Aquaporin 1 Facilitated Hepatocellular Carcinoma SMMC7221 Cell Migration Associated with Water Permeability

ZHANG Ai-li[1], LI Jiang[2], WANG Yan-qing[1], ZAKNROU Zohra[1], MA Tong-hui[1,4] and LI Xiao-meng[1,3*]

1. School of Life Sciences, Northeast Normal University, Changchun 130024, P. R. China;

2. Dental Hospital, Jilin University, Changchun 130021, P. R. China;

3. Bo'an Brothers Holding Pharmaceutical Co., Ltd.(China), Changchun 130012, P. R. China;

4. Central Research Laboratory, The Second Hospital of Jilin University, Changchun 130041, P. R. China

Abstract The authors investigated the regulation of human aquaporin 1(hAQP1) and the involvement of aquaporin 1(AQP1) in the migration of human hepatocellular carcinoma SMMC-7221 cells using RNA intereference technology. Firstly, two short hairpin RNA(shRNA) constructs in PBSU6 vector were reconstructed and their knockdown effects were identified in SMMC-7221 cells. Next, the involvement of endogenous hAQP1 in regulating the migration of SMMC-7221 cells was investigated *via* siRNA technology. HAQP1-shRNA can specifically inhibit AQP1 dependent osmotic water permeability. Meanwhile the migration of SMMC-7221 cells was inhibited remarkably after silencing AQP1 by performing transwell cell migration assay and *in vitro* wound healing assay. Furthermore, in the presence of an inhibitor $HgCl_2$, the water permeability of the cell membrane was remarkably decreased, the expression of AQP1 was upregulated after $HgCl_2$ treatment and the cell movement was decreased at the moment. Increased AQP1 cannot attenuate cell migration ability when cell membrane loses its water permeability function. This demonstrates that the cell migration was remarkably related to the transporting water function of cell membrane.

Keywords Aquaporin 1; shRNA; $HgCl_2$; Cell migration

Article ID 1005-9040(2011)-01-099-05

1 Introduction

Aquaporins(AQPs) are membrane water channels，which selectively transport water across cell plasma membranes. Previous studies have demonstrated that aquaporin 1(AQP1) is ubiquitously expressed in tumor vascular endothelium and is also expressed in many types of tumor cells, including liver cells, lung cancer cells, breast cancer tissue, glioma tissue, and malignant astrocytes[1—6]. But it is still unclear from expression studies whether this is an aberrant phenomenon or whether it plays a role in tumor biology. Compared with normal cells, whether tumor cells need more water permeability across plasma membrane to satisfy its multiplication and movement is still unsolved.

Hg^{2+} is a non-specific AQP blocker, which reacts with the free SH-group of critical cysteine(Cys189) located nearby. Before the discovery of AQP1, the channel-mediated transmembrane water passage of the human erythrocyte had been shown to be inhibited by mercurials. However, recent progress has shown this effect is due to mercurial reaction with the SH-group of Cys189 located near the constriction of the AQP1 "hour-glass" water channel[7]. As the inhibitor of AQP1, $HgCl_2$ inhibits the transporting water function of AQP1 according to previous researches[8—10]. But it is not clear whether $HgCl_2$ can inhibit the functions of AQP1 in tumorigenesis.

AQP1 is overexpressed in certain tumor cells. The major function of aquaporins was regarded to facilitate water transport across the membranes. Whether AQP1 plays certain roles in the tumorigenisis progress and whether this function relates to its water transport function are still not clear. The function of AQP1 in this process needs to be further discovered by different methods, including inhibitors and RNA interference.

Here, the function of AQP1 in the migration of hepatocellular carcinoma cells was discovered *via* siRNA technology and inhibitor $HgCl_2$. We investigated the relationship between the function of AQP1 in the cell migration and its transporting water role.

