

RESEARCH ARTICLE

Aquaporin 8 Involvement in Human Cervical Cancer SiHa Migration via the EGFR-Erk1/2 Pathway

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Abstract

Overexpression of aquaporins (AQPs) has been reported in several human cancers. Epidermal growth factor receptor (EGFR)-extracellular signal-regulated kinases 1/2 (Erk1/2) are associated with tumorigenesis and cancer progression and may upregulate AQP expression. In this study, we demonstrated that EGF (epidermal growth factor) induces SiHa cells migration and AQP8 expression. Wound healing results showed that cell migration was increased by 2.79-1.50-fold at 24h and 48h after EGF treatment. AQP8 expression was significantly increased (3.33-fold) at 48h after EGF treatment in SiHa cells. An EGFR kinase inhibitor, PD153035, blocked EGF-induced AQP8 expression and cell migration and AQP8 expression was decreased from 1.59-fold (EGF-treated) to 0.43-fold (PD153035-treated) in SiHa. Furthermore, the MEK (MAPK (mitogen-activated protein kinase)/Erk (extracellular signal regulated kinase)/Erk inhibitor U0126 also inhibited EGF-induced AQP8 expression and cell migration. AQP8 expression was decreased from 1.21-fold (EGF-treated) to 0.43-fold (U0126-treated). Immunofluorescence microscopy further confirmed the results. Collectively, our findings show that EGF induces AQP8 expression and cell migration in human cervical cancer SiHa cells via the EGFR/Erk1/2 signal transduction pathway.

Keywords: SiHa - aquaporin 8 - EGFR- Erk1/2 - migration

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Introduction

The cells of all living organisms are composed mainly of water, which is essential for the existence of life on the planet. Aquaporins (AQPs) are small transmembrane proteins that are expressed in a variety of epithelial tissues where they are responsible for regulating rapid water movement across epithelial barriers driven by osmotic gradients. Based on their permeability, mammalian AQPs are divided into three groups: a) water-selective permeable aquaporins (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8); B) aquaglyceroporins permeable to water, glycerol, urea, and other solutes (AQP3, AQP7, AQP9, and AQP10); C) subcellular aquaporins or super-aquaporins (AQP11 and AQP12), which have low homology with the other AQPs (Murai-Hatano et al., 2008). Tissue distribution and regulation studies have provided indirect evidence for roles of AQPs in a variety of physiological processes. Malignant tumorous cells have vigorous life activities and active metabolism, and their demand for water increases compared with normal cells. Current theories associate AQPs activity with osmotic pressure increase to form cell protrusions essential to form migration while aquaporins may play a key role in tumor energy metabolism (Hara-Chikuma et al., 2008; Verkman et al., 2008; Sekine et al., 2013). Recent evidences suggest that aquaporins are also involved in cell migration (Papadopoulos et al.,

2008), tumor angiogenesis (Zou et al., 2013), and tumor growth (Nico et al., 2011), strongly expressed in tumor cells of different origins, particularly aggressive tumors. A further, yet not sufficiently considered possibility for pro-tumorigenic functions of AQPs is the direct or indirect regulation of other genes, for example stabilization of hypoxia-inducible factor, which is facilitated by AQP1 expression (Echevarria et al., 2007) or the interaction of AQP5 with the Ras/extracellular signal regulated kinase (Erk1/2)/retinoblastoma protein signaling pathway (Kang et al., 2008). It is important to elucidate their tumor-specific expression patterns and to reveal their potential functions besides sole water transport in the different organs and tumor entities.

AQP8 gene encodes 261 amino acids that situates in chromosome 16 p12 (Viggiano et al., 1999), only participating in water metabolism. AQP8 is involved in the pathogenesis of inflammatory disease (Wang et al., 2013; Zhao et al., 2014). So far, however, AQP8 function is controversial in carcinogenesis. Epidermal growth factor receptor (EGFR)-Erk1/2 is a critical important signal transduction pathway in mitogen activated protein kinase (MAPK) family, closely related with tumorigenesis and progression (Tasioudi et al., 2012).

