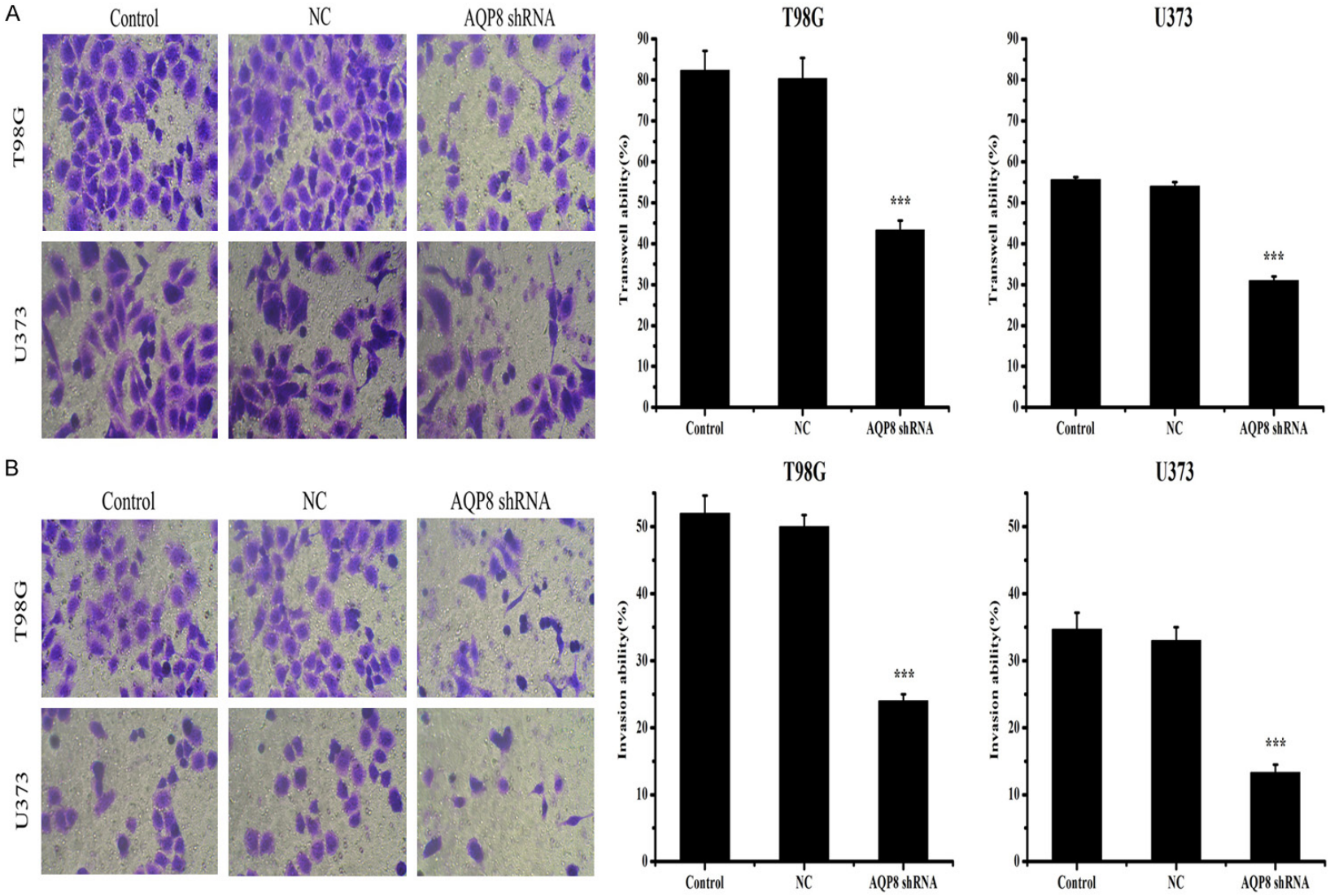


Figure 3. The effects of the down-regulation of AQP8 on the invasion and migration of glioma cells. Transwell assays for assessing the invasion and migration of the AQP8 shRNA-treated cells. Down-regulation of AQP8 suppressed the invasion and migration ability of glioma cells. The suppression of invasion and migration induced by AQP8 down regulation in U251 and U87 cells. Data were presented as mean ± SD in three biological replicates (***P < 0.001 vs. NC).

