Biochemical and Biophysical Research Communications 440 (2013) 173–177

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Expression of aquaporin8 in human astrocytomas: Correlation with pathologic grade

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ARTICLE INFO

Article history:
Received 9 September 2013
Available online 18 September 2013

Keywords:
Aquaporin-8
Astrocytic tumors
Pathologic grade

ABSTRACT

Aquaporin8 (AQP8), a member of the aquaporin (AQP) protein family, is weakly distributed in mammalian brains. Previous studies on AQP8 have focused mainly on the digestive and the reproductive systems. AQP8 has a pivotal role in keeping the fluid and electrolyte balance. In this study, we investigated the expression changes of AQP8 in 75 cases of human brain astrocytic tumors using immunohistochemistry, Western blotting, and reverse transcription polymerase chain reaction. The results demonstrated that AQP8 was mainly distributed in the cytoplasm of astrocytoma cells. The expression levels and immunoreactive score of AQP8 protein and mRNA increased in low-grade astrocytomas, and further increased in high-grade astrocytomas, especially in glioblastoma. Therefore, AQP8 may contribute to the proliferation of astrocytomas, and may be a biomarker and candidate therapy target for patients with astrocytomas.

1. Introduction

Astrocytomas are the most common primary brain tumors [1]. However, the pathological mechanisms of astrocytoma proliferation and growth are still unclear. Previous studies [2] have demonstrated three main variables that differentiate tumors from normal tissues: water content, regressive events, and vascular architecture. Most types of brain tumor typically exhibit increased water content due to increased cellularity [2]. Water crosses the plasma membrane through one of the following ways: directly through the lipid bilayer (a slow, unregulated process), or via protein water channels termed as aquaporins (AQPs). AQPs may accelerate glioma cell migration by facilitating rapid changes in cell volume that accompany the changes in cell shape. Within this family, the expression and role of AQP8 are still unknown in astrocytic tumors. Moreover, we investigated the expression pattern of AQP8 using immunohistochemistry (IHC), Western blotting, and reverse transcription polymerase chain reaction (RT-PCR) in different pathological grades of human astrocytic tumor tissues in this study.

2. Materials and methods

2.1. Patients and tumor specimens

Astrocytoma samples were obtained by surgical resection of tumors in patients at the Neurosurgical Department of the First Affiliated Hospital of Chongqing Medical University, Chongqing, China, from January 2008 to August 2012. The ages of the patients ranged from 15 to 75 years old. For this study, the pathologic grades of astrocytic tumors were established upon examination by a neuropathologist using the 2000 World Health Organization criteria. The pathological grades of the 75 tumor specimens were defined as follows: 9 cases of grade I, 20 cases of grade II, including 14 diffused fibrillary astrocytomas, 4 protoplasmic astrocytomas, and 2 gemistocytic astrocytomas; 25 cases of grade III, all anaplastic astrocytomas; and 21 cases of grade IV, all glioblastomas.

None of the patients received chemotherapy or radiotherapy before the procedure. The specimen bank of Chongqing Medical University provided five cases of normal brain tissue as control comparators.

This study was conducted in accordance with the Declaration of Helsinki. Procedures were approved by the ethics committee of the Department of Medical Research, Chongqing Medical University. Informed consent was obtained from the patients or from a family member previously designated by the patient if the patient was unable to provide consent.

The tissues were divided into two portions. One portion was fixed in 4% paraformaldehyde for H&E staining and IHC. The sections stained with H&E were examined to determine the quality and suitability of the tissues for immunostaining. The other fresh portion was frozen in cryovials for the analysis of protein and mRNA expression.

2.2. Immunohistochemistry

Paraffin-embedded specimens were serially cut into 5 μm thick sections for IHC. The sections were deparaffinized, rehydrated,

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0006-291X/$ - see front matter
http://dx.doi.org/10.1016/j.bbrc.2013.09.057

placed in citrate buffer (pH 6.0), and heated at 98 °C for 25 min to retrieve the antigens. Afterward, a kit (anti-mouse, anti-rabbit or anti-goat) from the ZhongShan Company involving a two-step technique for IHC was used. Sections were treated with 3% hydrogen peroxide for 10 min at room temperature, and then incubated overnight at 4 °C with primary antibody (mouse anti-human AQP8, 1:300, Abcam). After extensive washing with PBS, sections were incubated for 30 min at 37 °C with HRP-conjugated goat anti-mouse secondary antibodies (ZhongShan Company, Beijing, China), and then washed again in PBS. Staining was developed using a DAB kit (ZhongShan Company, Beijing, China). The sections were counterstained with hematoxylin, dehydrated, and then mounted. Control sections were prepared in parallel with omission of the primary antibody.

2.3. Staining interpretation and quantification of immunoreactivity for AQP8

The staining results were evaluated in a double-blinded manner by two independent neuropathologists. A third independent neuropathologist blinded to the experiment and patients would be asked to examine the sections if a dispute emerged in scoring the same section. Brown staining exhibited positive immunoreactivity for AQP. Five sections were selected from each sample. For the measurement of the AQP8 immunoreactivity score (IRS) in astrocytic tumors, 200 cells in the strongest immunoreactive region of every section were counted in 5–10 adjacent high-powered fields at 200×agnification. According to the method described by Friedrich et al. [3], the AQP8 IRS was determined by semiquantitative assessment. The AQP8 IRS was obtained by multiplying the values of the AQP8-positive cell percentage (0, <1%; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, >75%).

