Aquaporin 5 promotes tumor migration and angiogenesis in non-small cell lung cancer cell line H1299

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Abstract. Non-small cell lung cancer (NSCLC) constitutes the majority of all lung-cancer cases. Aquaporin 5 (AQP5) may be involved in NSCLC by promoting lung-cancer initiation and progression. The present study aimed to determine the role of AQP5 in migration and angiogenesis using NSCLC cells and HUVECs. AQPs 1, 3, 4, 5, 8 and 9 were screened in the NSCLC cell line H1299, and the present results showed that AQP5 mRNA was upregulated compared with the other AQP genes. At the protein level, AQP5 was significantly increased in H1299 cells compared with 16HBE cells. AQP5 knockdown in H1299 cells significantly decreased cell migration compared with untransfected cells, as demonstrated by both Transwell and wound closure assays. The present study further investigated H1299 ability to promote HUVEC vascularisation. The supernatants of both transfected and untransfected H1299 cells were used as conditioned medium for HUVECs, and tube formation was measured. The supernatant of AQP5-downregulated cells exhibited significantly low tube formation potential compared with untransfected cells. Similarly, vascular endothelial growth factor was significantly increased in control cells (si-NC) compared with cells transfected with small interfering RNA targeting AQP5. The present study found that AQP5 downregulation significantly decreased the phosphorylation level of epidermal growth factor receptor and the activity of the ERK1/2 pathway. In summary, the present study suggested that AQP5 influenced migration and angiogenesis in NSCLCs *in vitro* and may potentially exhibit similar *in vivo* effects.

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Introduction

Globally, lung cancer is ranked as the second most prevalent malignancy among all types of cancer and is a common cause of cancer-associated mortalities (1). It has been reported that >80% of diagnosed lung-cancer cases are non-small cell lung cancers (NSCLCs) (2). Lung-cancer treatment is promising if the disease is detected at its early stage, and less beneficial at advanced stage, though a relatively high risk of recurrence occurs even after exposure to available potent treatment (3). NSCLC migration and invasion contribute to poor treatment outcome and lung-cancer mortality (4). NSCLC cells aggressively proliferate and invade adjacent tissues, cross the basement membrane and migrate to establish colonies in distant organs of the body via either the vascular system or the lymphatic system (5). With advancements in molecular biology tools and the biological processes identified in the past decades, the basic biology of tumors is now well understood (6). Several oncogenes have been identified, including passenger and driver genes, such as KRAS, ERBB2 and BRAF (7). Various oncogenes modulating cancer angiogenesis and metastasis have been identified in recent years, providing novel potential therapeutic targets such as aquaporin (AQP), which was investigated in the present study.

AQPs are water channels, and are ubiquitous integral membrane proteins that control extra- and intra-cellular fluid passage (8). AQPs play a regulatory role in human carcinogenesis (9). The expression of human AQP1 has been found to be unaffected in brain tumors, renal cell carcinoma, colon cancer and pancreatic cancer, whereas AQP5 expression was identified to be increased in lung, salivary glands and kidney cancer (10). In addition, AQP5 is increased in ovarian and cervical cancer, esophageal squamous cell carcinoma, and hepatocellular carcinoma; however, lymphocyte activation stimulates the transcription of AQP1, AQP3 and AQP5 (11-14). AQPs play an important functional role in cell migration in various cell types, including brain, pancreas and colon cancer cells (15,16). A previous study examining NSCLC revealed that AQP5 level is associated with the progression of lung cancer (4). AQP5 upregulation was also observed in adenocarcinoma cells, but it is relatively less expressed in non-mucinous bronchioloalveolar carcinoma (11). The H1299 cell line is a human NSCLC cell line, which is derived from the lymph

node and has been widely used to investigate a number of disease-associated mechanisms, such as tumor necrosis and cancer cells apoptosis (17,18). A previous study indicated that AQP5 activates the EGFR1/2 and ERK1/2 pathway, which regulates cancer cell migration and proliferation (19,20). In the present study, the influence of AQP5 on H1299 cell migration and regulation of angiogenesis was investigated, and the present study suggested that AQP5 downregulation significantly decreased both migration and angiogenesis regulation of NSCLC H1299 cells.

Materials and methods

Cell culture. The NSCLC cell lines H1299 and 16HBE were purchased from the American Type Culture Collection. Cells were cultured in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Cells were passaged 3-5 times before collection. HUVECs were obtained from The Dalian Medical University, and cultured in M199 medium (Gibco; Thermo Fisher Scientific, Inc.). Both cultures were incubated at 37˚C with 5% CO_2.

