

Original Article

Influence of aquaporin-9 on migration ability of hepatocellular carcinoma cells

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Abstract: To explore the influence of aquaporin-9 (AQP9) on migration ability of hepatocellular carcinoma (HCC) cells. HepG2 cell lines were divided into 4 groups as follows: control, low-concentration oleic acid and high-concentration oleic acid groups which were treated with 0, 250 and 500 $\mu\text{mol/L}$ oleic acid respectively. Cells in silver nitrate group were treated with 3×10^{-5} mol/L silver nitrate at 37 °C for 30 min under the condition of 5% CO₂ saturated humidity and pre-treated with 500 $\mu\text{mol/L}$ oleic acid. After 4 groups were incubated at 37 °C under the condition of 5% CO₂ saturated humidity for 24 h, the expression of AQP9 was determined by western blotting assay and the migration ability of HCC cells were tested by the scratching experiment and the Transwell cell migration experiment. The expressions of AQP9 in oleic acid treated groups were up-regulated compared with the control group and the increasing tendency was in a dose-dependent manner ($P < 0.01$). The expression of AQP9 in silver nitrate group exhibited no change compared to the control group. Compared to the silver nitrate group, the cell migration ability in low-concentration oleic acid and high-concentration oleic acid groups presented the increasing trend ($P < 0.01$). The expression of AQP9 was negatively correlated with the migration rate of HepG2 cells ($r = -0.982$, $F = 190.098$, $P = 0.000$). The migration ability of HCC HepG2 cells was enhanced by the increased expression of AQP9, indicating that AQP9 might be related to the metastasis of HCC.

Keywords: Aquaporin-9, hepatocellular carcinoma, HepG2 cells, migration, western blotting assay, scratching experiment, transwell cell migration

Introduction

Hepatocellular carcinoma (HCC), one of the highest malignant degrees among malignant tumors, can be caused by intemperance, hepatitis B virus (HBV), aflatoxin and genetic factors. The common cause and inducement is post-hepatitic cirrhosis [1]. At present, surgery is the most effective treatment for HCC. As for patients who missed the best treatment period and can't undergo surgery or the intolerance of liver exposed to radical operation, the non-radical treatments such as chemotherapy, radio-frequency ablation, ablation and TCM therapy are widely used for improving the survival situation, prolonging lifetime, but the effects were limited [2, 3]. Recurrence of hepatocellular carcinoma is the main cause of worse effectiveness of HCC treatment because surgery can only remove the visible tumors, but can't eliminate the cancer cells at microcosmic level;

another reason is that chemotherapy as adjuvant therapy is limited in efficacy, leading to short-term postoperative recurrence [4, 5]. Aquaporin-9 (AQP9) is a kind of transmembrane protein expressed in brain, liver, spleen and adipocyte. It can be capable of conducting transmembrane transport of water, CO₂, glycerinum and carbamide and participating in a variety of physiological and pathological process of liver. Previous research has reported that AQP9 is differentially expressed between normal liver tissues and HCC tissues and its expression level is related to the development of HCC. Studies have shown that more than one member in aquaporins (AQPs) can promote migration of tumors [6-8]. Whether the abnormal expression of AQPs influencing the migration ability of HCC cells needs to be explored. Hence, the present study intended to explore the influence of AQP9 on the migration ability of HCC cells with the hope to provide the new target for treating HCC.

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Materials and methods

Culture of HCC HepG2 cells

Cryopreserved HepG2 cell strain (by Shanghai Cell Bank of Chinese Academy of Sciences) was melted rapidly in water bath at 37°C and centrifuged at 2,000 r/min for 5 min. Cells were suspended in DMEM (1 mL) culture medium containing 10% fetal calf serum (FCS) (by Hyelone Company) and cultured at 37°C under the condition of 5% CO₂ saturation humidity. Culture solution was replaced once every 2~3 days. Adherent cell growth condition was observed and cells were passaged when cells gathered together up to 75~80%. Generations 3~6 was used for experiments.

