

# Interactions of connexin 43 and aquaporin-4 in the formation of glioma-induced brain edema

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**Abstract.** Connexin 43 (Cx43) and aquaporin-4 (AQP4) have important roles in the formation of glioma-induced brain edema; however, the association between these two factors in the development of edema has remained to be elucidated. In the present study, immunofluorescence and western blot analysis revealed that in a rat model of intracranial C6 glioma, Cx43 expression levels were low to undetectable and AQP4 expression levels were low in glioma cells. Significantly higher Cx43 and AQP4 levels were detected in the tissue surrounding the glioma. To further investigate the potential interaction between Cx43 and AQP4, normal glial cells and C6 glioma cells were cultured in hypotonic medium. Reverse transcription quantitative polymerase chain reaction indicated that AQP4 and Cx43 mRNA expression levels increased as a function of time in normal glial cells and C6 glioma cells in a hypotonic environment. However, the increase observed in normal glial cells was significantly lower than that observed in C6 glioma cells. Furthermore, AQP4 expression levels changed prior to alterations in Cx43 expression. Following AQP4 silencing in C6 cells, the increase in Cx43 expression was significantly attenuated ( $P < 0.05$ ). In normal cells, Cx43 silencing did not influence AQP4 expression ( $P > 0.05$ ). Therefore, it was hypothesized that AQP4 and Cx43 had two distinct mechanisms underlying brain edema formation within and surrounding the glioma. Cx43 may be a downstream effector of AQP4. The elucidation of this pathway may aid in the development of drugs targeting the interaction between AQP4 and Cx43, providing novel therapeutic possibilities for glioma-induced brain edema.

## Introduction

Glioblastoma accounts for ~10% of intracranial tumors and ~22.3% of neuroepithelial tumors and is the second most common intracranial tumor (1,2), mainly occurring in

individuals aged 30-50 years. The ratio of male to female incidence is ~2.5:1 (1,3). Glioblastoma located under the cortex displays infiltrative growth and frequently encroaches upon multiple brain lobes, has a deep structure and spreads to the contralateral cerebral hemisphere via the corpus callosum (4). Glioblastoma is highly malignant, grows rapidly and has a short disease progression (5). Symptom appearance is commonly within three months, and within six months in ~70-80% of patients (6).

The rapid growth of glioblastoma is associated with extensive cerebral edema and marked intracranial hypertension (7,8). The majority of patients suffer from headaches, vomiting and papilledema. Computed tomography revealed lesions with inhomogeneous density, unclear boundaries and apparent brain edema surrounding the lesions (9). Enhancement scanning further revealed inhomogeneous enhancement or rim enhancement (10). At least one third of all glioblastoma mortalities are a result of brain edema and herniation (11). The hyperosmotic dehydrants, diuretics and hormones currently used for the symptomatic treatment of glioblastoma have poor outcomes (12); therefore, it is important to elucidate the molecular mechanisms underlying the development of glioblastoma-induced brain edema in order to aid in the development of more effective treatment plans.

Cell-cell communication is the basis of molecular transmission and transport among connecting cells (13-15). Connexin is composed of six polygonal subunits arranged in a circular shape to form a hydrophilic channel (16). This channel allows the transport of ions, sugars, nucleotides, fatty acids and low-molecular-weight polypeptides, as well as certain drugs and carcinogens whose molecular weight is below 1,000-1,600 Da (17-21). Connexin 43 (Cx43) is expressed in the brain with a high specificity and forms the structural basis for substance transfer among neurons and accessory cells, in particular, glial cells (22,23). Aquaporins are a family of water channel proteins that have critical roles in regulating the water content of cells under pathological and physiological conditions (24-26). Aquaporin-4 (AQP4) is specifically expressed in the brain and spinal cord, where it has an important role in maintaining the correct water balance in brain tissues (27,28).

Previous studies have mainly focused on the role of single genes in glioma-induced brain edema formation and have rarely analyzed the interaction between genes (29). The present study aimed to evaluate differences in Cx43 and AQP4 expression levels in glioblastoma tumors and the surrounding

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edema *in vivo*, in order to aid elucidation of the mechanisms underlying edema formation.