2 Experimental

2.1 Plasmids

PcDNA3-hAQP1 was kindly provided by Professor Verkman A. S.(University of California, San Francisco, USA). The expression plasmid pcDNA3.1 Hygro-EYFP, H148Q-V163S encoding the $C1^-$ sensitive EYFP mutant EYFP-H148Q-V163S(hygromycin resistant) was kindly provided by Dr. Haggie P.(Department of Medicine, University of California, San Francisco, USA). RNA interference target sequences of

*Corresponding author. E-mail: lixm441@nenu.edu.cn

Received January 13, 2010; accepted August 17, 2010.

Supported by the National Natural Science Foundation of China(Nos. 30871301，30700827), the Program of Ministry of Science and Technology of China(No.2010DFA31430), the Program of Jilin Provincial Science & Technology Department, China(Nos.20070719, 20080731 and 200905116) and the Fund of Northeast Normal University, China(No.NENU-STC07005).

human aquaporin 1(hAQP1) and emmprin gene were selected *via* "accctccagctggtgctat" and "attaaccctgctcggtcca" for AQP1, and then cloned into a pBluescript plasmid containing the U6 promoter[8].

2.2 Cell Cultures and Transient Transfections

SMMC-7221 human hepatocellular carcinoma cells(from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, China) were maintained in Dulbecco's modified Eagle's medium supplemented with 5% or 10% fetal bovine serum (HyClone, Denver, CO). These cells were originally established in 1980 and characterized by a particularly aggressive metastatic regulation. They were treated with $HgCl_2$(Chemical Plant of Beijing, China). Transient transfections were carried out with the help of a Lipofectamin 2000 kit(Invitrogen). Stably expressed hAQP1 CHO cells were established by the transfection of hAQP1-cDNA in CHO cells with Lipofectamine 2000(Invitrogen) reagent and selection *via* the addition of 1000 μg/mL G418(Life Technologies, Inc.) at final concentration.

2.3 Immunobloting

After treatment or transfection, the SMMC-7221 cells in the 6-well plate were rinsed twice with ice-cold PBS containing 1 mmol/L sodium orthovanadate, followed by addition of 50 μL of SDS sample buffer. The whole cell lysate was then treated in ice-cold water by sonication. The lysate was applied to SDS-PAGE, and the proteins in the gel were transferred to a polyvinylidene difluoride membrane. The membrane was incubated in the mixture of 5% skim milk, 25 mmol/L Tris/HCl (pH=7.6), 150 mmol/L NaCl, and 0.1% Tween-20(TBST) at room temperature for 1 h, and washed twice with TBST for 5 min each, then incubated with a primary antibody, including rabbit antibody against AQP1(α diagnostic), and mouse antibody against β-actin(Santa Cruz Biotechnology), at (500—1000)×dilution in TBST at 4 °C for 2 h. The membrane was washed three times with TBST for 10 min each, incubated with a second antibody of horseradish peroxidase (HRP)-conjugated anti-mouse IgG(Sigma) or HRP-conjugated anti-rabbit IgG(Sigma) at (1000—2000)×dilution in TBST at room temperature for 1 h, washed vigorously five times for 10 min each, and subjected to chemiluminescence(ECL-plus, Amersham Biosciences, Piscataway, NJ) to visualize HRP.

2.4 Cell Membrane Water Permeability Assay

Water permeability measurements were performed as described previously[9] on an FLUOstar Optima plate reader (BMG Technology) equipped with syringe pumps and HQ500/20X[(500±10) nm] excitation and HQ535/30M [(535±15) nm] emission filters. For water permeability measurement, the SMMC-7221-v163s cells were plated in the 96-well black-walled microplates(Costar) at a density of 20000 cells per well in DMEM medium supplemented with 10% FBS. They were transfected with AQP1-shRNA or treated with $HgCl_2$ for 24 h, the cells in the 96-well plates were washed twice in PBS buffer(200 μL/wash), leaving 100 μL of PBS after the last wash. Measurement was performed on an FLUOstar Optima plate reader(BMG Technology) equipped with syringe pumps and HQ500/20X[(500±10) nm] excitation and HQ535/30M[(535±15) nm] emission filters. Cells in every well of the plate were assayed individually for osmotically driven AQP1-mediated water influx across the plasma membrane that dilutes the cytoplasmic Cl^- by continuously recording fluorescence increase(0.2 s per point) for 2 s(baseline) and then for 21 s (water transport into the cells) after a rapid injection(<1 s) of 100 μL of distilled water. Water permeability was expressed as the slope when the cytoplasmic fluorescence reached the maximum. The higher slope represented the higher water permeability.

