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Localisation and expression of aquaporin subtypes in epithelial ovarian tumours

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Summary. To characterise AQP subtype localisation and expression in epithelial ovarian tumours, immunohistochemistry was used to assess the localisation and expression of AQP1-9 in 30 benign tumour cases, 30 borderline tumour cases, 50 malignant tumour cases and 20 normal ovarian tissue cases. Multiple AQP subtypes were expressed in epithelial ovarian tumours, with each AQP subtype displaying a different pattern of localisation and expression. AQP1 was mainly expressed in the microvascular endothelium, and AQP 2-9 were mainly expressed in tumour cells. Most AQP subtypes co-localised in the basolateral membranes of the epithelia of benign tumours and plasma membranes of malignant tumour cells. The positive rates for AQP1, 5, 6, 7, 8, and 9 were over 50%, but those for AQP2, 3 and 4 were only 10-40%. The expression of AQP1, 5 and 9 in malignant and borderline tumours was significantly higher than that in benign tumours ($P<0.05$) and normal ovarian tissue ($P<0.05$). However, AQP6 expression in ovarian malignant and borderline tumours was significantly lower than that in benign tumours ($P<0.01$) or normal ovarian tissue ($P<0.01$). AQP1 expression was increased in cases with ascites volumes greater than 1000 mL ($P<0.05$), AQP5 expression was greater in cases with lymph node metastasis ($P<0.05$), and more AQP9 expression was observed in G3 cases versus G1 and G2 cases ($P<0.01$). These results suggest that changes in the distribution and expression of AQP subtypes may be involved in ovarian carcinogenesis. This study presents a novel avenue of research that could illuminate the mechanism of ovarian carcinogenesis and treatment.

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Introduction

The aquaporins (AQPs) are a family of small membrane transport proteins that act primarily as water-selective pores. In humans, 13 AQPs are known, and these are divided into three functionally distinct groups (Magni et al., 2006; Rojek et al., 2008; Ishibashi et al., 2009): AQP0, 1, 2, 4, 5, and 8 are water channels, whereas the aquaglyceroporins AQP3, 7, 9, and 10 also transport glycerol and other small solutes. Finally, AQP6, 11, and 12, sometimes referred to as unorthodox aquaporins, have specific or yet unknown properties. Different AQP subtypes with specific patterns of organ, tissue, and cellular localisation are expected to function in virtually all physiological processes that involve water transport across a membrane (Walz et al., 2009; Fischbarg, 2010; Hachez and Chaumont, 2010).

Recent discoveries of the involvement of AQPs in cell migration and proliferation suggest that AQPs play a key role in tumour biology (Cao et al., 2006; Auguste et al., 2007; Verkman et al., 2008). AQPs are strongly expressed in tumour cells of different origins, particularly in aggressive tumours. AQP-expressing cancer cells show enhanced migration in vitro and greater local tumour invasion, tumour cell extravasation, and metastasis in vivo. AQP-dependent cell migration may involve AQP-facilitated water influx into lamellipodia at the front edge of migrating cells (Walz et al., 2009). Some studies suggested that AQP1 and AQP5 may play an important role in ovarian carcinogenesis (Yang et al., 2006a,b). However, the tissue distribution and cellular localisation of other AQP subtypes remains unclear in epithelial ovarian tumours.

Epithelial ovarian tumour is a disease that commonly afflicts women. Ovarian tumours commonly exhibit a simple-cyst or poly-cyst structure containing serous or mucous fluids, and ascites fluid is frequently found in progressive ovarian cancer (Heintz et al., 2006). The development of epithelial ovarian tumours clearly involves water transportion (Mobasheri et al., 2005; Yang et al., 2005, 2006a,b), but the relationship between AQP subtype expression and epithelial ovarian tumours remains unclear. Therefore, this study uses immunohistochemistry to characterise the localisation and expression of AQP subtypes 1-9 and their potential role in epithelial ovarian tumours.

Materials and methods

Tissue specimens

From November 2006 to December 2009, specimens of 110 primary ovarian epithelial tumours and 20 normal ovarian tissues with uterine myoma were collected from patients (ranging in age from 18 to 74, with a median of 45 years) who underwent surgical resection of the ovaries at the Second Hospital and Women's Hospital of Zhejiang University, Hangzhou, China. The 110 epithelial ovarian tumours included 30 cases of benign tumours (15 serous cystadenomas, 15 mucous cystadenomas) with no other diseases (e.g., inflammation or hypertension), 30 cases of borderline tumours (15 serous, 15 mucous), and 50 cases of malignant tumours (22 serous carcinomas, 25 mucous carcinomas and 3 endometrioid carcinomas), and 20 normal ovarian tissues with uterine myoma were also collected. Patient information was taken from the clinical records. The diagnoses of all patients were confirmed by pathology. All patients signed informed consent letters, and the experimental methods were approved by the local institutional review boards.

