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RNA interference mediated suppression of TRPV6 inhibits the progression of prostate cancer *in vitro* by modulating cathepsin B and MMP9 expression

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Purpose: The transient receptor potential vanilloid 6 (TRPV6) channel is overexpressed in prostate cancer and its silencing is known to inhibit the growth of LNCaP cells. However, the role of TRPV6 in the metastasis of prostate cancer cells and its relationship to the invasive markers, matrix metalloproteinase (MMP) and cathepsin B, is unclear. Thus, the present study was focused on understanding these tumor-related processes.

Materials and Methods: We performed a wound-healing assay and a Transwell migration and invasion assay to assess the migration and invasion of prostate cancer cells. Western blot analysis was used to measure the expression of cathepsin B, MMP2, and MMP9.

Results: TRPV6 siRNA significantly inhibited the proliferation of LNCaP prostate cancer cells. It also significantly attenuated the wound healing and migration capacities of LNCaP cells. Moreover, the invasiveness of LNCaP cells and the expression of MMP9 and cathepsin B in LNCaP cells were also significantly inhibited by TRPV6 siRNA.

Conclusions: The results indicate that TRPV6 may promote prostate cancer progression in association with MMP9 and cathepsin B, thereby validating further research into TRPV6 as a useful therapeutic target for local invasion or metastasis of advanced prostate cancer.

Keywords: Cathepsin B; Matrix metalloproteinase 9; Prostatic neoplasms; Transient receptor potential cation channel subfamily V member 6

INTRODUCTION

The incidence of prostate cancer is increasing because of societal changes including westernized eating habits, increased life expectancy, and national cancer screening and registration [1]. The prevalence is likely to be higher as the demographics are shifting to an older population. Prostate cancer initially responds to hormones and then progresses to a hormone-resistant phenotype [1]. Asian male exhibit a lower incidence of prostate cancer compared with Westerners, but the proportion of advanced prostate cancer patients is higher [2]. However, the treatment methods for advanced prostate cancer have not significantly changed. Therefore, research on effective new treatment modalities for advanced

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prostate cancer is needed and studies on various targets are ongoing.

It is known that calcium influx and calcium-related signaling pathways play an important role in the onset and progression of many cancers [3-5]. Many studies have been conducted on the effect of the transient receptor potential (TRP) channels, which are distributed throughout various tissues and are involved in the movement of cations including calcium through cell membranes during the initiation and progression of cancer [6-8]. Various types of TRP channels are known to be involved in the development and progression of prostate cancer [9,10]. The TRP channels are ubiquitously expressed cation permeable channels that share a high degree of structural homology [11]. Based on these criteria, mammalian TRP channels can be divided into six subfamilies: TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin), and TRPV (vanilloid) [11]. Of these, the TRPV channels are divided into six subtypes. Unlike the other four subtypes, the TRPV5 and TRPV6 channels exhibit significant nucleotide sequence homology and primarily act as calcium-selective channels [12]. It has been reported that TRPV6 is involved in the growth and apoptosis of prostate cancer cells through the Ca^{2+}-NFAT pathway [13]. In addition, TRPV6 mRNA is highly expressed in aggressive prostate cancer tissues and it has been implicated as a clinical outcome marker for prostate cancer [14-17]. However, little is known about the role of TRPV6 in the migration and invasion of cancer cells, which are involved in the metastasis of prostate cancer cells. In the present study, we examined the effects of small interfering RNA (siRNA) against TRPV6 on the migration and invasion of prostate cancer cells. We also measured the expression of the well-established invasion markers, matrix metalloproteinase (MMP), and cathepsin B, during TRPV6 siRNA treatment [18].

MATERIALS AND METHODS

1. Cell culture

Three prostate cancer cell lines (LNCaP, DU145, PC3) and cell culture media were purchased from the American Type Culture Collection (Rockville, MD, USA). Androgen-mediated cellular responses in LNCaP cells were evaluated using RPMI 1640 medium containing 5% charcoal-stripped fetal bovine serum (FBS) in place of FBS, to remove endogenous steroids to allow the cells to primarily respond to exogenously added 5α-dihydrotestosterone (DHT; Sigma-Aldrich Chemical Co, St. Louis, MO, USA). DU145 and PC3 cells were cultured in Eagle's minimum essential and F-12 media, respectively. Each medium contained 10% FBS (Gibco BRL, Grand Island, NY, USA), 1% penicillin/streptomycin solution and 1% L-glutamine solution (200 mM). The cells were cultured at 37℃ and 5% CO_2 and passaged every 48 hours to maintain the appropriate number of cells. The experiments were performed 24 hours after siRNA transfection and divided into an siRNA treatment group and a scrambled siRNA control group for TRPV6.

