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Original Article

Knockdown of AQP-1 restricts hypoxia-induced proliferation in human intestinal microvascular endothelial cells: role of HIF-1α, VEGF and PI3K/Akt pathway

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Abstract: Aquaporin-1 (AQP-1) acts as a novel prognostic biomarker for advanced colorectal cancer. It has been noted that AQP-1, in conjunction with vascular endothelial growth factor (VEGF), exerts a synergistic pro-angiogenic effect on human intestinal microvascular endothelial cells ($HIMEC_s$) and accelerates the neoangiogenesis of colorectal cancer. AQP-1 is abundantly expressed in endothelial cells and closely linked to cell proliferation and tube formation. However, the functional significance of AQP-1 in $HIMEC_s$ remains elusive. Accumulating evidence has shown the direct role of AQP-1 in cell proliferation that contributes to several main pathological events in intestinal tract, such as bowel inflammation and tumor angiogenesis. We therefore investigated the role of AQP-1 in HIMEC proliferation process induced by cobalt chloride ($CoCl_2$). The present study aimed to examine these actions of AQP-1, we investigated the effects on cell viability and proliferation, were evaluated by CCK8 assay and BrdU assay in vitro. The expression levels of hypoxia-inducible factor (HIF)-1α and VEGF were determined by western blot or ELISA analysis in the $HIMEC_s$. The effects of AQP-1 on the VEGF receptor 2 (VEGFR2) and the phosphoinositide 3-kinase (PI3K)/Akt signaling pathways in $HIMEC_s$ treated with $CoCl_2$ by western blot analysis. We found that $CoCl_2$-induced cell proliferation was parallel to a significant increase in endogenous AQP-1 protein expression. Silencing AQP-1 with small interfering RNA significantly inhibited $CoCl_2$-induced HIMEC viability and proliferation. Moreover, knockdown AQP-1 dramatically attenuated $CoCl_2$-induced increases of HIF-1α and VEGF expression levels, while decreased the activation of VEGFR2 phosphorylation through the PI3K/Akt pathway in the $HIMEC_s$. Therefore, our results demonstrated that knockdown AQP-1 restricts hypoxia-induced proliferation in $HIMEC_s$ through HIF-1α, VEGF and PI3K-dependent pathway, suggesting that inhibition of AQP-1 may be a novel therapeutic approach for colorectal tumor angiogenesis.

Keywords: AQP-1, proliferation, hypoxia, HIF-1α, VEGF, PI3K/Akt pathway

Introduction

Colorectal carcinoma is the second leading cause of cancer death in the world. Recent advances in cancer biology have defined a critical role for the microvascular endothelium and angiogenesis in tumor progression and metastasis [1]. Tumor angiogenesis, a hallmark of cancer phenotype, is the proliferation of a network of blood vessels from a pre-existing mature vasculature that predominates over antiangiogenic factors resulting in tumor vasculature growth [2]. The tumor microenvironment is characterized by hypoxia. The discrepancy between rapid tumor proliferation and insufficient blood supply leads to low oxygen partial pressure (pO_2) in tumors [3, 4].

Hypoxia-inducible transcription factor-1 alpha (HIF-1α), as a crucial regulator of the physiological and pathophysiological responses to hypoxia, induces the expression of a broad genetic

program, including angiogenic genes such as vascular endothelial growth factor (VEGF), by binding on the appropriate HIF-responsive elements (HREs) present on their promoter region [5]. Of all the known pro-angiogenic molecules, VEGF is the most important mediator that involves in blood vessel development as well as endothelial cell proliferation, survival, migration and vascular permeability, playing a central role in angiogenesis and neovascularization [6]. VEGF receptor 2 (VEGFR2) is an important receptor that transduces hypoxia-activated signaling in endothelial cells. The activation of VEGFR2 leads to the phosphorylation of specific downstream signal transduction mediators. The PI3K-dependent pathway is a central mediator of VEGF-driven angiogenesis [7]. Specific signaling cascades in gut endothelium associated with tumor angiogenesis have not been defined.

