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ORIGINAL ARTICLE

Anti-cancer effect of Aquaporin 5 silencing in colorectal cancer cells in association with inhibition of Wnt/β-catenin pathway

Wei Wang · Qing Li · Tao Yang · Dongsheng Li · Feng Ding · Hongzhi Sun · Guang Bai

Abstract Aquaporin 5 (AQP5) is a water channel protein that is over-expressed in many tumors. Elevated expression of AQP5 is associated with poor prognosis of colorectal cancer. Yet, whether AQP5 plays a role in epithelial–mesenchymal transition (EMT) of colorectal cancer has not been reported until now. Here we aim to investigate the function of AQP5 in the EMT process of colorectal cancer. We transfected HCT116 and SW480 cells with AQP5-specific shRNA and verified the knockdown efficiency through western blotting and real-time PCR. Afterwards, scratch wound healing assay, invasion assay, gelatin zymography, immunofluorescence staining and immunoblotting were performed to assess the effect of AQP5 silencing in these two cells. The ability of migration and invasion of colorectal cancer cells was significantly impaired after AQP5 silencing. Correspondingly, the activity and expression of Matrix Metallopeptidase (MMP)-2 and MMP-9 were reduced. Moreover, the expression levels of EMT-related factors were altered: E-cadherin, Tissue Inhibitor Of Metalloproteinases (TIMP)-1 and TIMP-2 were upregulated, whereas Vimentin, N-cadherin, Plasminogen Activator, Urokinase (uPA) and Snail were downregulated following knockdown of AQP5 in colorectal cancer cells. Furthermore, the expression of Wnt1 and β-catenin was markedly decreased after AQP5 knockdown. Interestingly, the alteration of EMT-related factors mediated by AQP5 knockdown could be reversed by upregulation of β-catenin. Taken together, silencing of AQP5 restrained the migration and invasion of colorectal cancer cells, and regulated the expression of EMT-related molecules in them by inhibiting Wnt/β-catenin pathway.

Keywords AQP5 · Colorectal cancer · EMT · Migration · Invasion · β-catenin

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Introduction

Colorectal cancer is a common malignant tumor with high morbidity and mortality worldwide (Torre et al. 2015). Multiple factors are associated with the development of colorectal cancer, such as genetics, polyposis and chronic inflammation (Cereda et al. 2016; Je et al. 2015; Rogler 2014). Despite the innovations in surgery and specific therapeutic agents, the 5-year overall survival of metastatic cases remains poor in patients with colorectal cancer (Brenner et al. 2014).

Therefore, understanding the molecular mechanisms underlying the metastasis of colorectal cancer and identifying novel potential therapeutic targets are urgent tasks for the improvement of colorectal cancer therapy.

Aquaporin 5 (AQP5) belongs to the family of membrane proteins termed aquaporins (AQPs) that facilitate the transport of water and some solutes through the biological membrane following an osmotic gradient (Takata et al. 2004). Physiologically, AQP5 plays an important role in the generation of saliva, tears and pulmonary secretions (Raina et al. 1995). Recently, increasing evidences have indicated a key role of AQP5 in tumor development and metastasis (Zhao et al. 2012). Overexpression of AQP5 was found in a variety of cancers, including non-small cell lung cancer, ovarian cancer, prostate cancer and cervical cancer (Chae et al. 2008; Pust et al. 2016; Tiwari et al. 2014; Zhang et al. 2012). Moreover, the expression level of AQP5 was negatively correlated with the prognosis of patients with multiple carcinomas (Huang et al. 2013; Jo et al. 2016; Lee et al. 2014; Li et al. 2014; Yan et al. 2014a). It has been demonstrated that AQP5 expression is elevated and proposed to be a novel biomarker that predicts poor clinical prognosis in colorectal cancer (Shan et al. 2014; Wang et al. 2012). However, the function and molecular mechanism of AQP5 in the epithelial–mesenchymal transition (EMT) process of colorectal cancer are unclear. EMT is a process that transforms epithelial cells into mesenchymal cells in order to obtain motility, invasiveness and stem-like properties (Profumo and Gandellini 2013). EMT is generally considered to be a leading factor in the early phase of cancer metastases (Seyfried and Huysentruyt 2013). The occurrence of distant metastases is mostly a lethal event during tumor progression, and liver metastasis is more frequent in colorectal cancer (Alberts et al. 2005; Folprecht 2016). The molecular mechanisms behind colorectal cancer EMT have been paid extensive attention in the recent studies in the hope of identifying novel therapeutic targets for effective cancer therapy.

