TRPM7 is overexpressed in bladder cancer and promotes proliferation, migration, invasion and tumor growth

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Abstract. Recent findings suggest that the melastatin transient receptor potential channel 7 (TRPM7) is overexpressed in many types of cancers and is involved in tumorigenesis. However, its expression pattern and the potential role in bladder cancer remain unclear. The aim of the present study was to investigate the expression status of TRPM7 and its relationship with the development of bladder cancer. In the present study, we observed that the expression of TRPM7 was significantly elevated in bladder cancer tissues compared with that noted in the adjacent non-tumor tissues. Furthermore, increased TRPM7 expression was significantly associated with recurrence, metastasis and prognosis. In addition, after knockdown of the expression of TRPM7 by siRNA, the proliferation and the motility of T24 and 5637 cells were obviously inhibited, and downregulation of TRPM7 was found to play an important role in bladder cancer cell apoptosis. In conclusion, our findings suggest that TRPM7 plays an important role in bladder cancer, and TRPM7 may serve as a potentially unfavorable factor and novel target for human bladder cancer.

Introduction

Bladder cancer is one of the most common genitourinary malignancies arising from the epithelial lining of the urinary bladder. In China, the incidence and mortality rate have been rapidly increasing in the last few decades (1,2). Tobacco use, *Schistosoma* infection, chemical exposure, diet and lifestyle trends and genetic susceptibility have been reported to be risk factors for the tumorigenesis and progression of bladder cancer (3-5). Standard treatment for bladder cancer patients includes surgical resection or adjuvant chemotherapy and radiation. However, despite advances in diagnosis and therapy, there has been no improvement in the overall survival rate for bladder cancer patients in recent decades. Therefore, identification of novel molecular markers is critical to refining our understanding of the pathogenesis of bladder cancer and for developing more efficient treatment and surveillance strategies.

The transient receptor potential (TRP) channel family includes biological transmembrane proteins that play an important role in various physiologic and pathologic processes by modulating cytoplasmic signaling and cellular responses (6). Recent studies have reported that TRPM7 is highly expressed in various types of cancers such as breast, ovarian and prostate cancer, glioblastoma and pancreatic cancer (9-13). Accumulating evidence has also shown that TRPM7 plays an important role in malignant progression including the regulation of cell proliferation, adhesion, apoptosis, gene expression, cell migration and metastasis (14-19). It has been found that TRPM7 regulates cancer cell proliferation and migration mainly by the channel activity mediating influxes of both Ca$^{2+}$ and Mg$^{2+}$ (20,21). Moreover, regulation of cell adhesion by TRPM7 includes the combined effect of Ca$^{2+}$ and kinase-dependent pathways on actomyosin contractility (22). However, the involvement of TRPM7 in the pathogenesis and progression of bladder cancer remains to be detected.

The aim of the present study was to characterize the expression and biological role of TRPM7 in bladder cancer. We found that TRPM7 was overexpressed in bladder cancer. In addition, TRPM7 was strongly correlated with clinicopathological characteristics and poor survival rates in bladder cancer. Furthermore, the effect of TRPM7 on the biological behaviors of bladder cancer was investigated by anti-TRPM7 small interfering RNA (siRNA) assays. The data revealed that TRPM7 promoted bladder cancer proliferation, migration and invasion.

Materials and methods

Tissue specimens. Bladder cancer and paired adjacent normal bladder tissues used for immunohistochemistry were collected from 74 bladder cancer patients who underwent surgical resection in The First Affiliated Hospital of China Medical University from 2008 to 2015. None of patients underwent chemotherapy, radiotherapy or adjuvant treatment before surgery. The present study was approved by the Ethics Committee of the China
Medical University. Informed consent was obtained from all patients. Twenty pairs of fresh bladder cancer and adjacent non-tumor bladder tissues used for quantitative PCR were collected from The First Affiliated Hospital of China Medical University and snap frozen in liquid nitrogen until use. The patients had not received any therapy before admission.

