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Expression and clinical significance of aquaglyceroporins in human hepatocellular carcinoma

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Abstract. Aquaglyceroporins (AQPs) are a subset of the aquaporin family, and are permeable to water and glycerol. The aim of the present study was to determine the expression and clinical significance of three AQPs, AQP3, 7 and 9 in hepatocellular carcinoma (HCC). Fresh HCC and adjacent non-tumorous liver tissues were collected from 68 patients diagnosed with HCC. The expression levels of AQP3, 7 and 9 were detected by reverse transcription-quantitative polymerase chain reaction, western blotting and immunohistochemical analysis. The association between the expression of AQPs and clinicopathological parameters of HCC were investigated. Compared with non-tumorous liver tissue, HCC tissues exhibited a significant ($P<0.05$) increase in the expression of AQP3 and a concomitant reduction in the expression levels of AQP7 and AQP9, at both the mRNA and protein levels. Immunohistochemistry revealed that AQP9 was dominantly localized on the plasma membrane of hepatocytes, while AQP3 and AQP7 exhibited a predominantly cytoplasmic and nuclear distribution. High expression of AQP3 was significantly ($P<0.05$) associated with low expression levels of AQP7 and AQP9. High expression of AQP3 was correlated with tumor grade ($P=0.017$), tumor stage ($P=0.010$) and lymphatic metastasis ($P=0.031$). Low expression of AQP7 was correlated with tumor grade ($P=0.043$). AQP3 was upregulated, and AQP7 and AQP9 were downregulated in HCC. A high expression of AQP3 and low expression of AQP7 was significantly associated with the aggressive features of HCC.

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Introduction

Aquaporins are integral membrane channel proteins that facilitate transcellular water movements (1). To date, 13 AQPs (AQP0-AQP12) have been cloned in mammals. AQP3, 7, 9 and 10 constitute the aquaglyceroporin (AQP) subfamily of the aquaporin family, and are permeable to both water and glycerol (2). Apart from the transport of small molecules, the AQPs are involved in a variety of biological processes, including tissue swelling (3), glucolipid metabolism (4), neural signal transduction (5) and cell migration (6). AQP9 is a unique AQP channel in hepatocytes, while AQP3 and AQP7 act as glycerol channels in adipocytes (7). The three AQP subtypes serve key roles in glucolipid metabolism (8,9).

Accumulating evidence indicates a close association between the expression of AQPs and carcinogenesis (10). For instance, AQP3 is expressed in human esophageal and oral squamous cell carcinoma, and contributes to tumor cell growth (11). Tan *et al* (12) reported that the expression of AQP9 is significantly higher in human astrocytic tumors compared with that in normal brain tissues, and is positively correlated with pathological grade. Hepatocellular carcinoma (HCC) is one of the most common gastrointestinal malignancies worldwide, with a particularly high incidence in Asian countries (13). It has been previously documented that combined overexpression of AQP3 and AQP5 is an independent poor prognostic factor for HCC (14). Decreased expression levels of AQP8 and AQP9 has been revealed to confer apoptosis resistance in a rat HCC line (15). These previous studies suggest that AQPs are involved in the development and progression of HCC. However, the expression and clinical significance of glycolipid metabolism-associated AQPs (AQP3, 7 and 9) in HCC remains to be fully elucidated.

Therefore, the present study assessed the mRNA and protein expression levels of AQP3, 7 and 9 in human HCCs and adjacent non-tumorous liver (NTL) tissues, and explored the association of AQP proteins with the clinicopathological features of HCC.

Materials and methods

Tissue specimens. The present study enrolled a total of 68 HCC patients who underwent hepatectomy at the affiliated hospitals