In this study, we investigated the function and molecular mechanism of AQP5 knockdown in colorectal cancer cells. We provided in vitro evidence that knockdown of AQP5 inhibited the migration and invasion of colorectal cancer cells, and regulated the expression of EMT-related molecules by suppressing Wnt/β-catenin pathway.

Materials and methods

Cell lines and cell culture

Colorectal cancer cell lines HCT116 and SW480 were obtained from Shanghai Cell Biology Institute, Chinese Academy of Sciences. HCT116 cells were cultured in RPMI 1640 medium (Gibco, Erie County, NY, USA) containing 10% fetal calf serum (Hyclone, Logan, UT, USA), and SW480 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone). The cells were maintained in an incubator with constant humidified atmosphere and 5% CO_2 at 37 °C.

Construction of a stable cell line and transient transfection

Recombinant plasmid vectors of pRNA-H1.1-AQP5 shRNA and pRNA-H1.1-NC were constructed. Briefly, the interference fragment (AQP5 shRNA) and negative control (NC) fragment were designed as followed: AQP5 sh-RNA: 5′-GATCCCCGCGCTCA ACAACAACACAATTCAAGAGATTGTGTTGTT GTTG AGCGCTTTTT-3′; NC shRNA: 5′-GATCCC CTTCTCCGAACGTGTCACGTTT CAAGAGAAC GTGACACGTTCGGAGAATTTTT-3′. The fragments were cloned into the pRNA-H1.1 vector. Recombinant plasmids were transfected into HCT116 and SW480 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 h, stably transfected cell lines were screened by G418 at a final concentration of 200 μg/ml (lasting a week). The β-catenin S33Y plasmid was purchased from AddGene (Cambridge, MA, USA) and transiently transfected into colorectal cancer cells with Lipofectamine 2000.

Real-time PCR

Total RNA was extracted from cells using a RNApure Kit (BioTeke, Beijing, China) according to the manufacturer's instruction. Reverse transcription was carried out by using Super M-MLV Reverse Transcriptase (BioTeke). Real-time PCR was performed in 96-well plates and in 20 μL reaction system containing 10 μL SYBR GREEN mastermix (2X), 1 μL template cDNA, and 0.5 μL forward and reverse primers (10 μM of each primer concentration). The

primer sequences were as follows, AQP5 forward: 5′-TGGCTGCCATCCTTTACTTCTACC-3′, AQP5 reverse: 5′-CCCAGTCCTCGTCAGGCTCATA-3′; β-actin forward: 5′-CTTAGTTGCGTTACACCCTT TCTTG-3′, β-actin reverse: 5′-CTGTCACC TTCACCGTTCCAGTTT-3′. The optimal reaction condition was set as follows: 10 min at 95 °C, then 40 cycles of 10 s at 95 °C, 20 s at 60 °C, and 30 s at 72 °C, followed by 5 min at 4 °C. Quantitative analysis was carried out by ExicyclerTM 96 (BIO-NEER, Daejeon, Korea) quantitative fluorescence instrument. The relative levels of the target mRNAs were calculated by normalizing the target to the respective reference (β-actin) based on the $2^{-\Delta\Delta Ct}$ method.

Immunoblotting and antibodies

Cells were harvested and lysed on ice with lysis buffer containing protease inhibitors. Proteins from the cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto Polyvinylidene Fluoride (PVDF) membranes, and immunoblotted respectively with antibodies against AQP5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-514022), β-catenin (BOSTER Biological Technology, Pleasanton, CA, USA, BA0426), MMP2 (BOSTER, BA0569), MMP9 (BOSTER, BA0573), E-cadherin (BOSTER, BA0474), Vimentin (Bioss Antibodies, Woburn, MA, USA, bs-8533R), N-cadherin (BOSTER, BA0673), uPA (Bioss, bs-1927R), TIMP-1 (Bioss, bs-0415R), TIMP-2 (Bioss, bs-10395R), Snail (Bioss, bs-1371R), Wnt1 (BOSTER, BA3158-2), β-actin (Santa Cruz Biotechnology, sc-47778). The target proteins were visualized using the enhanced chemiluminescence system (WD-9413B, Beijing, China) according to the manufacturer's instruction. The target bands were analyzed by densitometry with the Gel-Pro Analyzer software (Media Cybernetics, Bethesda, MD, USA) using β-actin as the internal control.