2.5 Transwell Migration Assays

A modified Boyden chamber assay was performed as an *in vitro* model of invasive cell migration. SMMC-7221 cells were transfected with hAQP1-shRNA or PBSU6 vector for 12 h and seeded on the top of a culture plate insert(Corning Costar) containing a polycarbonate filter(6.5 mm diameter, 8 μm pores) pre-coated with fibronectin(0.5 mg/mL). The upper chamber contained cells in DMEM plus 1% fetal bovine serum, and the lower chamber contained DMEM plus 10%(chemoattractant) or 1% fetal bovine serum(control). Cells were incubated for 12 h at 37 °C in an atmosphere containing 5% CO_2. The cells that did not migrate were wiped away from the top of transwell filter and the migrated cells on the bottom surface were counted after staining with Coomassie blue.

2.6 *In vitro* Wound Healing Assay

Assays were performed as described previously with modification[10]. Briefly, SMMC-7221 cells were treated with $HgCl_2$ or tumicamycin, or the cells were transfected hAQP1-shRNA or PBSU6 control for 12 h and cultured as confluent monolayer on 6-well plates and synchronized in 1% fetal bovine serum for 24 h. The monolayer was wounded by removing a 300—500 μm strip of cells across the well with a 200 μL pipette tip and then washed twice to remove non-adherent cells. Wound healing was quantified as the average linear speed of the wound edges over 24 h.

3 Results and Discussion

3.1 AQP1-shRNA Efficiently Knocking Down the Expression of AQP1 and Inhibiting the Water Permeability Across Plasma Membrane in SMMC7221 Cells

The AQPs assembled in membrane act primarily as water-selective pores, facilitating osmotically driven water transport across plasma membranes[11—17]. Firstly, we generated two short hairpin RNA(shRNA) constructs for hAQP1(50) and tested their knockdown effects in either stably expressed hAQP1-CHO cells or hepatocellular carcinoma SMMC7221 cell, which has an endogenous hAQP1 expression. Both AQP1 shRNA constructs reduced the expression of over-expressed

AQP1 protein in CHO cells[Fig.1(A)] and that of endogenous glycosylated hAQP1 expression AQP1 protein in SMMC-7221 cells[Fig.1(B)]. There was no change in *β*-actin expression, confirming the specificity of the hAQP1 shRNAs. Particularly, the hAQP1 shRNA construct 2 appeared most effective in this knockdown experiment. The hAQP1-shRNA 2 could also knock down the expression of AQP1 in the SMMC-7221 cells membrane confirmed by immunofluorescence analysis [Fig.1(C)].

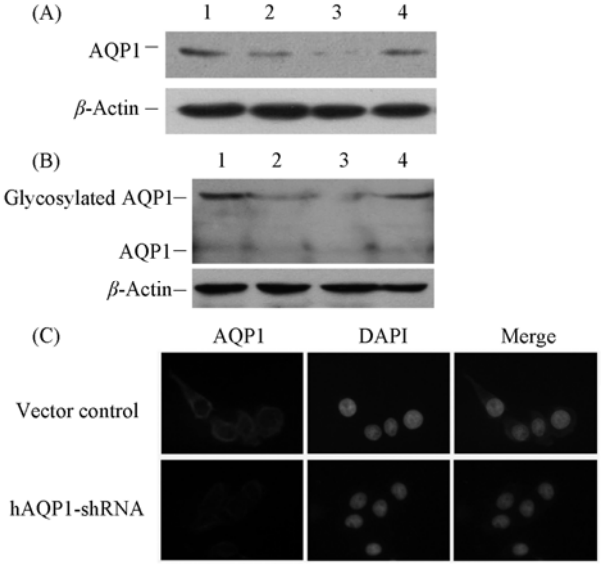

Fig.1 Expression analysis of AQP1 derived from different treatment of hAQP1-shRNA