of Chongqing Medical University (Chongqing, China) between October 2009 and May 2013. All patients were pathologically diagnosed with HCC. Fresh tumor samples, coupled with adjacent NTL tissues, were collected from each patient. Some of the excised tissues were placed immediately in liquid nitrogen and stored at -80˚C until gene expression analysis. The other tissue samples were fixed, paraffin-embedded and sectioned (4 μm; OML-QPA/QPB; Hubei OML Medical Science & Technology Co., Ltd., Xiaogan, China) for immunohistochemistry. Written informed consent was obtained from each patient and the study protocol was approved by the Ethics Committee of Chongqing Medical University.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was extracted from the tissue samples using the RNAiso Plus reagent (Takara Bio, Inc., Tokyo, Japan). cDNA was synthesized from the total RNA using the PrimeScript RT reagent Kit (Takara Bio, Inc.). RT-qPCR was performed on a CFX96 Real-Time System PCR detecting system (Bio-Rad Laboratories, Hercules, CA, USA) using the SYBR Premix Ex Taq II kit (Takara Bio, Inc.). The primers used are as follows: AQP3, sense: 5'-CCTCTG GACACTTGGATATGAT-3' and antisense: 5'-GGGACG GGGTTGTTGTAG-3'; AQP7, sense: 5'-CCGCATCTTCAC CTTCATTG-3' and antisense: 5'-CACCCACCACCAGTT CTC-3'; AQP9, sense: 5'-ATCCACCAGAAGTTGTTT-3' and antisense 5'-AGCAATGACAATAATCAGGAGGC-3'. For the control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified in a parallel reaction, with the following primers: GAPDH, sense: 5'-GGTGGTCTCCTCTGACTTCAACA-3' and antisense: 5'-GTTGCTGTAGCCAAATTCGTTGT-3'. The cycling conditions were as follows: Initial denaturation at 95˚C for 3 min, followed by 40 cycles of denaturation at 95˚C for 10 sec and annealing at 60˚C for 30 sec. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (16). The relative mRNA levels were calculated following normalization against GAPDH mRNA levels.

Western blot analysis. Tissue samples were homogenized in radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing 1% sodium dodecyl sulfate (SDS) and 1% phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA), a potent protease inhibitor. The protein concentrations were measured using the bicinchoninic acid Protein Assay kit (Pierce, Rockford, IL, USA). Equal quantities of the total protein (~100 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis and were transferred onto a polyvinylidene fluoride membrane. The membrane was blocked at room temperature for 1 h with 5% fat-free milk, and incubated overnight with primary antibodies at 4˚C. The following primary antibodies were used: Mouse monoclonal anti-GAPDH (1:500; cat. no. TA-08; ZSGB-BIO Co., Ltd., Beijing, China), rabbit polyclonal anti-AQP3 (1:500; cat. no. LS-B8185; LifeSpan Biosciences, Inc., Seattle, WA, USA), rabbit polyclonal anti-AQP7 (1:300; cat. no. ab85907;Abcam, Cambridge, MA, USA) and rabbit polyclonal anti-AQP9 (1:500; cat. no. ab85910; Abcam). The membranes were washed with Tris-buffered saline and Tween 20 buffer (ZSGB-BIO Co., Ltd.) and incubated for 1 h at room temperature with goat anti-rabbit (cat. no. ZB-2301) or anti-mouse immunoglobulin G (ZB-2305)(1:3,000; ZSGB-BIO Co., Ltd.). The proteins were visualized using an enhanced chemiluminescence detection kit (ECL plus; Beyotime Institute of Biotechnology). Relative band intensities (AQP/GAPDH protein ratios) were determined by densitometry using the Quality One software (version 4.62; Bio-Rad Laboratories).

Immunohistochemistry. Tissue specimens were embedded in paraffin and cut into 4 μm sections. The tissue sections were dewaxed in xylene, rehydrated, and heated in citrate buffer (ZSGB-BIO Co., Ltd.) for 20 min at 100˚C to retrieve antigen. Following the elimination of endogenous peroxidase, the tissue sections were blocked with 3% hydrogen peroxidase diluted with methyl alcohol for 20 min and washed with phosphate-buffered saline (PBS), and incubated overnight at 4˚C with anti-AQP3 (1:100), anti-AQP7 (1:200) or anti-AQP9 (1:200) primary antibodies. Negative controls were included by omitting the primary antibody. The membranes were then washed with PBS for 10 min, and the secondary antibody reaction was performed using the Polink-2 plus Polymer Horseradish Peroxidase Detection system (GBI Labs, Mukilteo, WA, USA), according to the manufacturer's protocol. The tissue sections were developed with 3,3-diaminobenzidine (ZSGB-BIO Co., Ltd.) and were counterstained with hematoxylin. The stained sections were independently assessed by two pathologists in a blinded manner. The median percentage of immunostained tumor cells (10%) was used as a cutoff. High expression of AQPs was defined as nuclear staining of ≥10% of the tumor cells and low expression of AQPs was defined as nuclear staining of <10% of the tumor cells or no nuclear staining.