Scratch wound healing assay

Cell migration was evaluated by scratch wound healing assay. In brief, cells were seeded in six-well plates. When the cells were cultured to full confluence, complete growth medium was replaced with serum-free medium and the cells were treated with 1 µg/ml Mitomycin C (Sigma, St. Louis, MO, USA) for 1 h. Subsequently, a scratch was created to the cell monolayer by scraping a straight line against the bottom of the culture dish with a pipette tip. The wound gaps were imaged by a phase-contrast microscope at 0, 12 and 24 h after scratching. The gap distances were measured, and the migration rate was calculated accordingly.

Invasion assay

Transwell chambers (Corning Inc., Corning, NY, USA) were used to perform cell invasion assay. Briefly, the cells were resuspended in serum-free medium and seeded into the chamber, the bottom membrane of which was coated with the mixture of serum-free medium and Matrigel (BD, Franklin Lakes, New Jersey, USA) at a ratio of 3:1. Twenty-four hour later, the cells invaded to the lower surface of the membrane were stained with 0.5% crystal violet, and the cell numbers were counted from five different fields of the surface.

Gelatin zymography

The activity of MMP-9 and MMP-2 was detected by gelatin zymography. Briefly, protein samples were separated by 10% SDS–PAGE (containing 0.1% gelatin). The activity of MMP-2 and MMP9 was restored in a buffer system containing divalent metal ions, and gelatin was hydrolyzed. Thereafter, the gel was stained with Coomassie Brilliant blue, and then decolorized. The un-dyed bands on the gel indicated gelatinolytic activity bands.

Immunofluorescence staining

The expression of E-cadherin and Snail was detected by immunofluorescence staining. The cells that were cultured on glass slides placed in 6-well plates were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% TritonX-100 for 30 min, blocked with goat serum for 15 min at room temperature, and incubated with primary antibodies against E-cadherin or Snail overnight at 4 °C. Thereafter, the cells were incubated with the goat anti-rabbit Cy3 fluorescently labeled secondary antibody (Beyotime, Beijing, China) for 60 min at room temperature, and then briefly stained with DAPI. The expression of E-cadherin and Snail was detected using a BX53 fluorescence microscope (OLYMPUS, Tokyo, Japan).

Fig. 1 Knockdown of AQP5 in HCT116 and SW480 cells. **a** The expression of AQP5 protein was assessed by western-blot assay after knockdown of AQP5, the bands were semi-quantified by densitometry, normalized to β-actin expression and expressed as fold change compared with the Mock group. **b** The expression of AQP5 mRNA was measured by real-time PCR after silencing AQP5. Data were presented as the mean ± SD. $**p < 0.01$ and $***p < 0.001$
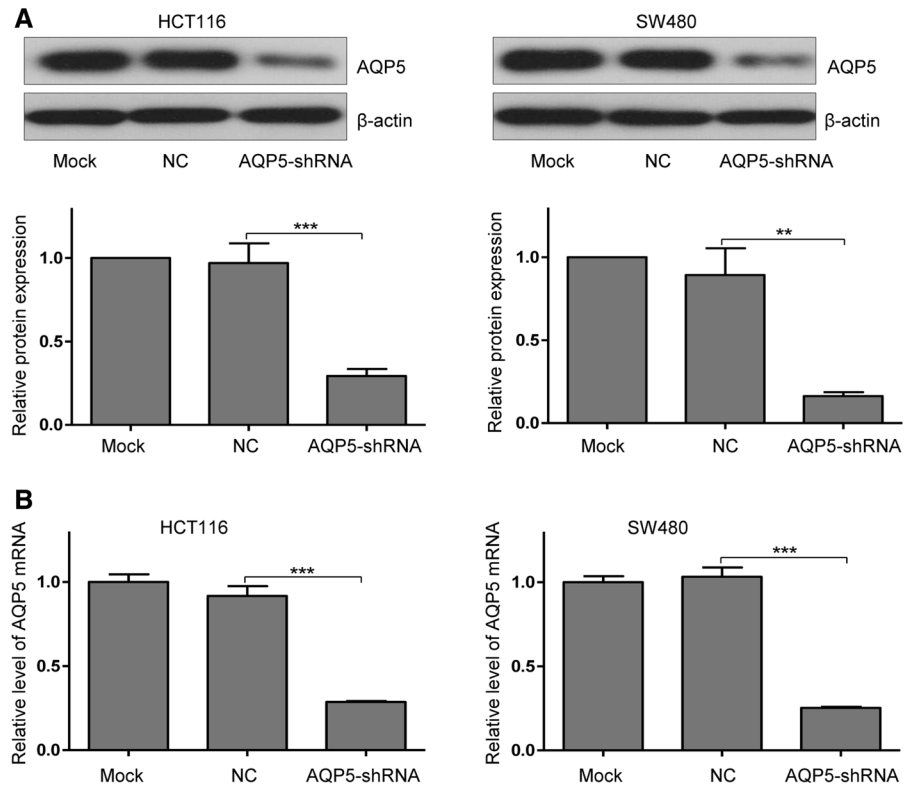
Statistical analysis

Each experiment was repeated three times. Student's t-test was used to compare the data between two independent groups. Statistical analyses were carried out using the SPSS 16.0 software. The results are presented as the mean ± SD. Differences with significance of $p < 0.05$, $p < 0.01$ and $p < 0.001$, denoted with "*", "**" and "***", respectively, in the figures, are considered statistically significant.

