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Aquaporin-8 is a novel marker for progression of human cervical cancer cells

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Abstract.
BACKGROUND: Role of aquaporin-8 (AQP8) in cervical cancer has not been fully elucidated.
OBJECTIVE: We aim to explore the impacts of AQP8 on viability, apoptosis and metastasis in cervical cancer cells.
METHODS: AQP8 protein expression in cervical carcinoma specimens and cell lines was detected by IHC and western blot analysis. Lentivirus-mediated transfection was used to upregulate and knockdown AQP8 in cells. Cell viability and apoptosis were assessed by CCK-8 and flow cytometry assays, respectively. Transwell experiments were conducted to investigate cell invasive and migratory capabilities. EMT-related markers were detected by western blot analysis.
RESULTS: A strong positive of AQP8 protein expression was observed in cervical cancer tissues. Western blot analysis confirmed overexpression and knockdown of AQP8 in SiHa cells. AQP8-overexpressed SiHa cells displayed an enhanced viability, reduced apoptotic rate, increased invasive and migratory abilities. Knockdown of AQP8 inhibited the viability, promoted the apoptosis, and suppressed invasion and migration. Furthermore, AQP8 overexpression significantly upregulated vimentin and N-cadherin, and downregulated E-cadherin, which were reversed by AQP8 knockdown.
CONCLUSIONS: AQP8 increases viability, inhibits apoptosis, and facilitates metastasis in SiHa cells. This may be associated with EMT-related markers regulated by AQP8. AQP8 could serve as a potential marker for cervical cancer progression.

Keywords: Aquaporins, AQP8, cervical cancer, invasion, migration, EMT

1. Introduction

Aquaporins (AQPs) are a family of membrane channels that facilitate transportation of water molecules and small solutes under an osmotic gradient [1,2]. Notably, accumulated evidences have shown that AQPs exist in numerous types of human cancer cells and are closely implicated into carcinogenesis [3–5]. AQP8 is one of the AQPs members and participates only in water metabolism [6]. Despite the recognized pathologic role of AQP8 in inflammatory diseases [7,8], its function in carcinogenesis remains rare and controversial. Intriguingly, AQP8 is reported to serve as oncoprotein in human esophageal cancer [9] but as a suppressor in colorectal cancer [10]. Our team has previously demonstrated that AQP8 was gradually upregulated in cervix as the disease progression [11]. Nonetheless, the exact role of AQP8 in cervical tumorigenesis has not been fully elucidated up to date.

Epithelial cells often lose their polarized characteristics and gain mesenchymal features through a remodel-

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ing process, named EMT [12]. This process is a fundamental event and intimately involved in various aspects of development, including embryogenesis, wound healing, as well as cancer progression [13,14]. During EMT, cancer cells display increased migratory and invasive phenotypes, which become a major cause of metastasis and relapse [15]. Studies have also reported the onset of EMT often leads to increased metastatic properties and drug resistance in cervical cancer [16]. However, there is a scarcity in studies that report AQPs regulating EMT process in human cancers so far.

In this study, AQP8 expression was detected in carcinoma tissues of cervix and different cell lines. Moreover, the function of AQP8 on the viability, apoptosis, and metastasis of cervical cancer cells was explored. Finally, whether AQP8 could regulate EMT process in cervical cancer cells was also investigated in this study.

2. Materials and methods

2.1. Clinical samples and immunohistochemistry

A total of 10 cervical cancer and matched normal cervix samples were gleaned from the Second Affiliated Hospital of Wenzhou Medical University from Jan 2020 to August 2020. All patients included in this study did not receive any chemoradiotherapies before surgery and provided an informed consent. The approval of this study had also been obtained from the ethical committee of the Second Affiliated Hospital of Wenzhou Medical University. The pathological classification and clinical stage were gained on the basis of FIGO.

IHC staining was performed on formalin-fixed and paraffin-embedded 4 μm tissue specimens. In brief, the sections were deparaffinized in xylene and rehydrated in a gradient of ethanol solutions. After that, the sections were submerged into citrate buffer (pH 6.0) at 95°C for 20 min in a microwave oven for antigen retrieval. Subsequently, sections were washed three times in PBS and blocked with 3% hydrogen peroxide solution. Slides were then incubated with primary antibodies against AQP8 (Boster, China, 1:25) overnight at 4°C. After washing, the sections were incubated with secondary HRP-conjugated antibodies at room temperature and counterstained with hematoxylin. PBS was used to replace the primary antibody as a negative control. A positive reaction was considered as cells showing yellowish brown in the cytoplasm.