Statistical analysis. The data are presented as the mean ± standard deviation. All statistical calculations were performed using SPSS version 18.0 (IBM SPSS, Chicago, IL, USA). Continuous data were compared using the paired Student's t-test. The correlation between the expression levels of AQP3, 7 and 9 and the association between AQP expression and the clinicopathological features of HCC were analyzed using the χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

mRNA expression levels of AQPs in HCC. RT-qPCR analysis revealed that compared with NTL tissue, HCC tissues exhibited a significant ($P<0.05$) increase in the AQP3 mRNA level and a concomitant reduction in the mRNA expression levels of AQP7 and AQP9 (Fig. 1).

Protein expression levels of AQPs in HCC. Western blot analysis confirmed an upregulation of AQP3 and downregulation of AQP7 and AQP9 in HCC compared with in the NTL tissue (Fig. 2). Immunohistochemistry was performed to determine the location and distribution of AQPs in HCC. AQP9 was localized on the plasma membrane and in the cytoplasm of hepatocytes, while AQP3 and AQP7 showed predominantly cytoplasmic and nuclear distribution (Fig. 3). The majority of HCC tissues exhibited a significant decrease

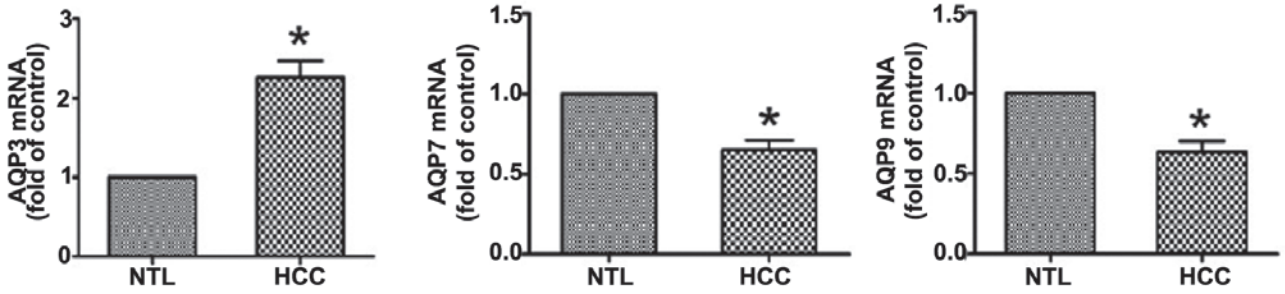

Figure 1. Reverse transcription-quantitative polymerase chain reaction analysis of the mRNA expression levels of AQP3, AQP7, and AQP9 in 68 pairs of HCC and NTL tissues. The data are presented as the mean ± standard deviation (*P<0.05). AQP, aquaglyceroporins; HCC, hepatocellular carcinoma; NTL, non-tumorous liver.