To further investigate the interaction between Cx43 and AQP4 in glioblastoma-induced brain edema formation, hypotonic medium was used to simulate the environment of brain edema *in vitro* and determine the effects on AQP4 and Cx43 messenger RNA (mRNA) expression levels in C6 glioma cells and normal glial cells. The effects of silencing of AQP4 in C6 glioma cells and Cx43 in glial cells under hypotonic conditions were also evaluated. The results of the present study may present a novel perspective on the mechanisms underlying glioma-induced brain edema formation and therefore aid in the development of drugs targeting the interaction between AQP4 and Cx43 for the treatment of glioma-induced brain edema.

## Materials and methods

**Reagents and animals.** Murine C6 glioma cells (Shanghai Cancer Institute, Shanghai, China), fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Huzhou, China), Dulbecco's modified Eagle's medium and trypsin (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA), Lipofectamine® 2000 (Invitrogen Life Technologies), short interfering RNA (siRNA) targeting AQP4 and Cx43 (synthesized by Wuhan Genesil Biotechnology Co., Ltd., Wuhan, China), reverse transcription quantitative polymerase chain reaction (RT-qPCR) kit (Beijing Zhongshan Co., Beijing, China), PCR primers (synthesized by Beijing Sunbiotech Company, Beijing, China), rabbit anti-rat Cx43 polyclonal antibody (Chemicon, Billerica, MA, USA) and rabbit anti-rat AQP4 polyclonal antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) were used in the present study. A total of 20 male Sprague-Dawley rats aged 7-8 weeks and weighing  $200 \pm 10$  g were purchased from the Animal Center of Academy of Military Medical Sciences (Beijing, China) and housed in the Experimental Animal Center (Tianjin Medical University, Tianjin, China) under controlled temperature ( $22 \sim 24^{\circ}\text{C}$ ) conditions with a 12 h light/dark cycle. The lights were on from 8:00 to 20:00. Food and water were made available *ad libitum*. Experiments were performed during the light phase of the cycle. All experimental procedures were carried out according to the regulations and internal biosafety and bioethics guidelines of Tianjin Medical University and the Tianjin Municipal Science and Technology Commission

**Establishment of intracranial glioma model and magnetic resonance imaging (MRI).** C6 glioma cells in the logarithmic phase were digested into a single cell suspension. Rats were anesthetized by intraperitoneal injection of ketamine/xylazine (100 g/kg ketamine, Parke-Davis, New Jersey, USA; 5 mg/kg xylazine, Bayer AG, Leverkusen, Germany), and then were fixed on benches with a stereotactic injection device and shaved. A C6 glioma cell suspension ( $1 \times 10^6$  cells in  $25 \mu\text{l}$ ) was injected into the brain in the right caudate nucleus. The duration of injection was  $>5$  min, following which the needle was maintained for at least three minutes and the incision was subsequently sutured. Cranial MRI was conducted at 3, 6, 9 and 12 days post-injection to observe tumor growth and edema.

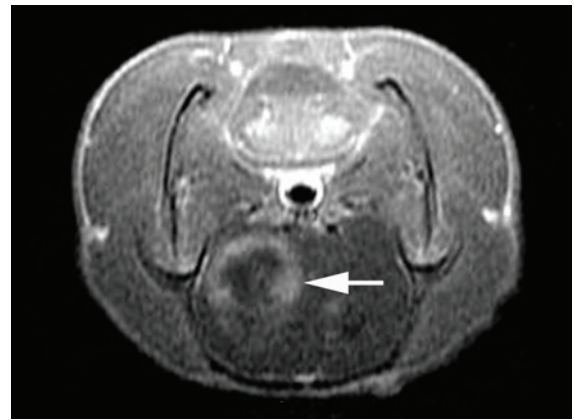


Figure 1. Magnetic resonance image of a rat intracranial glioma. Arrow indicates edema on the edge of the tumor.