(A) CHO cells stably transfected with pcDNA3-hAQP1 were transfected with different pBluescript(pBS)/U6-hAQP1-shRNA constructs(hAQP1-shRNA1 and hAQP1-shRNA2) or pBS/U6 vector only. Whole cell lysates were prepared after 48 h of transfection and analyzed by Western blotting with either AQP1 or *β*-actin antibody. Lane 1. vector control; lane 2. AQP1-shRNA1; lane 3. AQP1-shRNA2; lane 4. mock. (B) Different AQP-shRNA constructs or pBS/U6 vector was transfected into SMMC7221 cells. Cells were harvested, and cell lysates were analyzed as described in (A). Lane 1. vector control; lane 2. AQP1-shRNA1; lane 3. AQP1-shRNA2; lane 4. mock. (C) SMMC-7221 cells were transfected with 200 ng of AQP1-shRNA2 or pBS/U6 vector. 24 h after transfection, cells were fixed, incubated with an anti-hAQP1(1:500 dilution) polyclonal antibody, and then visualized with Rhoda mine-conjugated goat-anti-rabbit IgG at a dilution of 1:500 or with DAPI.

Using this construct, we further tested the role of endogenous hAQP1 in water permeability in SMMC-7221 cells. SMMC7221 cells stablely transfected with YFP-H148Q-v163S plasmid are shown in Fig.2(A), which is sensitive to the Cl^- concentration. And the cells in every well of the plate were assayed individually for osmotically driven water influx across the plasma membrane, the fluorescence decreased with the change of Cl^- concentration due to the dilution of the cytoplasm. This knockdown effect resulted in about a 40% reduction in the slope of the cell membrane osmotic water permeability after transfecting the hAQP1-shRNA[Fig.2(B)].

3.2 Silencing AQP1 Expression Inhibiting the Migration of SMMC-7221 Cell and hAQP1-CHO Cell

Next, we investigated the involvement of endogenous hAQP1 in regulating the migration of SMMC-7221 cells with RNA interference method using both Transwell cell migration assay and *in vitro* wound healing assay. As can be seen in Fig.3, Boyden chamber assay was performed as an *in vitro* model of

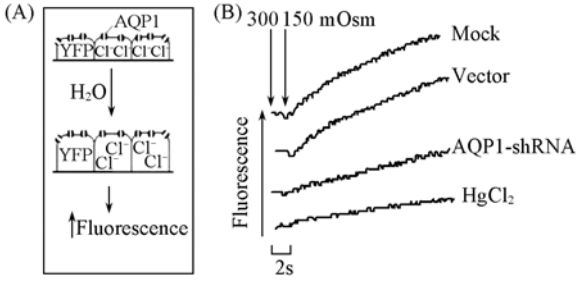

Fig.2 Reduction of water permeability due to knockdown AQP1 expression

(A) Schematic drawing of osmofic water permeability by Cl^- sensitive YFP-based fluorescence method. SMMC7221 cells were stablely transfected with YFP-H148Q-v163S plasmid, which is sensitive to the Cl^- concentration. And the cells in each well of the plate were assayed individually for osmotically driven water influx across the plasma membrane, the fluorescence decreases for the change of Cl^- concentration due to the dilution of the cytoplasm; (B) SMMC7221 cells stably transfected with YFP-H148Q-v163S were transfected with 0.5 μg of hAQP1-shRNA construct or pBS/U6 vector. A mock control and treatment with $HgCl_2$ of 20 μmol/L were set up as well. Cell membrane water permeability mediated by AQP1 was measured in above cells. Osm: Osmotic pressure with molar units.

