Histol Histopathol (2011) 26: 451-459

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Overexpression of Aquaporin-1 in lung adenocarcinomas and pleural mesotheliomas

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Summary. Aquaporin-1 (AQP1) is the main water channel responsible for water transport through many epithelia and endothelia. The latest evidence pointed toward an important role of this protein also in gas permeation, angiogenesis, cell proliferation and migration. In the present work we studied the expression of AQP1 by immunohistochemical staining of 92 lung biopsies from patients diagnosed with a pleuro-pulmonary tumor (71 lung and 21 pleural neoplasms). AQP1 expression was analyzed comparing the results among the different histological patterns and against 9 control cases (5 parenchyma and 4 healthy pleura). Clear staining of AQP1 was detected in 39 of the 92 tumors analyzed. In parenchyma, AQP1 was more frequently detected in primary lung adenocarcinomas (55%, $P<0.001$); in contrast, small cell carcinomas were the least AQP1 expressive tumors studied (93% of negative staining, $P<0.05$). Carcinomas analyzed in pleura (mesotheliomas and metastatic adenocarcinomas) also revealed strong expression of AQP1. High expression of this protein was detected in small capillaries in areas near or surrounding the tumor, and novel intense AQP1 immunostaining was detected over thicker alveolar walls in alveoli inside or next to the tumoral tissue regardless of the tumor type. An important role of AQP1 in tumor angiogenesis is sustained by the abundant expression of this protein in the endothelia of tumor capillaries. Further studies are necessary to elucidate the potential pathophysiological role of this protein in pleuro-pulmonary neoplasms.

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Key words: Lung neoplasms, Mesothelioma, Angiogenesis, Metastasis, Aquaporin-1

Introduction

The aquaporins (AQPs) are a family of small, highly hydrophobic, integral membrane proteins that function as specialized water channels to facilitate the movement of water across cell plasma membranes (Preston et al., 1992). At present, thirteen members of the AQP family named from 0 to 12 (AQP0- AQP12) have been identified in human. These proteins are widely distributed, and with few exceptions, they are present in practically all cell types in the human body (Takata et al., 2004). Besides their contribution to cell membrane water permeability recent studies implicate them in unexpected functions, such as neural signal transduction, skin flexibility, fat metabolism, membrane gas permeation and cell migration and proliferation (Amiry-Moghaddam and Ottersen, 2003; Hara-Chikuma et al., 2005; Saadoun et al., 2005; Echevarría et al., 2007; Herrera and Garvin, 2007; Hara-Chikuma and Verkman, 2008; Papadopoulos et al., 2008; Woo et al., 2008).

Today a large number of pathologies are been associated with alterations in either functioning or expression pattern of some AQPs, and the number of publications showing its correlation with different human carcinomas are growing. Thus, increased AQP1 expression was detected in reactive astrocytes and microvessel endothelia of human brain tumors (Saadoun et al., 2002), hemangioblastomas of the central nervous system (Longatti et al., 2006), active multiple myeloma (Vacca et al., 2001) and human glial tumours (Oshio et al., 2005). In different non-small cell lung cancer cell lines, as well as lung adenocarcinomas and

bronchoalveolar carcinomas, expression of AQP1 was also detected (Hoque et al., 2006), and overexpression of AQP1, but also AQP3 and 5, was found in colorectal cancer, both in cell lines and in human tissue samples (Moon et al., 2003).

The mechanisms by which AQPs participate in the origin and progression of the tumor are at the moment not fully established, but recent evidence pointed toward an important role of AQP1 in angiogenesis, cell proliferation and cell migration (Saadoun et al., 2005; Papadopoulos et al., 2008; Verkman et al., 2008; Woo et al., 2008). Thus, experiments done in AQP1-null mice demonstrated impaired angiogenesis of the tumoral tissue that leads to a faster tumor necrosis, and considerable growth reduction of melanoma cells subcutaneously implanted in mice lacking AQP1 was observed when compared to control animals (Saadoun et al., 2005). Likewise, the requirement of AQP1 expression has been confirmed for hypoxia-inducible angiogenesis in human retinal vascular endothelia cells cultured under hypoxia conditions (Kaneko et al., 2008). Considering all these findings, we were interested in exploring the presence of AQP1 in different lung carcinomas and analyzing whether the expression pattern of this protein changes in any other lung tissue apart from the tumor cells themselves. Inmunohistochemical staining of AQP1 in 92 biopsies obtained from patients diagnosed with different pleuro-pulmonary tumors was performed and AQP1 expression analyzed, comparing the results among the different histological patterns and against control cases of parenchyma and pleura.

