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Original Article

Zinc transporter 1 (ZnT1) is overexpressed in bladder cancer and promotes the proliferation and invasion of bladder cancer BIU87 cells

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Abstract: This study is to determine the expression status of Zinc transporter 1 (ZnT1) and its correlation with clinic-pathological parameters of bladder cancers and its role in the proliferation of bladder cancer cells. The mRNA and protein levels of ZnT1 in 62 bladder cancer tissue samples and 15 adjacent normal tissues were determined by real-time PCR and Western blot analysis, respectively. Immunohistochemistry (IHC) was performed to evaluate ZnT1 protein expression in 106 bladder cancer tissues and the correlation with different clinic-pathological factors. Small interfering RNA (siRNA) was used to investigate the effects of ZnT1 knockdown on the growth, migration and invasion of human bladder cancer BIU87 cells. The mRNA and protein levels of ZnT1 in bladder cancer tissues were significantly greater than those in corresponding normal bladder tissues. IHC analysis showed ZnT1 was upregulated in 71.7% (76/106) of bladder cancer samples, and was closely associated with gender, histological grade, tumor stage, and recurrence (P<0.01) of bladder cancer patients. Furthermore, silencing of ZnT1 by siRNA significantly inhibited the proliferation, migration and invasion of bladder cancer BIU87 cells (P<0.01). In conclusions, the findings of our study indicate that deregulation of ZnT1 may play an important role in the development and progression of bladder cancer.

Keywords: ZnT1, bladder cancer, proliferation, invasion, migration

Introduction

Bladder cancer is the ninth most common cancer worldwide and accounts for approximately 430,000 new cases and 145,000 deaths in 2012 [1]. Non-muscle invasive bladder cancer is the predominant subtype that constitutes approximately 75% of all new cases [2]. Despite recent progress in surgery, radiation and chemotherapy, it still recurs in 50-70% of patients within 5 years of treatment [3]. Therefore, it is crucial to identify and validate novel biomarkers for the diagnosis, treatment and prognosis of bladder cancer.

Zinc is an indispensable trace element that acts as an essential component of various human Zn metalloproteins, such as intracellular signaling enzymes and transcription factors [4, 5]. Zinc plays a vital role in many biological functions, including nucleic acid metabolism, cell division, tissue repair and normal growth. Recent evidence indicates that Zinc is involved in human cancer development and progression. The decreased Zinc levels were observed in the serum and tissue samples of patients with cancers of liver, gallbladder, digestive tracts and prostate gland [6-10]. Zinc transporter 1 (ZnT1) is a member of the zinc transporter family. It is present on the cell membrane and transports cytoplasmic Zinc ions across the membrane to the extracellular space [11]. It has been reported that dysregulation of the ZnT family is associated with the development of some human cancers [12, 13]. Nonetheless, whether ZnT1 plays a role in bladder carcinogenesis remains to be elucidated.

In the present study, we determined expression of ZnT1 in bladder cancer samples and investigated its association with clinico-pathological features. Additionally, the effects of ZnT1 knockdown by siRNA on cell growth, migration and invasion were evaluated in human bladder cancer BIU87 cells.

Table 1. Correlation between ZnT1 expression and clinico-pathological parameters of bladder cancer patients

Parameters	Group	No. of cases (%)	Cases with high ZnT1 expression (%)	X^2	*P*
Gender	Male	67 (63.2%)	42 (62.7%)	6.13	0.013
	Female	39 (36.8%)	34 (87.2%)		
Age (years)	<55	49 (46.2%)	41 (83.6%)	0.737	0.390
	≥55	57 (53.8%)	35 (61.4%)		
Histologic grade	G1	38 (35.8%)	20 (52.6%)		
	G2	52 (49.1%)	43 (82.7%)	8.070	0.0045
	G3	16 (15.1%)	13 (81.3%)	2.769	0.09
*p*T	<T1	52 (49.1%)	30 (55.8%)	8.559	0.003
	≥T1	54 (50.9%)	46 (87.0%)		
Tumor size	<3.6 cm	77 (72.6%)	53 (68.8%)	0.682	0.409
	≥3.6 cm	29 (27.4%)	23 (79.3%)		
Recurrence	Yes	44 (41.5%)	37 (84.1%)	4.697	0.030
	No	62 (58.5%)	39 (62.9%)		
Progression	Yes	24 (22.6%)	19 (79.5%)	0.443	0.505
	No	82 (77.4%)	57 (70.0%)		

Material and methods

Patients and tissue samples

After obtaining informed consents, tissues were collected from patients who underwent surgery at Department of Urology, The First Affiliated Hospital of China Medical University. This study was reviewed and approved by the Ethics Committee of China Medical University.

