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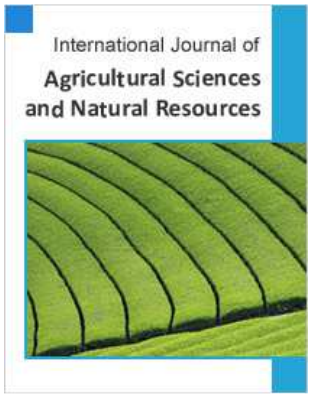

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The Regulation of Aquaporin 1 and Aquaporin3 by *Polyporus umbellate* in Rat Bladder Carcinoma

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Abstract

Bladder carcinoma is a common malignancy. Aquaporin 1 (AQP1) and aquaporin 3 (AQP3) are highly expressed in rat bladder endothelial and epithelial cells. Interestingly, *polyporus umbellate* effectively inhibits bladder carcinoma development in rats. In this study, we aim to examine how *polyporus umbellate* regulates AQP1 and AQP3 expression inbladder carcinoma model. Depending on the applied treatment, rats exhibiting bladder carcinoma were divided into the following five groups: saline (control), bladder carcinoma model, low dose (50 mg/kg), medium dose (250 mg/kg) and high dose (500 mg/kg) *polyporus umbellate*. Immunohistochemistry and RT-PCR were used to detect AQP1 and AQP3 expression after administration of *polyporus umbellate*. Bladder carcinoma was induced by N-butyl-n-(4-hydroxybutyl) nitrous amide (BBN). *Polyporus umbellate* inhibited bladder carcinoma development in rats. Compared with the model group, AQP1 and AQP3 protein expression decreased in *polyporus umbellate* treated rats. Moreover, with increasing dosage, ranging from250 to 500 mg/kg, *polyporus umbellate* decreased AQP1 and AQP3 mRNA expression ($P<0.05$, $P<0.01$, respectively). In conclusion，the *Polyporus umbellate* decreased bladder carcinoma growth and inhibited AQP1 and AQP3 expression.

1. Introduction

Bladder carcinoma is a common malignancy. In 2014, an estimated 74,960 Americans were diagnosed with bladder cancer, 15,580 of whom died [1]. Traditional Chinese medicine may offer promising alternatives for treating this disease. As a traditional Chinese medicine, *polyporus umbellate* (Zhuling in Chinese) is sweet, naturally-occurring, and is distributed to the kidney and bladder.

Polyporus umbellate effectively inhibits bladder carcinoma development [2] and promote surination by regulating kidney aquaporin 2 [3] and the host immune response [4]. A large family of aquaporins (AQPs) has been identified in diverse animal tissues, each AQP is special in the distribution and location of cell expression, and has different functions on different sites in order to meet the requirement of body metabolism and maintain the fluid equilibrium. AQP1 and AQP3 strongly expressed in rat bladder endothelial and epithelial cells [5]. Some scholars found that AQP1 was in close

relationship with the development of tumor angiogenesis [6], AQP3 protein expression is correlated with bladder carcinoma stage and grade, with reduced or lost AQP3 expression in tumors of a higher stage and grade [7].

In the present report, we investigate the pharmacodynamic evaluation of *polyporus umbellate* in rat bladder carcinoma, and explore the expression of AQP1 and AQP3 in mediating this process.

2. Materials and Methods

Polyporus umbellate (10g crude drug=1g pure drug) was purchased from Jiangyin Tianjiang Pharmaceutical Co., Ltd (Jiangsu, China). N-butyl-n-(4-hydroxybutyl) nitrous amide (BBN) was purchased from TCI Corporation in Japan. Trizol was purchased from Invitrogen (Carlsbad, CA, USA) and RT-PCR reagents were purchased from Takara (Mountain View, CA, USA). Chloroform, isopropanol, and absolute ethyl alcohol were all analytically pure. Antibodies of AQP1, AQP3 were purchased from Abcam (USA). Rabbit anti-rat IgG, DAB coloring kits, purchased from Santa Cruz (USA). Real-time PCR was performed using the ABI 7500 Real-time PCR system (Foster City, CA, USA) and evaluated on a UV-4000 UV-Ultraviolet and visible spectrophotometer (Ocean Optics, Dunedin, FL, USA). The Gel Dox XR Gel imaging system was purchased from Bio-Rad Co. (Hercules, CA, USA). Nikon Eclipse E microscope and NIS elements imaging software were obtained using a digital camera.