Material and methods

In the present study we performed immunohistochemical analysis of AQP1 expression on tissue sections of biopsies from 92 patients with different pleuropulmonary tumors (71 lung neoplasms and 21 pleural neoplasms). Except for small cell carcinomas and mesotheliomas that were obtained by endobronchial and thoracoscopic procedures, respectively, the rest of the tumors were lobectomy samples removed by surgery. To evaluate AQP1 distribution in normal lungs, samples from 5 age-matched individuals who underwent thoracic surgery for other reasons were taken. Control visceral pleura samples (n=4) were obtained from normal lungs of donors who died from non-respiratory problems. Kidney samples used for positive control of immunohistochemistry assays were obtained from patients that underwent kidney surgery for reasons other than cancer. Paraffin-embedded tissue slides were hematoxylin-eosin stained and evaluated for diagnosis by two independent pathologists.

The tumors were staged according to the tumor-node-metastasis classification, and histologically classified according to the World Health Organization guidelines (Travis et al., 2004). Data regarding lung function tests were also recorded. The study had the approval of The Ethics Committee of The University Hospital Virgen del Rocío (HUVR). Paraffin-embedded samples were obtained from the archives of the Department of Pathology, University Hospital Virgen del Rocío of Seville, Spain.

Immunohistochemistry

In order to set the immunohistochemistry conditions to detect AQP1, different dilutions of the antibody and developing times were tested in lung samples as well as in kidney ones for positive controls. All samples examined were obtained from formalin-fixed, paraffin-embedded pieces.

Tissue slices of 5 μm were cut with a microtome and mounted on microscope slides. Inmunohistochemical procedure started removing the paraffin from slices by immersion in xylene and rehydration through a series of decreasing dilutions of ethanol. Blocking of endogenous peroxidase activity was done by pre-incubation of slices on 3% H_2O_2. Heat-induced epitope retrieval was carried out by incubation of tissue sections at 65°C for 1 h in sodium citrate (10 mM, pH 6). Rabbit polyclonal anti-AQP1 (1:500 dilution, Abcam, Cambridge, UK) was used followed by the two steps EnVision + Dual Link System-HRP (DakoCytomation, Dako DenmarK) that contain goat anti-rabbit immunoglobulins conjugated to peroxidase-labelled polymer and DAB-sustrate-DAB-chromogen for the developing of brown precipitates. Qualitative evaluation of signal level was performed according to the following criteria: absence of brown precipitate indicated a "negative" result for immunoreactivity and the presence of brown precipitate over specific cell areas was considered a "positive" result. When the precipitate had a uniform distribution over a sizeable region of the tumor the sample was considered as "positive diffuse" and when the brown precipitate was present only in certain areas of the tumor, displaying a patchy distribution, the result was taken as "positive focal". Counter-staining with hematoxylin was done and analysis of sections was performed side by side with the pathologist by two independent observers. Sections were photographed using an AX70-Olympus microscope equipped with an Olympus DP10 camera. Omitting the primary antibody produced no staining.

Statistical analysis

Two statistical analyses for AQP1 staining were performed, one in which each neoplasm was compared against the rest of the histological patterns in each anatomical location and the other in which each neoplasm was compared against their respective control group, parenchyma or pleura. Data analysis was performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, Illinois), version 16.0. Continuous variables were described with the mean and standard deviation. Categorical data were summarized with the absolute and relative frequencies of each

category. The expression of AQP1 for each neoplasm and anatomical location was analyzed by χ^2 or Fisher exact test as appropriate. Alpha error was set at 0.05.

Results

Clinical and demographic information of patients included in the study, as well as the number of tumors distributed by carcinoma type, are summarized in Table 1. In normal lung tissues, AQP1 immunostaining was detected predominantly in endothelial cells of alveolar capillaries and major blood vessels (Fig. 1A,B), and a faint labeling was occasionally detected on pneumocytes of the alveolar wall (data not shown). Abundant AQP1 staining was observed over renal tubules (as positive control) (Fig. 1C) and no labeling was detected in absence of the primary antibody (as negative control) (Fig. 1D).

A summary of the immunohistochemical analysis of AQP1 in the different lung and pleura neoplasias is presented in Table 2. Clear staining of AQP1 was detected in 39 of the 92 tumors analyzed. On lung tumors the immunoreactivity of AQP1 varied depending on the tumor type and it was heterogeneous over the tumoral mass. Out of the different histological types analyzed in parenchyma, statistically significant labeling for AQP1 was more frequently detected in primary lung adenocarcinomas, 17 out of 31 (55%, P<0.001), whereas by contrast, small cell carcinomas were the least AQP1 expressive tumors studied (93% of negative staining, P<0.055). Given this specificity for adenocarcinomas to exhibit AQP1 expression we then decided to explore whether AQP1 could be taken as a hallmark useful to differentiate pleura metastatic adenocarcinomas from mesotheliomas (the most frequent pleura tumor) which sometimes can hardly be distinguished from one another. Nonetheless, our results indicated strong immunostaining for AQP1 in both tumor cells when compared to

Table 1. Summary of neoplasms included in the study .