In this study, we investigated the capacity of EGFR-Erk1/2 to induce AQP8 expression in human cervical cancer SiHa cells via EGFR-Erk1/2 pathway.

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Materials and Methods

Cell lines and reagents

Human cervical cancer SiHa cell line (bestowed by Zheng-hai Ma professor from Xinjiang University, Xinjiang, China).

Trizol (Invitrogen, USA); AQP8 primary rabbit polyclonal antibody (sc-28624, Santa Cruz, USA), EGFR primary rabbit monoclonal antibody (sc-03, Santa Cruz, USA), P-EGFR (Tyr1068) (3777, Cell Signaling Technology, USA); β -actin (BA2305, Boster, China); Pierce secondary goat antibody (31430, Thermo Scientific, USA); EGFR kinase inhibitor PD153035 (S1079, Selleckchem, USA); Erk1/2 inhibitor U0126 (PHZ1283, Gibco, USA).

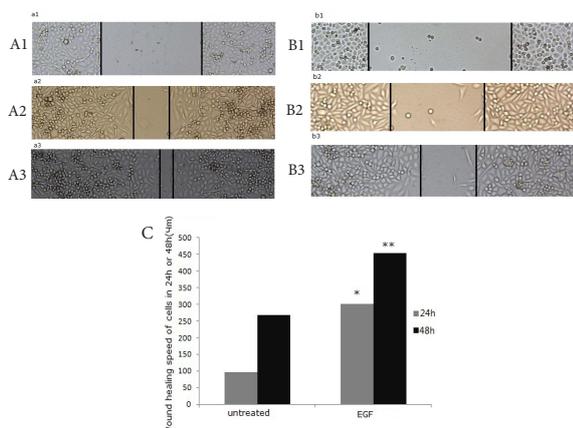


Figure 1. EGF Induces Cell Migration and Wound Healing in SiHa Cells. SiHa cells were treated with EGF at a concentration of 50ng/ml and cell migration was detected by wound healing assay at 0, 24, 48h, respectively. EGF-treated and untreated SiHa migration are shown in (A) and (B) respectively which are expressed as average gap in (C). (A1) and (B1): EGF group and untreated group at 0h; (A2) and (B2): EGF group and untreated group at 24h; (A3) and (B3): EGF group and untreated group at 48h. * $p < 0.05$ vs untreated group (24h), ** $p < 0.05$ vs untreated group (48h). Magnification (A) and (B): ($\times 50$)

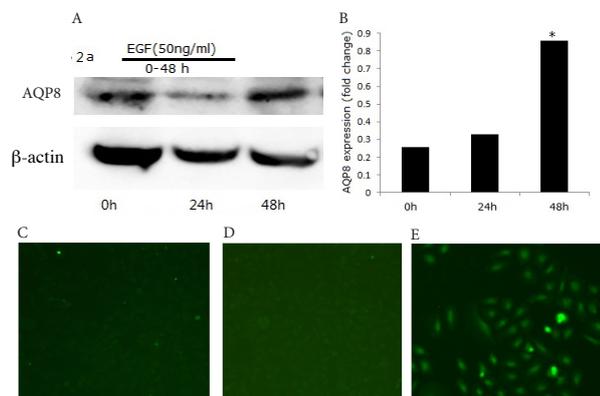


Figure 2. EGF Induces AQP8 Expression in SiHa Cells. SiHa cells were treated with EGF (50ng/ml) and harvested at different time points (0, 24, 48h), AQP8 expression was analyzed by Western blot as shown in (A) and (B). SiHa cells were also treated with 50ng/ml EGF and AQP8 expression was detected by immunofluorescence at different time points (0, 24, 48h) after EGF treatment as shown (C), (D) and (E). C: 0 h group; D: 24h group; E: 48h group. * $p < 0.05$ vs untreated group (48h). Magnification (C), (D) and (E): ($\times 100$)

Cell culture

SiHa cells were cultured at 37°C in a humidified incubator containing 5% CO₂ with Roswell Park Memorial Institute (RPMI)-1640 supplemented with 100 U ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin, and 10% fetal bovine serum (FBS).