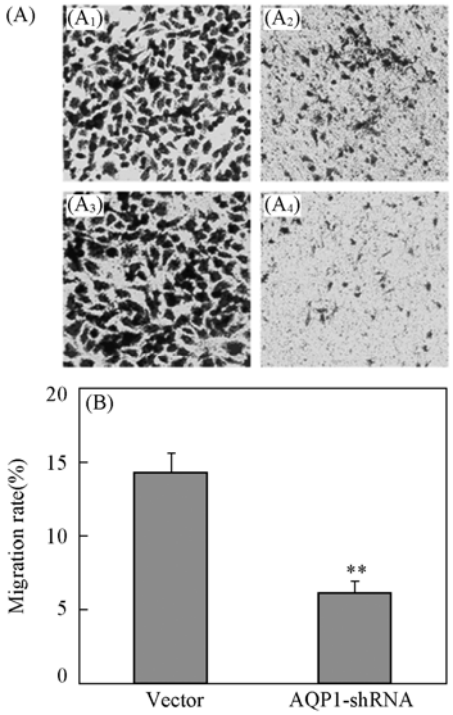

Fig.3 Inhibition of cell migration in transwell by knockdown AQP1 expression in SMMC-7221 cells

(A) SMMC-7221 cells were transfected with 0.5 μg of hAQP1-shRNA construct or pBS/U6 vector, 24 h after transfection, cells were plated in the Boyden chamber and cultured in DMEM with 1%(A_1, A_2) or 10%(A_3, A_4) fetal bovine serum. Cells migrated through the porous transwell filter before(A_1, A_3) and after(A_2, A_4) scraping of the non-migrated cells. Cells were stained with Coomassie blue. (A_1) vector control, total cells; (A_2) vector control, migrated cells; (A_3) AQP1-shRNA, total cell; (A_4) AQP-1-shRNA, migrated cells. (B) Summary of percentage of migrated cells at 12 h after plating. $^{**}P<0.01$.

invasive migration of SMMC-7221 transfected with control plasmid or hAQP1-shRNA plasmid. Cells will migrate from the upper 1% of fetal bovine serum to the lower 10% of fetal bovine serum through the 8 μm pores in the Boyden chamber. We discovered that the migration rate of transfected hAQP1-shRNA is obviously lower than that of the cells transfected with vector control, with a migration rate of (6.08+0.91)% in the AQP1-shRNA group and that of (14.31+1.25)% in the control group. In our privious result, AQP1-shRNA did not affect the SMMC cell profeliration by MTT experiment(data not shown here). So another wound closure experiments were performed to confirm the role of AQP1 in tumor cell migration. Significantly delayed wound closure in SMMC-7221 cells with silencing AQP1 is shown in Fig.4, compared to the SMMC-7221 cells transfected with control vector.

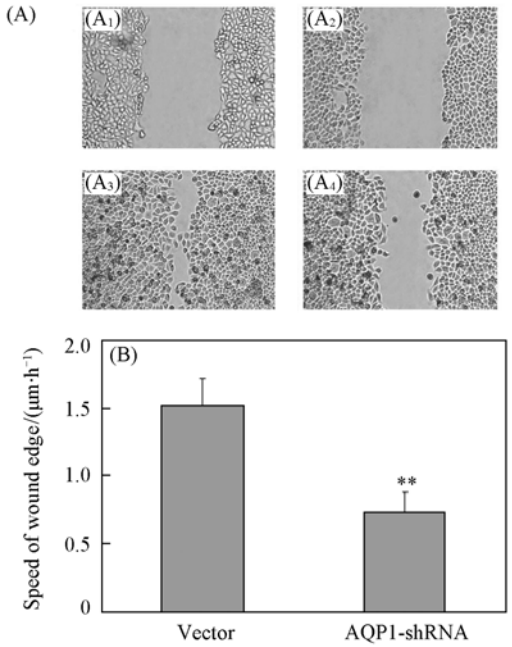

Fig.4 Inhibition of cell migration in wound healing of monolayer by knockdown AQP1 expression in SMMC-7221 cells