Location	Neoplasm type	N	%	Age	Male gender
Lung	Squamous cell carcinoma	12	13.0	67±5	11 (91.7%)
	Adenocarcinoma	31	33.7	63±8	20 (64.5%)
	- Bronchioloalveolar carcinoma	3	3.2	67±7	2 (66.6%)
	- Acinar adenocarcinoma	1	1.0	57	1 (100%)
	- Papillary adenocarcinoma	3	3.2	64±8	2 (66.6%)
	- Mucus-producing adenocarcinoma	1	1	55	1 (100%)
	- Solid adenocarcinoma	3	3.2	70±6	3 (100%)
	- Adenocarcinoma (mixed subtype)	20	21.7	62±9	11 (55%)
	Adenosquamous carcinoma	2	2.2	70±3	2 (100%)
	Large cell carcinoma.	11	12.0	63±7	6 (100%)
	Small cell carcinoma	15	16.3	68±9	13 (86.7%)
Pleura	Mesothelioma	12	13.0	70±11	9 (75%)
	Metastatic adenocarcinoma	9	9.8	60±15	5 (55.6%)
Total		92	100	65±11	65 (73%)

Table 2. Association between AQP1 staining and lung tumor types.

Location	Neoplasm type	Tumor cells AQ1 immunoreactivity			P value*	P value†
		Negative	Positive focal	Positive diffuse		
Lung	Adenocarcinoma (n = 31)	14 (45.2%)	14 (45.2%)	3 (9.7%)	0.001	0.074
	Adenosquamous carcinoma (n = 2)	2 (100%)	–	–	NS	N/A
	Squamous cell carcinoma (n = 12)	10 (83.3%)	2 (16.7%)	–	NS	NS
	Large cell carcinoma (n = 11)	8 (72.7%)	3 (27.3%)	–	NS	NS
	Small cell carcinoma (n = 15)	14 (93.3%)	1 (6.7%)	–	0.055	NS
Pleura	Mesothelioma (n = 12)	2 (16.7%)	7 (58.3%)	3 (25%)	NS	0.032
	Metastatic adenocarcinoma (n = 9)	3 (33.3%)	6 (66.7%)	–	NS	0.002

*: Calculated by χ^2 comparing each neoplasm against the rest of histological patterns in each anatomical location. †: Calculated by χ^2 comparing each neoplasm against the parenchyma and pleura control groups respectively

control tissues (83% positive mesotheliomas, P<0.032, and 67% positive metastatic adenocarcinomas, P<0.002) (Table 2) ruling out distinction among these two types of tumors just based on AQP1 immunostaining.

Representative examples of AQP1 immunoreactivity of each cancer type analyzed are shown in Figure 2 for lung carcinomas and in Figure 3 for primary pleural tumors (mesotheliomas) and for metastatic adenocarcinomas. In Figure 2A strong immunostaining of AQP1 is shown on adenocarcinoma tumor cells, while total absence of AQP1 staining was detected over adenosquamous carcinoma cells (Fig. 2B), large cell carcinoma (Fig 2C), or small cell carcinoma (Fig. 2D). In mesotheliomas and metastatic adenocarcinomas, immunostaining of AQP1 revealed strong expression of AQP1 in both types of pleura carcinomas. A high percentage (83%) of mesotheliomas studied were immunopositive for AQP1, with a level of signal clearly above the pleura background (Table 2, Fig. 3). Examples of three different mesotheliomas showing high intensity for AQP1 immunostaining are presented in Figure 3 (A,D,E). Cellular morphology and distribution varied among the three different mesotheliomas. In control pleura, immunostaining for AQP1 showed expression of this protein in mesothelial cells, with absence of staining in submesothelial fibroblasts (Fig. 3C). Reactive mesothelial cells (data not shown) showed a similar pattern of AQP1 expression. In Figures 3F,G, two examples of pleura metastatic lung adenocarcinomas are shown where AQP1 immunostaining of carcinoma cells was confirmed. The level of immunoreactivity for AQP1 in metastatic adenocarcinomas was in general greater (66% of positivity) than that observed for primary lung adenocarcinomas (55% of positivity), although differences were not statistically significant. Metastatic adenocarcinomas in pleura showed a focal AQP1 staining in all cases (100%), whereas in lung adenocarcinomas focal immunoreactivity was reached in 45 % of cases (Table 2).

