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ORIGINAL RESEARCH

Decreased expression of aquaporin 1 correlates with clinicopathological features of patients with cervical cancer

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Purpose: We aimed to investigate the expression dynamics of Aquaporin 1 (*AQP1*) in cervical cancer and evaluate correlations among *AQP1* levels and the clinicopathological features of patients with cervical cancer.

Patients and methods: *AQP1* mRNA and protein levels in cervical cancer and adjacent normal tissues were evaluated by quantitative reverse-transcription PCR (qRT-PCR) and western blot. Immunohistochemistry (IHC) for *AQP1* was performed with a tissue microarray of cervical cancer (containing 63 cases of squamous cell cervical cancers and 10 normal cervical tissues) to investigate clinicopathological outcomes. Cut-off scores for positive expression of *AQP1* were determined by receiver operating characteristic analysis. The χ^2 test was used to analyze correlations among *AQP1* expression and clinicopathological features of cervical cancer.

Results: The expression of *AQP1* was decreased in the majority of cervical cancer tissues by qRT-PCR and western blot analysis. Positive expression of *AQP1* was observed in 100% (10/10) of normal cervical tissues and in 42.86% (27/63) of cervical cancer tissues by IHC analysis. The cut-off score for positive expression of *AQP1* was determined to be 45% of cancer cells. Decreased expression of *AQP1* was correlated with clinicopathological features including; poor pathological grade (P=0.000), late International Federation of Gynecology and Obstetrics stage (P=0.008), and positive lymph nodes (P=0.002).

Conclusion: These data suggest that decreased expression of *AQP1* correlated with progressive features in patients with cervical cancer. *AQP1* levels may serve as a potential biomarker for the diagnosis of cervical cancer.

Keywords: aquaporin 1, cervical cancer, tissue microarray, immunohistochemistry

Introduction

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer-related deaths in women worldwide. Over 85% of newly diagnosed cases and cancer-related deaths occur in developing countries.[1] In recent years, cervical cytology screening has greatly improved the early diagnosis of cervical cancer.[2] However, incidence and mortality are still high, worldwide. Hence, there is an urgent need for further study of the molecular mechanisms of tumorigenesis and progression, which may identify new screening tools and biomarkers that facilitate early diagnosis as well as potential predictors of disease progression.

The aquaporins (*AQPs*) are a family of transmembrane water channel proteins expressed in many fluid-transporting tissues (eg, the glandular epithelia and kidney

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tubules) as well as in non-fluid-transporting tissues (eg, the epidermis). There are 13 known *AQPs* found in mammals. Their localization in the plasma membrane is crucial for the regulation of water transfer.[3] The first member of the family to be identified was *AQP1*, which is a membrane protein that controls the permeability of endothelial and epithelial barriers by facilitating water movement across the cell membrane.[4] In addition to its basic function, human *AQP1* expression is heterogeneous and found in many different human tumors.[5,6] Several studies have reported up-regulation of *AQP1* in malignancies of various organs and tissues,[7] such as glial tumors,[8] breast cancer,[9] and colorectal cancer.[10] In contrast, decreased expression of *AQP1* was observed in renal cell carcinoma (RCC),[11,12] with a correlation between unfavorable outcomes and lower *AQP1* expression in RCC,[11,12] intrahepatic cholangiocarcinoma,[13] and pleural malignant mesothelioma.[14] Despite growing evidence that *AQP1* is a crucial regulator of human cancers, its involvement in cervical cancer has not been assessed.

Therefore, in the present study, evaluations were made of *AQP1* expression levels in cervical cancers and adjacent normal tissues. We then investigated the relationships between *AQP1* expression and various clinicopathological parameters.