Results

Knockdown of AQP5 in colorectal cancer cells

To investigate whether AQP5 is involved in colorectal cancer cells metastasis, we designed and synthesized an interfering shRNA of AQP5 and a control shRNA, and then cloned them into pRNA-H1.1 vector. Afterwards, we tested knockdown efficiency in the stably transfected cells by western blotting and real-time PCR. The expression of AQP5 was markedly reduced

after AQP5 silencing as compared to control HCT116 and SW480 cells (Fig. 1a, b).

Knockdown of AQP5 significantly restrained the ability of migration and invasion in colorectal cancer cells

Previous literatures have documented that AQP5 is overexpressed in colorectal cancer and related to clinical outcomes (Shan et al. 2014; Wang et al. 2012). However, the study on the biological function of AQP5 in colorectal cancer cells is still limited. As evidenced by in vitro scratch and transwell assays, the ability of migration and invasion was significantly suppressed in AQP5-silenced HCT116 and SW480 cells ($p < 0.01$; Fig. 2a, b).

Moreover, we detected the expression of MMP-2 and MMP-9 in HCT116 and SW480 cells with and without AQP5 silencing by western blot assay, and found that the expression of MMP-2 and MMP-9 was reduced after AQP5 knockdown ($p < 0.05$; Fig. 2c). We also detected the activity of MMP-2 and MMP-9 by gelatin zymography and found that their activities were reduced following knockdown of AQP5 (Fig. 2d), which was
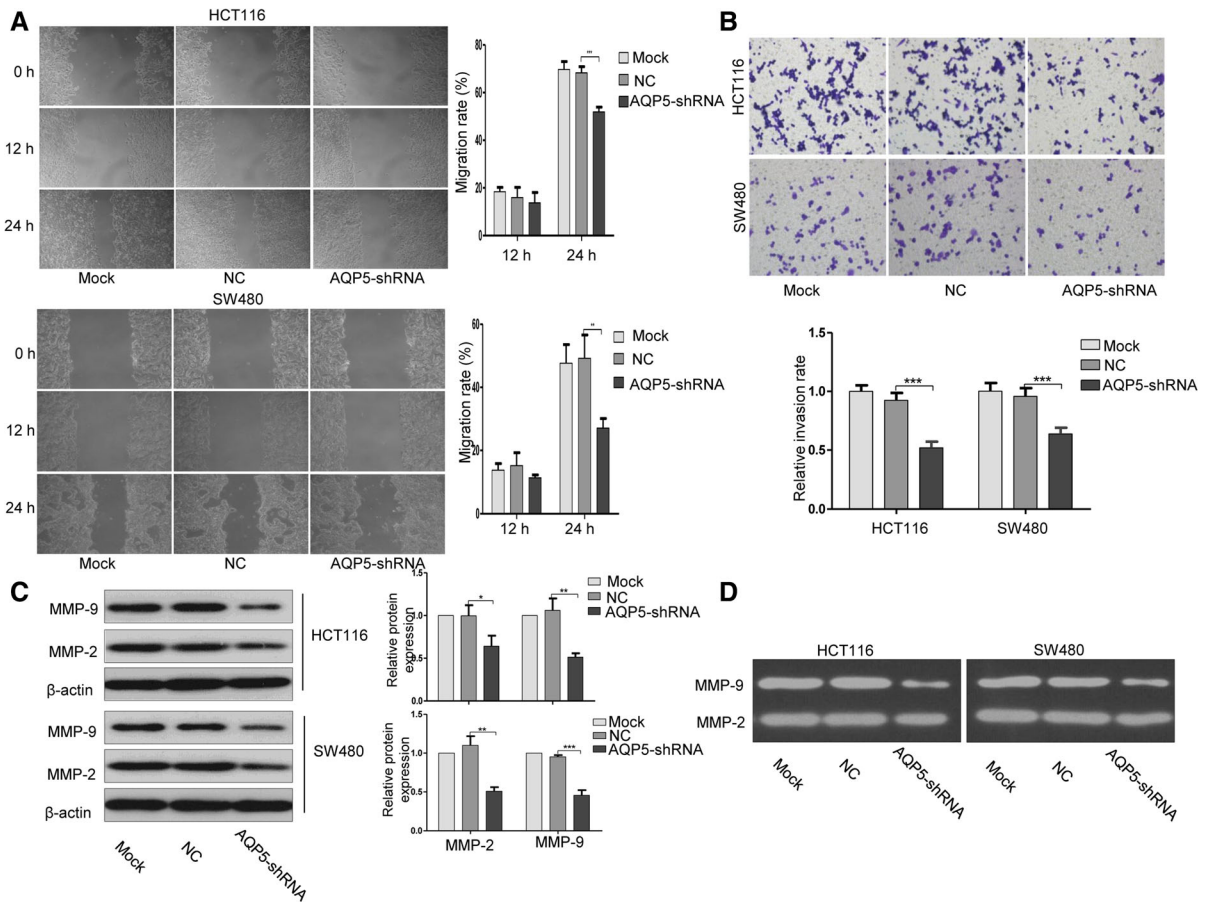
Fig. 2 Knockdown of AQP5 suppressed the ability of migration and invasion in HCT116 and SW480 cells. **a** The ability of migration was measured by scratch wound healing assay after knockdown of AQP5, the migration rate was determined. **b** The invasiveness of HCT116 and SW480 cells with and without AQP5 silencing was evaluated by transwell assay, invasive cells were counted and expressed as fold change compared to the Mock group. **c** The expression levels of MMP-2 and MMP-9 were detected by immunoblotting after knockdown of AQP5, the bands were semi-quantified by densitometry, normalized to β-actin expression and expressed as fold change compared with the Mock group. **d** The activities of MMP-2 and MMP-9 in AQP5-silenced and control cells were assessed by gelatin zymography. Data were presented as the mean ± SD. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$