In vitro wound healing (scratch) assay

The procedure was done as described (Hu et al., 2006). Shortly, SiHa cells were cultured as confluent monolayers in 6-well plates, synchronized in 1% FBS for 24h, and wounded by removing a 300~500µm-wide strip of cells across the well with a standard 10µl pipette tip, floating cells were removed by washing with PBS. Media containing 10% FBS without or with indicated

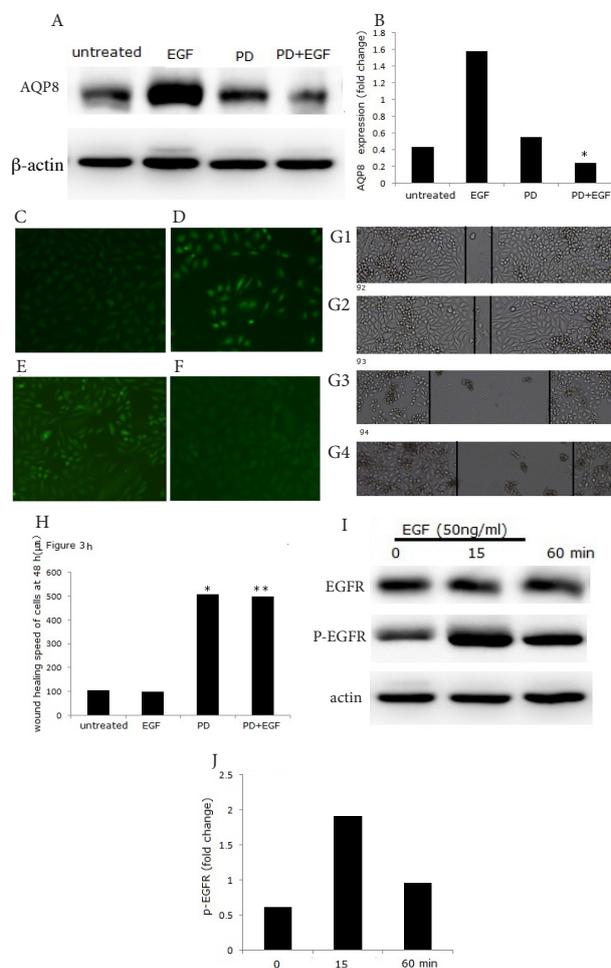


Figure 3. EGF-induced AQP8 Expression and Cells Migration are Blocked by PD153035. SiHa cells were treated with or without PD153035 for 48h. AQP8 expression in SiHa cells was analyzed by Western blot as shown in (A) and (B). Immunofluorescence was used to detect AQP8 expression (C), (D), (E) and (F). C: untreated group; D: EGF group; E: PD153035 group; F: EGF+PD153035 group. Cells migration was detected by wound healing assay as shown in (G) and (H). G1: untreated group; G2: EGF group; G3: PD153035 group; G4: EGF+PD153035 group. For EGFR phosphorylation, cells were treated with (50 ng/ml) and harvested at different points (0, 15, 60 min), p-EGFR and EGFR in cells were analyzed by Western blot (I) and was quantified in (J). * $p < 0.05$ vs untreated group; ** $p < 0.05$ vs untreated group. Magnification (C), (D), (E) and (F): ($\times 100$). Magnification (G): ($\times 50$)

concentrations of EGF, PD153035 and U0126 were added to the wells and incubated for an additional 0h, 24h, 48h. Five representative images of the scratched areas were photographed under microscope (ZEISS, Observer, Z1, magnification : $\times 50$) at 0h, 24h, 48h, respectively. Average gap was used to quantify the data.