3. Methods

3.1. Animals

Female Fisher-344 rats were purchased from Vital River Laboratories (Beijing, China), and used after 3 d acclimation. All rats were handled following the Guidelines for the Care and Use of Experimental Animals from Hebei University and approved by the institutional committee on animal care. Rats were maintained under standard environmental conditions (23±2°C, 55±5% humidity and 12 h/12 h light/dark cycle). All animals were allowed free access to tap water and standard laboratory rat food.

3.2. Groups and Treatment

All animals were divided into five groups with 10 rats per group: saline (control), bladder carcinoma model, and 50, 250, and 500 mg/kg *polyporus umbellate* groups. Food was available during the experimental periods. Bladder carcinoma was induced by N-butyl-n-(4-hydroxybutyl) nitrous amide (BBN), all rats had free access to water containing 0.05% BBN in the eighth growing week, with *polyporus umbellate* administrated in the 9th to 20th week (dose volume was 1mL/100 g). Following treatment, all animals were anesthetized with 10% chloral hydrate via intramuscular injection (0.3 mL/100 g), and bladder tissues were excised and cryopreserved at − 80°C.

3.3. Measure of Relative AQP1 and AQP3 mRNA Expression in Bladder

We separated fresh bladder tissue (50 mg) and extracted total RNA (3 μg), the concentration of which we then determined. The mRNA reverse was transcribed into cDNA in a 20-μL reaction. Fluorescent quantitative real-time PCR was used to determine AQP1 and AQP3 mRNA expression in bladder.

The amplification primers were: AQP1 sense，5′-T GCA GCG TCA TGT CTG AG-3′; antisense, 5′-GAA CTA GGG GCA TCC AAA C-3′; AQP3 sense, 5′-ACT CCA GTG TGG AGG TGG AC-3′; and antisense, 5′-GCC CCT AGT TGA GGA TCA CA-3′.

Primers were designed based on sequences obtained from GeneBank, AQP1-NM_012778.1, AQP3-NM_031703.1.The lengths of the amplified products were AQP1 (100bp), AQP3 (147bp), β-actin (150 bp). Primers were synthesized by Invitrogen. The PCR conditions were: 94°C, 60°C, and 68°C for 20s, 20s; and33s, respectively, for 30 cycles.

3.4. Detection of AQP1 and AQP3 in Bladder via Immunohistochemistry

Immunohistochemical method was adopted to detect the AQP1, AQP3 expression of the BUCC tissues and normal bladder tissues, carried out according to the manufacturer's instructions. The procedures were as follow: Samples were fixed in 4% paraformaldehyde, dehydratedand embedded in paraffin wax. Dewaxed 4μm tissue sections were subjected to antigen retrieval by boiling for 10min in tris-ethylenediaminetetraacetic acid (Tris-EDTA; pH 9) before labelling with titrated primary antibody(polyclonal anti-AQP1, polyclonal anti-AQP3, dilution 1:2000, Abcam, USA) for 12h at 4°C. Then the tissue section washed for 3 times by 0.1 mol/L phosphate buffer solutions (PBS). Second-antibody working solution (rabbit anti-rat IgG) marked with horseradish peroxidase (HRP) was added and incubated at room temperature for 2h. Diaminobenzidine (DAB) was added for color development. Yellow particles in the cytoplasm or nucleus were judged as positive.

3.5. Statistical Analysis

All results were evaluated using SPSS 15.0 (IBM, Chicago, IL, USA). Statistically significant differences were valued by one-way analysis of variance (ANOVA) followed by Dunnett's t-test. Expression was analyzed via the ΔCt method, and data analyzed by single factor variance. Values represent AQP1, AQP3 mRNA quantitated with 2-ΔΔCT method, $P<0.05$ was considered significant.

