AQP5: A novel biomarker that predicts poor clinical outcome in colorectal cancer

TAO SHAN¹, XIJUAN CUI², WEI LI³, WANRUN LIN⁴ and YIMING LI¹

¹Department of General Surgery, The Second Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an, Shaanxi 710004; ²Department of General Surgery, The First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an, Shaanxi 710061; ³Graduate School of The Fourth Military Medical University, Xi'an, Shaanxi 710032; ⁴Department of Pathology, Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021, P.R. China

Received April 5, 2014; Accepted June 12, 2014

DOI: 10.3892/or.2014.3377

Abstract. The aquaporins (AQPs) are water channel proteins that exhibit several properties related to tumor development. However, the expression and clinical significance of AQP5 in colorectal cancer, particularly the correlation with circulating tumor cells (CTCs), has not been elucidated. The aim of the present study was to determine whether or not the expression of AQP5 is a strong prognostic biomarker for colorectal cancer. The results showed that of 45 tumor specimens, 14 (31.1%) had high levels of expression of AQP5, 29 (64.4%) exhibited a moderate (intermediate) level of staining, and 2 (4.4%) had an absence of AQP5 staining. AQP5 was only occasionally detected in para-neoplastic [3/45 (6.67%)] and normal tissues [3/45 (6.67%)]. AQP5 protein overexpression frequently accompanied gene amplification detection with fluorescence *in situ* hybridization (FISH). Moreover, AQP5 expression in colorectal cancer cells was upregulated compared to normal colon cells. AQP5 overexpression was also associated with TNM stage ($P=0.002$), lymph node metastasis ($P=0.016$), and distant metastasis ($P=0.000$). The relationships between age, gender, histologic grade and tumor size with expression of AQP5 were not significant ($P>0.05$). A positive correlation between the number of CTCs and AQP5 expression ($P<0.05$) was demonstrated. In addition, patients who did not express AQP5 had a superior cumulative survival rate compared to patients with AQP5 positivity. AQP5 may be used as a novel biomarker for colorectal cancer aggressiveness and metastasis, but it does not reflect drug resistance.

Introduction

Colorectal cancer remains the second most common cause of cancer-related mortality in the United States. Of the patients with colorectal cancer who die, most succumb with a significant burden of metastatic disease (1). Colorectal cancer is an aggressive and intractable human malignant tumor, despite the advances in the diagnosis and treatment (2). Therefore, it is highly important to seek novel targets for therapeutic intervention.

Recently, increasingly more studies have shown that tumor growth, development, invasion and metastasis depend on the tumor microenvironment and tumor metabolism (3). Water molecules play a significant role in the progression of malignant epithelial tumors, an understanding of which is thus important in anticancer treatment strategy (4). Aquaporins (AQPs) are a family of membrane water channels that are required for the transport of water through many secretory and absorptive epithelia. Some subtypes of AQPs are also involved in the transport of other molecules, such as glycerol and urea. There are currently 13 AQP members which have been identified in mammals. Among them, AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8 are primarily water-selective, whereas AQP3, AQP7, AQP9, AQP10 and AQP12 also transport glycerol and other small solutes (5). An altered expression of AQPs has been revealed in several types of tumors based upon their specific tissue localization. Expression of AQP1 is frequently associated with brain tumors (6). In studies involving AQP3-null mice, AQP3 gene deletion induced resistance to carcinogen-induced skin tumors. Glycerol transport through AQP3 also contributes to the generation of ATP for cell proliferation and tumorigenesis (7). AQP5 is widely overexpressed in pancreatic cancer and appears to be involved in cell proliferation (8).