2.2. Cell lines and culture

Human cervical cancer cell lines (C33A, HeLa and SiHa) were purchased from ATCC (USA). The immortalized normal cervical epithelial cell lines H8 were donated by the Institute of Immunology, Wenzhou Medical University. All cells were cultivated in DMEM (Gibco, USA), supplemented with 10% FBS (Gibco, USA) and 1% penicillin-streptomycin solution (Invitrogen, UK). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

2.3. Lentiviral transduction

The AQP8 shRNAs and control plasmids were purchased from Shanghai Jiao Tong University School of Medicine. The AQP8 sequences were amplified via PCR and inserted into the lentiviral vector pLVX-IRES-ZsGreen1 (Origene, USA). After gene sequencing, pLVX-IRES-ZsGreen1-AQP8 and the null vector, AQP8 shRNAs and the negative plasmid, together with two packing plasmids psPAX2 and pMD2.G were transduced into 293 T cells for 48 h with the help of lipofectamine 2000 (Thermo Fisher Scientific, USA). Subsequently, SiHa cells were transfected with recombinant lentiviral particles together with polybrene (8 μg/ml). The AQP8-shRNA and the negative control cells were treated by 2 μg/ml puromycin (Sigma, USA) for selection. All cells after transfection were confirmed by western blot.

2.4. Cell viability assay

A CCK-8 kit (Dojindo, China) was used to measure viability of SiHa cells. Approximately 3×10^3 cells per well were seeded into 96-well plates, followed by incubation at 37°C for a set of times (0, 24, 48, 72, 96 h). Next, 100 μl working solution (mix of CCK-8 reagent and DMEM at ratio of 1:9) was added per well and cultivated for 3 h. Then, the absorbance at 450 nm was measured by Micro-plate Reader (Bio-Rad, USA). All experiments were repeated for three times. Cell viability was calculated as: average OD values of transfected group - average OD values of control group.

2.5. Flow cytometry analysis

The Annexin V-FITC/PI Apoptosis Detection kit (BD, USA) was used to evaluate cell apoptosis. The protocol used for analysis was previously described [17]. About 5×10^5 cells were harvested and washed with cold PBS. After that, the cells were incubated with PE and 7-AAD in dark condition for 15 min. The apoptotic rate was calculated using flow cytometry (Beckman, USA).

2.6. Transwell assay

The migratory and invasive abilities of cells were assessed using the 8 μm pore size filter (Corning, USA) in 24-well plates. For invasion assay, the filter was precoated with 60 μl extracellular matrix. Cells with the density of 1×10^5 cells/well in serum-free medium were seeded into the upper chamber. The bottom chamber was filled with complete DMEM. After incubation for 24 h, the inserts were fixed in 4% paraformaldehyde, stained with 1% crystal violet. Then, cells on the top surface that failed to penetrate the polycarbonate membrane were wiped off by a cotton swab. Finally, the filter was observed under a microscope (Leica, GER) for cell counting.

2.7. Western blot analysis

Cells were harvested and lysed in RIPA buffer containing protease inhibitors. After centrifugation, the supernatants were collected and determined by BCA assay for total protein concentration. Electrophoresis process was performed on SDS-PAGE with equal proteins (40 μg/lane). Then, the separated proteins were absorbed to PVDF membranes (Millipore, USA). The PVDF membranes were sealed with 5% skim milk and incubated with primary antibodies overnight at 4°C. After incubation with the secondary goat anti-rabbit/anti-mouse (Biosharp, China, 1:5000) antibody, membrane was treated with ECL kit (Advansta, USA) and visualized under GenoSens 2000 Touch (CLiNX, China). The intensity of blots was analyzed by Image Lab software and normalized to internal loading controls.

Primary antibodies used in this study included mouse anti-human AQP8 (Boster, China, 1:1000), PCNA (CST, USA, 1:2000), Bcl-2 (CST, USA, 1:1000), Bax (CST, USA, 1:1000), E-cadherin (CST, USA, 1:2000), N-cadherin (CST, USA, 1:2000), β-actin (CST, USA, 1:3000), α-Tubulin (Abcam, USA, 1:3000), and vinculin antibodies (CST, USA, 1:3000); rabbit anti-human Ezrin (CST, USA, 1:2000), Fascin (CST, USA, 1:2000), vimentin (CST, USA, 1:2000), and GADPH (Abcam, USA, 1:3000) antibodies.

2.8. Statistical analysis

Statistical analysis was performed by GraphPad Prism 8 software (GraphPad, USA). Data distribution was analyzed by the Kolmogorov-Smirnov test before comparison. Parametric or nonparametric tests were employed according to the results. Normally distributed data were displayed as the means $\pm$ SEM. The difference was compared by Student's t test (2 groups) and ANOVA analysis ($\geqslant$ 3 groups). Mann-Whitney testing was conducted to compare the difference between groups from non-normally distributed data. Statistically significant difference was defined as two-tailed $P < 0.05$.