Independently of the immunopositivity of AQP1 in a specific lung tumor, expression of this protein was detected in new blood vessels proliferating in areas near or surrounding the tumor (Figs. 2, 4, see arrows over the panels). Moreover, intense AQP1 immuno-staining over thicker alveolar walls was often observed in alveoli inside or next to the tumoral tissue regardless of the tumor type. In Figure 4 (see arrowheads), reactive type II pneumocytes of alveolus in the middle of a tumoral mass of lung adenocarcinoma (Fig. 4B) and large cell carcinomas (Fig. 4C,D), showed intense AQP1 expression, in spite of the complete absence of AQP1 staining in the tumor tissue.

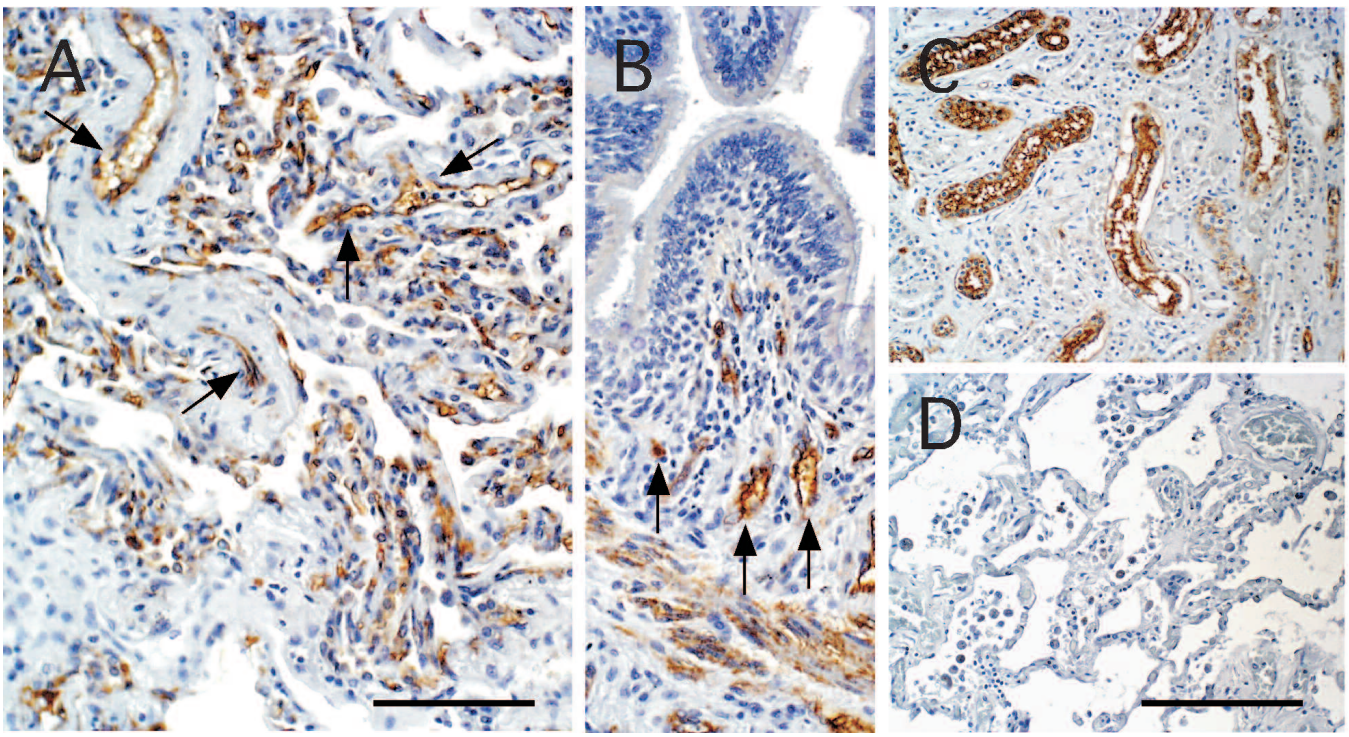

Fig. 1. Immunohistochemical analysis for AQP1 expression in control tissues. In normal lung tissue, AQP1 staining (brown precipitate) is observed in endothelia of alveoli capillaries (**A**, see arrows) and small blood vessels (**B**, see arrows). The epithelia lining the alveolar space is negative for AQP1 in normal lungs **(A, B)**. For a positive control, immunostaining of AQP1 in renal tubules was confirmed in a sample of human kidney **(C)**. As negative control, lack of AQP1 staining in lung was obtained when the primary AQP1 antibody was omitted **(D)**. Arrows indicate blood vessels. Scale bars: A, B, 100 μm; C, D, 200 μm.

