Full Length Research Paper

Down-regulation of aquaporins (AQP3) expression by RNA interference suppresses human lung cancer cell proliferation

Jing Zhang[1], Yun-Chao Huang[2]*, Qi Zhang[1], Zhi-Peng Hong[1], Yong-Chun Zhou[2], and Wan-Ling Chen[3]

[1]First Affiliated Hospital, KunMing Medical College, Yunnan, 650032, China.
[2]Third Affiliated Hospital, KunMing Medical College, 650032, China.
[3]Yunnan Provincial Corps Hospital, Chinese People's Armed Police Forces, 650032, China.

Aquaporins (AQPs) represent a family of homologous water channels expressed in many epithelial and endothelial cells. Most tumors have been shown to exhibit high vascular permeability and interstitial fluid pressure. Here, we tested the regulation on the expression of AQP3 by RNA interference (RNAi) in the human lung cancer cell line (XWLC-05) and observed the changes of proliferation and adhesion ability. The results show that AQP3 mRNA transcripts were decreased to 65 and 79%, respectively (P<0.05). Western blot analyses also revealed the AQP3 protein was decreased to 53 and 73% (P<0.05). MTS assay showed that proliferation of XWLC-05 was significantly inhibited by RNAi after 48 and 72 h transfection. The invasion assay demonstrated that the number of XWLC-05 cells penetrating the membrane in the transfected group was considerably lower than those in the untransfected and negative control group (P<0.05). These results indicate that vector-based shRNA could be used as a potential tool to inhibit the expression of AQP3.

Key words: Lung cancer, RNA interference, AQP5, cell adhesion ability, invasion ability.

INTRODUCTION

Lung cancer is a disease of uncontrolled cell growth in tissues of the lung. This growth may lead to metastasis, which means the invasion of adjacent tissues and infiltration beyond the lung. The vast majority of primary lung cancers are carcinomas of the lung, derived from epithelial cells. Lung cancer, the most common cause of cancer-related death in men and women, is responsible for 1.3 million deaths worldwide annually, as of 2004. Current therapies for lung cancer mainly take forms of surgery, radiotherapy and pharmacological approaches. Although, they are helpful to some degree none of them can offer a permanent cure. For patients with leading lung cancer, even extensive surgical operations combined with chemotherapy have not sufficiently brought about improved prognosis (Dovedi and Davies, 2009; Felip et al., 2010; Germain et al., 2010). In recent years, it has been demonstrated that gene therapy is one of the possible candidates for an innovative therapeutic approach for the treatment of advanced gastric carcinoma (Yamamoto et al., 2009; Li et al., 2010). Aquaporins (AQPs) are proteins embedded in the cell membrane that regulate the flow of water. They are "the plumbing system for cells" (Yan et al., 2010). Aquaporin genes can function in forming tetramers in the cell membrane to facilitate the transport of water and in some cases, other small solutes across the membrane. Their defects can lead to several human diseases (Agre and Kozono, 2003; Agre, 2006).

Human AQP1 is naturally expressed in erythrocytes and many epithelial and endothelial tissues, including kidney, choroids plexus, bile duct, gall bladder, eye lens,

---

*Corresponding author. E-mail: ling_zhangcn @126.com Tel: 0871-5324888 -2403.

Abbreviations: AQPs, Aquaporins; **RNAi,** RNA interference.

brain, and placenta (Cheng et al., 1997; Schrier, 2007). AQP3 acts as the membrane channel of water and other small solutes, which plays a major role in fluid homeostasis. AQP3 facilitates water transport in epidermal cell migration and glycerol transport in epidermal cell proliferation (Heymann et al., 1998). AQP3 plays an important role in the maintenance of water homeostasis. Inhibition of the AQP3 water channel can increase the sensitivity of prostate cancer cells to cryotherapy (Hara-Chikuma and Verkman, 2008). In addition, AQP3 is widely expressed in the normal respiratory tract. Lung carcinomas, especially adeno-carcinomas, can produce AQP3, possibly in connection with their functional and/or biological nature, although, the detailed mechanism of AQP3 expression in lung carcinomas remains to be clarified (Liu et al., 2007).

