Role of Cationic Channel TRPV2 in Promoting Prostate Cancer Migration and Progression to Androgen Resistance

Michaël Monet 1,2, V'yacheslav Lehen'kyi 1,2, Florian Gackiere 1,2, Virginie Firlet 3, Matthieu Vandenberghhe 1,2, Morad Roudbaraki 1,2, Dimitra Gkika 1,2, Albin Pourtier 3, Gabriel Bidaux 1,2, Christian Slomianny 1,2, Philippe Delcourt 1,2, François Rassendren 5, Jean-Pierre Bergerat 6, Jocelyn Ceraline 7, Florence Cabon 8, Sandrine Humez 2,8, and Natalia Prevarskaya 1,2

Abstract

Castration resistance in prostate cancer (PCa) constitutes an advanced, aggressive disease with poor prognosis, associated with uncontrolled cell proliferation, resistance to apoptosis, and enhanced invasive potential. The molecular mechanisms involved in the transition of PCa to castration resistance are obscure. Here, we report that the nonselective cationic channel transient receptor potential vanilloid 2 (TRPV2) is a distinctive feature of castration-resistant PCa. TRPV2 transcript levels were higher in patients with metastatic cancer (stage M1) compared with primary solid tumors (stages T2a and T2b). Previous studies of the TRPV2 channel indicated that it is primarily involved in cancer cell migration and not in cell growth. Introducing TRPV2 into androgen-dependent LNCaP cells enhanced cell migration along with expression of invasion markers matrix metalloproteinase (MMP) 9 and cathepsin B. Consistent with the likelihood that TRPV2 may affect cancer cell aggressiveness by influencing basal intracellular calcium levels, small interfering RNA–mediated silencing of TRPV2 reduced the growth and invasive properties of PC3 prostate tumors established in nude mice xenografts, and diminished expression of invasive enzymes MMP2, MMP9, and cathepsin B. Our findings establish a role for TRPV2 in PCa progression to the aggressive castration-resistant stage, prompting evaluation of TRPV2 as a potential prognostic marker and therapeutic target in the setting of advanced PCa. Cancer Res; 70(3); 1225–35. ©2010 AACR.

Introduction

Androgen ablation is initially beneficial to nearly all men suffering from prostate cancer (PCa) because androgen-dependent PCa cells undergo apoptotic death induced by such treatment (1). Unfortunately, androgen ablation is only palliative and patients relapse into castration-resistant PCa with dramatic consequences (2). Indeed, androgen-independent PCa cells do not enter the programmed cell death pathway following androgen ablation (3). Thus, the major reason for the ability of PCa cells to metastasize and kill the patient is not related to their rate of proliferation but to their extensive ability to survive once they have been disseminated to a distant site (4). These cells represent an androgen-insensitive and apoptosis-resistant cell phenotype in the prostate (5), and their abundance correlates with tumor malignancy, loss of sensitivity to androgens, and poor prognosis (6, 7). In addition, the emergence of castration-resistant phenotype is concomitant with an increase in overall tumor growth and progression associated with enhanced level of cell migration (8). However, the molecular and cellular mechanisms controlling hormone refractoriness, apoptosis resistance, and migration of these cells are only partially understood (9). Among the different mechanisms regulating cancer cell migration, those that involve calcium dependence have been supposed to play a primary role (10, 11). The family of transient receptor potential (TRP) cationic protein channels displaying extraordinarily diverse activation mechanisms (12) has recently received increasing attention, in particular, to their link to human diseases (13–15). Some of these TRP channels have been reported to be involved in cancerogenesis: TRPM1 in melanoma (13) and TRPV6 and TRPM8 in PCa.
Among them, the TRP vanilloid 2 (TRPV2) channel is the one for which the mechanisms of regulation or the role in carcinogenesis is not known thus far. Recent studies have shown that mRNA of TRPV2 is expressed in prostate (18). TRPV2 function may be regulated by some lysophospholipids identified and known to have a range of physiologic and pathologic effects, including stimulation of cancer cell migration (19–21). However, the question remains as to how TRPV2 is involved in tumor development and progression both in vitro and in vivo to an extremely aggressive castration-resistant stage and whether it could be a real hallmark of poor prognosis in patients suffering from PCa.