Aquaporins (AQPs), a family of water-selective membrane channel proteins, play a key role in transcellular and transendothelial water movement, fluid transport, cell proliferation and migration [8]. Moreover, AQP dysregulation has been implicated in a variety of diseases, including brain edema and colorectal cancer [9, 10]. The first member of this family, AQP-1, is widely expressed in vascular endothelial cells throughout the body. It was reported that AQP-1 is selectively expressed in colorectal cancer and it may be involved in tumor angiogenesis [11]. An *in vitro* study demonstrated that AQP-1-mediated plasma membrane water permeability is crucial for colon cancer cell migration and may be associated with tumor invasion and metastasis [12]. However, mechanisms of AQP-1 in gut-specific microvascular endothelial cell survival and proliferation are presently undergoing characterization [13]. We therefore hypothesized that AQP-1 might contribute to human intestinal microvascular endothelial cells ($HIMEC_s$) proliferation induced by hypoxia. To test the hypothesis, we silenced AQP-1protein expression by using a small interfering RNA. Our results collectively demonstrate that hypoxia evokes HIMEC proliferation and an increase in AQP-1 protein expression, whereas siRNA-mediated knockdown of endogenous AQP-1 restricted, hypoxia-evoked proliferation in $HIMEC_s$ through the HIF-1α, VEGF and PI3K-dependent pathway.

Materials and methods

Cell culture

$HIMEC_S$ (CHI Scientific Inc., USA) were isolated and cultured as previously described [14]. Briefly, cells were maintained in Dulbecco's modified essential medium (DMEM) containing 15% fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin, 0.584 g/L L-glutamine (Gibco BRL, USA) and 100 mg/L β-endothelial cell growth factor (β-ECGF; R&D Systems Inc., USA) at 37°C in 5% CO_2 humidified atmosphere. $HIMEC_S$ were identified by their morphology and immunostaining with monoclonal antibody specific for endothelium VIII factor. HIMEC passages between 3 and 6 were used in the present study.

Small interfering RNA transfection

The sequences of siRNA against human AQP-1 mRNA (GeneBank Accession No. NM_000385, 5'-CTCTTCTGGAGGGCAGTGG-3') was synthesized by Qiagen (Germany). A scrambled RNA (Qiagen, Germany) was used as negative control. $HIMEC_s$ were transfected with AQP-1 and negative control siRNA oligonucleotides by using HiPerfect transfection reagent according to the manufacturer's instructions (Qiagen, Germany). Briefly, $HIMEC_s$ were seeded at 2×10^5 cells/ml in 6-well plate in 700 μl normal culture medium. The siRNA was diluted in 100 μl culture medium without serum (the final siRNA concentration was 20 nM), and then 12 μl HiPerFect Transfection Reagent was added to the diluted siRNA. The samples were incubated for 20 min at room temperature to form transfection complexes. The complexes were added to the cells and all were swirled gently to ensure uniform distribution. After 3 h, 1600 μl culture medium was added to each well, and the cells were incubated with transfection complexes under normal culture conditions for 48 h.

Cell viability assay

Cell viability was measured by Cell Counting Assay Kit-8 (CCK-8, Dojindo Molecular Technologies, Japan). Following overnight incubation, $HIMEC_s$ were transferred into serum-free medium (without β-ECGF) and were further incubated for 24 h. The medium was then replaced with growth medium and cells were

exposed to 150 μM $CoCl_2$ (Sigma-Aldrich, USA) for another 48 h, which is known to trigger chemical hypoxia in cells. Finally, CCK-8 (10 μl/well) was added for 2 h and absorbance was read at 450 nm by Bio-Tek microplate reader (Winooski, USA).

Cell proliferation assay

The HIMEC proliferation detection was measured using BrdU Cell Proliferation Assay kit (Millipore, USA). Briefly, $HIMEC_s$ were seeded in 96-well plates for 24 h and then treated with 150 μM $CoCl_2$ for another 24 h. Ultimately, BrdU reagent (60 μM) was added for 24 h and absorbance was read at 450 nm by Bio-Tek microplate reader.

Enzyme-linked immunosorbent assay (ELISA)

To determine VEGF secretion by $HIMEC_s$ under hypoxic conditions, culture medium was assayed for VEGF using the Quantikine human VEGF ELISA kit (R&D Systems, USA). According to the manufacturer's instructions, the protein level of VEGF in the cultured medium was measured at 450 nm with a microplate reader and normalized to the total protein concentration.