(A) SMMC-7221 cells were transfected with 0.5 μg of hAQP1-shRNA construct or pBS/U6 vector, 24 h after transfection, the wound were scratched. Representative images of wound closure showing initial wound edge and wound edge after 0 and 24 h. (A_1) Vector control, 0 h; (A_2) AQP1-shRNA, 0 h; (A_3) vector control 24 h; (A_4) AQP1-shRNA, 24 h; (B) the quantified wound edge speed. $^{**}P<0.01$.

Taken together, the data above indicate an important role of AQP1 in facilitating tumor cell migration, and knockdown AQP1 expression reduces nearly 50%—60% of the cell migration ability in transwell migration or wound healing of the monolayer in SMMC-7221 cells.

3.3 Loss of AQP1 Function in Promoting Cell Migration During $HgCl_2$ Blocking AQP1-mediated Water Permeability

As an inhibitor of AQP1, it has been well documented that $HgCl_2$ can inhibit water permeability but few have investigated the change of hAQP1's expression after $HgCl_2$ treatment. To determine whether AQP1 exerts the ability to promote cell migration or whether it is merely dependent on its ostimic water permeability, we treated cells with 20 μmol/L $HgCl_2$ for 24 h. First, we detected the expression of AQP1 and cell movement after 24 h of $HgCl_2$ treatment and found that both glycosylated AQP1 and unglycosylated AQP1 expressions were significantly enhanced by more than two fold[Fig.5(A)]; whereas at this time point, membrane osmotic water permeability was still blocked in the presence of $HgCl_2$[Fig.5(B)].

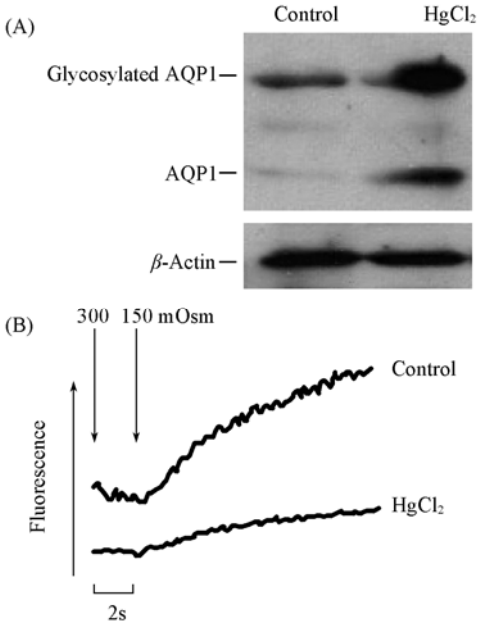

Fig.5 Expression analysis AQP1 losing its function in promoting cell migration when $HgCl_2$ blocks AQP1 mediated water permeability

(A) SMMC7221 cells were treated with $HgCl_2$ of 20 μmol/L for 24 h. Cell lysates were prepared and equalized by BCA kit. Then the lysates were analyzed by SDS-PAGE followed by Western blotting with anti-AQP1 and anti-β-actin; (B) SMMC7221 cells stably transfected with YFP-H148Q-v163S were treated with $HgCl_2$ of 20 μmol/L for 24 h, cell membrane water permeability mediated by AQP1 was measured in above cells. Osm: Osmotic pressure with molar units.

$HgCl_2$ treatment resulted in AQP1 expression and the blocking of water permeability. This contradictory phenomenon provided us a clue to discover the relationship between cell migration ability and water permeability dependent on AQP1. As shown in Fig.6, we discovered that the migration rate of $HgCl_2$ is obviously lower than that of the control group without treatment, with a migration rate of (4.81+1.59)% in the AQP1-shRNA group and that of (18.22+2.50)% in the control group.