Discussion

In the present study immunohistochemical analysis of AQP1 expression in a variety of different lung carcinomas confirmed the exclusive expression of this protein in mesotheliomas and adenocarcinomas. For the first time, the expression of AQP1 was demonstrated in mesothelial cells of control human pleura and concomitant with this, strong AQP1 staining of mesothelioma tumor cells was observed. AQP1 expression in pleura metastatic adenocarcinoma cells was also detected in a very robust way. According to our results, using AQP1 immunostaining as a diagnostic tool to distinguish both pleura carcinomas, mesotheliomas from pleura metastatic adenocarcinoma, would be unfeasible. Currently, an explanation for the exclusive expression of AQP1 in these tumor types is unavailable, but the normal expression of AQP1, albeit low in alveolar epithelial cells and in pleura mesothelia, from which adenocarcinoma and mesothelioma tumor cells derive, may help to understand why only these lung tumors overexpressed AQP1.

The identification and understanding of the links between AQP expression and tumor progression, malignance and metastasis is an active field nowadays. Increased expression of AQP1, AQP3 and AQP5 have

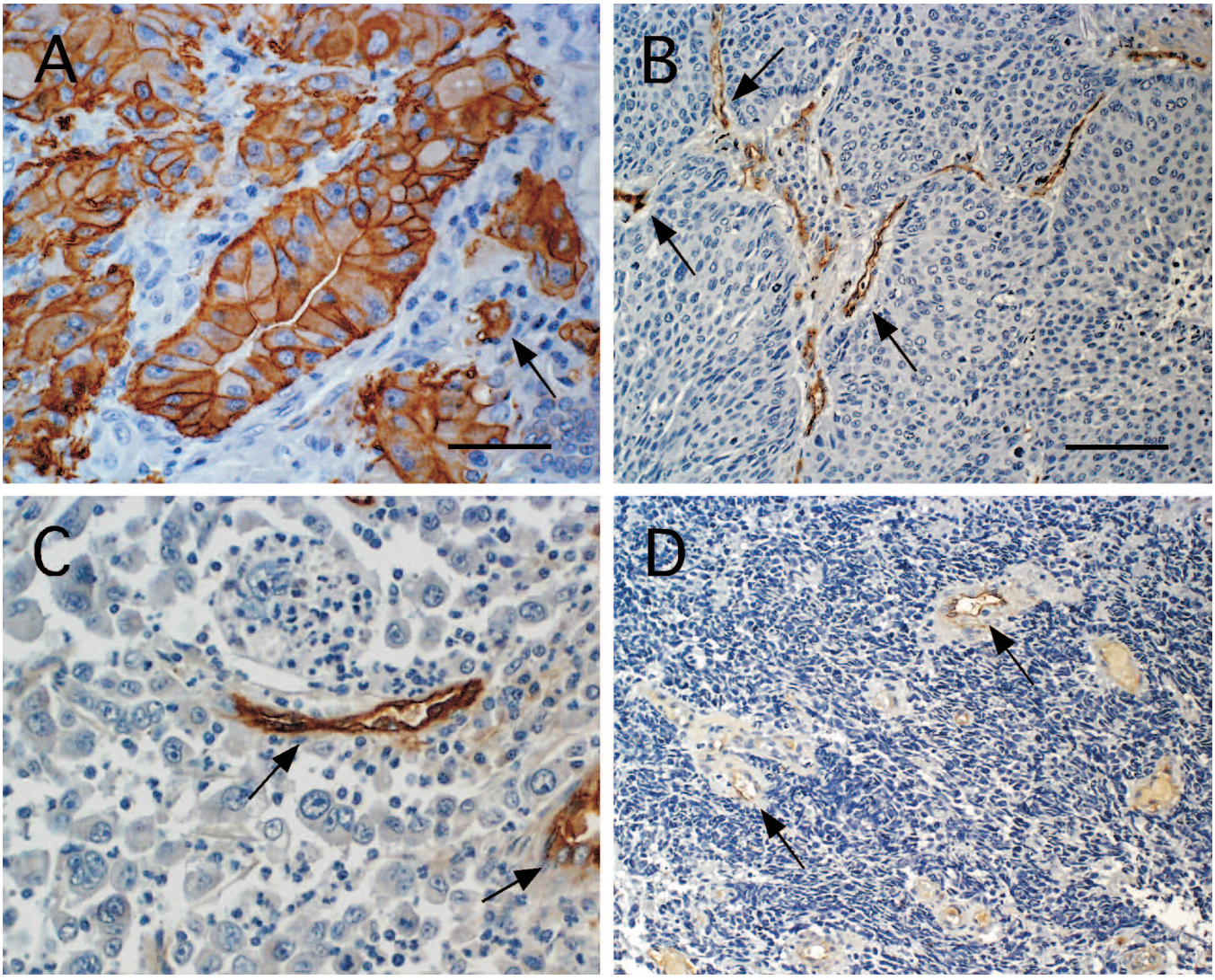

Fig. 2. Expression of AQP1 in different lung carcinomas. Positive immunostaining of AQP1 (brown precipitate) in lung carcinomas was only detected in Adenocarcinomas **(A)**. Squamous cell carcinoma **(B)**, large cell carcinoma **(C)** and small cell carcinoma **(D)** were overall negative for AQP1. Arrows indicate blood vessels. Scale bars: A, 50 μm; B-D, 100 μm.

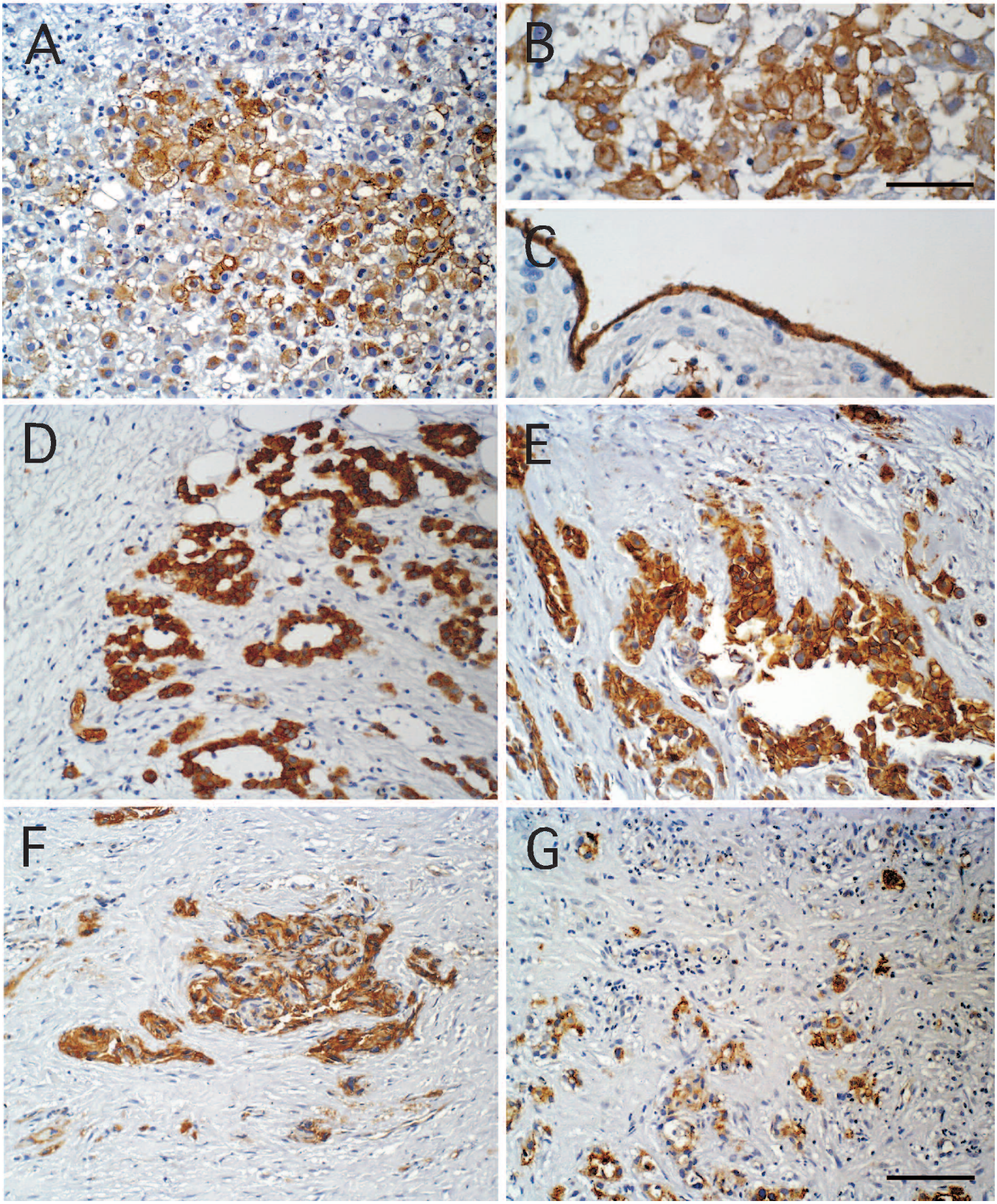

Fig. 3. Immunostaining of AQP1 seen as brown precipitate in mesotheliomas and metastatic adenocarcinomas. Different mesotheliomas are shown in panels **A, D and E**. **B** is a larger magnification of photograph **A**. Staining of AQP1 in control pleura is shown in **C**. Metastatic adenocarcinomas in pleura showed highly positive AQP1 immunostaining and two examples are shown in **F** and **G**, respectively. Scale bars: A, D-G, 100 μm; B, C, 50 μm.

