Correlation of Aquaporin-1 Water Channel Protein Expression with Tumor Angiogenesis in Human Astrocytoma

NICOLAI EL HINDY[1], AGNES BANKFALVI[2], ARNE HERRING[2], MICHAEL ADAMZIK[3], NICOLE LAMBERTZ[1], YUAN ZHU[1], WINFRIED SIFFERT[4], ULRICH SURE[1] and I. EROL SANDALCIOGLU[1]

[1]*Department of Neurosurgery,* [2]*Institue of Pathology & Neuropathology,*
[3]*Department of Anaesthesiology and Intensive Care Medicine,*
[4]*Institute of Pharmacogenetics, University Hospital Essen, Essen, Germany*

Abstract. *Aquaporin-1 (AQP1) is a water channel protein, widely expressed in epithelial and endothelial cells, known to be associated with invasion, angiogenesis, cell migration and formation of tumour oedema in several malignancies. We investigated the pattern of immunohistochemical expression of AQP1 in human astrocytomas and its role in tumour angiogenesis and infiltration. Immunohistochemical staining of AQP1 was performed in astrocytomas of grade II, III and IV. Intensity and pattern of expression were analysed. Non-neoplastic brain tissues served as control. There was a significant increase in the intensity of AQP1 expression from low-grade to high-grade astrocytomas ($p<0.0001$). Despite intense expression of AQP1 in astrocytoma grade IV, we observed strong regional differences. AQP1 up-regulation was predominantly located perivascularly, in areas of tumour infiltration, distant from the necrotic tumour core. AQP1 expression correlates with the grade of malignancy and is associated with angiogenesis, as well as with invasion of grade IV tumour in areas of tumour infiltration. Suppression of AQP1 expression could be a potential means of reducing invasion of glioma cells.*

Gliomas are the most common primary brain tumours in adults. These tumours derive from glial tissue and can be classified into ependymomas, oligodendrogliomas and astrocytomas. According to the WHO classification of tumours of the nervous system, astrocytomas can be further divided into four grades (grade I-IV) with increasing malignancy (1). Astrocytoma grade IV, also known as glioblastoma multiforme (GBM), is the most malignant brain tumour. Despite an improved survival by modern therapeutic strategies, treatment of high-grade astrocytomas remains challenging, mostly because of microvascular proliferation, intense anti-apoptosis, and diffuse invasion (2). A common characteristic of high-grade astrocytomas is the formation of peritumoral brain oedema, possibly facilitating tumour progression (3).

Aquaporins (AQPs) are involved in the formation of brain oedema. They are small transmembrane channel-forming proteins, facilitating rapid transport of water and small solutes across biological membranes (4). To date, 13 AQPs have been identified in humans (AQP0-AQP12). In addition to the physiological expression in many epithelia and organ systems, novel studies suggest a role for AQPs in the formation and progression of cancer (5-7).

Recent investigations reveal the importance of AQP1 for tumor growth and angiogenesis. The expression of AQP1 has been shown to influence the pathogenesis and outcome of various malignancies, comprising pleural malignant mesothelioma, breast carcinoma, and colorectal cancer (8-10). Besides the identification of *AQP1* as an early-response gene to mitogens and its expression in tumour microvessels, studies of AQP1-deficient mice revealed reduced tumour growth and angiogenesis (11, 12). AQP1 is further involved in the formation of peritumoral oedema and is consistently expressed in glioma cells (5, 12, 13). The aim of the present study was to elucidate the role of AQP1 expression on infiltration and angiogenesis in high-grade astrocytoma, with special respect to microvessels in GBM.