Sixty-two bladder cancer samples and 15 adjacent normal tissue samples were collected from March 2011 to Feb 2013 and were immediately stored at -80°C for RNA and protein extraction. Further, immunohistochemistry (IHC) analysis was performed on 106 paraffin-embedded primary bladder cancer samples and 23 matched normal bladder tissues collected between December 2008 and December 2012 in our hospital. All cancer tissues were pathologically confirmed to be bladder transitional cell carcinoma (BTCC). The clinico-pathological data of 106 bladder carcinoma patients are shown in **Table 1**. It was observed that the mean age of this study population was 55.7 years (range, 32-85 years) and comprised of more number of males as compared to females. There were more Grade 2 and advanced stage cancer samples. Based on the 1973 World Health Organization (WHO) guidelines, 35.8% (38/106) tumors were classified as Grade 1, 49.1% (52/106) Grade 2 and 15.1% (16/106) Grade 3. The pathologic staging was determined according to the Tumor-Node-Metastasis (TNM) classification of the International Union against Cancer. Of these 106 cases, 52 (49.1%) were stage Ta, 24 (22.6%) were stage T1, 11 (10.4%) were stage T2, 12 (11.3%) were stage T3, and 7 (6.6%) were stage T4.

Real-time PCR

Total RNA was extracted using Trizol reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. The amount of total RNA was determined by measuring absorbance at 260 nm with a spectrophotometer. One microgram of total RNA was reverse-transcribed to complementary DNA (cDNA) using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) according to the manufacturer's protocol. Real-time PCR was conducted by SYBR Green method on a Corbett Rotor-gene 3000 (Corbett Research, Concorde, Australia) real-time thermal cycler. PCR amplification included an initial denaturation at 95°C for 30 s, followed by 45 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s with detection of double stranded fluorescence products at the end of each cycle at 85°C. The sequences of the primers are as follows: ZnT1, forward: 5'-TACCAFCAACTCCAACGG-3', reverse: 5'-GAACCCAAGGCATCTCCA-3'; β-actin (control gene), forward 5'-TGGCACCCAGCACAATGAA-3'; reverse 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'. The specificity of the PCR was identified and confirmed by melt-curve analysis. PCR reactions were performed in triplicate, and data were analyzed through the comparative threshold cycle (CT) method [14].

Western blot analysis

Total proteins were extracted using radioimmunoprecipitation (RIPA) assay buffer (Wlaterson, Barcelona, Spain), and were quantified using a

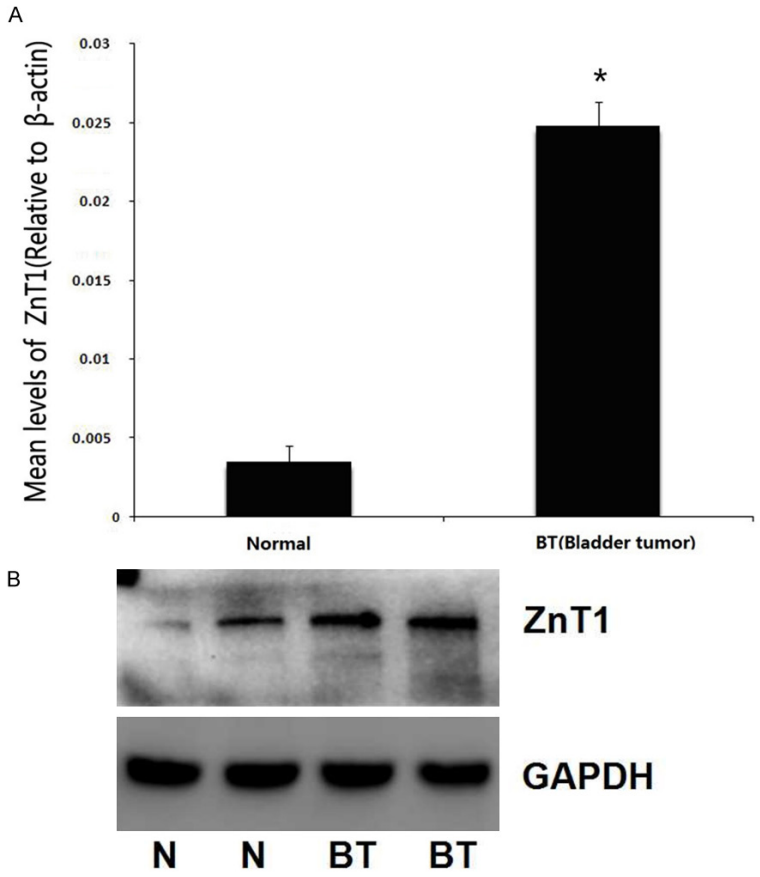

Figure 1. ZnT1 expression in clinical samples. A. ZnT1 mRNA expressions in primary bladder cancer and adjacent normal tissues were examined by RT-PCR. B. ZnT1 protein expressions in bladder cancer and adjacent normal tissues were evaluated by Western blot analysis.