Grouping and treatment of HCC HepG2 cells

HCC HepG2 cells were divided into 4 groups, including control group, low-concentration oleic acid and high-concentration oleic acid groups. Each group was added with 0, 250, 500 µmol/L oleic acid solution (Sigma-Aldrich Company, America) mixed with bovine serum albumin (BSA, Roche Company) with the molar ratio 3:1. Cells in silver nitrate group was added with 3×10⁻⁵ mol/L silver nitrate solution (Sigma-Aldrich Company, America), cultured at 37°C under condition of 5% CO₂ saturation humidity for 30 min, finally added with 500 µmol/L oleic acid solution. Samples of four groups were collected after incubation at 37°C under the condition of 5% CO₂ saturation humidity for 24 h.

Expression of AQP9 detected by Western blot assay

After HCC HepG2 cells of 4 groups sucked culture solution completely, cells were washed by precooling phosphate buffer solution (PBS) for twice. When PBS was sucked out, 200 µL lysis buffer containing phosphatase inhibitors (Sigma-Aldrich) and protease inhibitor (Roche) was added to cells. The adherent cells were taken, shaken for 4°C, and centrifuged at 12,000 r/min for 20 min. The liquid supernatant was removed. Total protein level was determined by bicinchoninic acid (CBA) Protein Assay Kit (Sigma-Aldrich Company).

Sample proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the membrane was

transferred with nitrocellulose membrane (NCM, Pierce Company) and then sealed by PBS containing 5% skimmed milk powder for 3 h at room temperature. Samples were incubated with the primary antibody (AQP9 rabbit anti-human antibody, Abcam Company, dilution rate 1:500) staying overnight at 4°C, and cultured with the second antibody (goat anti-rabbit IgG-HRP and rabbit anti-human β-actin IgG from Santa Company, dilution rate 1:500) for 1 h at room temperature. And then, proteins were performed with chemiluminescence color, tableting, exposure and photographic fixing. Integral optical density (IOD) of AQP9 band is measured using Gel-ProAnalyzer software, and compared with the β-actin. The ratio is the relative expression of AQP9.

Influence of AQP9 on the migration ability of HepG2

Scratching experiment: The 24-pore plate was precoated with 30 µg/mL polylysine, and sealed with bovine serum albumin (BSA). Each pore was added with 3×10⁵ cells and stayed overnight by serum-free starvation. 300~500 µm of acellular scratching area was made by the cusp of pipette (200 µL). The floating cells scratched were washed by PBS and were added with serum-free medium containing different concentrations of oleic acid. The scratch width was measured after 24 h. The rate of cell migration=(scratching distance of each group/scratching distance of control group) ×100%.

Transwell cell migration experiment: After HCC HepG2 cells in logarithmic phase were digested and centrifuged, the concentration of cells was adjusted to 5×10⁸/L. Each chamber was added with 100 µL cell suspension. 10% FBS and a serial of concentrations of oleic acid were added to DMEM culture in the lower chambers. After cultured at 37°C under the condition of 5% CO₂ saturation humidity for 24 h, cells in the upper of chamber were removed by cotton swab, fixed by 4% paraformaldehyde for 15 min at room temperature, washed by PBS, stained by 0.1% crystal violet and then washed by PBS. Five visual fields were randomly selected under the microscope and photographed.

Observational indexes

The relative expression amount of AQP9 and the rate of cell migration of each group were

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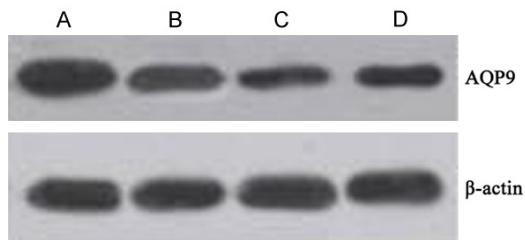


Figure 1. The Expression of AQP9 was detected by Western Blot Assay. Notes: A: High-concentration oleic acid group; B: Low-concentration oleic acid group; C: Silver nitrate group; D: Control group.

observed. The correlation between the relative expression of AQP9 and the migration rate of HepG2 cells.

Statistical data analysis

SAS9.3 software package was used for all data analysis. The measurement data was expressed as means \pm standard deviation, and the comparison of two groups was analyzed using t test and the comparison of multiple groups was analyzed by one-way analysis of variance. The correlation between the relative expression amount of AQP9 and the migration rate of HepG2 cells was used by linear-regression analysis. All statistical tests were evaluated by two-sided test and $\alpha=0.05$ was considered as inspection level.