mentioned and the specific mechanism of promote the growth of glioma remains not fully understood. Recent studies have already shown that the expression of AQP8 was related to the pathological grade of astrocytomas, and may have an important role in malignant progression of brain astrocytic tumors [5]. To analyze the expression of AQP8 in glioma, the mature AQP8 was detected in 35 pairs of glioma and adjacent nontumorous brain tissues using RT-PCR. The results showed that the expression of AQP8 was significantly upregulated in glioma when compared to the nontumorous tissues ($P < 0.001$) (**Figure 1A**). Then we structured three interference targets of AQP8 gene shRNA to transfect U373 and T98G cells, After transfection AQP8 shRNAs targeting these three genes respectively into glioma cells, we found that the best sits of interference effectiveness was AQP8-2 (**Figure 1B**).

Down-regulation of AQP8 suppresses proliferation and cell cycle progression of glioma cells

Studies have shown that AQP8 can promote the growth of tumor cells, in this study, to evaluate the contribution of AQP8 down-regulation by AQP8 shRNA to cell growth and cycle regulation, AQP8 shRNA was transfected into U373 and T98G cells separately. The results indicated that low-expression of AQP8 in glioma cells suppresses growth and cell cycle progression. More importantly, our results revealed the inhibition of growth of glioma cells by AQP8 shRNA, and this effect can change with time ($P < 0.01$) (**Figure 1C**). Furthermore, the percentage of cells in individual cell-cycle phases was assessed, and representative histograms are shown in (**Figure 2A**). These data suggested that down-regulation of AQP8 can inhibit the growth, the percentage of cells in S phase were declined indicating that DNA synthesis was retarded of U373 and T98G cells.

Down-regulation of AQP8 inhibits the migration and invasion of glioma cells

The migration of cells to the wound area was analyzed at 48 h after injury, and the results revealed that the low-expression of AQP8 led to a marked inhibition of wound healing compared with the negative control, indicating impaired migration ($P < 0.001$, **Figure 3A**). As shown in **Figure 3B**, the low-expression of AQP8 significantly impaired the invasion of both U373 and T98G cells across a Transwell chamber compared with the negative control and scramble group ($P < 0.001$). Collectively, these data indicate that AQP8 play a role in glioma cell migration and invasion in vitro. (**Figure 3**).