2. DHT treatment

LNCaP cells were cultured in red-free RPMI medium containing 10 % charcoal stripped FBS for 24 hours after plating. TRPV6 siRNA was transfected, and the cells were allowed to grow for 24 hours at 37℃ in 5% CO_2. For the WST-1 assay, wound healing assay, and Transwell migration and invasion assay, the LNCaP cells were treated with 1 nM or 100 nM DHT 24 hours after transfection, and all experiments were performed after 0–2 days. The DHT powder was dissolved in 0.1% ethanol and diluted into the required concentrations with the culture medium.

3. TRPV6 siRNA transfection

In order to suppress TRPV6 gene expression, siRNA for TRPV6 was designed and synthesized by Bioneer (Daejeon, Korea). In a preliminary experiment, the inhibitory effect of three TRPV6 siRNAs was confirmed by RT-PCR, and the most effective # (#2) TRPV siRNA was used for the experiments (Fig. 1A). The base sequences of the siRNAs were as follows: scrambled siRNA, sense 5"-CCUACGCCACCAAUUUCG-3", antisense 5"-ACGAAAUUGGUGGCGUAGG-3"; TRPV6 siRNA, sense 5"-CUAAUUCUCUGCCUAUGGA-3", antisense 5"-UCCAUAGGCAGAGAAUUAG-3"; siRNA against androgen receptor as a positive control, sense 5"-CACAAGUCCCGGAUGUACA-3", antisense 5"-UGUACAUCCGGGACUUGUG-3".

4. Reverse transcription polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized with M-MLV reverse transcriptase (Promega, Madison, WI, USA). The cDNA was amplified with Taq polymerase (Finnzymes, Espoo, Finland) in a DNA thermal cycler (MJ Research, Watertown, MA, USA). The following cycling conditions were applied: 30 cycles of 1 minute at 95℃, 45 seconds at the annealing temperature, and 1 minute at 72℃. The annealing temperatures were 57℃ for TRPV6 and 60℃ for β-actin. The primers for TRPV6 were 5′-CAAGTTCTGCAGATGGTTCC-3′ (forward) and 5′-GCAAAGGTTTTGTTGGGCTG-3′ (reverse).

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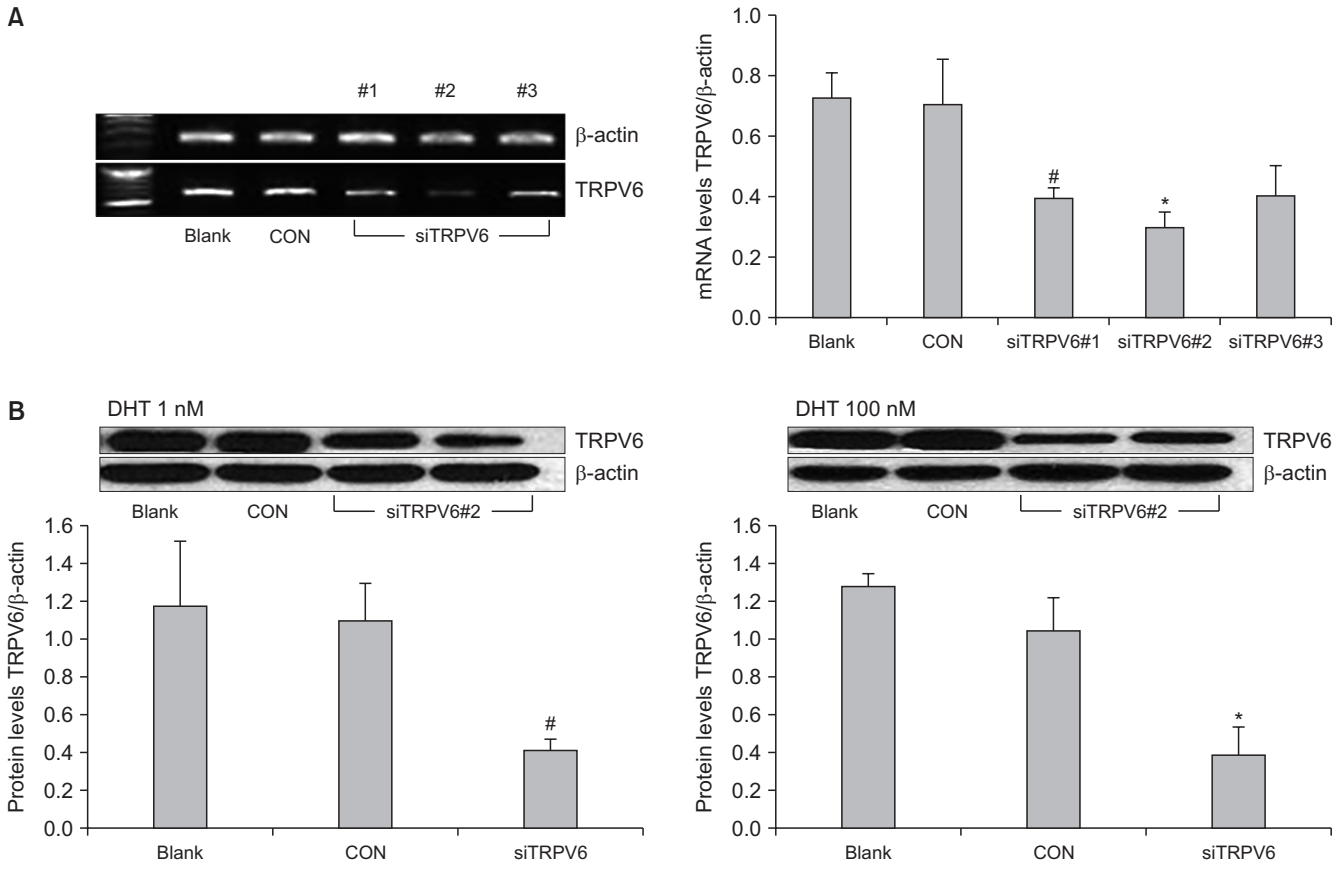