Materials and methods

Patients and tissue specimens

For quantitative reverse-transcription PCR (qRT-PCR) and western blot analysis, 18 pairs of fresh squamous cell cervical cancer and adjacent normal cervical tissue were collected from patients between September 2013 and February 2014. Immediately after surgery, the tissues were placed in RNAlater (Ambion, Austin, TX, USA). Following the manufacturer's instruction, the samples were kept submerged in RNAlater for at least 24 hrs at 4°C, and afterwards RNAlater was discarded and samples stored at −80°C until used. For tissue microarray (TMA) construction and immunohistochemistry (IHC) analysis, 73 paraffin-embedded tissues diagnosed between 2010 and 2013 were retrieved. All samples were collected from the Department of Obstetrics and Gynecology, the General Hospital of Guangzhou Military Command (Guangzhou, China). These samples were pathologically diagnosed cases of cervical cancer, having received no prior chemotherapy or radiotherapy before surgery. The age of these 63 patients ranged from 32 to 70 years (median, 44 years). Clinicopathological features of these patients included age at diagnosis, histological grade, clinical stage, and pTNM stage. Tumor clinical stage was according to the Federation International of Gynecology and Obstetrics staging of cervical carcinomas. Written informed consent was obtained from all patients for the use of tissue samples and clinical records. The study protocol was approved by the Ethics Committee of the General Hospital of Guangzhou Military Command, in accordance with the Declaration of Helsinki. Histopathological grading was by experienced pathologists.

qRt-PCR

Total RNA from the 18 pairs of frozen tissue was extracted by homogenization in RNAiso Reagent (Takara, Dalian, China), according to the manufacturer's protocol. RNA was reverse-transcribed to generate cDNA by using a PrimeScript RT-PCR kit (Takara, Dalian, China). The PCR reaction included 90 ng of cDNA template, 0.4 μM of the forward and reverse primers each, 25 μL of the 2× SYBR Premix Ex Taq™ II (Takara, Dalian, China) buffer, and ddH_2O to a total volume of 50 μL; *β-actin* was used as an internal control. For the *AQP1* gene, the forward primer was 5′- ATGGCAACAGAAACCAAGAGACA-3′, and the reverse primer was 5′-TGAGAAGCTGGAAATG AGGGAA-3′. For *β-actin*, the forward primer was 5′-TGGCACCCAGCACAATGAA-3′, and the reverse primer was 5′-CTAAGTCATAGTCCGCCTAG AAGCA-3′. PCR was performed in an ABI 7500 real-time PCR amplifier (Applied Biosystems, Foster City, USA) with a predenaturation step of 95°C for 30 s, followed by 40 cycles with a denaturation step of 95°C for 5 s and an elongation step of 60°C for 34 s. Ct values were acquired using the 7,500 system SDS software (Applied Biosystems, Foster City, USA) with manual thresholds. The $2^{-\triangle\triangle Ct}$ values were calculated as fold change between paired cervical cancer and normal cervical tissue. To acquire stable results, each PCR reaction was performed in triplicate. The qRT-PCR products were identified by electrophoresis in 2% agarose gels and visualized with UV light after staining with ethidium bromide. A Tanon 1,600 image station (Tanon, Shanghai, China) was used to capture band images.

Western blotting

Total protein from 18 paired cervical cancer and adjacent normal tissues was extracted in radio-immunoprecipitation assay buffer containing 1 mM phenylmethanesulfonyl fluoride. After centrifugation, supernatants were collected

and protein concentration determined with a BCA Protein Assay Kit (Beyotime, Haimen, China) at 562 nm with a TECAN Infinite 200 microplate reader (TECAN, Austria). Tissue homogenates (35 μg of protein per sample) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were transferred onto a polyvinylidene difluoride membrane (Millipore, USA) with a Trans-Blot SD semi-dry transfer machine (Bio-Rad, USA). The blots were washed with 1× TBST buffer (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, and 0.05% Tween-20), and membranes were blocked overnight with 5% skim milk in TBST and incubated with primary antibody at room temperature for 2 hrs. Polyclonal rabbit anti-human antibody reactive with *AQP1* (Merck Millipore, Temecula, USA, at a dilution of 1:500) and monoclonal rabbit anti-human antibody reactive with β-actin (Cell Signaling Technology, USA, at a dilution of 1:3,000) were used as primary antibodies. The membranes were then washed with 1× TBST, and primary antibodies were detected with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, USA, at a dilution of 1:5,000). Immunoreactive bands were visualized with a BeyoECL Plus Kit (Beyotime, Haimen, China) and scanned with an Image Station 4000R PRO analyzer (Carestream Health, USA).