Immunohistochemistry

Standard hematoxylin and eosin-stained slides from the files of the Department of Pathology were examined, and the best sections were chosen by two pathologists for immunohistochemical reactions. The expression of AQP1, 2, 3, 4, 5, 6, 7, 8, and 9 were examined by immunohistochemistry. Paraffin-embedded, 4 μm-thick tissue sections were deparaffinised in xylene and then rehydrated through a graded series of alcohol solutions. The endogenous peroxidase was blocked with 10% hydrogen peroxidase. The antigen retrieval reaction was performed by a 1.5 min incubation in 10 mM boiling sodium citrate buffer (pH 6.0), and nonspecific binding was reduced by normal nonimmune serum. The samples were incubated for 60 min at room temperature with the primary antibodies AQP1, 5, 6, 8 and 9 at a 1:100 dilution or AQP2, 3, 4, and 7 at a 1:50 dilution (Santa Cruz Biotechnologies, Santa Cruz, CA), and then with the secondary antibody for 30 min at room temperature (UltraSensitive™ SP kits, Zymed Laboratories Inc., South San Francisco, CA). The reaction was then visualised with diaminobenzidine, and the slides were counterstained with hematoxylin. Positive reaction controls (kidney and liver tissues that were known to be reactive to the AQP antibodies) and negative reaction controls (tissues of ovarian tumour samples that were not incubated with the primary antibodies) were used in all the immunohistochemical reactions performed in this study. The immunoreactivity was evaluated by two investigators using a 3-level scale (-, 1+ and 2+): -, negative or positive in <10% of cancer cells or microvessels; 1+, positive in 10-49% of cancer cells or microvessels; 2+, positive in 50-100% of cancer cells or microvessels.

Statistical analysis

All data were analysed using SPSS13.0 software. The χ^2 test was used for statistical analysis of the immunohistochemical results. $P<0.05$ was regarded as statistically significant.

Results

The localisation and expression of AQP subtypes in normal ovarian tissue

The immunohistochemical results reveal that AQP1 is mainly expressed in the microvascular endothelium of normal ovarian tissue, but not in normal ovarian epithelia (Fig. 1). AQP 4, 5, 6, 7 and 8 were observed in epithelia of normal ovarian tissue, whereas AQP 2, 3, and 9 were seldom found (Figs. 2-9).

Of nine AQP subtypes, strong expression of AQP1, 6, 7, and 8 was found in normal ovary tissue, with positive rates over 50%. Of these, the highest positive rate was for AQP6 at 95%, followed by AQP8 at 75%, AQP7 at 65%, AQP1 at 55%. AQP5 and 4 had very low positive rates: 40% and 30%, respectively. Finally, AQP 2, 3, and 9 had the lowest positive rates: only 10% (Tables 1, 2).

The localisation of AQP subtypes in epithelial ovarian tumours

The immunohistochemical results show that AQP1 is strongly expressed in the microvascular endothelium of various epithelial ovarian tumours, but seldom in tumour cells (Fig. 1).

AQP 2, 3, 4, 5, 6, 7, 8 and 9 staining was all observed in tumour cells, although their localisation differed among benign, borderline and malignant tumours. In the epithelia of benign tumours, five subtypes (AQP3, 4, 5, 8 and 9) were mainly expressed in the basolateral membranes, with AQP6 and AQP7 expressed in the plasma membranes. In borderline tumour cells, AQP2 and AQP9 staining was observed in the basolateral membranes, AQP3, 4, 5, 6 and 8 in the

plasma membrane, and AQP7 in the nuclear membrane. In malignant tumours, AQP2, 3, 4, 5, 6, 8 and 9 were scattered in the plasma membrane and AQP7 was observed in the nuclear membrane (Figs. 2-9).

Expression of AQP subtypes in epithelial ovarian tumours

Of nine AQP subtypes, AQP1 had the highest positive rate, at 66.67% in benign tumours and 100% in borderline and malignant tumours. The group with the next highest expression was AQP5, 6, 7, 8 and 9. Finally, AQP2, 3, and 4 had very low positive rates in epithelial ovarian tumours: less than 40% (Tables 1, 2).

AQP1 expression in malignant and borderline tumours was significantly higher than that of benign tumours ($P<0.01$) and normal ovarian tissue ($P<0.01$). The expression of AQP1 in cases with an ascites volume greater than 1000 mL was greater than in cases with an ascites volume less than 1000 mL ($P<0.05$) (Table 3).

AQP5 expression in ovarian malignant and borderline tumours was significantly higher than that in benign tumours ($P<0.01$, $P<0.05$, respectively) and normal ovarian tissue ($P<0.01$, $P<0.05$, respectively),

