

## Original Article

# Expression of zinc- $\alpha$ 2-glycoprotein (AZGP1) is associated with clinical prognosis of bladder cancer a tumor marker?

Shaochen Shen<sup>1\*</sup>, Jianlong Li<sup>2\*</sup>, Hongwei Huang<sup>1</sup>, Yi Yu<sup>1</sup>, Ruihai Xiao<sup>1</sup>, Renrui Kuang<sup>1</sup>, Longwang Wang<sup>1</sup>

<sup>1</sup>Department of Urology, The Second Affiliated Hospital of Nanchang University, Nanchang, China; <sup>2</sup>Department of Urology, Xi'an No. 3 Hospital, Xi'an, China. \*Equal contributors and co-first authors.

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**Abstract:** Zinc- $\alpha$ 2-glycoprotein 1 (AZGP1) is a multidisciplinary protein that participates in many important functions in the human body, including fertilization, immunoregulation and lipid mobilization. Recently, it has been shown that AZGP1 is also involved in carcinogenesis and tumor differentiation. In this study, expression levels and prognostic value of AZGP1 was investigated in bladder cancers. Samples from enrolled 10 paired fresh frozen samples were collected including both adjacent noncancerous tissues and urothelium carcinoma, and total 174 cases of each grade urothelial carcinoma samples. RT-PCR was utilized to detected 10 paired BCa samples and adjacent non-cancerous tissues. Subsequently, 174 samples were determined by immunohistochemistry analysis. Expression levels of AZGP1 in clinical bladder cancer were significantly lower than that in paired adjacent noncancerous tissues ( $P < 0.05$ ). Low level expression of AZGP1 was closely related with differentiation ( $P = 0.013$ ) and tumor style ( $P = 0.028$ ) of patients with bladder cancer. Additionally, Kaplan-Meier analysis revealed that the expression of AZGP1 was closely correlated with overall survival ( $P = 0.019$ ) of bladder cancer, while, recurrence-free survival ( $P = 0.006$ ). Moreover, Cox multivariate regression analyses showed that AZGP1 expression was an independent predictor of overall survival (HR=3.214, 95% CI: [1.008-9.341],  $P = 0.046$ ) and recurrence-free survival (HR=4.507, 95% CI: [1.730-12.412],  $P = 0.005$ ). This study suggests that AZGP1 might serve as a candidate tumor suppressor and a potential prognostic biomarker in bladder carcinogenesis.

**Keywords:** AZGP1 expression, bladder cancer, prognosis

## Introduction

Bladder cancer (BCa) is the second most common genitourinary malignancy after prostate cancer and the ninth most common cancer in the world. Urothelial carcinoma of the bladder is a common pathologically histological type of BCa throughout the world. In 2012, there were 430,000 new cases diagnosed worldwide with a male to female ratio of 3:1 [1]. At presentation, 85% of patients have non-muscle invasive bladder cancer (NMIBC) which comprises stages Tis, Ta and T1 [2]. Despite complete gross resection, bladder cancer has a high rate of recurrence (50-70%) within 5 years, and up to 20% of NMIBC will progress to muscle-invasive disease and require radical treatment [3]. Along with the deeply understanding of tumor molecular mechanism, many tumor biomarkers

have been found that can be used to evaluate prognosis of BCa. Previously described, markers include BLCA-4, CYFRA 21-1, Survivin, and DD23. Other promising, separate Kaplan-Meier analyses for NMIBC and MIBC identified a prognostic potential for miR-141 only in MIBC, but not NMIBC. This initial translational evidence created a rigorous platform implicating a potential value for miRNAs as possibly prognostic markers in BCa [4]. However, the relatively rapid rate of discovery of potential markers has resulted in confusion of which markers or combinations to use in certain clinical situations.