Fig. 1. Knockdown efficiency of siTRPV6 in LNCaP cells. (A) The efficacies of three different kinds of small interfering RNA against TRPV6 (siTRPV6#1, siTRPV6#2, and siTRPV6#3) were analyzed by RT-PCR in LNCaP cells. siTRPV6#2 was selected for the following experiments because it blocked more TRPV6 compared with the other variants. The expression of TRPV6 is normalized to β-actin gene expression. (B) The degrees of inhibition of TRPV6 protein levels were analyzed by western blot in siTRPV6#2 transfected LNCaP cells treated with 1 nM or 100 nM DHT. The expression of TRPV6 is normalized to β-actin expression. siTRPV6, small interfering RNA targeting transient receptor potential cation channel vanilloid subfamily number 6; Blank, mock; CON, control scrambled siRNA; DHT, dihydrotestosterone. The data are presented as the mean±standard error of the mean of three independent experiments. *$p<0.01$, #$p<0.05$ vs. control.

For β-actin, the primers were 5′-GGACTTCGAGCAAGAGATGG-3′ (forward) and 5′-AGCACTGTGTTGGCGTACAG-3′ (reverse).

5. Western blot analysis

Using an appropriate amount of lysis buffer (20 mM Tris-HCl, pH 7.5) containing 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/mL leupeptin), the proteins were extracted from the reactions at 4℃ for 1 hour in the presence of 1 mM phenymethylsulfonyl fluoride. Equivalent amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and blocked with skim milk solution. After washing the membranes several times, diluted primary antibody was added and incubated at 4℃ for 16 to 18 hours. After washing the membranes were incubated with secondary antibody (goat anti-rabbit or goat anti-mouse, IgG) at room temperature for 2 hours. The membranes were developed using a chemiluminoesence solution (Amersham Life Science Corp, Little Chalfont, UK). The primary antibodies against TRPV6 used in this experiment were purchased from Alomon Labs (Jerusalem, Israel), whereas the others were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The dilution for each primary antibody was as follows: anti-TRPV6 (1:500); anti-MMP2 (1:1,000); anti-MMP9 (1:1,000); anti-cathepsin B (1:10,200), and anti-β actin (1:2,000).

6. WST-1 assays

LNCaP cells were trypsinized, counted, and seeded onto 96-well plates at densities of 3,000 cells/100 μL per well. Twenty-four hours after cell seeding, cells were transfected with siRNA against TRPV6. The original medium was removed and replaced with prewarmed DHT-containing medium 24 hours after transfection. For each measurement, 10 μL of WST-1 reagent was added to each 100-μL well, and the plate was incubated for 2 hours at 37℃. After the incu-

bation period, the absorbance was measured at 440 nm on a microplate reader. Further measurements were made at DHT addition (day 0) and two days later.

