



# Aquaporin-4 Expression in Meningioma Malignancy Progression

## *Expressão da aquaporina-4 na progressão da malignidade dos meningiomas\**

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### Abstract

**Objectives** The aim of the present study is to analyze if aquaporin-4 (AQP4) may also be a tumor progression marker for meningiomas.

**Methods** This is an immunohistochemistry study realized at the Universidade de São Paulo, São Paulo, state of São Paulo, Brazil: frozen meningioma samples from 81 patients (57 females and 24 males, age range from 22 to 81 years old, average  $56.5 \pm 14.1$  years old), including 57 meningiomas World Health Organization (WHO) grade I (GI); 19 grade II (GII), and 5 grade III (GIII) were analyzed. The relative expression level of AQP4 was analyzed by quantitative polymerase chain reaction (qPCR), using the SYBR Green approach and for staining detection. Tissue sections were routinely processed and subjected to antigen retrieval.

**Results** The expression of AQP4 in meningioma samples ranged from 0 to 10.26, with a median of 0.001 in GI cases, of 0.008 in GII cases, and of 0.006 in GIII cases. Although not statistically significant ( $p = 0.942$ ), GI meningiomas have a lower median AQP4 expression level than higher malignant grade cases.

**Conclusion** The AQP4 gene and protein expressions presented no association with meningioma malignant progression.

### Keywords

- ▶ aquaporin-4
- ▶ malignancy
- ▶ meningioma

\* The present study was performed at the LIM 15 – Hospital de Clínicas da Universidade de São Paulo (USP).

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## Resumo

**Objetivo** O objetivo do presente estudo é analisar se a aquaporina-4 (AQP4) também pode ser um marcador de progressão tumoral para meningiomas.

**Métodos** Trata-se de um estudo imunohistoquímico realizado na Universidade de São Paulo, SP, Brasil. Amostras congeladas de meningioma de 81 pacientes (57 mulheres e 24 homens, faixa etária de 22 a 81 anos, média de  $56,5 \pm 14,1$  anos), incluindo 57 meningiomas grau I (GI) da Organização Mundial da Saúde (OMS); 19 grau II (GII) e 5 grau III (GIII) foram analisados. O nível de expressão relativa de AQP4 foi analisado por reação em cadeia de polimerase quantitativa (qPCR, sigla em inglês), usando a abordagem SYBR Green e para detecção de manchas. As seções de tecido foram rotineiramente processadas e sujeitas a recuperação de antígeno.

**Resultados** A expressão de AQP4 em amostras de meningioma variou de 0 a 10,26, com mediana de 0,001 nos casos GI; 0,008 nos casos GII; e 0,006 nos casos GIII. Embora não sejam estatisticamente significantes ( $p = 0,942$ ), os meningiomas GI apresentam mediana mais baixa do nível de expressão de AQP4 do que os casos de grau maligno mais alto.

**Conclusão** Expressões de genes e proteínas AQP4 apresentadas na associação com progressão maligna do meningioma.

## Palavras-chave

- ▶ aquaporina-4
- ▶ malignidade
- ▶ meningioma

## Introduction

Meningiomas are brain tumors derived from arachnoidal cap cells in the meningeal coverings of the spinal cord and the brain.<sup>1</sup> They are the most common benign intracranial tumors,<sup>2</sup> and account for between 13 and 34% of all surgically removed primary brain tumors.<sup>3</sup>

Peritumoral brain edema in patients with meningiomas has been associated with aquaporin 4 (AQP4) expression levels.<sup>4-7</sup>