Materials and Methods

**Cell culture.** The androgen-dependent human PCa cell line LNCaP and androgen-independent human PCa cell lines LNCaP C4-2, DU145, and PC3 were obtained from the American Type Culture Collection and maintained in culture in RPMI 1640 (Life Technologies) supplemented with 10% FCS (Seromed, Poly-Labo), 5 mmol/L L-glutamine (Sigma), and 100 μg/mL kanamycin.

Cells were grown at 37°C in a humidified atmosphere containing 5% CO2. Before fluorescence measurements, the cells were trypsinized and transferred onto glass coverslips. Cells were used 2 to 4 d after trypsinization. The medium was replaced every 48 h.

**Generation of shTRPV2 vectors.** Hybridized oligonucleotides were cloned into pSilencer 4.1-CMV-puro vector (Ambion) following the manufacturer’s instructions and defined as shTRPV2 I (sense, 5′-GCTAGACTGTGCTGATGA-3′) and shTRPV2 II (sense, 5′-TAAAGCTACTCCATGAC-3′).

**Nucleofection.** Transfection of LNCaP or PC3 cells with TRPV2-GFP or with shTRPV2 I or II was carried out using Nucleofector (Amaxis GmbH) according to the manufacturer’s instructions. Briefly, 3 μg of vector were transected into 2 millions of trypsinized cells, which then were plated into a T75 flask or onto the glass coverslips for 72 h.

**Small interfering RNA transfection.** LNCaP and LNCaP C4-2 cells were transfected with 50 nmol/L small interfering RNA (siRNA) against TRPV2 (siTRPV2; synthesized by Dharmacon, Inc.) using 6 μL TransIT-TKO transfection reagent (Mirus, Inc.) following the manufacturer’s instructions (see Table 1 for the siRNA sequence).

**Ca2+ measurements using Fura-2 acetoxyethyl ester.** Before fluorescence measurements, the cells were trypsinized and plated onto glass slips. The medium was replaced every 48 h. Cells were used 3 d after trypsinization. The culture medium was replaced by a HBSS solution containing 142 mmol/L NaCl, 5.6 mmol/L KCl, 1 mmol/L MgCl2, 2 mmol/L CaCl2, 0.34 mmol/L Na2HPO4, 0.44 mmol/L KH2PO4, 10 mmol/L HEPES, and 5.6 mmol/L glucose. The osmolality and pH of this solution were adjusted to 310 mOsm/L and 7.4, respectively. When a calcium-free medium was required, CaCl2 was omitted and replaced by equimolar MgCl2. Dye loading was achieved by transferring the cells into a standard HBSS solution containing 1 mmol/L Fura-2 acetoxyethyl ester (Calbiochem) loaded (45 min) for 40 min at 37°C, as described previously (11), using a photomultiplier-based system (Photon Technologies) and a double-wavelength (340 and 380 nm) excitation protocol to quantify the absolute value of calcium concentration (22). All recordings were made at 37°C. The cells were continuously perfused with HBSS solution via a whole-chamber perfusion system. The flow rate of the whole-chamber perfusion system was set to 1 mL/min and the chamber volume was 500 mL. The basal Ca2+ was measured using HBSS solutions containing 0 and 2 mmol/L CaCl2. The relative increase in [Ca2+]c was measured following consecutive switch from free Ca2+ medium to 2 mmol/L Ca2+ medium.

**Reverse transcription-PCR.** Total mRNA was isolated from cells as previously described (23). DNA amplification conditions included an initial denaturation step of 7 min at 95°C, 36 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C; and finally 7 min at 72°C. Primers used are listed in Table 1.

**Antibody production.** Rabbit polyclonal antibody anti-TRPV2 was produced in the laboratory headed by Dr. Rassen (Centre National de la Recherche Scientifique UMR 5203, Institut National de la Santé et de la Recherche Médicale U661, Montpellier, France). The polypeptide AEE-NTVPVQLQS corresponding to 740 to 763 amino acids of the COOH-terminal sequence of the human TRPV2 channel (NM_016113) was injected into rabbit to produce a polyclonal anti-TRPV2 antibody.