Zinc- $\alpha$ 2-glycoprotein (AZGP1) is a 40-kDa single-chain polypeptide, which is secreted in various body fluids [5]. AZGP1 was first reported in human serum and subsequently purified [6]. The presence of AZGP1 in human seminal fluid

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**Table 1.** Expression of AZGP1 expression in bladder cancer patients according to clinicopathologic characteristics

	NO. (n)	AZGP1 expression		P
		High N (%)	Low N (%)	
Sex				0.839
Male	145	70	75	
Female	29	15	14	
Age (y)				1.000
≤45	22	11	11	
>45	152	74	78	
Differentiation				0.013*
Grade I/II	104	59	45	
Grade III	70	26	44	
Tumor style				0.028*
NMIBC	144	76	68	
MIBC	30	9	21	
Distant metastasis				0.139
Negative	148	76	72	
Positive	26	9	17	
Recurrence				0.761
Negative	81	41	40	
Positive	93	44	49	

Statistical analyses were performed by the Pearson  $\chi^2$  test.  
\* $P < 0.05$  was considered significant.

was reported in 6-fold molar excess compared with human serum [7]. AZGP1 has also been reported to stimulate epididymis fat cells in mice for lipolysis function and then consume the fatty acid in fatty tissue, therefore it is called the fat mobilization factor [8]. Additionally, it has been found in secretory epithelia cells of skin, breast, the gastrointestinal tract [9-11], etc. Subsequently AZGP1 was found to be over-expressed in certain malignant tumors such that it may serve as a cancer marker in ovarian, gastric, prostate carcinomas [12-14]. In addition, Irmaks et al. [15] reported that AZGP1 expression of BCa patients is higher than that of volunteers in urine. AZGP1 may thus be related to the development of superficial bladder cancer and to its switch to an invasive phenotype.

Here, expression levels of AZGP1 between BCa and adjacent non-cancerous tissues utilizing real-time quantitative PCR (RT-PCR) were investigated to detect expression of AZGP1 in clinical low and high-grade carcinoma samples with immunohistochemistry. Furthermore, the relationship between AZGP1 expression and clinical

copathological features was identified and evaluated for its prognostic value for post-operation survival in BCa.

### Methods and materials

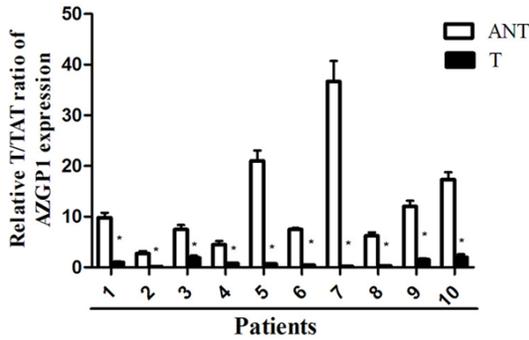
#### Ethics statement

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Review Board and Ethics Committee of the Second Affiliated Hospital of Nanchang University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written consent for using the samples for research purposes was obtained from all patients after surgery and the Ethics Committee approved the consent procedure.

#### Patients and tissue samples

In total, 174 patients (145 males and 29 females) with urothelium carcinoma were treated at the Department of Urology, the Second Affiliated Hospital of Nanchang University between 2011 and 2014. Enrolled 10 paired fresh frozen samples include both adjacent noncancerous tissues and urothelium carcinoma, which had a histologically and pathologically confirmed diagnosis of BCa after operation or under transurethral cystoscopic biopsy, and the adjacent noncancerous tissues were carefully checked without any evidences of malignancy. The age distribution of patients' samples was from 29 to 88 years old, and the mean age was 60.8. According to the 2009 WHO 7th Edited TNM Classification System, 144 patients were primary NMIBC and 30 were MIBC. Pathological grades were classified according to WHO Classification Criteria of Urothelial Carcinoma. Grade I, Grade II and Grade III are well, moderately and poorly differentiated tumors, respectively. Grade I and Grade II were classified as low-grade urothelial carcinomas, Grade III was classified as high-grade ones. In all, 174 cases of urothelial carcinomas were divided into 70 cases of high-grade urothelial carcinoma and 104 cases of low-grade urothelial carcinoma according to the pathological results. All relevant clinical pathologic features of enrolled patients are shown in **Table 1**.

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**Figure 1.** Depressed AZGP1 expression in bladder cancer. Real-time PCR analysis of AZGP1 expression in 10 paired bladder cancer tissues (T) and their adjacent normal tissues (ANT).  $\beta$ -actin was used as loading control.