Patients and Methods

Tissue specimens. Tissue specimens from 40 Caucasian patients were retrieved from the Institute of Pathology and Neuropathology, University Hospital Essen. All patients had undergone surgery between 2008 and 2010 at the Neurosurgical Department, University Hospital Essen. The study was strictly carried out according to the Declaration of Helsinki and was approved by the local Ethics Committee of the University Hospital of Essen. Informed consent was obtained from the patients;

Correspondence to: Nicolai El Hindy, Department of Neurosurgery, University Hospital Essen, Hufelandstraße 55, 45122 Essen, Germany. Tel: +49 2017231201, Fax: +49 2017235909, e-mail: nicolai. elhindy@uk-essen.de

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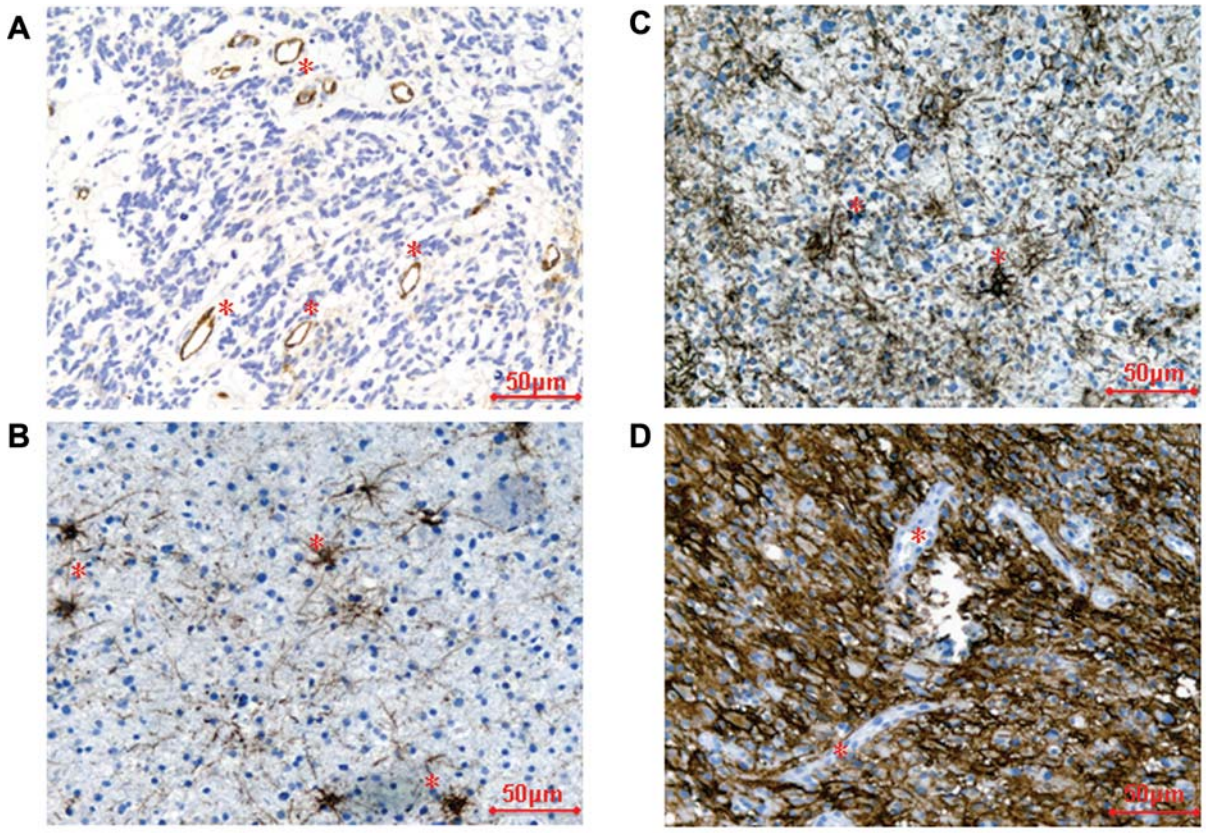

Figure 1. *Immunhistochemical staining (brown) for Aquaporin1 (AQP1). Normal brain (A) revealed solely capillary staining of AQP1 (*). Astrocytoma grade II (B) exhibited mainly membranous staining of some tumour cells, presenting the typical astrocytic appearance (*). Anaplastic astrocytoma grade III (C) revealed denser AQP1 staining of tumour cells. Besides membranous staining, intracytoplasmatic staining was stronger (*). Grade IV astrocytoma (D) exhibited massive membranous and intracytoplasmatic AQP1 expression, with a lack of endothelial expression (*).*

patients' anonymity was preserved. Tumour grading was assessed according to the WHO classification of tumours (1), comprising astrocytoma grade II (n=5), astrocytoma grade III (n=5) and primary astrocytoma grade IV (GBM) without oligodendroglial component (n=25). Non-neoplastic brain tissue (n=5), obtained from temporal lobe resection of patients with intractable epilepsy served as control.

