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Potential role of melastatin-related transient receptor potential cation channel subfamily M gene expression in the pathogenesis of urinary bladder cancer

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Abstract. Urinary bladder cancer is one of the most common malignancies of the urinary tract. Ion channels and calcium homeostasis are involved in almost all basic cellular mechanisms. The transient receptor potential cation channel subfamily M (TRPM) takes its name from the melastatin protein, which is classified as potential tumor suppressor. To the best of our knowledge, there have been no previous studies in the literature investigating the role of these ion channels in bladder cancer. The present study aimed to determine whether bladder cancer is associated with mRNA expression levels of TRPM ion channel genes, and whether there is the potential to conduct further studies to establish novel treatment modalities. The present study included a total of 47 subjects, of whom 40 were bladder cancer patients and 7 were controls. Following the histopathological evaluation for bladder carcinoma, the mRNA and protein expression of TRPM were examined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry in tumor and normal tissues, in order to determine whether there is a difference in the expression of these channels in tumor and normal tissues. Immunoreactivity for TRPM2, TRPM4, TRPM7 and TRPM8 was observed in epithelial bladder cells in the two groups. RT-qPCR revealed a significant increase in TRPM7 expression in bladder cancer tissue compared to the controls (healthy bladder tissue), whereas no differences in TRPM2 or TRPM4 expression levels were observed. There were significant reductions in the expression levels of TRPM5 and TRPM8 in bladder cancer tissues. In the present study, the effects of TRP ion channels on the formation of bladder cancer was investigated. This study is instructive for TRPM2, TRPM4, TRPM5, TRPM7 and TRPM8 and their therapeutic role in bladder cancer. The results support the fact that these gens can be novel targets and can also be tested for during the treatment of bladder cancer.

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Abbreviations: TRPM, transient receptor potential cation channel subfamily M; PCR, polymerase chain reaction

Key words: melastatin, transient receptor potential cation channel subfamily M, bladder cancer, gene, expression, immunohistochemistry, quantitative polymerase chain reaction

Introduction

Urinary bladder cancer is one of the most common malignancies of the urinary tract, and the ninth most common cancer worldwide. A high frequency of bladder tumors (77%) occur in men; it is 7th most common cancer type in men and 17th in women (1,2). With a steady increase in its incidence with age, bladder cancer is most frequently diagnosed in the 5 to 7th decades (3). Almost 75% of all bladder cancers are confined to mucosa (Ta) and submucosa (T1) at the time of initial diagnosis, whereas the remainder have progressed to muscle-invasive disease. Until recently, Ta and T1 bladder tumors were identified as 'superficial bladder cancers'; however, with the revelation that certain subgroups can progress to invasive disease, they are now categorized as 'non-muscle-invasive diseases'. To date, numerous etiological factors, including chemical agents, smoking, diet and genetic factors, have been identified as potential causes of bladder cancer (4,5). In addition, the effects of various biochemical markers have been considered (6).

Ion channels and calcium homeostasis play a role in all basic cellular mechanism, including apoptosis, proliferation and differentiation, to maintain homeostasis (7,8). Transient receptor potential (TRP) channels were first described in *Drosophila* (9). TRP channels are cation-selective channels, which are predominantly localized to the plasma membrane (10,11) and are associated with various diseases,

such as cancer (7,8). Variable expression levels of one or more TRP proteins are important for the initiation and progression of certain cancer types (8).

At the cellular level, TRP channels act as multifunctional sensors; they may be activated by physical or chemical stimuli, or by the binding of specific ligands (8). More than 50 different TRP channels have been identified among different species, and >20 TRP channel types may be found in mammals (8). Depending on sequence homology, the 28 mammalian TRP channels are categorized into 6 subfamilies: Vanilloid receptor (TRP cation channel subfamily V); canonical (TRP cation channel subfamily C); melastatin [or long; TRP cation channel subfamily M (TRPM)]; polycystin (TRP cation channel subfamily P); mucolipin (TRP cation channel subfamily ML); and ankyrin (TRP cation channel subfamily A) (12,13).

The TRPM subfamily includes 8 subgroups (TRPM1, TRPM2, TRPM3, TRPM4, TRPM5, TRPM6, TRPM7 and TRPM8), which are considered to have roles in tumorigenesis, cell proliferation and differentiation (11,14). The TRPM subfamily takes its name from the melastatin protein, which is defined as potential tumor suppressor. Most members of TRPM subfamily are permeable to calcium (4,11). Changes in the expression levels of TRPM ion channels can affect various physiological functions and lead to the initiation of pathological processes (4).