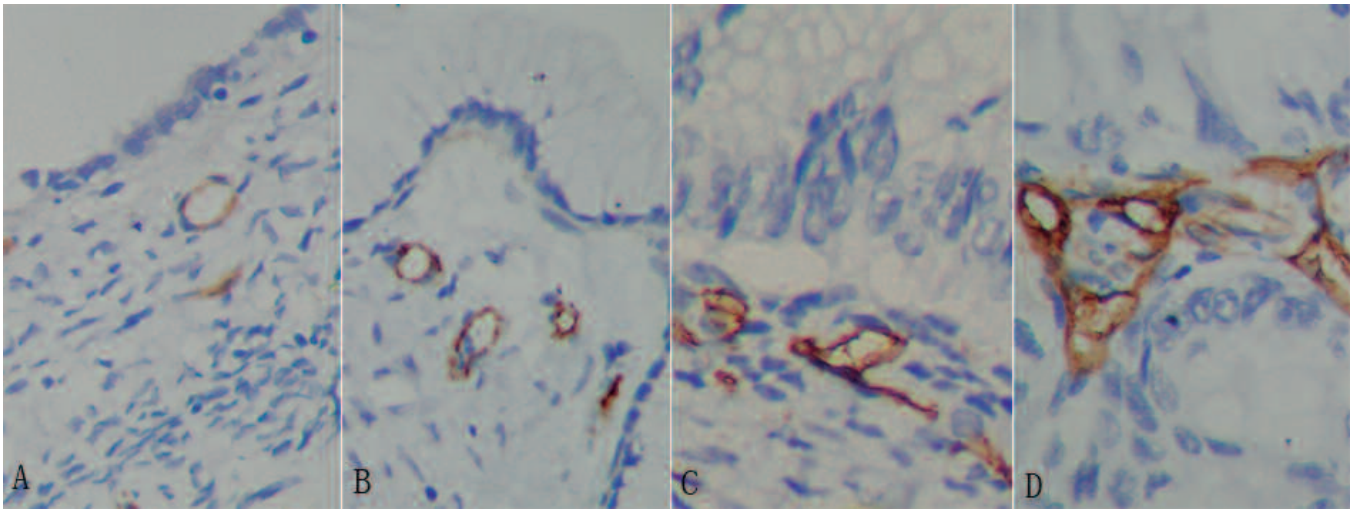

Fig. 1. Immunohistochemical localisation of AQP1 in epithelial ovarian tumours and normal ovarian tissue. Strong AQP1 is restricted to the microvascular endothelium. Normal ovarian tissue **(A)**, benign ovarian tumour **(B)**, borderline ovarian tumour **(C)** and malignant ovarian tumour **(D)**. x 400

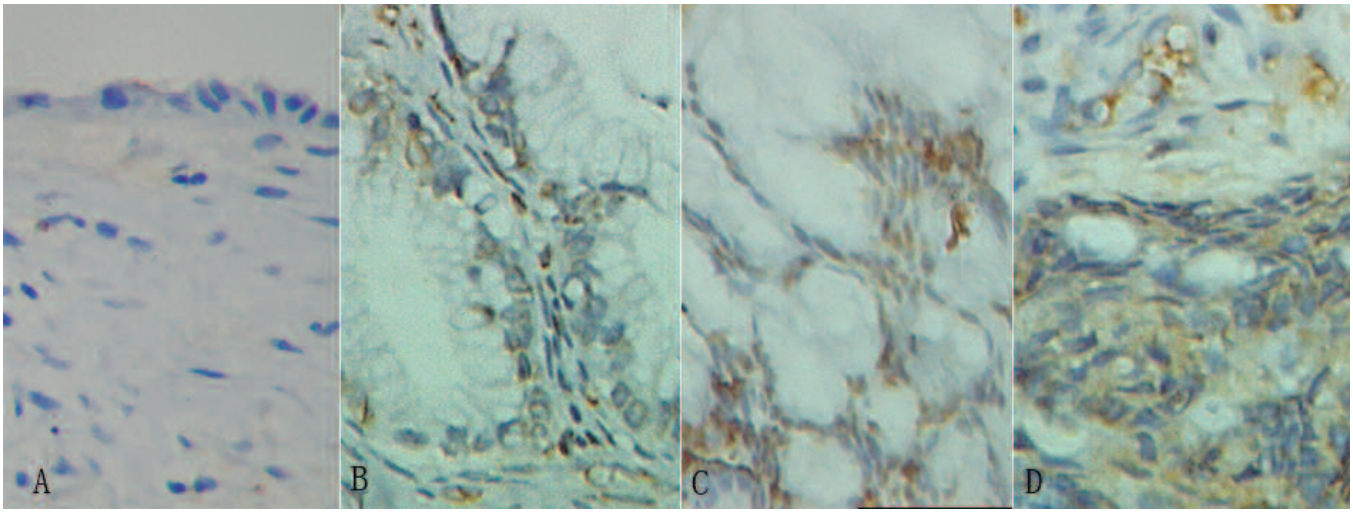

Fig. 2. Immunohistochemical localisation of AQP2 in epithelial ovarian tumours and normal ovarian tissue. Normal ovarian tissue **(A)**, benign ovarian tumour **(B)**, borderline ovarian tumour **(C)** and malignant ovarian tumour **(D)**. x 400

but no significant difference was observed between borderline and malignant tumours ($P<0.05$) (Table 1). Increased AQP5 expression was found in cases with lymph node metastasis ($P<0.05$) (Table 3).

AQP6 expression in ovarian malignant and borderline tumours was significantly lower than that in benign tumours ($P<0.01$, $P<0.01$, respectively) and normal ovarian tissue ($P<0.01$, $P<0.01$, respectively), but no significant difference was observed between borderline and malignant tumours ($P<0.05$) (Table 3).

AQP9 expression was significantly higher in malignant tumours than in borderline tumours ($P<0.01$) and higher in borderline tumours than in benign tumours ($P<0.01$) and normal ovaran tissue ($P<0.01$) (Table 2). AQP9 expression was significantly higher in undifferentiated tumours (G3) compared with well-

Table 1. Expression of AQP1, 2, 4, 5 and 8 in epithelial ovarian tumours and normal ovarian tissue.

	n	AQP1		AQP2		AQP4		AQP5		AQP8	
		- (%)	+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)	+ (%)
Normal (1)	20	9 (45.0)	11 (55.0)	18 (90.0)	2 (10.0)	14 (70.0)	6 (30.0)	12 (60.0)	8 (40.0)	5 (25.0)	15 (75.0)
Benign (2)	30	10 (33.33)	20 (66.67)	23 (76.67)	7 (23.33)	17 (56.67)	13 (43.33)	16 (53.33)	14 (46.67)	6 (20)	24 (80)
Borderline (3)	30	0 (0.00)	30 (100.0) **	22 (73.33)	8 (26.67)	20 (66.67)	10 (33.33)	8 (26.67)	22 (73.33) ^	8 (26.67)	22 (73.33)
Malignant (4)	50	0 (0.00)	50 (100.0) **	31 (62.0)	19 (38.0)	34 (68.0)	16 (32.0)	7 (14.0)	43 (86.0) **	7 (14.0)	43 (86.0)

**: $P<0.01$ versus 1 and 2; ^: $P<0.05$ versus 1 and 2.

Table 2. Expression of aquaglyceroporins AQP3, 7 and 9, as well as the unorthodox subtype AQP 6, in epithelial ovarian tumours and normal ovarian tissue.