**Fluorescence experiments.** LNCaP cells nucleofected with TRPV2-GFP were washed twice with PBS and subjected to fluorescence analysis using a Zeiss LSM 510 confocal microscope and analysis software (AIM 3.2, Zeiss), as previously described (23).

**Western blotting.** The experiments were carried out as described previously (19). Membranes were rebotted twice: first with the anti-NSE mouse monoclonal antibody (1:250; Dako) and then with the anti-β-actin mouse monoclonal antibody (1:500; NeoMarkers).

**Migration assay.** Cells were seeded onto the top of Transwell cell culture inserts with 8.0-μm pore size (Falcon) at a density of 30,000 per well (24-well format) in serum-free culture medium. Cells were stimulated to migrate across the filters by providing 10% FCS as a chemoattractant in the assay chambers beneath the inserts. After 2 h, almost two thirds of the cells were adherent to each filter in the inserts. After 26 h of incubation at 37°C, nonmigratory cells were removed from the top of the filter by scraping, whereas cells that had migrated through the filter pores to the lower face of the inserts were fixed in 4% paraformaldehyde in PBS and stained with Hoechst (5 mg/L in PBS). Cells under each filter were counted on five random examination fields (x200) using a Leica DMIRB. Data are expressed as means of four wells ± SE.

**Cell proliferation assay.** Cells were plated at the initial density of 500 per well for PC3 cells and 2,500 per well for LNCaP cells in 96-well plates (Poly-Labo). After 48 h, cells were cultured in the treatment medium (day 0). From day 0, half of the medium was changed daily for each condition.
The CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega Corp.) was used to determine the number of viable cells (11).

**Cancer tissue sampling and total RNA extraction.** After informed consent, patients with clear localized T2a and T2b stages of PCa for whom a prostatectomy has been scheduled, as well as patients with metastatic PCa having a stage M1 who were previously subjected to androgen ablation therapy, have been included in a clinical research program (protocol ID: 2897) funded by the Hôpitaux Universitaires de Strasbourg (Strasbourg, France). A total volume of 3 mL of bone marrow was aspirated at a scintigraphic-indicated metastatic site. Microscopic observations were made from an aliquot of each bone marrow aspirate to confirm the presence of metastatic PCa cells. Total RNAs were extracted from small pieces of localized tumor by the use of the RNeasy Midi kit (Qiagen) and from bone marrow aspirates by the use of the QIAamp RNA Blood kit (Qiagen) according to the manufacturer’s protocol. Nine samples from both localized and metastatic PCa were used in our study.

**Quantitative real-time PCR.** Quantitative real-time PCR of TRPV2 mRNA transcript was done using MESA GREEN qPCR MasterMix Plus for SYBR Assay (Eurogentec) on the Bio-Rad CFX96 Real-Time PCR Detection System. The sequence of primers is indicated in Table 1. The 18S rRNA gene was used as an endogenous control to normalize variations in RNA extractions, the degree of RNA degradation, and variability in RT efficiency. To quantify the results, we used the comparative threshold cycle method described by Livak and Schmittgen (24).

**Animals, siRNA injection, and tumorigenicity assays.** Studies involving animals, including housing and care, method of euthanasia, and experimental protocols, were conducted in accordance with the local animal ethical committee in the Institut André Lwoff in Villejuif, France. Animals, siRNA injection, and tumorigenicity assays. Studies involving animals, including housing and care, method of euthanasia, and experimental protocols, were conducted in accordance with the local animal ethical committee in the Institut André Lwoff in Villejuif, France. Tumor cells (2 × 10⁶ per mouse) were injected s.c. in 50% (v/v) Matrigel (BD Biosciences) to 6- to 8-wk-old male nude mice. Once tumors were exponentially growing, mice were randomized for treatment (at least six animals per group) and received daily i.p. either siRNA control (siCTL) or siTRPV2 at a dose of 120 μg/kg diluted in PBS. Mice were sacrificed after 17 d of treatment, and tumors were dissected and weighed.

**Data analysis.** Results were expressed as mean ± SE. Plots were produced using Origin 7.0 (MicroCal Software, Inc.). Each experiment was repeated at least three times. n indicates the number of the cells per experiment. N indicates the number of experiments performed. The Turkey-Kramer test was used for statistical comparison among means and differences, and P < 0.05 was considered significant.