Results

Expression of AQP9 in HCC HepG2 cells of different groups

The expressions of AQP9 in oleic acid treated groups were up-regulated compared with the control group and the increasing tendency was in a dose-dependent manner ($P<0.01$). The expression of AQP9 exhibited no change after giving the AQP9 inhibitor in the high concentration of oleic acid group. There was no difference compared with control group ($P>0.05$) (Figure 1 and Table 1).

Comparison of migration ability of HepG2 cells of groups

Transwell cell migration experiment showed that the cell number in the lower chamber was increased in response to oleic acid, indicating the migration ability of HepG2 cells enhanced. As shown in Figure 2, the number in the lower

chamber with silver nitrate pretreatment was lower than oleic acid groups. The scratching experiment showed that the cell migration ability in silver nitrate group, low-concentration oleic acid and high-concentration oleic acid groups presented the increasing trend and there were significant differences among groups ($P<0.01$) (Table 2 and Figure 3).

Correlation between the expression of AQP9 and the migration rate of HepG2 cells

Under the condition that the expression of AQP9 as independent variable and the migration rate of HepG2 cells as dependent variable, linear-regression analysis was conducted. The results showed that the expression of AQP9 was negatively correlated with the migration rate of HepG2 cells ($r=-0.982$, $F=190.098$, $P=0.000$), that is, the migration rate of HepG2 cells was enhanced by the increasing expression of AQP9.

Discussion

Surgery is presently as an optimal efficacy treatment among comprehensive treatments for HCC. HCC is characterized by the high invasiveness, early metastasis and high recurrence, however, the migration and diffusion of HCC cells were accompanied after surgery, with 3-year recurrence of 40%~60% [9-11]. There was only poor prognosis once it reoccurs, therefore the control of invasion and metastasis of HCC is the key and difficult point for treating HCC. Research on pathological process, mechanism and development of drugs for directly preventing cancer cells from being divorced from primary focus to reduce the metastasis of HCC is of vital importance for improving the survival rate of HCC patients.

AQP9, one of transmembrane aquaporins which was expressed in abundance in the liver cell membrane, participates in the multiple physical and pathological process of the liver. The previous study has showed that AQP9 is involved in the process of the liver transporting glycerol and plays an important role in gluco-lipid metabolism [12]. Under normal condition, the liver takes in free glycerol in blood by AQP9 as raw material of gluconeogenesis substrate and for synthesis of triglyceride to maintain the stability of gluco-lipid metabolism. However, when fasting, AQP9 is increasingly expressed in

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Table 1. Expression of AQP9 in HCC HepG2 cells of different groups

Groups	Expression amount of AQP9	F	P	t	P
Silver nitrate group	0.682±0.011	356.907	7.4222E-9	—	—
Control group	0.686±0.026			0.25	0.8182*
Low-concentration oleic acid	0.844±0.021			8.19	0.0012#
High-concentration oleic acid	1.131±0.016			18.83	0.0000 ^Δ

*: $P > 0.05$ compared with silver nitrate group; #: There was significance compared with control group; ^Δ: There was significance compared with low concentration acid group.