	n	AQP3		AQP7		AQP9		AQP6	
		- (%)	+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)	+ (%)
Normal (1)	20	18 (90.0)	2 (10.0)	7 (35.0)	13 (65.0)	18 (90.0)	2 (10.0)	1 (5.0)	19 (95.0)
Benign (2)	30	24 (80.00)	6 (20.0)	10 (33.33)	20 (66.67)	20 (66.67)	10 (33.33)	1 (3.33)	29 (96.67)
Borderline (3)	30	23 (76.67)	7 (23.33)	3 (10.0)	27 (90.0)	7 (23.33)	23 (76.67) #	20 (66.67)	10 (33.33) #
Malignant (4)	50	32 (64.0)	18 (36.0)	10 (20.0)	40 (80.0)	1 (4.0)	49 (98.0) * ^	37 (74.0)	13 (26.0) *

*: $P<0.01$ versus 1 and 2; ^: $P<0.01$ versus 3; #: $P<0.01$ versus 1 and 2.

Table 3. The expression of AQP1, AQP5 and AQP9 according to FIGO stage, histological type, grade, lymph node metastasis, and ascites volume.

	AQP1 (n =50)		AQP5 (n =43)		AQP9 (n =49)	
Group	1+ (%)	2+ (%)	1+ (%)	2+ (%)	1+ (%)	2+ (%)
FIGO stage						
I II	12 (57.14)	9 (42.86)	8 (50.0)	8 (50.0)	13 (65.0)	7 (35.0)
III IV	18 (62.07)	11 (37.93)	15 (55.56)	12 (44.44)	14 (48.28)	15 (51.82)
Histology						
serous	12 (54.55)	10 (45.45)	10 (52.63)	9 (47.37)	11 (50.0)	11 (50.0)
mucous	16 (64.0)	9 (36.0)	12 (54.55)	10 (45.45)	14 (58.33)	10 (41.67)
endometrioid	2 (66.67)	1 (33.33)	1 (50.0)	1 (50.0)	2 (50.0)	1 (50.0)
Grade						
G1	11 (68.75)	5 (31.25)	9 (60.0)	6 (40.0)	14 (87.5)	2 (12.5)
G2	7 (77.78)	2 (22.22)	3 (50.0)	3 (50.0)	5 (55.56)	4 (44.44)
G3	12 (48.0)	13 (52.0)	11 (50.0)	11 (50.0)	8 (33.33)	16 (66.67) **
Lymph node metastasis						
no	15 (68.28)	7 (31.82)	13 (62.22)	5 (27.78)	13 (59.09)	9 (40.91)
yes	15 (53.67)	13 (46.43)	10 (40.0)	15 (60.0) *	14 (51.85)	13 (48.15)
Ascites						
<1000ml	25 (73.63)	9 (26.47)	14 (50.0)	14 (50.0)	19 (57.58)	14 (42.42)
≥1000ml	5 (31.25)	11 (68.75) *	9 (60.0)	6 (40.0)	7 (50.0)	8 (50.0)

*: $P<0.05$; **: $P<0.01$.

differentiated tumours (G2 and G1) ($P<0.01$) (Table 3).

AQP8 was highly expressed in epithelial ovarian tumours, but no significant differences in expression were observed among benign tumours, borderline tumours, malignant tumours and normal ovarpan tissue ($P>0.05$) (Table 1).

Although the localisation of AQP7 varied according to tumour type, no significant difference of expression was found among benign tumours, borderline tumours, malignant tumours and normal ovaran tissue ($P<0.05$) (Table 2).

The positive rates of AQP2, 3 and 4 in epithelial ovarian tumours was low (only 10-40%), and no significant differences were observed in these levels among benign tumours, borderline tumours, malignant tumours and normal ovaran tissue ($P>0.05$) (Tables 1, 2).

Discussion

A growing body of reports have demonstrated that AQPs play a crucial role in maintaining water