RNA interference (RNAi) is an evolutionarily conserved process in which recognition of double-stranded RNA (dsRNA) ultimately leads to posttranscriptional sup-pression or gene expression. This suppression is mediated by dsRNA (21 to 23 nucleotides), which induces degradation of mRNA based on complementary base pairing (Elbashir et al., 2001; Brummelkamp et al., 2002).

In this study, we attempted to observe the down-regulation of AQP3 gene induced by vector-based small hairpin RNA (shRNA) in human lung cancer cells, and detected the changes of proliferation and adhesion ability after AQP3 gene was suppressed.

MATERIALS AND METHODS

Cells and culture conditions

The human lung cancer cell lines XWLC-05 were supplied by the Institute of Clinical Cancer, Kunming Medical College, China. These cell lines were cultured in RPMI 1640 (Sigma, St. Louis, MO, USA) at 37°C in an atmosphere of 5% CO_2 in the air with 10% fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA, USA).

Construction of pAQP3-siRNA

DNA sequence of AQP3 (GenBank no: BC013566) was inputed into Dharmacon web-based software for selecting target sequences. The following criteria were used to identify targets for siRNAs from the AQP3 cDNA coding sequence: (a) start with an AA dinucleotide; (b) 21 nucleotides in length; (c) G/C content of < 50% and (d) no sequence homology to other coding sequences on BLAST search. Using these criteria, two siRNA sequences from human AQP3 were chosen as follows: 5′-CGAGGAAGAGAAUGUGAAG-3′ (A1), 5′-GAGAAUGUGAAGCUGGCCC-3′ (A2). One negative control siRNA containing a scrambled sequence with the same nucleotide composition was also selected. Sense and antisense primers containing the sense siRNA sequence, 9 bp loop sequence, antisense siRNA sequence, and RNA polymerase III terminator sequence were created at *Bam*H I and *Hin*d III restriction sites on the 5′ and 3′ ends, respectively. These primers were annealed and inserted into pSilencer 4.1-CMV neo (Ambion, Inc., Austin, TX) downstream of the H1RNA polymerase III promoter following the manufacturer's instructions. The resulted plasmids containing

siRNA sequences A1, A2 and negative control sequence NC were named pAQP3-siRNA1, pAQP3-siRNA2 and pAQP3-N.

Cell transfection

Cells were cultured in the RPMI 1640 medium, seeded at 1×10^6 /well in 6 well plates. When the cells reached 80 to 90% confluence, transfection of dsRNA was performed with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Briefly, one day before transfection, the attached cells in logarithmic growth phase were respectively implated into 12-well plates at density of 1×10^5 /well. Transfection mixes containing Solution A (5 μg plasmid + 100 μl OptiMEM medium) and Solution B (10 μl Lipofectamine^{TM} 2000 + 100 μl OptiMEM medium) were prepared in 96-well plates. When cells reached 70% confluence, the plasmid DNA was transfected into the cells. The transfection efficiency was observed by fluorescence microscopy after 24 h. Cells were transfected for 6 h per day, lasted for 3 days and were collected in the 4th day. The transfection concentration of the experimental group shRNA was 1.0, 2.5 and 5.0 µg/L respectively.

