



Hydropenia may accelerates the progression of orthotopic bladder cancer induced by N-methyl-N-nitrosourea by increasing the expression levels of AQP1, AQP3, and AQP4

Jun-Wei Pan, Xiang Zhang, Xing-Wei Jin, Xian-Jin Wang, Wei-Chao Tu, Bao-Xing Huang, Da Xu, Yuan Shao

Shanghai Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Department of Urology, Shanghai, China

Contributions: (I) Conception and design: JW Pan; (II) Administrative support: Y Shao; (III) Provision of study materials: X Zhang, XW Jin; (IV) Collection and assembly of data: X Zhang, XW Jin; (V) Data analysis and interpretation: JW Pan, X Zhang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Yuan Shao. Shanghai Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Department of Urology, No. 999 Xi Wang Road, Shanghai 201801, China. Email: shaoyuan15@126.com.

Background: Increasing evidence has demonstrated aquaporins (AQPs) to be critical players in carcinogenesis. Here, we aimed to explore the role of hydropenia in the progression of bladder cancer (BCa), as well as to assess the expression of AQP1, AQP3, and AQP4 in bladder tissues from hydropenic and N-methyl-N-nitrosourea (MNU)-treated rats.

Methods: An orthotopic BCa model was induced by administering Sprague Dawley rats with MNU. A hydropenic rat model was established by administrating rats with 2/3 of the amount of water given to the control group. At week 8, the rats were sacrificed and their bladder tissues were collected. Then, pathological alterations in the rat bladders were assessed by hematoxylin and eosin staining. The RNA and protein expression levels of AQP1, AQP3, and AQP4 were determined by using qRT-PCR and western blot assays.

Results: All of the rats (100%) administrated with MNU developed tumors, of which 5 were large (diameter, 0.5–1.0 cm), 10 were medium (diameter, 0.2–0.5 cm), and 5 were small (diameter, <0.2 cm) in size. The tumors were nodular and cauliflower shaped, with multiple satellite focus, and were accompanied by bleeding, ulcers, stones, and residual urine. Hematoxylin and eosin staining revealed that the bladder mucosa was incomplete, with a large amount of necrotic tissue and obvious leukocytic infiltration. The tumor volume in the MNU + hydropenia group was significantly larger than that in the MNU group. Noticeably, hydropenia exacerbated pathological changes induced by MNU administration. QRT-PCR and western blot analysis revealed that the MNU group, hydropenia group, and MNU + hydropenia group had significantly increased levels of AQP1, AQP3, and AQP4 compared to the control group, with the most dramatic increase seen in the MNU + hydropenia group.

Conclusions: Hydropenia exacerbates pathological alterations induced by MNU in rats with orthotopic BCa by increasing the expression levels of AQP1, AQP3, and AQP4. This study reveals a possible mechanism of the occurrence of BCa.

Keywords: Bladder cancer (BCa); hydropenia; aquaporin 1 (AQP1); AQP3; AQP4

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Introduction

Bladder cancer (BCa) ranks as the 6th most commonly occurring malignancy globally, and is the 4th most frequently diagnosed malignancy among males in developed countries (1). In the majority of cases (70%), BCa does not invade the muscle of the bladder, and transurethral resection followed by intravesical instillation of chemotherapy is considered as the primary treatment (2). Although numerous studies have attempted to uncover the mechanisms of BCa tumorigenesis and progression, their results have been inconsistent.

Water was first reported to be transported across epithelia in erythrocytes by Agre *et al.* in the 1980s (3). In subsequent studies, water-transport channels, referred to as aquaporins (AQPs), were also discovered in renal epithelial cells (4,5). The AQP family comprises transmembrane proteins which control the passage of water or water together with other small, uncharged molecules (e.g., urea and glycerol) along hydrostatic and osmotic gradients. The expression of AQPs is ubiquitous in cells of humans, animals, and bacteria, and the essentiality of AQPs for cellular functions has been recognized (6). In mammals, there are 13 AQPs localized in particular tissues (7). Research has revealed AQPs to play roles in multiple pathological conditions as well as their promise as therapeutic targets (8). It has been strongly speculated that AQPs participate in oncogenesis, namely in the tumor angiogenic process and cell migration (9). For example, one study observed a relationship between AQP3 knockdown and an increase in the migratory and proliferative ability of gastric cancer cells (10).