Western blot analysis

Cultured human SiHa cells with or without treatment were washed with cold PBS and harvested by scraping into 500 μ l RIPA buffer. Cell lysates were incubated at 4 $^{\circ}$ C for 30 min. After centrifugation at 10000g for 10 min at 4 $^{\circ}$ C, protein concentration was determined by BCA assay. Then proteins were denatured in 5 \times SDS-PAGE loading buffer for 5 min at 100 $^{\circ}$ C. The proteins were separated by SDS/10% PAGE or SDS/5% PAGE and transferred on to PVDF membrane (IPVH00010, Millipore, USA) for 50 min at 4 $^{\circ}$ C. Non-specific binding was blocked with 5% dry milk. After blocking, membranes were incubated with specific antibodies against AQP8 (1:200), EGFR (1:800), phspho-EGFR (1:1000) and β -actin (1:300) in dilution buffer overnight at 4 $^{\circ}$ C. The blots were incubated with secondary antibody at room temperature for 1h. Antibody binding was detected using the ECL (enhanced chemiluminescence) detection system.

Immunofluorescence analysis

Cells after treatment were fixed in cold acetone for 10 min at 4 $^{\circ}$ C, washed 3 times with PBS, 5 min /time. Fixed cells were stained with primary antibody of anti-AQP8 overnight at 4 $^{\circ}$ C, followed by fluorescein isocyanate-labeled secondary antibody at 37 $^{\circ}$ C for 45 min, PBS instead of primary antibody was used as negative control. After washing, AQP8 immunofluorescence was observed in a Zeiss fluorescence microscope.

Statistical analysis

Data were expressed as the mean \pm standard deviation.

Statistical differences among groups were compared using the one-ANOVA for measurement data. $P < 0.05$ were considered to be statistically significant.

Results

EGF induces cell migration in SiHa cells

Invasion of malignant tumors is correlated with cell migration, thus we tested the capacity of EGF to induce migration in SiHa cells. SiHa cells were cultured in 6-well plates and treated with EGF at a certain concentration of 50ng/ml. Wound healing was monitored and digitized at 0, 24 and 48h after EGF treatment. The results showed that EGF induced cell migration in SiHa. Cell migration was increased by 2.79-, 1.50-fold at 24 and 48h in SiHa (Figure 1 A-C, $p < 0.05$) after treatment with EGF when compared with untreated cells. These results clearly demonstrate that EGF strongly induces human SiHa migration.

EGF induces AQP8 expression in SiHa cells

The study above demonstrated that induced cell migration in human SiHa. Recent studies indicated that AQP8 expression displayed downregulation and increased apoptotic responsiveness in carcinoma (Jablonski et al., 2007; Wang et al., 2012), and thus decreased cell migration. We next investigated whether AQP8 is expressed in human SiHa and whether EGF induces AQP8 expression. Cells treated with EGF (50ng/ml) were collected at 0, 24 and 48h and analyzed for AQP8 by western blotting and immunofluorescence. As shown in Figure 2, AQP8 was expressed in human SiHa cells and EGF induced AQP8 expression in a time-dependent manner. AQP8 expression was increased remarkably at 48h and reached 3.33 fold at 48h in SiHa (Figure 2 A-B). Immunofluorescence microscopic results shown in Figure 2 C-E further confirmed that EGF induced AQP8 expression in human SiHa cells (Figure 2 C-E).

