Down-regulation of AQP4 Inhibits Proliferation, Migration and Invasion of Human Breast Cancer Cells

(breast cancer / AQP4 / siRNA / E-cadherin / ERK pathway)

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Abstract. Aquaporins (AQPs), proteinaceous water channels, have been proposed as mediators of tumour development and progression. However, the role of aquaporin 4 (AQP4), a member of the AQP family, in breast cancer has not been distinctly evaluated. The aim of the present study was to examine the effect of AQP4 down-regulation on proliferation, migration and invasion in human breast cancer. To determine this effect, siRNA interference was used to knock down its expression in T47D and MCF-7 cell lines. Down-regulation of AQP4 resulted in increased expression of E-cadherin along with an inhibitory effect on the proliferation, migration and invasion in breast cancer cells. In addition, AQP4 regulation of cell proliferation could be related with the ERK/E-cadherin pathway. In conclusion, the present data have suggested that down-regulation of AQP4 inhibits breast cancer cell proliferation, migration and invasion.

Introduction

Breast cancer (BC) is estimated to be the most commonly diagnosed cancer in women, affecting millions of women worldwide (Siegel et al., 2014). Like other cancers, breast cancer is a chronic and a multiple-step process in which accumulation of genetic and epigenetic alterations is involved. Although various achievements have been obtained in breast cancer research, the detailed understanding of its mechanism remains to be elucidated. To achieve a more effective treatment of human breast cancer, understanding the mechanisms that drive breast cancer progression is essential.

Aquaporins (AQPs) are a family of small hydrophobic, integral membrane proteins ranging from 26–34 kDa in size and have been discovered in many organisms including mammals (Agre et al., 1993). At present, 13 transcellular water movement proteinaceous aquaporin (AQP) isoforms have been identified in mammals (Agre et al., 2002). Aquaporin 4 (AQP4), a member of the AQP family, has a key role in maintaining water and ion homeostasis, which is thought to be associated with tumour development (Verkman et al., 2008). AQP4 is known to participate mainly in the central nervous system disorders, such as brain oedema after injury or some other brain diseases (Siegel et al., 2014). Recent studies of AQP4 are not restricted to the central nervous system, and many researchers have found a new role for AQP4 in other biological processes. A previous study indicates that AQP4 knockout inhibits proliferation, migration and neuronal differentiation of adult neural stem cells derived from the subventricular zone of adult mice (Kong et al., 2008). Siegel et al. (2014) reported that AQP4 deficiency impairs migration and invasion of human glioma cells. Shi et al. (2012) screened the expression profiles of AQP0–12 in breast cancer tissues, and these results show a significantly higher level of AQP4 expression in normal than in cancer tissues, which implies that AQP4 may play a role in breast carcinogenesis. However, up to date, there have been little reported attempts to study the relationship between AQP4 and breast cancer.

E-cadherin belongs to the cadherin family of cell adhesion molecules, playing an important role in the maintenance of epithelial structure. Besides acting as an epithelial marker, E-cadherin also plays a role in controlling tumour growth and metastasis (Jeannes et al., 2008; Onder et al., 2008). Loss of E-cadherin has been frequently correlated with poor prognosis in human cancer and metastasis in the mouse model (Birchmeier and Behrens, 1994; Thiery, 2002). To get insight into the function of AQP4 in the mechanism of breast cancer development, further investigations are required. Therefore, in this study, using down-regulation of AQP4 through small interference RNA (siRNA) we tested the hypothesis whether AQP4 may have a direct role in human breast cancer cell proliferation, migration and invasion. Our results may provide a new insight into the mechanism of breast cancer development.
Material and Methods

Cell culture and reagents

Human breast cancer cell lines MDA-MB-231, T47D, MCF-7 and ER-ZR-70 were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 1% pen/strep and 10% FBS (Biological Industries, Kibbutz Beit-Haemek, Israel) and incubated in a humidified atmosphere at 37 °C in 5% CO₂. When they reached ~70-80 % confluency, cells were trypsinized and resuspended in an antibiotic-free medium.