**Results**

**TRPV2 channel expression and function in human PCa cell lines.** We have studied the expression of TRPV2 channel in the androgen-dependent human PCa cell line LNCaP, the androgen-independent human PCa cell line LNCaP C4-2 derived from androgen-dependent LNCaP cells, and the castration-resistant human PCa cell lines DU145 and PC3 (Fig. 1A).

Short hairpin RNA (shRNA) TRPV2 I and II (PC3 shV2 I and II) were nucleofected into PC3 cells to specifically inhibit the expression of TRPV2 (Fig. 1B). TRPV2 knockdown with shTRPV2 I or II in PC3 cells led to significant suppression of both mRNA and protein of TRPV2 and NSE (Fig. 1B). We have shown that the proliferation rate of PC3 cells treated with shTRPV2 I or II evaluated during 6 days is not statistically different as compared with PC3 pSilencer–treated cells (N = 4; Fig. 1C). However, the cell migration is decreased in PC3 cells treated with shTRPV2 I or II (59 ± 11 cells for PC3 treated with shTRPV2 I and 58 ± 12 cells for PC3 treated with shTRPV2 II, n = 4, N = 4; Fig. 1C) than in PC3 pSilencer cells (89 ± 5 cells, n = 4, N = 4). The basal [Ca²⁺]cyt in PC3 treated with shTRPV2 I or II is lower (120 ± 13 nmol/L and 110 ± 9 nmol/L, n = 120,

**Table 1.** List of primers used for RT-PCR amplifications and a siRNA-TRPV2 sequence

<table>
<thead>
<tr>
<th>5′-forward-3′</th>
<th>5′-reverse-3′</th>
<th>Product size (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV2 (a)</td>
<td>TCCACTCTTGGTGGCAAGGG</td>
<td>351</td>
<td>NM_016113.3</td>
</tr>
<tr>
<td>TRPV2 (b)</td>
<td>TCCACTCTTGGTGGCAAGGG</td>
<td>475</td>
<td>NM_016113.3</td>
</tr>
<tr>
<td>NSE</td>
<td>CTGTGATAGCTTCCTTCAC</td>
<td>553</td>
<td>NM_001975.2</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGCATGAAGTGGTGTGATGA</td>
<td>237</td>
<td>NM_002046.3</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GGTGAAGGTCTCACAAGCATGTC</td>
<td>210</td>
<td>NM_001101</td>
</tr>
<tr>
<td>siRNA-TRPV2:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5′-UAAGAGUCAACCUCACUACUdTdT-3′</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRPV2</td>
<td>TGGAGGGAGAGATGACCTCA</td>
<td>99</td>
<td>NM_016113</td>
</tr>
<tr>
<td>MMP2</td>
<td>ATGCCCGCTTACCTGAGGA</td>
<td>103</td>
<td>NM_004530</td>
</tr>
<tr>
<td>MMP9</td>
<td>ACGACTTGTCCAGTCAAGGA</td>
<td>95</td>
<td>NM_004994</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>CTTAAAGGCCAGGCTCCACTAC</td>
<td>99</td>
<td>NM_001908</td>
</tr>
<tr>
<td>18S</td>
<td>CAGCTTCGGGAAAACAAAGTC</td>
<td>111</td>
<td>NR_003286</td>
</tr>
</tbody>
</table>