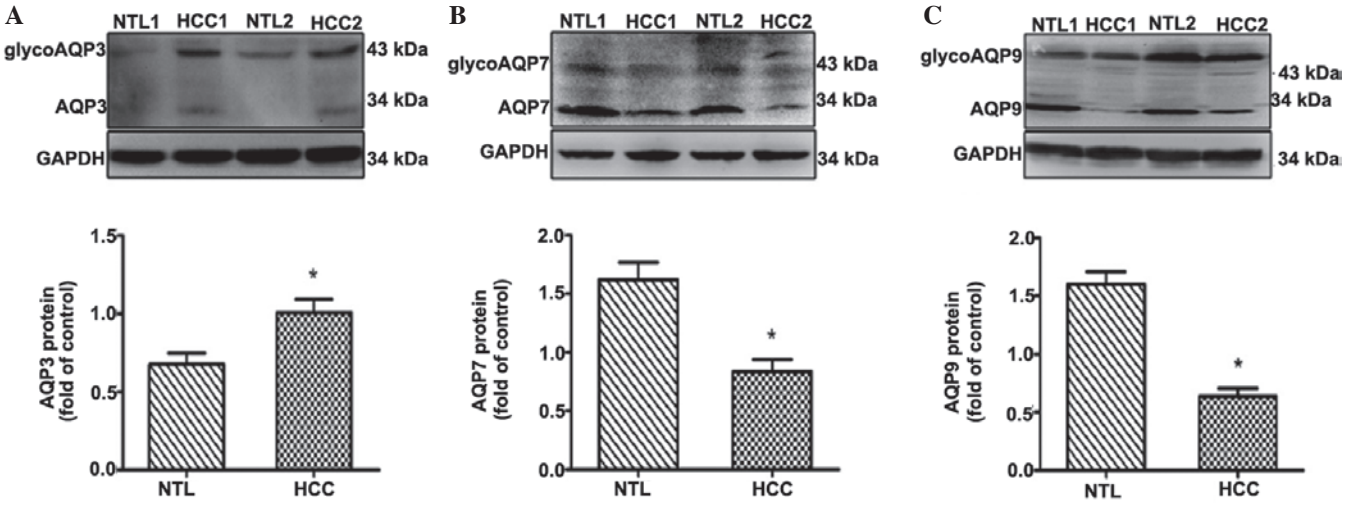

Figure 2. Western blot analysis of the protein expression levels of (A) AQP3, (B) AQP7 and (C) AQP9 in 68 pairs of HCC and adjacent NTL tissues. Representative western blotting is shown in the upper panels. Bar graphs (lower panels) represent densitometric analysis of the blots from three independent experiments. The data are presented as the mean ± standard deviation (*P<0.05). The antibodies detect glycosylated and non-glycosylated forms of AQP. AQP, aquaglyceroporins; HCC, hepatocellular carcinoma; NTL, non-tumorous liver.

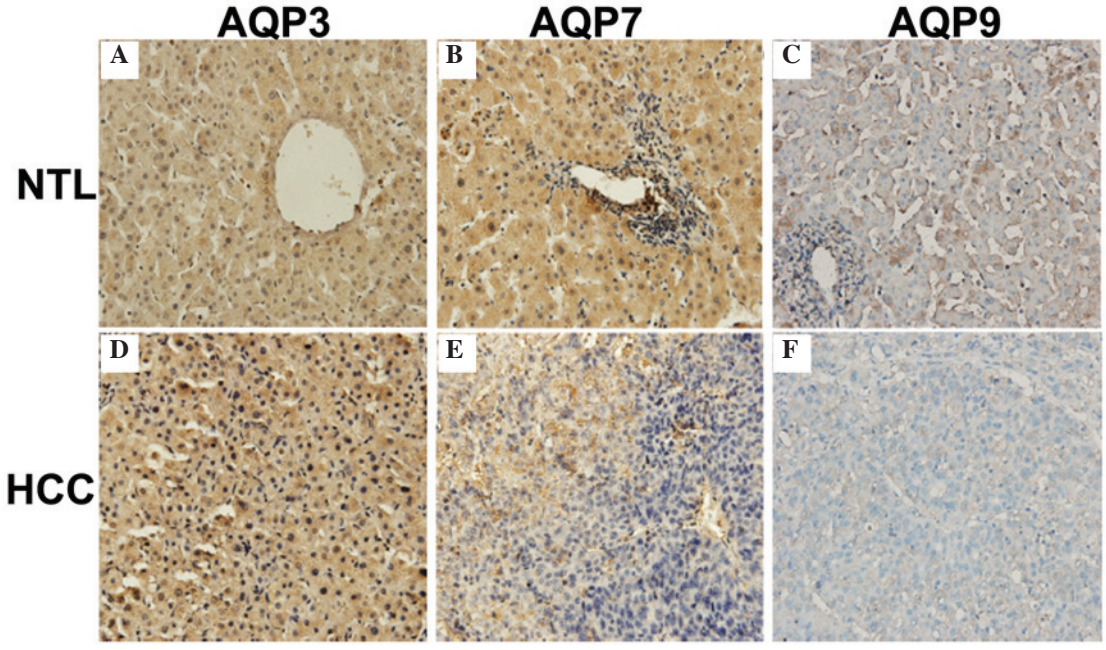

Figure 3. Assessment of histological location and distribution of AQPs by immunohistochemistry. Representative images of NTL tissues stained with (A) anti-AQP3, (B) anti-AQP7 and (C) anti-AQP9 antibodies, and HCC tissues stained with (D) anti-AQP3, (E) anti-AQP7 and (F) anti-AQP9 antibodies. Magnification, x400. AQP, aquaglyceroporins; HCC, hepatocellular carcinoma; NTL, non-tumorous liver.