Total mRNA extraction. H1299 cells were grown until they reached 80-90% confluency and were washed twice with sterile PBS and harvested using RNAiso Plus (Takara Bio, Inc.) according to the manufacturer's protocol. Cells were resuspended in RNAiso Plus, put on ice for 5 min and centrifuged at 4˚C at 12,000 x g for 15 min. The supernatant was added to an equal volume of chloroform, incubated on ice for 10 min and centrifuged at 4˚C at 12,000 x g. In total, 550 μl isopropanol was added to the aqueous phase, and the solution was mixed gently and centrifuged at 12,000 x g for 15 min at 4˚C. The sediment was washed in 1 ml ethanol (75%) in diethyl pyrocarbonate (DEPC)-treated H_2O, and centrifuged for 10 min at 4˚C at 12,000 x g. The pellet was air-dried, dissolved in 10 μl RNase-free H_2O, and the concentration was determined using a NanoDrop 2000 (Thermo Fisher Scientific, Inc.).

Reverse transcription PCR (RT-PCR). RNA (~1 μg) was converted to cDNA using the PrimeScript first strand cDNA Synthesis kit (Takara Bio, Inc.) according to the manufacturer's protocol. A mixture containing oligo dT primer, dNTP mixture (Takara Bio, Inc.), RNase-free ddH_2O and the extracted RNA was incubated at 65˚C for 5 min and immediately cooled on ice. The buffer 5X PrimeScript (Takara Bio, Inc.), RNase inhibitor, reverse transcriptase and RNase-free ddH_2O (Takara Bio, Inc.) were added to the previous mixture in a total volume of 20 μl, according to the manufacturer's protocol. The mixture was homogenized gently using a micro-centrifuge at 6,000 x g at room temperature for 5 min, and incubated for 60 min at 42˚C. The enzyme was inactivated by incubating the samples at 95˚C for 5 min. Subsequently, the samples were cooled on ice. PCR was performed using PCR premix (Takara Bio, Inc.) as follows: 30 cycles of 94˚C for 5 min, 94˚C for 30 sec, 56˚C for 30 sec, 72˚C for 1 min, 72˚C for 7 min and final hold at 12˚C, the PCR product was loaded in 2% agarose gel and visualized using EthidiumBromide, and GAPDH was used as an internal control to evaluate cDNA synthesis efficiency. The RT bands were determined by IMAGEJ software (version 1.46; National Insitutes of Health). The primers used in the present study were purchased from Takara Bio, Inc. (Table I).

Protein extraction. H1299 cells cultured to 100% confluence were scraped using ice-cold PBS and a cold plastic scraper and harvested following a centrifugation at 12,000 x g for 3 min at 4˚C. RIPA lysis buffer (Nanjing KeyGen Biotech Co., Ltd.) containing a proteinase inhibitor, phosphate inhibitor and PMSF, and cells were lysed according to the manufacturer's protocol. The mixture was then vortexed for 40 min at room temperature and centrifuged at 12,000 x g for 15 min at 4˚C. The supernatant was used as the total protein extract. Total protein concentration was measured using a bicinchoninic acid assay (Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's protocol. A standard absorbance curve was established using known concentrations of protein, and the protein concentration in the samples was extrapolated from the standard curve. Total protein (~20 μg) was used for SDS-PAGE and western blot analysis.

Western blot analysis. Total protein (~20 μg/lane) extracted from H1299 or 16HBE cells was loaded on 12% SDS-PAGE, transferred to Hybond-C nitrocellulose membranes (GE Healthcare Life Sciences), and blocked with 5% skimmed milk in 0.5% TBS-Tween 20 (TBST) at room temperature for 1 h. The membranes were incubated with rabbit anti-AQP5 (cat. no. ab78486), EGFR (cat. no. ab52894), p-EGFR (cat. no. ab5644), ERK (cat. no. ab54230) and p-ERK (cat. no. ab65142), (dilution, 1:1,000) and β-actin (dilution, 1:1,000; cat no. ab6276) (all from Abcam) at 4˚C overnight. After the membranes were washed three times with TBST, they were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (dilution 1:100, catalog no. ab205718; Abcam) for 1 h at room temperature. The protein was visualized using an enhanced chemiluminescence detection system (Bio-Rad Laboratories, Inc.). Band density was quantified using the Image Lab software (version 4.0; Bio-Rad Laboratories, Inc.).

AQP5 gene silencing. Small interfering (si)RNAs against AQP5, negative (NC) and positive (PC) controls were purchased from Shanghai GenePharma Co., Ltd. siRNA sequences are presented in Table II. The siRNAs were dissolved in 125 μl DEPC-treated water (Shanghai GenePharma Co., Ltd.) and transfected into H1299 cells at 70-90% confluence. According to the manufacturer's protocol, cells were transfected with 20 nmol of siRNA using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The effect of the transfection was confirmed by RT-PCR, western blot analysis and fluorescence microscopy 48 h after transfection.

Transwell migration assay. Transwell migration assay was performed to measure tumor cell migration. Transwell inserts (8-μm pores; Corning, Inc.) in 24-well plates were used. H1299 cells (~100 μl; 1x10^5 cells/ml) in 100 μl serum-free RPMI, were added to the upper chambers, and the lower chambers were filled with 350 μl RPMI medium containing 20% FBS as the attracting agent. After 24 h of incubation at

Table I. Primer sequences used for cDNA amplification.