7. Wound-healing assay

Cells (1×10^5) were seeded into 6-well plates, incubated for 48 hours, transfected with siRNA for TRPV6, and 24 hours later, a constant sized artificial gap was created with a pipette tip in the cell monolayer that was more than 90% saturated. Afterwards, the degree of movement of the cells filling the gap was compared by photographing at 0, 24, and 48 hours with a microscope (magnification ×100).

8. Cell migration assay

After 24 hours of transfection with TRPV6 siRNA or scrambled siRNA, prostate cancer cell migration was measured. A Transwell chamber (Corning, New York, NY, USA) with an 8 μm pore was washed with culture media and seeded with 1.5×10^5 cells in the upper compartment at 24 hours post-transfection. Cell migration was carried out and the cells that moved to the bottom compartment were stained with 0.2% crystal violet solution (Sigma-Aldrich Chemical Co). To obtain an average sum of the cells that migrated through the membrane to the lower compartment, an inverted microscope (magnification ×400) was used to count the number of cells in different fields of view.

9. Cell invasion assay

Changes in the invasive activity of the three prostate cancer line cells were measured 24 hours after transfection with the TRPV6 siRNA-treated and scrambled siRNA-treated controls. A Transwell chamber with an 8 μm pore was coated with Matrigel (BD Biosciences, San Jose, CA, USA) and allowed to dry. The lower compartment was filled with culture media containing 20% FBS. Matrigel-coated Transwell chamber was washed with cell culture media and the upper compartment was seeded with 1.5×10^5 cells 24 hours after transfection. After 6 hours, the cells that moved to the lower part of the Transwell chamber were stained with a 0.2% crystal violet solution. Using an inverted microscope (magnification ×400), the number of cells in different fields of view was counted to obtain an average sum of the cells that migrated through the Matrigel and attached to the underside.

10. Statistical analysis

The experiments were performed in triplicate unless otherwise stated and expressed as the mean±standard error of the mean. Statistical significance was analyzed using a Student t-test and a significance level of 0.05 (two-tailed p-value). The statistical analyses were conducted using SPSS version 20.0 (IBM Corp., Armonk, NY, USA) software.

RESULTS

1. Regulation of prostate cancer cell proliferation by TRPV6 siRNA

LNCaP cells were transfected with 10 nM of each of the three TRPV6 siRNAs and the expression of TRPV6 mRNA was measured after 24 hours. Among the siRNAs used, the #2 TRPV6 siRNA inhibited TRPV6 mRNA expression the most (Fig. 1A, $p<0.01$). The expression of TRPV6 protein in LNCaP cells treated with two different concentrations of DHT and the #2 TRPV6 siRNA was also effectively suppressed compared with the control group (Fig. 1B). In the 1 nM DHT treatment group, TRPV6 expression was suppressed to 37%±6% of the scrambled siRNA treatment control group (Fig. 1B, $p<0.05$), and in the 100 nM treatment group, it was suppressed to 38%±13% of the control group (Fig. 1B, $p<0.01$). Therefore, TRPV6 protein expression in LNCaP cells was inhibited by >60% when the #2 TRPV6 siRNA was present.

The #2 TRPV6 siRNA had the highest blocking effect as in LNCaPand DU145 cells, whereas #3 TRPV6 siRNA had the highest blocking effect in PC3 cells (Supplementary Fig. 1). However, the blocking effect of TRPV6 siRNAs on DU145 cells and PC3 cells was low (10%–25%), and subsequent experiments were mainly performed in LNCaP cells using the #2 TRPV6

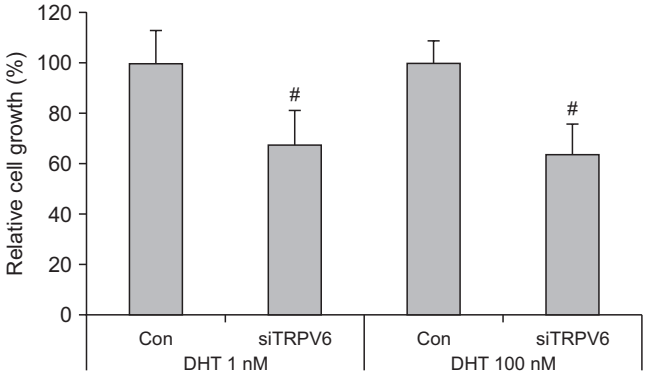

Fig. 2. Effects of TRPV6 siRNA on the proliferation of LNCaP cells. The viability of LNCaP cells was measured by WST-1 assay at 48 hours after transfection with TRPV6 siRNA. Cells were treated with 1 nM or 100 nM DHT as described in the "MATERIALS AND METHODS" section. Cell growth is expressed relative to the value of the negative control, which was set to 100%. siTRPV6, small interfering RNA targeting transient receptor potential cation channel vanilloid subfamily number 6; CON, control scrambled siRNA; DHT, dihydrotestosterone. Data are presented as the mean±standard error of the mean of three independent experiments. #p<0.05 vs. control.