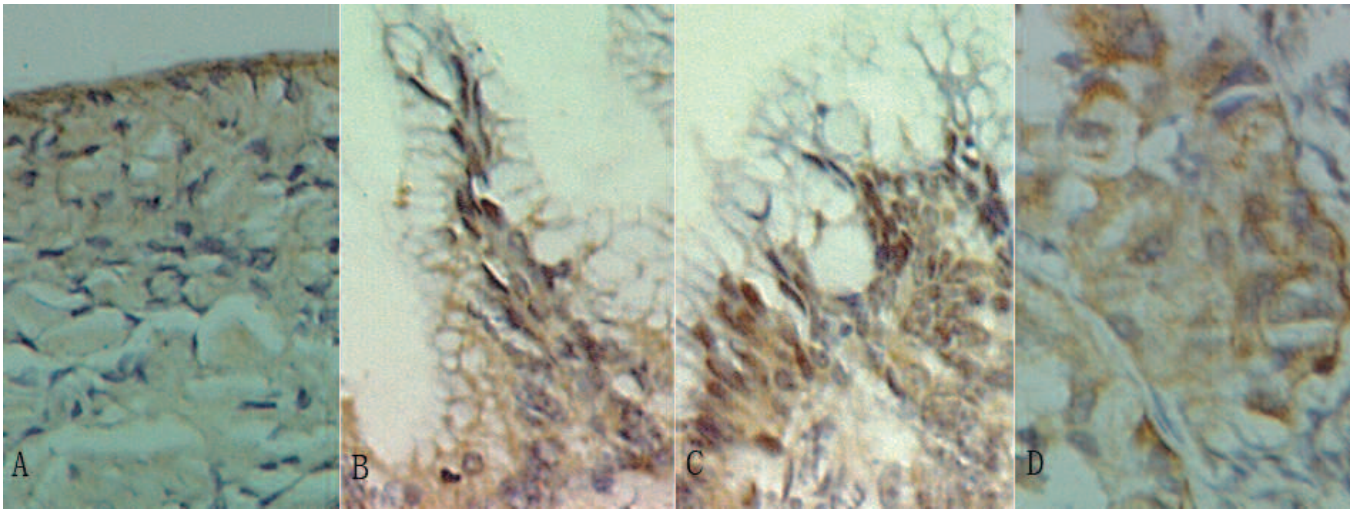

Fig. 3. Immunohistochemical localisation of AQP3 in epithelial ovarian tumours and normal ovarian tissue. Normal ovarian tissue **(A)**, benign ovarian tumour **(B)**, borderline ovarian tumour **(C)** and malignant ovarian tumour **(D)**. x 400

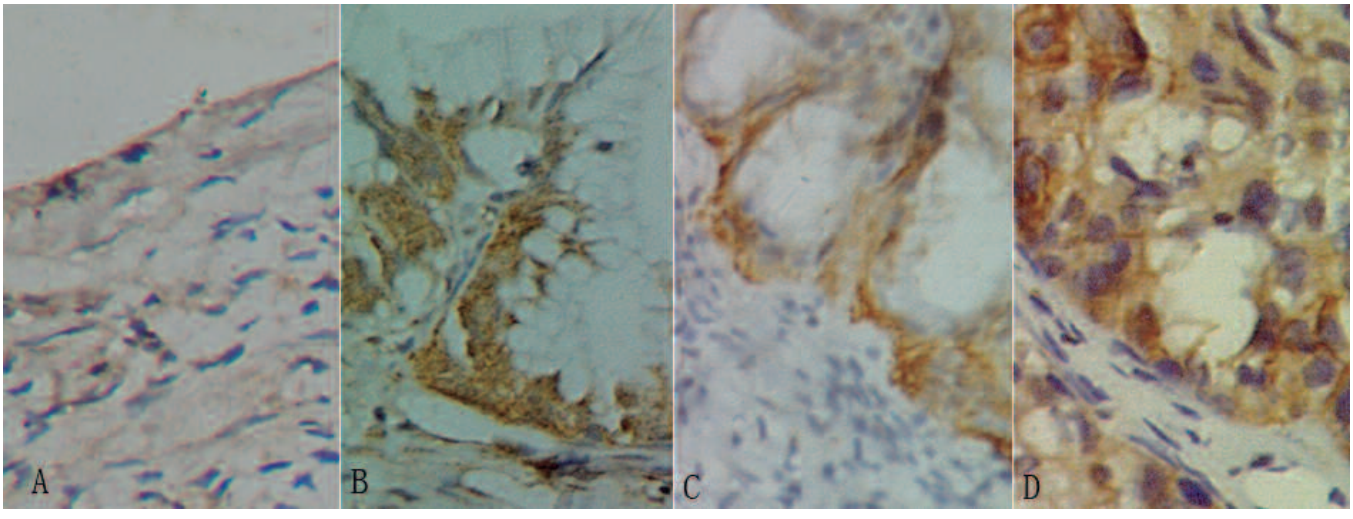

Fig. 4. Immunohistochemical localisation of AQP4 in epithelial ovarian tumours and normal ovarian tissue. AQP4 is localised to the epithelia of normal ovarian tissue **(A)**, the basolateral membrane of benign tumour epithelia **(B)** and borderline tumour cells **(C)**, and the plasma membranes of malignant cells **(D)**. x 400

homeostasis and that they are involved many human tumours (Yang et al., 2006b; Warth et al., 2007; Verkman et al., 2008; Miao et al., 2009; Xu et al., 2009; Badaut, 2010). Recent studies found that AQP1 is located mainly in microvessels of epithelial ovarian tumor and AQP5 in epithelial ovarian tumour cells, and the data suggested that overexpression of AQP1 and AQP5 plays an important role in ovarian carcinogenesis, progression, and ascites formation (Yang et al., 2005, 2006a,b). However, localisation and expression of other AQP subtypes in epithelial ovarian tumours is still unclear. The current study investigated the nine AQP subtypes using immunohistochemistry. The results show that all nine subtypes were expressed in epithelial ovarian tumours, with each AQP subtype displaying a different pattern of localisation and expression. Of the nine AQP subtypes, only AQP1 was observed in the microvascular endothelia of ovarian tumours, the remaining eight AQP subtypes were found in epithelial ovarian tumour cells: AQP2, 3, 4, 5, 6, 7, 8, and 9.

This study revealed strong expression of AQP1, 5, 6, 7, 8, and 9 with positive rates over 50% in epithelial