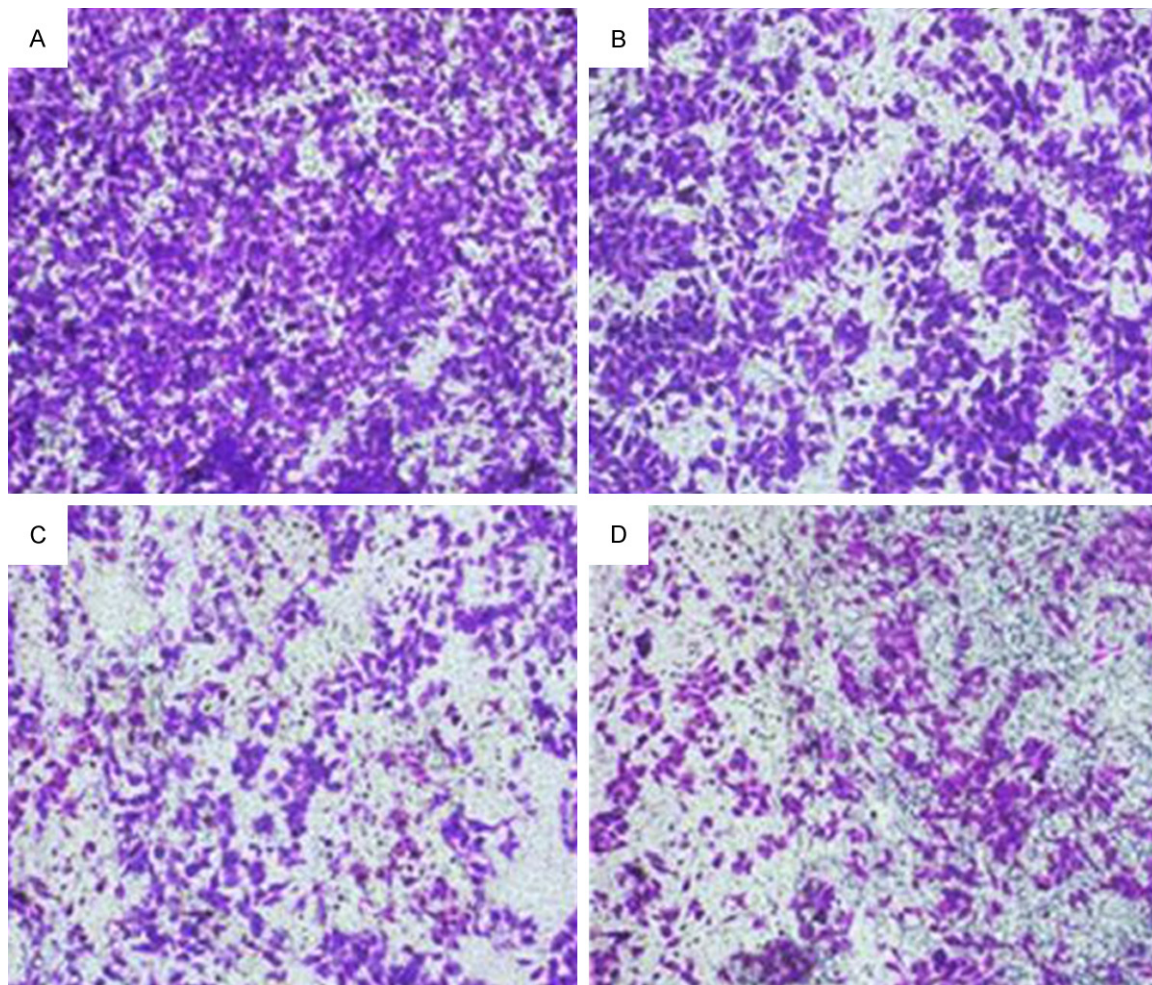


Figure 2. Cell migration ability of different groups was detected by transwell cell migration experiment. Notes: A: High-concentration oleic acid group; B: Low-concentration oleic acid group; C: Silver nitrate group; D: Control group.

the liver cells, which increases the uptakes of glycerol, promotes gluconeogenesis and raises blood glucose. The expression of AQP9 is down-regulated by insulin, which reduces the uptakes of glycerol, lower blood glucose and thus maintains the stability of blood glucose after eating. In addition, AQP9 is related to the secretion of biliary secretion. Previous study has showed that extrahepatic cholestasis caused by bile

duct ligation can downregulate the expression of AQP9 in base serosa of liver cells [2].

HCC, similarly to other tumor, is caused by the imbalance of regulatory mechanism of the cell proliferation and apoptosis. Cell apoptosis is a highly conserved controlled cell removal process and the speed of the process is directly influenced by permeability of serosa to water.

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Table 2. The migration rate of HepG2 cells in different groups

Groups	Migration Rate	<i>F</i>	<i>P</i>	<i>t</i>	<i>P</i>
Silver nitrate group	1.003±0.021	235.343	0.0000	—	—
Low-concentration oleic acid	0.806±0.021			11.49	0.0003*
High-concentration oleic acid	0.528±0.036			11.55	0.0003#

*: There was significance compared with silver nitrate group; #: There was significance compared with low concentration acid group.

