Effect of selective inhibition of aquaporin 1 on chemotherapy sensitivity of J82 human bladder cancer cells

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Abstract. The occurrence of resistance to mitomycin C (MMC) often limits its clinical effectiveness. Combination therapy thus is employed to overcome this treatment resistance. The present study aimed to establish a novel J82 bladder cancer cell line so as to study the effect of inhibition of aquaporin 1 (AQP-1) on chemotherapy sensitivity of J82 bladder cancer cells. A novel J82 bladder cancer cell line whose expression of AQP-1 is inhibited was established through transfection of J82 cells with newly constructed recombinant plasmid. The resulting cell line was designated J82-short hairpin (sh)AQP1 and was subjected to further analyses together with J82 cell line. Reverse transcription-polymerase chain reaction was used to quantify the expression of AQP-1mRNA in the cells; cell viability was analyzed with MTT assay and apoptosis was measured by flow cytometry. The expression of cell proliferation and cell apoptosis-associated proteins, proliferating cell nuclear antigen (PCNA), B cell lymphoma 2 (Bcl-2), Bcl-2 associated X protein (Bax) and caspase-3, were detected by Western blot. A statistically significant decrease in the transcription and expression of AQP1 was observed in the J82-shAQP1 cells as compared with J82 cells. J82-shAQP1 cells treated by MMC, also had a lower cell viability than J82 cells treated by MMC and showed enhanced apoptosis. Western blot analysis revealed J82-shAQP1 cells treated by MMC had less expression of PCNA, lower bcl-2/Bax ratio and more expression of caspase-3 as compared with the J82 cells treated by MMC. Selective inhibition of AQP-1 enhanced MMC chemotherapy sensitivity of J82 bladder cancer cells, suggesting combination of AQP-1 inhibition with MMC treatment as a promising treatment strategy to overcome bladder cancer treatment resistance.

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Introduction

Bladder cancer is a common urothelial cancer and the ninth most common type of cancer in the world with 430,000 new cases and 165,000 associated mortalities reported in 2012 (1-5). Bladder cancer may be divided into early stage, non-muscle invasive and higher stage muscle invasive disease (6). Mitomycin C (MMC) is a mitomycin that is often used as a chemotherapeutic agent for treatment of non-muscle invasive bladder cancer (7-11). It functions by binding to the DNA of cancer cells to prevent cell division and thereby inhibit the growth of the cancer. However, its clinical effectiveness is limited bythe occurrence of resistance to MMC (12-14). To overcome this treatment resistance, MMC is often combined with other agents to increase MMC chemotherapy sensitivity (15).

Aquaporins (AQPs) are small integral membrane proteins that function as molecular water channels that allow water to transported rapidly into the cell rather than diffusing slowly through the cell membrane (16). Presently, 13 members of AQPs have been reported (17). In addition, previous studies have demonstrated that AQPs are associated with the development of cancer (18,19). AQP1, a member of the AQP family, has been demonstrated to be involved in tumor angiogenesis (20). More specifically, a recent study revealed that the expression level of AQP1 in bladder uroepithelium cell carcinoma tissue was significantly increased compared with that in normal bladder tissue (21). As combination therapies possess great potential in overcoming treatment resistance, the present study hypothesized that a combination of AQP1 inhibition alongside MMC treatment may increase MMC chemotherapy sensitivity, which has not yet been reported, to the best of our knowledge. In the present study, AQP1 inhibition was combined with MMC treatment and it was revealed that inhibition of AQP-1 enhanced MMC chemotherapy sensitivity in J82 bladder cancer cells.

Materials and methods

Mammalian cell culture conditions. J82 human bladder cancer cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA)

supplemented with 10% fetal calf serum (FCS; Invitrogen; Thermo Fisher Scientific, Inc.) and incubated at 37˚C in a cell culture incubator with 5% CO_2.