Previous studies have reported on the associations between certain cancer types and TRPM ion channels (7,15-21); however, to the best of our knowledge, there have been no studies in the literature that have investigated the role of these ion channels in bladder cancer. In this original study, we aimed to determine whether bladder cancer is associated with the mRNA expression levels of TRPM ion channel genes, and whether this could merit further study with the aim of establishing novel treatment modalities.

Materials and methods

Subjects. After obtaining local ethics committee approval, we conducted a prospective controlled study involving patients who were admitted to Urology Clinics of Turkey Yuksek Ihtisas Education and Training Hospital (Ankara, Turkey) between January 2012 and December 2013 with painless gross hematuria and lower urinary tract symptoms. Patients with a tumor-like lesion in the urinary bladder that was histopathologically confirmed to be a benign disorder were excluded from the study. A total of 47 subjects were included in the study, of whom 40 were bladder cancer patients and 7 were controls. Informed consent was obtained from all patients prior to their inclusion in the study. Transurethral resection was performed if a bladder tumor was detected, and biopsy was taken if there was a suspicious field in the bladder. Patients for whom pathological examination revealed transitional cell carcinoma of the urinary bladder constituted the patient group, whereas patients with benign pathology examination results constituted the control group. Following the histopathological evaluation for urothelial carcinoma, the mRNA and protein expression levels of the TRPM genes were examined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry in tumor and normal tissues, in order to determine whether the expression of these channels differed between tumor and normal tissues.

RT-qPCR analysis of TRPM expression. A PureLink™ RNA Mini kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to isolate RNA from fresh bladder tumor samples obtained from patients, according to the manufacturer's instructions. RNA samples were stored at -80˚C until processing. A Qubit® RNA Assay kit and a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Inc.) were used for the measurement of RNA, with results presented as μg/ml. Subsequently, cDNA synthesis was performed using a High-Capacity RNA-to-cDNA kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), with a 20 μl reaction volume containing 10 μl RNA sample, 2 μl 10XRT random primers, 2 μl 10XRT buffer, 0.8 μl 25X dNTP mix, 4.2 μl nuclease-free water and 1 μl MultiScribe™ Reverse Transcriptase enzyme. The PCR for cDNA synthesis was performed at 25˚C for 10 min, 37˚C for 120 min and 85˚C for 5 min. cDNA obtained by RT was amplified by qPCR in the presence of a gene-specific assay. PCR was performed at 50˚C for 2 min, 95˚C for 10 min, 95˚C for 15 sec and 6˚C for 1 min, for 40 cycles. The Taqman Gene Expression Assays (Table I) were used to determine the expression levels of TRPM genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RT-qPCR experiments were performed three times. Gene expression levels were normalized according to GAPDH. The expression of this housekeeping gene was presented as $2^{-\Delta Cq}$, where $\Delta Cq(C_{qTime\ X} - C_{qTime\ 0})$ and time 0 represented 1x expression of this gene (22).

Immunohistochemistry. Immunohistochemical staining was performed on paraffinized tissue blocks taken from patients. TRPM2, TRPM4, TRPM5, TRPM7 and TRPM8 primary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and a Lab Vision biotinylated goat anti-polyvalent (anti-mouse/rabbit IgG; catalog no. TP-125-BN; Thermo Fisher Scientific, Inc.) The primary antibodies were diluted at 1:200 and they were all polyclonal (TRPM2 catalog no., SC-19,198; TRPM4 catalog no., SC-27539; TRPM5 catalog no., SC-27367; TRPM7 catalog no., SC-19562; TRPM8 catalog no., SC-130903). The incubation time and temperature for the primary and secondary antibodies were 60 min at 37˚C and 30 min at 37˚C, respectively. Secondary antibodies [biotinylated goat anti-polyvalent (anti-mouse/rabbit IgG); catalog no., TP-125-BN; Lab Vision Corporation] were used in the process. After secondary antibody application, the tissues were washed for 5 min, 3 times each, with phosphate-buffered saline. Streptavidin peroxidase (catalog no., TS-125-HR; Lab Vision Corporation) and 3-amino-9-ethylcarbazole (AEC) Substrate (catalog no., TA-015 ve HAS) +AEC Chromogen (catalog no., TA-002-HAC) (Lab Vision Corporation) were used for visualization.