**Immunofluorescence to analyze the spatial expression of Cx43 and AQP4 within and surrounding the glioma.** Following the final MRI examination (12 days post-injection), the rats were sacrificed by cervical dislocation. Brain tissues containing the glioma were embedded in paraffin (Zhongshan Biocorp., Beijing, China), sliced into sections and analyzed using immunofluorescence. Briefly, sections were boiled for 5 min in Tris (10 mM)/ethylene diamine tetra acetic acid (1 mM, Zhongshan Biocorp.; pH 9.0) buffer for antigen retrieval, treated by Triton X-100 (Zhongshan Biocorp.), and were blocked with serum (Hangzhou Sijiqing Biological Materials Co., Ltd., Hangzhou, China) for one hour, treated with rabbit anti-rat Cx43 polyclonal antibody (1:500) and rabbit anti-rat AQP4 polyclonal antibody (1:1,000) at  $4^{\circ}\text{C}$  overnight. The sections were subsequently incubated with fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TRITC)-labeled secondary antibodies (FITC or TRITC goat anti-rabbit IgG; Santa Cruz Biotechnology Inc.), followed by mounting and observation under a fluorescence microscope (Olympus DP 70, Tokyo, Japan).

**Western blot analysis for quantitative detection of Cx43 and AQP4 expression levels within and surrounding the glioma.** The remaining brain tissues containing gliomas were dissected. Tumor tissue and 5 mm of the surrounding tissue were stored in liquid nitrogen prior to extraction of total proteins. Total protein was extracted using standard lysis buffer (Sigma, St. Louis, MO, USA) for 10 min. The proteins were quantified using the Bradford method (30), separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with skimmed milk (Zhongshan Biocorp.) for one hour, washed with phosphate-buffered saline (Zhongshan Biocorp.) and incubated with primary antibodies (anti-Cx43, 1:500; AQP4, 1:1,000) at  $4^{\circ}\text{C}$  with horizontal shaking overnight. The following day, the membranes were treated with horseradish peroxidase-labeled secondary antibodies (1:500) at  $37^{\circ}\text{C}$  for two hours and visualized using a Chemiluminescence Detection Kit (Weifang Kanghua Biotech Co., Ltd., Shandong, China). Bio-Rad imaging system (Bio-Rad Laboratories, Hercules, CA, USA) was used to measure absorbance values at a wavelength of 570 nm. Quantity One software version 4.4.1 (Bio-Rad Laboratories) was used to

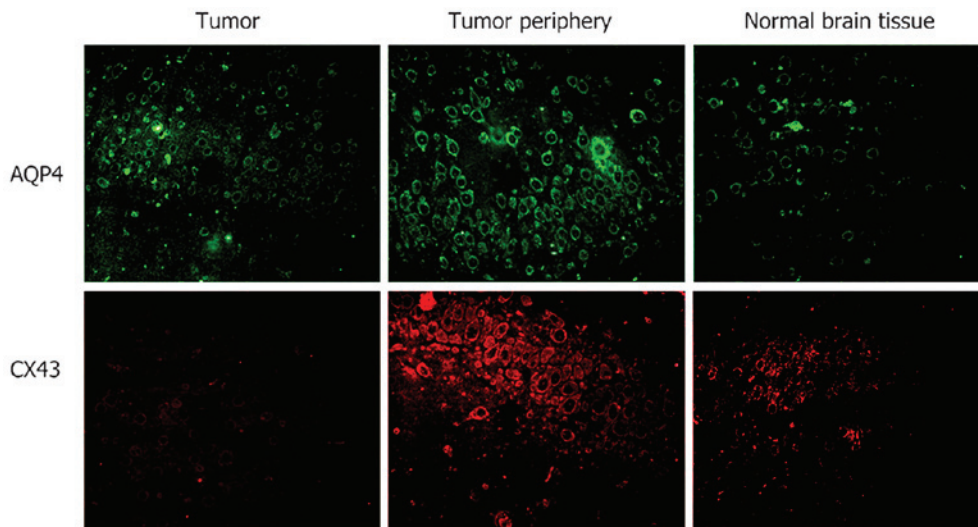


Figure 2. Expression of AQP4 and CX43 in glioma and the tumor periphery *in situ* (magnification, x200). Green fluorescence, fluorescein isothiocyanate-labeled AQP4; red fluorescence, tetramethylrhodamine isothiocyanate-labeled Cx43. AQP4, aquaporin-4; CX43, connexin 43.

analyze the data. The dilution of the control  $\beta$ -actin antibody was 1:200.