RNA isolation and RT-PCR analysis

Total RNA was extracted with Trizol reagent according to the protocol described by the supplier (TakaRa, Dalian, China). cDNA was obtained from the total RNA by reverse transcriptase polymerase chain reaction (RT-PCR) according to the standard protocols (Wang et al., 2009). PCR primers were designed as follows: (300bp) upstream: 5'- AGGCGATTAAGTTGGGGTA-3' and downstream: 5'- CGGTAGGCGTGTACGGTG-3'; Amplification reactions (25 µl) contained 1µl (50 ng) of cDNA, 2 µl (10 µmol/L) of each primer, 0.5 µl (10 µmol/L) of probe, 12.5 µl of PCR mix and 7 µl of deionized water. Mixed them gently and amplified by real time RT-PCR, cycling parameters were 55°C for 2 min, then 35 cycles of 93°C for 10 min, 93°C for 10 min and 60°C for 55 min. β-actin was used as the control. The targeted DNA amplified specifically was confirmed by electrophoresis and sequencing. PCR products were analyzed using GelWorks software, after the ethidium bromide-stained 1.5% agarose gel was scanned.

Western blot

Cells transfected with various vectors were lysed in RIPA buffer (1% NP-40, 50 mmol/L Tris, 150 mmol/L NaCl) 48 h after transfection. Proteins in an equal amount were separated by gel electrophoresis and transferred into a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk and then incubated with 1:1000 dilution of primary antibody overnight at 4°C. Subsequently, the membrane was washed and incubated with a secondary peroxidase-conjugated antibody for 1 h after being washed. Antibody binding was detected by using an enhanced chemiluminescence detection system. Western immunoblotting films were digitized, and band intensities were quantified by a Millipore Digital Bioimaging System (Bedford, MA). β-actin protein ratio was calculated for each protein.

MTS assay

Cell proliferation was assayed by the CellTiter 96 AQ non-radioactive cell proliferation assay (MTS) from Promega (Madison, WI). After 24, 48 and 72 h, 20 µl of freshly prepared MTS/PMS solution was added to each well, and the mixture was incubated for 2 h at 37°C. Optical density was read directly at 490 nm using the ELISA plate reader. All samples were assayed at least in quadruplicate with an appropriate blank.
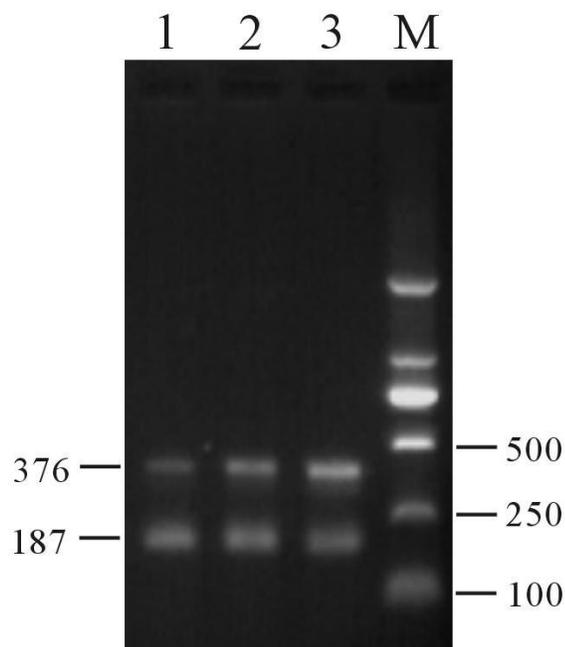
Figure 1. Regulation of the expression of AQP3 mRNA by shAQP3 in XWLC-05 cells. The inhibitory effects on pAQP3-siRNA1 group (Lane 1), pAQP3-siRNA2 (Lane 2) and non-transfected control group (Lane 3) were analyzed, among which AQP3 mRNA was measured over a 2-day post-transfection period. β-actin was used as the internal control.

Matrigel invasion assay

Matrigel matrix (Becton Dickinson Labware, Franklin Lakes, NJ) was applied and polymerized in 24-well 9-mm inserts containing polyethylene terephtphalate (PET) membranes with 8-µm pores to create invasion chambers as directed by the supplier (Becton Dickinson). Matrigel was thawed out at 4°C overnight on ice. Pipettes, plates and tips were chilled at -20°C. Matrigel (5 mg/ml) in serum-free cold DMEM was diluted with pre-cooled pipettes. 100 µl (h = 100 ul/200 mm 2 = 0.5 mm) of the diluted matrigel per well may be gently pipetted using a pre-cooled pipette to ensure homogeneity. The transwell was incubated at 37°C for 30 min to 5 h for gelling.