been detected in different cancer cell lines and human tumor samples and previous works associate at least three other AQPs to diverse tumor types (Vacca et al., 2001; Saadoun et al., 2002; Moon et al., 2003; Oshio et al., 2005; Hoque et al., 2006; Longatti et al., 2006; Hara-Chikuma and Verkman 2008; Verkman et al., 2008; Woo et al., 2008). An important role of AQP1 in angiogenesis, cell proliferation and cell migration has been demonstrated. The proposed mechanisms to explain how AQP1 facilitates these processes always allude to a more rapid water flow across the plasma membrane into the front end of migrating cells (Saadoun et al., 2005; Verkman et al., 2008) that would facilitate cellular changes in volume and shape. However, more recent functions of AQP1, for instance its ability to increase O_2 and CO_2 membrane's permeability (Echevarría et al., 2007; Endeward et al., 2006), might also contribute to explain its participation in tumor progression. In PC12 cells, overexpression of AQP1 accelerates the loss of cytosolic oxygen in hypoxia leading to a faster stabilization of the O_2-dependent hypoxia-inducible transcription factor (HIF) and expression of its target genes (Echevarría et al., 2007). Furthermore, the participation of HIF1α in the hypoxia regulation of AQP1 has recently been reported (Abreu-Rodríguez et al., 2007), thus supporting previous works

