RESEARCH ARTICLE

Increased Migration and Local Invasion Potential of SiHa Cervical Cancer Cells Expressing Aquaporin 8

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Abstract

Overexpression of several aquaporins (AQPS) has been reported in different types of human cancer but roles in human carcinogenesis have yet to be clearly defined. Here, we up-regulated expression of the AQP8 gene in SiHa human cervical cancer cells with a lentivirus transfection system and investigated its effects as a potential therapeutic target for cervical cancer. Results showed AQP8 overexpression did not affect their substrate adherence and proliferation, but accelerated migration as assessed by transwell migration and wound healing assays. Moreover, AQP8 overexpression significantly enhanced local invasion of SiHa cells in nude mice. These findings altogether indicate that AQP8 overexpression increases migration of SiHa cells and probably participates in the process of tumor local invasion.

Keywords: Lentivirus - cell transfection - AQP8 - cell migration

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Introduction

Cervical cancer is generally the most common cancer in developing countries and the second most common in women globally (Farivar et al., 2012), metastasis and invasion are the important factors of mal-curative effect and unfavourable prognosis for cancer of cervix. Tumor cell migration is a key step of tumor invasion and metastasis (Harlozinska, 2005). The aquaporins (AQPs) are a family of hydrophobic, small, and integrated transmembrane proteins (30kDa monomer) that distribute widespreadly in the bodies of animals and plants. Up to now, the discovery of AQPs, 13 mammalian homologs (AQP 0~12) have been identified (Verkman et al., 2008). Recent studies indicated the key role of AQPs in tumor cell migration, proliferation, and invasion. Gene therapy is an experimental treatment that introduces genetic material into tissues or cells for treatment of diseases. Lentivirus vectors, which derived from HIV-1, show exceptional promise as gene transfer agents and have been proven to be effective vehicles for transduction of cancer cells of various organs (Castellani et al., 2010).

In this study, we delivered AQP8 into SiHa human cervical carcinoma cells via lentivirus vectors labeling red fluorescent protein(RFP) in order to explore AQP8 effects on SiHa cells migration and invasion potential in vitro and in vivo.

Materials and Methods

Materials

Lentiviral expression vector pLVX-IRES-tdTomato-RFP (Clontech, America), and Lentiviral Packaging Mix (K4975-00, Invitrogen) contains a mixture of the pLP1, pLP2, and pLP/VSVG plasmids. Plasmid extract kit (Qiagen, Germany). BD chamber (354480, America).

Animals 5-week female inbreeding line nude mice with the background of BALB/C, grade SPF (Specific pathogen Free, SPF), all animal experiments were approved by Animal Experiments Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University.

Cell culture

SiHa (bestowed by Ma Zheng-hai professor from Xinjiang University, China) and 293T cells were cultured at 37°C in a humidified incubator containing 5% CO_2 with Dulbecco's modified Eagle medium(DMEM) supplemented with 100 U ml-1 penicilin, 0.1 mg ml^{-1} streptomycin, and 10% FBS.

Cell stable transfection mediated by lentivirus

Build recombinant lentivirus plasmids: according to AQP8 mRNA sequence (NM_ 001169.2) in genebank, AQP8 gene was synthesized to the vector pUC57, the size was 807bp. The building process was done according to manufacture's instruction. Shortly, pUC57-AQP8 were linked with the plasmid pLVX-IRES-tdTomato-RFP digested with EcoR I and BamH I, then linked products were transducted into competent cells JM109, and pLVX-IRES-tdTomato-RFP-AQP8 plasmids were extracted from positive clones and identified by electrophoresis and sequencing, and the sequencing results were compared to BLAST. Package and determine the titer of lentivirus: The lentivirus particles were produced by cotransfecting 293T