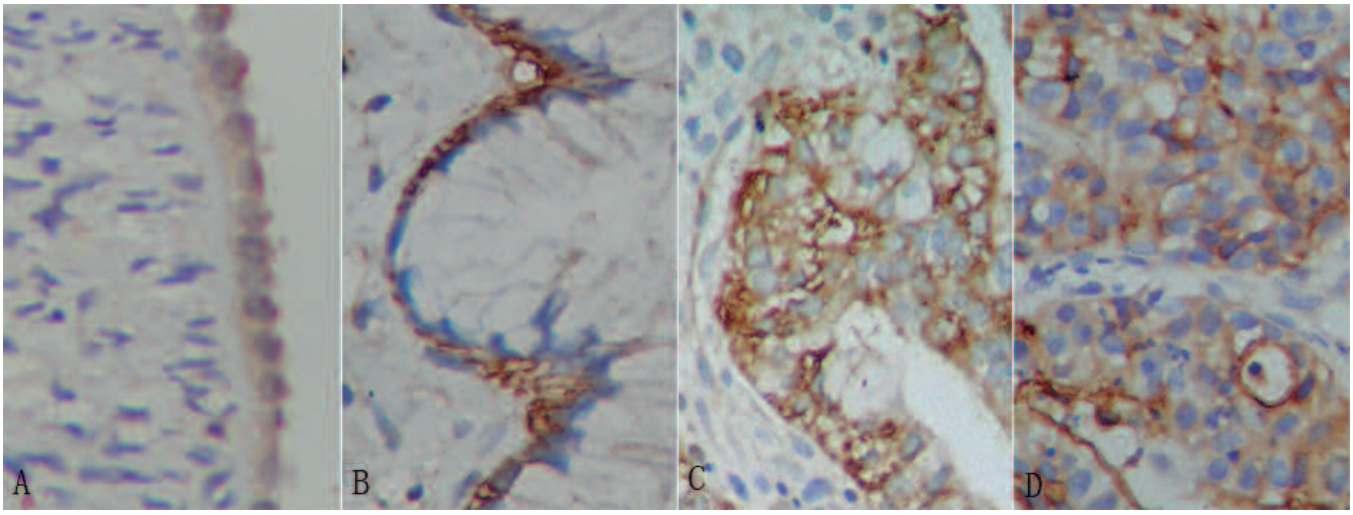

Fig. 5. Immunohistochemical localisation of AQP5 in epithelial ovarian tumours and normal ovarian tissue. AQP5 is expressed in the epithelia of normal ovarian tissue **(A)**, in the basolateral membranes of the epithelia of benign tumours **(B)**, the plasma membrane of borderline cells **(C)** and malignant cells **(D)**. x 400

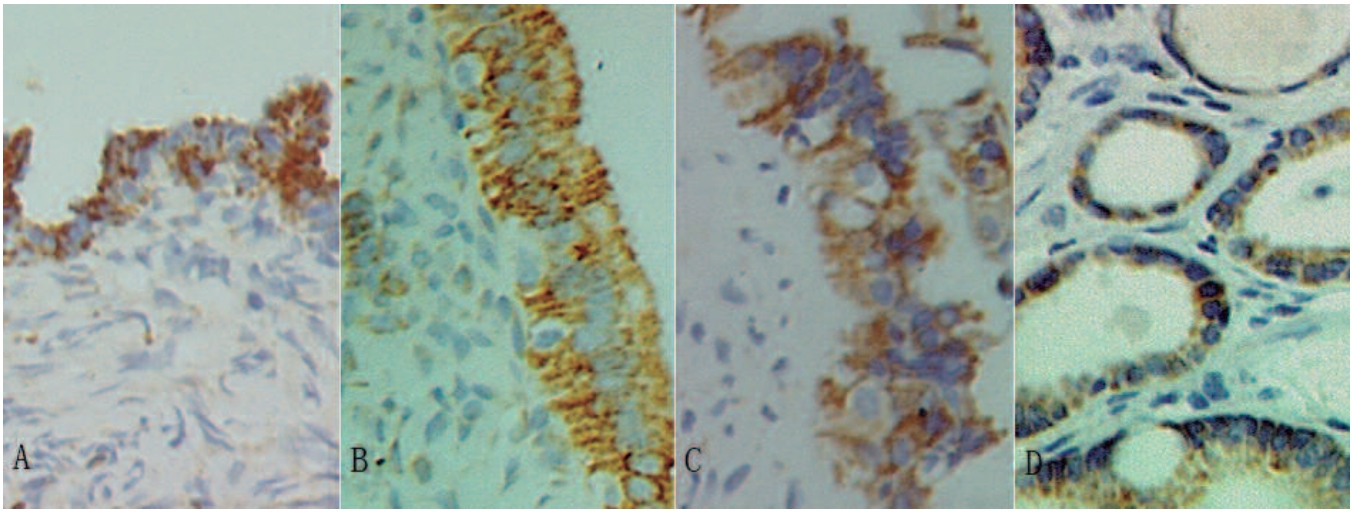

Fig. 6. Immunohistochemical localisation of AQP6 in epithelial ovarian tumours and normal ovarian tissue. Normal ovarian tissue **(A)**, benign tumour **(B)**, borderline **(C)**, malignant tumour **(D)**. x 400

ovarian tumours, whereas AQP2, 3 and 4 were expressed in only 10-40% of tumour cells. However, significant differences in expression were observed among benign tumours, borderline tumours, malignant ovarian tumours and and normal ovaran tissue for AQP1, 5, 6 and 9. The expression of AQP1, 5 and 9 in malignant and borderline ovarian tumours was significant higher than in benign ovarian tumours and normal ovarian tissue, whereas the expression of AQP6 was lower than that in benign ovarian tumours and normal ovarian tissue. These results suggest that AQP1, 5 and 9 may be involved in ovarian carcinogenesis, with AQP6 playing more of a role in benign ovarian tumours and normal ovarian tissue. The importance of AQP7 and 8 in epithelial ovarian tumours needs to be further investigated. The weak expression levels of AQP2, 3 and 4 in the present sample suggest that these subtypes may play little to no role in ovarian carcinogenesis or tumour biology.