In particular, AQP5 expression in colon cancer tissues is associated with metastasis, suggesting that AQP5 overexpression plays a role in cancer progression (9). Ras signal transduction has been suggested to enhance cell proliferation in AQP5-overexpressed NIH3T3 cells (10). Moreover, a molecular study revealed that AQP5 binds to the SH3 domains of c-Src, a non-receptor cytoplasmic tyrosine kinase associated with invasive and metastatic phenotypes in various tumors (11). However, the expression and clinical significance of AQP5 in

Correspondence to: Dr Tao Shan, Department of General Surgery, The Second Affiliated Hospital of Medical College, Xi'an Jiaotong University, 157 Xiwu Road, Xi'an, Shaanxi 710004, P.R. China
E-mail: shantao820304@163.com

Key words: colorectal cancer, aquaporin 5, prognosis, circulating tumor cells, fluorescence *in situ* hybridization

colorectal cancer, particularly the correlation with circulating tumor cells (CTCs), has not been elucidated. To evaluate the potential of AQP5 as a novel prognostic marker of colorectal cancer, we used immunohistochemical, RT-PCR, real-time PCR, western blotting and fluorescence *in situ* hybridization (FISH) methods to detect the expression and amplification of the AQP5 gene in clinical samples of colorectal cancer, and immunofluorescence *in situ* hybridization (imFISH) staining. We then analyzed the correlations between the expression of AQP5 and clinicopathologic features, CTCs and prognosis of colorectal cancer.

Materials and methods

Patient specimens. From January 2008 to December 2013, colorectal cancer tissues (including adequately sized tumor tissue samples and tissue samples obtained from areas within 2.0 cm around the tumor) were obtained from 45 patients with colorectal cancer at the Department of General Surgery, Second Affiliated Hospital of Xi'an Jiaotong University. Samples were fixed with 4% formalin for histological studies. Of the 45 patients, 24 were male and 21 were female. Median age at the time of surgery was 58.3 years (range, 40-78 years). The histological type in all 45 patients was colorectal adenocarcinoma. Tumor stage and histopathological grading were recorded according to the classification of the International Union Against Cancer. There were 3 stage I, 11 stage II, 27 stage III, and 4 stage IV tumors. Histological grades for the patients were as follows: 7 patients grade I, 20 grade II and 18 grade III. All patients were followed up and the median duration of follow-up was 23 months (5-53 months). All the studies were approved by the Human Subjects Committee of Xi'an Jiaotong University, China. Consent forms signed by all patients recruited in this study were approved by the Ethics Review Committee of the Human Subjects Committee of the Xi'an Jiaotong University, China.

Immunohistochemistry. AQP5 protein was detected immunohistochemically using a standardized streptavidin-peroxidase (SP) method. Tissue sections (4 μ m) were incubated overnight with primary antibody at a proper concentration. The next day, the slides were incubated for 30 min with biotinylated goat anti-rabbit IgG, followed by incubation with peroxidase-conjugated streptavidin for 20 min at room temperature. Color was developed using 0.02% 3, 3'-diaminobenzidine (DAB) in 50 mM Tris-HCl buffer (pH 7.6) for 5-7 min. Finally, the sections were counterstained with hematoxylin, rinsed with water, dehydrated, cleared and coverslipped. Negative controls for immunostaining replaced the primary antibody with nonimmune goat or rabbit serum. The number of stained cells per 1,000 was determined under a microscope (Olympus Optical, Tokyo, Japan) in three visual fields, at a x400 magnification. When the total number of cells observed under the microscope was <1,000, all cells were counted. The staining was scored semiquantitatively as negative (0, no staining); moderate (1, either diffuse weak staining or strong staining in <30% of cells per core); or strong (2, strong staining of $\geq$ 30% of the cells). The antibodies against AQP5 and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

RT-PCR and real-time PCR. Total RNA was extracted from colorectal tumor, peri-tumor and normal tissue using TRIzol reagent (Gibco-BRL). First-strand cDNA was synthesized from 2 μ g of total RNA using the RevertAid Kit (Fermentas MBI, Amherst, NY, USA). The PCR primer sets were designed: i) for AQP5, forward, TGACGAGGACTGGGAGG AGC-3' and reverse, 5'-GGCGGCATTCAATGAACCA-3'; ii) for β -actin, forward, 5'-ATCGTGCGTGACATTAAGGAG AAG-3' and reverse, 5'-AGGAAGGAAGGCTGGAAGA GTG-3'. The PCR conditions included an initial denaturation step for 5 min at 95 °C followed by 22 cycles of amplification: 30 sec at 95°C, 30 sec at 57°C and 30 sec at 72°C. After the last cycle, a final extension was performed at 72°C for 10 min. The housekeeping gene β -actin was used as an internal control.