The aquaporins (AQPs) are members of a family of molecular water channels, and at least 13 isoforms have been identified in mammals.<sup>8</sup> They are small membrane spanning proteins (monomer size 30 kDa), expressed in several cell types and involved with water transport.<sup>9,10</sup> Among them, AQP4 is the most abundant water channel in the central nervous system (CNS), particularly abundant on astrocytes, where AQP4 expression is mostly restricted to endfoot membranes contacting the basal lamina of capillaries.<sup>11</sup> The loss or reduction in this highly polarized AQP4 distribution on astrocytes forming the glia limitans of the blood-brain barrier has been implicated in the pathogenesis of normal pressure hydrocephalus, *pseudotumor cerebri* and brain edema.<sup>12</sup> The arrangements of AQP4 tetramers in a high-order complex, known as orthogonal array particles (OAP), are related to the water gating mechanism.<sup>13</sup> Also, the size of the resulting OAP structure is related to the ratio of the splice variants of AQP4, M1 isoform (323 amino acids long) and M3 isoform (22 amino acid shorter at the N-terminus than M1).<sup>14</sup>

Under pathological conditions, such as cancer, AQP4 presents upregulation, and its redistribution is not strictly located in the perivascular endfoot membrane; it is also inserted into nonendfoot membrane domains in high-grade astrocytomas. These findings were not observed in low-grade astrocytomas and, thus, it has been reported as a

tumor progression marker in World Health Organization (WHO) grade II-IV astrocytomas.<sup>15,16</sup> However, the AQP4 expression pattern was not related with patient survival.<sup>16</sup>

The aim of the present study is to analyze if AQP4 may also be a tumor progression marker for meningiomas.

## Methods

Frozen meningioma samples from 81 patients (57 females and 24 males, age range from 22 to 81 years old, average  $56.5 \pm 14.1$  years old), including 57 meningiomas WHO grade I (GI); 19 grade II (GII), and 5 grade III (GIII)<sup>17</sup> were analyzed. The samples were collected during therapeutic surgery of patients treated by the Neurosurgery Group of the Department of Neurology at the Hospital das Clínicas at the School of Medicine of the University of São Paulo, São Paulo, State of São Paulo, Brazil, in the period of 2000 to 2007.<sup>18</sup> The samples were macrodissected and immediately snap-frozen in liquid nitrogen upon surgical removal. A 4 $\mu$ m-thick cryosection of each sample was analyzed under a light microscope after hematoxylin-eosin staining for assessment of necrotic, cellular debris and hemorrhagic areas, followed by removal from the frozen block by microdissection prior to RNA extractions.<sup>19,20</sup> Written informed consent was obtained from all patients according to the ethical guidelines approved by the Department of Neurology of the School of Medicine of the University of São Paulo (0599/10).

All donors signed an informed consent form, and the present study was approved by our Institutional Review Board under the registration number CAPPESq # 200/05. The present study had financial support by the São Paulo Research Foundation (FAPESP, in the Portuguese acronym), under the registration numbers: 2004/1233-6, 2013-02162-8, 2013/06315-3, 2013/07704-3.

### Sample Preparation

Total RNA was extracted from frozen tissue using an AllPrep Mini Kit (Qiagen, Hilden, Germany). A conventional reverse transcription reaction was performed to yield single-stranded cDNA. The first strand of cDNA was synthesized from 1 µg of total RNA previously treated with 1 unit of DNase I (FPLC-pure, GE Healthcare, Uppsala, Sweden) using random and oligo (dT) primers, RNase inhibitor, and SuperScript III reverse transcriptase according to the recommendations of the manufacturer (Thermo Fisher Scientific, Carlsbad, USA). The resulting cDNA was subsequently treated with 1 unit of RNase H (GE Healthcare, Uppsala, Sweden), diluted with TE buffer, and stored at -20°C until later use.