bicinchoninic acid (BCA) protein assay kit (Wlaterson, Barcelona, Spain). Equal amounts of protein (60 μg) were boiled at 100°C for 5 min, separated on 12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE), and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 10% skimmed milk (w/v) at room temperature for 2 h and immunoblotted overnight at 4°C with rabbit anti-human monoclonal ZnT1 antibody (1:500; Abcam) or with mouse anti-human monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, protein bands were visualized by an ECL plus chemiluminescence kit (Beyotime Institute of Biotechnology, Haimen, China). Densitometric analysis was performed using Image J software.

Immunohistochemistry (IHC)

Immunohistochemistry was performed using standard streptavidin peroxidase complex technique on 4 μm-thick paraffin-embedded tissue sections of 106 cancer samples and 23 matched normal samples. After pretreatment and blocking, ZnT1 immunostaining was performed using rabbit anti-human monoclonal antibody (1:200, Abcam, Cambridge, MA, USA) as a primary antibody and streptavidin-biotinylated anti-rabbit immunoglobulin G as the secondary antibody. This was followed by incubation with streptavidin horseradish peroxidase enzyme conjugate. The sections were visualized using 3,3'-diaminobenzidine (DAB) and finally counterstained with hematoxylin. Negative controls were subjected to IHC by replacing the primary antibody with PBS solution. The expression of ZnT1 protein was determined by staining the membrane of tumor cells and was evaluated using a semi-quantitative scoring system where both intensity and percentage of positive tumor cells were analyzed. The intensity of staining was scored as follows: 0, negative; 1+, weakly positive; 2+, moderately positive; and 3+, strongly positive. The percentage of positive tumor cells was graded as follows: 1 for 1-10%, 2 for 11-50%, 3 for 51-75%, and 3 for >75%. A tissue with a total score ≥3 was considered to be positive of ZnT1 expression, and a total score ≥5 was considered to be high expression of ZnT1.

Cell culture and siRNA transfection

Simian virus-40-immortalized normal uroepithelial cell line (SV-HUC-1) was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The human bladder cancer cell lines (BIU87, T24 and 5637) were provided by the Institute of Urology, the First Affiliated Hospital of China Medical University. All cells were propagated in RPMI 1640 medium (Hy-

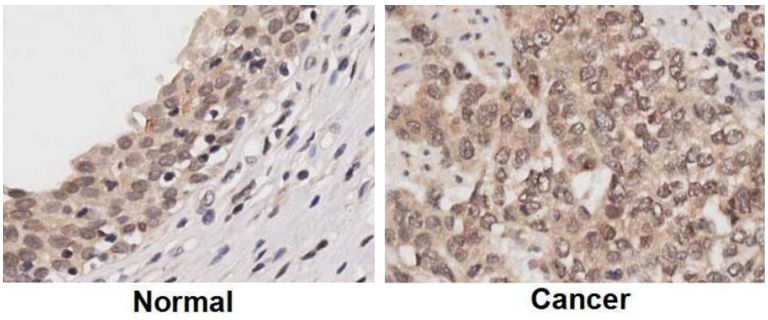

Figure 2. Immunohistochemical staining of ZnT1 protein expression in primary bladder cancer tissue samples. ZnT1 expression in 106 paraffin-embedded bladder cancer samples was detected by IHC. The results showed representative ZnT1 protein expression in adjacent normal tissue and primary bladder cancer.

Table 2. Univariale and multivariale analysis of prognostic factors for bladder cancer

Variables	Hazard ratio (95% CI)	*p* value
Univariate analysis		
ZnT1 expression (low vs high)	2.431 (1.196-3.985)	0.001
Gender (male vs female)	1.205 (0.946-1.601)	0.063
Age (>55 vs ≤55 years)	1.505 (0.938-1.864)	0.726
Tumor size (>3.6 vs ≤3.6 cm)	2.142 (0.886-3.982)	0.452
pT (<T1 vs ≥T1)	1.563 (1.123-4.284)	0.001
Histologic grade (G1 vs G2 vs G3)	1.962 (1.016-3.583)	0.006
Multivariate analysis		
ZnT1 expression (low vs high)	2.241 (1.262-3.889)	0.012
Histologic grade (G1 vs G2 vs G3)	2.126 (1.132-3.968)	0.022
pT (<T1 vs ≥T1)	1.896 (0.946-3.588)	0.384