www.aacrjournals.org Cancer Res; 70(3) February 1, 2010 1227

Published OnlineFirst January 26, 2010; DOI: 10.1158/0008-5472.CAN-09-2205

Downloaded from cancerres.aacrjournals.org on July 13, 2017. © 2010 American Association for Cancer Research.
Figure 1. TRPV2 expression and function in PCa cells. A, expression of TRPV2 mRNA in different PCa cell lines at the mRNA and protein levels. B, TRPV2 silencing downregulates NSE expression. PC3 cells were nucleofected with either pSilencer (PC3 pSil) or shRNATRPV2 I or II (PC3 shTRPV2 I or PC3 shTRPV2 II) for 72 h. C, effects of TRPV2 silencing on cell proliferation and migration of PC3 cells. Cell proliferation was measured after 0, 1, 2, 3, or 6 d of transfection with shRNATRPV2 I or II and compared with PC3 pSilencer cells. Points and columns, mean; bars, SD. The number of migrating cells was counted in four fields per Transwell membrane, four membranes counted per condition (N = 3). *, P < 0.05, compared with control cells. D, TRPV2 affects basal [Ca^{2+}]_{cyt} in PC3 cells (n = 120, N = 4 per condition). **, P < 0.05, compared with control cells.
TRPV2 channel contributes to hormone refractoriness of LNCaP C4-2 cells. Semiquantitative reverse transcription-PCR (RT-PCR) was used to show that LNCaP C4-2 cells express TRPV2 mRNA in the control (transfected with a vehicle) or with 50 nmol/L siCTRL for 72 hours (Fig. 2A). To specifically silence TRPV2 mRNA, we transfected LNCaP C4-2 cells with 50 nmol/L siTRPV2 for 72 hours. We have also shown that in LNCaP C4-2 cells treated with siTRPV2, the NSE expression is downregulated (Fig. 2A). A Western blot experiment was used to confirm that transfection with 50 nmol/L siTRPV2 for 72 hours drastically diminished the expression of TRPV2 and NSE proteins as compared with siCTRL (Fig. 2B). The basal [Ca^{2+}]_{cyt} is lower in LNCaP C4-2 cells transfected with siTRPV2 (77 ± 2 nmol/L, n = 120, N = 4) than in LNCaP C4-2 cells transfected with siCTRL (102 ± 12 nmol/L, n = 120, N = 4) in the presence of Ca^{2+} in the medium (Fig. 2C). Our results also show that LNCaP C4-2 cell migration is drastically decreased in LNCaP C4-2 cells transfected with siTRPV2 (44 ± 2 cells, n = 4, N = 4) than in LNCaP C4-2 cells transfected with siCTRL (73 ± 5 cells, n = 4, N = 4; Fig. 2D).

The role of TRPV2 channel in PCa aggressiveness. As we have shown, mRNA and protein of TRPV2 are not expressed in human PCa cell line LNCaP (Fig. 3A); however, LNCaP cells nucleofected with TRPV2-GFP (LNCaP TRPV2-GFP) expressed TRPV2 mRNA as compared with the cells

Figure 2. TRPV2 contributes to the oncogenic potential of LNCaP C4-2 cells. A, TRPV2 silencing downregulates NSE expression. LNCaP C4-2 cells were transfected with either 50 nmol/L siTRPV2 or siCTRL for 72 h or untreated (vehicle) used as a control. B, expression of TRPV2 and NSE proteins in LNCaP C4-2 cells. LNCaP C4-2 cells were transfected with either 50 nmol/L siTRPV2 or siCTRL for 72 h. C, TRPV2 affects basal [Ca^{2+}]_{cyt} in LNCaP C4-2 cells (n = 120, N = 4 per condition). *, P < 0.05, compared with control cells. D, TRPV2 silencing by siTRPV2 decreases LNCaP C4-2 cell migration (N = 3). *, P < 0.05, compared with control cells.
nucleofected with a control plasmid pSilencer (LNCaP pSilencer). We have shown that the growth of LNCaP cells is not affected by the presence of TRPV2 channels (N = 4; Fig. 3B) measured during 7 days. The cell migration is strongly increased in LNCaP TRPV2-GFP cells (46 ± 2 cells, n = 4, N = 4) than in LNCaP pSilencer cells (34 ± 2 cells, n = 4, N = 4; Fig. 2D). Basal [Ca^{2+}]_{cyt} in LNCaP cells (n = 120, N = 4 per condition) D, quantitative real-time PCR for LNCaP cells nucleofected with pTRPV2 plasmid as compared with pcDNA3 plasmid. The expression of TRPV2, MMP2, and cathepsin B is normalized to 18S gene expression. Columns, mean; bars, SD. *, P < 0.05, compared with control cells.