TMA construction and IHC

Based on hematoxylin-eosin staining, representative sections of cervical cancer and normal cervical tissue in pre-existing paraffin-embedded tissue blocks were identified. A 1.5 mm diameter cylinder was punched from a representative section of each block and placed into a recipient paraffin block to construct the TMA. The TMA block was then sliced into 5 μm thick multiple sections and mounted on microscope slides for IHC. The TMA consisted of 63 cases of cervical cancer and 10 cases of normal control paraffin-embedded tissue. Clinical characteristics of the patients are summarized in Table 1. TMA slides were dried overnight at 37°C, deparaffinized in xylene, rehydrated through graded alcohol, immersed in 3% hydrogen peroxide for 15 mins to block endogenous peroxidase activity, and antigen-retrieved by microwave heating with sodium citrate buffer (pH 6.0) at 100°C for 20 mins. The slides were pre-incubated with 10% normal goat serum in TBST at room temperature for 30 mins to reduce non-specific reactions. The primary rabbit anti-*AQP1* polyclonal antibody (Merck Millipore, Temecula, USA) was diluted (1:500) with 1× phosphate buffered saline and incubated with the TMA overnight in a humidity chamber at 4°C. The slide was sequentially incubated with a polymer peroxidase labeled secondary antibody (ZSGB-Bio, Beijing, China) for 30 mins at room temperature and then stained with a DAB HRP Color Development Kit (Beyotime, Haimen, China). The sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. As a negative control, the primary antibody was replaced with normal murine IgG. A known melanoma expressing high levels of *AQP1* was used as a positive control.

IHC evaluation

Immunoreactivity for the *AQP1* protein was scored by a semi-quantitative method as the proportion of positive tumor cells over the total number of tumor cells. Scores were assigned in 5% increments (0%, 5%, . . ., 100%). The scores were accepted if all three investigators (MW, RS, and JZ) agreed. Otherwise, the values were re-estimated until a consensus was obtained. Conclusions were in complete agreement for 85% of the cases, indicating a high degree of reproducibility.

Selection of cut-off scores

Receiver operating characteristic (ROC) curve analysis was utilized to determine the cut-off score by using the 0.0, 1.0 criterion.[15] At different *AQP1* scores, the sensitivity and specificity for each outcome were plotted, generating various ROC curves. The score closest to the point with both maximum sensitivity and specificity was selected as the cut-off score. Tumors designated as "negative" for *AQP1* were those with values below or equal to the cut-off score, whereas "positive" for *AQP1* were values above the cut-off score.[16,17] In order to perform ROC curve analysis, the clinicopathological features were dichotomized: age (< median age or ≥ median age), histological grade (low G_1 or high G_2+G_3), clinical stage (I_a~I_b or II_a~III_b), pT stage (early T_1 or moderate and late T_2+T_3), N stage (N_0 [no lymph node involvement], N_1+N_2 [any lymph node involvement]), M stage (M_0 [no distant metastasis], or M_1 [distant metastasis]).

Statistical analysis

Statistical analysis was performed with the SPSS statistical software program (standard version 13.0, SPSS,

Table 1 Relationship between *AQP1* expression and clinicopathological features in cervical cancer

Clinicopathological feature	All cases	AQP1 expression		Pearson Chi-square	*P*-value‡
		Positive	Negative		
Age (years)					
<44^	31	13	18	0.021	0.884
≥44	32	14	18		
Tumor grade					
G1	11	10	1	18.572	0.000
G2	33	15	18		
G3	19	2	17		
Clinical stage					
Ia	6	6	0	15.609	0.008
Ib	12	7	5		
IIa	3	1	2		
IIb	16	8	8		
IIIa	4	1	3		
IIIa	22	4	8		
pT status					
$T_1(T_{1a}+T_{1b})$	27	14	13	3.902	0.142
$T_2(T_{2a}+T_{2b})$	28	12	16		
$T_3(T_{3a}+T_{3b})$	8	1	7		
pN status					
N_0	40	23	17	9.593	0.002
N_1	23	4	19		
pM status					
M_0	63	27	36	N/A	N/A
M_1	0	0	0		

Notes: ^Median age. ‡*P*-value are from Chi-square test.

