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T-type calcium channels drive the proliferation of androgen-receptor negative prostate cancer cells.

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KEY WORDS: TTCC; PCa; NEPC; CACNA1G; MTT assay.

Running head: T-type calcium channels in prostate cancer

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Abstract

Background. Androgen deprivation therapy (ADT) is the treatment of choice for metastatic prostate cancer (PCa). After an initial response to ADT, PCa cells can generate castration resistant (CRPC) or neuroendocrine (NEPC) malignancies, which are incurable. T-type calcium channels (TTCCs) are emerging as promising therapeutic targets for several cancers, but their role in PCa progression has never been investigated.

Methods. To examine the role of TTCCs in PCa, we analyzed their expression level, copy number variants (CNV) and prognostic significance using clinical datasets (Oncomine and cBioPortal). We then evaluated TTCC expression in a panel of PCa cell lines and measured the effect of their inhibition on cell proliferation and survival using MTT and caspase assays.

Results. TTCCs were up-regulated in PCas harboring androgen receptor (AR) mutations; CNV rate was positively associated with PCa progression. Higher expression of one TTCC isoform (CACNA1G) predicted poorer post-operative prognosis in early stage PCa samples. Pharmacological or siRNA-based inhibition of TTCCs caused a decrease in PC-3 cell survival and proliferation.

Conclusions. Our results show that TTCCs are overexpressed in advanced forms of PCa and correlate with a poorer prognosis. TTCC inhibition reduces cell proliferation and survival, suggesting that there may be possible value in the therapeutic targeting of TTCCs in advanced PCa.

Introduction

Androgen-deprivation therapy (ADT) is employed as first-line treatment for metastatic prostate cancers (PCas). Unfortunately, ADT itself can promote the proliferation of castration-resistant PCa (CRPC) cells\(^1\), which triggers cancer recurrence. Most CRPCs are androgen receptor (AR)-positive
and rely on the aberrant activation of the AR-pathway, which favours cell survival and proliferation. Less frequently, CRPC cells are AR-negative and can trans-differentiate into neuroendocrine PCa (NEPC). NEPC is highly metastatic and resistant to all available therapies. Despite some progress in the treatment of these diseases, CRPC and NEPC are invariably fatal. Hence, the research of new therapies against advanced PCa is crucial. Recent studies have highlighted the importance of T-type calcium channels (TTCCs) in cancer progression. TTCCs are low voltage-activated calcium channels that play important roles during embryonic development, and in adults are known to participate in the generation of calcium signals in electrically excitable cells. There are three isoforms of TTCCs, encoded by \textit{CACNA1G, CACNA1H} and \textit{CACNA1I} genes. In healthy adults, the expression of TTCCs is largely restricted to excitable cell types, but numbers of studies have reported an aberrant expression of TTCCs in different cancers. In these neoplasms, TTCCs are expressed at similar levels than during embryonic development. TTCC inhibition via chemical antagonists (e.g. Mibefradil) or siRNAs was shown to arrest proliferation and induce apoptosis in glioblastoma, colon, breast, and ovarian cancers. In PCa, TTCCs seem to be involved in the acquisition of neuroendocrine features and in the calcium-dependent secretion of mitogenic factors. However, the functional role of TTCCs in CRPC and NEPC initiation and progression has never been investigated. Here we examine the expression of TTCCs in a panel of PCa cell lines and the effect of their inhibition on cell proliferation. Based on our findings, we hypothesize that TTCCs can be valuable therapeutic targets for the treatment of advanced PCa.

\textbf{Materials and Methods}

\textbf{Clinical database analysis}

We queried the online microarray databases Oncomine\textsuperscript{6} and cBioPortal\textsuperscript{7} to evaluate \textit{CACNA} expression levels in relation with AR-status and to compare \textit{CACNA} copy number variants (CNVs) among 112 hormone-naïve PCa (HNPC), 70 CRPC-AR\textsuperscript{+} and 44 NEPC samples. To evaluate the prognostic impact of \textit{CACNA} overexpression, we investigated a cohort of 131 primary PCa samples.
by querying cBioPortal (dataset: MSKCC, Cancer Cell 2010) for mRNA expression of \textit{CACNA1G-1H-II} (over-expression threshold fixed to 2, 0.5 and 1.8 standard deviations (SDs) above the mean for \textit{CACNA1G, 1H and 1I} respectively).