Cells were harvested when they grew to near confluence by trypsinization, which was inactivated with medium containing bovine calf serum, and cells were subsequently washed twice in DMEM without added serum or proteinase inhibitor. 100 µl of the cell suspension was put onto the matrigel. Lower chamber of the transwell was filled with 600 µl (3 mm) of culture medium containing 5 µg/ml fibronectin, as an adhesive substrate. Incubated at 37°C for 48 h, then removed transwells from 24-well plates and stained with Diff-Quick solution. Noninvaded cells were scraped off on the top of the transwell with a cotton swab, and invaded cells were counted on the transwell under a light microscope.

Statistical analysis

Experiments were performed at least in duplicate, and a typical set of data was indicated. Differences were statistically evaluated by Student's t-test. A P-value of less than 0.05 was considered to be statistically significant.
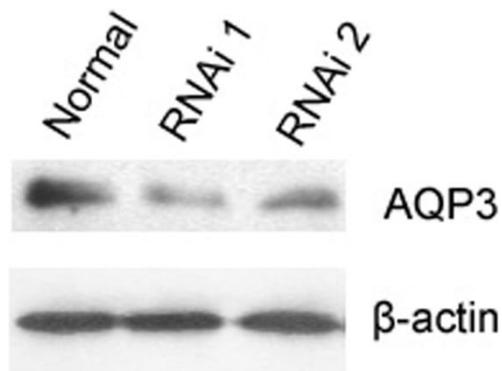
Figure 2. Suppression of the expression of AQP3 by shRNA in XWLC-05 cells. shRNA-expressing vector, pAQP3-siRNA1 or pAQP3-siRNA2 was transfected into XWLC-05 cell. After 2 days of tranfection, cell proteins were extracted and subjected to 12% SDS-PAGE. The separated proteins were transferred onto PVDF membrane and detected using antibodies against AQP3 and β-actin.

RESULTS

Inhibition of AQP3 expression by pAQP3-siRNA

To determine the inhibitory effect of shRNA on AQP3, three groups were divided: (1) non-transfected control group, (2) pAQP3-siRNA1 transfected group and (3) pAQP3-siRNA2 transfected group. AQP3 mRNA levels were assessed by RT- PCR, using β-actin as the internal control. 2 days after transfection, AQP3 mRNA was reduced by 35 and 21% in pAQP3-siRNA1 and pAQP3-siRNA2 groups, respectively compared to the control group (P<0.05), and the inhibition level of pAQP3-siRNA1 was significantly better than that of pAQP3-siRNA2 (Figure 1). Western blot analysis showed the suppression effect on AQP3 protein appeared at 48 h post-transfection. Total proteins from the non-transfected control group, pAQP3-siRNA1 and pAQP3-siRNA2 transfected groups were extracted 2 days after transfection and Western blot analyses were performed (Figure 2). Expression of AQP3 decreased significantly in the presence of XWLC-05 shRNA. The inhibitory rates were 47 and 27% by pAQP3-siRNA1 and pAQP3-siRNA2, respectively (P<0.05, compared to the control group). The results show that the inhibition level of pAQP3-siRNA1 was significantly better than that of pAQP3-siRNA2, which was similar to the result of mRNA analysis.