### Extraction of total RNA and RT-PCR analysis

According to the manufacturer's protocol, Trizol Reagent (TransGen) was utilized for extracting total RNA from 10 pairs of fresh surgical bladder cancer tissues and adjacent non-cancerous tissues. The RNA was pretreated with RNase-free DNase (Promega), and 2  $\mu$ l RNA was used for cDNA synthesis primed with random hexamers. Quantitative real-time PCR was performed through the Applied Biosystems 7500 Sequence Detection system. Sequences of the primers were: 5'-ATAACAGAAGCAGCGGAGCA-3' and reverse: 5'-TATTTCCGAGAGTCGAGG-3';  $\beta$ -actin forward: 5'-CATGGATGATGATATCGC-3' and reverse: 5'-AGGAATCCTTCTGACCCATGC-3'. Expression data were normalized to the housekeeping gene  $\beta$ -actin as a loading control.

### Immunohistochemistry (IHC) analysis

Paraffin-embedded specimens were cut into 5- $\mu$ m sections and mounted onto poly-L-lysine-coated slides, for immunohistochemical detection. The sections were deparaffinized, rehydrated, and then boiled for 10 minutes in 10  $\mu$ mol/L citrate buffer solution (pH6.0) using a microwave oven. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 minutes, and non-specific staining was blocked by treating the slides with 1% FSG for 30 minutes at room temperature. Subsequently, slides were incubated overnight with primary antibodies against AZGP1 (Proteintech, 1:100). After washing with PBS, the slides were incubated with pre-diluted secondary antibody (Sigma-

Aldrich), followed by further incubation with diaminobenzidine (DAB). Finally, sections were counterstained with hematoxylin and mounted.

For AZGP1, the IHC score was defined by Germany Semi-quantitative Scoring System. The mean percentage of positive IHC staining cells were scored as "0" (0-5%), "1" (6%-25%), "2" (26-50%), "3" (51%-75%) or "4" (76%-100%). The staining intensity was scored as "0" (no staining), "1" (weakly stained), "2" (moderately stained), or "3" (strongly stained). The score of each IHC slice was the product of mean percentage of positive IHC staining cells and the staining intensity score. The total AZGP1 immunostaining score ranged from 0 to 12. We defined the AZGP1 expression levels as follows: "-" for a score of 0, "+" for a score of 1-5, which were defined as low expression; "++" for a score of 6-8, and "+++" for a score 9-12, which were defined as high expression cells by the intensity.