\textit{In-vitro experiments}

\textbf{Cell cultures:} we employed a panel of five PCa cell lines (LNCaP, C4-2, 22Rv1, Du145 and PC-3) representative of various stages of PCa. All cells used were bought from ATCC and maintained in RPMI Medium 1640 (GIBCO, Life Technologies, UK) with 10% FBS and 1% penicillin-streptomycin, at 37°C and 5% CO₂.

\textit{Gene expression analysis:} RT-qPCR was performed as described using pre-designed TaqMan gene expression assays: \textit{GAPDH:} Hs02786624_g1; \textit{HAPRT1:} Hs02800695_m1; \textit{CACNA1I:} Hs01096207_m1; \textit{CACNA1H:} Hs01103527_m1; and \textit{CACNA1G:} Hs00367969_m1 (Life Technologies, UK). Normalised gene expressions were calculated using $2^{-\Delta\Delta CT}$ and using the mean Ct values of \textit{HPRT1} and \textit{GAPDH} genes as references.

\textit{Inhibition of TTCCs:} to investigate the functional role of TTCCs in PCa, we treated PC-3 cells with increasing concentrations of two TTCCs antagonists: Mibefradil and Ethosuximide. In addition, we also exposed PC-3 cells to a specific \textit{CACNA1G}-targeting siRNA (hs.Ri.CACNA1G.13.2, IDT). We then evaluated the consequences of these inhibitions on cell proliferation and survival by MTT and caspase assays.

\textit{MTT and Caspase assays:} PC-3 cells were seeded in 96-well plates and transfected with 13.2-siRNA or negative control NC-siRNA at a final concentration of 10nM using Lipofectamine 2000 (Invitrogen, UK). Alternatively, after seeding, cells were incubated for 24h at 37°C and 5% CO₂ and then treated with increasing concentrations of Mibefradil or Ethosuximide (1.23 - 33.75µM and 3.90mM - 100mM respectively). MTT-assays were performed at different time points, by adding MTT solution (5mg/ml) in each well (incubation time: 3h at 37°C). Upon removal of culture medium, MTT crystals were dissolved in 50µl of DMSO and absorbance at 570 and 630-690 was measured. Caspase assay was carried out using Caspase-Glo 3/7 Assay (Promega Corporation, UK)
on PC-3 cells transfected, as described above, with 10nM of 13.2-siRNA or NC-siRNA. Three days
after siRNAs exposure, Caspase-Glo reagent was added to the cells and total luminescence was
quantified following the manufacturer’s instructions and normalized to treatment-match cell
absorbance (MTT).

**In-silico prediction of CANCA1G-dependent molecular pathways**

To investigate the possible molecular pathways through which TTCCs exert their effect on cancer
progression, we first identified genes (protein coding and one non-coding RNA) that were
associated with CANCA1G expression in 49 prostate (CRPC and NEPC) clinical samples from the
cBioPortal mRNA (dataset: Trento/Cornell/Broad 2016). Using R programming with the Weighted
Correlation Network Analysis, WGCNA package, we selected the strongest interactions with
CANCA1G. These interactions were selected by being the most significant (Pearson correlation,
p<0.01) and most direct interaction (removing secondary or intermediate genes in the gene
network), according to an adjacency matrix and weighted interaction filter. Gene networks were
then visualized using Cytoscape 3.6.1. We identified the cellular pathways associated with the
CANCA1G gene network using Reactome, KEGG, and UniProtKB.