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siRNA.

TRPV6 siRNA significantly reduced cell proliferation ($p<0.05$, Fig. 2). The proliferation inhibition rates were approximately 30%–35% in LNCaP cells (Fig. 2).

2. Regulation of wound healing by TRPV6 siRNA

Using a wound-healing assay (Fig. 3A), 1 nM DHT-treated LNCaP cells treated with scrambled siRNA resulted in a gap width narrowing to 61.6%±2.5% of the initial value 24 hours after scratching and 35.1%±4.6% after 48 hours. TRPV6 siRNA significantly slowed wound healing compared with the scrambled siRNA-treated control group by maintaining the area of the gap at 78.5%±6.1% after 24 hours and 52.6%±7.3% after 48 hours ($p<0.01$, Fig. 3). For 100 nM DHT-treated LNCaP cells, the gap narrowed to 76.7%±5.8% of the initial value 24 hours after scratching and 51.3%±12.1% after 48 hours. In addition, TRPV6 siRNA significantly slowed the wound-healing process to 95.2%±2.6% of the initial value after 24 hours ($p<0.05$, Fig. 3B) and 82.4%±6.4% after 48 hours ($p<0.01$, Fig. 3B), similar to 1 nM DHT-treated LNCaP cells.

3. Regulation of migration and invasion by TRPV6 siRNA

The number of LNCaP cells that migrated through the Transwell insert at 48 hours after TRPV6 siRNA treatment was significantly reduced to 68±12.21 cells compared with 8.92±7.65 for the control group treated with scrambled siRNA ($p<0.01$, Fig. 4). In addition, TRPV6 siRNA decreased the invasiveness of LNCaP cells through the Matrigel-coated Transwell insert significantly compared with scrambled siRNA ($p<0.05$, Fig. 5A, B). Expression of MMP9 and cathepsin B protein, markers of cancer cell invasion, was also decreased by treatment with TRPV6 siRNA compared with the control group (Fig. 5C).

DISCUSSION

Calcium plays an important role in the proliferation, migration, invasion, metastasis and apoptosis of cancer cells