Chicago, IL, USA). ROC curve analysis was used to determine the cut-off score for positive expression of *AQP1*, and areas under curves (AUCs) were calculated. The relationships among *AQP1* protein expression and clinicopathological features of cervical cancer patients were estimated using the χ^2 test. $P<0.05$ was considered statistically significant by two-tailed test.

Results

Expression of AQP1 mRNA and protein in paired cervical cancer and adjacent normal tissue

The qRT-PCR results showed that in the majority (17/18) of the sample pairs, fold changes (the $2^{-\triangle\triangle Ct}$ values) were less than 1 between cervical and adjacent normal cervical tissue (Figure 1A), which indicated that *AQP1* mRNA expression was decreased in cervical cancer tissues compared to adjacent normal tissue. Western blot analysis also demonstrated reductions in *AQP1* protein for 15 of the 18 cervical cancer tissues compared to their adjacent normal counterparts (representative results are shown in Figure 1B). The mean fold change in *AQP1* mRNA expression in cervical cancer tissue compared to adjacent normal tissue was 0.434. Paired *t*-test showed the difference between the two groups to be statistically significant ($P=0.000$, Figure 1C). The mean fold change in *AQP1* protein level between the cervical cancer tissue and the adjacent normal tissue was 0.428 by semi-quantitative analysis. The difference between the two groups was also statistically significant by paired *t*-test ($P=0.000$, Figure 1D).

Expression levels of AQP1 in cervical cancer tissues by IHC

To investigate the *AQP1* expression levels in cervical cancer, we examined *AQP1* protein expression in 63