Real-time quantitative PCR was carried out with Platinum SYBR-Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA, USA) using the Rotor-Gene RG-3000 (Corbett Research, Doncaster Victoria, Australia). For each amplicon, the amount of AQP5 and β -actin was determined from a standard curve generated by serial dilution. Prior to amplification, the samples were incubated at 95°C for 10 min, and each amplification cycle consisted of denaturation for 45 sec at 95°C, annealing for 30 sec at 57°C and extension for 30 sec at 73°C. The amount of target genes in the cDNA samples was calculated based on the threshold cycle (Ct). The PCR signals were quantitated by densitometric analysis using Quantity One® analysis software.

Western blotting. Colorectal tumor tissues were minced and incubated on ice for 30 min in 0.5 ml of ice-cold whole-cell lysate buffer (10% NP-40, 5 M NaCl, 1 M HEPES, 0.1 M EGTA, 0.5 M EDTA, 0.1 M PMSF, 0.2 M sodium orthovanadate, 1 M NaF, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin). The minced tissue was homogenized using a Dounce homogenizer and centrifuged at 16,000 x g at 4°C for 10 min. The protein was separated by 10% SDS-PAGE and electro-transferred onto nitrocellulose membranes. After being blocked with 5% non-fat milk in TBST (20 mM Tris, 150 mM NaCl, 0.2% Tween-20, pH 7.6), the membranes were incubated with primary antibodies at 4°C overnight, followed by 1:2,000 horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The western blotting signals were quantitated by densitometric analysis using Total Lab Nonlinear Dynamic Image® analysis software (Nonlinear, USA). Final histogram results = Target gene absolute value/ β -actin absolute value.

Isolation of normal colon cells and culture of colorectal cancer cells. Normal colon cells were isolated from colorectal site in normal colorectal specimens. Briefly, each specimen was collected and transferred to the laboratory. After several washings with sterile phosphate-buffered saline (PBS), 1-cm² pieces of tissues were placed into the wells of culture flasks. Once the tissue appeared to attach flasks (5-6 h), Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS was added gently to the tissue pieces. Specimens were inspected daily and the medium was exchanged after 24 h for the first time and every third day thereafter. Tissue samples were then removed from the cultures and cells were transferred to larger

tissue culture vessels once they had reached 70% confluence, after approximately 2 weeks.

The COLO 205 and SW480 colorectal cancer cell lines (the American Tissue Type Collection, USA) were maintained in DMEM, (Gibco-BRL) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 0.1 mM non-essential amino acids, 0.2 mM glutamine, 1 mM pyruvate, and 10% heat-inactivated fetal bovine serum (FBS) and incubated in a 5% CO₂ humidified atmosphere at 37°C.

Immunofluorescence assay. Exponentially growing cells were seeded on 25-mm square glass cover slips placed in 35-mm diameter culture dishes. The cells were fixed with 4% formaldehyde for 5 min, permeabilized with 0.2% solution of Triton X-100 in PBS, and blocked with 2% bovine serum albumin-PBS 30 min. Slides were incubated with anti-AQP5 for overnight. Fluorescent imaging was obtained with a confocal laser scanning microscope (Carl Zeiss Micro Imaging).