**Immunohistochemical staining.** Immunohistochemistry for TRPM7 expression in bladder cancer tissues was performed using standard methods. Sections were deparaffinized in xylene and hydrated in a graded ethanol series. The sections were then processed in 10 mmol/l citrate buffer (pH 6.0) and heated at 120°C for 5 min to retrieve the antigen. After that, sections were soaking in 3% hydrogen peroxide for 20 min, which served as a blocking agent for endogenous peroxidase activity. After being rinsed in phosphate-buffered saline which served as a blocking agent for endogenous peroxidase activity, sections were incubated with anti-TRPM7 (1:50; Abcam) antibody in 2% bovine serum at 4°C. After 1 h at room temperature, sections were rinsed in phosphate-buffered saline with Tween-20 (TBST) buffer for 2 h at room temperature and incubated overnight at 4°C with a secondary antibody. Horseradish peroxidase-conjugated anti-goat IgG was used as a secondary antibody. After washing, the peroxidase reaction was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen solution. Finally, the sections were counterstained with hematoxylin, dehydrated and coverslipped.

All the slides were evaluated independently by two pathologists using a conventional semi-quantitative scoring system according to a previously defined scoring system (16). Briefly, the intensity of the staining in each section was assessed as very strongly positive (3), moderately positive (2), weakly positive (1), or negative (0). The percentage of positive tumor cells was scored as <5% (0), 5-20% (1), 21-50% (2) and >50% (3). The final score was calculated by multiplying the percentage and the intensity score. A score of ≥4 was defined as high TRPM7 expression and scores of <4 were defined as low TRPM7 expression.

**Cell culture and siRNA transfection.** Human bladder cancer cell lines (BIU87, 5637 and T24) were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) (both from HyClone, Logan, UT, USA) at 37°C in 5% CO2 incubator. We obtained TRPM7 siRNA and the negative control siRNA (NC_siR) from GenePharma Co. Ltd. (Shanghai, China). Before transfection, cells were plated overnight and grew to 30-50% confluency. According to the manufacturer's instructions, the siRNAs were transfected into 5637 and T24 cells using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA). The sequences of siRNAs are shown in Table I. The cells were then incubated with FITC-labeled secondary antibody for 2 h at room temperature. Nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI) (2 µg/ml). Fluorescence was visualized with a confocal microscope (Carl Zeiss Inc., Gottingen, Germany).

**Quantitative real-time PCR.** Total RNA was isolated from bladder cancer and adjacent non-tumor bladder tissues with TRIzol reagent (Invitrogen). cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis kit (Roche, Mannheim, Germany). Then, SYBR-Green Real-Time PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used. Real-time PCR was performed using Thermal Cycler Dice™ Real-Time System TP800 (Takara, Tokyo, Japan). Amplification conditions consisted of 2 min at 50°C for reverse transcription, 5 min at 95°C for Taq activation followed by 45 cycles of 94°C for 40 sec, one cycle of 58°C for 20 sec and elongation at 72°C for 30 sec. The sequence of primers designed for TRPM7 and gAPDH are listed in Table I. gAPDH served as the internal control for mRNA determination of TRPM7. Results were normalized to gAPDH. The relative gene expression was calculated using 2-ΔΔCt.

**Western blot analysis.** Cells were lysed by ice-cold cell RIPA buffer. Protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA). Total cellular protein (30 µg) was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) filter membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST) buffer for 2 h at room temperature and incubated overnight at 4°C with a specific primary antibody against TRPM7 (ab85016; 1:500 dilution; Abcam) and mouse-anti-human actin (1:2,000 dilution; Abcam). TRPM7, transient receptor potential channel 7; F, forward; R, reverse; S, sense; A, antisense.
Table II. Relationship between the expression of TRPM7 and clinicopathological factors in 74 bladder cancer patients.