Construction of recombinant plasmid. Invitrogen BLOCK-iT RNAi Designer (rnaidesigner.lifetechnologies.com/rnaiexpress) was used for obtaining the following target sequence from AQP1 gene: 5'-GCTGTACTCATCTACGACTTC-3' (724-744). The pSuper RNAi System manual was followed for short hairpin (sh)RNA design (22). The primers designed are as follows: Sh-AQP1 forward, 5'-GATCCCCGCTGTACT CATCTACGACTTCTTCAAGAGAGAAGTCGTAGATGA GTACAGCTTTTTA-3'; and reverse, 5'-AGCTTAAAAAGC TGTACTCATCTACGACTTCTCTCTTGAAGAAGTCGT AGATGAGTACAGCGGG-3'; Sh-AQP1 negative control forward, 5'-GATCCCCGCCAGCTTAGCACTGACTCT TCAAGAGAGAGTCAGTGCTAAGCTGGCTTTTTA-3'; and reverse, 5'-AGCTTAAAAAGCCAGCTTAGCACTGAC TCTCTCTTGAAGAGTCAGTGCTAAGCTGGCGGG-3'.

Primers were synthesized at GenePharma Co., Ltd (Shanghai, China), dissolved and diluted to 1 μg/μl. Reverse primer (5 μl) and forward primer (5 μl) were mixed and annealed at 95˚C for 4 min, 70˚C for 10 min and then allowed to cool to 4˚C. The resulting double stranded DNA and the plasmid pSUPER-retro-puro (Cell BioLabs, Inc., San Diego, CA, USA) were then double digested by Bgl II and Hind III restriction enzymes at 37˚C overnight, ligated together and transferred into E.coli DH5α. Colony PCR was performed to identify the colonies harboring the recombinant plasmid. The reaction system included: 23 μl double distilled H_2O, 1 μl reverse primer, 1 μl forward prime rand 25 μl Taqmix. Pre-denaturation occurred at 95˚C for 6 min, denaturation occurred at 95˚C for 20 sec, annealing occurred at 55˚C for 30 sec and extension occurred at 72˚C for 30 sec, for 30 cycles. Primers were then purified, sequenced at GenePharma Co., Ltd and used to transfect 293FT cells. Prior to transfection, log phase 293FT cells were collected and seeded ($2x10^5$ cells) to 6 well plates. After 24 h incubation at 37˚C, cells were transfected with liposome Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). At 48 h post-transfection, cells were harvested and interference analysis was performed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The resulting recombinant plasmid was designated pSUPER-retro-puro-shAQP1 and was transferred to 293FT cells using calcium phosphate co-precipitation. At 48 h post-transfection, the culture supernatant containing recombinant virus was collected and the virus titrations were determined using multiple proportion dilutions. J82 cells were then seeded to T25 cell culture flask. Polybrene was added to the culture supernatant collected above to a final concentration of 4 μg/μl. The culture media for J82 cells were replaced by 5 ml culture supernatant collected above and the cells were cultured in a 37˚C incubator for 24 h. The transfection was repeated 3 times at 3 h intervals followed by replacement of the current media with DMEM supplemented with 10% FCS. After 48 h, 0.5 μg/ml puromycin was added to the culture for screening of positive clones and culture amplification. This was to establish a J82 cell line (designated as J82-shAQP1) whose expression of AQP1 gene is inhibited. Another J82 cell line transfected with the empty plasmid pSUPER-retro-puro was used as the control (designated as J82-ctrl).

RT-qPCR. The transcription of AQP1 gene was analyzed using RT-qPCR analysis and quantified using the $2^{-\Delta\Delta Cq}$ method (23). RT-qPCR was performed according to the manufacturer's protocol (Bio-Rad Laboratories, Inc., Hercules USA). Total RNA from J82 cells was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). GAPDH was used as an internal control. RT was performed using PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China). CFX96 Real Time PCR detection system (Bio-Rad Laboratories, Inc.) with SYBRGreen II (Bio-Rad Laboratories, Inc.) was used for qPCR. Primers used were as follows: AQP-1 forward, 5'-ACCCGCAACTTCTCAAAC-3; and reverse, 5'-AGGCCAAGCCTCCTCTAT-3'; GAPDH forward, 5'-ACCACAGTCCATGCCATCAC-3', and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'. Thermocycling conditions were as follows: Pre-denaturation at 94˚C for 1 min, denaturation at 94˚C for 20 sec, annealing at 55˚C for 20 sec, extension at 72˚C for 20 sec, for 40 cycles.

MTT assay. Cell viability was assessed using an MTT assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). J82-shAQP1 cells and J82-ctrl cells ($2x10^3$ cells/plate) were seeded into 96 well plates and incubated for 24 h in culture media with or without MMC (1 mg/ml). A total of 200 μl MTT (5 mg/ml) was then added to each well and cells were incubated for a further 4 hat 37˚C, and 150 μl dimethyl sulfoxide was added to each well and incubated at 37˚C for 5 min. A Versamax microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) was used for reading optical density at 490 nm.