Western blot analysis

Western blot was performed as previously described [15]. Briefly, $HIMEC_s$ were rinsed with ice-cold PBS and lysed with RIPA lysis buffer containing protease inhibitor cocktail (Merk, Germany). The protein content was quantified with BCA kit. Protein was separated with 10% SDS-PAGE and was transformed to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were blocked in 5% non-fat dry milk diluted with TBST (in mM: Tris-HCl 20, NaCl 150, pH 7.5, 0.1% Tween 20) at room temperature for 1 h. The membranes were incubated with primary antibody: rabbit polyclonal anti-AQP-1, rabbit polyclonal anti-HIF-1α (Santa Cruz Biotechnology Inc., USA), and rabbit polyclonal anti-phosphorylated (p-)VEGFR2, anti-VEGFR2, anti-p-PI3K, anti-PI3K, anti-p-Akt, anti-Akt (Cell Signaling Technology, USA) at 4°C overnight, and then were incubated for 1 h with anti-rabbit IgG conjugated to horseradish peroxidase (Cell Signaling Technology, USA) at room temperature. Incubation with polyclonal rabbit α-tubulin antibody (Santa Cruz Biotechnology Inc., USA) or polyclonal mouse β-actin antibody (Cell Signaling Technology, USA) was performed as the loading sample control. Bands were detected with Pierce ECL western blotting substrate (Thermo Scientific, USA) and quantified with the computer-aided 1-D gel analysis system.

Statistical analyses

All data are expressed as mean ± SEM, and n value represents the number of independent experiments. Statistical analyses were performed using an unpaired two-tailed Student's t test or one way ANOVA followed by a post hoc comparison using the least significant difference test (SPSS 22.0). The correlation analyses were determined by the Pearson correlation test. Values of $P<0.05$ were considered statistically significant.

Results

Effect of $CoCl_2$ on AQP-1 expression in $HIMEC_s$

AQP-1 is widely expressed, and has been found in human intestinal microvascular endothelial cells, colonic microvascular endothelial cells, where AQP-1 has been related with regulation of angiogenesis [11, 13]. Expression of AQP-1 protein in $HIMEC_s$ was detected by immunoblotting with the use of a polyclonal antibody against rabbit AQP-1. The anti-AQP-1 antibody recognized a major band at 28 kDa (**Figure 1A**).

Then we detected whether the $CoCl_2$ could functionally affect endogenous AQP-1 expression. As shown in **Figure 1A**, treatment with $CoCl_2$ for 24 h significantly induced the expression of AQP-1 in a concentration-dependent manner. $CoCl_2$ at 150 μM significantly increased the AQP-1 protein expression compared to control group (**Figure 1A**). The proliferation effects of $CoCl_2$ on $HIMEC_s$ were examined by BrdU Cell Proliferation Assay as described in our previous studies. As shown in **Figure 1B**, **1C**, the proliferation rate (induced by $CoCl_2$ treatment) was positively correlated with AQP-1 protein expression.

Effects of AQP-1 on $CoCl_2$-induced cell viability and proliferation in $HIMEC_s$

To specifically reduce the endogenous AQP-1 protein expression, we performed gene-silencing experiments using transfection with siRNA