Gene	Sequences (5'→3')
AQP1	F: TCTGGAGGCTGTGGTGGCT
	R: AAGTGAGTTCTCGAGCAGGGA
AQP3	F: AGCGAGTTTGGATGAGCAGCAGA
	R: AAGGAGACGGCAAGCAGGGTGTA
AQP4	F: ATGGTGGCTTTCAAAGGGGT
	R: GATGGGCCCAACCCAATATAT
AQP5	F: TGGGTCTTCTGGGTAGGGCCTATTGT
	R: GCCGGCTTTGGCACTTGAGATACT
AQP8	F: TCATTGGAGATGGGAAG ACC
	R: TGAGAAGCAAGGAAGTG GC
AQP9	F: CTCAGTCCCAGGCTCTTCAC
	R: CTCAGTCCCAGGCTCTTCAC
VEGF	F: TTCTGGGCTGTTCTCGCTTC
	R: CTCTCCTCTTCCTTCTCTTCTTCC
GAPDH	F: TGACCACAGTCCATGCCATCAC
	R: CGCCTGCTTCACCACCTTCTT

F, forward; R, reverse; AQP, aquaporin.

Table II. Sequences of siRNA used to knockdown AQP5 expression.

siRNA	Sequence (5'→3')
AQP5-560	F: CCGUGUUCGCAGAGUUCUUTT
	R: AAGAACUCUGCGAACACGGTT
AQP5-883	F: GCGUGUGGCCAUCAUCAAATT
	R: UUGUGUUGUUGUUGAGCGCTT
AQP5-1224	F: GCGUGUGGCCAUCAUCAAATT
	R: UUUGAUGAUGGCCACACGCTT
Negative siRNA	F: UUCUCCGAACGUGUCACGUTT
	R: ACGUGACACGUUCGGAGAATT
Positive siRNA	F: UGACCUCAACUACAUGGUUTT
	R: AACCAUGUAGUUGAGGUCATT

F, forward; R, reverse; AQP, aquaporin; siRNA, small interfering RNA.

37˚C with 5% CO_2, the non-migrated cells were removed from the upper side, whereas the migrated cells were fixed with 70% methanol for 10 min at room temperature, and stained with 0.1% crystal violet at room temperature for 10 min. The cells were visualized using a light inverted microscope (Olympus 1X71; Olympus Corporation) at x10 magnification.

Wound-healing assay. H1299 cells were cultured into six-well plates for 24 h until they reached 100% confluence, and then the middle of each well was scratched using a 200 μl pipette tip. The scratched layers were washed with PBS to remove non-adherent cells, and the medium was replaced with serum-free RPMI. Wound healing was observed using light inverted microscope (Olympus 1X71; Olympus Corporation) at x10 magnification, and evaluated by comparing the cell-free area with the initial wound region.

HUVEC tube formation assay. HUVEC tube formation was evaluated using a previously described protocol (12). The supernatant of H1299 cells was collected and stored at -20˚C before use. Growth factor-reduced Matrigel (BD Biosciences) was added into 48-well plates (100 μl each) and allowed to solidify at 37˚C for 30 min. ($1x10^2$ cells/ml) HUVECs with serum-free RPMI medium were resuspended in H1299 culture supernatants with 1% FBS RPMI (total 200 μl) at a 1:1 ratio, added onto a Matrigel layer and incubated at 37˚C with 5% CO_2. Following cell seeding, tube formation was observed every hour using light inverted microscope (Olympus 1X71; Olympus Corporation) using x10 magnification, and cells were imaged after 8 h.

Statistical analysis. GraphPad Prism (version 6.07; GraphPad Software, Inc.) was used for all statistical analyses. Data are presented as mean ± standard deviation. Student's t-test was used to compare differences between two groups, and one-way ANOVA was employed to compare ≥3 groups, and the multiple comparisons were performed using one-way analysis, followed by Tukeys post hoc test. All experiments were performed in triplicate and $P<0.05$ was considered to indicate a statistically significant difference.

Results

AQP5 is upregulated in H1299 cells. To assess the pattern of AQP expression in NSCLC, the expression of six AQPs genes, including AQP 1, 3, 4, 5, 8 and 9, was evaluated in H1299 cells via RT-PCR. Among the six genes investigated, AQP5 was upregulated in H1299 cells (Fig. 1A). The expression of AQP5 was also assessed in the normal human bronchial epithelial cells 16HBE, and it was observed that AQP5 gene was expressed at a lower level compared with AQP3, with no detectable expression levels of the other AQPs genes (1, 4, 8 and 9), (Fig. 1B). The protein levels of AQP5 were assessed in both H1299 and 16HBE cells by western blot analysis. AQP5 protein was significantly upregulated in H1299 cells compared with 16HBE cells (Fig. 1C). AQP5 was therefore selected as a target for gene silencing.