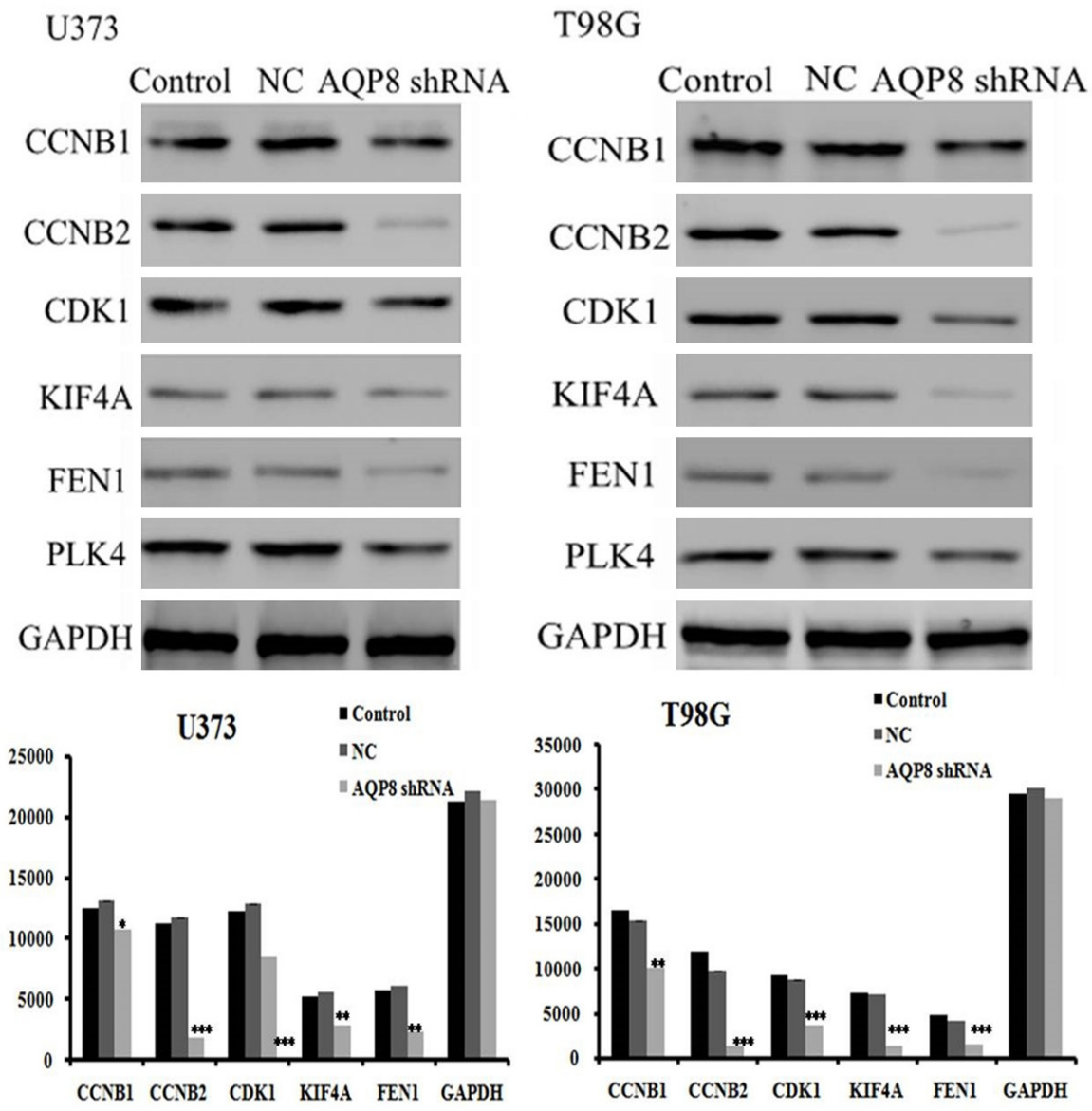
Down-regulation of AQP8 suppresses the cell cycle protein expression of glioma cells

To explore the possible underlying molecular mechanism, the cell cycle protein expression was examined in U373 and T98G cells after transfection (**Figure 4**). Our data showed that down-regulation of AQP8 significantly decreased the expression of cyclin B1 (CCNB1), cyclin B2 (CCNB2), cyclin-dependent kinase 1 (CDK1), chromokinesin KIF4A, Flap endonuclease-1 (FEN1) and Polo-like kinase 4 (PLK4) (**Figure 4**).

Discussion

AQPs, a family of hydrophobic, small, and integrated transmembrane glucoproteins (30 kDa monomer) [11], are involved in multiple physiological and pathological processes in the human body, and are closely associated with many types of human tumors [12, 13]. So far found in the body of water channel protein in mammals, a total of 13 kinds, namely AQP0 ~ AQP12 [14, 15], only 6 of them distributed in brain tissue of a total of 6 kinds of AQP1, AQP3, AQP4, AQP5, AQP8 and AQP9. Zhang et al. [8] reported that AQP8 was mainly distributed in the cytoplasm of astrocytoma cells. The expression levels and immunoreactive score of AQP8 further increased in high-grade astrocytomas, especially in glioblastoma. Therefore, AQP8 may contribute to the proliferation of glioma, however, the molecular mechanism of AQP8 during the growth of glioma have not been fully established yet. In this study, we detected the expression level of AQP8 mRNA in 35 pairs of human glioma and matched adjacent nontumorous tissues, and found that AQP8 mRNA was significantly up-regulated in glioma tissues than matched adjacent nontumorous tissues. Then we structured three interference targets of AQP8 gene shRNA to transfect U373 and T98G cells to find the best sits of interference effectiveness.