Figure 3. The effects of TRPV2 overexpression in androgen-dependent LNCaP cells. A, expression of TRPV2 in LNCaP cells nucleofected with either pSilencer (LNCaP pSil) or TRPV2-GFP for 72 h at the mRNA and protein level using Western blotting and confocal imaging; B, effects of TRPV2 overexpression on LNCaP cell proliferation and migration (N = 3). Points and columns, mean; bars, SD. C, TRPV2-GFP transfection affects basal [Ca^{2+}]_{cyt} in LNCaP cells (n = 120, N = 4 per condition). D, quantitative real-time PCR for LNCaP cells nucleofected with pTRPV2 plasmid as compared with pcDNA3 plasmid. The expression of TRPV2, MMP2, and cathepsin B is normalized to 18S gene expression. Columns, mean; bars, SD. *, P < 0.05, compared with control cells.

nucleofected with a control plasmid pSilencer (LNCaP pSilencer). We have shown that the growth of LNCaP cells is not affected by the presence of TRPV2 channels (N = 4; Fig. 3B) measured during 7 days. The cell migration is strongly increased in LNCaP TRPV2-GFP cells (46 ± 2 cells, n = 4, N = 4) than in LNCaP pSilencer cells (34 ± 2 cells, n = 4, N = 4; Fig. 2D). Basal [Ca^{2+}]_{cyt} is higher in LNCaP TRPV2-GFP cells (167 ± 7 nmol/L, n = 120, N = 4) than in LNCaP pSilencer (103 ± 6 nmol/L, n = 120, N = 4) in the presence of Ca^{2+} in the medium (Fig. 3C). Such markers of invasion as cathepsin B and matrix metalloproteinase (MMP) 9 were shown to be induced on the expression of TRPV2 in these cells (Fig. 3D).

Induction of TRPV2 expression during transition to castration-resistant phenotype. A semiquantitative PCR experiment showed that, under conditions of androgen deprivation, the expression of TRPV2 mRNA is detected
starting from 24 hours (Fig. 4A) and that this expression lasts for several days of the treatment. We have shown that the addition of Bt2cAMP and IBMX to the culture medium induces TRPV2 mRNA expression in LNCaP cells after only 8 hours of treatment (Fig. 4B). Further, the siTRPV2 transfection into these cells reduces TRPV2 and NSE mRNA expression in LNCaP treated with 1 mmol/L Bt2cAMP and 100 μmol/L IBMX for 6 days (Fig. 4C). We observed that both TRPV2 channel and NSE proteins were expressed (Fig. 4C), being absent in LNCaP CTL. siTRPV2 transfection of LNCaP cells treated with Bt2cAMP and IBMX showed the consequent knockdown of both TRPV2 and NSE proteins. The basal [Ca2+]cyt is higher in LNCaP cells (vehicle) treated with Bt2cAMP and IBMX than in LNCaP CTL cells (vehicle; 125 μmol/L Bt2cAMP + 100 μmol/L IBMX; Fig. 4D).

Figure 4. The involvement of TRPV2 channel in androgen ablation of LNCaP cells. A. TRPV2 transcripts are upregulated in LNCaP cells treated with a steroid-deprived medium. B. TRPV2 transcripts are upregulated in LNCaP cells treated with 1 mmol/L Bt2cAMP and 100 μmol/L IBMX (LNCaP + Bt2cAMP + IBMX). C. Effects of siRNA TRPV2 on the mRNA and protein levels. siRNA TRPV2 (50 μmol/L; siTRPV2) treatment for 72 h downregulated TRPV2 transcripts as well as NSE transcripts in LNCaP + Bt2cAMP + IBMX cells. Nontransfected LNCaP + Bt2cAMP + IBMX cells (Vehicle) or cells transfected with 50 μmol/L siRNA CTL (siCTL) for 72 h were used as control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. D. Basal [Ca2+]cyt in LNCaP + Bt2cAMP + IBMX cells and untreated LNCaP cells (n = 120 per condition, N = 4). *, P < 0.05.
± 10 nmol/L and 78 ± 3 nmol/L, n = 120, N = 4 per condition, respectively) in the presence of Ca²⁺ in the medium (Fig. 4D). Cell transfection with siCTL does not significantly modify the basal [Ca²⁺]_{cyt} (135 ± 10 nmol/L for LNCaP treated with Bt²cAMP and IBMX and 80 ± 6 nmol/L for LNCaP CTL, respectively, n = 120, N = 4 per condition) in the presence of Ca²⁺ in the medium. It should be noted that transfection of LNCaP cells with siTRPV2 had no effect on basal [Ca²⁺]_{cyt} (80 ± 3 nmol/L, n = 120, N = 4) in the presence of Ca²⁺ in the medium. However, in LNCaP treated with Bt²cAMP and IBMX, the transfection with siTRPV2 significantly decreased the [Ca²⁺]_{cyt} (96 ± 4 nmol/L, n = 120, N = 4) in the presence of Ca²⁺ in the medium as compared with LNCaP treated with Bt²cAMP and IBMX and transfected with siCTL.