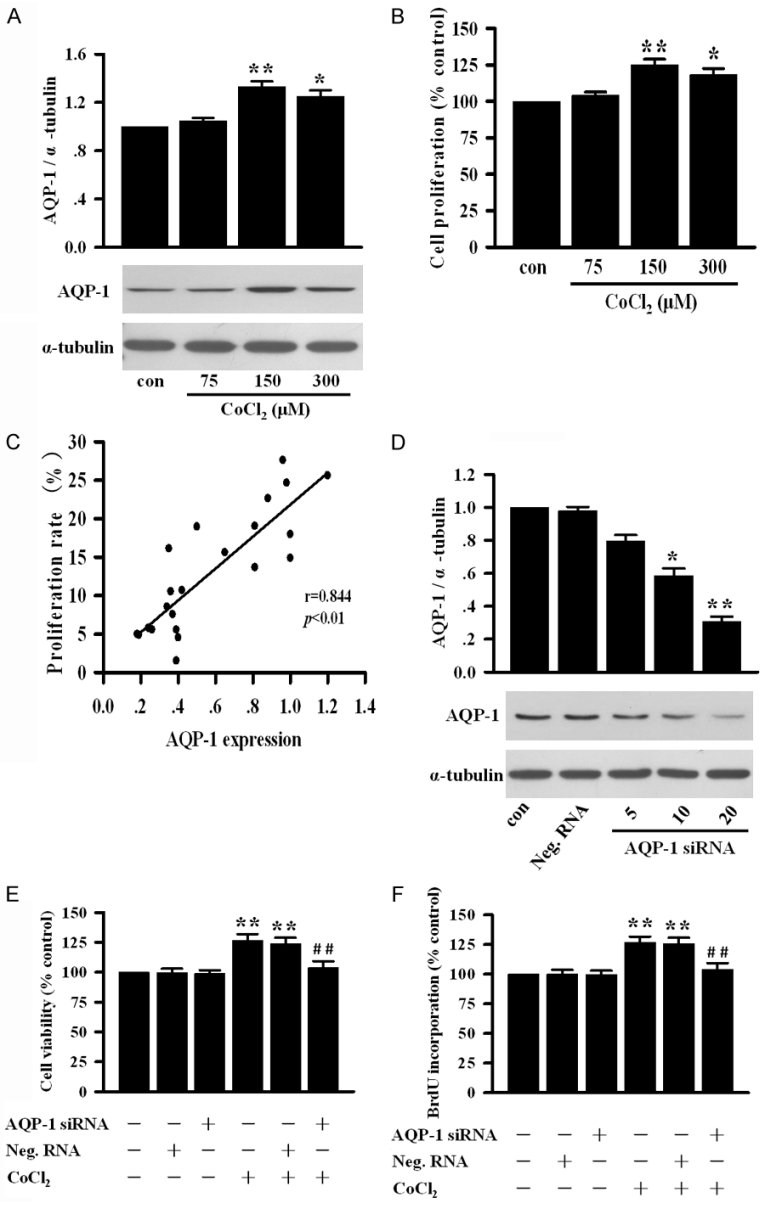

Figure 1. Treatment with $CoCl_2$ enhanced AQP-1 protein expression and the effects of AQP-1 on $CoCl_2$-induced HIMEC proliferation. A. Densitometric analysis showed that AQP-1 expression was enhanced after treatment with 75 μM, 150 μM and 300 μM $CoCl_2$ for 24 h, representative western blot images were shown in the bottom. B. Cell proliferation following 24 h of treatment with 75 μM, 150 μM and 300 μM $CoCl_2$ was assessed using the BrdU assay. C. Correlation between AQP-1 expression and proliferation rate in HIMECs was analyzed by Pearson method. D. Densitometric analysis showed infection with specific AQP-1 siRNA at multiplicity of infection (nM) of 5, 10 and 20 for 48 h significantly decreased endogenous AQP-1 expression, but infection with negative control siRNA (Neg. siRNA) did not significantly change AQP-1 expression. Representative western blot images were was shown in the bottom. E. Cell Counting Assay Kit-8 (CCK-8) results showed that treatment with $CoCl_2$ significantly increased cell viability, AQP-1 knockdown further decreased cell viability. F. DNA synthesis was assessed using BrdU incorporation. (n = 6 in each test; $^{*}P<0.05$, $^{**}P<0.01$ vs. control, $^{\#\#}P<0.01$ vs. $CoCl_2$ group).

specific for AQP-1 or negative control. AQP-1 specific siRNA (AQP-1 siRNA, 20 nM, 48 h) effectively reduced endogenous level of AQP-1 protein expression to ~70 % of the negative control group (Neg. RNA, 20 nM, **Figure 1D**), so in the following study, we used AQP-1 siRNA at 20 nM for 48 h.

To analyze if change in AQP-1 protein expression would affect $CoCl_2$-induced hypoxia in $HIMEC_s$, cell viability was evaluated by CCK-8 assay. $HIMEC_s$ were treated with 150 μM $CoCl_2$ for 48 h, the cell viability rate was significantly induced as compared with the $CoCl_2$-untreated group. Knockdown of AQP-1 reduced $CoCl_2$-induced cell viability rate (**Figure 1E**).

To determine the functional role of AQP-1 in $CoCl_2$-induced HIMEC proliferation, the proliferation rate was measured by BrdU Cell Proliferation Assay. $CoCl_2$ remarkably enhanced the proliferation rate of $HIMEC_s$, which could be significantly inhibited by AQP-1 siRNA (**Figure 1F**).

Effects of AQP-1 on $CoCl_2$-induced activation of VEGFR2 in $HIMEC_s$

It has been reported that the sensitization of VEGFR2 phosphorylation is a key signaling step during endothelial cell proliferation and tube formation. As shown in **Figure 2A**, $CoCl_2$-treatment significantly increased the phosphorylation of VEGFR2 in $HIMEC_s$. However, AQP-1 knockdown dramatically attenuated $CoCl_2$-induced increases