Glioma, one of the common malignant tumors of the primary central nervous system, which was cured by clinical surgery combined radiotherapy, chemotherapy and other adjuvant



Group	U373			T98G		
	G1	S	G2	G1	S	G2
Control	45.96	33.39	17.16	43.65	34.98	18.10
	48.31	33.16	14.64	42.46	35.39	17.76
	46.09	33.35	17.64	43.35	33.29	19.74
AVERAGE	46.79	33.30	16.48	43.15	34.55	18.53
STDEV	1.32	0.12	1.61	0.62	1.11	1.06
NC	48.13	35.97	12.29	42.62	37.24	14.30
	49.23	34.61	11.12	45.52	34.45	14.69
	48.90	35.15	11.94	43.36	35.46	15.28

Figure 4. The cell cycle protein expression was examined in U373 and T98G cells after transfection. Down-regulation of AQP8 significantly decreased the expression of CCNB1, CCNB2, CDK1, KIF4A, FEN1 and PLK4.

therapy integrated mode, however the effect was unsatisfactory. Highly aggressive was a major cause of the high fatality and recurrence rate [16]. Therefore, it is of great significance to

suppress glioma cells growth and invasion/migration. Migration is the precondition of tumor cell invasion and metastasis [17]. Our study found that down-regulation of AQP8 suppresses U373 and T98G cells invasion/migration via suppressing the cell cycle protein expression.

In previous studies, the investigations of suppressing in glioma cell lines were focused on cell cycle arrest, mitochondrial pathway or oxidative stress. Our results indicated that down-regulation of AQP8 in U373 and T98G cells showed a significant inhibitory effect on cell proliferation and invasion/migration, which accompanied with cell cycle S arrest as well as decreased the expression of CCNB1, CCNB2, CDK1, KIF4A, FEN1 and PLK4.

CCNB1 and CCNB2 have unique amino-terminal segments but are highly homologous over the last 300 residues [18-20]. Both proteins activate Cdk1 and are expressed in most cell lines, tissues and organs with a high mitotic index, are the key molecule to start the mitotic [21, 22]. B-type cyclin levels are low in G1, gradually increase during S and G2, and peak during mitosis [23, 24]. Although CCNB1 is a cell cycle protein of M phase, acting on the G2/M checkpoint, it also can appear in different periods of cycle arrest [25]. KIF4A is a key protein which ensures normal cell division, the abnormal expression of KIF4A may lead to the occurrence of tumor. KIF4A is also one of motor proteins in cells with tubes for orbit, May regulation of cell migration through the directional transfer the key protein molecules in the process of migration of cell movement [26]. The expression level of FEN1 is associated with tumor malignant degree and regulates cell cycle of S phase [27].

The results of the present study, for the first time, support a model in glioma U373 and T98G cells whereby down-regulation of AQP8 suppressing the growth and invasion/migration via cell cycle pathway, In conclusion, our study demonstrated that AQP8 is significantly up-regulated in glioma cells. Thus, down-regulation of AQP8 in glioma could be further investigated which allow for more effective therapeutic strategy, as well as additional targets for the clinical drugs development.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shengzhong Tao Tao, Department of Neurosurgery, The Second Affiliated Hospital of Zhengzhou University, 2 Jingba Road, Zhengzhou 450014, Henan, China. Tel: 86-371-63620617; Fax: 86-371-63620617; E-mail: taoshengzhou321@163.com

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