consistent with western blot results. Collectively, these results demonstrated that AQP5 was required for the migration and invasion of colorectal cancer cells.

Knockdown of AQP5 regulated the expression of EMT-related molecules of colorectal cancer cells

Next, we investigated whether AQP5 was involved in the EMT process in colorectal cancer. EMT is a process that delivers epithelial cancer cells with mesenchymal properties including decreased adhesion and increased motility (Thiery et al. 2009).

Cell adherin is tightly controlled by cadherin family proteins including E-cadherin, N-cadherin and P-cadherin. Elevated expression of N- or P-cadherin at the expense of E-cadherin is a hallmark of EMT (Gheldof and Berx 2013). Here, western blot analysis revealed that the expression of Vimentin and N-cadherin was remarkably reduced in AQP5-silenced HCT116 and SW480 cells, whereas the expression of E-cadherin was upregulated (Fig. 3a). Similar changes were observed in immunofluorescence assay, the fluorescence intensity of E-cadherin was augmented after AQP5 silencing as compared to control cells
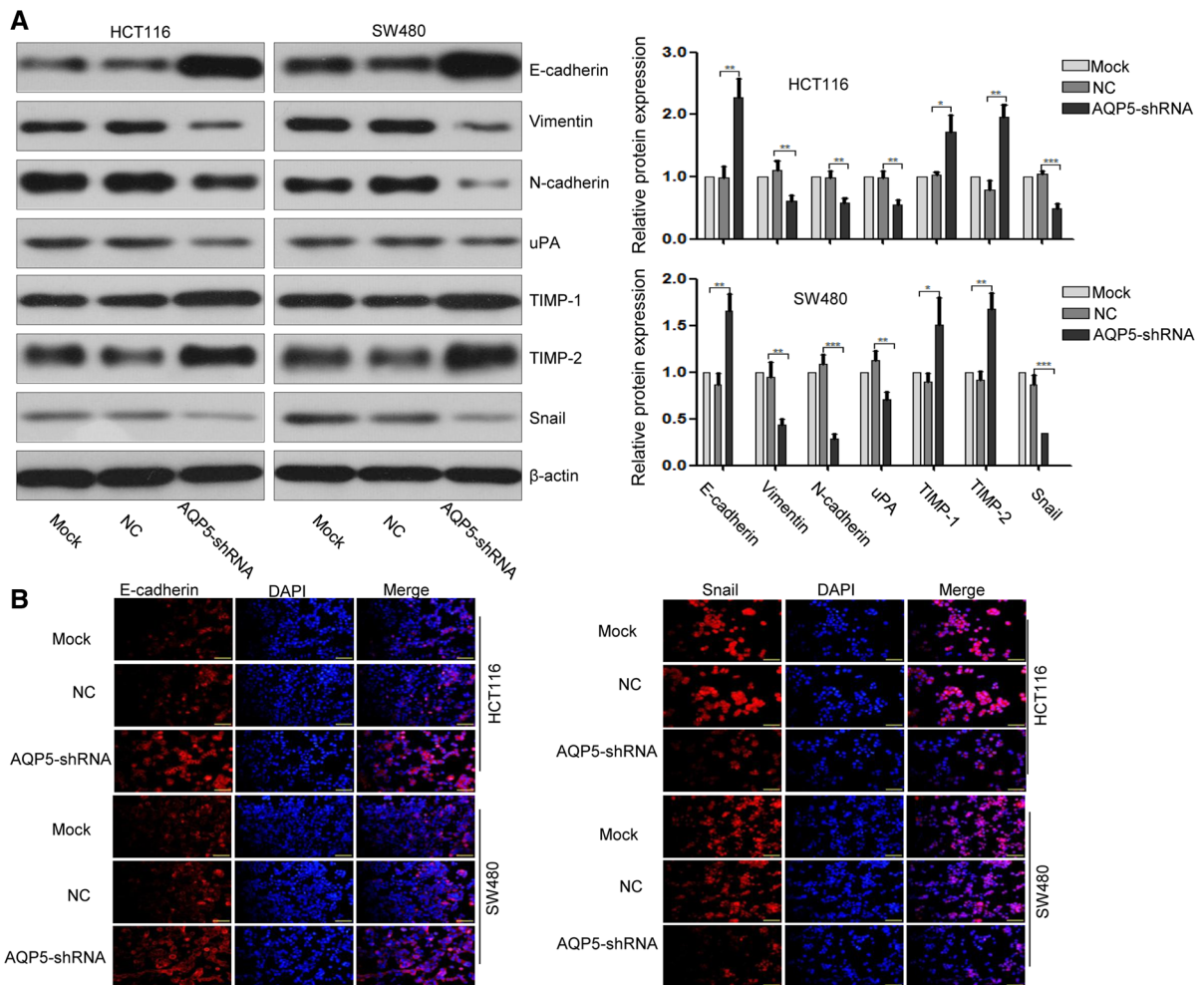
Fig. 3 Knockdown of AQP5 regulated the expression of EMT-related proteins in HCT116 and SW480 cells. **a** The expression levels of E-cadherin, Vimentin, N-cadherin, uPA, TIMP-1, TIMP-2 and Snail were measured by western-blot analysis after knockdown of AQP5, the bands were semi-quantified by densitometry, normalized to β-actin expression and expressed

as fold change compared with the Mock group. **b** Immunofluorescence staining was performed to detect the expression of E-cadherin and Snail in HCT116 and SW480 cells with and without AQP5 silencing. Data were presented as the mean ± SD. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$

(Fig. 3b). ZEB, Twist, Snail, and Slug are known to inhibit E-cadherin expression, and the induction of these transcription factors promotes cell migration, tissue morphogenesis and cancer development (Chan and Wang 2015). We found that the expression of Snail was significantly reduced after silencing of AQP5 in HCT116 and SW480 cells (Fig. 3a). Consistently, the fluorescence intensity of Snail was markedly weaker in AQP5-silenced HCT116 and SW480 cells than that in the control cells (Fig. 3b). Vimentin and Fibronectin are overexpressed in the cells undergoing EMT, and they facilitate epithelial cells to acquire a mesenchymal