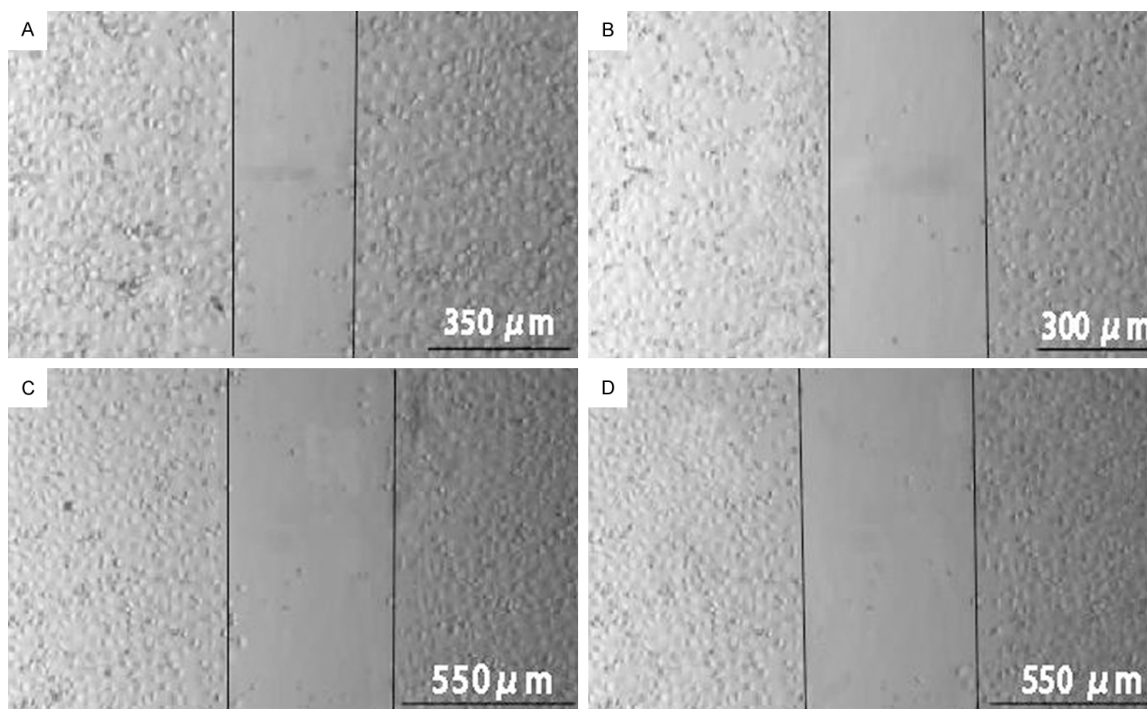


Figure 3. Cell migration ability of different groups were detected by the scratching experiment. Notes: A: High-concentration oleic acid group; B: Low-concentration oleic acid group; C: Control group; D: Silver nitrate group.

Jablonski et al. [13] pointed that dependent water flow of AQP9 inhibited the formation of apoptosis body, and the overexpression of AQP9 increased the permeability of serosa to water and apoptosis rate. Our preliminary study has proved that the expression of AQP9 in HCC cells is obviously lower than in para-carcinoma tissue, indicating that HCC cells failed to complete the normal physical function of the liver with the reduced expression of AQP9, thus resulting in the enhancement of resistance to apoptosis stimulation and the inhibition of apoptosis body formation. Those research results have proved that the expression of AQP9 influences the occurrence and development of HCC, therefore whether mechanism is associated with migration ability of HCC cells or not? At present, multiple researches have indicated that AQPs such as AQP3, AQP4 and AQP5

members can promote the migration of tumors [14-18]; however, studies on the influence of AQP9 on migration ability of HCC cells are rarely reported.

The present study explores the influence of the up-regulated AQP9 on migration ability of HCC cells by the scratching experiment and Transwell cell migration experiment. The results showed that with the concentration of oleic acid increasing, the expression of AQP9 was increased in dose-dependent manner and the migration ability of HCC HepG2 enhanced. Additionally, the migration ability after pre-treating with AQP9 inhibitor in oleic acid treatment group is similar to the control group, indicating that the AQP9 was upregulated by oleic acid and enhanced the migration ability of cells. These results indicated that the increase of AQP9

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expression can enhance the migration ability of HCC HepG2 cells.

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Disclosure of conflict of interest

None.

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