Here, we aimed to explore the role of hydropenia in the tumorigenesis of BCa, as well as to assess the expression levels of AQP1, AQP3, and AQP4 in bladder tissues from hydropenic and N-methyl-N-nitrosourea (MNU)-treated rats.

We present the following article in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/tau-21-273>).

Methods

Animals and ethics statement

Forty adult female Sprague Dawley rats (age, 6 weeks old; weight, 200±10 g) were obtained from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China). The rats were divided equally into the following 4 groups (n=10):

the control, MNU, hydropenia, and MNU + hydropenia groups. All animal experiments were performed in accordance with the guidelines for animal care and approved by Animal Care and Use Committee of Shanghai Ruijin Hospital.

Construction of the BCa rat model

The rats were housed under a 12-hour light-dark cycle and given ad libitum access to food and water. The rats were anesthetized through the delivery of 3% isoflurane in a closed chamber. After transfer of the rats to a nose cone, the surgical area was prepared, and the urethra was placed into a 20-gauge angiocatheter. Then, 0.30 mL 1 M sodium citrate (pH 6.0) with MNU (1.5 mg/kg) was instilled, the catheter was removed, and the rats were kept sedated continuously for 45 minutes to avoid the occurrence of spontaneous micturition and to enable absorption. As previously described, MNU was instilled once a week for 4 weeks (11). The hydropenia and MNU + hydropenia group was given 2/3 of the amount of water given to the control group. At week 8, the rats were sacrificed, and the pathological alterations and the expression levels of AQPs were assessed.

Pathological assessment

The bladder tissues were collected from the rats and subjected to 24-hour immersion in a solution containing 4% paraformaldehyde (Dulbecco's Phosphate Buffered Saline; Mediatech, Inc., Herndon, VA, USA) for fixation. Each specimen underwent rinsing in phosphate-buffered saline, followed by dehydration in a graded ethanol series and paraffinization. The specimens were then sectioned at a thickness of 5-µm, after which hematoxylin and eosin (HE) staining was performed. Finally, a light microscope was used to evaluate each tissue section.

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, USA), and then used for cDNA synthesis with the help of PrimeScript RT Master Mix kit (RR036A; Takara). Next, the cDNA was applied to PCRs detection with 2× SYBR Green PCR Mastermix (Solarbio, Beijing, China) in 7500 Real-Time PCR System (Applied Biosystems, USA). The reactions were as follows: 95 °C for 1 min, 39 cycles

Table 1 Primer sequences

Gene	Forward (5'-3')	Reverse (5'-3')
AQP1	TGCCGTTAACCATGTCGTGA	GTCCAGGCAGAAACGGAGAA
AQP3	CAGCTCGTGACTTTGGACCT	ACCTAATCCCCTAGCTGGCA
AQP4	ACCTGTGATAGCACCTTGCC	TGCTGAGTCCAAAGCAGAGG
GAPDH	GCATCTTCTTGTGCAGTGCC	GATGGTGATGGGTTTCCCGT

Table 2 Characteristics of rats in each group

Characteristics	Control (n=10)	MNU (n=10)	Hydropenia (n=10)	MNU + hydropenia (n=10)
Weight (g)				
Before	201.5±10.4	199.5±9.8	202.0±10.3	200.5±10.2
After	260.6±12.5	248.9±11.2	249.6±11.0	243.5±12.0
Food intake (g/day)				
Before	21.2±2.3	22.0±2.5	21.6±2.5	21.8±2.3
After	25.3±2.8	24.2±3.1	23.5±3.0	23.1±3.2

including 95 °C for 15 s and 60 °C for 1 min. The relative expressions of mRNA were calculated based on the $2^{-\Delta\Delta C_t}$ method (12). Primers were shown in *Table 1*.