Small interference RNA (siRNA) treatment

T47D and MCF-7 cells were seeded into 6-well plates for 24 h before transfection in the complete medium. Transient transfection was performed with Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Human breast cancer cells were transfected with 5 μM control siRNA or human AQP4 siRNA oligos (5’-GCTCAATAGCTTTAGCAATTG-3’, Genechem Corp., Shanghai, China). The transfected cells were cultured in complete medium and incubated at 37 °C for 24 and 48 h.

RNA isolation, reverse transcription and real-time PCR

Total RNA was extracted using the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Reverse transcription was performed with 1 μg total RNA per reaction using the PrimeScript RT-PCR Kit (Takara Bio, Tepco, Japan). The amount of cDNA equivalent to 5 ng total RNA was included in each PCR reaction. Expression analysis of AQP4 and the housekeeping gene GAPDH was performed in T47D and MCF-7 cells by quantitative real-time PCR (ABI Prism 7900HT Sequence Detection System; Applied Biosystems, Weiterstadt, Germany). Gene expression differences between sample groups were analysed using t-test.

Western blotting analysis

Cells were lysed in ice-cold 1× RIPA lysis buffer (containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% sodium deoxycholate), 1 mM NaF, 10 mM Na₃VO₄, 1 mM PMSF (phenylmethylsulphonylfluoride), and protease inhibitor cocktail (10 mg/ml leupeptin, 10 mg/ml aprotinin, and 1 mM pepstatin). After incubation at 4 °C for 30 min, the lysates were centrifuged at 15,000 g for 10 min at 4 °C. Protein concentrations were determined using a BCA Kit (Takara Bio, Tepco, Japan). The amount of cDNA was equivalent to 5 ng total RNA was included in each PCR reaction. Reverse transcription was performed with the PrimeScript RT-PCR Kit (Takara Bio, Tepco, Japan). The amount of cDNA equivalent to 5 ng total RNA was included in each PCR reaction. Expression analysis of AQP4 and the housekeeping gene GAPDH was performed in T47D and MCF-7 cells by quantitative real-time PCR (ABI Prism 7900HT Sequence Detection System; Applied Biosystems, Weiterstadt, Germany). Gene expression differences between sample groups were analysed using t-test.

Transwell invasion assay

Cell invasion was measured by assessing the invasion of cells through Matrigel-coated transwell inserts (Costar, Cambridge, MA). Briefly, T47D and MCF-7 cells (2 × 10⁵) in culture medium with 5% FBS were loaded in the upper chamber of a modified Boyden chamber. The lower chamber contained culture medium including 20% FBS as chemoattractant. Cells were incubated for 24 h and the cells remaining on the bottom surface were fixed in 95% ethanol for 15 min, and stained with haematoxylin and eosin. Observations were performed using a phase-contrast microscope in five predetermined fields.

Statistical analysis

Statistical analysis was carried out using Prizm 6 from GraphPad Software (San Diego, CA). Statistical significance for comparison between groups was determined by using Student’s t-test. All samples were tested in triplicate, and the data are expressed as means ± SD.
Results

Characterization of AQP4 expression in human breast cancer cell lines

We first examined the expression of AQP4 and E-cadherin in breast cancer cell lines (MDA-MB-231, T47D, MCF-7 and ER-ZR-70) by Western blotting. AQP4 was expressed in all four human breast cancer cell lines, and the expression of AQP4 in T47D was the highest. The differences in E-cadherin expression were not significant (Fig. 1 A). We therefore chose T47D and MCF-7 cell lines for further investigation.