3. Results

3.1. AQP8 is upregulated in cervical cancer tissues and cells

To understand the expression pattern of AQP8 in cervical cancer, AQP8 protein levels were first detected in normal cervix and cervical cancer tissues by immunohistochemistry. Results showed that AQP8 staining was strong positive in cancer but negative in adjacent normal samples. The representative images are shown in Fig. 1A and AQP8 localized in the cytoplasm. Cervical cancer tissues (8.3 ± 1.6) showed a higher mean IHC score than that in normal cervical tissues (1.3 ± 0.5) (Fig. 1A; $P < 0.05$). Next, western blot analysis revealed a higher expression level of AQP8 in SiHa and HeLa than that in H8 cells (Fig. 1B; $P < 0.05$). No significant difference of AQP8 expression between C33A and H8 cells was observed ($P > 0.05$). Notably, C33A is the cell lines from the cervical carcinoma in situ. SiHa and HeLa cells are derived from invasive cervical squamous cell carcinoma and adenocarcinoma of high-risk HPV infection, respectively. Based on these findings, it can conclude that AQP8 may play an oncogenic role in cervical tumorigenesis. A moderate expression of AQP8 was observed in SiHa cells as it was higher in HeLa and lower in C33A cells. Thus, AQP8 was stably overexpressed by AQP8-cDNA and knocked down using AQP8-shRNA in SiHa cells. The overexpression and knockdown efficacies of AQP8 were confirmed by western blot (Fig. 2A and B).

3.2. AQP8 increases viability and inhibits apoptosis in cervical cancer cells

Overexpression of AQP8 in SiHa cells led to a significantly higher cell viability and lower apoptotic rate than cells with control-vector (Fig. 3A and B; $P < 0.05$). Conversely, SiHa cells with AQP8 knockdown exhibited a remarkably reduction in viability and increase in apoptosis (Fig. 3C and D; $P < 0.05$). Western blot analyses for PCNA (proliferative marker) and

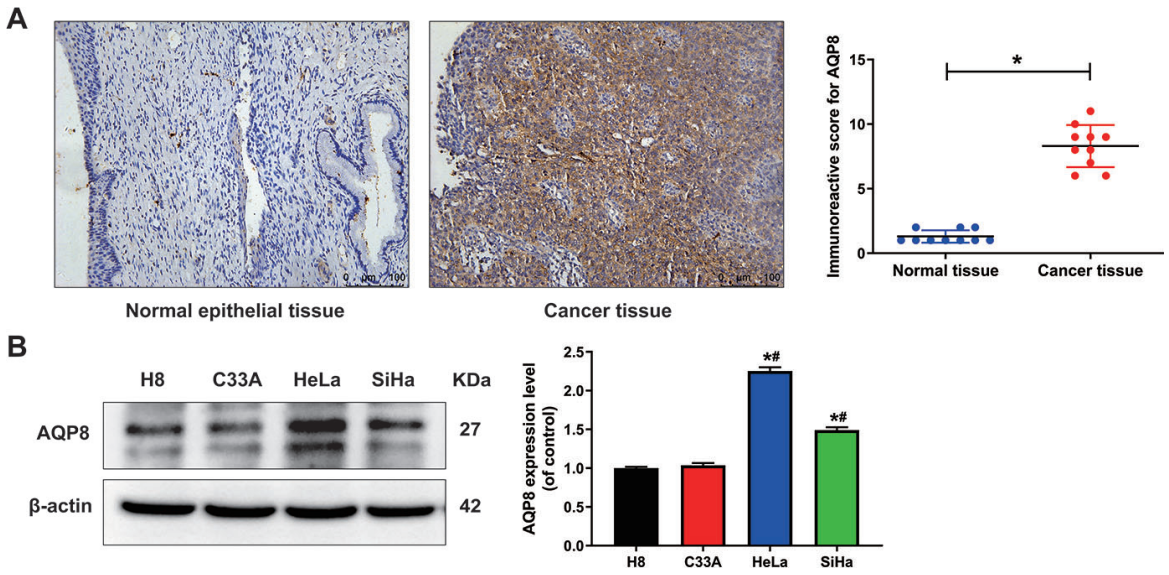

Fig. 1. The expression of AQP8 in human cervical cancer tissues and cell lines. A, Immunohistochemistry analysis showed a strong positive staining of AQP8 protein in cervical cancer samples but negative in adjacent normal cervix (magnification, 200 ×). The data shown were the IHC scores of the AQP8 and the means ± standard error between cervical cancer and normal cervical tissues. $^{*}P < 0.05$ vs normal cervical tissue group; B, The expression of AQP8 protein in normal cervical epithelial cell lines (H8) and cervical cancer cell lines (C33A, HeLa and SiHa) was detected using western blot analysis. The data shown were the ratios of the AQP8/β-actin and the means ± standard error of each cell lines. Each experiment was performed three times. $^{*}P < 0.05$ vs H8 group; $^{\#}P < 0.05$ vs C33A group.