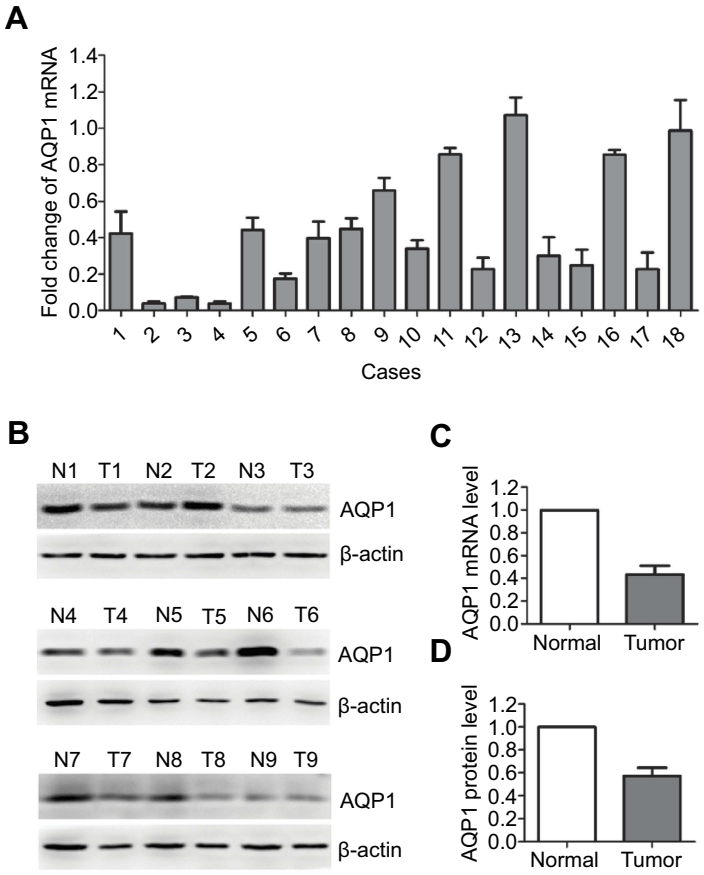

Figure 1 qRT-PCR and western blot analysis of *AQP1* expression in paired cervical cancer and adjacent normal cervical tissue.
Notes: (**A**) Fold changes ($2^{-\triangle\triangle Ct}$ values) by qRT-PCR showed a down-regulation of AQP1 mRNA in a majority of cervical cancer cases, when compared with paired normal cervical tissue. Expression levels were normalized for *β-actin*. (**B**) Western blotting indicated down-regulation of *AQP1* protein in cervical cancer compared to adjacent normal cervical tissue. β-Actin was used as an internal control. T, cervical cancer; N, normal. (**C**) Significant differences in *AQP1* mRNA expression between cervical cancer and adjacent normal cervical tissue (P=0.000). (**D**) Significant difference in *AQP1* protein expression between cervical cancer and adjacent normal cervical tissue (P=0.000). *P<0.05 by paired two-sided *t*-test.
Abbreviation: qRT-PCR, quantitative reverse-transcription.

cases of cervical cancer and 10 adjacent cervical tissues by IHC and TMA. Immunoreactivity was observed primarily in the membrane of cells, especially in vascular endothelial cells, with occasional yellowish granules in the cytoplasm (Figure 2). Decreased expression of *AQP1* was detected in poorly differentiated and advanced stage cervical cancer tissue (Figure 2A) compared to lower grade and stage cervical cancer tissue (Figure 2B, 2C, and 2D). Normal cervical epithelia showed very high *AQP1* expression (Figure 2E).

Selection of cut-off scores for AQP1 IHC expression

ROC curve analysis was used to determine the cut-off score for expression of *AQP1*. The ROC for each clinico pathological parameter (Figure 3) clearly identified points on the curves closest to (0.0, 1.0), which were maximal for both sensitivity and specificity for each outcome. Tumors with values below the obtained cut-off score were considered negative for *AQP1* protein. The corresponding AUCs and cut-off scores are shown in Figure 3 and Table 2, respectively. Histology grade had the shortest significant distance from the curve to the point (0.0, 1.0). Hence, the cut-off score was determined by histology grade. The cut-off score for positive expression of *AQP1* was defined as positive staining for *AQP1*≥45% of all cancer cells. *AQP1* negative expression was observed in 57.14% (36/63) of cervical cancer tissue by IHC analysis. Negative expression of *AQP1* has detected in 35/52 (67.3%) of grade G_2+ G_3 cervical cancers and in 21/45 (46.67%) of stage II + III cervical cancers. Positive expression of *AQP1* was observed in 10/10 (100%) of normal cervical tissue, 10/