4. Results

Bladder carcinoma is graded based on four pathological stages: hyperplasia, pTa, pT1, and pT2. At pT2, cancer has spread deeply into the bladder. In the bladder carcinoma rat

model, 1 rat was found at hyperplasia, 3 were pTa, 1 was pT1, and 4 were pT2, indicating that bladder carcinoma had been induced successfully (Table 1). After administering *polyporus umbellate* (50 mg/kg), 3 rats exhibited bladder at hyperplasia, with 1 each stages pTa and pT1,2 at pT2, whereas the250 mg/kg dose yielded 6 rats with bladder hyperplasia, with 2 at pTa and1 pT2. The highest dose(500 mg/kg)generated 9 rats with bladder hyperplasia, with only 1at pTa. All data were processed via Radit analysis, which indicated that *polyporus umbellate* inhibits BBN-induced bladder carcinoma(Table 1).

Table 1. *Morphology of bladder cancer stages in each experimental group.*

Group	Number	Normal	Hyperplasia	pTa	pT1	pT2
saline group	9	9	0	0	0	0
BBN group	9	0	1	3	1	4
50mg/kg group	10	0	3	1	1	2
250mg/kg group	9	0	6	2	0	1
500mg/kg group	10	0	9	1	0	0

Microscopic images were obtained after H&E staining of the paraffin-embedded bladder tissues. pTa, pT1, and pT2 refer to noninvasive superficial tumors,invasion of the lamina propria, and invasion of the muscularis propria, respectively.

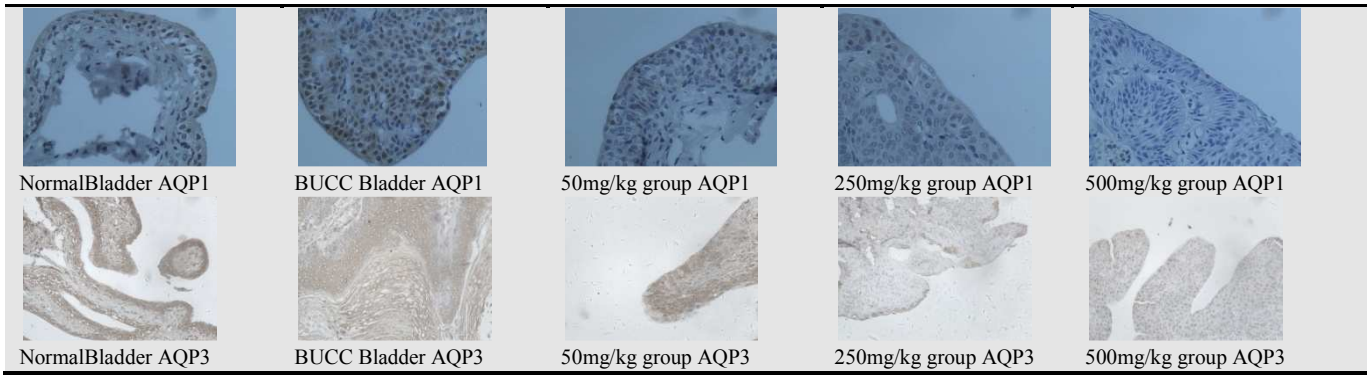
Compared with the bladder carcinoma model, AQP1 and AQP3 mRNA expression reduced following both the 250 and 500 mg/kg *polyporus umbellate* treatments (P<0.05 and P<0.01, respectively) (Table 2). Immunohistochemical analysis indicated that AQP1 and AQP3 protein expression is reduced in bladder carcinoma, which was consistently with the pathological results (Table 3).