**Results and discussion**

**Alteration of CACNA variants in different types of prostate cancer**

To investigate the possible involvement of TTCCs in PCA progression, we first analyzed their
expression levels and genomic status in public databases. Oncomine analysis revealed that the
expression levels of CANCA1G and CANCA1I were significantly higher in PCA samples harbouring
a mutant AR gene, than in samples with wild type AR (Figure 1A). We then queried cBioPortal to
evaluate the presence of CANCA-1G-1H-II CNVs in HNPC, CRPC-AR+ and NEPC. CNVs
appeared to be very rare in samples derived from HNPC. Compared with HNPC, CRPC-AR+ samples showed an increase in CNVs: CANCA1G and CANCA1H were amplified in 11 of 70 cases
(15.7%) and CANCA1I in 4/70 cases (5.7%). A further increase in CANCA1G and CANCA1H CNV
rate was identified in NEPC samples (Figure 1B). While this observation is consistent with the hypothesis that TTCCs may play a role in the progression from HNPC to CRPC and NEPC, it is important to consider that this pattern of genetic amplifications is not limited to CACNA loci (Supplementary Table 1). To evaluate the prognostic power of TTCCs in PCa, we investigated a previously described cohort of 131 primary PCa samples with extensive post-operative follow-up. We found that CACNA1G up-regulation was significantly associated with shorter relapse-free survival (Figure 2). Our analysis indicates that CACNA1G expression is progressively up-regulated in CRPC and NEPC samples, and that higher CACNA1G expression levels predict poorer prognosis in earlier stages of the disease. These data are in accordance with the observation that TTCCs are generally unregulated in the majority of PCa samples.15,16

In-vitro analysis of the role of CACNA genes

To investigate the functional role of TTCCs in PCa cells, we measured the expression of CACNA mRNAs in a panel of PCa cell lines. As shown in Figure 3A, CANCA genes were generally up-regulated in AR- cells. This up-regulation was particularly marked for CACNA1G, which showed the highest expression levels in PC-3 cells; a model of NEPC.17 Given these observations, we decided to employ PC-3 cell line as an in vitro model to assess the effect of the inhibition of TTCCs and, more specifically, of CACNA1G, on aggressive forms of PCa. Treating PC-3 cells with increasing concentrations of TTCCs antagonists led to a significant decrease in cell viability starting from a concentration as low as 6.67µM of Mibefradil, and 5.85mM of Ethosuximide (relative viability 0.74 ± 0.21 St.Dev, p-value<10^-5 and 0.83 ± 0.06 St.Dev, p-value=0.045, respectively)(Figure 3B; Supplementary Table S2). Even if these results seemed compatible with an on-target effect of these two drugs, as suggested by electrophysiology experiments,18,19 we decided to further confirm the gene-specificity of this effect by exposing PC-3 cells to a CACNA1G-targeting siRNA or negative control. We first showed that 13.2-siRNA induced a significant reduction in CACNA1G mRNA expression (fold change: 0.41; p-value<0.05) (Figure 3C). Then we analyzed the effect CACNA1G silencing by MTT and caspase assays, and found that
CACNA1G-knockdown was able to significantly reduce cell proliferation and survival (Figure 4A and 4B). Based on our results, and previous observations, we can hypothesize that the inhibition of CACNA1G induced cell death on PC-3 cells. Probably, both pharmacological antagonists and the specific siRNA exerted these effects of PC-3 cells by activating the p38-MAPK pathway through an upregulation of p53 as already observed in other cell lines (as reviewed by Sallan et al). Nonetheless, further experiments will be necessary to evaluate this hypothesis and to extend our observations to a larger panel of PCa cell lines.

In-silico prediction of CANCA1G-dependent molecular pathways

An obvious outcome of expressing more calcium channels would be a cytosolic calcium increase, which can have a number of effects on cellular activities. Alterations in cellular calcium handling has been implicated in cancer cell differentiation and growth. However it is unclear exactly how increased expression of TTCCs would lead to PCa progression. We therefore investigated the possible molecular pathways activated by TTCCs in cancer cells, by a previously described “guilt-by-association” analysis. We identified genes associated with CACNA1G expression in CRPC and NEPC clinical samples (Figure 4C) and the cellular pathways that are associated with the CACNA1G gene network (Table I). G protein-coupled receptor (GPCR) signalling was the most represented pathway, with 5 different genes of this pathway associated with CACNA1G expression. Notably, GPCRs are emerging as key mediators of cell survival in PCa. Among the genes showing a strong association with CACNA1G, Wnt-11 was of particular interest as it is reported to be involved in PCa progression by promoting neuroendocrine differentiation, cell survival and cell migration. CACNA1G network genes are also associated with known oncogenic functions (DNA replication, cell growth, apoptosis, metabolism, immune system and further calcium signalling) and pathways (WNT, NOTCH, JNK and MAPK signalling). Further analyses will be necessary to dissect the mechanistic correlation between CACNA1G and these pathways. In conclusion, our study demonstrates that CACNA genes are progressively up-regulated during PCa progression, and
that both pharmacological and siRNA-mediated inhibition of CACNA expression reduces the proliferation of PCa cells.