Western blotting assay

Following culture as indicated, protease inhibitor cocktail-containing RIPA lysis buffer was used to lyse the rat bladder tissues. Centrifugation of cell lysates was carried out at 3,500 rpm for 10 minutes at 4 °C. The resulting supernatant was used for protein extraction, and a BCA assay kit (Sigma-Aldrich) was employed for the determination of protein concentration. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to separate proteins (25 µg) before their transfer onto a polyvinylidene difluoride membrane. After blocking with 5% nonfat skim milk, the membrane was incubated overnight with primary antibodies against AQP1 (Chemicon, Temecula, CA, USA), AQP3 (Abcam, Cambridge, MA, USA), AQP4 (Abcam), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Sigma, St. Louis, MO, USA) at 4 °C. Next, following the addition of horseradish peroxidase-conjugated secondary antibody, the membrane was subjected to incubation once more. After washing, the protein bands were visualized with an enhanced chemiluminescent detection system (Thermo Scientific, Carlsbad, CA, USA).

Statistical analysis

The data were presented as mean ± standard deviation (SD). Statistical analyses were performed with GraphPad Prism 5 (GraphPad Inc., La Jolla, CA, USA). Student's *t* test or one-way analysis of variance followed by the Bonferroni post-hoc test were used for between-group comparison. $P < 0.05$ was deemed to indicate statistical significance.

Results

Baseline data

During of the experiment, most of the rats showed slightly poor spirit and decreased appetite. The weights of rats in MNU, hydropenia and MNU + hydropenia groups were decreased as compared with the control group, but the difference was not statistically significant (P value > 0.05). The relevant characteristics were listed in *Table 2*.

Pathological assessment

The tumorigenic rate was 100%, with all MNU-treated rats developing tumors. Of the tumors, 5 were large (diameter, 0.5–1.0 cm), 10 were medium (diameter, 0.2–0.5 cm), and 5 were small (diameter < 0.2 cm) in size. The tumors were nodular and cauliflower shaped, with multiple satellite