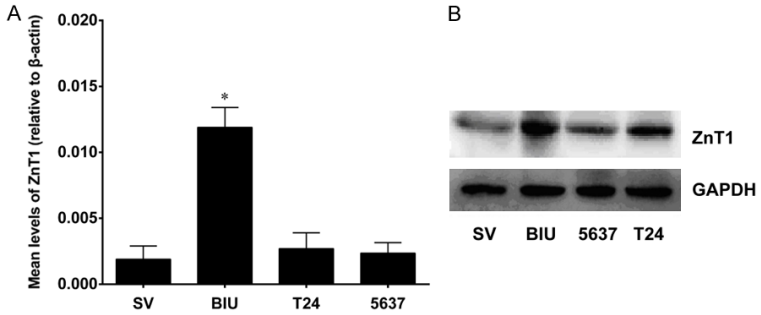

Figure 3. ZnT1 expression in bladder cancer cell lines. qRT-PCR (A) and Western blot (B) analysis of ZnT1 in a panel of bladder cell lines.

Clone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone) at 37°C in a humidified atmosphere of 5% CO_2. Three chemically synthesized oligonucleotides encoding ZnT1 siRNAs were inserted into downstream of the U6 promoter. The three targeting sequences of human ZnT1 are 5'-GTGAGCAGTGGCAATGATTCTGCTTCCAG-3' (ZnT1 siRNA1), 5'-TGAGCAGTGGCAATGATTCTGCTTCCAGC-3' (ZnT1 siRNA2), and 5'-AATGGTAGTAGCGTGAATTCCGTGATTA-3' (ZnT1 siRNA3). siRNA with a scrambled sequence served as a negative control. Transient transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Immunofluorescence

Cells were grown on coverslips, fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and incubated overnight at 4°C with a rabbit monoclonal antibody against human ZnT1 (1:25, Abcam), followed by incubation with a Cy3-labeled secondary antibody. Finally, the nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). The coverslips were examined under a laser confocal microscopy and number of nuclear staining per visual field was quantified.

Methyl thiazolyl terazolium (MTT) assay

Cell viability was evaluated using the MTT assay. In brief, cells were plated at a density of 1×10^4 cells/well in 96-well plates and grown at 37°C for different time intervals. Then, 150 μL of MTT stock solution (0.5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added to each well, and cells were incubated at 37°C for 4 h. Following aspiration of the supernatant, the formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the absorbance was recorded on a microplate reader at 490 nm.

Wound healing assay

Cells were seeded in 6-well plates and incubated to 80% confluence. The cell monolayer was