FISH. AQP5 gene amplification was detected with dual-color FISH using a Passvision AQP5 DNA probe kit (Vysis Inc., Downers Grove, IL, USA), according to the manufacturer's instructions. Tissue sections (4-µm thick) were baked overnight at 56°C, and were handled with deparaffinization, enzyme digestion and fixation. The slides were then denatured in 70% formamide/2X standard saline citrate (SSC), at 72°C for 5 min. After a buffer wash, 10 µl of a mixture of two directly labelled probes (AQP5 specific sequence probe) were added to the tissue sections and hybridization was carried out at 37°C for 14-18 h. The slides were then washed in a post-hybridization wash at 72°C, counterstained with DAPI, mounted and stored in dark before signal enumeration. AQP5-spectrum red probe contains a DNA sequence specific for the AQP5 gene. Chromosome enumeration probe 17 (CEP17)/spectrum green probe containing α-satellite DNA that hybridizes to the D17Z1 locus (centromere region of chromosome 17) was used as a control. The slides were observed under a fluorescence microscope equipped with a digital camera (DP50; Olympus, Tokyo, Japan). For each specimen, gene amplification was scored when a minimum of 20 cancer cell nuclei exhibited AQP5/CEP17 ratio ≥2, or when AQP5 signal cluster was observed.

Blood sampling and enrichment of CTCs. Peripheral blood (7.5 ml) collected in a BD Vacutainer tube (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) was washed with PBS. In order to avoid epithelial cell contamination during venipuncture, all samples were collected after discarding the first 2 ml blood. Red blood cells (RBCs) were mixed with 45 ml lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA), followed by rotation for 8 min and centrifugation (600 x g for 5 min) to remove RBCs. Resulting cell pellet was resuspended in PBS and subsequently incubated with 0.5 ml of antileukocyte surface marker CD45 monoclonal antibody coated magnetic beads for 30 min, followed by separation of magnetic beads using a magnetic stand (Promega, Madison, WI, USA). Supernatants were transferred into a new tube, and subsequently centrifuged at 800 x g for 3 min. Cell pellets were spotted on glass slides, followed by imFISH staining.

imFISH staining (CEP8-CD45-DAPI). Tumor cells were negative enriched by the immunomagnetic beads method, followed by identification with cytology analysis. FISH was performed using centromere DNA probes of chromosome 8 (yellow) (Vysis), and immunofluorescence assay was performed using anti-CD45 (red) (Santa Cruz, CA, USA). The slides were washed three times with TBS (10 mM Tris, 2.8 mM KCl, 137 mM NaCl, pH 7.4) containing 0.2% BSA for 3 min, and subsequently rinsed with TBS once. Cells were mounted with mounting medium containing the nuclear dye DAPI. A blinded review of the fluorescent images by three technicians confirmed the identity of the CTCs from 3-color fluorescent images that were magnified x400. Evaluation criteria for CTC identification from fluorescent images included both CEP8 ≥3 and CD45 (-) staining pattern overlying the DAPI staining of the nucleus.

Statistical analysis and patient outcome. Data were analyzed by the χ^2 or two-sided Fisher's exact test, as appropriate. Pearson's correlation coefficient was used to measure the strength of the association among AQP5 gene amplification, enumeration of CTCs and AQP5 expression levels. Survival rate was calculated by the Kaplan-Meier method, and differences were examined by the log-rank test. Factors found to be significant were then selected for a stepwise Cox's multivariate proportional hazard model to determine their prognostic values. Gene SNP was analyzed using the software of pyro-sequencing equipment (PyroMark Q24, Qiagen, Germany). P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS® ver. 13.0 statistical software (SPSS, Chicago, IL, USA).

Results

Protein expression and gene amplification of AQP5 in colorectal cancer. It is known that AQP5 is expressed on the membrane and in the cytoplasm of cells. To determine the expression status of AQP5 in colorectal cancer, we first used immunohistochemistry to evaluate patient specimens in the colorectal cancer, peri-tumor and normal groups. The results highlighted the difference observed in AQP5 immunostaining in the compartment (Fig. 1A); specifically, 14/45 (31.1%) of the patients had strong AQP5 expression and 29/45 (64.4%) of the patients had moderate AQP5 expression in the cancer group. AQP5 was only occasionally detected in the peri-tumor [3/45, (6.67%)] and normal tissues [3/45, (6.67%)].