**RT-qPCR for the characterization of AQP4 and Cx43 expression in a hypotonic environment.** Subcultured C6 glioma cells and primary cultured normal glioma cells were separately incubated in hypotonic medium at 250-260 mOsm/kg. AQP4 and Cx43 mRNA expression was determined in C6 cells and normal glial cells at 0, 12, 24, 36, 48, 60 and 72 hours. The primers used for analysis were as follows: AQP4 forward, 5'-CCA GCT GTG ATT CCA AAA CGG AC-3' and reverse, 5'-TCT AGT CAT ACT GAA GAC AAT ACC TC-3' (product size, 305 bp); Cx43 forward, 5'-TGT AAC ACT CAA CAA CCT GGC-3' and reverse, 5'-GAT CTT GAT CTT CAT GGT GCT AGG-3' (product size, 764 bp). Reaction conditions were as follows: 32 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 min and extension at 72°C for 1 min, followed by extension at 72°C for 10 min. Time x represents an arbitrary time-point. Time 0 represents a 1-fold target gene expression following  $\beta$ -actin correction.  $\Delta\Delta CT$  was calculated using the formula  $(C_{T,Target} - C_{T,U6})_{Time\ x} - (C_{T,Target} - C_{T,U6})_{Time\ 0}$ .

**AQP4 and Cx43 gene silencing.** AQP4 and Cx43 full-length mRNAs were retrieved from the NCBI gene database (<http://www.ncbi.nlm.nih.gov/gene>). siRNA was designed with gene silencing design software in the coding regions. The oligonucleotide sequence targeting AQP4 was 5'-AGA TCA GCA TCG CCA AGT C-3', and that targeting Cx43 was 5'-UUG AAG UUA UGU AUC CUC CUU-3'. C6 glioma cells and glial cells that were growing successfully were selected for transfection and their media was replaced with fresh media on the day prior to transfection. For transfection, 1  $\mu$ g plasmid DNA and 100  $\mu$ l LipoVec<sup>TM</sup> (Santa Cruz Biotechnology, Inc.) were mixed for 20 min. The mixture was added to the medium for 4 h and subsequently the medium was replaced by medium containing 20% serum. Following 48 h of transfection, fresh medium containing 200 ng/l geneticin (Zhongshan Biocorp.) was used for selection. The selective medium was replaced every three days. Following 14 days of culture, cell colonies

were visible in successfully transfected groups, whereas all non-transfected cells had died. When the number of positive cells reached a certain number, cell clones were transferred into 24-well plates for further passaging. These cells were incubated in hypotonic conditioned medium (Zhongshan Biocorp.) at 250-260 mOsm/kg. AQP4 and Cx43 mRNA expression levels were detected at 0, 12, 24, 36, 48, 60 and 72 h using the method described above.

**Statistical analysis.** Experiments were repeated three times. Data were processed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Values are expressed as the mean  $\pm$  standard deviation. To evaluate differences amongst the groups,  $\chi^2$  analysis was performed. A value of  $P < 0.05$  was considered to indicate a statistically significant difference between values.

## Results

**Establishment of intracranial glioma models and MRI examination.** As displayed in Fig. 1, which was captured on the 10th day post-C6 glioma cell injection, the examination revealed circular tumor shadows in the brain of Sprague-Dawley rats. Cystic degeneration or necrosis was observed in the center of certain tumors. Edema ( $>5$  mm; Fig. 1, red arrow) was observed on the periphery of the majority of tumors evaluated.