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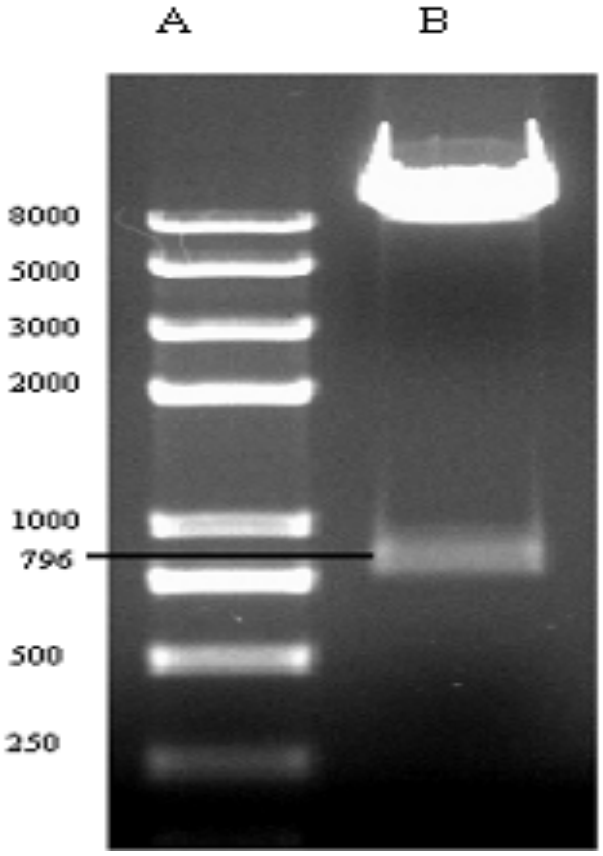

Figure 1. Electrophotogram of Lentiviral Expressing Plasmids with AQP8 (A: marker, B: AQP8)

cells with the transfer vector and three packaging vectors: pLP1, pLP2, and pLP/VSVG. Subsequent purification, concentration and titer determination of lentivirus were performed. Cells stable transfection: SiHa cells were cultured in 6-well plates, lentivirus particles were added into 6-well plates at MOI (multiplicity of infection, MOI)=50. Monoclonal cells with the strongest red flourescence were picked by limiting dilution assay and then expanded to culture. SiHa cells containing negative sequence were obtained by same methods. SiHa cells were divided into 3 groups: the non-transfected control group, the lentivirus-RFP-AQP8 transfected group (lentivirus-RFP-AQP8) and the lentivirus-RFP transfected group (containing the negative sequence).

Transwell migration and invasion assays

Using BD chamber containing a PET (polyethylene terephthalate)membrane with a thin layer of matrigel basement membrane matrix (6.5 mm diameter, 8 um pore size). The procedure was done according to manufacturer's instructions.

Wound healing assay

The procedure was done as described (Hu et al., 2006). Shortly, cells were cultured as confluent monolayers in 6-well plates, synchronized in 1% FBS for 24 h, and wounded by removing a 300~500 μm-wide strip of cells across the well with a standard 10μl pipette tip, followed to culture in 10% FBS for 24 h. Take photoes under microscope(10×5) at 0 h, 24 h, respectively, and counting wound healing mean speed.

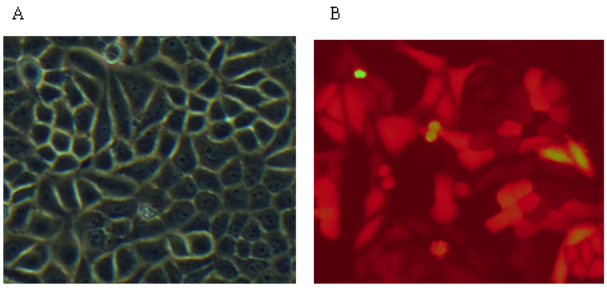

Figure 2. Stable Transfection of AQP8 Gene into SiHa Cells by Lentivirus (A: the graph under white light; B: under fluorescence microscope, red flourescence represents AQP8 expression in SiHa cells)

Cell proliferation and substrate adherence assays

Seeding cells in 24-well plates at a density of 2×10^4/ml and counting triplicate wells every 24 h for 7 days, cells proliferation curves were generated. For measurements of cell substrate adherence, seeding cells at a density of 2×10^4/ml in 24-well plates coated with 40 μg/ml fibronectin for 1 h at room temperature, plates were incubated for 2 h, at 37 °C in 5% CO_2, and nonadherent cells were gently washed away 3 times using D-Hanks. Adherent cells were trypsinized, counted with a blood counting chamber.