Table I. Altered mRNA expression levels of AQP3, 7 and 9 in human HCC tissues and pair-matched NTL tissues.

Group	AQP3	AQP7	AQP9
NTL (n=68)	1.000±0.000	1.000±0.000	1.000±0.000
HCC (n=68)	2.259±0.210	0.652±0.487	0.636±0.069
t-test	3.025	4.928	6.596
P-value	<0.0001	<0.0001	<0.0001
R-value	0.120	0.266	0.394
95% CI	(-0.546)-(-0.112)	0.467-1.104	0.672-1.256

Paired sample t-test (NTL vs HCC). NTL, non-tumorous liver tissue; HCC, hepatocellular carcinoma; CI, confidence intervals.

Table II. Correlation analysis of the protein expression levels of AQP3, AQP7 and AQP9 in HCC tissues (n=68).

	AQP3				
	High (n=46)	Low (n=22)	χ^2	r	P-value
AQP7					
High (n=12)	5	7	4.494	-0.479	<0.05
Low (n=56)	41	15			
AQP9					
High (n=10)	4	6	4.095	-0.448	<0.05
Low (n=58)	42	16			

in the expression levels of AQP7 and AQP9, compared with adjacent NTL tissues (P<0.0001 for each comparison; Table I). By contrast, an increased expression of AQP3 was observed in HCC compared with the NTL tissues (P=0.0035; Table I).

Correlation between the expression levels of AQP3, 7 and 9. To determine whether a correlation exists between the protein expression levels of AQP3, 7 and 9 in HCC tissues, a χ^2 test was performed. As shown in Table II, high expression of AQP3 was significantly (P<0.05) associated with low expression of AQP7 (r=0.479; P<0.05) and AQP9 (r=0.448; P<0.05) in HCC tissues. However, no significant correlation was observed between AQP7 and AQP9 (data not shown)

Correlation between AQP proteins and the clinicopathological parameters of HCC. Clinicopathological features and the expression of AQP proteins in the 68 HCC patients were summarized in Table III. High expression of AQP3 correlated with tumor grade (χ^2=5.740; P=0.017), tumor stage (χ^2=6.680; P=0.010) and lymphatic metastasis (χ^2=4.636; P=0.031). Low expression of AQP7 was correlated with tumor grade (χ^2=4.091; P=0.043). However, the expression of AQP9 protein revealed no association with any of the clinicopathological factors studied.

Discussion

As water transporters, AQPs are expressed in a variety of tissues and cells (1,17). Accumulating evidence indicates that AQPs are frequently dysregulated in cancer and serve critical roles in tumor development and progression (10). The expression of AQP3 has been shown to be upregulated in colorectal carcinoma (18), gastric cancer (19), cervical cancer (20) and HCC (14). Guo *et al* (14) reported that elevated expression of AQP3 and AQP5, as determined by immunohistochemistry, is significantly associated with tumor progression and prognosis in patients with HCC. The present data confirmed the upregulation of AQP3 in HCC compared with that in adjacent NTL tissue. Additionally, it was revealed that the mRNA and protein expression levels of AQP3 were consistently increased in HCC, suggesting that the upregulation of AQP3 likely occurs at the transcriptional level. It has been documented that the expression of AQP9 is significantly decreased in HCC tissues compared with NTL tissues (21). In agreement with this previous study, the present results demonstrated that both the mRNA and protein expression levels of AQP9 were reduced in HCC compared with the adjacent NTL tissue. The downregulation of AQP9 has been shown to induce apoptosis resistance in HCC cells (21). Notably, AQP7 has been identified in human urothelial carcinoma (22). The present study provided the first evidence, to the best of our knowledge, that AQP7 was downregulated in HCC compared with adjacent NTL tissue. This downregulation may be due to transcriptional inhibition, since the mRNA and protein expression levels of AQP7 were consistently decreased. Taken together, the present data demonstrated that HCCs exhibited coordinated expression of AQPs. However, the exact mechanisms for their dysregulation require further clarified.