**AQP4 and Cx43 expression levels are higher in the tumor periphery than those in the center.** In Fig. 2, green fluorescence indicates FITC-labeled AQP4, and red fluorescence indicates TRITC-labeled Cx43. AQP4 and Cx43 were expressed at low levels in normal brain tissues. In glioma cells, AQP4 was expressed at low levels and Cx43 was expressed at low to non-detectable levels. In the 5-mm area of tissue surrounding the glioma, significantly higher expression levels of AQP4 and Cx43 were detected.

**AQP4 and Cx43 expression levels are altered in glioblastoma tissues.** As exhibited in Fig. 3, relative expression levels of AQP4 were significantly higher in tumor tissues than those in



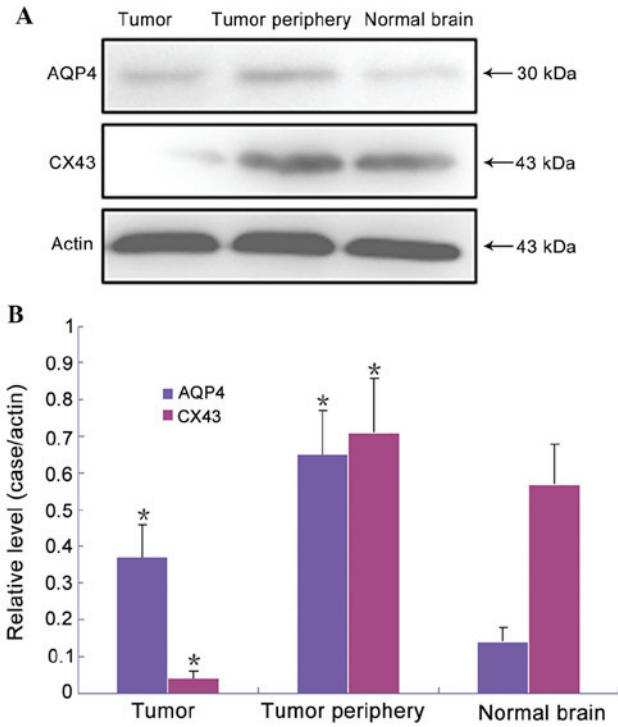


Figure 3. Relative expression of AQP4/CX43 proteins in various regions. (A) Protein expression levels indicated by by western blot analysis. (B) Protein expression levels exhibited as a column diagram. Values are expressed as the mean  $\pm$  standard deviation. \*P<0.05 vs. normal brain tissue. AQP4, aquaporin-4; CX43, connexin 43.

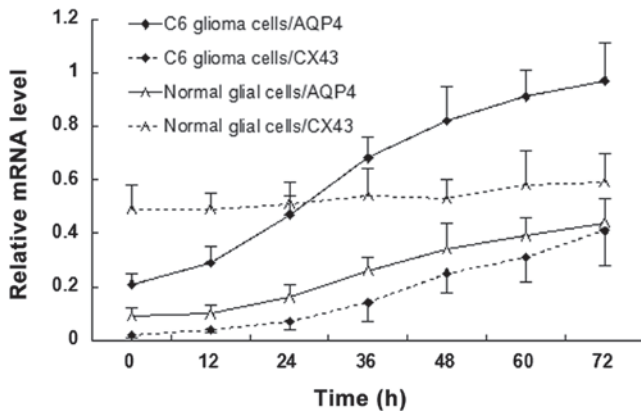


Figure 4. Changes in AQP4 and CX43 mRNA expression levels in C6 glioma cells and normal glial cells cultured under hypotonic conditions. Values are expressed as the mean  $\pm$  standard deviation. AQP4, aquaporin-4; CX43, connexin 43; mRNA, messenger RNA.

normal brain tissues ( $0.37 \pm 0.09$  vs.  $0.14 \pm 0.04$ ), whereas Cx43 expression levels were significantly lower in tumor tissues than those in normal brain tissues ( $0.04 \pm 0.02$  vs.  $0.57 \pm 0.11$ ). In the edema tissue surrounding the tumor, the relative expression levels of AQP4 were significantly higher than those in normal brain tissues ( $0.65 \pm 0.12$ ). Expression levels of Cx43 were higher than those in normal brain tissues, while there was no significant difference ( $0.71 \pm 0.15$ ). Significant differences were detected between the experimental groups and the control group, as determined by paired comparisons ( $P < 0.05$ ).