**TRPV2 gene expression increases in PCa metastasis and is involved in tumor development in vivo.** A quantitative real-time PCR showed that mRNA expression is ∼12 times higher in metastatic PCa samples than in localized PCa samples (n = 9 for each condition; Fig. 5A). The most pertinent question was whether TRPV2 silencing could inhibit tumor development in vivo. Previously, a RNA interference in vivo has been used to show that the androgen receptor is still required for the development of castration-resistant prostate tumors (25). Similarly, the model of mice bearing xenografted PC3 cell–provoked tumors has been created. Then, the mice were treated on a daily basis during 17 days with either siCTL or siTRPV2 at a dose of 120 μg/kg diluted in PBS. The weight of tumors was significantly smaller in mice treated with siTRPV2 than in siCTL-treated animals (Fig. 5B). To search for the mechanisms that might account for the profound effect of TRPV2 knockdown on the tumors, such markers of invasion as MMP2, MMP9, and cathepsin B were shown to be substantially downregulated on the TRPV2 knockdown in vivo (Fig. 5C).

**Discussion**

In the present study, we report three major findings that allow the understanding of human PCa development and progression to castration-resistant phenotype. We have shown for the first time that (a) the PCa tumor progression to castration-resistant phenotype is characterized by the expression de novo of a cationic nonselective TRPV2 channel, which may be served as a reliable prognostic marker of the most aggressive castration-resistant stage; (b) the role of TRPV2 in cancer cells derived from castration-resistant tumors is to maintain an elevated level of cytosolic calcium due to the constitutive activity of the channel; (c) the main feature of the PCa metastatic cells—their ability to migrate and invade the adjacent tissues—could be mediated by TRPV2 activity via the direct regulation of such proteins as...
MMP2, MMP9, and cathepsin B used by the cancer cell to invade.

The fact that the hormone refractoriness of PCs is featured by the emergence of malignant cell phenotypes characterized by uncontrolled proliferation, resistance to apoptosis, and enhanced potential to invade has led us to the search of some specific molecular intermediators helping PCs cells to escape a natural regulation by hormones. In the context of the present work, we report a nonselective cationic channel TRPV2 as a unique and distinguished feature of castration-resistant PCs. As the evidence, TRPV2 is ultimately expressed in metastatic castration-resistant PCs samples rather than in localized hormone-responsive PCs tumors. Indeed, until now, TRPV2 channel has never been associated with the development and progression of PCs. Unlike some other TRP channels (e.g., TRPM8 channel), the expression of TRPV2 channel does not require the presence and/or activation of an androgen receptor (26). Therefore, it seems likely that TRPV2 expression being confined to the castration-resistant phenotype, which is no longer under AR control, may be used by the cancer cell to increase its oncogenic potential.

It has been previously shown that TRPV2 channels were constitutively activated in TRPV2-overexpressing cell systems and induced an increase in basal [Ca^{2+}]_{cyt} (27). Our results show that the basal [Ca^{2+}]_{cyt} is higher in hormone-resistant PCa cell lines, which express TRPV2, as compared with androgen-dependent LNCaP cell line, which does not express TRPV2. This increase in calcium is ultimately mediated by TRPV2 because, on the one hand, this high level may be achieved by TRPV2 transfection into LNCaP cells and, on the other hand, shRNA of TRPV2 prevents calcium increase. Thus, by increasing basal [Ca^{2+}]_{cyt}, which is apparently temporally and spatially regulated in the cancer cell, TRPV2 channels contribute to the progression to castration-resistant phenotype.