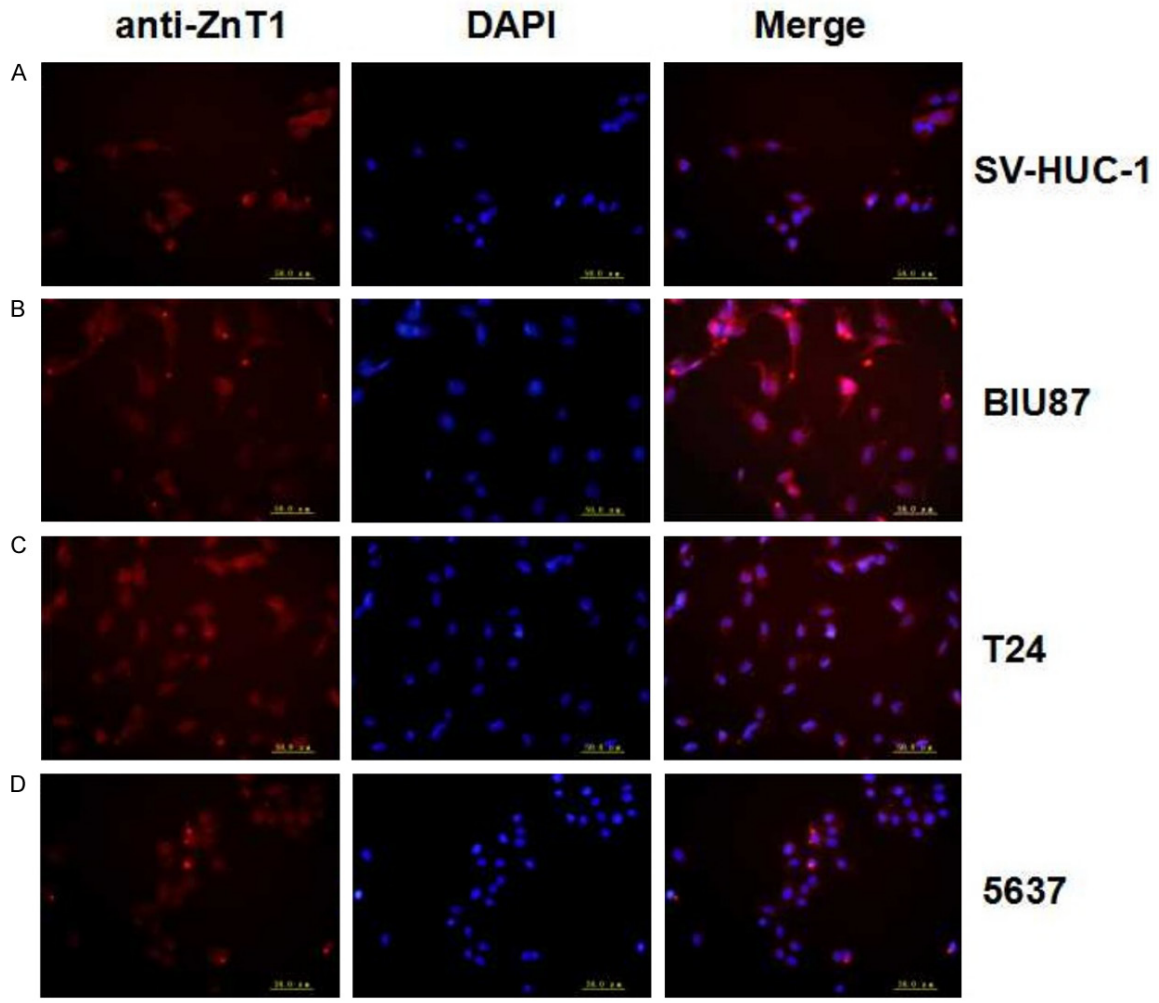

Figure 4. Immunofluorescence staining of ZnT1 in a panel of bladder cell lines. A-D. Immunofluorescence staining for ZnT1 (cytoplasmic and nuclei stain) and nuclei counterstained with DAPI in SV-HUC-1, BIU-87, T24 and 5637, respectively.

gently scraped with a 10 μL pipette tip and washed three times with PBS solution and incubated at 37°C. The scraped cell monolayer was photographed at various time intervals (0, 12, 24, 36 h). Images were acquired using computer-assisted microscopy and the wound width was measured at various time points.

Transwell invasion assay

Cell invasion was determined by the Transwell assay. In brief, the inserts were pre-coated with Matrigel (Becton Dickinson, San Jose, CA, USA) and allowed to solidify at 37°C for 30 min. Cells in FBS-free medium were seeded at a density of 3×10^4 in the upper chambers, and lower wells were loaded with 500 μL culture medium containing 10% FBS. After incubation at 37°C for 24 h, non-migratory cells on the upper surface of the insert were gently removed using a cotton-tipped swab, and cells that had entered the lower surface of the membrane were fixed and stained with crystal violet at room temperature for 20 min. For quantification, five randomly selected fields on the lower side of the insert were photographed.

Statistical analysis

Data are presented as mean ± standard deviation (SD), or alternatively as median and range. The association between ZnT1 expression and clinico-pathological parameters were analyzed by the Chi-square test. Statistical differences between groups for the in vitro cell assays were determined by one-way analysis of variance (ANOVA) and Bonferroni test. Cox's proportional hazards model was used to determine the inde-