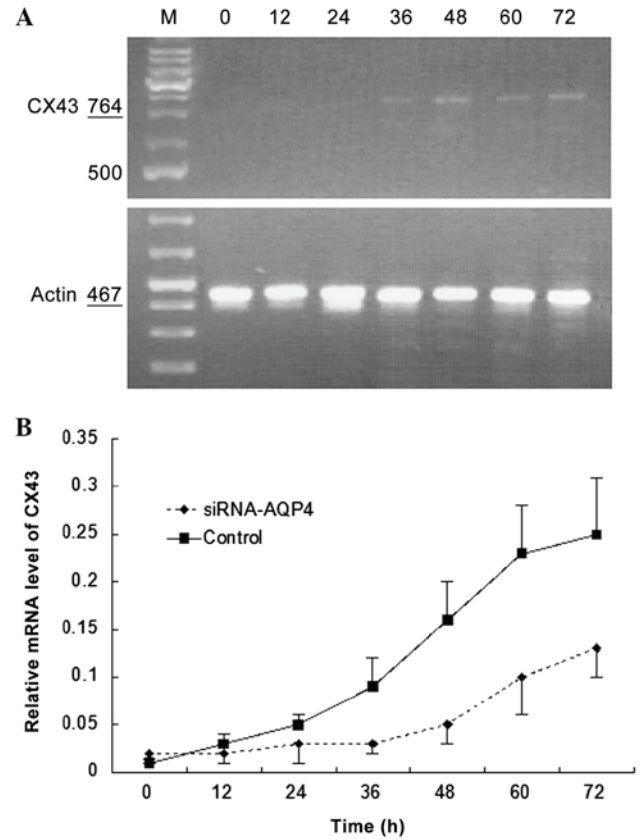


Figure 5. Effects of AQP4 silencing on CX43 mRNA expression in C6 glioma cells cultured under hypotonic conditions. (A) Representative image of polymerase chain reaction gel. M, marker, 0, 12, 24, 36, 48, 60 and 72 h culture under hypotonic conditions. (B) Comparison of relative mRNA expression level curves between the small interfering RNA-AQP4 and control groups. Values are expressed as the mean  $\pm$  standard deviation. AQP4, aquaporin-4; CX43, connexin 43; mRNA, messenger RNA.

*AQP4 and Cx43 mRNA expression levels change with time under hypotonic conditions.* The mRNA expression levels of AQP4 and Cx43 in C6 glioma cells and normal glial cells are revealed in Fig. 4. AQP4 mRNA expression was detected in C6 glioma cells ( $0.21 \pm 0.04$ ) and rapidly increased with time under hypotonic conditions. AQP4 mRNA expression levels were lower in normal glial cells ( $0.09 \pm 0.03$ ) than those in C6 glioma cells and slowly increased with time under hypotonic conditions. Cx43 mRNA expression levels in C6 glioma cells were initially lower than those in normal glial cells ( $0.02 \pm 0.01$  vs.  $0.49 \pm 0.09$ ). Although Cx43 mRNA expression levels in C6 glioma cells increased with time under hypotonic conditions, the expression levels remained lower than those of Cx43 and AQP4 in C6 glioma cells. An inflection point appeared 24 h following hypotonic cultivation, which was later than that of C6 glioma cells (12 h). No significant increase in Cx43 mRNA expression was detected in normal glial cells with prolonged time under hypotonic conditions.