As shown in Fig.7, the healing edge experienced almost no change after the $HgCl_2$ treatment, whereas the control group without $HgCl_2$ treatment showed a healing trend with a faster closure. Even though some cells experienced toxicity due to $HgCl_2$ treatment, these results suggest the function of AQP1 in promoting tumor cell migration may be closely related to its role in transporting water. When the cell membrane lost its water permeability function by $HgCl_2$ block, the acceleration of cell migration mediated by AQP1 also disappeared.

The relationship between accelerated tumor cell migration by AQP1 and the AQP1-mediated water transporting function was revealed by $HgCl_2$ treatment. $HgCl_2$, as a good tool to study the function of AQPs, inhibited the transporting of water

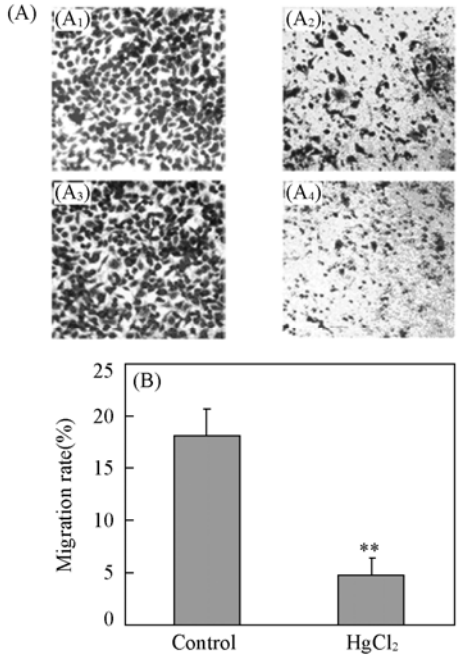

Fig.6 Inhibition of cell migration by AQP1 losing its function in promoting cell migration when $HgCl_2$ blocks AQP1 mediated water permeability

(A) SMMC-7221 cells were treated with $HgCl_2$ of 20 μmol/L, 24 h after transfection, cells were plated in the Boyden chamber and cultured in DMEM with 1%(A_2, A_4) or 10%(A_1, A_3) fetal bovine serum. Cells migrated through the porous transwell filter before(A_1, A_3) and after(A_2, A_4) scraping of the non-migrated cells. Cells were stained with Coomassie blue. (A_1) Total cells, control; (A_2) migrate cells, control; (A_3) total cells, $HgCl_2$; (A_4) migrate cells, $HgCl_2$; (B) summary of percentage of migrated cells at 12 h after plating. ***P*<0.01.

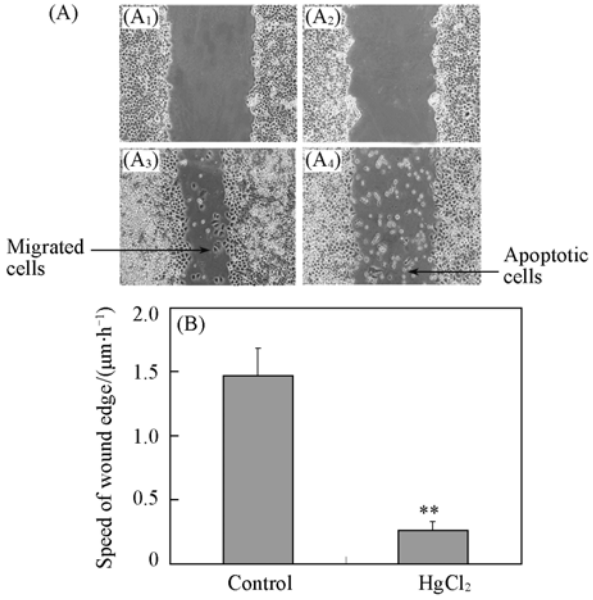

Fig.7 Wound healing of AQP1 losing its function in promoting cell migration when $HgCl_2$ blocks AQP1 mediated water permeability

(A) SMMC-7221 cells were scratched and treated with $HgCl_2$ of 20 μmol/L and cultured for 24 h. Representative images of wound closure showing wound edge and wound edge after 0 and 24 h. (A_1) Control, 0 h; (A_2) $HgCl_2$, 0 h; (A_3) contral, 24 h; (A_4) $HgCl_2$, 24 h; (B) the quantified wound edge speed. ***P*<0.01.

by binding SH in the amino acid of AQPs. Interestingly, our experiments show that the expression of AQP1 was upregulated after 24 h of $HgCl_2$ treatment. At this period, the water permeability of the cell membrane remarkably decreased, while the cell movement was still decreased.