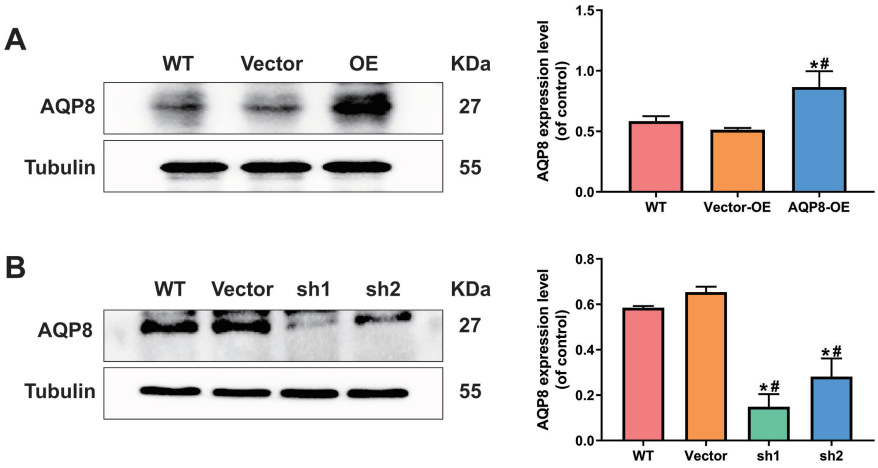

Fig. 2. AQP8-overexpression and knockdown SiHa cell lines were constructed by lentivirus transduction. A, Stably upregulated AQP8 expression in SiHa cells were identified by western blot analysis. B, Stably downregulated AQP8 expression in SiHa cells were confirmed by western blot analysis. The data shown were the ratios of the AQP8/Tubulin and the means ± standard error of each group. Each experiment was performed three times. $^{*}P < 0.05$ vs WT group; $^{\#}P < 0.05$ vs vector group.

Bcl-2/Bax (apoptotic marker) were also conducted. As shown in Fig. 3E, despite no change in PCNA expression ($P > 0.05$), AQP8 overexpression significantly enhanced the ratio of Bcl-2/Bax in SiHa cells ($P < 0.05$). Furthermore, the PCNA expression and Bcl-2/Bax ratio were significantly reduced by AQP8 knockdown in SiHa cells ($P < 0.05$). These results demonstrate that AQP8 enhances the viability and inhibits apoptosis of cervical cancer cells.

3.3. *AQP8 promotes invasion and migration in cervical cancer cells*

Transwell assays were performed to explore the effect of AQP8 in invasion and migration of cervical can-