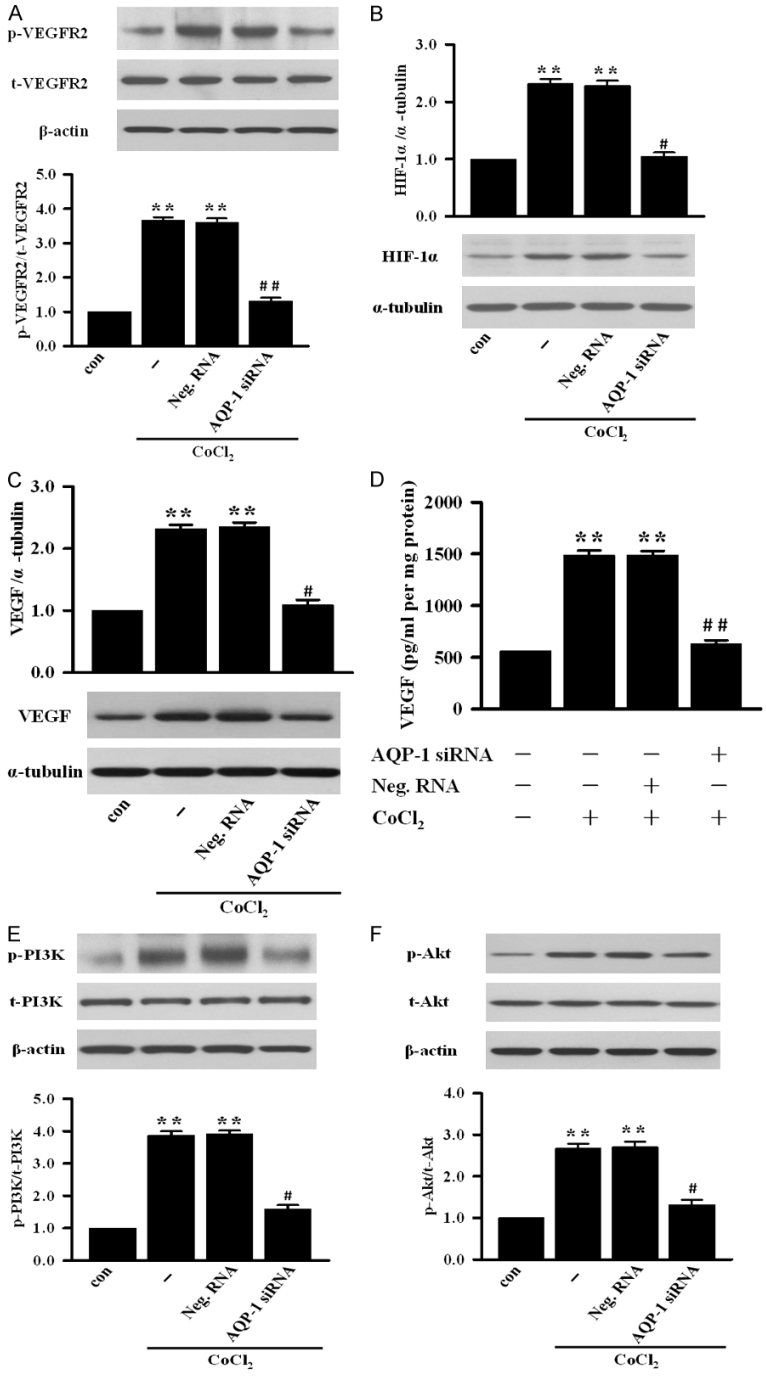

Figure 2. Mechanisms of AQP-1 on hypoxia-induced proliferation process in human intestinal microvascular endothelial cells (HIMECs). A-E. $CoCl_2$ (150 μM) induced expression of the phosphorylation of VEGFR2 (p-VEGFR2), hypoxia-inducible factor (HIF)-1α, vascular endothelial growth factor (VEGF), phosphoinositide 3-kinase (PI3K) and Akt from HIMECs transfected with negative siRNA (Neg. RNA) or AQP-1 siRNA. A. Knockdown AQP-1 inhibited p-VEGFR2 induced by $CoCl_2$ in HIMECs. Representative western blot images were was shown in the top. B, C. The HIF-1α and VEGF levels are increased by $CoCl_2$, and repressed by AQP-1 deficiency. Representative western blot images were was shown in the bottom. D. ELISA results showed that treatment with $CoCl_2$ significantly increased the secretion level of VEGF in the culture supernatants; AQP-1 knockdown further decreased the secretion level of VEGF. E, F. AQP-1 deletion restricted $CoCl_2$-induced PI3K/Akt signaling in HIMECs. Representative western blot images were was shown in the top. (n = 6 in each test; $^{**}P<0.01$ vs. control, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. $CoCl_2$ group).

of VEGFR2 phosphorylation, which indicated that AQP-1 exerted its angiogenic effect by directly targeting VEGFR2 (**Figure 2A**).