*AQP4 silencing attenuates the increase in Cx43 expression characteristic of C6 glioma cells.* Fig. 5A reveals Cx43 mRNA expression following selective AQP4 silencing in C6 glioma cells. Cx43 mRNA expression levels increased with prolonged time in C6 glioma cells under hypotonic condi-

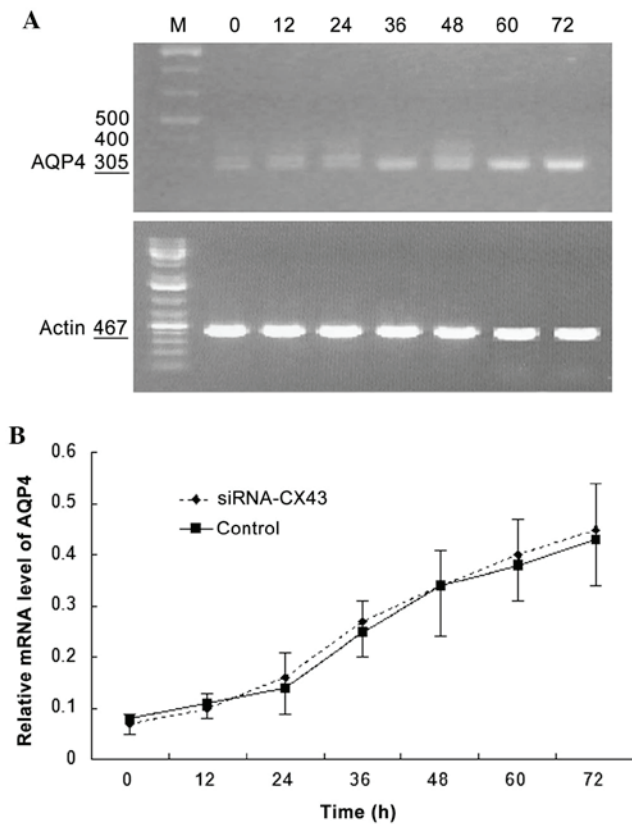


Figure 6. Effect of CX43 silencing on AQP4 mRNA expression levels in normal glial cells cultured under hypotonic conditions. (A) Representative image of polymerase chain reaction gel. (B) Comparison of relative mRNA expression level curves between the siRNA-CX43 and control groups. Values are expressed as the mean  $\pm$  standard deviation. CX43, connexin 43; AQP4, aquaporin-4; mRNA, messenger RNA; siRNA, small interfering RNA.

tions and the proportion of increase was significantly higher than that of the control group ( $P < 0.05$ ; Fig. 5B).

*Cx43 silencing has no significant effect on AQP4 expression in normal glial cells.* Fig. 6A shows AQP4 mRNA expression in normal glial cells following selective Cx43 silencing. AQP4 mRNA expression gradually increased over time in normal glial cells under hypotonic conditions and the increase was similar to that of the control group ( $P > 0.05$ ; Fig. 6B).

## Discussion

Brain edema is characterized by an excessive accumulation of fluid in the intracellular or extracellular spaces of the brain (31). Various intracranial pathological changes, including hypoxia, poisoning, metabolic disturbance, craniocerebral injury, tumors, abscesses, blood vessels and inflammation, may induce increased fluid content in the brain parenchyma, resulting in intracranial hypertension, which may induce alterations in cerebral metabolism, blood supply and aggravated brain edema (10). When glioblastoma initially develops, edema in neoplastic cells and the tumor nidus is not apparent (32). Following increased tumor nidus and tumor blood vessel growth, the blood-brain barrier in the focal zone is damaged, capillary permeability increases and plasma components and water are released, triggering angioedema (33). Endothelial cells are subsequently

destroyed and tight connections in the vascular endothelial cell membrane are opened. Water and partially ionic compositions overflow and enter the tumor periphery (34). Normal cells at the tumor periphery are subjected to hypotonic conditions for extended time-periods; therefore, the dynamic configuration of the membrane is damaged, resulting in dysfunction of the membrane transport system for water and electrolytes;  $\text{Ca}^{2+}$  release from the mitochondria and endoplasmic reticulum, which increases the intracellular  $\text{Ca}^{2+}$  concentration; activation of phospholipase A and C and induced phospholipase degradation, resulting in arachidonic acid release, increased cell permeability and cellular dysfunction (34,35). Therefore, the mitochondria, lysosomes, microsomal membranes and subcellular organelles are destroyed, resulting in dysfunction of neurons and accessory cells and aggravation of brain edema (36). It was hypothesized that glioblastoma-induced brain edema is a combination of angioedema and cytotoxic brain edema (37). In the present study, angioedema was mainly detected in the tumor nidus and cytotoxic edema was mainly detected in the tumor periphery.