| Parameters                      | No. case | Low | High | P-value |
|---------------------------------|----------|-----|------|---------|
| Sex                             |          |     |      | 1.0     |
| Male                            | 46       | 17  | 29   |         |
| Female                          | 28       | 10  | 18   |         |
| Age, years                      |          |     |      | 0.432   |
| <60                             | 21       | 6   | 15   |         |
| ≥60                             | 53       | 21  | 32   |         |
| Histological grade              |          |     |      | 0.226   |
| Low                             | 45       | 19  | 26   |         |
| High                            | 29       | 8   | 21   |         |
| Tumor stage                     |          |     |      | 0.333   |
| Ta-T1                           | 40       | 17  | 23   |         |
| T2-T4                           | 34       | 10  | 24   |         |
| Multiplicity                    |          |     |      | 0.054*  |
| Unifocal                        | 35       | 17  | 18   |         |
| Multifocal                      | 39       | 10  | 29   |         |
| Recurrence                      |          |     |      | <0.01*  |
| Yes                             | 47       | 3   | 44   |         |
| No                              | 27       | 24  | 3    |         |
| Metastasis                      |          |     |      | 0.021*  |
| Yes                             | 17       | 2   | 15   |         |
| No                              | 57       | 25  | 32   |         |

*Statistical analyses were performed using the Pearson's χ² test. P<0.05 was considered significant. TRPM7, transient receptor potential channel 7.

Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as an internal control. Next, the membranes were incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2,500 dilution; Santa Cruz Biotechnology, Inc.), and signals were developed using Western Blotting Luminol Reagent (Gene Company Ltd., Hong Kong).

Cell proliferation assays. Cell proliferation was determined using the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Tokyo, Japan). The cells (3x10³ cells/well) were seeded into 96-well plates. At time points of 0, 6, 12, 24, 36 and 48 h, 10 µl of CCK-8 was added/well and was incubated at 37°C for 1 h. The absorbance was measured at 450 nm using a plate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA) to determine the number of viable cells. All of the experiments were performed three times.

Wound healing assay. Cells (5x10³ cells/well) were seeded into 6-well plates. After the cells were grown to 80% confluency, wounds were created by scraping the cells with a 100-µl pipette tip. The 6-well plates were incubated at 37°C and a microscope was used to observe the migrated distance every 12 h.

Flow cytometric analysis for alteration in apoptosis. For apoptosis analysis, after transfection for 48 h, cells were collected, washed with PBS, and stained with FITC/Annexin V Apoptosis Detection Kit I (BD Biosciences), and analyzed by flow cytometric analysis.

Cell cycle analysis. Cells were trypsinized and washed in ice-cold PBS, and then fixed in ice-cold 75% ethanol in PBS overnight. PI/RNase staining buffer (BD Biosciences) was added, and the cells were incubated at 4°C for 30 min. Cell cycle profiles were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Calculation and statistical analysis. All data are presented as means ± standard deviation (SD) and analyzed using SPSS 21.0 statistical software (SPSS, Inc., Chicago, IL, USA). t-test was used to determine the significance of differences in multiple comparisons. Survival curves were estimated by Kaplan-Meier analysis and compared by the log-rank test. All tests performed were two-sided. P<0.05 was considered statistically significant.

Results

TRPM7 is overexpressed in bladder cancer tissues. To characterize the expression of TRPM7 in human bladder cancer tissues, we firstly examined the mRNA and protein levels of TRPM7 in 20 pairs of human bladder cancer, and the adjacent non-tumor bladder tissues by qRT-PCR and western blotting. As shown in Fig. 1A and B, the mRNA and protein levels of TRPM7 were significantly increased in the bladder cancer tissues compared with levels in the paired adjacent non-tumor tissues (P<0.05). Next, we performed immunohistochemical analysis to assess the protein expression and subcellular localization of TRPM7 in 74 paraffin-embedded bladder cancer tissues (Fig. 1C). High TRPM7 expression was detected in 47 (63.5%) of the 74 bladder cancer tissues and in 16 (25.80%) of the 62 adjacent non-tumor tissues (P<0.05). These data suggest that TRPM7 is overexpressed in bladder cancer tissues and may be a potential biomarker for bladder cancer.