Effects of AQP-1 on $CoCl_2$-induced HIF-1α and VEGF expression in HIMEC$_s$

HIF-1α is a key regulator of cell proliferation during hypoxia, and VEGF is a major pro-angiogenic growth factor, which is mediated by HIF-1α. As shown in **Figure 2B-D**, the HIF-1α and VEGF expression was significantly increased by $CoCl_2$ stimulation, whereas dramatically attenuated by AQP-1 knockdown.

Effects of AQP-1 on $CoCl_2$-induced PI3K/Akt signaling pathway in HIMEC$_s$

To further investigate the mechanisms how AQP-1 modulates $CoCl_2$-evoked cell proliferation, we analyzed the phosphorylation of PI3K and Akt by western blot. PI3K/Akt pathway is an important role in endothelial cell proliferation, which functions upstream of HIF-1α and VEGF. As shown in **Figure 2E**, **2F**, the $CoCl_2$-treatment significantly enhanced PI3K and Akt phosphorylation, while knockdown AQP-1 abolished $CoCl_2$-induced activation of PI3K and Akt phosphorylation.

Discussion

Endothelial cells in the vicinity of tumors make an essential contribution to tumor growth

and metastasis [16]. The elucidation of the mechanisms of tumor angiogenesis will certainly provide more precise and effective anticancer therapies. Expression of several AQPs was found in a wide range of tumor cells in addition to the ubiquitous expression of AQP-1 in the micro vessels of solid tumors [17]. The aim of this study was to characterize the expression and function of AQP-1 water channel in hypoxia-induced HIMEC proliferation. We first used western blot to demonstrate endogenous AQP-1 protein was significantly enhanced by hypoxia in a concentration-dependent manner. Then we found that hypoxia-evoked HIMEC proliferation was accompanied by an increase in AQP-1 protein expression. Silencing of AQP-1 could alleviate hypoxia-evoked HIMEC proliferation, decrease cell viability rate, down-regulate HIF-1α and VEGF through PI3K-dependent pathway. These results provide the first and compelling evidence that AQP-1 is critically linked to hypoxia-induced proliferation in $HIMEC_s$.

AQPs are widely distributed in the digestive system. Since AQPs play a key role in water homeostasis by regulating cellular water transport, alterations in the AQP function have been implicated in neoplastic transformation, as well as physiologic changes in the digestive tract [18, 19]. According to specific AQP isoforms, AQP-1 is present in the endothelial cells of capillaries and small vessels as well as in the central lacteals in the intestines [13]. Several studies have suggested that this may be due to the involvement of AQP-1 in cell signaling, ion channel regulation, cell proliferation and differentiation as well as angiogenesis [20-22]. This series of processes may also be involved in the development and progression of in colorectal cancer. Therefore, several recent studies have focused on the clinical significance of AQP-1 expression for predicting the prognosis of colorectal cancer [23].

HIF-1α is essential for the microvascular endothelial cell proliferation progression, and the enhancing progression of tumor angiogenesis is dependent on its expression [24]. HIF-1α plays a key role in regulating cell transport and the secretion of numerous pro-angiogenic growth factors, such as VEGF [25]. Furthermore, the angiogenic effect of VEGF is mainly mediated by binding and activating the phosphorylation of its receptors especially VEGFR2. The VEGFR2 pathway acts as a positive mediating loop to further enhance cell proliferation and angiogenesis. Upon VEGFR2 activation, the downstream PI3K-dependent signaling incidents are initiated by its phosphorylation in endothelial cells [26, 27]. It has been indicated that PI3K/AKT activity markedly promoted hypoxia-induced HIF-1α expression and VEGF secretion, strongly supporting the notion that the induction of HIF-1α and VEGF is largely regulated by the PI3K/AKT pathway [28]. Accordingly, the present study provides a new insight into the effects of AQP-1 on this pathway in $HIMEC_s$.

In conclusion, our findings here demonstrate for the first time that AQP-1 in HIMEC cell proliferation, which may provide a functional explanation for the expression of AQP-1 in gut angiogenesis. Knockdown AQP-1 retards hypoxia-evoked proliferation process in $HIMEC_s$ by blocking the activation of HIF-1α and VEGF through the PI3K/Akt signaling pathway. In summary, downregulation expression of AQP-1 may be novel therapeutic approaches for treating colorectal cancer.

Disclosure of conflict of interest

None.

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