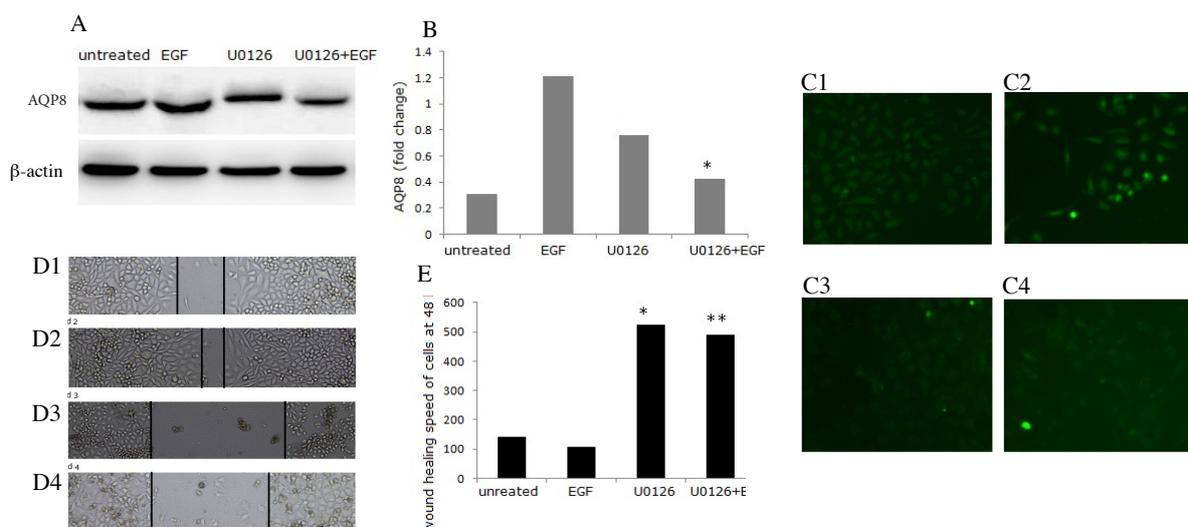


Figure 4. EGF-induced AQP8 Expression and Cells Migration are Blocked by U0126. SiHa cells were treated with or without U0126 for 48h. AQP8 expression in SiHa cells was analyzed by Western blot as shown in (A) and was quantified (B). Immunofluorescence was used to detect AQP8 expression (C). C1: untreated group; C2: EGF group; C3: U0126 group; C4: EGF+U0126 group. Cells migration was detected by wound healing assay as shown in (D) and (E). D1: untreated group; D2: EGF group; D3: U0126 group; D4: EGF+U0126 group. * $p < 0.05$ vs untreated group; ** $p < 0.05$ vs untreated group. Magnification (C): ($\times 100$). Magnification (D): ($\times 50$)

EGFR mediates EGF-induced AQP8 expression and cell migration in SiHa

To further understand the role of EGFR in EGF-induced AQP8 expression and cell migration, we used an EGFR kinase inhibitor, PD153035 (5 μ M). Western-blot data showed that pretreatment with PD153035 at 48h inhibited EGF-induced AQP8 expression. AQP8 expression was decreased from 1.59-fold (EGF-treated) to 0.43-fold (PD153035+EGF-treated) in SiHa (Figure 3 A-B). This effect was confirmed by immunofluorescence analysis (Figure 3 C-F). Moreover, cell migration induced by EGF also was inhibited by PD153035 (Figure 3 G-H). EGF induced EGFR phosphorylation in a time-dependent, reaching a peak at 15 min post-EGF treatment and remaining elevated for 1h (Figure 3 I-J).

Erk1/2 mediates EGF-induced AQP8 expression and cell migration in SiHa cells

Abundant studies have shown that activation of EGFR results in phosphorylation and activation of various effector proteins (Wang et al., 2014), among which are MAPK (mitogen-activated protein kinase) (Fan et al., 2014; Qu et al., 2014). The data presented here indicated that activation of EGFR is required for EGF-induced AQP8 expression and cell migration. The MEK (MAPK/Erk)/Erk inhibitor U0126 (30nM) was used to further clarify the cell signaling pathway leading to EGF-induced AQP8 expression and cell migration. Western-blot data showed that pretreatment with U0126 at 48h partially inhibited EGF-induced AQP8 expression. AQP8 expression were decreased from 1.21-fold (EGF-treated) to 0.43-fold (U0126-treated) in SiHa (Figure 4 A-B). Immunofluorescence analysis further confirmed that Erk1/2 inhibitors inhibited EGF-induced AQP8 expression (Figure 4C). Moreover, cell migration induced by EGF also was inhibited by U0126 (Figure 4 D-E).

Discussion

Invasion and metastasis of malignant tumor is bound up with cell migration. Cell migration is a dynamic, well-organized and complex process requiring co-ordination of many molecules in cells. Numerous studies have demonstrated that EGF activates its receptor, following resulting in increased cell migration in human tumorous cells (Cho et al., 2014; Jeong et al., 2014; Magi et al., 2014), thus EGF, via its receptor, plays an important role in cell migration and tumor invasion. Our research, using *in vitro* wound healing assay, clearly showed that EGF induced human cervical cancer SiHa cell migration (Figure 1).