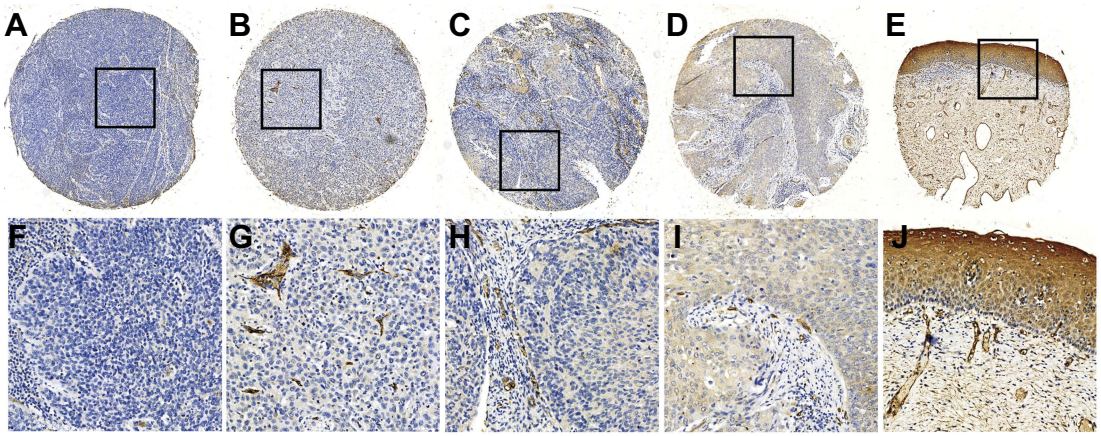

Figure 2 Expression levels of *AQP1* protein in cervical cancer and adjacent normal cervical tissue.
Notes: (**A**) Negative expression of *AQP1* in a cervical cancer case (case 19, grade III), in which none of the tumor cells showed immunoreactivity for the *AQP1* protein (×100). (**B**) Negative *AQP1* expression was observed in a cervical cancer sample (case 15, grade II), in which 10% of the tumor cells revealed positive immunostaining for *AQP1* in the membrane (×100). (**C**) Negative *AQP1* expression was observed in a cervical cancer sample (case 30, grade II) in which 35% of the tumor cells revealed positive immunostaining for *AQP1* in the membrane (×100). (**D**) Positive *AQP1* expression was observed in a cervical cancer sample (case 61, grade I), in which 85% of the tumor cells revealed positive immunostaining for *AQP1* in the membrane (×100). (**E**) Positive expression of *AQP1* protein in normal cervical tissue (×100). The lower panels (**F–J**) indicate higher magnification (×400) of areas in the boxes of (**A–E**), respectively.