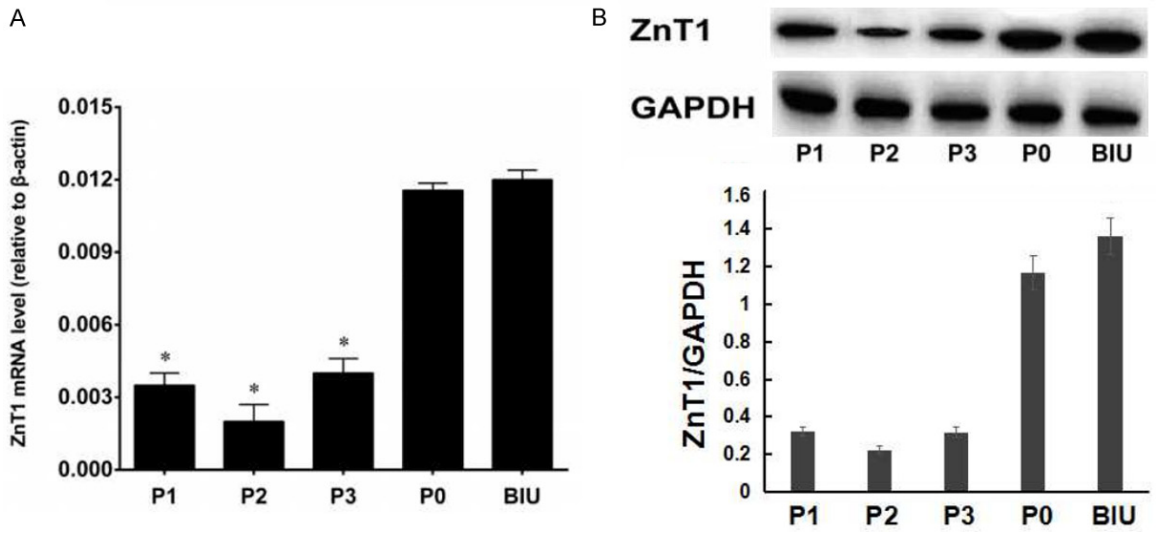

Figure 5. ZnT1 knockdown by plasmid expressing siRNA in BIU87 cells. ZnT1 expression in siRNA-transfected BIU87 cell line by qRT-PCR (A) and Western blot analysis (B) 24 h after transfection.

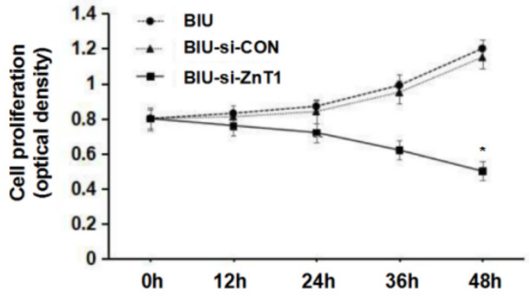

Figure 6. ZnT1 knockdown inhibits proliferation of BIU87 cells. BIU87 cells were transfected with ZnT1-siRNA-2 (BIU-si-ZnT1), siRNA-conrol (BIU-si-CON) or without treatment (BIU) and cell proliferation was assessed by MTT assay at 24, 36 and 48 h post transfection. The data are presented as the mean ± S.D. of triplicate experiments. *P<0.001, compared with BIU group and BIU-siCON.

pendent prognostic factors. All statistical analyses were performed using SPSS software version 17 (SPSS Inc., Chicago, IL, USA). A *P* value <0.05 was considered statistically significant.

Results

Upregulation of ZnT1 in bladder cancers and its correlation with clinico-pathological parameters of bladder cancer patients

Real time PCR analysis revealed that the relative expression of ZnT1 mRNA in 62 bladder cancer tissues was significantly higher than that in the 15 normal adjacent tissues (**Figure 1A**). Consistent with the results of real-time PCR analysis, Western blot analyses demonstrated that there were generally higher levels of ZnT1 protein in the bladder cancer tissues than that in the normal adjacent tissues (**Figure 1B**). We next determined ZnT1 expression status in 106 paraffin-embedded bladder cancer samples by IHC. The results showed that ZnT1 protein was predominantly observed in the cytoplasm of the cancer cells (**Figure 2**). Positive ZnT1 staining was observed in 71.7% (76/106) of cancer samples and only 34.8% (8/23) of adjacent normal tissues. Statistical analyses showed that upregulation of ZnT1 was significantly associated with gender, histological grade, tumor stage, and recurrence, but not with age, tumor size, and progression (**Table 1**). Furthermore, high expression of ZnT1 could predict poor prognosis as a prognostic factor (**Table 2**). Taken together, our data suggest that ZnT1 overexpression might be involved in the development and progression of bladder cancer.

ZnT1 expression is increased in bladder cancer cells

To explore the underlying mechanism of the bladder cancer tumorigenesis by ZnT1, we first assessed the expression of ZnT1 in bladder cancer cell lines (BIU87, T24 and 5637) and normal bladder SV-HUC-1 cells. The real time PCR showed that the mRNA level of BIU87 cells was significantly higher than bladder cancer cell lines T24 and 5637, and normal bladder SV-HUC-1 cells (**Figure 3A**, *P*<0.05). Western blot analysis revealed that ZnT1 protein was relatively higher in bladder cancer cell lines