Tumor xenografts in nude mice

Five-week-old female nude mice were injected subcutaneously with 1×10^7 SiHa cells transduced with lentivirus-RFP -AQP8 or lentivirus-RFP(eight mice each group). Tumor length (L) and width(W) were measured with a caliper for estimation of tumor volumes as 0.52×L×W2 every 3 days for 27 days . Nude mice were killed after 27 days, xenografts were fixed in 4% paraformaldehyde and stained using H.E.

Statistical Analysis

Using SPSS 13.0 statistical software package, data were compared using one-way ANOVA, Student's T test and Fisher exact test; $P < 0.05$ was considered to be statistically significant.

Results

Characterization of SiHa cells stably expressing AQP8

Plasmids were extracted from positive clones of JM109 cells and identified by electrophoresis, the fragment size linked with AQP8 was 796bp (Figure 1). After package and transfection of lentivirus, Monoclonal SiHa cells labeling RFP were picked by limiting dilution assay and cultured. Transfection efficiency was about more than 90% (Figure 2 A, 2B).

Figure 3 summarizes proliferation and growth studies. Cell proliferation assays showed that differences of proliferation after cultured for 1~7 days of SiHa cells among three groups were not statistically significant (Figure 3, $P>0.05$). Adherence rates among lentivirus-RFP-AQP8-SiHa, lentivirus-RFP- SiHa and SiHa cells groups were 61.30%, 58.21%, 57.62%, respectively, the differences were not statistically significant ($P>0.05$).

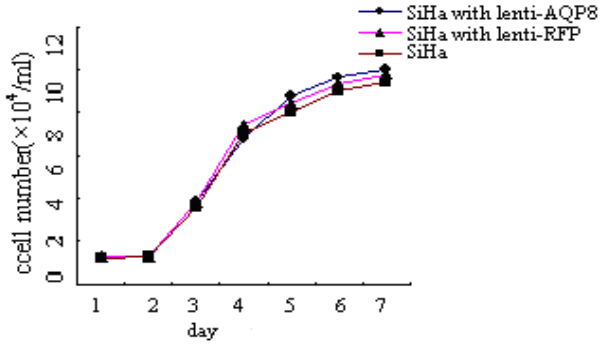

Figure 3. Cell Proliferation Assay. Cell numbers of cultured lentivirus-RFP -AQP8-SiHa in 7 days were not different from SiHa with lentivirus-RFP and SiHa (n=3, x±s, *P*>0.05)

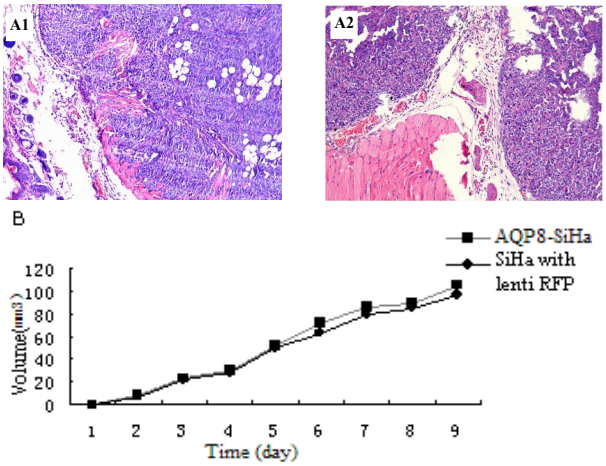

Figure 4. Tumor Xenografts in Nude Mice. A1) Image under microscope of subcutaneous xenograft with lentivirus-RFP -AQP8, xenografts invasion into subcutaneous adipose and muscle were seen, A2) Image under microscope of subcutaneous xenograft with lentivirus-RFP (H.E. staining, 10×10); B)

AQP8 expression did not significantly effect the adherence or growth of SiHa cells.