Relationship between TRPM7 expression and clinicopathological variables in bladder cancer. To investigate the potential role of TRPM7 in bladder cancer, the relationship between
TRPM7 expression level and clinicopathological factors was analyzed and is summarized in Table II. As shown, TRPM7 overexpression was associated with recurrence (P<0.01), and metastasis (P=0.021) of patients with bladder cancer. However, TRPM7 exhibited no significant association with other clinicopathological characteristics, such as age, sex, histologic grade, tumor stage and multiplicity (all P>0.05).

High TRPM7 expression predicts poor prognosis of bladder cancer patients. The prognostic value of TRPM7 was evaluated by Kaplan-Meier analysis. The survival curves indicated that high TRPM7 expression was significantly associated with poor survival of bladder cancer patients. Patients with TRPM7 high expression had worse prognosis than those with low expression of TRPM7 (Fig. 2) (P<0.05).

Furthermore, univariate and multivariate Cox regression analyses were applied to the clinicopathological characteristics in regards to TRPM7 expression levels. As shown in Table III, multiplicity (P=0.017), recurrence (P<0.01), metastasis (P=0.049) and TRPM7 expression (P<0.01) were all significantly related to bladder cancer patient poor survival. Multivariate analysis showed that high TRPM7 expression...
was independently associated with the poor prognosis of bladder cancer patients (P=0.035) (Table IV).

Table IV. Contribution of various potential prognostic factors to survival by Cox regression analysis on 74 human bladder cancer tissues.

| Parameters | Hazard ratio | P-value | 95% CI       |
|------------|--------------|---------|--------------|
| Multiplicity | Unifocal vs. multifocal | 0.930   | 0.838       | 0.466-1.857 |
| Recurrence | Yes vs. no | 5.542   | 0.043*       | 1.052-29.194 |
| Metastasis | Yes vs. no | 1.384   | 0.379       | 0.671-2.853 |
| TRPM7 expression | Low vs. high | 0.089   | 0.035*       | 0.009-0.846 |

Statistical analyses were performed using the Cox regression analysis; *P<0.05 was considered significant. CI, confidence interval; TRPM7, transient receptor potential channel 7.

TRPM7 mRNA and protein levels are higher in 5637 and T24 cells than BIU87 cells. To examine the mRNA and protein levels of TRPM7 in BIU87, 5637 and T24 bladder cancer cell lines, RT-PCR and western blotting were carried out, respectively. Fig. 3A shows that the levels of TRPM7 mRNA in 5637 and T24 cells (normalized to GAPDH, 1.38±0.7, 1.45±0.6; P<0.05) were significantly higher than that noted in the BIU87 cells (0.75±0.4; P<0.05). Western blotting demonstrated that TRPM7 protein levels (normalized to GAPDH) were also higher in the 5637 and T24 cells (1.25±0.41, 1.36±0.49; P<0.05) compared to that noted in the BIU87 cells (0.58±0.32) (Fig. 3B). TRPM7 protein levels were further determined in bladder cancer cell lines using immunofluorescent staining. As shown in Fig. 3C, higher levels of TRPM7 protein were observed in the 5637 and T24 cells compared to that noted in the BIU87 cells in cell culture, and the TRPM7 protein trend was to localize around the nuclear membrane in the 5637 and T24 cells. The fluorescence intensity in the 5637 and T24 cells was 25 or 30% higher than that in the BIU87 cells (P<0.05).