Flow cytometry analysis. Apoptosis was measured using flow cytometry with propidium iodide (PI) and the annexin V-fluorescein isothiocyanate (FITC) staining kit (BD Biosciences, Franklin Lakes, NJ, USA). J82-shAQP1 and J82-ctrl cells ($2x10^3$ cells/plate) were seeded onto 96 well plates and incubated at 37˚C for 24 h in DMEM with or without 1 mg/ml MMC. Then, the cells were detached and rinsed with DMEM and cold PBS twice. Cells were then centrifuged at 300 x g for 5 min and resuspended in 1x binding buffer at $1x10^6$ cells/ml. In total, 100 μl of the cell buffer solution ($1x10^5$ cells) was transferred to a round bottom tube and incubated with 5 μl of PI and annexin V-FITC for 15 min in the dark. Finally, 400 μl of 1x binding buffer was added to each sample tube and analyzed using a BD FACSCalibur cytometer (BD Biosciences) according to the manufacturers protocol. Data were analyzed using FlowJo software (version 7.2.5; FlowJo LLC, Ashland, OR, USA).

Western blot analysis. For Western blot analysis, J82-shAQP1 and J82-ctrl cells ($2x10^3$ cells/plate) were seeded into 96 well plates and incubated at 37˚C for 24 h in DMEM with or without 1 mg/ml MMC. Total cellular proteins were extracted in EBC lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.5% NP40; Sigma-Aldrich; Merck KGaA) containing a proteinase inhibitor cocktail (100 μg/ml lysozyme, 10 μg/ml aprotinin and 10 μg/ml lepupetin; Sigma-Aldrich; Merck KGaA), as described

previously (24). Protein concentrations were measured using Bradford protein assay. A total of 30 μg protein samples were then subjected to SDS-PAGE (12% gel). Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes, where they were subsequently blocked by TBST and 5% non-fat dry milk at 4˚C for 2 h, followed by incubation with primary antibodies at 4˚C for 12 h. The primary antibodies used were: Anti-AQP-1 (1:100; cat. no. BM5035, Wuhan Boster Biological Technology, Ltd., Wuhan, China), anti-proliferating cell nuclear antigen (PCNA; 1:5,000; cat. no. sc7907; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Bcl-2 associated X protein (Bax; 1:2,000; cat. no. ab32503; Abcam, Cambridge UK), anti-B cell lymphoma 2 (Bcl-2; 1:500; cat. no. ab692; Abcam), anti-caspase-3 (1:5,000; cat. no. sc-98758; Santa Cruz Biotechnology, Inc.) and anti-β-actin (1:1,000; cat. no. sc-130656; Santa Cruz Biotechnology, Inc.). Following incubation with corresponding primary antibody at 4˚C overnight, the membranes were washed and incubated with either horseradish peroxidase-conjugated-goat anti-rabbit immunoglobulin G secondary antibody (1:2,000; cat. no. ab6721; Abcam) for 1 h at 4˚C and developed with electrochemiluminescence (ECL) Western Blotting Substrate (BD Biosciences). Data was analyzed using Quantity One 1D image analysis software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Each experiment was performed ≥3 times and analyzed using SPSS (v19; IBM Corp., Armonk, NY, USA) was used for statistical analysis of the data collected. A two-tailed Student's t-test was adopted for comparison of the expressions of AQP-1 mRNA between J82-ctrl and J82-shAQP1 cells, whereas comparisons among groups were assessed using one-way analysis of variance with a Student-Newman-Keuls post-hoc test. Data were presented as the mean ± standard deviation. P<0.05 was considered to indicate as statistically significant difference.

Results

AQP-1mRNA expression was inhibited in J82-shAQP1 cells. The expression of AQP-1 mRNA was detected by RT-qPCR. As presented in Fig. 1, the expression of AQP-1 mRNA in J82-shAQP1 cells decreased significantly and was only 36% of that in J82-ctrl cells. In addition, the expression of GAPDH mRNA in the 2 cell types remained consistent. GAPDH is a commonly used loading control for RT-PCR as the GAPDH gene is a housekeeping gene often stably and constitutively expressed in the majority of tissues and cells.