### Quantitative Real Time PCR (qPCR)

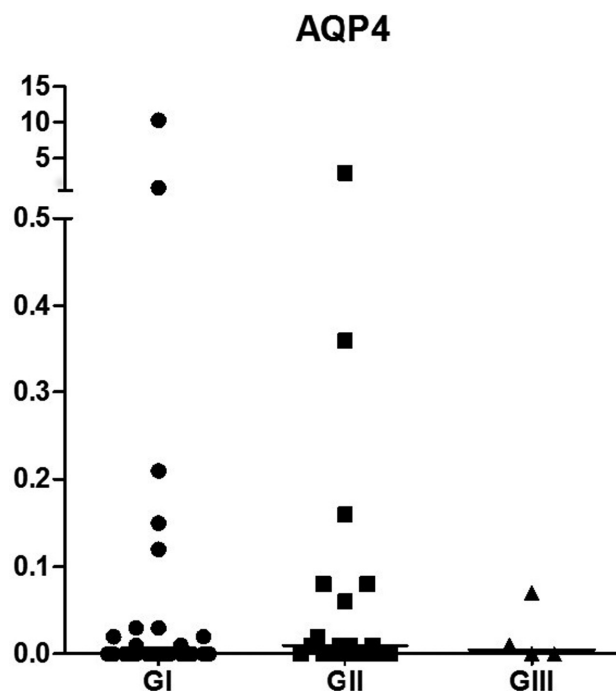
The relative expression level of *AQP4* was analyzed by quantitative polymerase chain reaction (qPCR), using the SYBR Green approach. Quantitative data was normalized in relation to the geometric mean of two housekeeping genes, suitable for the analysis: hypoxanthine phosphoribosyltransferase (*HPRT*), and glucuronidase β (*GUSB*), as previously demonstrated by our group.<sup>21</sup> Primers were designed to amplify 80–130 bp amplicons, with a melting temperature of 60°C, and were synthesized by IDT (Integrated DNA Technologies, Coralville, USA) as follows (5' to 3'): *AQP4* F: ATAGCAGCGAACAGGGAATGAC, *AQP4* R: ATGGCTCACAGATTCCTGGAGA, *HPRT* F: TGAGGATTTGAAAGGTGT, *HPRT* R: GAGCACACAGAGGGCTACAA; *GUSB* F: GAAAATACGTGGTTGGAGAGCTCATT, *GUSB* R: CCGAGTGAAGATCCCCTTTTA. All the reactions were performed in duplicate. The *AQP4* expression level was calculated according to  $2^{-\Delta Ct}$ ,<sup>22</sup> where  $\Delta Ct = Ct \text{ specific gene} - \text{geometric mean Ct of housekeeping genes for each grade of meningioma}$ .

### Immunohistochemistry

For staining detection, tissue sections were routinely processed and subjected to antigen retrieval. Briefly, slides were immersed in 10 mM citrate buffer, pH 6.0, and incubated at 122°C for 3 minutes using an electric pressure cooker (Bio-Care Medical, Walnut Creek, California, USA). Specimens were then blocked and further incubated with antibody raised against human AQP4 (mouse monoclonal, Clone 2456C1a, ab66495, Abcam, Cambridge, UK, 1:25) at between 16 and 20°C for 16 hours. Development of the reaction was performed with a commercial kit (Novolink; Novocastra, Newcastle-upon-Tyne, UK) at room temperature, using diaminobenzidine and Harris hematoxylin for nuclear staining. Optimization using positive control suggested by the manufacturer of the antibody was performed to obtain optimal dilution (normal lung tissue). The staining intensity of the tissue sections was evaluated independently by two observers. Digital photomicrographs of representative fields were captured and processed using Picasa 3 (Google, Mountain View, CA, USA).

### Statistical Analysis

The statistical analysis of relative gene expression in different grades of meningioma were assessed using the Kolmogorov-Smirnov normality test and the nonparametric



**Fig. 1** AQP4's expression according to histological grade of the meningioma.

Kruskal-Wallis and Dunn tests. Differences were considered statistically significant when  $p < 0.05$ . Calculations were performed using IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, USA).