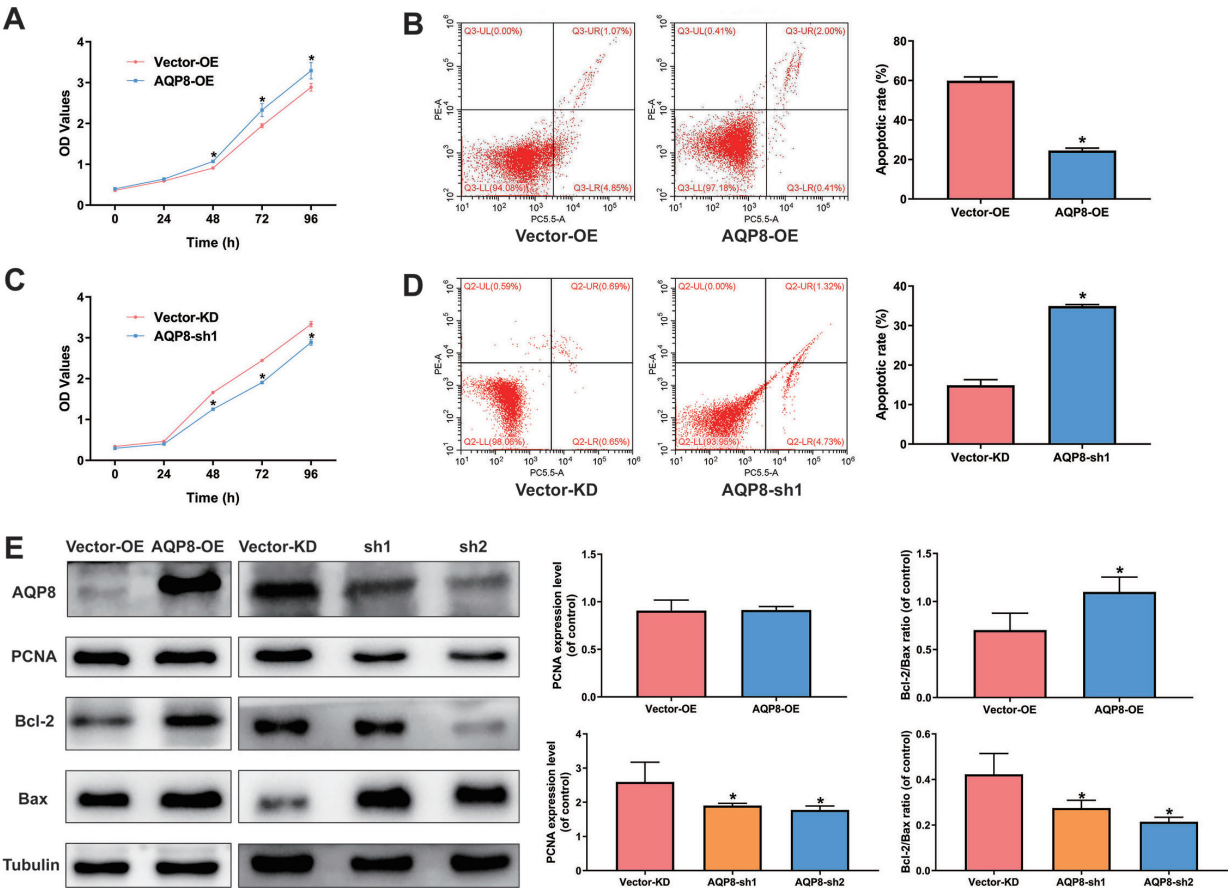

Fig. 3. AQP8 increases viability and inhibits apoptosis in SiHa cells. A and C, Cell viability of SiHa cells were determined by CCK-8 assay after AQP8 overexpression or knockdown for 0, 24, 48, 72, and 96 h. B and D, Apoptosis of SiHa cells after AQP8 overexpression or knockdown for 48 h was evaluated by flow cytometry assay. The data were shown as mean ± standard error of each group. Each experiment was performed three times. $^*P < 0.05$ vs vector group. E, Expression of PCNA, Bcl-2, and Bax proteins in SiHa cells after AQP8 overexpression or knockdown were identified by western blot analysis. The data shown were the ratios of the PCNA/Tubulin or Bcl-2/Bax. The data were shown as the means ± standard error of each group. Each experiment was performed three times. $^*P < 0.05$ vs vector group.

cer cells. It was obvious that the invaded and migrated cell numbers developed from SiHa cells with AQP8 overexpression were remarkably more than cells from control groups (Fig. 4A and B; $P < 0.05$). In contrast, knockdown of AQP8 in SiHa cells exerted a significant reduction in invasive and migratory abilities, as compared to control groups (Fig. 4C and D; $P < 0.05$).

To further investigated potential mechanisms of AQP8 in regulating migration of SiHa cells, levels of migration-related proteins Ezrin and Fascin, were evaluated by western blot analysis. Nevertheless, either AQP8 overexpression or AQP8 knockdown did not alter the level of Ezrin and Fascin proteins (Fig. 5A and B; $P > 0.05$). Altogether, these results indicate that AQP8 could enhance metastatic capabilities of cervical cancer cells but not through modulating Ezrin and Fascin proteins.

3.4. AQP8 induces EMT in cervical cancer cells

To test whether the metastasis-promoting function of AQP8 in SiHa cells was due to its ability to modulate EMT process, western blot analysis was employed again to detect several classic EMT-specific markers level, including E-cadherin, N-cadherin and vimentin. Figure 6 revealed that AQP8 overexpression significantly suppressed E-cadherin expression and elevated N-cadherin and vimentin (Fig. 6A; $P < 0.05$). Conversely, the EMT process was reversed via AQP8 knockdown in SiHa cells, as manifested by upregulation of E-cadherin and downregulation of N-cadherin and vimentin (Fig. 6B; $P < 0.05$). Collectively, these data suggest that AQP8, through inducing EMT process, promotes metastasis in cervical cancer cells.