AQP5 gene silencing. H1299 cells were transfected with various siRNAs against AQP5. In total, three different siRNA sequences were tested to determine the optimal siRNA to knockdown AQP5 expression. siRNA-560 exhibited the highest downregulation of AQP5 gene, as evidenced by fluorescence imaging for determine the transfection efficiency using (FAM) conjugated to the 5' end of the siRNAs (Fig. 2A), RT-PCR (Fig. 2B) and western blot analysis (Fig. 2C).

AQP5 is involved in lung cancer cell migration. To evaluate the migration of H1299 cells following AQP5 downregulation, Transwell migration and wound closure assays were performed. A significant reduction in the closure of the wound gap was observed after AQP5 knockdown (siAQP5) compared with the NC cells (Figs. 3A and 3B). Similarly, the Transwell assay indicated that siAQP5 significantly decreased

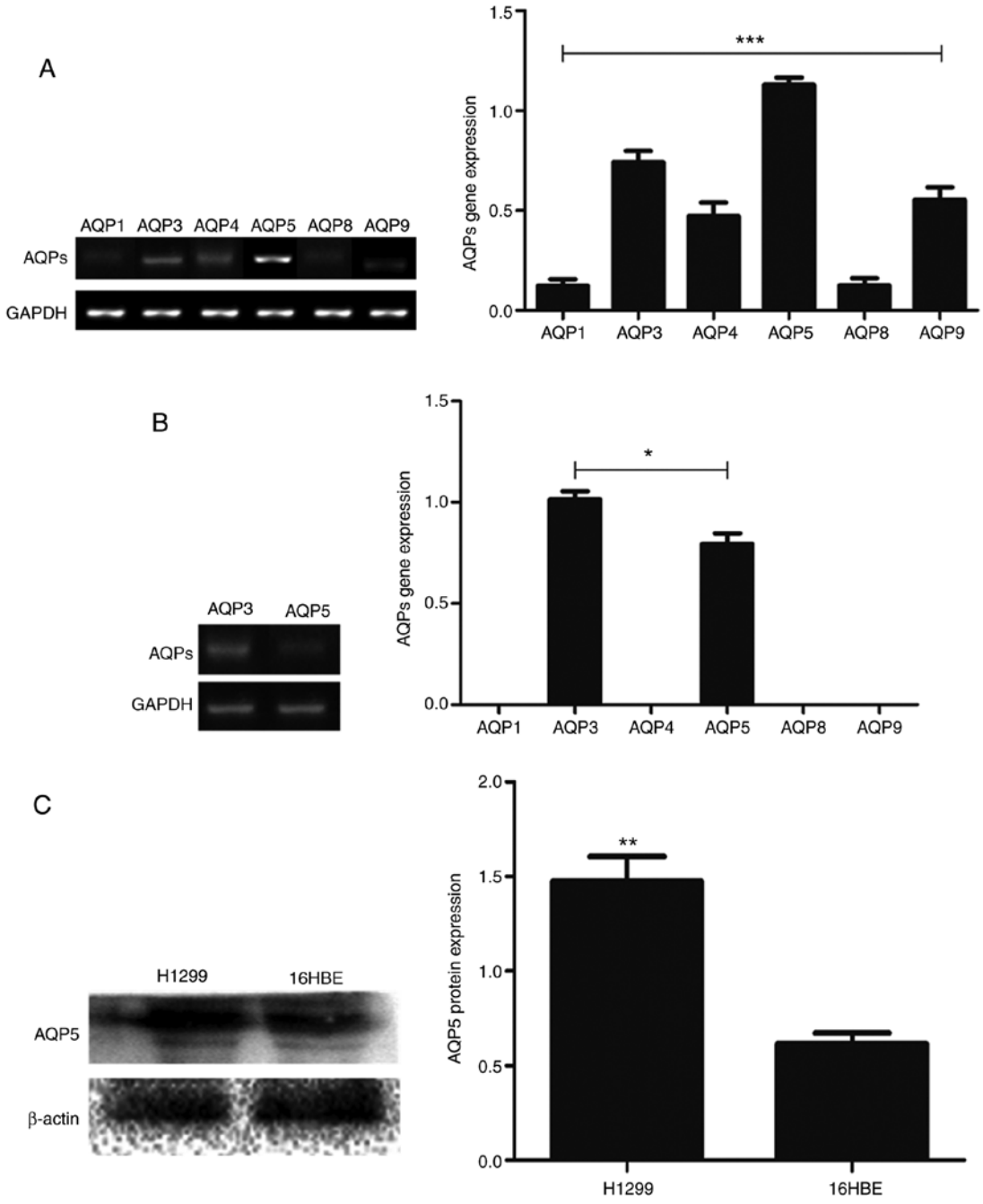

Figure 1. Expression of AQP genes in H1299 and 16HBE cells. (A) Gene expression levels of various AQPs in H1299 cells. (B) Gene expression of various AQPs in 16HBE cells. Only AQP3 and AQP5 showed detectable bands. (C) Protein expression of AQP5 in H1299 and 16HBE cells. Results shown are representative of three independent experiments, $^{*}P<0.05$, $^{**}P<0.001$, $^{***}P<0.0001$. GAPDH was used as internal control for gene expression, and β-actin was used as reference protein. AQP, aquaporin.