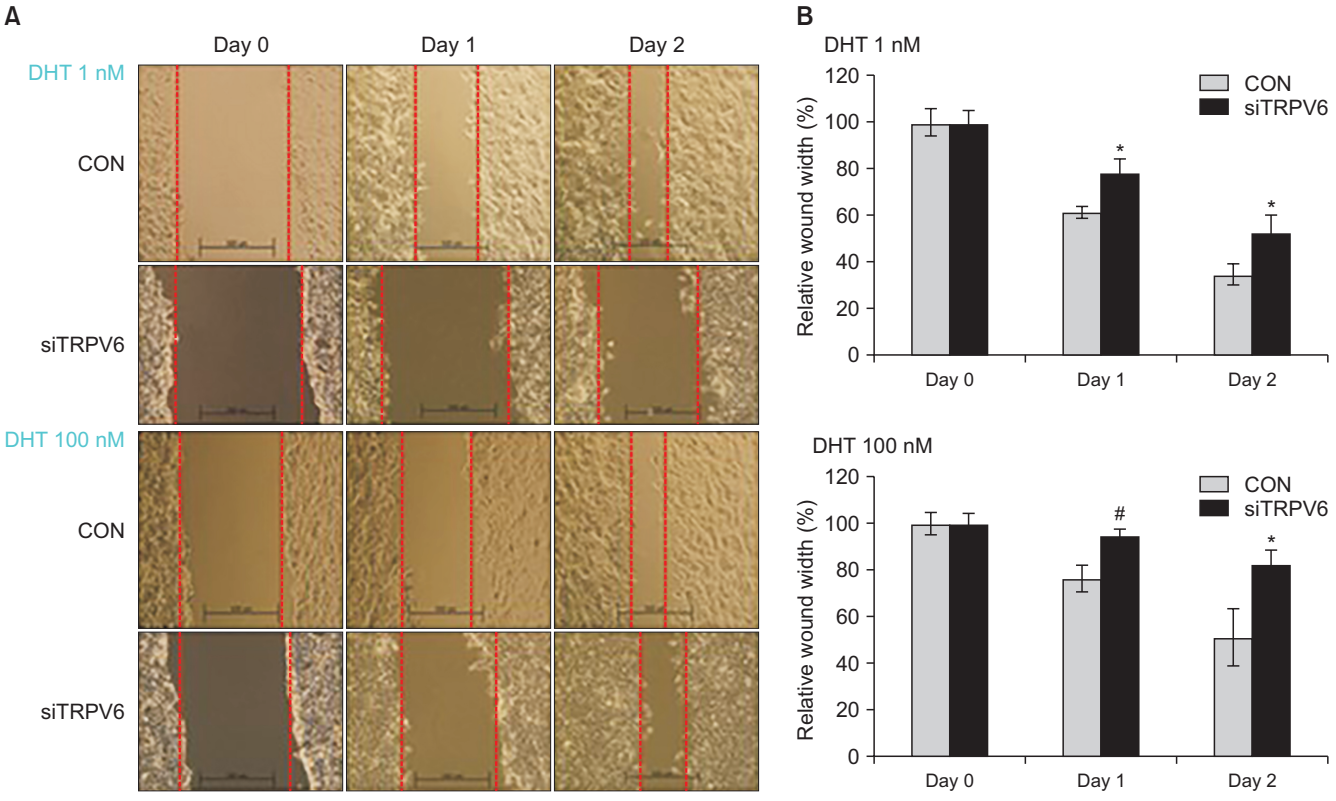

Fig. 3. Effects of TRPV6 siRNA on the cell motility rate of LNCaP cells. Representative images (A) and graph (B) of the scratch wound-healing assays show that knockdown of TRPV6 suppressed cell motility in LNCaP cells. Cells (1×10^5) were seeded into 6-well plates, incubated for 48 hours, transfected with siRNA for TRPV6, and 24 hours later, a constant sized artificial gap was created with a pipette tip in the cell monolayer that was more than 90% saturated. Afterwards, the degree of movement of the cells filling the gap was compared by photographing at 0, 24, and 48 hours with a microscope (magnification ×100). Cells were treated with 1 nM or 100 nM DHT as described in the "MATERIALS AND METHODS" section. DHT, dihydrotestosterone; siTRPV6, small interfering RNA targeting transient receptor potential cation channel vanilloid subfamily number 6; CON, control scrambled siRNA. Data are presented as the mean±standard error of the mean of three independent experiments. *$p<0.01$, #$p<0.05$ vs. control.

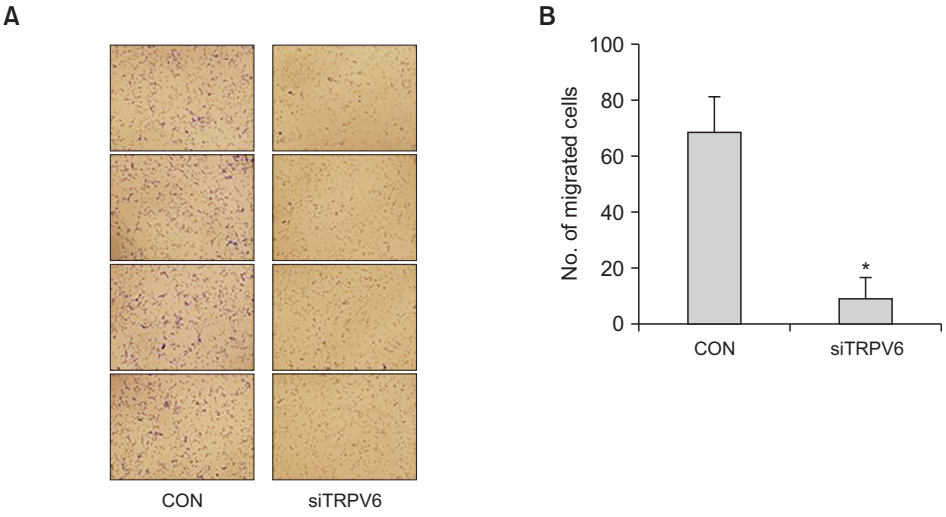

Fig. 4. Effect of TRPV6 siRNA on the migratory capability of LNCaP cells. Representative images (A) and graph (B) of the Transwell migration assay of LNCaP cells with TRPV6 siRNA, showing suppressed migration, compared with the scrambled siRNA control. Cells were treated with 1 nM DHT as described in the "MATERIALS AND METHODS" section. siTRPV6, small interfering RNA targeting transient receptor potential cation channel vanilloid subfamily number 6; CON, control scrambled siRNA. Data are presented as the mean±standard error of the mean of three independent experiments. *p<0.01 vs. control.