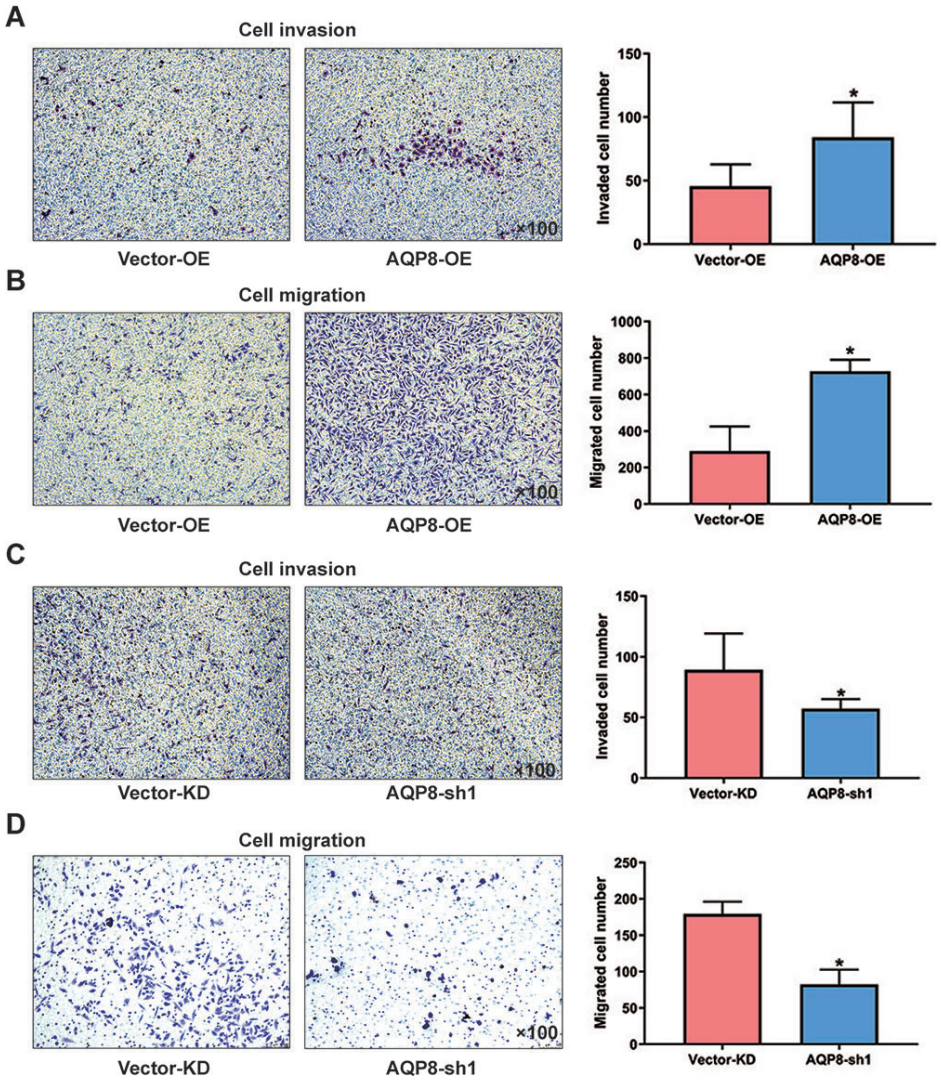

Fig. 4. AQP8 promotes invasion and migration in SiHa cells. A and B, Invaded and migrated SiHa cells with AQP8 overexpression were detected by transwell assays. C and D, Invaded and migrated SiHa cells with AQP8 knockdown were detected by transwell assays. Representative images for cell invasion and migration on SiHa cells were observed under a microscopy (magnification, 100 ×). The data were shown as mean ± standard error of each group. Each experiment was performed three times. $^{*}P < 0.05$ vs vector group.

4. Discussion

Patterns of upregulation of specific classes of AQPs have been repeated for clusters of cancers [3,18,19]. In contrast to the common AQPs subtypes such as AQP1, AQP3, AQP5, and AQP9, AQP8 seems to serve as a tumor suppressor in human colorectal cancer [10]. However, the role of AQP8 in most other types of human cancers such as cervical cancer is poorly understood. Herein, we thoroughly investigated the function of AQP8 in viability, apoptosis, invasion, migration and EMT in cervical cancer cells.

In the current study, IHC analysis first revealed that AQP8 staining was strong positive in cervical cancer specimens but weak positive in adjacent normal cervical tissues. High expression level of AQP8 was further confirmed in different cervical cancer cell lines by western blot analysis. SiHa cells showed a moderate expression pattern of AQP8, as compared with other cell lines. Thus, SiHa cells was constructed with AQP8 overexpression and knockdown in the following experiments.

Due to various tumorigenic factors, a loss of normal cell growth at the genetic level is frequently occurred in tumor development. A growing body of evidence have