H1299 cell migration compared with the NC cells (Fig. 3C). Collectively, the present results suggested that AQP5 may be involved in NSCLC cell migration, and reducing its expression may represent a potential therapeutic treatment.

Downregulation of AQP5 inhibits angiogenesis and decreases activation of the epidermal growth factor receptor (EGFR)/ERK pathway. Vascular supply is required for cancer cells to proliferate and survive (21). Therefore, the role of AQP5 in tumor vascularisation was investigated using HUVECs. These cells exhibit the ability to grow and form tube-like structures, depending on the growth factors contained in the supernatant (22). HUVECs were treated with the supernatant of NC cultures as control medium (Fig. 4A). The supernatant of H1299 cells transfected with siAQP5 was used as the experimental sample. Increased tube formation was observed in HUVECs treated with the supernatant collected from NC H1299 cells compared with siAQP5-treated H1299 cells (Fig. 4A). The present results suggested that AQP5 knockdown reduced H1299 cell vascularisation. The expression of vascular endothelial growth factor (VEGF) in NC and siAQP5-treated H1299 cells was also assessed. The present results suggested that the expression level of VEGF was reduced in siAQP5 cells compared with NC cells (Fig. 4B).

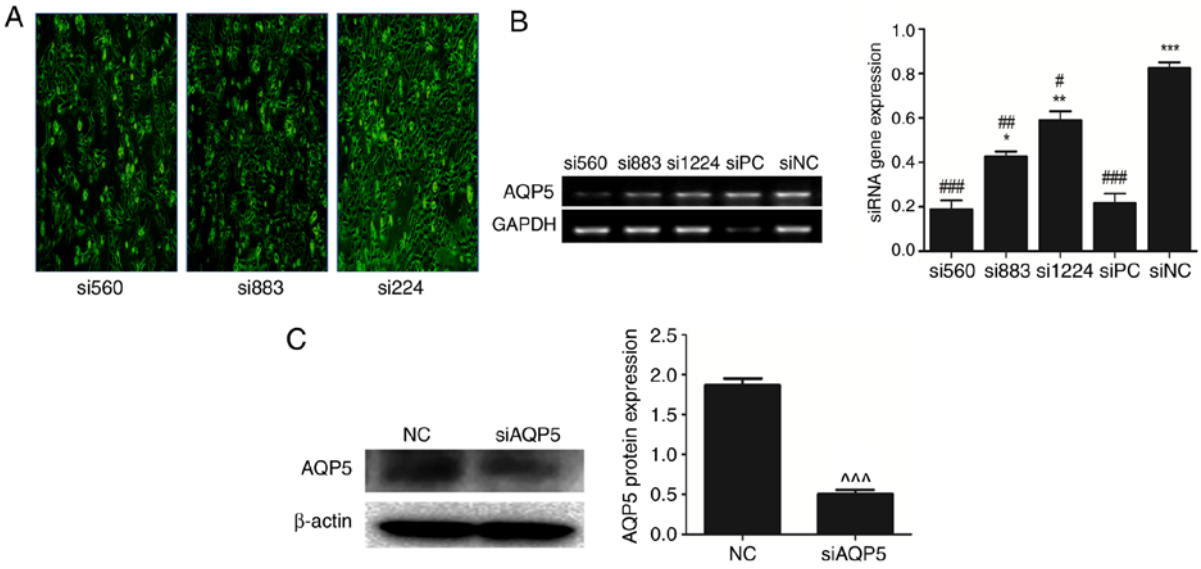

Figure 2. Knockdown of AQP5 in H1299 cells and AQP5 expression. (A) H1299 cells were transfected with different siRNAs. Images were obtained using a fluorescence microscope, magnification, x10. (B) Gene expression of AQP5 following transfection with different siRNAs. (C) Protein expression of AQP5 in H1299 cells with and without transfection. GAPDH was used as internal control for gene expression, and β-actin was used as reference protein. Results shown are representative of three independent experiments. *P<0.05, **P<0.001, ***P<0.0001 vs. siPC; $^{\#}$P<0.05, $^{\#\#}$P<0.001, $^{\#\#\#}$P<0.0001 vs. siNC; $^{\wedge\wedge\wedge}$P<0.0001 vs. NC. siRNA, small interfering RNA; siPC, siRNA positive control targeting GAPDH; siNC, siRNA negative control; NC, untransfected cells; AQP, aquaporin.