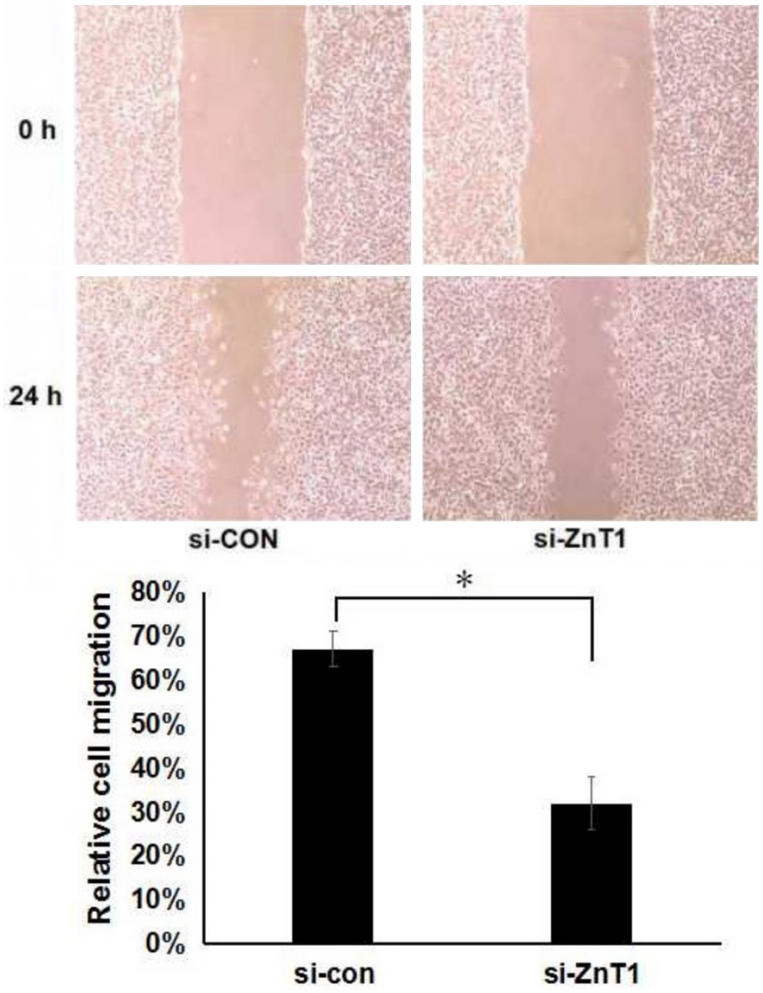

Figure 7. Wound healing assay of BIU87 cell migration after transfection with ZnT1 siRNA. BIU87 cells were transfected with ZnT1-siRNA-2 (si-ZnT1) or si-Control (si-CON), followed by wound healing assays. Representative pictures (upper) and quantitative analyses results (lower) are shown. The data are presented as the mean ± S.D. of triplicate experiments. *P<0.05.

than that in normal bladder SV-HUC-1 cells, with the highest level in BIU87 cells (**Figure 3B**). Immunofluorescence staining revealed that ZnT1 protein was primarily expressed in the cytoplasm of SV-HUC-1 cells, while it was detected in nuclear and perinuclear region of bladder cancer cell lines (**Figure 4**). Since BIU87 cells had the highest levels of ZnT1 mRNA and protein, they were used for the subsequent experiments.

ZnT1 knockdown by plasmid expressing siRNA in BIU87 cells

In order to explore the biological role of ZnT1 in bladder cancer cells, we transfected BIU87 cells with specific siRNAs to knockdown ZnT1 expression. Our data revealed that 24 h post-transfection with ZnT1-siRNA-1, siRNA-2, and siRNA-3 containing vectors, ZnT1 mRNA levels were significantly down-regulated by 68.3%, 83.2% and 66.2%, respectively (**Figure 5A**; *P*<0.01). Similarly, Western blot analysis demonstrated apparent decrease of ZnT1 protein levels in BIU87 cells transfected with ZnT1-siRNA-1, siRNA-2, and siRNA-3 when compared to the control vector-transfected cells (**Figure 5B**). These findings demonstrated that ZnT1-specific siRNAs effectively inhibited ZnT1 expression at both mRNA and protein levels in BIU87 cells. Since it showed the highest knockdown efficiency, ZnT1-siRNA-2 was used for further experiments.

ZnT1 knockdown inhibits proliferation of BIU87 cells

To test the effects of ZnT1 knockdown on bladder cancer cell proliferation, BIU87 cells were transfected ZnT1-siRNA-2 and cell proliferation was assessed by MTT assay at 24, 36 and 48 h post transfection. It was observed that silencing of ZnT1 expression by siRNA lead to a significant decrease in the proliferation of BIU87 cells (**Figure 6**, *P*<0.01), suggesting a role of ZnT1 in the proliferation of BIU87 cells.