By immunohistochemistry, AQPs exhibited different localization patterns in HCC. It was found that AQP9 was localized on the plasma membrane and in the cytoplasm of hepatocytes, while AQP3 and AQP7 exhibited cytoplasmic

Table III. Correlation between AQP proteins and clinicopathological parameters of HCC (n=68).

	AQP3 protein				AQP7 protein				AQP9 protein			
Variable	Low (n=22)	High (n=46)	χ^2	P-value	Low (n=56)	High (n=12)	χ^2	P-value	Low (n=58)	High (n=10)	χ^2	P-value
Age (year)												
<50	8 (36.4%)	19 (41.3%)	0.152	0.697	21 (37.5%)	6 (50.0%)	0.229	0.633	23 (39.7%)	4 (40%)	0.109	0.742
≥50	14 (63.6%)	27 (58.7%)			35 (62.5%)	6 (50.0%)			35 (60.3%)	6 (10%)		
Gender												
Male	15 (68.2%)	37 (80.4%)	0.654	0.419	41 (73.2%)	11 (91.7%)	0.985	0.321	44 (75.9%)	8 (80%)	0.014	0.906
Female	7 (31.8%)	9 (19.6%)			15 (26.8%)	1 (8.3%)			14 (24.1%)	2 (20%)		
Tumor size (cm)												
<3	6 (27.3%)	15 (32.6%)	0.199	0.656	18 (32.1%)	3 (25.0%)	0.020	0.887	19 (32.8%)	2 (20%)	0.190	0.663
≥3	16 (72.7%)	31 (67.4%)			38 (67.9%)	9 (75.0%)			39 (67.2%)	8 (80%)		
Tumor number												
Single	18 (81.8%)	39 (84.8%)	0.002	0.967	46 (82.1%)	11 (91.7%)	0.145	0.703	47 (81.0%)	10 (100%)	1.080	0.299
Multiple	4 (18.2%)	7 (15.2%)			10 (17.9%)	1 (8.3%)			11 (19.0%)	0 (0%)		
Tumor grade												
Moderate or well	20 (90.9%)	29 (63.0%)	5.740	0.017	37 (66.1%)	12 (100.0%)	4.091	0.043	39 (67.2%)	10 (100%)	3.065	0.080
Poor	2 (9.1%)	17 (37.0%)			19 (33.9%)	0 (0.0%)			19 (32.8%)	0 (0%)		
Tumor stage												
I-II	19 (86.4%)	25 (54.3%)	6.680	0.010#	33 (58.9%)	11 (91.7%)	3.315	0.069	37 (63.8%)	7 (70%)	0.000	0.983
III-IV	3 (13.6%)	21 (45.7%)			23 (41.1%)	1 (8.3%)			21 (36.2%)	3 (30%)		
PVTT												
Yes	2 (9.1%)	8 (17.4%)	0.290	0.591	10 (17.9%)	0 (0.0%)	1.290	0.256	10 (17.2%)	0 (0%)	0.881	0.348
No	20 (90.9%)	38 (82.6%)			46 (82.1%)	12 (100.0%)			48 (82.8%)	10 (100%)		
LM												
Yes	0 (0.0%)	11 (23.9%)	4.636	0.031	11 (19.6%)	0 (0.0%)	1.550	0.213	10 (17.2%)	1 (10%)	0.012	0.913
No	22 (100.0%)	35 (76.1%)			45 (80.4%)	12 (100.0%)			48 (82.8%)	9 (90%)		
TM												
Yes	1 (4.5%)	13 (28.3%)	3.772	0.052	13 (23.2%)	1 (8.3%)	0.583	0.445	13 (22.4%)	1 (10.0%)	0.224	0.636
No	21 (95.5%)	33 (71.7%)			43 (76.8%)	11 (91.7%)			45 (77.6%)	9 (90.0%)		
HBsAg												
Positive	19 (86.4%)	36 (78.3%)	0.217	0.642	45 (80.4%)	10 (83.3%)	0.028	0.868	46 (79.3%)	9 (90%)	0.129	0.720
Negative	3 (13.6%)	10 (21.7%)			11 (19.6%)	2 (16.7%)			12 (20.7%)	1 (10%)		

Table III. Continued.