Immunhistochemistry and scoring. Tissue specimens were embedded in paraffin, cut into 2-µm sections and mounted on SuperFrost Plus slides (Menzel, Braunschweig, Germany). After heat-based antigen retrieval (30 min at 95˚C in citrate buffer, pH 6.0), immunostaining with anti-AQP1 (clone B-11; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a dilution of 1:1000 was performed. Automated immunohistochemistry was performed using the Dako Autostainer Plus System (DakoCytomation, Carpinteria, CA, USA) with the anti-mouse IgG EnVision Plus detection kit for secondary and tertiary immunoreactions. Reaction products were developed with diamino benzidine (DAB) according to general protocols. Positive and negative control sections were included in each run, which showed appropriate results. Assessment of sections was performed in a blinded fashion by two independent investigators. AQP1 expression was scored as positive, if at least 25% of tumour cells showed any positive membranous staining. The intensity of staining was scored as mild (1+) when the AQP1 expression was obvious in 25%-50% of tumour cells, moderate (2+) with an expression of 50%-75%, and finally as strong expression (3+) with AQP1 expression in over 75% of tumour cells.

For assessment of microvessels, laminin staining for depiction of basement membrane was performed. Paraffin embedded sections were de-paraffinised in xylene followed by dehydration in graded alcohol, and rinsed in water for 15 min. After antigen retrieval, sections were incubated with mouse anti-human laminin (1:50; VectorLabs, California, USA).

Statistical analysis. Comparison of AQP1 expression intensity was carried out using Pearson's χ^2 test. Differences were regarded significant at $p<0.05$. The statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software, California, USA) and SPSS-Software 17.0 (IBM, New York, USA).

Results

Sections of normal brain revealed AQP1 expression on the endothelium of microvessels, whereas AQP1 expression on glial cells was totally absent (Figure 1A). In contrast,