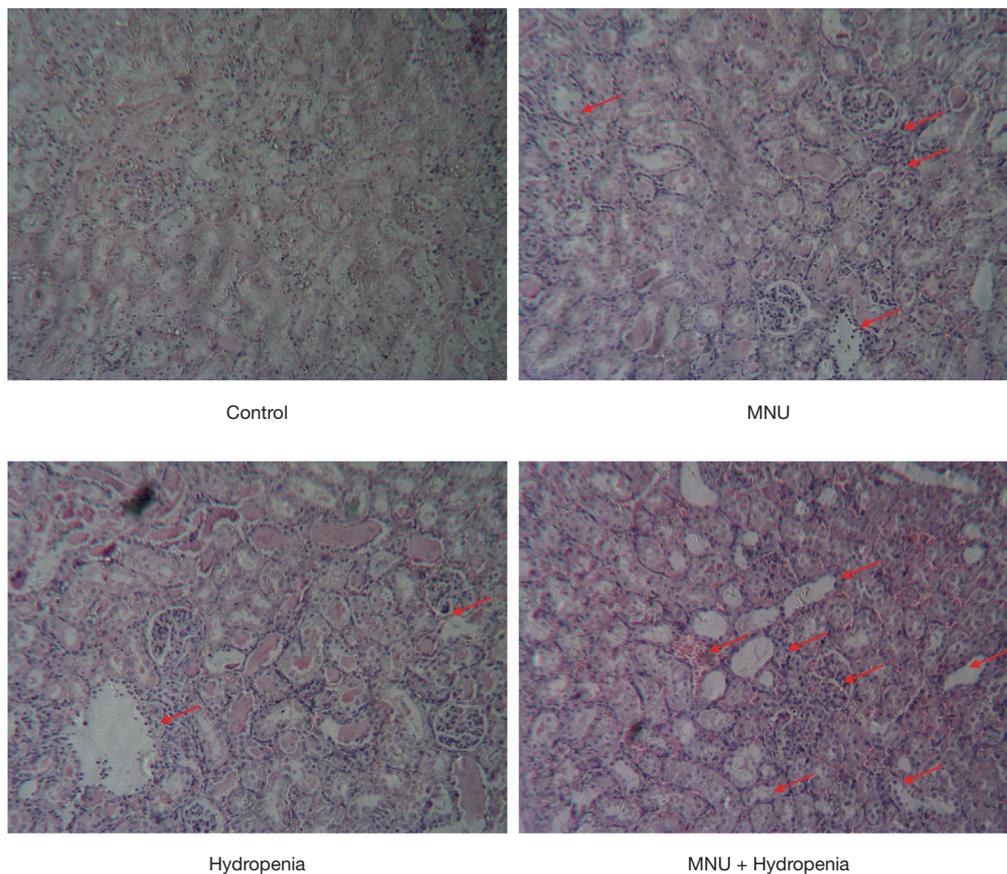


Figure 1 Bladder tissues by H&E staining ($\times 100$). Hematoxylin and eosin staining was applied to assess the histopathology of bladder tissues for rats in different groups (the control, MNU, hydropenia, MNU + hydropenia groups). H&E, hematoxylin and eosin; BCa, bladder cancer. A large amount of necrotic tissue and obvious leukocytic infiltration were seen in the MNU + hydropenia group.

focus, and were accompanied by bleeding, ulcers, stones, and residual urine. The tumor volume in the MNU + hydropenia group was significantly greater than that in the MNU group. The results of HE staining showed that the bladder mucosa of the rats was incomplete, with a large amount of necrotic tissue and obvious leukocytic infiltration. Noticeably, hydropenia exacerbated the pathological changes induced by MNU administration (*Figure 1*).

Evaluation of AQP1, AQP3, and AQP4 expression

Next, we assessed the expression of AQP1, AQP3 and, AQP4 in the bladder tissues from the rats. The MNU

group, hydropenia group, and MNU + hydropenia group, exhibited significantly increased mRNA (*Figure 2A*) and protein (*Figure 2B*) levels of AQP1, AQP3, and AQP4 compared to the control group, with the most dramatic increase seen in the MNU + hydropenia group.

Discussion

More and more research has demonstrated the importance of AQPs to cancer progression. For example, Deb *et al.* (13) found AQP1 to be closely associated with tumor angiogenesis, indicating it to potentially be a predictive indicator of tumor development and prognosis. Liu and colleagues (14) found the expression levels of AQP1 in