shape and increased motility (Zeisberg and Neilson 2009). Here we found that silencing of AQP5 decreased the expression of Vimentin in HCT116 and SW480 cells compared with the control cells (Fig. 3a). The tissue inhibitors of matrix metalloproteinases (TIMPs) consist of TIMP-1, TIMP-2, TIMP-3, and TIMP-4, of which TIMP-1 and TIMP-2, the most extensively researched TIMPs, have been demonstrated to possess the capability of inhibiting the activity of MMPs. Here, knockdown of AQP5 in HCT116 and SW480 cells promoted the expression of TIMP-1 and TIMP-2 as compared with the control cells (Fig. 3a). Urokinase type
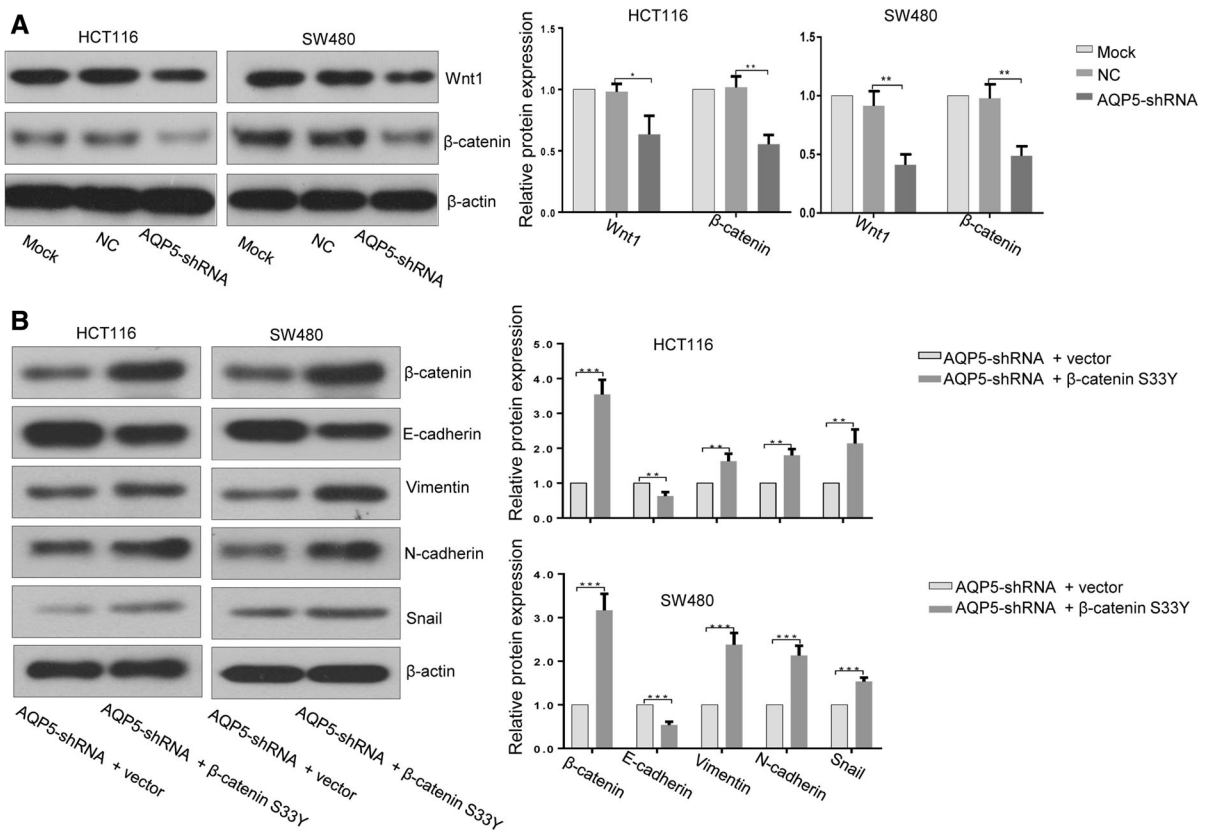
Fig. 4 Silencing of AQP5 inhibited Wnt/β-catenin signal transduction. **a** The expression levels of Wnt1 and β-catenin were detected by western blotting after silencing AQP5 in HCT116 and SW480 cells. **b** Following transfection of β-catenin S33Y in AQP5-silenced HCT116 and SW480 cells, the expression of β-catenin, E-cadherin, Vimentin, N-cadherin and Snail was determined by western blotting. The bands were semi-quantified by densitometry, normalized to β-actin expression and expressed as fold change compared with the Mock group. Data were presented as the mean ± SD. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

plasminogen activator (uPA) is a secreted serine protease that converts plasminogen to plasmin. Increased expression of uPA or its activator would retard the invasion of cancer cells (Wang 2001). We found that knockdown of AQP5 reduced the expression of uPA in HCT116 and SW480 cells compared with the control cells (Fig. 3a).

Knockdown of AQP5 downregulated Wnt/β-catenin signaling pathway

The Wnt/β-catenin pathway is one of the major signaling pathways that mediate EMT by transactivating EMT-related genes in malignant tumors (Armstrong 2011). We examined the expression levels of Wnt1 and total β-catenin in HCT116 and SW480 cells with and without AQP5 knowckdown, and found that

their expression were markedly decreased after knockdown of AQP5 (Fig. 4a). Moreover, following transfection of AQP5-silenced HCT116 and SW480 cells with β-catenin S33Y, the alteration of β-catenin, E-cadherin, Vimentin, N-cadherin and Snail caused by AQP5 silencing was reversed (Fig. 4b). Overall, these data suggested that AQP5 knockdown could regulate the expression levels of EMT-related molecules of colorectal cancer cells by targeting Wnt/β-catenin pathway.