MTS assays

In this study, the effect of AQP3-shRNA on the growth of XWLC-05 at 24, 48 and 72 h after transfection were investigated (Figure 3). The results show that cell proliferation was not significantly inhibited at 24 h after
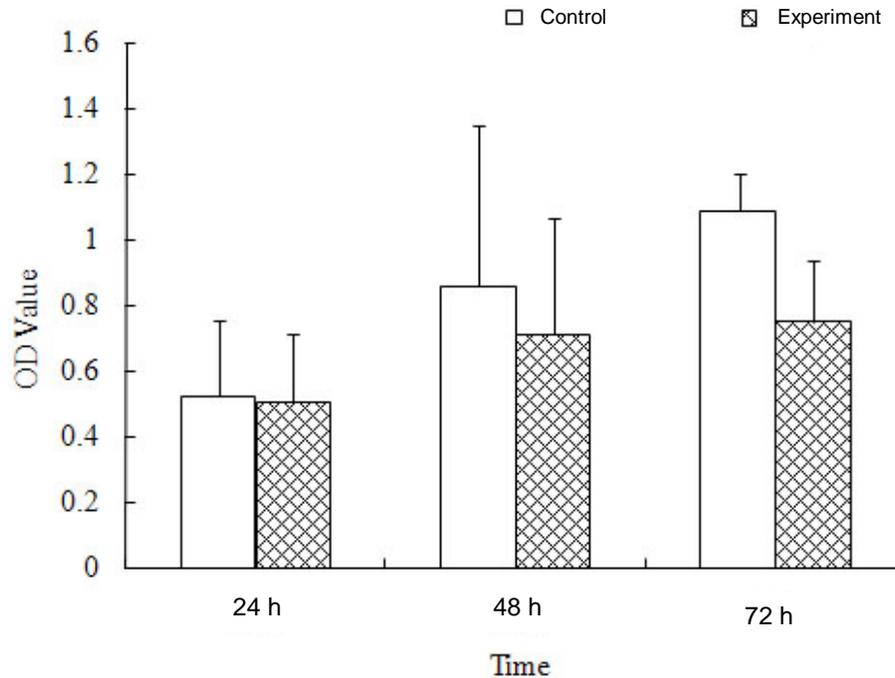
Figure 3. MTT assays were performed to measure the survival rate of XWLC-05 cells after treatment with shAQP3. Data are expressed by the mean of percent cell viability compared to control. Each bar represents the mean ± standard deviation of 4 to 6 experiments (P<0.05).

transfection. However, at 48, and 72 h post-transfection, the cell proliferation was significantly inhibited (P<0.05), suggesting silencing of AQP3 could inhibit the growth of lung cancer XWLC-05.
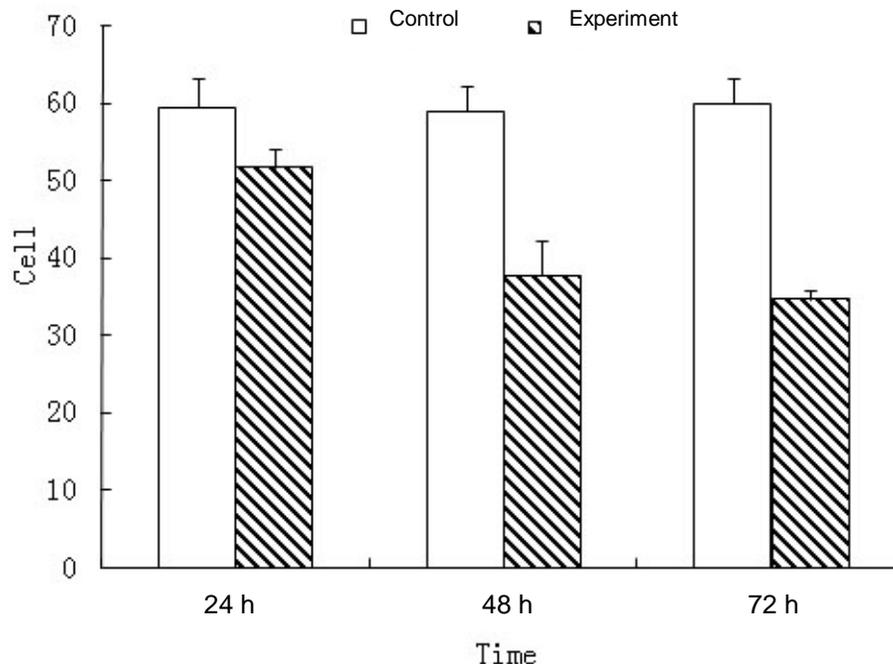
Matrigel invasion assays