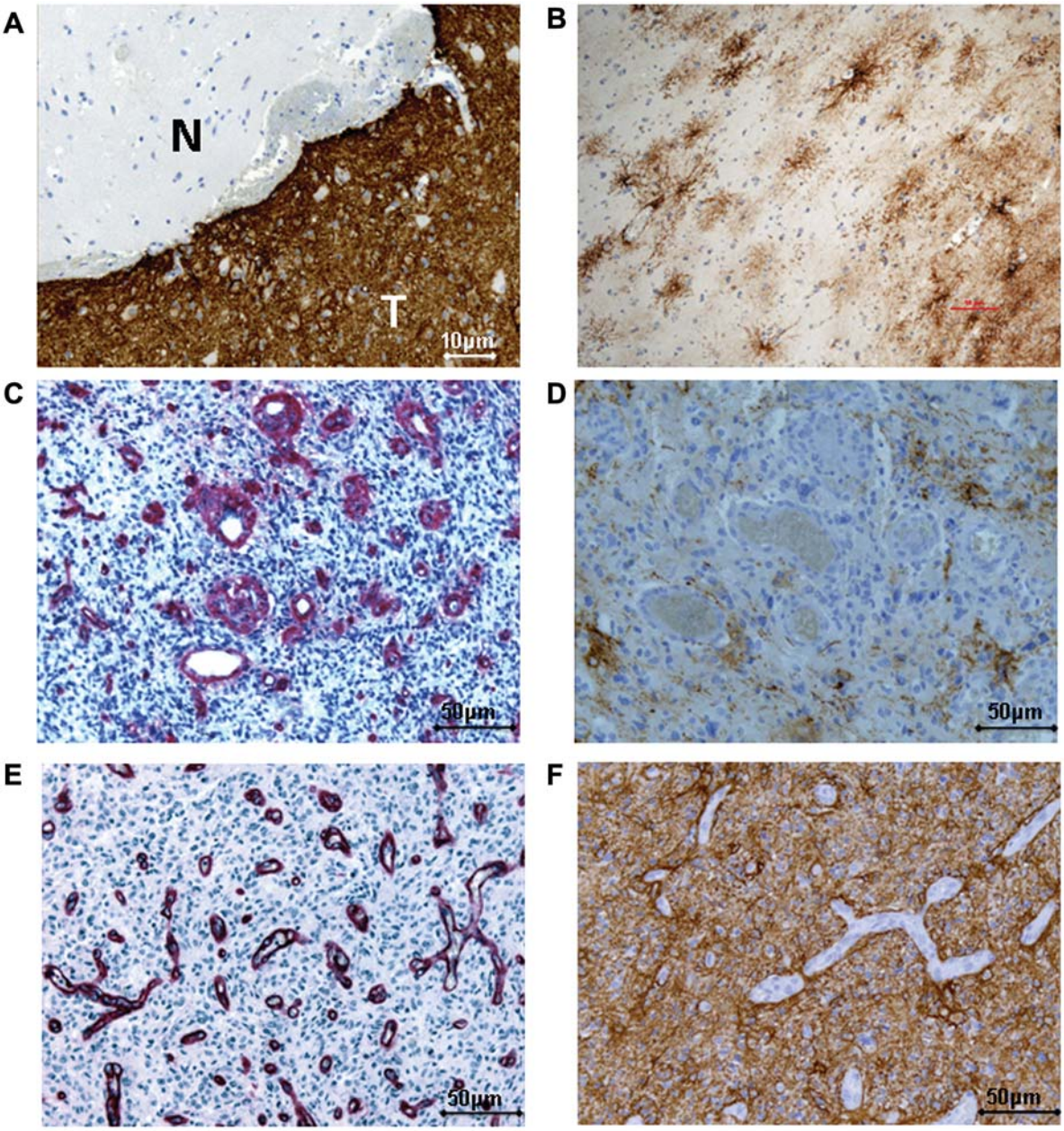

Figure 2. *Immunhistochemical staining of Aquaporin1 (AQP1) (brown, A, B, D, F), and laminin (blue, C, E) in grade IV astrocytoma: The difference between massive AQP1 up-regulation (A) in tumour (T) and lack of expression in normal brain (N) was obvious. The infiltration zone (B) revealed expression of AQP1 similar to that of low-grade astrocytoma (I) in contrast to dense tumour (T). Laminin revealed two different neovascularization patterns: Glomeruloid neovascularization was mainly found within the necrotic tumour core (C). Many branching, connected microvessels were found distant from the tumour core in the periphery, near infiltrating areas (E). There was a remarkable difference in AQP1 expression among the different patterns. Great overexpression was visible, especially perivascularly in the tumour periphery (F), in contrast to only weak AQP1 expression in the necrotic tumour core (D).*

throughout all tumour sections there was a lack of endothelial AQP1 immunostaining on microvessels, but a positive AQP1 expression on astrocytic tumour cells. We furthermore observed a significant increase in the expression of AQP1 from low-grade (Figure 1B) to high-grade astrocytomas (Figure 1C and D). While all (n=25, 100%) GBM revealed a strong expression (>75%) of AQP1, only 20% (n=1) of grade III astrocytomas, and none (0%) of grade II astrocytomas exhibited a strong expression of AQP1 ($p<0.0001$; Table I). AQP1 expression in low-grade

Table I. *Aquaporin 1 (AQP1) expression according to tumour grading.*

	AQP1 expression n (%)				
Astrocytoma	0	1+	2+	3+	*p*-Value
Grade II	0 (0%)	5 (100%)	0 (0%)	0 (0%)	
Grade III	0 (0%)	1 (20%)	3 (60%)	1 (20%)	0.002
Grade IV	0 (0%)	0 (0%)	0 (0%)	25 (100%)	<0.0001

Expression grading: 0 : <25% cells; 1+ : 25-50% cells; 2+ : 50-75% cells; 3+ : >75% cells.

astrocytomas was mainly located on the cell membrane, while in high-grade astrocytomas, AQP1 immunoreactivity was detected on the cell membrane, as well as throughout the cytoplasm of tumour cells (Figure 1C and D). In astrocytoma grade III, AQP1 expression was positive on many, but not all, tumour cells (Figure 1C).