ZnT1 knockdown suppressed cell migration and invasion of BIU87 cells

To test the role of ZnT1 in the migration and invasion of bladder cancer cells, BIU87 cells were transfected ZnT1-siRNA-2, followed by wound healing and Transwell assays. We observed a dramatic inhibition in migration of cells to the wounded area of cells transfected with ZnT1 siRNA (*P*<0.01; **Figure 7**). Moreover, Transwell assay showed an obvious decrease in the number of invasive cells when compared

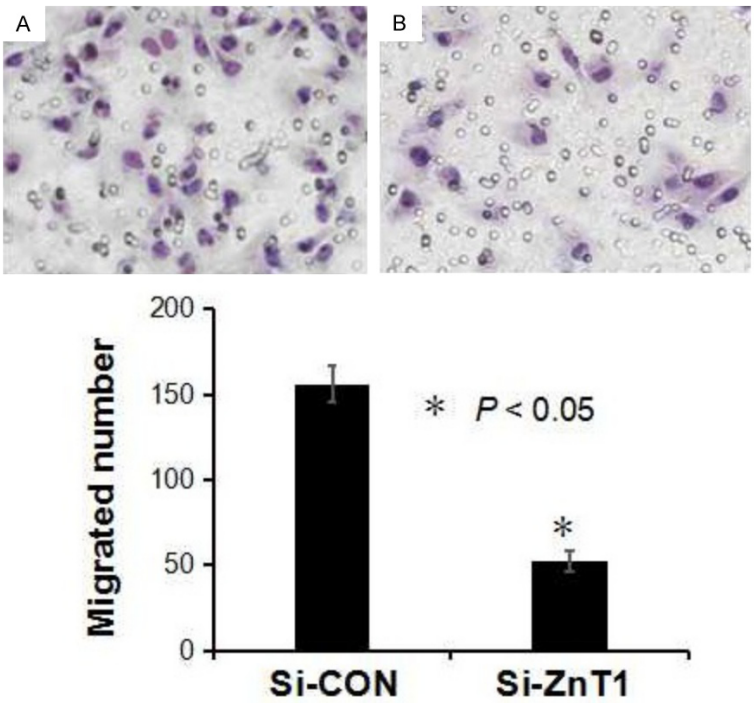

Figure 8. Transwell migration assay of BIU87 cell invasion after transfection with ZnT1 siRNA. BIU87 cells were transfected with ZnT1-siRNA-2 (si-ZnT1) or si-Control (si-CON), followed by Transwell migration assays. Representative pictures (upper) and quantitative analyses results (lower) are shown. The data are presented as the mean ± S.D. of triplicate experiments. *P<0.05.

to the control vector-transfected cells (**Figure 8**). These results indicate that ZnT1 may have a role in the invasion and migration of bladder cancer cells.

Discussion

In the present study, we found that ZnT1 was upregulated in bladder cancer tissues and was significantly associated with clinico-pathological parameters such as gender, histological grade, tumor stage, and recurrence. In vitro, silencing of ZnT1 inhibited the growth, migration and invasion of bladder cancer BIU87 cells. Our results suggest that ZnT1 may play an important role in bladder carcinogenesis.

Zinc is a trace element that has been shown to play an important role in various human biological processes, including tumor growth [15]. It has been reported that zinc acts as an anticancer agent in esophageal [16], ovarian [17], and prostate cancers [18]. The two families of zinc transporters involved in zinc homeostasis in the body are zinc transporter (ZnT) and zinc influx transporter (ZIP) [19, 20]. The ZIP family members promote transport (influx) of extracellular zinc into the cytosol of the cells and have been demonstrated to be down-regulated in some human cancers [21]. For example, Zou et al. have reported that overexpression of Ras responsive element binding protein-1 (RREB-1) contributes to ZIP1 down-regulation in prostate cancer [22]. Moreover, the downregulation of ZIP14 is likely to be involved in the depletion of zinc and the progression of hepatocellular cancer [23]. In contrast, the ZnT family is responsible for transport of cytoplasmic zinc ions across the membrane to the extracellular space. Previous reports have documented that ZnT family is involved in the development of some human cancers [12, 13]. However, there are no such reports on bladder cancer. Earlier reports have shown that zinc inhibits cell growth, promotes cell apoptosis, and suppresses cell migration and invasion in prostate cancer cells [24-26]. In consistent, we found that ZnT1 upregulation is associated with the progression of bladder cancers. Thus, it is possible that ZnT1 transporter overexpression may cause loss of Zn accumulation in cells thereby promoting bladder carcinogenesis.

In summary, our observations provide preliminary evidence of the potential role of ZnT1 in bladder cancer development and progression. However, these results need to be confirmed in a larger cohort of population to obtain statistical correlations for ZnT1 to be used in future as a promising biomarker for the diagnosis, treatment and prognosis of bladder cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

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