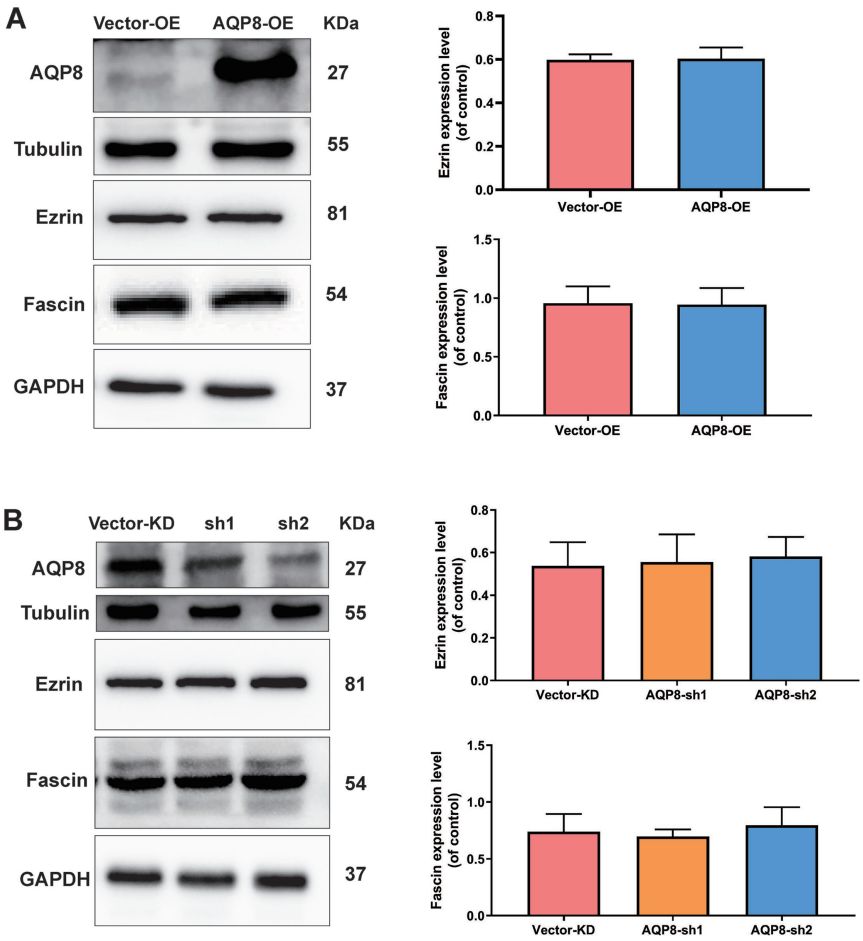

Fig. 5. AQP8 does not alter the expression level of Ezrin and Fascin proteins in SiHa cells. A, Expression of Ezrin and Fascin in SiHa cells after AQP8 overexpression were identified by western blot analysis. B, Expression of Ezrin and Fascin in SiHa cells after AQP8 knockdown were identified by western blot analysis. The data shown were the ratios of the Ezrin/GAPDH or Fascin/GAPDH. The data were shown as the means ± standard error of each group. Each experiment was performed three times. $^{*}P < 0.05$ vs vector group.

indicated that AQPs play a vital role in cell proliferation by different mechanisms [20]. Our study revealed that AQP8 upregulation significantly enhanced viability and inhibited apoptosis in SiHa cells, while these effects were reversed by AQP8 knockdown. Moreover, western blot analysis confirmed the pro-growth and anti-apoptosis function of AQP8 in SiHa cells. Specifically, AQP8 upregulated the expression of proliferation-related protein PCNA and reduced the Bcl-2/Bax ratio that was closely related to anti-apoptosis. Indeed, tumor growth relies on various metabolic processes including water molecules that can be specifically transported by AQPs [4,21]. However, it is reported that the AQPs-mediated cell growth is not completely dependent on their water permeability. In this scenario, impairment of AQP8 permeability to H_2O_2 by stress is followed by growth arrest and death in HeLa cells [22]. Marchissio et al. have also showed that mitochondrial AQP8 knockdown causes loss of viability in hepatoma HepG2 cells through inhibiting mitochondrial H_2O_2 release [23]. These findings indicate both permeability to various molecules and localization of AQP8 in cells may provide a pivotal role in tumorigenesis.

Metastasis is the most frequent cause of death for cancerous patients [24]. Studies from the last two decades have demonstrated AQPs-facilitated rapid osmolality and cell volume changes are indispensable for cancer cell migration [25–28]. Specifically, this change may involve water influx into lamellipodia at the front edge of migrating cells, which is triggered by AQPs [18]. Our results confirmed the function of AQP8 in promoting invasion and migration of SiHa cells, which was impaired by AQP8 knockdown. Interestingly, western blot analysis showed no alterations in