Combined effect of recombinant virus transfection and MMC chemotherapy on J82 cell proliferation and apoptosis. As presented in Table I, there was a significant decrease in cell viability for J82-ctrl cells following treatment with MMC (P<0.05). The cell viability was also significantly decreased in J82-shAQP1 cells compared with that in J82-ctrl cells (P<0.05). Treatment of J82-shAQP1 cells with MMC further decreased cell viability, which was significantly decreased compared with that in J82-ctrl cells treated by MMC (P<0.01). A similar association was also observed in cell apoptosis. As presented in Fig. 2, when cells were treated with MMC, J82-shAQP1 cells exhibited a significantly increased level of cell apoptosis compared with J82-ctrl cells (P<0.05).

Table I. Cell viability of J82-shAQP1 and J82-ctrl cells.

	Cell viability (% of the control), mean ± standard deviation	
MMC (mg/ml)	J82-ctrl	J82-shAQP1
0	100±8.39	77.42±9.26[a]
2	81.58±6.30[a]	60.05±6.77[b,c]

[a]Statistically significant difference vs. J82-ctrl cells (P<0.05). [b]Statistically significant difference vs. J82-ctrl cells (P<0.01).[c]Statistically significant difference vs. J82+MMC group (P<0.05). MMC, mitomycin C; ctrl, control; sh, short hairpin; AQP, aquaporin.

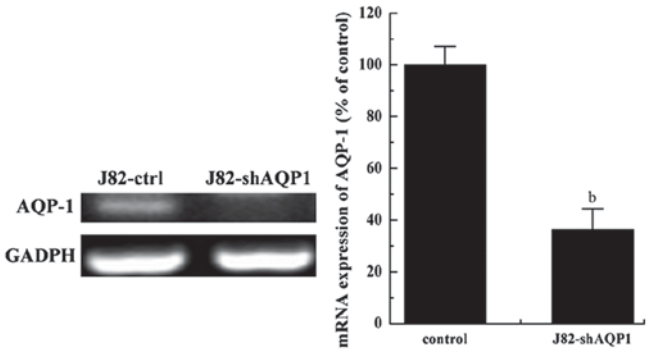

Figure 1. AQP-1mRNA expression decreased significantly in J82-shAQP1 cells. Data were presented as mean ± standard deviation. Statistically significant difference was observed for AQP-1mRNA expression in J82-shAQP1 cells and J82-ctrl cells. [b]P<0.01 vs. control cells. AQP, aquaporin; ctrl, control; sh, short hairpin.

The combined effect of recombinant virus transfection and MMC treatment on expressions of AQP-1, Bax, Bcl-2, caspase-3 and PCNA. As presented in Fig. 3, significantly decreased expression of AQP-1 was observed in J82-shAQP1 cells compared with J82-ctrl cells (P<0.05). Treatment of J82 cells with MMC also significantly decreased AQP-1 expression (P<0.05). Additionally, AQP-1 expression was decreased in J82-shAQP1 cells treated with MMC compared with J82-ctrl cells treated by MMC and was decreased more as compared with J82 cells without MMC treatment.

To further assess how MMC treatment and inhibition of AQP-1 expression affected J82 cell viability and apoptosis, the expressions of PCNA, Bcl-2, Bax and Caspase-3 in J82-shAQP1 cells and J82-ctrl cells were measured. PCNA, Bcl-2, Bax and Caspase-3 are cell proliferation- and apoptosis-associated proteins. As presented in Fig. 3, PCNA was most highly expressed in J82-ctrl cells and was decreased significantly by MMC treatment. Significantly decreased expression of PCNA was also observed in J82-shAQP1 cells compared with J82 cells and was further decreased by MMC treatment (P<0.05). Significantly decreased expression of PCNA was observed between J82-shAQP1 cells treated with MMC (P<0.01) and