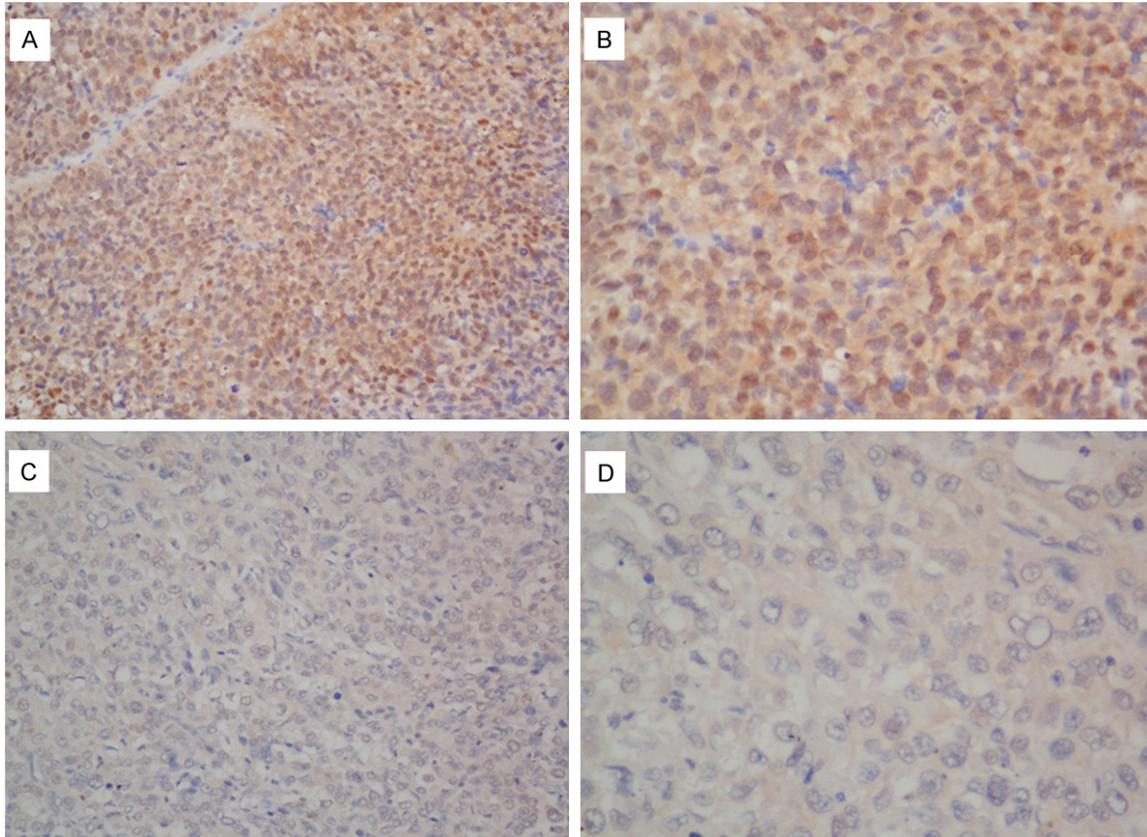
### Statistical analysis

All statistical analyses were carried out by SPSS 19.0 statistical software package. The  $\chi^2$  test or Fisher's exact test and paired-samples t test were used to analyze the correlation between AZGP1 expression and clinicopathologic features. Survival of patients, including overall survival and recurrence-free survival was estimated by Kaplan Meier analysis, and differences were compared by the log-rank test. The Cox proportional hazards regression model was used to assess the relationship between the expressions of AZGP1 and pathological features for the prediction of recurrence for bladder cancer. In all cases,  $P < 0.05$  was considered statistically significant.

## Results

### mRNA expression of AZGP1 analyzed by RT-PCR in BCa samples and adjacent non-cancerous tissues

mRNA expression level of AZGP1 was determined by RT-PCR assays in 10 paired bladder cancer tissues (T) and their adjacent normal tissues (ANT) (**Figure 1**). The AZGP1 expression level was exactly lower in the tumor-bearing tissues, compared with the matched adjacent non-cancerous bladder tissues ( $P < 0.05$ ). The-



**Figure 2.** Representative immunostaining of AZGP1 in low-grade urothelial carcinoma samples (A, B), and high-grade urothelial carcinoma samples (C, D). High expression of AZGP1 (A, B) is showed in low-grade urothelial carcinoma samples and low expression of AZGP1 (C, D) is showed in high-grade urothelial carcinoma samples. (A and C 10×20; B and D 10×40).

refore, results of RT-PCR analysis showed that AZGP1 mRNA expression in BCa samples were significantly decreased than that in adjacent non-cancerous tissues.

*The protein of AZGP1 expression analyzed by immunohistochemistry in low and high-grade urothelial carcinoma samples*