In this study, AQP1 was mainly detected in the microvascular endothelium of ovarian tumours of all

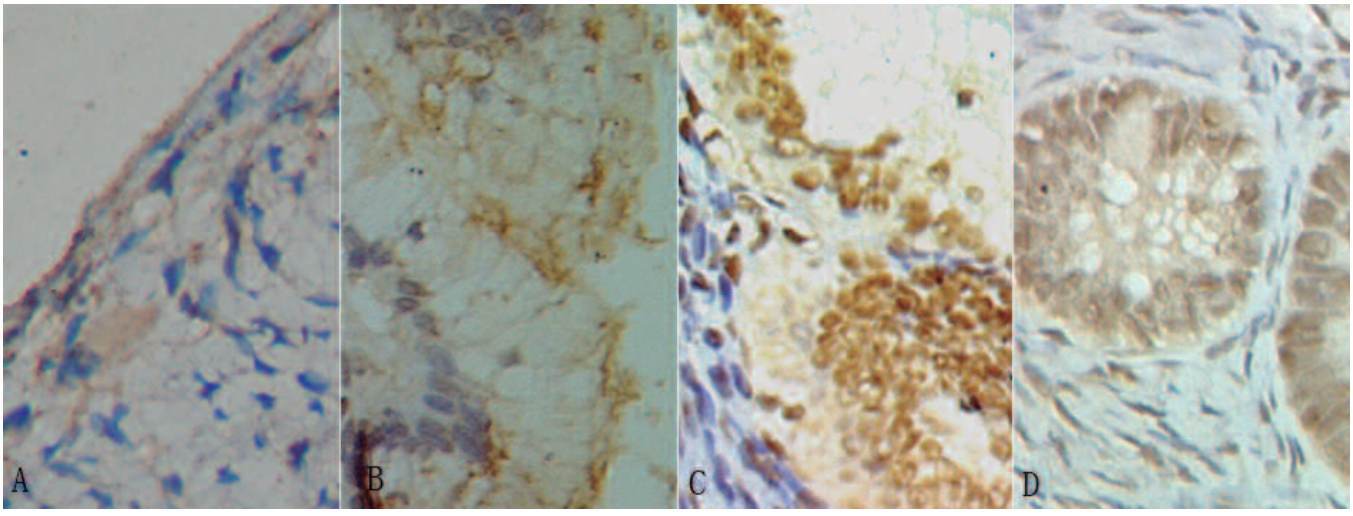

Fig. 7. Immunohistochemical localisation of AQP7 in epithelial ovarian tumours and normal ovarian tissue. AQP7 is expressed in the epithelia of normal ovarian tissue **(A)**, in the plasma membranes of benign tumour epithelia **(B)**, but selectively stained in the nuclear membrane of borderline **(C)** and malignant tumour cells **(D)**. x 400

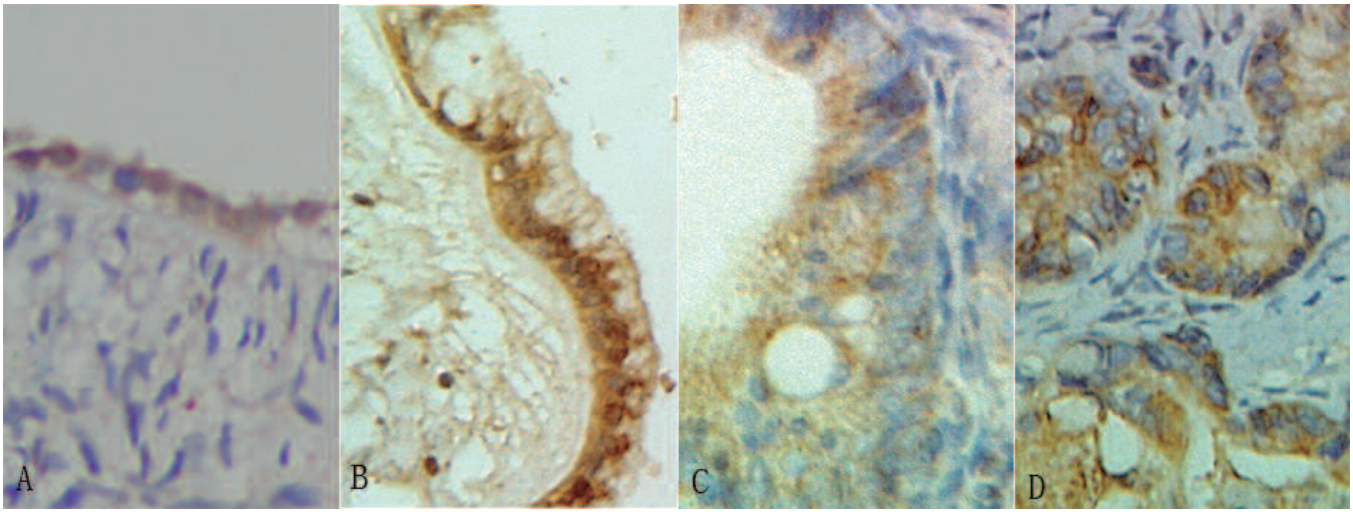

Fig. 8. Immunohistochemical localisation of AQP8 in epithelial ovarian tumours and normal ovarian tissue. It is mainly localised to the epithelia of normal ovarian tissue **(A)** and the basolateral membranes of benign tumour epithelia **(B)**, and scattered in the plasma membrane of borderline **(C)** and malignant cells **(D)**. x 400