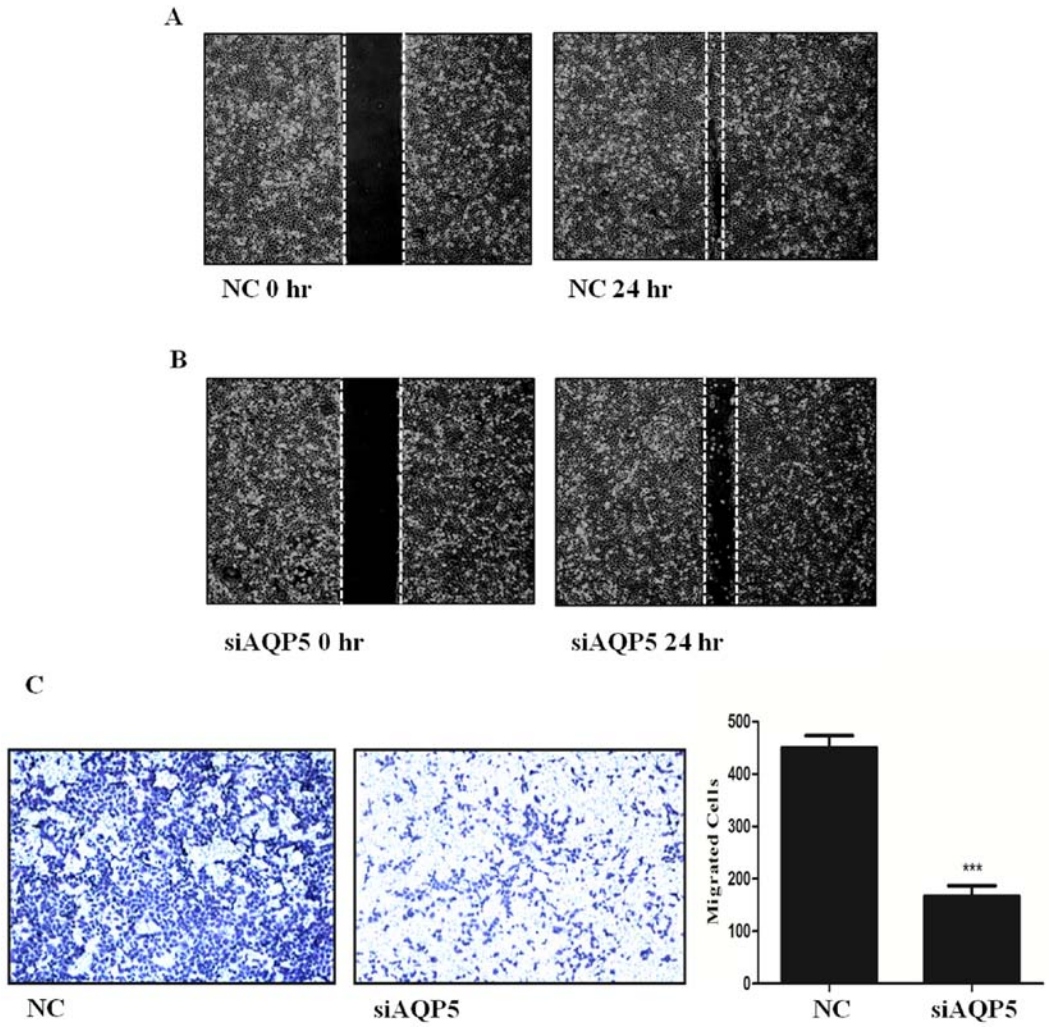

Figure 3. AQP5 downregulation decreases migration of H1299 cells. Wound-healing assay of (A) non-transfected and (B) transfected H1299 cells. (C) NC and transfected H1299 cells migration across Transwell membrane at 24 h, magnification, x10. ***P<0.0001 vs. NC. siRNA, small interfering RNA; NC, untransfected cells; AQP, aquaporin.