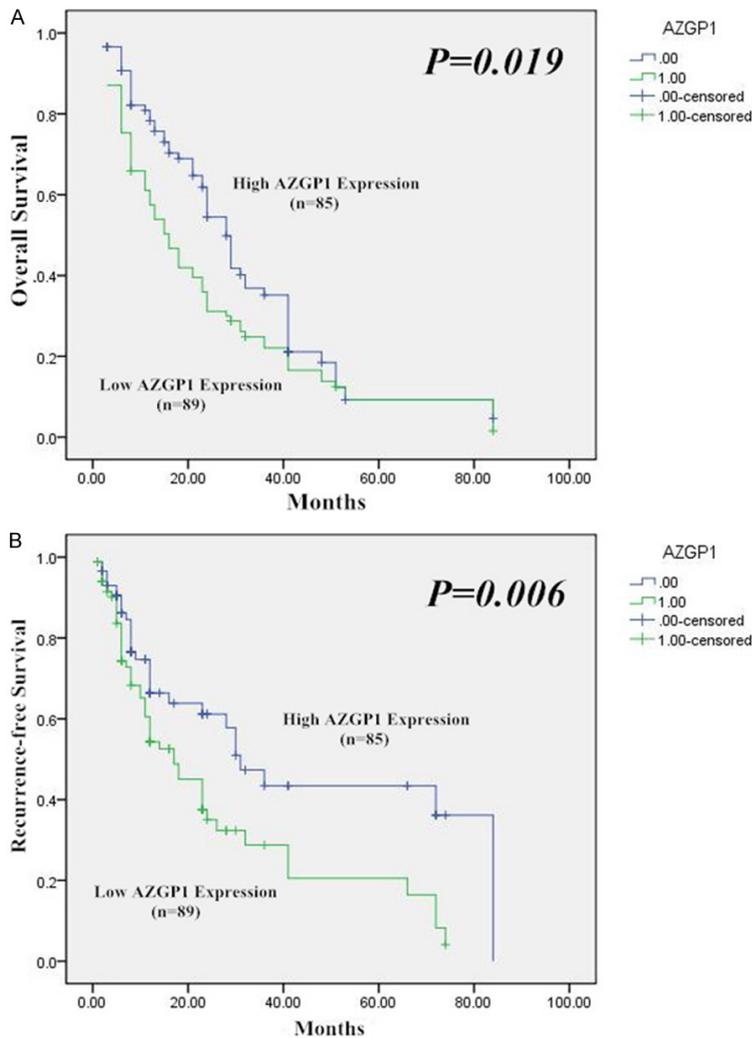
In our study, to investigate the potential roles of AZGP1 in BCa, expression of AZGP1 in 174 sections was examined by immunohistochemistry (**Figure 2A-D**). High-grade urothelial carcinoma samples were found to have high expression of AZGP1 in 26 cases of 70 (37.14%) and low expression of AZGP1 in 44 cases of 70 (62.86%). Furthermore, low-grade urothelial carcinoma samples had high expression of AZGP1 only in 59 cases of 104 (56.73%), and the remaining had low expression of AZGP1 in 45 cases of 104 (43.27%). These data show that the level of AZGP1 expression is strongly

correlated with differentiation ( $P=0.013$ ) and tumor style ( $P=0.028$ ) of patients with bladder cancer, while it is not correlated with sex ( $P=0.839$ ), age ( $P=1.000$ ), distant metastasis ( $P=0.139$ ) and recurrence ( $P=0.761$ ) (**Table 1**). Taken together, these data indicate that AZGP1 expression is commonly elevated in human urothelial carcinoma samples.

*Correlation of AZGP1 expression with the overall survival (OS) and recurrence-free survival (RFS) of bladder cancer*

To determine whether AZGP1 expression level is an independent prognostic factor of patient outcomes, Kaplan-Meier analysis was also applied to calculate the effect of AZGP1 expression and clinical features of bladder cancer. As **Figure 3A** and **3B** shown, statistical analysis revealed that expression level of AZGP1 was closely correlated with OS ( $P=0.019$ ) of BCa, respectively, RFS ( $P=0.006$ ). Moreover, multi-

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**Figure 3.** Patients were divided into two groups: high AZGP1 expressers (n=85) and low AZGP1 expressers (n=89). Kaplan-Meier analysis of overall survival (A) and recurrence-free survival (B) in relation to AZGP1 expression in 174 bladder cancer patients showed a highly significant separation between curves.

ivariate Cox regression analyses showed (as **Table 2**) that expression of AZGP1 was an independent prognostic factor in patients with BCa for OS (HR=3.214, 95% CI: [1.008-9.341],  $P=0.046$ ) and RFS (HR=4.507, 95% CI: [1.730-12.412],  $P=0.005$ ).