Table 2. *Expression of AQP1and AQP3 mRNA in bladder carcinoma rat tissue.*

Groups	n	Expression and relative content of mRNA	
		AQP1	AQP3
The saline group	9	1.0 [0.7, 1.4]	1.0 [0.5, 1.9]
The bladder carcinoma model group	9	1.3 [0.8, 1.9]	2.0 [0.9, 4.4]
The 50 mg/kg polyporus umbellate group	10	0.8 [0.5, 1.4]	2.4 [1.2, 4.7]*
The 250 mg/kg polyporus umbellate group	9	0.5 [0.3, 1.0]**$^{\triangle\triangle}$	0.6[0.2, 1.7]$^{\triangle}$
The 500 mg/kg polyporus umbellate group	10	0.6 [0.4, 1.0]$^{\triangle}$	0.9 [0.4, 2.0]$^{\triangle}$

Notes: Statistical analyses of RT-PCR data were performed using ΔCt values in each group. Compared to saline group,$^{*}P < 0.05$; $^{**}P < 0.01$; $^{\triangle}P < 0.05$,$^{\triangle\triangle}P < 0.01$, compared to BBN group; using Dunnett's t-test. Values represent AQP1, AQP3 mRNA quantitated with 2-ΔΔCT method.

Table 3. *Expression of AQP1 and AQP3 in Bladder Cancer by immunohistochemistry (Positive gray scale value).*



5. Discussion

Bladder carcinoma develops from hyperplasia to invasive bladder carcinoma in rats, similar to the process observed in humans [8]. Studies have demonstrated that induced bladder carcinoma may serve as a useful model to understand how to block bladder carcinoma development [9].

The mechanism which traditional Chinese medicine works in cancer treatments are actively being researched [10]. In recent years, the diuretic function and chemical components of *polyporus umbellate* had been active areas of research, and results indicate that *polyporus umbellate*may be able to prevent bladder carcinoma [2]. This potential function has been attributed to some hepatic microsomal enzyme [11], while others have hinted that it is related to immune activation [12, 13].

In the bladder tissues, AQP1 is expressed in the capillary endothelial and arteriole endothelial cells beneath the bladder

mucous membrane, while AQP3 is distributed in the cytomembrane and the cytoplasm of bladder mucous membrane epithelial cells [5]. A research showed the 5-year survival rate of patients with positive expression of AQP1 was evidently lower than those with negative expression [14].Additionally, AQP1, which expressed excessively in multiple tumors, such as breast cancer [15], adenoid cystic carcinoma, renal cell carcinoma and glioblastoma multiforme [16], etc., could be used as a potential biological marker for the early diagnosis and prognostic predication of tumors [17,18]. The expression level of AQP1 in BUCC tissues, which could be used to predicate the invasive and metastatic severity of tumors primarily, was of certain significance in guiding the clinical diagnosis and treatment as well as prognostic evaluation [19]. Lack of AQP3 protein expression in pT1 tumours was shown to be associated with progression towards muscle-invasive disease, AQP3 maybe an independent predictor of tumour progression from stage pT1 towards stage pT2 tumours [20].

In this study, rats with bladder carcinoma induced by BBN, RT-PCR, especially immunohistochemical results showed AQP1 and AQP3 mRNA and protein expression been activated by BBN while the bladder carcinoma development. Rats with bladder carcinoma were treated with *polyporus umbellate*, RT-PCR results suggested that *polyporus umbellate* inhibits the up-regulation of AQP1 and AQP3 mRNA in bladder carcinoma, the immunohistochemical results indicated that AQP1 and AQP3 protein expression was lower in treated rats compared with control rats. The research results suggested that bladder carcinoma development depended on AQP1 and AQP3 expression, the *polyporus umbellate* could inhibit bladder carcinoma development by inhibit the expression of AQP1 and AQP3 in mediating this process.

6. Conclusions

In conclusions, *Polyporus umbellate* decreased bladder carcinoma growth and inhibited AQP1 and AQP3 expression.

Author Contributions

ZGW initiated the study plan and revised this manuscript. CSY performed all assays and drafted the manuscript. LZB and ZJY provided guidance during the study.

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