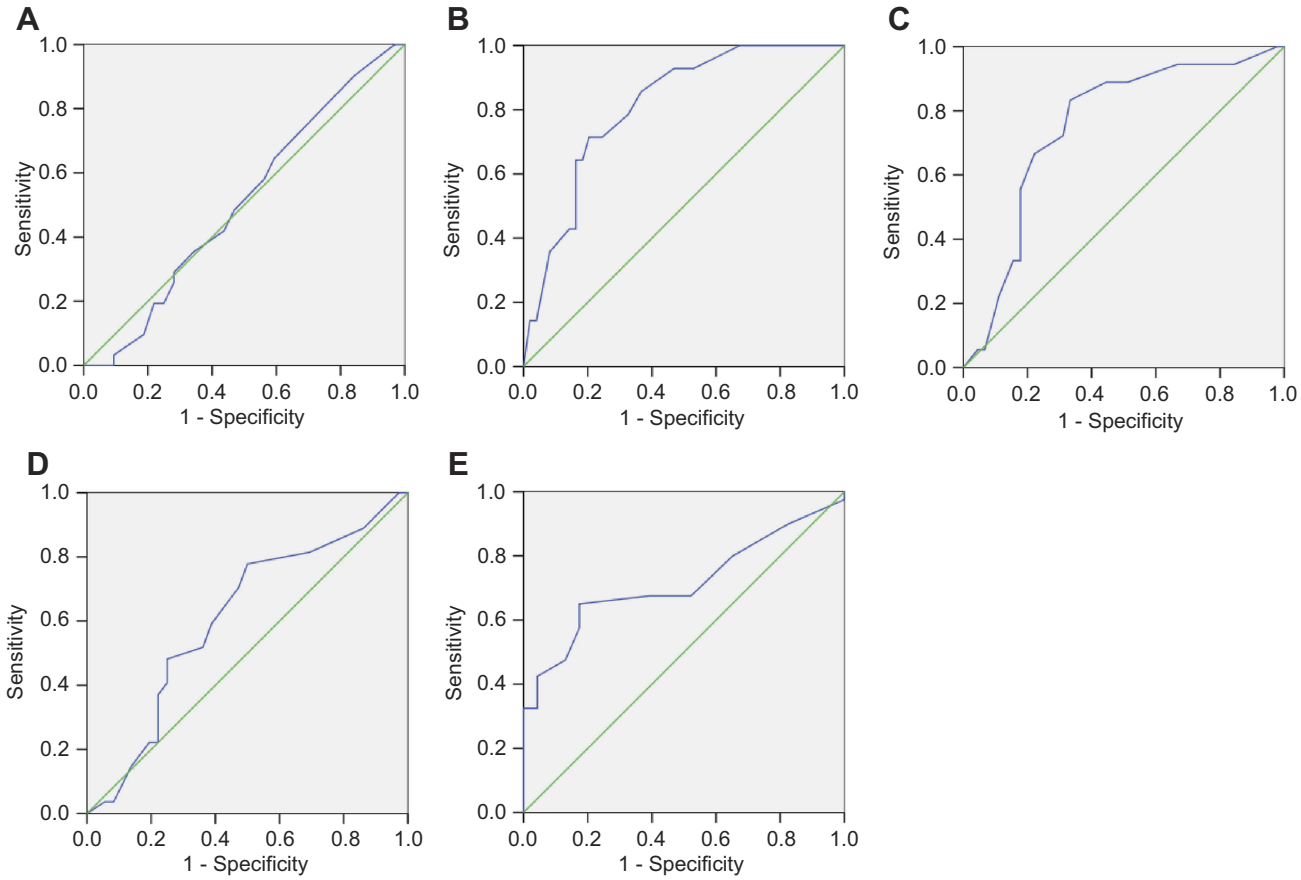

Figure 3 Receiver operating characteristic (ROC) curves were used to determine the cut-off score for positive *AQP1* expression. The sensitivity and specificity for each outcome were plotted, and the areas under ROC curve presented.
Notes: (**A**) Age, (**B**) histological grade, (**C**) clinical stage, (**D**) pT stage, (**E**) pN stage.

Table 2 Area under ROC curve for each clinicopathological feature

Feature	AUC (95% CI)	*P*-value
Age	0.507 (0.363–0.651)	0.923
Pathological grade	0.810 (0.697–0.924)	0.000
Clinical stage	0.752 (0.622–0.882)	0.002
T stage	0.610 (0.467–0.752)	0.139
N stage	0.715 (0.590–0.840)	0.005
M stage	N/A	N/A

Abbreviations: AUC, area under the curve; ROC, receiver operating characteristic.

11(90.91%) of grade G_1, 6/6 (100%) of stage I_a, and 7/12 (58.3%) of stage I_b cervical cancers (Table 1).

Decreased expression of AQP1 correlates with clinicopathological features of cervical cancer

AQP1 expression in cervical cancer with respect to several standard clinicopathological features is shown in Table 1. The χ^2 test showed *AQP1* expression to be lower in patients with a poorly differentiated tumor grade (χ^2=18.572, *P*=0.000), advanced clinical stage (χ^2=15.609, *P*=0.008), and in patients with positive lymph nodes (χ^2=9.593, *P*=0.002).

Discussion

AQPs have been reported in at least 13 different tumor cell types,[18] with dysregulation observed in tumor cells and vascular endothelial cells. For some tumors, positive correlations have been established between *AQP* expression and histological grade, metastatic potential, and cancer prognosis. Up-regulation of *AQP3* and *AQP5* was reported in gastric carcinoma, with the level of expression found to correlate with lymph node metastasis and lympho-vascular invasion.[19] Down-regulation of *AQPs* has been observed for *AQP8* in colorectal cancer,[20] *AQP8* and *AQP9* in hepatocellular carcinoma,[21] and *AQP4* in pleural mesothelioma.[22]