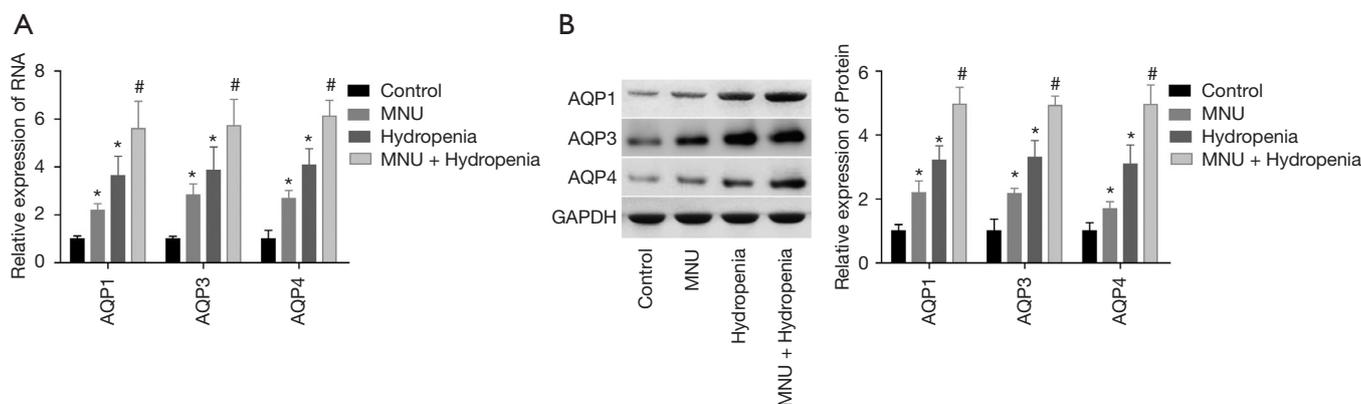


Figure 2 AQP1, AQP3 and AQP4 expression levels in bladder tissues. (A) QRT-PCR and (B) Western blotting analysis of the expression levels of AQP1, AQP3, and AQP4 in the bladder tissues of the different groups of rats (the control, MNU, hydroponia, MNU + hydroponia groups). *, P value <0.05, vs. control group; #, P value <0.05, vs. MNU group. BCa, bladder cancer; AQP, aquaporin.

normal bladder tissues and bladder uroepithelium cell carcinoma specimens to be 2.175 ± 0.693 and 3.689 ± 0.701 , respectively, with a high expression of AQP1 being closely linked to a higher tumor grade and stage, lymph node metastasis, and recurrent disease. Their findings demonstrated the potential significance of AQP1 to guide bladder carcinoma diagnosis and treatment. Furthermore, Zhang *et al.* (15) reported that selective inhibition of AQP1 enhanced the sensitivity of J82 BCa cells to mitomycin C. Moreover, AQP3, AQP4, AQP7, AQP9, and AQP11 transcripts were detected in both freshly isolated urothelial cells and cultured urothelial cells. At the protein level, an unequivocal expression of AQP3 was also observed, with immunohistochemistry revealing intense staining of cell membranes in both the basal and intermediate layers of normal bladder urothelial tissue (10). Rubenwolf *et al.* (16) detected the expression of AQP3 and AQP9 transcripts in low-grade RT4 and RT112 BCa cells; however, they were not detected in high-grade T24 cells. Meanwhile, although there was an absence of AQP4 messenger RNA in RT4 cells, its expression was observed in RT112 and T24 cells. All of these findings point toward AQPs potentially being players in BCa progression.

The current study explored the role of hydroponia in the progression of BCa and assessed the expression levels of AQP1, AQP3 and AQP4 in the bladder tissues of hydroponic and/or MNU-treated rats. We found the bladder mucosa of the rats to be incomplete, with a

large amount of necrotic tissue and obvious leukocytic infiltration in the MNU group compared to the control group. Noticeably, hydroponia exacerbated the pathological changes induced by MNU administration. The tumor volume in the MNU + hydroponia group was significantly larger than that in the MNU group. It was revealed that hydroponia accelerated the pathological alterations and contributed to the progression of tumorigenesis.

Although it was considered that the urothelium was impermeable to water and solutes, but recent researched showed that urine constituents could be reabsorbed during urinary tract transit and storage (17). Our results that hydroponia induced the expression of AQP1, AQP3 and AQP4 in bladder tissues supported this conclusion. Through the upregulated expression of AQPs, hydroponia was thought to further induce the cell liquid permeation and reabsorption, even the transport of MNU as assumed. The changes of intravesical and intracellular concentration of MNU might accelerated the tumorigenesis.

Furthermore, western blotting analysis showed that compared with the control group, the MNU group, hydroponia group, and MNU + hydroponia group exhibited significantly increased expressions of AQP1, AQP3, and AQP4, with the highest levels seen in the MNU + hydroponia group. These results verify our view to a certain extent.

In conclusion, this study has demonstrated that hydroponia exacerbates MNU-induced pathological

alterations in rats with orthotopic BCa by increasing the expression levels of AQP1, AQP3, and AQP4, which may be a possible mechanism of the occurrence of BCa.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <http://dx.doi.org/10.21037/tau-21-273>

Data Sharing Statement: Available at <http://dx.doi.org/10.21037/tau-21-273>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tau-21-273>). The authors reported this study was supported by: (I) Fund of Shanghai Ruijin Hospital North (2016ZY07) and (II) Youth Talents Plan of Shanghai Ruijin Hospital North (2019RCPY-B12), of which funders had no role in this study. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal experiments were performed in accordance with the guidelines for animal care and approved by Animal Care and Use Committee of Shanghai Ruijin Hospital.

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