Since FISH is a more accurate and sensitive assay, and is generally regarded as the gold standard for detecting gene amplification compared to gene expression using immunohistochemistry (12), we further detected AQP5 amplification status using FISH. Notably, strong expression of AQP5 had a positive correlation with AQP5 gene amplification ($r = 0.712$, $P = 0.000$; Fig. 2B; Table I). In contrast, the expression of AQP5 was low or absent in peri-tumor and normal samples, while the AQP5 gene was not amplified.

Finally, we continued to determine the expression status of AQP5 in samples by RT-PCR and real-time PCR. The AQP5 mRNA level was significantly upregulated in tumor samples when compared to peri-tumor and normal tissues (Fig. 1C and D; $P < 0.05$). Similarly, western blotting results

Figure 1. (A) Representative immunohistochemistry results of AQP5 expression in colorectal cancer. Paraffin sections were immunostained as described in Materials and methods. (B) AQP5 amplification status using FISH. (C and D) mRNA expression of AQP5 in samples by RT-PCR and real-time PCR. (E and F) Protein expression of AQP5 in samples by western blotting. Data from at least three independent experiments with duplicate determinations are expressed as the means $\pm$ SEM. N, normal tissue; P, paraneoplastic tissue; C, tumor tissue; magnification, $\times 400$.

Figure 2. Immunodetection of AQP5 in colorectal cancer (COLO 205 and SW480) and normal cells (magnification, $\times 200$). AQP5 protein expression stained with CY3-labeled and FITC labeling-IgG antibody and analyzed by confocal microscopy.

showed that AQP5 protein was upregulated compared to peritumor and normal tissues (Fig. 1E and F; $P < 0.05$).

Expression of AQP5 in colorectal cancer and normal colon cells. We further determined AQP5 protein expression status in colorectal cancer cells (COLO 205 and SW480) and colon normal cells using an immunofluorescence assay and analyzed by confocal microscopy. The AQP5 fluorescence signal in colorectal cancer cells was increased compared to normal

colon cells (Fig. 2). These findings further indicate that AQP5 is upregulated in colorectal cancer.

Relationship between AQP5 expression and clinicopathologic parameters. Table II summarizes the associations between AQP5 protein expression and clinicopathologic parameters in colorectal cancer. AQP5 was strongly expressed in 1/14 stage I-II cases (7.1%), which was significantly lower than stage III [33.3%, (9/27)] and stage IV [100.0% (4/4)] cases

Table I. Relationship between expression of AQP5 and amplification.

Gene	Case	AQP5			P-value
		0 n (%)	1 n (%)	2 n (%)	
Total	45	2	29	14	0.000 ^a
AQP5					
Amplification	15	0 (0.0)	2 (6.9)	13 (92.9)	
Normal	30	2 (6.7)	27 (90.0)	1 (3.3)	

^aP<0.05; AQP, aquaporin.

Figure 3. Kaplan-Meier analysis of the overall post-operative survival curves in colorectal cancer cases according to their immunohistochemical staining as positive or negative of AQP5 protein expression.

(P=0.002). AQP5 was also associated with lymph node (P=0.016) and distant metastases (P=0.000). We also determined the relationships between age, gender, histologic grade, and tumor size with expression of AQP5, however no significant relationships were observed (P>0.05).

Prognostic value of AQP5 in patients with colorectal cancer. To determine the prognostic value of AQP5 for colorectal cancer, we analyzed the cumulative survival of patients based on AQP5 status (Fig 3). AQP5 weak and no staining were merged as negative, while strong staining was regarded as positive. The cumulative survival rate in AQP5-negative patients (n=31) at 3 years was 24.3% (median time, 21.2 months). In contrast, the cumulative survival rate in AQP5-positive patients (n=14) was 7.4% (median time, 7.7 months), a difference that was highly statistically significant (P<0.05).