Variable	AQP3 protein				AQP7 protein				AQP9 protein			
	Low (n=22)	High (n=46)	χ^2	P-value	Low (n=56)	High (n=12)	χ^2	P-value	Low (n=58)	High (n=10)	χ^2	P-value
AFP (μg/l)												
<400	19 (86.4%)	36 (78.3%)	0.217	0.642	46 (82.1%)	9 (75.0%)	0.028	0.868	46 (79.3%)	9 (90%)	0.129	0.720
≥400	3 (13.6%)	10 (21.7%)			10 (17.9%)	3 (25.0%)			12 (20.7%)	1 (10%)		
Liver cirrhosis												
Yes	6 (27.3%)	18 (39.1%)	0.471	0.493	17 (30.4%)	7 (58.3%)	2.273	0.132	20 (34.5%)	4 (40%)	0.000	0.983
No	16 (72.7%)	28 (60.9%)			39 (69.6%)	5 (41.7%)			38 (65.5%)	6 (60%)		

PVTT, portal vein tumor thrombosis; LM, lymphatic metastasis TM, tumor metastasis; AFP, alpha-fetoprotein.

and nuclear distribution. Nihei *et al* (23) reported that AQP9 is normally localized on the surface of rat hepatocytes and Leydig cells. Similarly, Elkjaer *et al* (24) demonstrated an immunolocalization of AQP9 on the plasma membrane of liver hepatocytes. The data from knockout mice support the essential role for AQP9 in glycerol transport (25). Previous studies, combined with the present findings, suggested that AQP9 may facilitate the transport of water, glycerol and other small molecules in HCC cells. The cytoplasmic and nuclear expression pattern of AQP3 and AQP7 suggested that the two proteins may be involved in the regulation of gene expression. Indeed, Xie *et al* (26) demonstrated that AQP3 has a protective activity against ultraviolet A-induced human skin fibroblast apoptosis via the upregulation of B-cell lymphoma-2. AQP3 depletion has been shown to induce the expression of p21 and FAS in cancer cells (27). Forced expression of AQP7 leads to improved insulin resistance by increasing the phosphorylation of protein kinase B (28). Taken together, different expression and localization patterns of AQPs in HCC indicate their distinct roles in tumor progression.

The present data demonstrated that high expression of AQP3 correlated with tumor grade, tumor stage and lymphatic metastasis in HCC, suggesting its favorable role in HCC progression. The tumor-promoting effects of AQP3 have also been previously described in several other cancer types. For instance, Chen *et al* (29) reported that AQP3 facilitates the epithelial-mesenchymal transition in gastric cancer. Li *et al* (18) revealed that AQP3 overexpression promotes colorectal carcinoma cell migration and is significantly associated with tumor metastasis. Different from AQP3, low expression of AQP7 was found to be significantly correlated with tumor grade in HCC patients, implying that AQP7 may exert suppressive effects on HCC. Although the protein expression of AQP9 exhibited no significant association with HCC clinicopathological features, low expression of AQP9 was significantly associated with high expression of AQP3 in patients with HCC. It has been documented that AQP9 and AQP7 are implicated in the uptake of certain chemotherapeutic agents into cancer cells (30,31). Decreased expression of AQP9 is associated with increased resistance to apoptosis in HCC cells (15). Therefore, the dysregulation of AQP3, 7 and 9 may cooperatively contribute to the pathogenesis of HCC. However, their biological functions in HCC requires further elucidation.

In conclusion, the present data demonstrated that AQP3 is upregulated, and that AQP7 and AQP9 are downregulated in HCC. It was also revealed that the three investigated AQPs exhibit different intracellular localizations in HCC hepatocytes. High expression of AQP3 was significantly associated with tumor grade, tumor stage and lymphatic metastasis in patients with HCC, while low expression of AQP7 was significantly correlated with tumor grade. The present results suggest a complex role for AQPs in the development and progression of HCC. Additional direct studies are required to determine the biological functions of AQPs in this malignancy.

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