Increased in vitro migration of AQP8-expressing SiHa cells

Analysis of migration was done by using transwell migration and invasion and wound healing assays. Migration rate of lentivirus-RFP-AQP8-SiHa was 43.68%, significantly faster than 33.46% of lentivirus-RFP- SiHa and 36.24% of SiHa cells (*P*=0.000), the difference between lentivirus-RFP-SiHa and SiHa cells was not significant (*P*>0.05). In agreement with the transwell assay, a complementary wound healing assay of cell migration in vitro showed that wound closure speed in lentivirus-RFP-AQP8-SiHa was 285.28 Чm, obviously faster than 222.99 Чm of lentivirus-RFP- SiHa and 232.34 Чm of SiHa cells at 24 h after scratch (*P*=0.000), the difference between lentivirus-RFP-SiHa and SiHa cells was not significant (*P*>0.05).

Increased local invasion of SiHa cells expressing AQP8 in xenografts of nude mice

The effects of AQP8 overexpression on the invasion of SiHa cells were investigated. Nude mice were injected subcutaneously with SiHa transduced with lentivirus-RFP or lentivirus-RFP -AQP8. By 27 days after cells injection, tumor growth ,as assessed by tumor volume at different times, was not affected by AQP8 expression (*P*>0.05). However, SiHa cells invasion into subcutaneous adipose and muscle were seen with lentivirus-RFP-AQP8 but not control cells (*P*=0.041, Figure 4A, 4B).

Discussion

Recent Studies showed that AQPs overexpressed in diverse tumor tissues and theirs vascular endothelial cells, tumor cell lines, which alluded to the role of AQPs in the development of human cancers. For example, AQP1 overexpression promoted human colon cancer cell line HT20 migration (Jiang, 2009), and was also related to lung cancer and angiogenesis (Li et al., 2010; Xie et al., 2012). AQP3 expression increased in esophageal and oral cancers, as did AQP5 in chronic myelogenous leukemia (Chae et al., 2008; Kusayama et al., 2011). But there is few reports concerning the expression and role of AQPs in human cervical carcinogenesis so far.

We previously demonstrated that AQP8 expression upregulated in CIN (cervical intraepithelial neoplasia, CIN) and cervical carcinoma tissues by real-time PCR and immunohistochemisty. In this study, we upregulated AQP8 expression in SiHa human cervical cancerous cells by lentivirus vector, and further investigated AQP8 effects on SiHa cells migration, proliferation, substrate adherence in vitro and local invasion potential in vivo. We found that AQP8 overexpression in SiHa cells predominantly increased the migration and wound closure speed of SiHa cells by transwell migration and invasion and wound healing assays. Moreover, stable expression of AQP8 gene led to the xenografts invading into subcutaneous adipose and muscle in vivo. The above indicated that AQP8 overexpression in SiHa cells accelerated the migration and local invasion potential of SiHa cells in vitro an in vivo, and hinted that AQP8 perhaps possessed some clinical significance as a oncotherapy target potential. Galamb et al. (2007) also pointed that AQP8 overexpressed in colorectal cancer by using gene chips. Cell culture confirmed that cultured aortic endothelial cells from wild-type and AQP1 null mice had comparable morphology, growth, and adherence to different surfaces but showed remarkable impairment in their migration (Saadoun et al., 2005). SPC-A1 cells, a lung adenocarcinoma cell line in which AQP5 gene was silenced showed a 55.3% decrease in migration and a 28.4% decrease in invasion potential (Chen et al., 2011). It was reported that the positive rate of AQP8 in cervical squamous-cell carcinoma was 98% (Yao et al., 2008). Our results coincided with the above reports. But Jablonski et al. (2007) reported that AQP8 expression down- regulated in mouse hepatocellular carcinoma model. AQP8 was mainly expressed in paraneoplastic normal tissues and barely expressed in colorectal carcinoma cells (Wang et al., 2012). These contradictory reports indicate the necessity of further research on effects of AQPs in tumorigenesis.