Prepared tissues were examined with an Olympus BX50 microscope and photographed. Quantification of immunohistochemical staining results was conducted as previously described (5). In brief, the extent and intensity of the staining were graded on an arbitrary scale ranging from 0 to 3, representing negative (score 0), weak (score 1), moderate (score 2) and strong (score 3) staining. Negative and weak staining was

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Catalog number
GAPDH	Hs02758991_g1
TRPM2	Hs01066071_m1
TRPM4	Hs00214167_m1
TRPM5	Hs00175822_m1
TRPM7	Hs00918956_m1
TRPM8	Hs00375481_m1

TaqMan Gene Expression Assay Gex: AB Applied Biosystems. TRPM, transient receptor potential cation channel subfamily M; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

classified as low-level, whereas moderate and strong staining was classified as high-level expression. Tissue sections incubated in the absence of anti-TRPM antibodies were processed in parallel, and did not exhibit any detectable TRPM-specific immunoreactivity.

Statistical analysis. Statistical analysis was performed via a commercially available licensed statistics program (IBM SPSS 22.0; IBM SPSS, Inc., Armonk, NY, USA). A Student's t-test was used to determine the difference in expression levels between groups. An independent samples t-test was used to evaluate the difference in immunostaining between groups. $P<0.05$ was considered to indicate significance in statistical analysis (23).

Results

Patient characteristics. Among the 40 patients, 33 were male and 7 were female; in the control group, 5 were male and 2 were female. The mean age of the patient group was 67.3 years, with age ranging from 56 to 78 years, and the mean age of the controls was 64.6 years (range, 59-70 years). Of the 40 patients with bladder cancer, 28 (70%) had low-grade urothelial carcinoma, and the remaining 12 (30%) had high-grade urothelial carcinoma. All patients in the control group were smokers, whereas 92.5% of the bladder cancer patients were smokers. The demographic characteristics of the patients in the two groups are shown in Table II.

Immunohistochemical findings. The expression levels of TRPM2, TRPM4, TRPM5, TRPM7 and TRPM8 were examined in normal bladder tissues and in malignant cells invading the smooth muscle and the tunica media layer of blood vessels of the bladder. For each antibody, 4 control and 4 transitional bladder cancer samples were studied. TRPM2 was expressed in the epithelial, muscle and mucosal bladder cells in the control patients and in the invasive malignant cells (score 3) (Fig. 1A and B), whereas TRPM4 (score 2), TRPM7 (score 2) and TRPM8 (score 3) were expressed only in the epithelial cells of the bladder in the two groups. There was no immunoreactivity against TRPM5 (score 0) in the bladder tissues of cancer patients or control subjects.

RT-qPCR analysis findings. There were no statistically significant differences in TRPM2 ($P=0.174$) or TRPM4 ($P=0.551$) gene expression levels between patients and controls. By contrast, there were statistically significant reductions in the mRNA levels of TRPM5 ($P=0.003$) and TRPM8 ($P<0.001$), and a statistically significant increase in the mRNA levels of TRPM7 ($P<0.001$) in bladder cancer patients compared with controls (Fig. 2).

Discussion

TRP channels are involved in cell differentiation, cell growth and apoptosis, and increases in Ca^{2+} induce apoptosis in cells (10). Therefore, TRP channels localized on the tumor cell membrane are predicted to be novel targets for drug therapy, immunotherapy and gene therapy. There is only a small amount information available regarding changes in the expression of these channels during cell differentiation and carcinogenesis (10). Further research studies on the expression pathways of these channels are required to contribute to the understanding of carcinogenesis and the implementation of TRP agonists in cancer treatment (10).

Intracellular calcium regulation is one of the basic mechanisms by which cell replication and apoptosis are controlled. It has an essential role in tumor formation (24). Although the selective suppression of TRPM protein expression or transmigration to the nucleus does not exert any effects on normal cells, this is an important therapeutic target for inducing apoptosis of cancer cells (24).