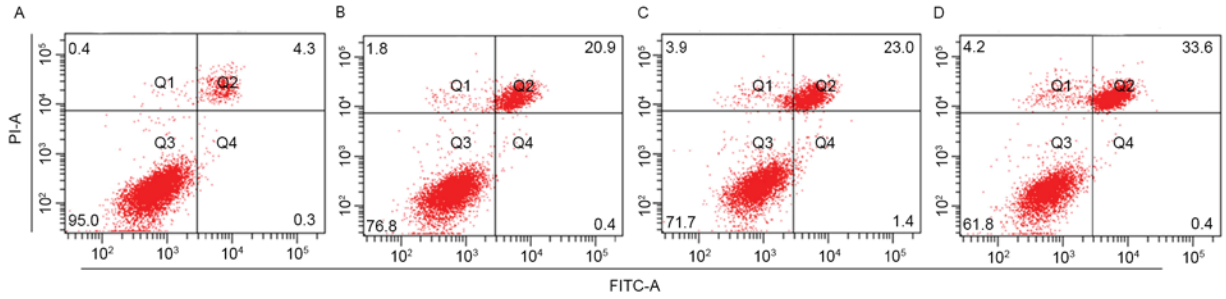

Figure 2. Combined effect of recombinant virus transfection and MMC treatment on J82 cell proliferation and apoptosis. Flow cytometry analysis of (A) J82-ctrl cells, (B) J82-shAQP1, (C) J82-ctrl cells treated with MMC and (D) J82-shAQP1 cells treated with MMC. MMC, mitomycin C; ctrl, control; PI, propidium iodide; FITC, fluorescein isothiocyanate; sh, short hairpin; AQP, aquaporin.

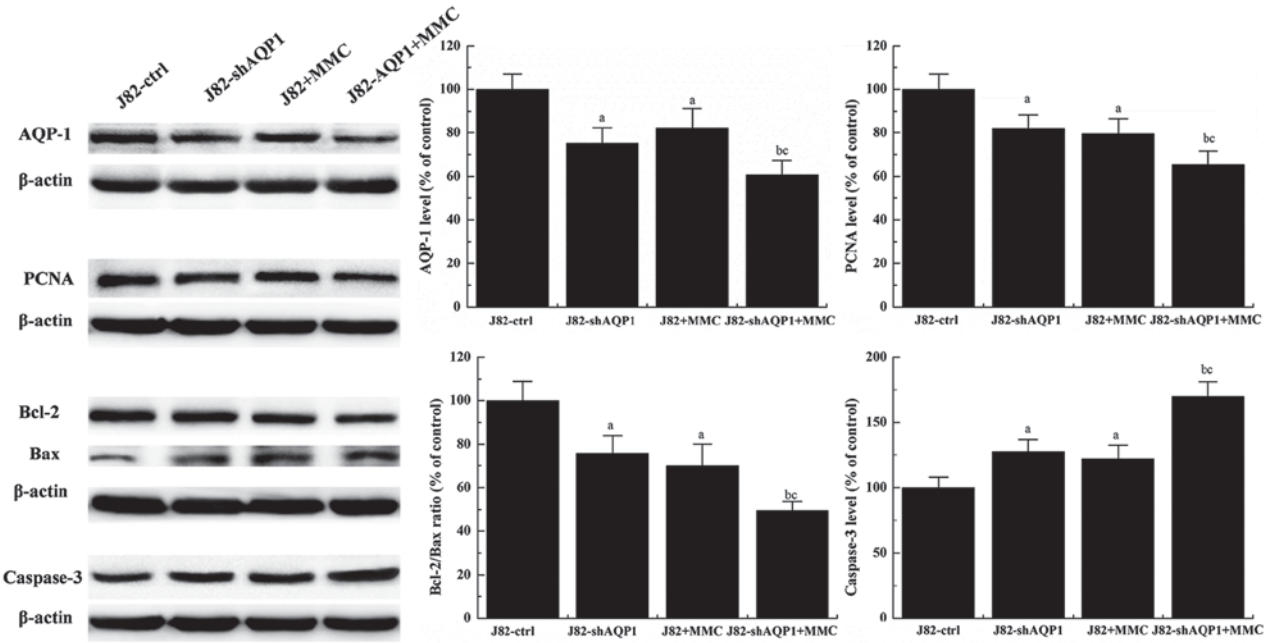

Figure 3. Western blot analysis of expression of AQP-1, Bax, Bcl-2, caspase-3 and PCNA. Data were presented as mean ± standard deviation. [a]Statistically significant difference vs. J82-ctrl cells (P<0.05); [b]Statistically significant differencevs. J82-ctrl cells (P<0.01); [c]Statistically significant difference vs. J82+MMC group (P<0.05). AQP, aquaporin; ctrl, control; Bcl-2, B cell lymphoma 2; Bax, Bcl-2 associated X protein; PCNA, proliferating cell nuclear antigen; MMC mitomycin C; sh, short hairpin.