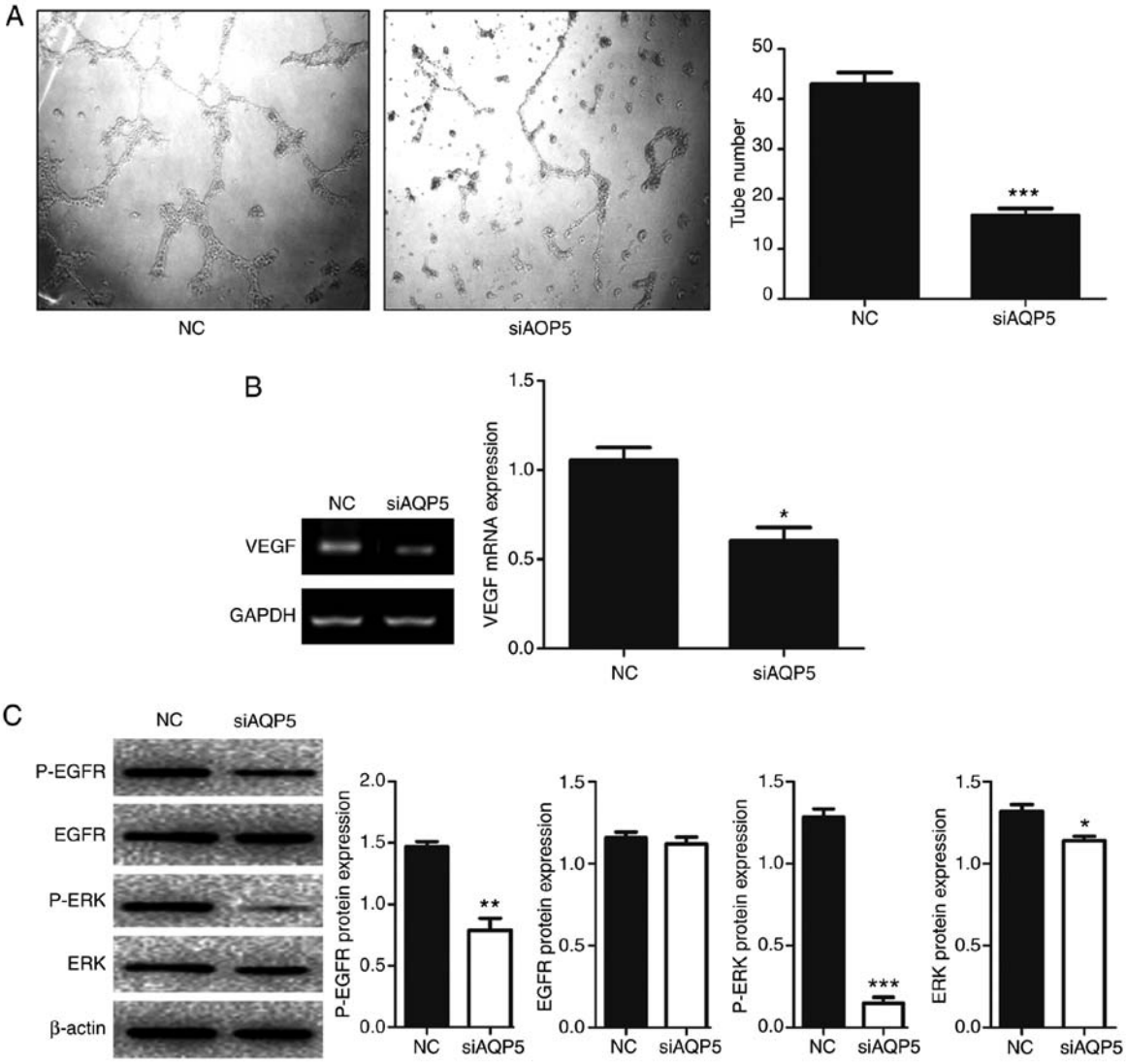

Figure 4. AQP5 downregulation inhibits angiogenesis and the activity of the EGFR/ERK signaling pathway. (A) HUVEC cultured in conditioned medium isolated from untransfected and transfected H1299 cells, magnification, x10. (B) VEGF mRNA levels in untransfected and transfected H1299 cells. (C) Knockdown of AQP5 caused a significantly decreased activity in the expression levels of protein associated with the EGFR/ERK pathway in H1299 cells. GAPDH was used as internal control for gene expression and β-actin was used as reference protein. *P<0.05, **P<0.001, ***P<0.0001 vs. NC. siRNA, small interfering RNA; HUVEC, human umbilical vein endothelial cell; NC, untransfected cells; AQP, aquaporin; EGFR, epidermal growth factor receptor; p-, phosphorylated; VEGF, vascular endothelial growth factor.

NC cells showed significantly increased EGFR and ERK1/2 phosphorylation, whereas AQP5 downregulation significantly decreased EGFR/ERK pathway activation in H1299 cells (Fig. 4C).

Discussion

AQPs serve important roles in vascular permeability and interstitial fluid pressure in tumors (23). An increased expression of AQP genes was found in different types of tumors, suggesting their potential to influence tumor activities in various tissues (8,23,24). Low AQP expression can inhibit the outgrowth of capillaries by decreasing VEGF expression in NSCLC (21). Therefore, AQPs may be partly responsible for tumor metastasis, and reducing their biological activity in tumors may be a promising therapeutic option (25-27). The growth, development, invasion and migration of NSCLC depend on an efficient supply of nutrients and metabolic activity (11). Water molecules are indispensable in cellular activities, especially in tumors, and an increased nutrition and water supply is required by cancerous cells. Consistent with previous findings (21), six different types of AQPs were found to be expressed in NSCLC and H1299 cells in the present study. Specifically, AQP1, 3, 4, 5, 8 and 9 were identified to be expressed in NSCLC cells. However, only AQP3 and 5 were detected in the normal lung cell line 16HBE, in contrast to other studies reporting that multiple AQPs are expressed in normal lung cells (28-30). In normal lung cells, AQP3 was significantly increased compared with AQP5; however, AQP3 and AQP5 exhibited opposite trends in NSCLCs. The present study did not investigate whether knockdown of AQP3 in NSCLC could increase the expression level of AQP5 in tumours. However, Machida *et al* (11) have reported that the expression of AQP3 and AQP5 in lung cancer cells is generally associated with cellular differentiation.