Certain Ca^{2+} and Na^{2+} permeable members of TRP channel family are expressed at various levels in cancer cells; however, to date, there have been few studies reporting this expression of the TRP channel family in cancer cells (25). Such alterations in the expression of TRP have not yet been fully elucidated, and it is presently unclear whether this is one of the cornerstones of cancer progression or whether it occurs secondary to other changes (25). As an answer to this question, some other markers in bladder and prostate cancer, such as BLCA-4 (a nuclear matrix protein that has homology with the ELK-3 gene, a member of the ETS transcription factor family) could predict the course of the cancer. This suggests that some TRP proteins may also be of value in the course of bladder cancer (26). The existence of ion channels in bladder cancer and their potential effects on malignant phenotypes have not been studied previously (15).

TRPM7 channels are Ca^{2+}-permeable, non-selective cation channels. The activation of TRPM7 channels has been shown to be involved in cellular Mg^{2+} homeostasis, Ca^{2+}-associated neuronal damage under ischemic conditions, and diseases caused by abnormal magnesium absorption (27). TRPM2 channels are non-voltage-gated cation channels and have a significant permeability to an important second messenger: Ca^{2+} (27).

In the present study, a significant increase was observed in TRPM7 expression in bladder cancer tissues compared to healthy bladder tissue, whereas no differences were observed with regard to TRPM2 and TRPM4. Furthermore, significant decreases in the expression levels of TRPM5 and TRPM8 were found in bladder cancer tissues. This finding was in accordance with a study conducted by Mizuno *et al* (16), who

Table II. Demographic data of the patients in the control and study groups.

Group	Total, n	Mean age ± standard deviation, years	Male/female	No. of smokers
Patients	40	67.35±6.439	33/7	37
Transitional tumor	30	67.57±5.823		
Invasive tumor	10	66.70±7.056		
Controls	7	64.57±4.117	5/2	7
Total	47	66.94±5.870	38/9	40

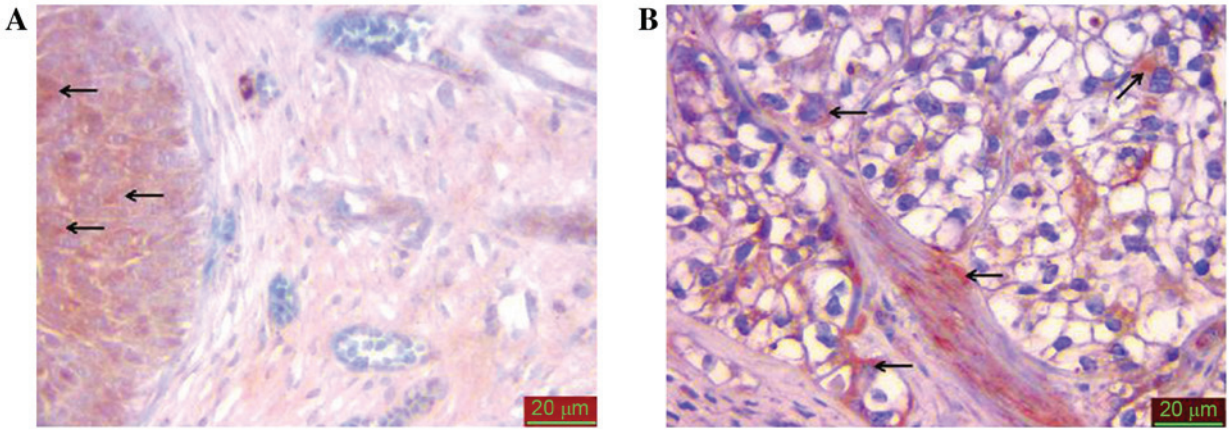

Figure 1. Immunohistochemical staining of TRPM2 protein in bladder cancer and control samples. (A) Typical image of a normal tissue sample with high expression of TRPM2 protein. (B) Typical image of a bladder cancer sample with high expression of TRPM2 protein in bladder smooth muscle, and malignant cells invading the smooth muscle and the tunica media layer of blood vessels in a bladder cancer patient. TRPM2, transient receptor potential cation channel subfamily M member 2.