The lung cancer cell invasion of Matrigel matrix was inhibited when the human lung cancer XWLC-05 cell was transfected by the pAQP3-siRNA1 (Figure 4). It could effectively decrease invasion at 24, 48 and 72 h after tranfection (P<0.05 as compared to control). In addition, there was a statistically significant difference between 24 h group, and the post-transfection 48 and 72 h groups (P<0.05). However, such significant difference did not appear between the 48 and 72 h transfected groups (P>0.05).

DISCUSSION

Aquaporins are membrane water channels that play critical roles in controlling the water contents of cells. Aquaporins have been proposed as a novel target in cancer and oedema, and are associated with surprising arrays of important processes in the brain and body, such as angiogenesis, cell migration, development and neuropathological diseases. Based on the fact that

current therapies for both cancer and brain are limited, new pharmacological approaches focusing on AQPs may offer exciting potentials for clinical advance (Yool et al., 2010). AQP1-null mice remarkably impaired tumor growth after subcutaneous or intracranial tumor cell implantation, with reduced tumor vascularity and extensive necrosis (Saadoun et al., 2005). AQP1 expression was intensely up-regulated in all glioblastomas studied (Oshio et al., 2005). AQP3 acts as the membrane channel of water and other small solutes and plays a major role in fluid home-ostasis. It can play an important role in the maintenance of water homeostasis. AQP3 is widely expressed in the normal respiratory tract. Lung carcinomas, especially adenocarcinomas, can produce AQP3, possibly in connection with their functional and/or biological nature (Liu et al., 2007).

Moreover, some reports showed that down-regulating the expression of AQP5 using siRNA approaches may inhibit the proliferation and invasion of cancer cells (Chen et al., 2006; Frigeri et al., 2007). RNA interference has become widely used *in vivo* knockdown of genes in cancer therapy. This study aimed to determine whether the silencing of AQP3 RNA interference may inhibit the growth of human lung cancer cell. The AQP3 gene expression was markedly decreased by RNAi based on RT-PCR and Western blot results, the proliferation of XWLC-05 was significantly inhibited at 48 and 72 h post-transfection, and the adhesion ability of XWLC-05 cells was also significantly decreased compared with the

Figure 4. Inhibition of XWLC-05 lung cancer cells in the Matrigel invasion assay. Assays were performed at least 3 times, and in triplicate wells. Data represents the percentage of cells migrating through the Matrigel-coated filters compared to uncoated filters seeded with the same number of cells, expressed as mean ± SEM.

untransfected group and negative control group (P<0.05). The invasion assays demonstrated that the number of XWLC-05 cells penetrating the membrane in the transfected group was significantly lower than those in the untransfected and negative control groups (P<0.05). In summary, AQP3 gene expression silenced by shRNA could inhibit the growth and invasion ability of lung cancer cells.

REFERENCES

Agre P (2006). The aquaporin water channels. Proc. Am. Thorac. Soc. 3: 5-13.

Agre P, Kozono D (2003). Aquaporin water channels: molecular mechanisms for human diseases. FEBS Lett. 555: 72-78.

Brummelkamp TR, Bernards R, Agami R (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science, 296: 550-553.

Chen Z, Zhu R, Bai L, Bai C (2006). Downregulation of aquaporin 5 induced by vector-based short hairpin RNA and its effect on MUC5AC gene expression in human airway submucosal gland cells. Respir. Physiol. Neurobiol. 152: 197-203.