2.4. Immunoblotting

Lysates from tissues were prepared in ice-cold lysis buffer containing a cocktail of protease inhibitors (Sigma–Aldrich, St. Louis, Mo, USA). Crude protein lysates (50 μg) were separated on 12% SDS–PAGE gel, transferred to a nitrocellulose membrane (Bio-Rad), and then blocked for 2 h with 5% nonfat powdered milk dissolved in tris-buffered saline with 0.05% Tween 20 at RT with shaking. The following commercial antibodies were used for Western blot analysis: mouse monoclonal anti-AQP8 antibody (1:1200; Abcam) and anti-β-actin antibody (1:5000; Sigma, USA). Anti-mouse horseradish peroxidase-conjugated secondary antibodies (Sigma, USA) were used at 1:3000. Immunoreactive bands were visualized using a DAB kit (ZhongShan, Beijing, China).

2.5. RT-PCR

Total RNA from both normal brain and tumor specimens was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was performed using Superscript III reverse-transcriptase (Invitrogen) to transcribe poly(A)+ RNA with oligo $(dT)_{18}$ as primers. PCR was carried out using the Taq DNA Polymerase kit (TaKaRa, Dalian, China). The sequences of the PCR primers used were as follows: AQP8 gene 5′-tcctgaggagaggttctgga-3′ (sense) and 5′-agagggccctttgtcttctc-3′ (antisense), providing a product of 159 bp. The internal loading control was β-actin, and PCR primers were 5′-ctgccgcatcctcttcctc-3′ (sense) and 5′-ctcctgcttgctgatccacat-3′ (antisense), providing a product of 398 bp. PCR was performed as follows: initial denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 20 s, and elongation at 72 °C for 20 s; ending with an extension at 72 °C for 2 min, and then cooling and holding indefinitely at 4 °C. The resulting PCR products were visualized after separation on a 1.5% agarose gel.

2.6. Bands analysis for immunoblotting and RT-PCR

The optical densities of the AQP8 and β-actin bands were quantitatively analyzed with gel densitometry (Bio-Rad, Hercules, USA). Statistical data were obtained using AQP8/β-actin. The western and mRNA bands were quantified by gel densitometry (Bio-Rad, Hercules, USA). The value of the individual AQP protein (or mRNA) band was divided by the value for beta-actin for the same sample, and the ratio of protein: β-actin for each sample was obtained. Bands were normalized with the β-actin loading control, and each group was normalized to the ratio of the corresponding control for analysis.

2.7. Statistical analysis

All statistical analysis was performed with the SPSS 13.0 software package (Chicago, IL, USA). Values are presented as means ± SD. Differences of AQP8 expression in the specimens of different pathologic groups were compared using analysis of variance (ANOVA); and then, Tamhane's *t2* test was used to compare differences between groups produced by ANOVA. All reported *p*-values were two-sided. Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. AQP8 protein expression

The IHC results showed that AQP8 was mainly detected in the cytoplasm in normal brain tissue and tumor cells. In anaplastic astrocytoma tissue, AQP8 labeling was found in cytomembrane of a few tumor cells. AQP8 labeling was found in almost all glioblastoma cells. However, AQP8 positive cells in grade II were scattered (Fig. 1A–E). In addition, we also found that the IRS of AQP8 in astrocytic tumors was higher ($p < 0.05$) compared with the normal brain tissues. AQP8 expression is still the strongest elevated in glioblastoma. For the 75 cases of tumor specimens, the IRS of AQP8 increased significantly in low-grade tumor tissues (grade I, $p = 0.015$; grade II, $p = 0.011$), and then increased further in high-grade tumor tissues (grade III, $p = 0.011$; grade IV, $p = 0.008$), especially in glioblastoma. Compared with the normal brain tissues (0.8 ± 0.06), the IRS of AQP8 in tumor tissues increased to 1.7 ± 0.11 (grade I), 2.2 ± 0.24 (grade II), 5.3 ± 0.38 (grade III), and 7.6 ± 0.61 (grade IV) (Fig. 2).

To further quantitatively analyze the expression of AQP8 protein, Western blotting was used to detect the level of AQP8 protein in tumor and normal brain specimens. Compared with normal brain tissues, the level of AQP8 protein in the tumor specimens increased to 181.94 ± 11.41 for grade I, 194.28 ± 12.43 for grade II, 302.23 ± 32.56 for grade III, and 327.36 ± 33.58 for grade IV (Fig. 3). However, no significant correlations were found between the expression levels of AQP8 protein and age, gender, or tumor site.

3.2. Expression of AQP8 mRNA

To examine the changes in the expression of AQP8 mRNA in tumor tissues with different pathological grades, RT-PCR was used to detect the mRNA level. Compared with normal brain tissues, the expression level of AQP8 mRNA in tumor specimens were stranger ($p < 0.05$). The expression rule of AQP8 mRNA corresponds to the AQP8 protein. The levels of AQP8 mRNA in tumor tissues increased to 188.57 ± 13.17 for grade I, 214.21 ± 16.18 for grade II, 377.17 ± 38.23 for grade III, and 417.32 ± 40.25 for grade IV tumors