We observed strong regional differences in the expression of AQP1 in GBM: Despite the massive up-regulation of AQP1 in all GBM, in contrast to normal brain (Figure 2 A), some areas presented dense AQP1 expression virtually on all tumour cells (Figure 2 F), while on other areas there was only weak AQP1 immunoreactivity (Figure 2 D).

For further analysis of the different regional expression patterns of AQP1 in GBM and its possible impact on angiogenesis, we used laminin staining for the assessment of microvessels. Interestingly laminin staining displayed differences in microvessel appearence in GBM. In areas within the tumour core characterized by dense necrosis, microvessels exhibited a typical glomeruloid appearance (Figure 2C). In contrast, tumour regions distant from the necrotic core displayed multiple, larger and branching tumour vessels (Figure 2E), often located directly next to the infiltration zone in the normal brain area. Furthermore, while AQP1 expression was weak within the tumour core around necrosis (Figure 2D), it was strongly overexpressed at the border of the tumour. This strong overexpression was predominantly found perivascularly around multiple, branching microvessels (Figure 2F). The expression pattern of AQP1 in areas with infiltration into adjacent brain revealed similarity to that of low-grade astrocytoma, with positive staining of AQP1 on only some tumour cells (Figure 2B). No positive nuclear immunoreaction was observed in any of the tumour sections.

Discussion

The results of the present study reveal an increase of AQP1 expression from low-grade to high-grade astrocytomas, which is in line with previous studies (13, 14). Under normal conditions, AQP1 in the brain is mainly expressed on epithelial cells located on the choroid plexus and ependyma, whereas AQP3, AQP4, AQP5 and AQP9 are most abundantly expressed in astrocytes (15, 16). Interestingly, there is a close connection between AQP1-expressing ependymal endothelium and the subventricular zone (SVZ) during brain development and residual SVZ cells in the mature brain (17). These SVZ cells contain stem and progenitor cells, which have properties similar to glioma-initiating cells with respect to signalling pathways, growth factors, cell markers, and the capacity for migration, invasion and self-renewal (18). The increasing up-regulation of AQP1, normally located in the ependymal endothelium of the SVZ, from low-grade to malignant astrocytoma may therefore represent properties of infiltrating glioma-initiating cells, leading to de-differentiation processes during tumour formation. This would also be in line with the finding that ectopic expression of AQP1 induces phenotypic changes in tumour cells, being characteristic of transformation and cell proliferation (19). Moreover, we observed AQP1 expression predominantly within the perivascular area of the tumour periphery. Glioma invasion is known to occur mainly along blood vessels, corresponding to the occurrence of glioma-initiating cells in close approximation to blood vessels and capillaries (20).

To the best of our knowledge, we are the first to report local differences in AQP1 expression associated with different angiogenic properties in GBM. AQP1 was mainly expressed in the tumour periphery, where infiltration into adjacent brain and the need for a rapid blood supply is obvious. In these areas, many large and branching microvessels were found. In contrast, the hypoxic tumour core, characterized by dense necrotic areas and glomeruloid-like microvessels, revealed only mild AQP1 expression. This finding supports earlier results, where up-regulation of AQP1 led to increased blood vessel formation and AQP1 deletion impaired tumour microvessel proliferation (13). AQP1 expression in the brain is known to be induced under hypoxic conditions by a hypoxia-inducible transcription factor-1α-dependent mechanism (21). Hypoxia-inducible transcription factor-1α is mainly expressed around the necrotic and hypoxic tumour core and increases AQP1 expression, while promoting tumour invasion and angiogenesis, as well as modifying cell membrane water permeability. Unlike other malignancies that spread haematogenously, gliomas

invade the brain by active cell migration, which requires cells to undergo coordinated cell volume changes, facilitated by AQP1 (22).

In conclusion our data suggest a direct correlation between AQP1 expression and the grade of malignancy of astrocytoma, as well as an association with angiogenesis and invasion of GBM into the adjacent brain. Despite multimodal treatment, recurrence of malignant astrocytoma is currently inevitable, as invasion into normal brain has already occurred along blood vessels. Blockade of AQP1 could be useful in reducing the invasiveness of glioma cells.

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