Multivariate analysis revealed that lymph node metastasis (P=0.018) and TNM stage (P=0.031) were independent prognostic factors for overall survival in patients with colorectal cancer; tumor diameter and other clinical parameters were not independent prognostic factors.

Expression of AQP5 correlates with CTC enumeration. CTCs are tumor cells that are shed from the primary tumor into the circulation. The presence of CTCs in the peripheral blood of patients has long been associated with metastasis and poor survival, although some authorities debate the biologic

Table II. Association between AQP5 protein expression and clinicopathologic factors in colon cancer.

Variables	Case	AQP5			P-value
		0 n (%)	1 n (%)	2 n (%)	
Total	45	2	29	14	
Age (years)					0.848
>60	28	1 (3.6)	18 (64.3)	9 (32.1)	
≤60	17	1 (5.9)	11 (64.7)	5 (29.4)	
Gender					1.000
Female	21	1 (4.8)	14 (66.7)	7 (33.3)	
Male	24	1 (4.2)	15 (62.5)	7 (29.2)	
Histologic grade					0.913
I	7	2 (28.6)	4 (57.1)	2 (28.6)	
II	20	0 (0.0)	12 (60.0)	6 (30.0)	
III	18	0 (0.0)	13 (72.2)	6 (33.3)	
Tumor size (cm)					0.263
≤2	15	1 (6.7)	10 (66.7)	4 (26.7)	
2-5	26	1 (3.8)	18 (69.2)	8 (30.8)	
>5	4	0 (0.0)	1 (25.0)	2 (50.0)	
Lymph node metastasis					0.016
Negative	17	2 (11.8)	8 (47.0)	3 (17.6)	
Positive	28	0 (0.0)	19 (67.9)	11 (39.3)	
Distant metastasis					0.000
Negative	39	2 (5.1)	29 (74.3)	8 (20.5)	
Positive	6	0 (0.0)	0 (0.0)	6 (100.0)	
TNM stage					0.002
I/II	14	1 (7.1)	12 (85.7)	1 (7.1)	
III	27	1 (3.7)	17 (62.9)	9 (33.3)	
IV	4	0 (0.0)	0 (0.0)	4 (100.0)	

AQP5 staining score 0 (no staining), 1 (weak) were counted as negative and score 2 (strong) counted as positive; the two groups were compared using the χ^2 test. AQP, aquaporin.

significance of CTCs due to tumor genomic instability and potential metastatic inefficiency. In view of the obvious clinical relevance, CTCs have been recently recommended by the American Society of Clinical Oncology as an acceptable cancer marker. To further determine whether or not AQP5 is an adverse prognostic biomarker, the correlation between AQP5 expression and the number of CTCs was analyzed. CTCs were detected in 78.57% (11/14) of colorectal cancer patients with strong AQP5 expression vs. 45.16% (14/31) with low and absent AQP5 expression (P<0.05; Fig. 4); Moreover, a significantly greater number of CTC enumeration was observed in patients with strong expression of AQP5 compared to patients with low and absent expression of AQP5 (19.5±2.0 in 7.5 ml blood vs. 5.5±1.5 in 7.5 ml blood). In conclusion, AQP5 is positively correlated with the number of CTCs.

Figure 4. CTCs stained with anti-CD45-CEP8-DAPI in peripheral blood of colorectal cancer patients (magnification, x400).

Discussion

Colorectal cancer is characterized by early lymph node metastasis and poor prognosis (1). In the present study, we determined the clinical significance of AQP5 expression in colorectal cancer patients, and thereby better defined the potential role for AQP5 in novel therapeutic modalities. Our results revealed that AQP5 is upregulated in colorectal cancer compared with peri-tumor and normal tissues accompanied by AQP5 DNA amplification. Furthermore, AQP5 was closely correlated with advanced TNM stage and poor prognosis. Markedly, AQP5-overexpressing cancer was shown to be prone to metastasis; clearly, there are more CTCs circulating in patients with AQP5 overexpression. These findings extend our understanding of the role of AQP5 as a diagnostic marker. To our knowledge, this is the first study to show AQP5 in colorectal cancer and its association with CTCs.