Cell migration is a multistep process involving numerous growth factors, cytokines and adhesion molecules as well as extracellular-matrix proteins. Recent studies have implicated another important process, namely, AQP-dependent cell migration involves AQP-facilitated water influx into dynamic cellular protrusions (lamellapodia) at the leading edge of migrating cells (Hu et al., 2006; Shi et al., 2013). At least the discovery of AQPs, 13 mammalian homologs (AQP0~12) have been identified to date (Verkman et al., 2008). Previous studies have shown

that AQP8 mainly distribute in gastrointestinal epithelial cells (especially colon), liver, pancreas, male and female reproductive system. However, there are controversial literature reviews on the role of AQP8 in carcinogenesis. Some experiments demonstrated that AQP8 contributed to tumor cell migration and proliferation (Shi et al., 2013; Zhu et al., 2013), while some research reported that AQP8 induced to tumor cell apoptosis and its expression was downregulated in carcinoma (Fischer et al., 2001; Jablonski et al., 2007). Here we provide the evidence that AQP8 expressed in human cervical cancer SiHa and that EGF, which induced cells migration, also induced AQP8 expression in human SiHa cells (Figure 2). Our findings support AQP8 positive contribution to carcinogenesis. There are consistent discoveries about the roles of other subtypes of AQPs in tumorigenesis, such as that AQP1 increases the migration speed of B16F10 melanoma cells and 4T1 mammary gland tumor cells, and AQP3 is favor of FGF-2-induced migration of human breast cancers (Hu et al., 2006; Cao et al., 2013). Our results suggest that up-regulation of AQP8, in response to EGF accelerates human SiHa cell migration by enhancing plasma-membrane water permeability, which in turn boosts transmembrane water fluxes that occur during cell migration.

AQPs family has provided insight into molecular mechanisms of membrane water permeability and tumor cell migration. However, the mechanisms through which EGF up-regulates AQP8 expression in SiHa cells remain poorly understood. In this study, we have provided the evidence that EGFR and Erk1/2 mediate EGF-induced AQP8 expression and SiHa migration. our experiments demonstrated that EGF transiently induced EGFR phosphorylation, and EGFR kinase inhibitor, PD153035 inhibited cell migration induced by EGF in human SiHa. It has been well proved that EGFR is an essential regulator in tumorigenesis, including tumor cell migration (Gándola et al., 2014; de Melo Maia et al., 2014; Moerkens et al., 2014). Here we presented the results demonstrating that both EGFR and its kinase activity were required for EGF-induced AQP8 expression and cell migration in human cervical cancer SiHa cells (Figure 3).

It has been estimated that Erk1/2 targets more than 180 different molecules that are responsible for cell growth, survival, and differentiation; thus, aberrant regulation greatly affects cell growth. Erk1/2 kinase couples the signals from cell surface receptors to molecules that transmit cell proliferative signals. Following a cascade reaction, Erk1 phosphorylates Erk1/2, leading to the upregulation of various transcription factors such as Ets-1, c-Jun, c-Myc, and HIF1 α (Chetram et al., 2012). Our study also showed that Erk1/2 inhibitor U0126 inhibited cell migration induced by EGF in human SiHa and that Erk1/2 inhibitor U0126 effectively restrained EGF-induced AQP8 expression (Figure 4A-4E) in human SiHa cells. These results clearly demonstrate that EGFR/Erk1/2 signaling pathways are involved in EGF-induced AQP8 expression and cell migration in human SiHa cells. We contend that Erk1/2 is a critical signal molecule selectively regulating AQP8 expression and/or function in human SiHa cells.

In conclusion, our findings demonstrate for the first time that AQP8 is expressed in human cervical cancer

SiHa cells, and that EGF induces AQP8 expression and cell migration via EGFR/Erk1/2 signal transduction pathway in human SiHa cells. Our findings also provide an explanation as to why AQP8 has a positive function in carcinogenesis. It is envisioned that development of AQP8 openers or forced expression of AQP8 may be beneficial for human malignant tumor cells invasion.

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