Despite higher expression in the microvascular, Mobasheri et al,[9] reported that *AQP1* was heterogeneously expressed in different human tumors and not necessarily expressed in all neoplastic cells as judged by IHC and TMA of prostate, colon, lung, breast, and ovarian cancer. These findings have been confirmed by other reports. Up-regulation of *AQP1* was found in glioma, laryngeal cancer, hemangioblastoma, and colorectal cancer,[4,23,24,25,26] with clinicopathological feature correlations. Otterbach et al,[27] reported that *AQP1* was strongly expressed on the membrane of breast cancer cells, with elevated expression significantly associated with poor prognosis. Down-regulation of *AQP1* in RCC was reported as a potential prognostic factor for unfavorable outcomes in several cancers. By using different methods, both Takenawa et al, and Huang et al, reported that *AQP1* expression was reduced in RCC,[12] and that expression levels of *AQP1* provided useful prognostic information for patients with RCC. Aishima et al,[13] reported that down-regulation of *AQP1* correlated with large tumor size, poorly differentiated histology, and positive lymph node metastasis in intrahepatic cholangiocarcinoma. Kao et al,[14] suggested that expression of *AQP1* by ≥50% of tumor cells was associated with significantly enhanced survival and could be used as an independent prognostic factor for pleural malignant mesothelioma. Interestingly, heterogeneous expression of *AQP1* exists among different histopathologic tumor subtypes of RCCs. Huang et al,[12] showed that although the majority of RCC subtypes express *AQP1* at a lower level than the normal kidney, *AQP1* levels were statistically higher in papillary and clear-cell RCCs than in all other subtypes. Median *AQP1* expression in papillary RCCs was even higher than that in the normal kidney. Thus, the relationship between *AQP1* expression and cancer is complex and requires careful interpretation.

Moreover, *AQP1* has been reported to be involved in tumor angiogenesis,[28,29] with overexpression of *AQP1* in vitro increasing migration and metastasis of certain tumor cell lines.[30,31] Another study suggested that *AQP1* may serve as a therapeutic target for lung and glial tumors.[10] In a recent study, we demonstrated *AQP1* to promote cell differentiation of the human erythroleukemia, K562, by inducing the expression of erythroid differentiation related genes.[32]

The expression level of *AQP1* in cervical cancer and its correlation with the clinicopathological features of cervical cancer was poorly recognized. Shi et al,[33] found that AQP1 expression significantly increased in the advanced stage, deeper infiltration, metastatic lymph nodes and larger tumor volume in cervical carcinoma in Xinjiang Uygur women of China. Shen et al,[34] found that AQP1 showed a higher positivity rate in intraepithelial neoplasia (CIN) than in squamous cervical cancer (SCC) and normal cervical tissues. And there was a significant increase in the expression of AQP1 in stage I than that in stage II of SCC. Herein, qRT-PCR, western blotting, and IHC were used to evaluate expression and immune-localization of *AQP1* in patients with cervical cancer. Results showed that *AQP1* mRNA and protein levels were decreased in human cervical cancer tissues when compared to corresponding adjacent normal tissues. IHC demonstrated

AQP1 protein mainly on the cell membrane of tumor cells and in vascular endothelial cells, with expression reduced in cervical cancer tissue when compared to normal cervical epithelial tissue. *AQP1* was found to be associated with tumor status. Decreased expression of *AQP1* was significantly correlated to poor pathological grade, late clinical stage, and positive lymph node metastasis. Hence, decreased expression of *AQP1* in squamous cell cervical cancer may be related to the process of tumor progression. These findings are consistent with *AQP1* expression in RCCs,[11,12] in that decreased expression of *AQP1* was also observed. *AQP1* expression levels in these cancers may be used as a predictive prognostic indicator for these patients. Validation of the levels of *AQP1* and other *AQPs* in cervical cancer requires further large-scale clinical investigations. Detailed investigations into the role of *AQP1* in the carcinogenesis and differentiation of cervical cancer are necessary in order to evaluate the use of *AQP1* as a biomarker for diagnosis and prognosis of cervical cancer.

Conclusion

This study demonstrates *AQP1* expression to be reduced in cervical cancer when compared to adjacent normal tissue. Decreased expression of *AQP1* was significantly correlated with poor cancer cell differentiation and unfavorable clinical features in patients with cervical cancer. As such, *AQP1* may serve as a potential biomarker for the diagnosis of cervical cancer.

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Disclosure

The authors report no conflicts of interest for this work.

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