Down-regulation of AQP4 in human breast cancer cells by siRNA

First, we sought to down-regulate the mRNA expression of AQP4 in T47D and MCF-7 cells using siRNA techniques. AQP4 siRNA sequences were transfected into T47D and MCF-7 cells, which were designated as siAQP4/T47D and siAQP4/MCF-7 cells. A scrambled siRNA sequence was also transfected to the T47D cells and designated as scr/T47D cells. Control cells were designated as siCtrl cells. The transfected cells were screened for AQP4 expression by RT-PCR and Western blotting analyses. The expression of AQP4 mRNA and protein was significantly reduced after silencing with siRNAs (Fig. 1 B-E)

AQP4 modulates breast cancer cell proliferation

The results of the cell proliferation tested by CCK-8 showed that AQP4-siRNA-transfected T47D and MCF-7 cells exhibited decreased cell growth ability (P < 0.05) compared with the control group (Fig. 2A-B, P < 0.05). The inhibition rate of AQP4 knockdown on the proliferation was ~25%. The CCK-8 experiment confirmed that T47D and MCF-7 cells were sensitive to down-regulation of AQP4, which displayed an inhibitory effect on the proliferation of human T47D cells (Fig. 3).

AQP4 reduces breast cancer cell migration and invasion

Migration is a fundamental property of cells that occurs during many physiological and pathological pro-

Fig. 1. Down-regulation of AQP4 in human breast cancer cells by siRNA. (A) AQP4 expressed in human breast cancer cell lines. (B-E) RT-PCR and Western blot analysis of AQP4 expression in siCtrl, scr/T47D and siAQP4/T47D cells. β-actin was used as a loading control.
cesses including organogenesis in the embryo, repair of damaged tissue after injury and the spread of cancer (Papadopoulos et al., 2008). We examined the potential role of AQP4 in cell migration according to the hypothesis of water channel role in volume regulation associated with migration. As shown in Fig. 2, we found that the time required for wound closure of AQP4-knockdown breast cancers was significantly longer than the time required for the corresponding control cells. Our results obviously demonstrated that knockdown of AQP4 greatly impaired the migratory ability of breast cancer cells (Fig. 3).

Reduction of the cell migratory ability usually leads to reduction of their invasive ability (Siegel et al., 2014). To determine whether AQP4, which we showed to regulate migration of breast cancer cells, also affects cell invasion, we assessed the invasiveness of the cells with AQP4 down-regulation in transwell assays. The invasion assay showed significant differences in the invasive ability between the siAQP4 cells and siCtrl cells (P < 0.01; Fig. 4). Quantitative analysis of the cell numbers revealed that siCtrl cells had a 2.3-fold higher rate of invasion than siAQP4/T47D cells, while the MCF7 cells also displayed 2.0-fold increase (Fig. 5). Collectively, these results indicate that AQP4 plays an important role in the migration and invasion of breast cancer cells.

Down-regulation of AQP4 enhances E-cadherin expression

E-cadherin is an important cell growth inhibitor (Stockinger et al., 2001). Given that our data indicate AQP4 regulation of cell growth in E-cadherin-expres-

Fig. 2. Scratch assay in T47D cells and MCF-7 cells. (A-B) siCtrl and siAQP4 cells were grown to confluence in a 35-mm tissue dish, and the monolayer was wounded with a 200-μl pipette tip. Images were captured at 0, 12 h and 24 h.

Fig. 3. Silencing of AQP4 significantly inhibits proliferation in human breast cancer cell lines. (A-B) Silencing of AQP4 significantly inhibited proliferation of T47D cells and MCF-7 cells at 12 h, 24 h, 48 h compared to the control, as determined by CCK-8 test (P < 0.05).
sing cells, we attempted to understand whether AQP4 regulates the expression of E-cadherin. As shown in Fig. 5A, knockdown of AQP4 consistently increased E-cadherin expression at both the transcript and protein levels; these results indicate that the expression of E-cadherin may be regulated by AQP4 in human breast cancer cells.

It has been reported that the expression of E-cadherin can be regulated through the PI3K/AKT or MAPK/ERK pathway. To investigate whether these pathways are affected by AQP4 modulation, we knocked down AQP4 and determined the phosphorylation status of AKT and ERK1/2 in T47D and MCF-7 cells. As shown in Fig. 5B, we did not see any effect of AQP4 down-regulation on AKT phosphorylation. However, down-regulation of AQP4 resulted in increased phosphorylation of ERK 1/2, indicating that AQP4 is able to inhibit the ERK pathway in human breast cancer cells. These data suggest that AQP4 insufficiency may promote human breast cancer cell growth through activation of the ERK pathway.