TRPM7 siRNA effectively suppresses TRPM7 expression. To investigate the role of TRPM7 in bladder cancer, TRPM7 was knocked down using RNAi method. T24 cells were transfected with three siRNAs targeting TRPM7. As illustrated in Fig. 4, the mRNA (Fig. 4A) and protein (Fig. 4B) expression levels of TRPM7 were both significantly inhibited by TRPM7_siR1. TRPM7 knockdown inhibits cellular proliferation and induces cell apoptosis. We transfected the 5637 and T24 cells with TRPM7_siR1 and NC_siR. Compared with NC_siR and control groups, flow cytometric analysis revealed that downregulation of TRPM7 significantly induced apoptosis (Fig. 5A). Statistical analysis of apoptosis is shown in Fig. 5B. CCK-8 assay was carried out to assess the effects of TRPM7 knockdown on cell proliferation. The CCK-8 assay showed that after downregulation of TRPM7 with TRPM7_siR1, T24 and 5637 cells exhibited a significant decrease in cell proliferation compared with that noted in the control siRNA group (Fig. 5C). Flow cytometric cell cycle analysis revealed that 5637 and T24 cells were blocked in the G2/M phase after transfection with TRPM7_siR1 (Fig. 6A and B).

TRPM7 knockdown inhibits cell motility. We next investigated the effect of TRPM7 on cell motility with wound-healing and Transwell assays. After being incubated with physical-wound and cultured in serum-free medium to exclude the interference of proliferation, the percentage of wound closure at 48 h was significantly lower in the TRPM7_siR1-treated cells than that...
Figure 3. The difference in mRNA and protein levels of TRPM7 in bladder cancer cell lines. (A) The amount of TRPM7 mRNA in 5637 and T24 cells was significantly higher than that in BIU87 cells (*P<0.05). (B) Western blotting demonstrated that TRPM7 protein level (normalized to GAPDH) was also higher in the 5637 and T24 cells compared to that in the BIU87 cells (P<0.05). (C) Higher level of TRPM7 protein was observed in 5637 and T24 cells compared to that in the BIU87 cells by immunofluorescent staining (P<0.05), and the TRPM7 protein trend was to localize around the nuclear membrane in the 5637 and T24 cells.

Figure 4. Expression of TRPM7 in T24 cells is effectively downregulated by specific TRPM7 siRNAs. (A) Interference efficiency at the mRNA level was detected by RT-qPCR. (B) Interference efficiency at the protein level was detected by western blotting compared with NC_siR; *P<0.05.
Figure 5. Effect of TRPM7 on cell proliferation and apoptosis. 5637 and T24 cells were transfected with TRPM7_siR1 for 48 h and compared with NC_siR and parental cells. (A and B) Apoptotic cells stained with Annexin V and PI were revealed by flow cytometric analysis and the apoptotic rates were statistically analyzed; *P<0.05. (C) Cell proliferation of T24 and 5637 cells was measured by CCK-8 assay. The inhibition rate of T24 and 5637 cells with different treatments; *P<0.05 compared with NC_siR and control group.

Figure 6. Flow cytometric cell cycle analysis of bladder cancer cells. (A and B) 5637 and T24 cells in the G2/M phase were markedly increased in the TRPM7_siR1 group compared with the control and NC_siR groups.
noted in the control cells (Fig. 7A and B). Transwell assays showed that the silencing of TRPM7 measurably inhibited cell migration and invasion in the Transwell assays (Fig. 7C and D). All the data support that TRPM7 stimulates bladder cancer cell migration and invasion.

**Discussion**

It is believed that bladder tumorigenesis and development are multistep pathologic processes involving numerous genetic alterations, of which the inactivation of tumor suppressors and activation of oncogenes are critical events in the initiation of bladder tumors. As one of the most fatal carcinomas worldwide, the molecular mechanisms of bladder cancer remain unknown. It is urgent to identify novel molecules which may serve as prognostic factors and therapeutic targets for bladder cancer. In the present study, we found that TRPM7 is highly expressed in bladder cancer and is closely correlated with clinical stages and poor prognosis of bladder cancer patients. However, TRPM7 accelerated bladder cancer cell growth and migration. Taken together, these results suggest that TRPM7 may be a potential regulator in bladder cancer progression.