J82-ctrl cells treated with MMC (P<0.05), compared with the untreated control.

In contrast with PCNA expression, an opposite expression pattern was observed for caspase-3. As presented in Fig. 3, the expression of caspase-3 in J82-shAQP1 cells was significantly increased compared with that in J82-ctrl cells (P<0.05), suggesting recombinant virus transfection of J82 cells increased caspase-3 expression. Treatment of J82 cells with MMC also increased caspase-3 expression. Of all the conditions tested, the highest expression level of caspase-3 was observed in J82-shAQP1 cells treated with MMC.

In addition, the Bcl-2/Bax ratio was also measured. As presented in Fig. 3, the highest Bcl-2/Bax ratio was observed in J82 cells and was significantly decreased following MMC treatment and recombinant virus transfection (P<0.05). The Bcl-2/Bax ratio in J82-shAQP1 cells was further decreased by MMC treatment. A significantly decreased Bcl-2/Bax ratio was observed in J82-shAQP1 cells treated with MMC compared with that in J82 cells treated with MMC (P<0.05).

Discussion

Bladder cancer is associated with an increased morbidity rate and common relapses and metastases. It may be treated either by removal of the bladder or by radiation therapy and chemotherapy (25). MMC, anaziridine-containing natural product (26), is often applied as a chemotherapeutic agent for the treatment of bladder cancer (7-11). Numerous individuals with heart disease in China also take MMC (27,28). However, occurrence of resistance to MMC often limits its clinical effectiveness (12-14). To increase MMC chemotherapy sensitivity, MMC is often combined with other agents (17). Previous studies have demonstrated that multiple AQPs, particularly AQP1, are associated with cancer development and progression, including bladder cancer (29-31). In the present study, a novel J82 bladder cancer cell line, J82-shAQP1 cell line, was established. RT-qPCR analysis demonstrated that AQP1expression in J82-shAQP1 cells was inhibited. Investigating cell viability and apoptosis demonstrated that, when treated with MMC, J82-shAQP1

cells exhibited decreased cell viability and increased apoptosis compared with that exhibited by J82 cells. In addition, it was observed that J82-shAQP1 cells exhibited decreased expression of PCNA, decreasedBcl-2/Bax ratio and increased expression of caspase-3 compared with that exhibited by the J82 cells.

The association of AQPs with cancer development and progression has been previously reported (29-31). For example, AQP1 has been revealed to be overexpressed in glioblastoma multiforme, adenoid cystic carcinoma, renal cell carcinoma and breast cancer (32-37). There have also been previous studies using AQP inhibitor or RNA interference to inhibit AQP1 expression in cancer cells (38). Therefore, AQP1 may represent an important therapeutic target for cancer treatment.

As demonstrated in the present study, a combination of AQP1 inhibition with MMC treatment decreased cell viability and increased the apoptosis rate in human J82 cells. Similar changes were observed in the expression of PCNA, Bcl-2, Bax and caspase-3, which are proteins associated with cell proliferation and apoptosis (39). PCNA has sustained activity and functions as a processivity factor for eukaryotic DNA polymerase, serving an essential function in DNA replication, cell proliferation and apoptosis (40). Inhibition of PCNA as a cancer treatment strategy has been reported previously (41). Caspases (cysteine-dependent aspartate-directed proteases) are an evolutionarily conserved family of cysteine proteases that serve central functions in cell apoptosis and inflammation (42). It has been reported that activated caspase 3 stimulates tumor growth, while inactive caspase 3 caused substantial tumor sensitivity to radiotherapy (43). Bcl-2is a member of the Bcl-2 family proteins that regulate cell apoptosis (44,45). Bcl-2 is an anti-apoptotic protein; Bax is a pro-apoptotic protein in the Bcl-2 family (46,47).

Taken together, the results of the present study demonstrated that inhibiting AQP1 expression decreased cell viability and increased cell apoptosis in human J82 bladder cancer cells, suggesting that a combination of MMC1 treatment with AQP1 inhibition may be a promising treatment strategy to enhance MMC1 chemotherapy sensitivity for bladder cancer treatment.

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