### Discussion

BCa remains one of the most deadly human malignancies. Even with advances in diagnosis and therapy, the prognosis for it is still dismal [16]. According to the current investigation, features of tumor, such as growth, differentiation, invasion and distant metastasis are regulated

by a variety of related genes and tumor proteins for the clinical outcome of BCa. However, there is still no much reliable tumor marker to estimate the risk and predict the prognosis of BCa currently.

Previously, AZGP1 has been reported to act as tumor suppressive properties in some other malignant tumors yet. Nevertheless the role and the specific mechanisms of AZGP1 in BCa have not been evaluated. An ocean of investigators made no efforts illustrating it. AZGP1 belongs to an ancient and conservatively evolutionary macroglobulin family linking to the immune system. Zorin et al. [17] reported that the macroglobulin has the ability to combine the hydrolase, which may inhibit tumor erosion mediated by the hydrolase. Moreover, excessive macroglobulin/hydrolase complexes can activate the cell apoptosis [18]. He et al. [19] found that AZGP1 may down-regulate the cell cycle protein kinase, which is responsible for regulating the limitation step in the cell cycle of the G2-M transition. AZGP1 may indirectly inhibit tumor progress. Additionally, the research Kong et al. [20] has shown that AZGP1, a tumor suppressor, can induce transdif-

ferentiation of ectomesenchymal cells of the pancreas through ERK signal pathway mediated by inhibition of TGF- $\beta$  *in vitro*. These are in agreement with our present study. However, so far the expression status of AZGP1 and prognostic value of this protein in BCa have not been reported.

In our study, after investigating AZGP1 mRNA expression in BCa specimens by RT-PCR, AZGP1 mRNA expression was found in bladder cancer samples to be significantly lower than that in adjacent noncancerous tissues, which was consistent with Huang's and Kong's findings [13, 20]. In addition, Irmak's [15] study

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**Table 2.** Multivariate Cox regression analysis of Overall survival (OS) and Recurrence-free survival (RFS) in patients with bladder cancer

Prognostic variables	OS		RFS	
	HR (95% CI)	P	HR (95% CI)	P
Age (>45 vs ≤45)	1.201 (0.200-5.451)	0.926	1.372 (0.296-6.209)	0.637
Differentiation (Grade III vs I/II)	1.428 (0.873-4.361)	0.712	1.517 (0.562-4.243)	0.748
Tumor style (NMIBC vs MIBC)	2.783 (0.915-7.285)	0.501	2.649 (1.726-6.953)	0.738
Distant metastasis (Yes vs No)	3.572 (1.748-8.481)	0.392	2.817 (1.502-6.858)	0.670
Recurrence (Yes vs No)	3.086 (1.934-7.459)	0.027*	2.923 (1.544-6.741)	0.019*
AZGP1 (high vs low)	3.214 (1.008-9.341)	0.046*	4.507 (1.73-12.412)	0.005*

Statistical analyses were performed by the Cox regression analysis. \* $P < 0.05$  was considered significant.

confirmed our results from immunohistochemistry on BCa tissue and revealed the tumor cells were mostly low expression for AZGP1. Furthermore, the correlation of AZGP1 expression with survival by Kaplan-Meier revealed that AZGP1 expression was closely correlated with OS and DFS of BCa. Furthermore, the results of multivariate Cox regression analysis revealed that the expression of AZGP1 is an independent prognostic factor for a poor prognosis of bladder cancer patients. Overall, our study suggests that AZGP1 may play a crucial role in bladder carcinogenesis and low level expression might represent a novel indicator for the poor prognosis of bladder cancer. However, the limitation of our previous reports was continued here. Little is known regarding the specific regulatory mechanisms and signaling pathways of AZGP1 in regulating bladder cell generation, invasion, metastasis, and apoptosis. In conclusion, the present study evaluated the expression levels of AZGP1 and suggests that AZGP1 might serve as a candidate tumor suppressor for early diagnosis and evaluation of prognosis in bladder carcinogenesis.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Longwang Wang, Department of Urology, The Second Affiliated Hospital of Nanchang University, No. 1 Minde Road, East Lake Area, Nanchang 330006, Jiangxi Province, China. Tel: +86 15180161378; E-mail: 252423414@qq.com

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