These results demonstrate that the function of AQP1-accelerated cell migration was remarkably related to the ability of AQP1 to transport water. Increased AQP1 can not attenuate the cell migration when the cell membrane lost its water permeability function.

4 Conclusions

In conlusion, we demonstrated that the migration of SMMC-7221 cells was inhibited remarkably after silencing AQP1 by hAQP1-shRNA. Our experiments show that the expression of AQP1 was upregulated after 24 h of $HgCl_2$ treatment. The water permeability of the cell membrane remarkably decreased, while the cell movement was still decreased. Our results suggest that the tumor cell migration relates to its function of transporting water. Our experiments partly provide a good explanation that upregulated AQP1 expression in tumor cells may meet the requirement of water for the movement of tumor cells.

References

[1] Mobasheri A., Airley R., Hewitt S. M., Marples D., *Int. J. Oncol*., **2005**, *26*(5), 1149

[2] Endo M., Jain R. K., Witwer B., Brown D., *Microvasc. Res*., **1999**, *58*(2), 89

[3] Verkman A. S., Hara-Chikuma M., Papadopoulos M. C., *J. Mol. Med.*, **2008**, *86*(5), 523

[4] Hoque M. O., Soria J. C., Woo J., Lee T., Lee J., Jang S. J., Upadhyay S., Trink B., Monitto C., Desmaze C., Mao L., Sidransky D., Moon C., *Am. J. Pathol.*, **2006**, *168*(4), 1345

[5] Yin T., Yu S., Xia L., Zhang J., Liu C., Lu Y., Liu C., *J. Huazhong Univ. Sci. Technology Med. Sci*., **2008**, *28*(3), 346

[6] McCoy E., Sontheimer H., *Glia*., **2007**, *55*(10), 1034

[7] Kuang K., Haller J. F., Shi G., Kang F., Cheung M., Iserovich P., Fischbarg J., *Protein Sci*., **2001**, *10*(8), 1627

[8] Lahajnar G., Pecar S., Sepe A., *Bioelectrochemistry*, **2007**, *70*(2), 462

[9] Yang B., Kim J. K., Verkman A. S., *FEBS Lett*., **2006**, *580*(28/29), 6679

[10] Umenishi F., Schrier R. W., *J. Biol. Chem.*, **2003**, *278*(18), 15765

[11] Sui G., Soohoo C., el Affar B., Gay F., Shi Y., Forrester W. C., Shi Y., *Proc. Natl. Acad. Sci. USA*, **2002**, *99*(8), 5515

[12] Jin S. Y., Liu Y. L., Xu L. N., Jiang Y., Wang Y., Yang B. X., Yang H., Ma T. H., *World J. Gastroenterol*, **2006**, *12*(7), 1092

[13] Saadoun S., Papadopoulos M. C., Hara-Chikuma M., Verkman A. S., *Nature*, **2005**, *434*(7034), 786

[14] Arima H., Yamamoto N., Sobue K., Umenishi F., Tada T., Katsuya H., Asai K., *J. Biol. Chem.*, **2003**, *278*(45), 44525

[15] Hoffert J. D., Leitch V., Agre P., King L. S., *J. Biol. Chem*., **2000**, *275*(12), 9070

[16] Harlozinska A., *Anticancer Res*., **2005**, *25*(5), 3327

[17] Bogenrieder T., Herlyn M., *Oncogene*, **2003**, *22*(42), 6524