The role of AQP5 in lung cancer migration *in vitro* has been investigated in the present study. Tumor migration is a major feature of cancer, and suppressing this process is essential to reduce the spread of tumors (31). Both AQP3 and AQP5 are involved in malignant tumors (23). Woo *et al* (13) observed that AQP5 is involved in promoting tumor cells proliferation, whereas other researchers have reported the role of elevated AQP5 in the metastasis of colorectal, hepatocellular, squamous cell, cervical and early breast cancer (8,9,13,23,25,27,32). A high level of AQP5 has been observed in NSCLC, which positively correlates with lymph node metastasis; in addition, AQP5 expression is significantly higher in stages III and IV NSCLC (2), compared with that in stages I and II, suggesting its role in lung cancer progression (2). Woo *et al* (13) reported that AQP5 is a promising therapeutic target and may be involved in tumor establishment and progression more predominantly than other AQPs. AQP5 upregulation is associated with cellular differentiation and serves a major role in invading lung-cancer cells (19).

In the present study, it was observed that AQP5 was highly expressed in H1299, a NSCLC cell line. This observation is in line with previous findings suggesting the upregulation of AQP5 in lung adenocarcinoma cells (14). In clinical settings, AQP5 upregulation is considered as a sign of poor cancer prognosis (2,19). Patients with NSCLC exhibiting AQP5 upregulation present high rates of recurrence and AQP5 upregulation is associated with early disease progression, supporting the oncogenic roles of AQP5 in tumor cells (10). The EGFR/ERK signalling pathway is crucial in lung cancer metastasis (19). AQP5 expression levels are directly associated with the activity of the EGFR/ERK signalling pathway, in addition, p-ERK activates hypoxia inducible factor (HIF)-1α, which leads to the degradation of inhibitors of AQP5 transcription, and enhances the transcriptional activators of AQP5, modulating AQP5 transcription (33). An association between AQP5 and mucin 5 subtype AC gene expression has been observed, and increased AQP5 expression results in mucin hypersecretion in human pulmonary tracts (19). The present study identified a reduced migration of H1299 cells following AQP5 knockdown, suggesting that AQP5 may serve a regulatory role in the expression of migration-associated genes.

The present study suggested that AQP5 knockdown decreased tube formation in HUVECs, suggesting a role for AQP5 in angiogenesis. AQPs are water channels and exhibit significant diagnostic and prognostic potential, particularly in tumours (34). AQP3 and AQP5 were found to play crucial roles in tumour vascularisation, and AQP3 knockdown reduces the density of microvessels in NSCLC via HIF-2α (26). Inhibition of AQP5 significantly decreases the expression of VEGF, which is critical in tumour angiogenesis, suggesting that increased expression of VEGF is positively associated with angiogenesis (21). In a previous study, AQP5 downregulation in colorectal cancer cells resulted in decreased expression of VEGF and a corresponding inhibition of angiogenesis in HUVEC (35). In line with this previous study, the present study found that supernatant collected from H1299 cells following AQP5 knockdown exhibited reduced HUVEC angiogenesis. Tumors exhibit an increased vascularisation compared with normal tissues, the increased vascularisation in tumors can reduce the nutrition supply of normal tissues, decreasing their metabolic activities (21). This effect suggests that suppressing tumour-specific angiogenesis may facilitate cancer treatment. As identified in the present study, downregulation of AQP5 may repress tumor-specific vascularization. The present study did not investigate whether concomitant knockdown of AQP3 and AQP5 may synergistically decrease tumor vascularization, particularly in NSCLC. Collectively, the present results suggested that AQP5 may be involved in the regulation of lung cancer cells migration and angiogenesis via the EGFR/ERK1/2 signaling pathway, and that AQP5 knockdown may be used as a potential target for the prevention of lung cancer metastasis and angiogenesis. However, the association between AQP5 and NSCLC remains elusive, and further studies are required to verify the role of AQP5 *in vivo*.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

AE, MA and WW designed and performed the experiments, analyzed the data and wrote the manuscript. HL, XO, XS, YT and BW interpreted the experiment and analyzed the data. XL and JW planned the experiments, analyzed data, modified the paper and approved the final version of the manuscript submitted for publication. All authors read and approved the final version of manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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