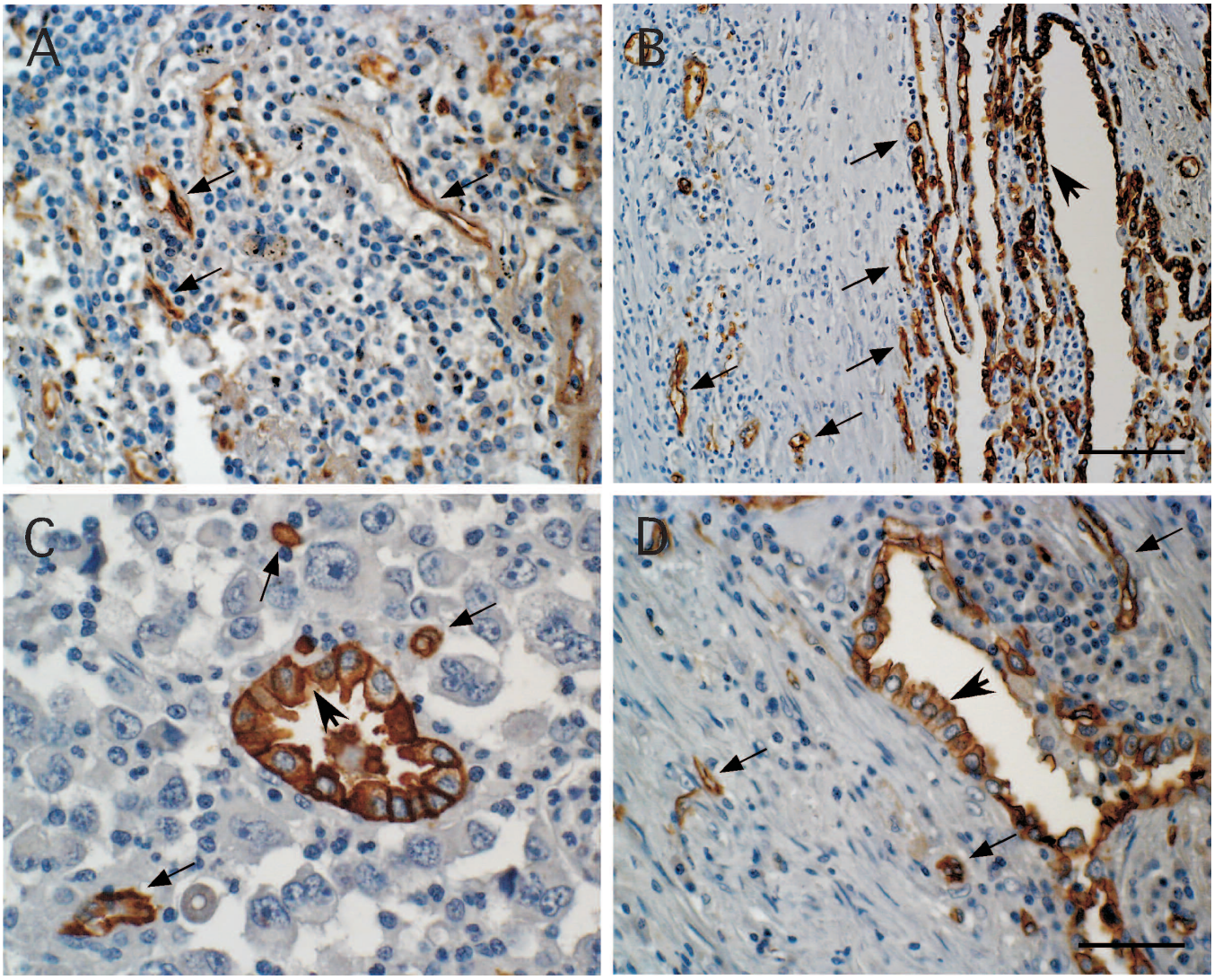

Fig. 4. Blood vessels and hypertrophic pneumocytes inside the tumoral area highly express AQP1. Abundant presence of small blood vessels (arrows) with intense labelling for AQP1 in squamous cell carcinoma **(A)**, adenocarcinoma **(B)** and large cell carcinoma (**C and D**). Reactive type II pneumocytes (arrow head) of alveolus showed intense AQP1 expression in areas near the tumoral mass of either lung adenocarcinoma (**B)** or large cell carcinomas (**C and D**). Scale bars: A, C, D, 50 μm; B, 100 μm.

that indicated a significant increase in AQP1 expression under hypoxic conditions (Echevarría et al., 2007; Hayashi et al., 2007). The clear association observed between HIF1α and tumor progression in many tumor types (Jeffs et al., 2009; Yoo et al., 2009) could then provide us with a putative clue to link AQP1 with tumor growth and metastatic potential. Taking together all this evidence one may hypothesize that AQP1 overexpression may confer to tumoral cells a competitive phenotype (faster water influx for shape remodeling and gas equilibration across the membrane, stronger stabilization of hypoxia transcription factors and hence expression of targets genes) that could explain its implications in human cancerogenesis.

The abundant expression of AQP1 observed in the small blood vessels endothelia surrounding all primary lung neoplasias confirms similar observations (Vacca et al., 2001; Saadoun et al., 2002; Oshio et al., 2005; Longatti et al., 2006; Verkman et al., 2008) and strengthens the case for AQP1 as having essential role as tumor angiogenesis promoter (Saadoun et al., 2005; Clapp and Martínez de la Escalera, 2006). In addition to its role as a structural element of capillaries endothelia necessary for new blood vessel formation in tumors, AQP1 may importantly contribute to the high vascular permeability and interstitial fluid pressure found in various carcinomas (Mobasheri et al., 2005).

The detection of strong induction of AQP1 expression in hyperplasic pneumocytes of alveolar epithelia in pneumonitis peritumoral areas of all tumor types analyzed constitutes another novel finding of the present work. Thickening of the alveoli wall evidences a hyperreactivity response in which an increase of AQP1 expression was a remarkable feature. Again, since induction of the hypoxia inducible factor HIF1α has been demonstrated in alveolar epithelial cells of hypoxic lungs (Tzouvelekis et al., 2007; Clerici and Planes, 2009) one could hypothesize that HIF might be involved in this epithelial overexpression of AQP1. In addition, it has recently been demonstrated that inflammatory mediators like cytokines IL-1ß or TNFα (Frede et al., 2007) induce HIF1α in vascular smooth muscle cells and tumor cells of different tissue origin, and thus one also could hypothesize that AQP1 lung epithelia induction may occur in a HIF dependent manner. In vivo experiments with animal models and cellular culture lines of lung tumors and alveolar epithelial cells will be necessary to further address some of the questions and hypotheses raised from the present work.

In conclusion, the data presented here indicates that AQP1 is overexpressed in several pleura-pulmonary tumors. The potential role of this expression as a means to facilitate tumor growth and spread of lung adenocarcinomas and mesotheliomas through the activation of tumor angiogenesis and perhaps tumor cell migration deserves further scrutiny. If these pathways are finally addressed, new antitumor therapies may be potentially derived from AQP1 inhibition in the near future.

Acknowledgements. This work was funded by grants from the "Instituto de Salud Carlos III" PI PI060511 and PS09/00605, and by grants from "La Junta de Andalucía", Consejería de Salud (exp. # 0144 and # 0192) and Consejería de Innovación Ciencia y Empresa (P08-CTS-03574).

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Accepted October 27, 2010