Aquaporins play a crucial role in maintaining water homeostasis and modulating a variety of physiologic and pathologic processes (13). Notably, the present study suggested that AQPs, including AQP5, are involved in tumorigenesis. AQP5, a 21-24 kDa protein, was initially described as the main structural protein in caveolae and was believed to be a key molecule involved in oncogenic transformation and malignant progression (14,15). AQP5 plays an important regulatory role in several signaling pathways leading to cellular transformation, including those mediated by the Src family of tyrosine kinases, epidermal growth factor receptor, Wnt, and Erk1/2 (10,16,17). Recently, Lee *et al* (11) reported that AQP5 overexpression is significantly associated with lymph node involvement and a poorer prognosis in patients with breast cancer, suggesting the value of AQP5 as a prognostic marker in breast cancer. Huang *et al* (18) also verified that AQP5 promotes the proliferation and migration of human gastric carcinoma cells. Wang *et al* (19) performed a study to show that AQP5 was mainly expressed in colorectal carcinoma cells and barely expressed in paraneoplastic normal tissues. The clinical significance of AQP5 in colorectal carcinoma is unknown. Our results showed that AQP5 is mainly expressed

in colorectal cancer cells and minimally expressed in peri-tumor and normal tissues. The expression patterns of AQP5 in colorectal tissues detected herein are consistent with the results of previous studies. Furthermore, we analyzed the correlations of AQP5 expression with the clinicopathologic features of colorectal cancer and showed that AQP5 expression is not significantly associated with the gender or age of patients with colorectal cancer, but is closely associated with the differentiation, TNM stage and distant lymph node metastasis of colorectal cancer.

CTCs are tumor cells shed from the primary tumor into the circulation. The presence of CTCs in the peripheral blood of patients has long been associated with metastasis and poor survival and is now considered an acceptable cancer marker. However, current techniques are limited (20,21). The only commercially available CTC test (CellSearch; Veridex, Raritan, NJ, USA) has a detection rate of 50% in late-stage patients (22,23). In the present study, we detected CTCs harboring negative enrichment using an immunomagnetic beads method, followed by identification with cytologic analysis, immunofluorescence and imFISH. This combination of methods resulted in detection rates up to 78.57% in patients with AQP5 overexpression, suggesting that imFISH staining could be used as a detection method for CTCs in future studies. In addition, our study verified that AQP5 overexpression is associated with the possibility of metastasis compared to lower expression of AQP5.

In summary, the AQP5 protein is upregulated in colorectal cancer and is closely related to advanced TNM stage, lymph node metastasis and poor prognosis. AQP5 strong overexpression is highly correlated with gene amplification. Thus, AQP5 may be used as a novel biomarker for colorectal cancer aggressiveness and metastasis.

Acknowledgements

The authors thank the staff of the Biology and Genetics Laboratory of Xi'an Jiaotong University for their technical assistance in these studies.