Malignant tumor cells usually exhibit local invasiveness or infiltration. Tumor cells invasion into surrounding tissue involves the active migration of tumor cells across the endothelial barrier and penetration through underlying basement membrane. We found here AQP8-

overexpressing SiHa cells showed increased migration and invasiveness potential compared to control cells, but similar proliferation, adherence to basement membrane and similar growth in nude mice. Some mechanisms may be proposed to explain how AQPs facilitate cell migration and thus their invasion potential: AQPs facilitate rapid water flow across the plasma membrane into the front end of migrating cells. The rapid transmembrane water influx is driven by changes in osmolality produced by transmembrane ion flux and actin depolymerization. Cells migration through the irregularly shaped extracellular space undergo rapid changes in their volume, accompanied by rapid changes in transmembrane water fluxes (Verkman et al., 2008).

Here We confirmed that AQP8 overexpression can obviously increase SiHa cells migration in vitro and local invasion potential in vivo, but has no effects on SiHa cells proliferation and substrate adherence. It will further require to be confirmed in more tumor cell lines and studies in vivo in future.

Acknowledgements

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References

Castellani S, DiGioia S, Trotta T, et al (2010). Impact of lentiviral vector-mediated transduction on the tightness of apolarized model of airway epithelium and effect of cationic polymer polyethylenimine. *J Biomed Biotechnol*, **2010**, 103976.

Chae YK, Kang SK, Kim MS, et al (2008). Human AQP5 plays a role in the progression of chronic myelogenous leukemia(CML). *PLoS One*, **3**, e2594.

Chen Z, Zhang Z, Gu Y, et al (2011). Impaired migration and cell volume regulation in aquaporin 5-deficient SPC-A1 cells. *Respir Physiol Neurobiol*, **176**, 110-7.

Farivar TN, Johari P, Shafei S, et al (2012). Lack of association between herpes simplex virus type 2 infection and cervical cancer--Taq Man realtime PCR assay findings. *Asian Pac J Cancer Prev*, **13**, 339-42.

Galamb O, Gyorffy B, Sipos F (2007). Identification of colorectal cancer, adenoma, and inflammatory bowel disease specific gene expression patterns using whole genomic -oligonucleotide microarray system. *Orv Hetil*, **148**, 2067-79.

Harlozinska A (2005). Progress in molecular mechanisms of tumor metastasis and angiogenesis. *Anticancer Res*, **25**, 3327-33.

Hu J, Verkman AS (2006). Increased migration and metastatic potential of tumor cells expressing aquaporin water channels. *FASEB J*, **20**, E1228-36.

Jablonski EM, Mattocks MA, Sokolov E, et al (2007). Decreased aquaporin expression leads to increased resistance to apoptosis in hepatocellular carcinoma. *Cancer Lett*, **250**, 36-46.

Jiang Y(2009). Aquaporin-1 Activity of Plasma Membrane Affects HT20 Colon Cancer Cell Migration. *IUBMB Life*, **61**, 1001-9.

Kusayama M, Wada K, Nagata M, et al (2011). Critical role of aquaporin 3 on growth of human esophageal and oral squamous cell carcinoma. *Cancer Sci*, **102**, 1128-36.

Li Q, Zhang W, Zhang B (2010). The correlation between the expression of AQP1 and angiogenesis ability in nasopharyngeal carcinoma. *Lin Chuang Er Bi Yan Hou Tou Jing Wai Ke Za Zhi*, **4**, 1115-8.

Saadoun S, Papadopoulos MC, Hara-Chikuma M, et al (2005). Impairment of angiogenesis and cell migration by targeted aquaporin-1 gene disruption. *Nature*, **434**, 786-92.

Verkman AS, Hara-Chikuma M, Papadopoulos MC(2008). Aquaporins new players in cancer biology. *J Mol Med*, **86**, 523-29.

Wang W, Li Q, Yang T, et al (2012). Expression of AQP5 and AQP8 in human colorectal carcinoma and their clinical significance. *World J Surg Oncol*, **13**, 242.

Xie Y, Wen X, Jiang Z, et al (2012). Aquaporin 1 and aquaporin 4 are involved in invasion of lung cancer cells. *Clin Lab*, **58**, 75-80.

Yao JF, Zhou CY, Wei LF, et al (2008). Expression of aquaporin-8 and bcl-2 protein in human cervical carcinoma and their correlations. *Zhonghua Fu Chan Ke Za Zhi*, **43**, 205-8.