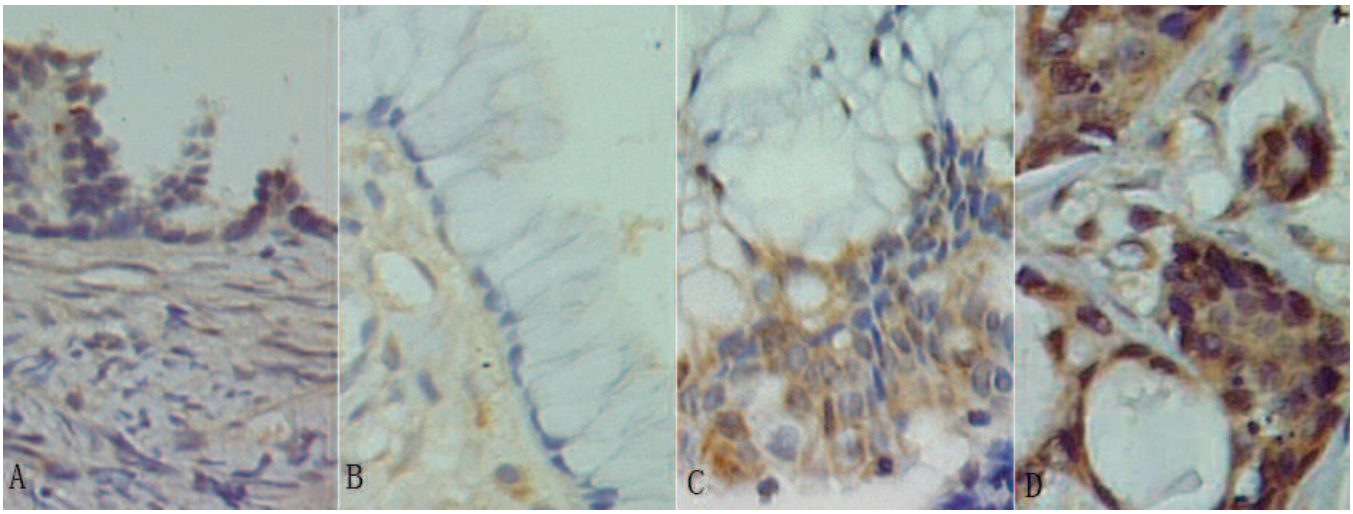

Fig. 9. Immunohistochemical localisation of AQP9 in epithelial ovarian tumours and normal ovarian tissue. AQP9 is localised to the epithelia of normal ovarian tissue **(A)**, in the basolateral membranes of benign tumour epithelia **(B)** and borderline tumour cells **(C)**, and scattered in the plasma membrane of malignant tumour cells **(D)**. x 400

types, which suggests that AQP1 may play a crucial role in water transport across the vascular endothelium in epithelial ovarian tumours. Previous studies have shown that strong AQP1 expression in tumour microvascular endothelial cells facilitates tumour angiogenesis and endothelial cell migration (Saadoun et al., 2005), and that AQP1 is involved in carcinoma development and brain oedema (Badaut, 2010). In the present study, high AQP1 expression in malignant tumours and in cases with ascites volumes greater than 1000 mL indicates that AQP1 expression is associated with ovarian carcinogenesis and ascites formation (Yang et al., 2006a).

Five AQP subtypes exhibit high expression in epithelial ovarian tumour cells: AQP5, 6, 7, 8, and 9. These highly expressed subtypes display different patterns of cellular localisation. In the epithelia of benign tumours, AQP5, 8 and 9 are mainly located in the basolateral membranes, and AQP6 and AQP7 are found in the plasma membranes. In borderline tumour cells, AQP9 is observed in the basolateral membranes, AQP6 and AQP8 in the plasma membrane, and AQP7 in the nuclear membrane. In malignant tumour cells, AQP5, 6, 8 and 9 are scattered in plasma membranes, and AQP7 is observed in nuclear membranes. It is well known that AQP5 and 8 are only permeable to water (Magni ey al., 2006; Ishibashi et al., 2009), while AQP7 and AQP9 are aquaglyceroporins permeable to glycerol as well as water (Rojek et al., 2008). Recent studies have proven that AQP-expressing cancer cells show enhanced migration, tumour cell extravasation, and metastasis (Cao et al., 2006; Auguste et al., 2007; Warth et al., 2007; Badaut, 2010). The present findings suggest that AQP5, 6, 7, 8 and 9 are involved in water and glycerol transporting in epithelial ovarian tumours, and the varying patterns of localisation may be associated with ovarian cancer biology (Verkman et al., 2008). The translocation of the above AQP subtypes in malignant ovarian tumour cells may facilitate the rapid penetration of water and glycerol into the growing tumour mass, which could be related to tumour development (Fischer et al., 2001; Yang et al., 2006b; Xu et al., 2009), and may be associated with energy metabolism (Saito et al., 2004; Sohara et al., 2005). The detailed mechanism of water and glycerol transport in ovarian tumours is unclear, and should be addressed in future studies.

In addition, the correlations between AQPs expression and the clinicopathologic characteristics of patients were evaluated. Increased AQP1 expression was observed in cases with ascites volumes greater than 1000 mL, high AQP5 expression was observed in cases with lymph node metastasis, and more AQP9 expression was observed in cases with undifferentiated tumours (G3). Together, these results suggest that these AQP subtypes may be involved in the differentiation, the development and the metastasis of malignant ovarian tumour cells, and thus may be related to a poor prognosis (Tan, 2008; Leung et al., 2009).

In summary, multiple AQP subtypes were expressed in epithelial ovarian tumours, with each AQP subtype displaying a distinct pattern of localisation and expression. Increased AQP1, 5 and 9 expression and decreased AQP6 were observed in malignant ovarian tumours, suggesting that these subtypes may be involved in ovarian carcinogenesis. This study presents a novel avenue of research that could illuminate the mechanism of ovarian carcinogenesis and elucidate new treatment targets (Frigeri et al., 2007; Miao et al., 2009; Takeda

and Taguchi, 2009).

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