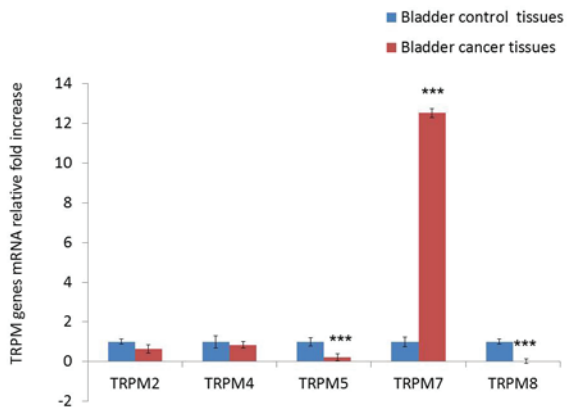

Figure 2. Fold changes in the mRNA levels of the TRPM genes. A comparison of the mRNA levels of TRPM2, TRPM4, TRPM5, TRPM7 and TRPM8 ion channel genes in bladder cancer patients and controls is shown. Gene expression levels were normalized according to the expression of glyceraldehyde 3-phosphate dehydrogenase. Results are presented as the mean ± standard deviation for all groups. ***P<0.05 vs. bladder control tissues. TRPM, transient receptor potential cation channel subfamily M.

reported increased expression of TRPM7 mRNA in bladder cancer cells compared with normal urothelial cells. TRPM7 was also suggested to be associated with breast and gastric cancers (17,18). Additionally, TRPM7 has been used as a therapeutic target in the treatment of cancers such as breast and gastric cancer (17,18). Overexpression of these channels could be a novel marker for carcinogenic and tumor cells (24). The increased expression of TRPM7 in bladder cancer in the current study may confirm this concept.

TRPM8 channels are Ca^{2+}-permeable channels that are widely expressed and may be used as prognostic and diagnostic markers, particularly in prostate cancer (19). In a recent study by Xiao *et al* (20), the increased expression of TRPM8 in metastatic bladder cancer was reported. By contrast, a significant decrease in the expression of TRPM8 mRNA in bladder cancer cells was observed in the present study. This difference may be due to the low patient number or the difference between the two patient cohorts regarding to tumor stage. For example, all cancer patients in the present study were diagnosed with superficial bladder cancer, whereas the study by Xiao *et al* comprised patients with metastatic cancer. These findings demonstrate the importance of the current study.

TRPM4 and TRPM5 channels are impermeable to Ca^{2+}. However, Ca^{2+} has an important role in their activities, as these two channels are activated by the increase of intracellular calcium. Thus, TRPM4 and TRPM5 are calcium-activated, non-selective cationic channels that are found in numerous tissue types (28). The expression levels of TRPM4 and TRPM5 are known to increase in certain types of cancer (21). It has been hypothesized that various expression levels (high and low) of TRPM5 may be associated with tumorigenesis:

For example, an increase in the expression of TRPM5 mRNA was previously detected in cases of Wilms tumor and rhabdomyosarcoma (29); however, in the present study, TRPM5 immunoreactivity was revealed to be extremely low in bladder cancer tissues and normal bladder tissues. Recent studies have suggested that TRPM4 is one of the predominant regulators of the excitation-contraction mechanisms of bladder smooth muscle (30,31) and, thus, it may serve as an interesting therapeutic target in the treatment of overactive bladder. However, an association between TRPM4 and bladder cancer has not yet been determined. The present results are consistent with the literature (15), as no difference in TRPM4 expression between bladder cancer tissue and normal tissue was detected.

In the literature, limited data is available regarding the immunostaining and expression of TRPM channels in normal and cancerous bladder tissues. In the present study, intense expression of TRPM2 was observed in bladder epithelium, mucosal and smooth muscle cells in controls and cancer patients, whereas TRPM7 expression was found to be restricted to epithelial cells in the two study groups and was not observed in other cell types. Despite the absence of TRPM5 immunoreactivity in any of the tissues, a limited level of mRNA was detected by RT-qPCR in each group. TRPM4 and TRPM8 immunoreactivity was also found to be extremely limited and confined to epithelial cells.

In conclusion, to the best of our knowledge, the present study is the first in the literature to focus on TRP channels in bladder cancer. As shown in the aforementioned studies, TRPM7 may have a role in tumor pathogenesis, progression, migration and invasion. With the aid of TRPM7 analysis, it may be possible to predict the progression of low-grade tumors to high-grade tumors. In this study, due to the small number of patients, it was not possible to make significant comparisons between the cases categorized as non-muscle invasive, muscle invasive, low-grade and high grade. In future studies with larger patient volumes, the correlation of TRPM7 expression with bladder cancer progression may be established.

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