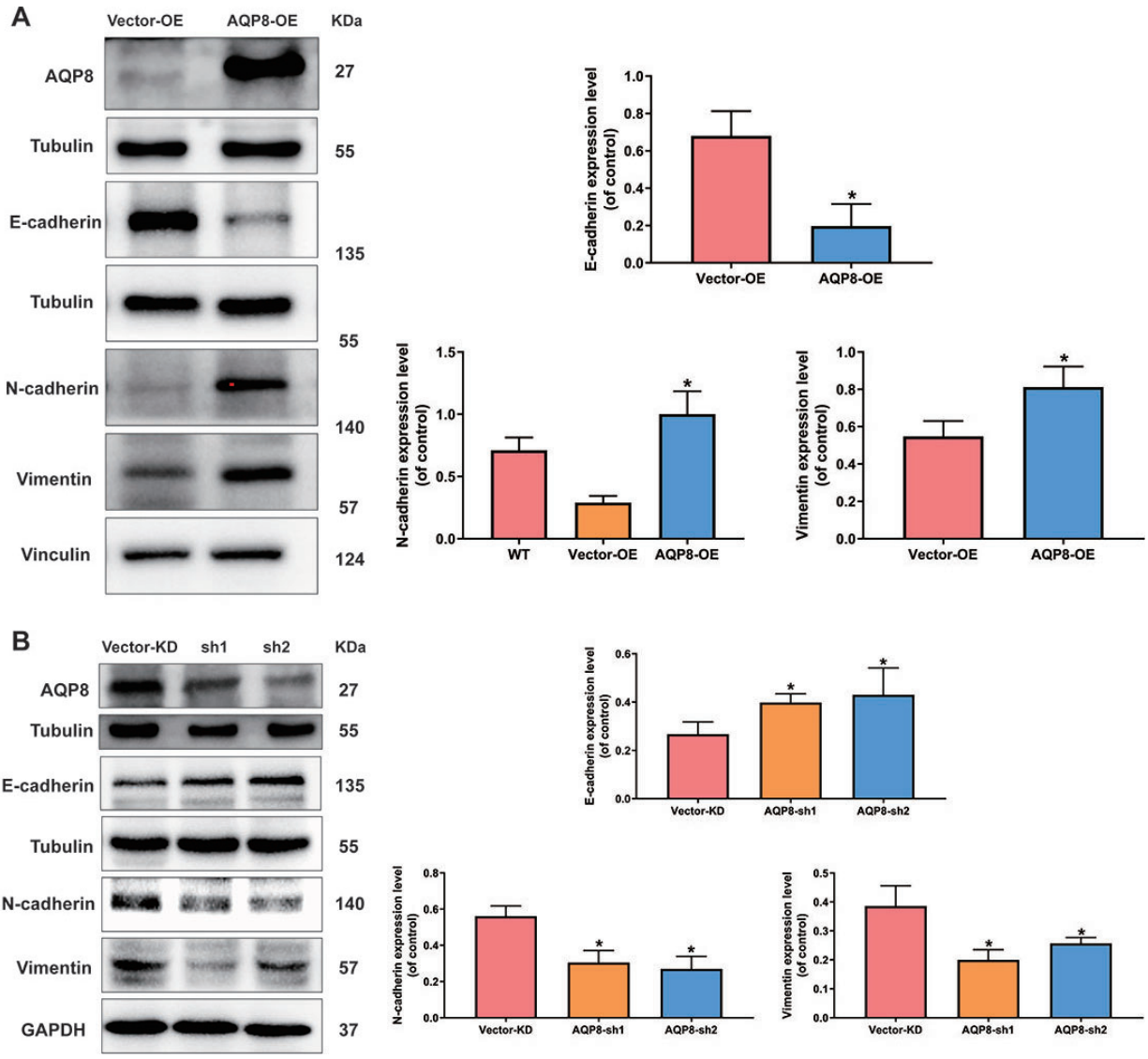

Fig. 6. AQP8 induces expression of EMT-related markers in SiHa cells. A, Expression of E-cadherin, N-cadherin and vimentin in SiHa cells after AQP8 overexpression were identified by western blot analysis. B, Expression of E-cadherin, N-cadherin and Vimentin in SiHa cells after AQP8 knockdown were identified by western blot analysis. The data shown were the ratios of the E-cadherin/Tubulin, N-cadherin/Vinculin, N-cadherin/GAPDH, Vimentin/Vinculin or Vimentin/GAPDH. The data were shown as the means ± standard error of each group. Each experiment was performed three times. $^{*}P < 0.05$ vs vector group.

Ezrin and Fascin proteins, irrespective of AQP8 upregulation or downregulation. This finding indicated that these molecules did not contribute to AQP8-dependent SiHa cells migration.

EMT process is an embryonic program that loosens cell-cell adherence complexes and endows cells with enhanced metastatic properties [29]. Notably, EMT is critically involved in cervical cancer progression and cancer cells that undergo EMT are more aggressive and invasive [16]. Herein, levels of EMT-related central markers were detected in the present study, including E-cadherin, N-cadherin and vimentin. Results found that expression of the epithelial marker E-cadherin was significantly reduced after AQP8 overexpression and rescued by AQP8 knockdown in SiHa cells. Moreover, the two mesenchymal-specific markers N-cadherin and vimentin were upregulated when AQP8 was overexpressed and downregulated after AQP8 knockdown in SiHa cells. These results suggested that AQP8-activated EMT process was a novel hallmark that led to invasion

and migration in SiHa cells. However, it is unknown whether a crosstalk exists between water/ion channel functions of AQP8 and EMT process.

5. Conclusion

In conclusion, this study found that AQP-8 may serve as a potential marker for cervical cancer progression. Furthermore, these is a need for new experiments designed specifically to address more potential mechanisms under the AQP8-mediated cervical cancer tumorigenesis.

Abbreviation

7-AAD, 7-amino-actinomycin D; ANOVA, analysis of variance; AQP8, aquaporin-8; AQP8-KD, AQP8 knockdown; AQP8-OE, AQP8 overexpression; AQPs, aquaporins; ATCC, American Type Culture Collection; BCA, bicinchoninic acid; CCK-8, cell counting kit-8; DMEM, Dulbecco's Modified Eagle Medium; ECL, efficient chemiluminescence; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; FIGO, International Federation of Gynecology and Obstetrics; FITC, fluorescein isothiocyanate isomer; HPV, human papilloma virus; HRP, horseradish peroxidase; IHC, immunohistochemistry; PBS, phosphate buffer saline; PE, phycoerythrin; PI, propidium iodide; PVDF, polyvinylidene fluoride; RIPA, radioimmunoprecipitation assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of mean; shRNA, short hairpin RNA; WT, wild type.

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Author contributions

Conception: WBL, YZS, JAZ, and XQZ.
Interpretation or analysis of data: WBL, YZS, CYP, and JHY.
Preparation of the manuscript: WBL and YZS.
Revision for important intellectual content: JAZ and XQZ.
Supervision: XQZ.

Conflict of interest

None.

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