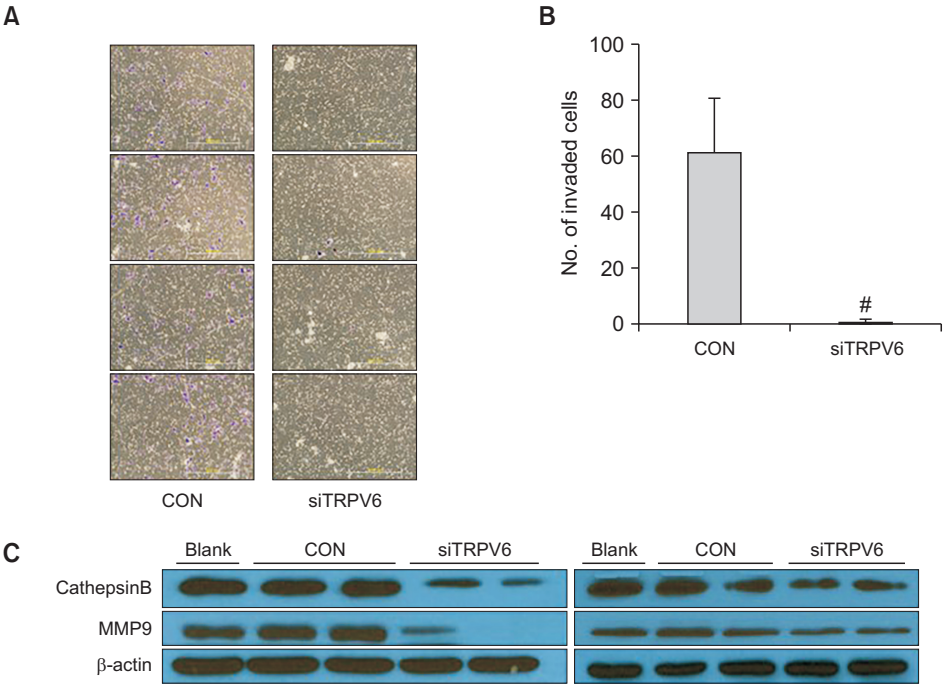

Fig. 5. Effect of TRPV6 siRNA on the invasive capability of LNCaP cells. Representative images (A) and graph (B) of the Transwell invasion of LNCaP cells with TRPV6 inhibition and respective scrambled siRNA control. (C) Effect of TRPV6 siRNA on the protein expression of cathepsin B matrix metalloproteinase 9 (MMP9) in LNCaP cells. Both cathepsin B and MMP9 expression were reduced by TRPV6 siRNA transfection in LNCaP cells. siTRPV6, small interfering RNA targeting transient receptor potential cation channel vanilloid subfamily number 6; Blank, mock; CON, control scrambled siRNA. Data are presented as the mean±standard error of the mean of three independent experiments. #p<0.05 vs. control.

[3,4]. It has been implicated in the development and progression of prostate cancer [9,10,14,17]. TRPV6, which belongs to the vanilloid family of the TRP channel, is involved in the development and progression of pancreatic, breast, and ovarian cancer [7,19,20]. The role of TRPV6 in the occurrence, growth, migration, invasion and metastasis of prostate cancer has been studied [14,17]. TRPV6 expression in human prostate cancer tissues has been reported to increase as prostate cancer progresses [14,17]. As shown in this study (Fig. 1A, Supplementary Fig. 1), the blocking effect by the same concentration of the #2 TRPV6 siRNA was lower in more advanced cell lines than the LNCaP cell line. Therefore, it is possible that the blocking effect by the #2 TRPV6 siRNA of the same concentration was lower in DU145 and PC3 cells because TRPV6 expression was higher in these than the LNCaP cells. The #3 TRPV6 siRNA showed a higher blocking effect in the PC3 cells than the #2 TRPV6 siRNA, which showed the greatest effect in LNCaP and DU145 cells. Therefore, it may also be possible that the blocking effect of certain types of TRPV6 siRNA differed between cell lines due to differences in transfection efficacy between prostate cancer cell lines.