Calcium (Ca\(^{2+}\)) and magnesium (Mg\(^{2+}\)) are two important metal elements that contribute to a variety of tumor cell

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**Figure 7.** Effect of TRPM7 on cell migration and invasion. (A and B) T24 and 5637 cells transfected with TRPM7 siRNAs showed a slower wound closure rate than cells transfected with non-specific siRNA or control. Scale bar, 50 µm. (C and D) Knockdown of TRPM7 inhibited cell migration and invasion as detected by Transwell assays. Number of cells that invaded through the membrane was counted in 10 fields under magnification, x200; *P<0.05 compared with NC_siR and control group.
processes such as proliferation, migration and apoptosis. Studies have reported that TRP channels are involved in a variety of basic cellular processes and are crucial for carcinogenesis and cancer development (22,23). TRPM7, belonging to the TRP channel family, is a non-selective cation channel mediating both Ca²⁺ and Mg²⁺ flow (7,8). TRPM7 is ubiquitously expressed and essential for diverse physiological and pathophysiological processes such as excitability, gene expression, muscle contraction, cell volume regulation and hormone secretion (20,24). Accumulating studies have confirmed that TRPM7 is aberrantly overexpressed and plays a vital role in various types of cancers (25-28). For example, TRPM7 is high expressed in pancreatic cancer and correlates with tumor size and stage. Moreover, TRPM7 is required for pancreatic cancer invasion (13). However, the contribution of TRPM7 to bladder cancer carcinogenesis remains largely unidentified and needs to be determined.

In the present study, we firstly detected TRPM7 expression in human bladder cancer tissues by RT-PCR, western blot and immunohistochemical analyses. The expression levels of TRPM7 mRNA and protein were significantly higher in bladder cancer than levels in the non-tumor tissues. In addition, a similar consequence was detected in bladder cancer cell lines (BIU87, 5637 and T24). Next, the correlation analysis demonstrated that higher TRPM7 expression level was closely associated with recurrence (P<0.01) and metastasis (P=0.021). All these data suggest that TRPM7 functions as a potential oncogene and plays an important role in the progression of bladder cancer.

Previous research has demonstrated that TRPM7 is correlated with the poor prognosis of various malignancies such as neuroblastoma (19,29). Thus, we performed Kaplan-Meier analysis to investigate the prognostic role of TRPM7 in bladder cancer patients. We found that overexpression of TRPM7 was correlated with the patient overall survival time. In addition, univariate and multivariate analyses revealed that TRPM7 is a significant independent prognostic predictor for bladder cancer patients.

Thus, to further explore the functions of TRPM7 in bladder cancer, we determined the effect of TRPM7 on the behaviors of bladder cancer cells (T24 and 5637). TRPM7 was downregulated via RNAi strategy, the siRNAs targeting TRPM7 were designed and screened in bladder cancer cells, and the most efficient silencing siRNA (TRPM7_siR1) was used for following observations. After TRPM7 was silenced, the CCK-8 assay showed that the proliferation of T24 and 5637 cells was significant inhibited and flow cytometric cell cycle analysis exhibited that cells were blocked in the G2/M phase, and flow cytometric analysis revealed that downregulation of TRPM7 induced apoptosis.

Bladder cancer has a tendency to metastasize, and metastasis is an important characteristic which influences bladder cancer patient prognosis. We used wound-healing and Transwell assays to study the role of TRPM7 in bladder cancer migration and invasion. The loss-of-function experiments showed that the silencing of TRPM7 expression inhibited cell migratory and invasive abilities. All the results suggest that TRPM7 may be a potential tumor promoter in bladder cancer.

In summary, our data confirmed that TRPM7 is overexpressed in bladder cancer tissue. Furthermore, high TRPM7 expression may be involved in the clinical development and poor prognosis of bladder cancer patients. Moreover, we showed that TRPM7 may be involved in cell proliferation, apoptosis, migration and invasion abilities of bladder cancer cells. The present study indicated that TRPM7 has potent oncogenic activity in bladder cancer and TRPM7 channel functions may uncover new strategies in the future to prevent the progression of bladder cancer diseases.

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