In the present study, a Sprague-Dawley rat model of intracranial glioma was established. MRI examination revealed tumor formation and edema at the tumor periphery. Immunofluorescence analysis and western blot assay verified marked differences in AQP4 and CX43 expression in the tumor bed and the tumor periphery. In cells of the tumor bed, AQP4 expression levels increased, whereas Cx43 expression decreased or was absent in comparison to those of normal brain tissues. However, AQP4 expression levels were significantly increased and Cx43 expression levels were higher in the edema region at the tumor periphery compared with those of normal brain tissues.

Aside from a small number of infiltrative glioma cells, abundant normal glial cells were visible at the tumor periphery. Normal glial cells also expressed AQP4 and Cx43 to a certain extent. The present study hypothesized that edema formation in the tumor bed was associated with glioma cells, whereas edema at the tumor periphery was associated with normal glial cells. Therefore, C6 glioma cells and normal glial cells were separately incubated under hypotonic conditions to simulate edema, in order to characterize AQP4 and Cx43 expression in both cell types. The results demonstrated that AQP4 mRNA expression rapidly increased with time under hypotonic conditions in C6 glioma cells, whereas it increased slowly in normal glial cells. Simultaneously, Cx43 mRNA expression levels were low in C6 glioma cells and increased with prolonged time under hypotonic conditions; however, the increase in expression levels was not so marked as that observed in that of AQP4. An inflection point appeared at 24 h following hypotonic cultivation, which was later than that observed in C6 glioma cells (12 h). No increase in Cx43 mRNA expression levels was detected in normal glial cells with time under hypotonic conditions.

To further identify the association between AQP4 and Cx43, Cx43 expression under hypotonic conditions following AQP4 silencing in C6 glioma cells was observed. AQP4 expression levels under hypotonic conditions following Cx43 silencing in normal glial cells were also evaluated. The results confirmed that the increased Cx43 expression observed in C6 glioma cells was significantly attenuated

following AQP4 silencing, while AQP4 expression was not altered following Cx43 silencing in normal glial cells. Based on these results, it was concluded that AQP4 and Cx43 within and surrounding the glioma had separate mechanisms underlying brain edema and that Cx43 may be a downstream effector of AQP4. Due to the impairment of glioblastoma cell-cell junctions and communication, numerous micro-molecular substances and ions are restricted in single tumor cells (38). The block in communication results in disordered transport of water molecules, ions and micromolecular substances (39). Although water transport is not a major function of cell-to-cell junctions and communication, intracellular and extracellular osmotic pressure gradients induced by the disordered transport of ions and micromolecular substances affect intracellular and extracellular water distribution, resulting in impaired intracellular water excretion and the formation of glioblastoma-induced brain edema (40).

A previous study confirmed that a hypotonic environment caused AQP4 expression levels to increase in glial cells (41). Li *et al* (25) found that a hypotonic environment led to astrocyte edema and a reduction in viability, with increased AQP4 mRNA and protein expression levels. Yang *et al* (27) suggested that when glial cells in the edema region at the tumor periphery were in a hypotonic environment, increased AQP4 expression promoted the entrance of extracellular water to cells. Intracellular water overloading would therefore induce the occurrence of cytotoxic edema. These studies did not clearly explain the mechanism underlying glioma-induced brain edema within and around the edge of the tumor. The results of the present study enhanced the available knowledge in this field and provided a novel insight into the roles of AQP4 and Cx43 in brain edema. However, further study is required in order to fully elucidate this complex pathway.

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