### Results

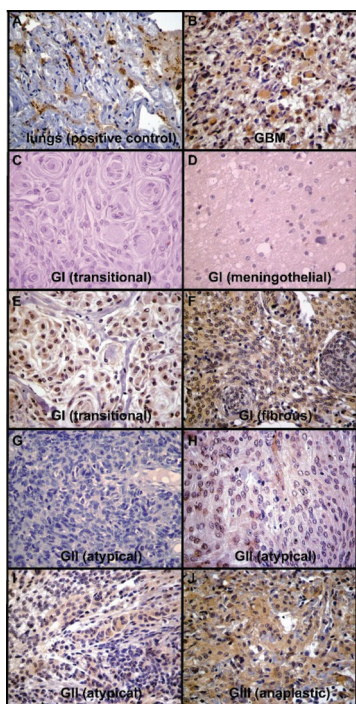
The expression of *AQP4* in meningioma samples ranged from 0 to 10.26, with a median of 0.001 in GI cases, of 0.008 in GII cases, and of 0.006 in GIII cases. (► **Fig. 1**). Although not statistically significant (► **Table 1**), GI meningiomas have a lower median *AQP4* expression level than higher malignant grade cases.

The high level of heterogeneity found in gene expression data could also be observed in protein levels. Immunohistochemistry of AQP4 was first assessed in normal, non-CNS tissue (lungs), where there is a clear polarized distribution of the protein (► **Fig. 2A**). Next, we verified that in glioblastoma (GBM) tissue, AQP4 expression is high and homogeneous, and that the polarized state is lost (► **Fig. 2B**). In meningioma samples from grades I (► **Fig. 2C-F**), II (Figure G-I) and III

**Table 1** Aquaporin-4 distribution according to meningioma grade

	Medium	p-value
AQP 4		
Grade I	0.21	0.9410
Grade II	0.22	
Grade III	0.02	

Abbreviation: AQP-4, aquaporin-4.



**Fig. 2** Immunohistochemistry- A: AQP4 expression normal, non-CNS tissue (lungs); B: AQP4 expression in glioblastoma tissue is high and homogeneous, and the polarized state is lost; C-F: AQP4 expression in different meningioma grades I; G-I: AQP4 expression in atypical meningiomas; J: AQP4 expression in anaplastic meningioma.

(► Fig. 2J), we observed that, although there are GI cases with none or low expression of AQP4, there are also cases expressing high levels of the protein. Grade II cases showed a similar pattern. There was only one available grade III sample, as those tumors are quite rare. Still, the same level of expression of AQP4 in this anaplastic case was found in grade I samples.

## Discussion

Tumor surgical resection extent, histological grade, and angiogenesis are parameters addressed as indicators of tumor progression in meningiomas. However, a specific factor associated with meningioma malignancy has not been identified yet. Aquaporin-4 was described as a tumor progression marker among grade II to IV astrocytomas.<sup>15,16</sup> In meningiomas, AQP4 has been associated with peritumoral edema.<sup>4-7</sup> Although AQP4 expression was lower in meningiomas WHO grade I, no statistical difference was observed compared with meningiomas of higher grades, further corroborating previous immunohistochemistry results with this protein.<sup>4</sup> Interestingly, a diffuse AQP4 cytoplasmic staining was observed in positive meningiomas cases, as has been described previously in astrocytomas of high grade of malignancy. The characteristic polar distribution on glia limitans was also lost in meningiomas, similar to the observation on GBM<sup>15</sup>. The pathological impact of the redistribution of this protein in the tumor cell might be interesting to pursue to better understand its role in tumorigenesis.

## Conclusion

Aquaporin-4 gene and protein expressions presented no association with the progression of malignant meningioma.

### Disclosure

The authors have no personal financial or institutional interest in any of the drugs, materials, or devices described in this article.

### Ethical Approval

All procedures performed in the present study were in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The present study was approved by our Institutional Review Board under the registration number CAPPEsq # 200/05.

### Informed Consent

All authors agree to the publication guidelines of the São Paulo medical Journal

The present article does not contain any studies with human participants performed by any of the authors.

### Conflict of Interests

The authors have no conflict of interests to declare.

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