Discussion

AQPs have been shown to have pleiotropic biological functions. AQP1 and AQP4 are involved in invasion of lung cancer cells (Xie et al., 2012). AQP2, 3, 4, 5 are related with the apoptosis process (Choi et al., 2009; Flamenco et al., 2009; Tang et al., 2011; Xie et al., 2013). AQP1 could promote cell migration in 4T1 breast cancer cells (Ridley et al., 2003), and AQP5 is a marker protein for proliferation and migration of human breast cancer cells MCF-7 (Xie et al., 2012). Recent studies indicated a correlation of AQP4 with glioma proliferation (Warth et al., 2004). AQP4 is involved in the control of glioblastoma cell migration and invasion (Amos et al., 2007). AQP4 is differentially expressed in breast cancer tissues (Shi et al., 2012). However, little is known about the significance of AQP4 in breast cancer. Thus, the aim of our study was to study the relationship between AQP4 and breast cancer development.

In the present study, we decreased the expression level of AQP4 in T47D and MCF-7 cells. The efficiency and specificity of this decrease was confirmed by RT-PCR analysis. Our results demonstrated that down-regulation of AQP4 in T47D and MCF-7 cells could significantly inhibit cell proliferation. It seems that AQP4 probably functions as a positive regulator in breast cancer development. Reduction of AQP4 can impair the T47D and MCF-7 cell migration and invasion. Cell migration is a multistep process involving numerous soluble growth factors, cytokines, proteases and extracellular matrix proteins (Ridley et al., 2003). We found that AQP4 reduction slows down T47D and MCF-7 cell migration in cell chemotaxis and wound healing assays, suggesting that AQP4 is involved in T47D and MCF-7 cell migration. Similar to migration, invasion through the extracellular matrix is an important step in tumour invasion (Amos et al., 2007). It is also strongly associated with the infiltration and invasion of tumours (Maki et al., 2000). Our Matrigel invasion assay shows that reduc-
tion of AQP4 led to a decrease of invasiveness in the T47D and MCF-7 cells.

Loss of E-cadherin function is a common event in cancer (Nollet et al., 1999). E-cadherin is known to suppress tumour cell growth and migration in various malignancies (Yanagisawa and Anastasiadis, 2006; Soto et al., 2008). We have demonstrated in this study that knockdown of AQP4 can enhance E-cadherin expression. ERK kinases can activate several transcription factors and further regulate expression of specific genes (Zhang et al., 2008; Hsu et al., 2011). In our study, we found that AQP4 deficiency up-regulates E-cadherin expression by activation of the ERK pathway, as reflected by the finding that AQP4 knockdown enhanced phosphorylation of ERK1/2. These results suggest that AQP4 may be a potential therapeutic target of human breast cancer.

Understanding the molecular mechanisms of human breast cancer is critical for the development of novel therapeutic strategies or treatments. In the present study, we discovered the function of AQP4 in human breast cancer cells. Hopefully, improved chemotherapy targeting AQP4 may be available to patients with breast cancer in the next few years.

Conclusion

In this study, we provide evidence for a new role of AQP4 in breast cancer. Our results emphasize that decreased AQP4 expression inhibits proliferation, migration and invasion of human breast cancer cells. Furthermore, knockdown of AQP4 expression enhanced E-cadherin expression and, at least in part, AQP4-regulated cell proliferation in breast cancer cells may be related with the ERK/E-cadherin pathway. In conclusion, the presented data suggest that AQP4 may play an important role in breast cancer.

Fig. 5. Reduction of AQP4 by siRNA impairs the invasive ability in vitro. siCtrl and siAQP4 cells invading through Matrigel-coated 8-μm pore size transwell inserts were evaluated as described in manufacturer’s instructions. The number of invading cells was quantified by counting the stained cells in random fields of the membrane.
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Conflicts of interest – none.

References