Discussion

The EMT process is the molecular basis of cancer metastasis. During EMT, epithelial cells lose their cell polarity and cell–cell adhesion, and acquire migratory

and invasive capabilities (Kang and Massague 2004). It has been reported that EMT is associated with tumor cell metastasis in multiple tumors (Smith and Bhowmick 2016), and plays a key role in the progression and metastasis of colorectal cancer. Blocking EMT process can effectively inhibit metastasis of colorectal cancer (Li et al. 2016; Su et al. 2016). AQP5 is elevated in colorectal cancer cells, and its overexpression is correlated with poor prognosis of the patients with colorectal cancer (Shan et al. 2014). In this study, we demonstrated that knockdown of AQP5 inhibited migration and invasion of colorectal cancer cells, and induced expression alteration of EMT-related genes via downregulation of Wnt/β-catenin pathway.

In the present study, we knocked down AQP5 in HCT116 and SW480 cells and found that the ability of migration and invasion was decreased after AQP5 silencing. Meanwhile, the expression and activity of MMP-2 and MMP-9 were reduced in AQP5-silenced cells. EMT is the process during which epithelial cells dramatically alter their morphology and mobility while differentiating into mesenchymal cells (Profumo and Gandellini 2013). Matrix metalloproteinase (MMP) family proteins have been widely studied and demonstrated to be associated with tissue remodeling, organogenesis, and regulation of inflammation and cancer, MMPs are divided into six categories: collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10 and MMP-11), matrilysins (MMP-7 and MMP-26), membrane-type MMPs (MT-MMPs: MMP-14, -15, -16, -17, -24 and -25), and other MMPs that are not categorized into any of the above groups (MMP-12, -19, -20, -21, -23, -27 and -28). It has been reported that MMP-2 and MMP-9 are associated with the invasive capacity of tumor cells (Velinov et al. 2010). Abnormal expression of MMP-2 and MMP-9 has been demonstrated to be associated with cancer cell proliferation, invasion and EMT in various cancers (Chen et al. 2012; Liu et al. 2016). Taken together, our data suggest that AQP5 plays an important role in the migration and invasion as well as the regulation of MMP2 and MMP9 in colorectal cancer cells, thus it may be involved in EMT of colorectal cancer cells.

As previously reported (Chan and Wang 2015; Gheldof and Berx 2013; Wang 2001; Zeisberg and Neilson 2009), E-cadherin, Vimentin, N-cadherin, uPA, TIMP-1, TIMP-2, and Snail were closely related to EMT of cancer cells. In our study, the expression levels of these EMT-related proteins were altered after knockdown of AQP5 in a way that dampened the potential of EMT. These results implied that AQP5 could regulate the expression of EMT-related genes.

The EMT process is mediated via a number of signaling pathways, including nuclear factor-kappa B (NF-kB), transforming growth factor-β (TGF-β), Wnt, and Notch signaling pathways (Fender et al. 2015; Peng et al. 2015; Yan et al. 2014b). Wnt/β-catenin signal pathway plays a critical role in the EMT process, and aberrant Wnt/β-catenin signaling increases the malignancy of various human cancers (Gonzalez and Medici 2014; Gu et al. 2016). In this signaling pathway, β-catenin functions as a pivotal signaling mediator, and activation of Wnt/β-catenin signaling leads to nuclear translocation of β-catenin, which ultimately activates the transcription of downstream target genes, such as C-myc, Cyclin D1, and MMP-7 (Anastas and Moon 2013). β-catenin is degraded in the cytoplasm by the destruction complex in the absence of Wnt 1 engagement to the receptor. Activation of β-catenin is tightly regulated by glycogen synthase kinase-3 (GSK-3) and the phosphorylation status of β-catenin (Yost et al. 1996). Here, expression levels of Wnt1 and total β-catenin were significantly decreased after AQP5 silencing, and overexpression of β-catenin reversed the alteration of EMT-related factors mediated by AQP5 knockdown, suggesting that silencing of AQP5 regulated the expression of EMT-related factors through inhibiting Wnt/β-catenin pathway.

In summary, our study demonstrates that silencing of AQP5 inhibits the migration and invasion of colorectal cancer cells, which is accompanied with altered expression of EMT-related molecules and weakened Wnt/β-catenin signaling transduction. The cell phenotypic changes induced by AQP5 silencing are partly restored when Wnt/β-catenin pathway is reactivated. Our findings suggest that AQP5 may serve as a potential therapeutic target in the treatment of colorectal cancer.

Compliance with ethical standards

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