Cheng A, van Hoek AN, Yeager M, Verkman AS, Mitra AK (1997). Three dimensional organization of a human water channel. Nature, 387: 627-630.

Dovedi SJ, Davies BR (2009). Emerging targeted therapies for bladder cancer: a disease waiting for a drug. Cancer Metastasis, Rev. 28: 355-367.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) . Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature, 411: 494-498.

Felip E, Rosell R, Maestre JA, Rodríguez-Paniagua JM, Morán T, Astudillo J, Alonso G., Borro JM, González-Larriba JL, Torres A,

Camps C, Guijarro R, Isla D, Aguiló R., Alberola V, Padilla J, Sánchez-Palencia A, Sánchez JJ, Hermosilla E, Massuti B (2010). Preoperative chemotherapy plus surgery versus surgery plus adjuvant chemotherapy versus surgery alone in early-stage non-small-cell lung cancer. J. Clin. Oncol. 28: 3138-3145.

Frigeri A, Nicchia GP, Svelto M (2007). Aquaporins as targets for drug discovery. Curr. Pharm. Des. 13: 2421-2427.

Germain MA, Bonvalot S, Rimareix F, Missana CM (2010). Locally advanced soft-tissue sarcomas. An innovating triad to avoid amputation: isolated limb perfusion, TNFalpha, and free microsurgical flap. Bull. Acad. Natl. Med. 194: 51-65.

Hara-Chikuma M, Verkman AS (2008). Aquaporin-3 facilitates epidermal cell migration and proliferation during wound healing. J. Mol. Med. 86: 221-31.

Heymann JB, Agre P, Engel A (1998). Progress on the structure and function of aquaporin 1. J. Struct. Biol. 121: 191-206.

Li Y, Sun DL, Duan YN, Zhang XJ, Wang N, Zhou RM, Chen ZF, Wang SJ (2010). Association of functional polymorphisms in MMPs genes with gastric cardia adenocarcinoma and esophageal squamous cell carcinoma in high incidence region of North China. Mol. Biol. Rep. 37: 197-205.

Liu YL, Matsuzaki T, Murata SZ, Murata S, Nakamura N, Kondo T, Iwashina M, Mochizuki K, Yamane T, Takata K, Katoh R (2007). Expression of aquaporin 3(AQP3) in normal and neoplastic lung tissues. Hum. Pathol. 38: 171.

Oshio K, Devin K, Yu L, Bollen A, Feuerstein B, Berger MS, Manley G.T (2005). Expression of the aquaporin-1 water channel in human glial tumors. Neurosurgery, 56: 375-381.

Saadoun S, Papadopoulos MC, Hara-Chikuma M, Verkman AS (2005). Impairment of angiogenesis and cell migration by targeted aquaporin-1 gene disruption. Nature, 434: 786-792.

Schrier RW (2007). Aquaporin-related disorders of water homeostasis. Drug News Perspect. 20: 447-453.

Wang T, Xue L, Ji X, Li J, Wang Y, Feng Y (2009). Cloning and characterization of the 14-3-3 protein gene from the halotolerant alga *Dunaliella salina*. Mol. Biol. Rep. 36: 207-214.

Yamamoto K, Fujiwara Y, Nishida T, Takiguchi S, Nakajima K, Miyata H, Yamasaki M, Mori M, Doki Y (2009). Induction chemotherapy with docetaxel, 5-FU and CDDP (DFP) for advanced gastric cancer. Anticancer Res. 29: 4211-4215.

Yan S, Zhang H, Xie Y, Sheng W, Xiang J, Ye Z, Chen W, Yang J (2010). Recombinant human interleukin-24 suppresses gastric carcinoma cell growth *in vitro* and i*n vivo*. Cancer Invest. 28: 85-93.

Yool AJ, Brown EA, Flynn GA (2010). Roles for novel pharmacological blockers of aquaporins in the treatment of brain oedema and cancer. Clin. Exp. Pharmacol. Physiol. 37: 403-409.