References

1. American Cancer Society: Cancer facts and figures, 2004. <http://acs.org>.
2. Kinzler KW and Vogelstein B: Lessons from hereditary colorectal cancer. *Cell* 87: 159-170, 1996.
3. Koontongkaew S: The tumor microenvironment contribution to development, growth, invasion and metastasis of head and neck squamous cell carcinomas. *J Cancer* 4: 66-83, 2013.
4. Sekine S, Shimada Y, Nagata T, Moriyama M, Omura T, *et al*: Prognostic significance of aquaporins in human biliary tract carcinoma. *Oncol Rep* 27: 1741-1747, 2012.
5. Moon C, Soria JC, Jang SJ, Lee J, Obaidul HM, *et al*: Involvement of aquaporins in colorectal carcinogenesis. *Oncogene* 22: 6699-6703, 2003.
6. Wang D and Owler BK: Expression of AQP1 and AQP4 in paediatric brain tumours. *J Clin Neurosci* 18: 122-127, 2011.
7. Hara-Chikuma M and Verkman AS: Prevention of skin tumorigenesis and impairment of epidermal cell proliferation by targeted aquaporin-3 gene disruption. *Mol Cell Biol* 28: 326-332, 2008.
8. Burghardt B, Elkaer ML, Kwon TH, Racz GZ, Varga G and Steward MC: Distribution of aquaporin water channels AQP1 and AQP5 in the ductal system of the human pancreas. *Gut* 52: 1008-1016, 2003.
9. Kang SK, Chae YK, Woo J, Kim MS, Park JC, *et al*: Role of human aquaporin 5 in colorectal carcinogenesis. *Am J Pathol* 173: 518-525, 2008.
10. Woo J, Lee J, Kim MS, Jang SJ, Sidransky D and Moon C: The effect of aquaporin 5 overexpression on the Ras signaling pathway. *Biochem Biophys Res Commun* 367: 291-298, 2008.
11. Lee SJ, Chae YS, Kim JG, Kim WW, Jung JH, *et al*: AQP5 expression predicts survival in patients with early breast cancer. *Ann Surg Oncol* 21: 375-383, 2014.
12. Shpiz S, Lavrov S and Kalmykova A: Combined RNA/DNA fluorescence in situ hybridization on whole-mount *Drosophila* ovaries. *Methods Mol Biol* 1093: 161-169, 2014.
13. McCoy E and Sontheimer H: Expression and function of water channels (aquaporins) in migrating malignant astrocytes. *Glia* 55: 1034-1043, 2007.
14. Jung HJ, Park JY, Jeon HS and Kwon TH: Aquaporin-5: a marker protein for proliferation and migration of human breast cancer cells. *PLoS One* 6: e28492, 2011.
15. Zhang Z, Chen Z, Song Y, Zhang P, Hu J and Bai C: Expression of aquaporin 5 increases proliferation and metastasis potential of lung cancer. *J Pathol* 221: 210-220, 2010.
16. Chen XJ, Yang JH and Zheng W: Effect of topotecan on expression of aquaporin protein 5 and nuclear factor-kappaB in ovarian cancer SKOV3 cells. *Ai Zheng* 28: 856-860, 2009 (In Chinese).
17. Choi JH, Wu HG, Jung KC, Lee SH and Kwon EK: Apoptosis and expression of AQP5 and TGF-beta in the irradiated rat submandibular gland. *Cancer Res Treat* 41: 145-154, 2009.
18. Huang YH, Zhou XY, Wang HM, Xu H, Chen J and Lv NH: Aquaporin 5 promotes the proliferation and migration of human gastric carcinoma cells. *Tumour Biol* 34: 1743-1751, 2013.
19. Wang W, Li Q, Yang T, Bai G, Li D, Li Q and Sun H: Expression of AQP5 and AQP8 in human colorectal carcinoma and their clinical significance. *World J Surg Oncol* 10: 242, 2012.
20. Onstenk W, Gratama JW, Foekens JA and Sleijfer S: Towards a personalized breast cancer treatment approach guided by circulating tumor cell (CTC) characteristics. *Cancer Treat Rev* 39: 691-700, 2013.
21. Attard G, Crespo M, Lim AC, Pope L, Zivi A, *et al*: Reporting the capture efficiency of a filter-based microdevice: a CTC is not a CTC unless it is CD45 negative - letter. *Clin Cancer Res* 17: 3048-3050, 2011.
22. Coumans FA, Ligthart ST, Uhr JW and Terstappen LW: Challenges in the enumeration and phenotyping of CTC. *Clin Cancer Res* 18: 5711-5718, 2012.
23. Lustberg M, Jatana KR, Zborowski M and Chalmers JJ: Emerging technologies for CTC detection based on depletion of normal cells. *Recent Results Cancer Res* 195: 97-110, 2012.