The transition of PCa cells to hormone-insensitive phenotype is a common feature of human prostate carcinoma (28, 29) and the appearance of neuroendocrine markers is considered to be associated with a poor prognosis and reduced long-term survival (30). We have shown that TRPV2 expression is associated with the NSE expression in PC3 and LNCaP C4-2 cells. It has been already shown that other neuroendocrine cell markers, PGP9.5 and chromogranin A, are expressed in PCs derived from metastatic (lymph node and bone) adenocarcinomas, suggesting that these cells had undergone specific PCa neuroendocrine differentiatation (31). This is usually accompanied by the loss of a nuclear androgen receptor (5) or, in the case of LNCaP C4-2, by the loss of androgen sensitivity (32). Both steroid deprivation and Bt-cAMP and IBMX treatments were used to restore in the experimental conditions the consequences of androgen ablation therapy in men in vivo. Interestingly, both treatments resulted in the expression of TRPV2 channels in androgen-deprived LNCaP cells, in increase in cytosolic calcium levels, and in overexpression of NSE. The latter has already been used as a marker because serum NSE in metastatic PCa patients who underwent endocrine therapy is significantly higher than that in nonmetastatic patients (33). Our data were confirmed by the clinical in vivo studies using the biopsies of the patients subjected to hormone ablation therapy and who finally developed a castration-resistant metastatic PCa.

TRPV2 was suggested to be a physiologic sensor of hot temperatures (34). It is also expressed in nonneuronal cells, such as prostate cells (19, 35), or in human blood cells, suggesting that, in addition to its role as a noxious heat sensor (34), this channel may encompass other cellular functions (36). One of such function of TRPV2 in cancer cells is the control of cell migration. As we have shown, TRPV2 is primarily involved in PCs cell migration and not in cell proliferation. TRPV2 silencing drastically decreased the migration of the cancer cells derived from castration-resistant tumors and that TRPV2 overexpression increased cell migration of androgen-dependent LNCaP cells. Such a promigrative role of TRPV2 has been also previously shown on macrophage cells (37). To have an insight into the possible molecular mechanisms responsible for TRPV2-induced enhanced potential to invade, such markers of invasion as MMP2, MMP9, and cathepsin B (38–40) were studied. We have shown that the expression of the above enzymes directly participating in the process of invasion is strongly dependent on TRPV2 expression. Indeed, the transfection of TRPV2 channel into androgen-dependent LNCaP cells induces the expression of MMP9 and cathepsin B, and at the same time, the siRNA knockdown in vivo leads to progressive downregulation of these markers in the xenografted tumors.

Moreover, our data obtained in vivo using a nude mice xenograft model show that TRPV2 silencing in mice bearing TRPV2-expressing PC3 cell tumors significantly decreased the tumor weight, suggesting that tumors void of TRPV2 channel are likely to undergo necrosis. The fact that siRNA against particular target does not inhibit cancer cell proliferation or is implicated in cell survival in vitro, preserving its strong antitumor effects in vivo, comes from the specific suppression of this cancer cell migration. Indeed, the continuous cell division inside of the tumor without any possibility to invade the host in addition to restriction in oxygen and nutrition element supply leads eventually to tissue necrosis (see ref. 41 for review).

Thus, the involvement of TRPV2 in the invasion process by enhancing cell migration is evident for more aggressive PCa cells and therefore seems vital for the PCa tumor in general. In conclusion, we have discovered a new feature of castration-resistant PCa characterized by de novo expression of a nonselective cationic TRPV2 channel. The latter has been shown to manifest its activity via maintaining an elevated intracellular calcium concentration. As a result of TRPV2 function, it contributes to enhanced cancer cell migration by induction of key proteases—MMP2, MMP9, cathepsin B—and is necessary for castration-resistant tumor development and progression, as we have shown in vivo. Thus, TRPV2 channel could be a prospective prognostic marker and potential therapeutic target for future interventions to increase the life expectancy of the patients.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Thierry Capiod, Etienne Dewailly, and Gilbert Lepage for helpful advice.

References