Lehen'kyi et al. (2007) [13] showed that Ca^{2+} introduced into the cell through TRPV6 in LNCaP cells activated the nuclear factor of activated T-cell transcription factor and contributed to cell proliferation and apoptosis inhibition. Similarly, we found that the proliferation of LNCaP cells was significantly inhibited by TRPV6 siRNA compared

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with the scrambled siRNA-treated control group.

A recent study determined that the expression of TRPV2, a non-specific cationic channel belonging to the vanilloid family, was increased with prostate cancer progression [21]. In LNCaP cells, overexpression of TRPV2 promoted cell migration along with the expression of cathepsin B and MMP9 [21]. Furthermore, the growth and invasion of PC3 cell xenografts in nude mice and the expression of the invasive enzymes, MMP2, MMP9, and cathepsin B, were all reduced by treatment with TRPV2 siRNA [21]. In this study, we observed that wound healing of these prostate cancer cells was significantly inhibited by TRPV6 siRNA compared with scrambled siRNA-treated controls. Furthermore, the migration of LNCaP cells through the Transwell insert was reduced in the TRPV6 siRNA-treated group compared with the control group, and the invasiveness of LNCaP cells was also significantly inhibited by TRPV6 siRNA. Finally, the expression of the MMP9 and cathepsin B proteins, which are known to be involved in the process of cancer migration, invasion, and metastasis in various tissues [18], was significantly reduced by treatment with TRPV6 siRNA.

The MMPs are involved in the degradation of the extracellular matrix and basement membrane components, and are directly involved in essential cellular processes such as cell growth and differentiation, angiogenesis, and apoptosis [22]. In addition, the expression of MMP2 and MMP9 correlates with the aggressiveness and overall survival of cancer [23,24]. In prostate cancer, MMP2 and MMP9 have been identified as biomarkers associated with the invasion and metastasis of prostate cancer [24]. Androgen deprivation therapy induces a paradoxical elevation of MMP, including MMP9, leading to an aggressive tumor phenotype in many prostate cancer patients [25]. Furthermore, an oligomer MMP9 antisense attenuated DU145 cell proliferation and invasion, and angiogenesis [25].

Cathepsin B, a lysosomal cysteine protease, is linked to various processes including autophagy, antigen presentation, and apoptosis [26]. Upregulation of cathepsin B induces remodeling or degradation of the extracellular matrix and leads to the invasion and metastasis of prostate cancer [26,27]. In addition, a previous study in PC3 and DU145 cells suggests that increasing the activity of cathepsin B and cathepsin L promotes the invasion of these cells through Matrigel [28].

This study examined the effects of TRPV6 on the migration and invasion of cancer cells in prostate cancer cell lines, and there are some limitations in directly applying the results to the progression of prostate cancer in patients. Based on the results of this study, there is a need to clarify the correlation between increased TRPV6 expression and cancer progression by analyzing TRPV6 expression in the tissues of prostate cancer patients at different stages of progression.

In a recent phase I clinical trial performed in 22 patients with advanced solid cancers, including one patient with prostate cancer, a potential TRPV6 inhibitor stabilized the course of the disease for several months to tens of months in more than half of the patients [29]. These findings combined with the results found here, make researching TRPV6 as an effective therapeutic target for local invasion or metastasis of advanced prostate cancer especially interesting.

CONCLUSIONS

In this study, we discovered for the first time that the migration and invasion of prostate cancer cells were inhibited by TRPV6 siRNA along with a reduction of cathepsin B and MMP 9 protein expression. These results suggest that TRPV6 may be involved not only in the growth of prostate cancer, but also in the progression of prostate cancer to an advanced hormone-refractory stage through the regulation of cathepsin B and MMP9 expression.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

AUTHORS' CONTRIBUTIONS

Research conception and design: Duk Yoon Kim and Eun Kyoung Yang. Data acquisition: Soon Hee Kim. Statistical analysis: Soon Hee Kim and Eun Kyoung Yang. Data analysis and interpretation: Duk Yoon Kim and Eun Kyoung Yang. Drafting of the manuscript: Duk Yoon Kim and Eun Kyoung Yang. Critical revision of the manuscript: Duk Yoon Kim, Eun Kyoung Yang. Supervision: Eun Kyoung Yang. Administrative, technical, or material support: Soon Hee Kim. Approval of the final manuscript: Duk Yoon Kim, Soon Hee Kim, and Eun Kyoung Yang.

SUPPLEMENTARY MATERIAL

Supplementary material can be found via https://doi.org/10.4111/icu.20200511.

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