Bibliography


Legends

**Figure 1**: box-plot showing CACNA1G, CACNA1H and CACNA1I mRNA levels in prostate cancer tissues expressing wild-type vs. mutated AR (Oncomine) [Grasso Prostate, Nature 2012] (A); percentage of hormone naive (HNPC), castration resistant (CRPC) and neuroendocrine (NEPC) prostate cancer samples showing an amplification of CACNA genes (cBioPortal) [Broad/Cornell, Nat. Genet. 2012; Trento/Cornell/Broad 2016] (B).

**Figure 2**: progression-free survival in 131 hormone sensitive prostate cancer patients based on CACNA1G, CACNA1H and CACNA1I expression (top 5% vs all other samples; cBioPortal) [MSKCC, Cancer Cell 2010].

**Figure 3**: mRNA expression of the three CACNA variants in a panel of AR positive and AR negative prostate cancer cell lines (A). Effect of increased concentrations of Mibefradil and Ethosuximide on PC3 cells viability (B). CACNA1G mRNA levels upon treatment with 10nM negative control siRNA (NC-siRNA) or 10nM CACNA1G-targeting siRNA (13.2-siRNA) (C).

**Figure 4**: effect of CACNA1G silencing on PC3 cells proliferation rate assessed by MTT-assay (*p<0.05 unpaired T test). The interaction plot, with 95% confidence intervals, shows how the inhibition of CACNA1G affects the absorbance level over time (**p<0.01; *p<0.05; two-way
ANOVA and Sidak’s post-test) (A). Effect of CACNA1G-knockdown on caspase 3/7 activity; the barchart shows the fold-change in the caspase 3/7 activity 3 days after siRNAs exposure. Unpaired student’s t-test was used for statistical significance (****p<0.0001; FC 13.2-siRNA = 1.7) (B). CACNA1G gene networks of direct (primary) strong interactions using a node-to-node (gene to gene) edge (line) threshold of 0.2 to view interactions. The length of the line corresponds to the strength of the interaction i.e. genes closer to the CACNA1G gene have a stronger interaction to the CACNA1G gene than those further away (C).
Figure 1

(A) Comparison of Log2 median-centered intensity for 3 genes (CACNA1G, CACNA1H, CACNA1L) between AR wild type and AR mutated groups. 

- **CACNA1G**
  - p-value = 0.034
  - Fold change = 2.352

- **CACNA1H**
  - p-value = 0.327
  - Fold change = 1.170

- **CACNA1L**
  - p-value = 0.029
  - Fold change = 3.150

(B) Bar graphs showing the percentage of samples with gene amplification for HN, CRPC, and NEPC for each gene.

- **CACNA1G**

- **CACNA1H**

- **CACNA1L**
Figure 2

**CACNA1G**

- Logrank Test
  - p-value = 0.0308

**CACNA1H**

- Logrank Test
  - p-value = 0.579

**CACNA1I**

- Logrank Test
  - p-value = 0.238

Legend:
- Red: Cases with a high level of expression for the selected gene
- Blue: Cases with a low level of expression for the selected gene
Figure 3

A

CACA\textsubscript{1}G \hspace{1cm} CACA\textsubscript{1}H \hspace{1cm} CACA\textsubscript{1}I

<table>
<thead>
<tr>
<th></th>
<th>LNCaP</th>
<th>C4-2</th>
<th>22Rv1</th>
<th>Du145</th>
<th>PC-3</th>
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<tbody>
<tr>
<td>AR+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AR-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Relative cell viability

Log\textsubscript{10} concentration of Mibefradil [\mu M]

C

<table>
<thead>
<tr>
<th></th>
<th>NC-siRNA</th>
<th>13.2-siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative mRNA expression</td>
<td></td>
<td>*</td>
</tr>
</tbody>
</table>

Log\textsubscript{10} concentration of Ethosuximide [mM]
Table I: cell signalling pathways associated with the *CACNA1G* gene network. Genes associated with known oncogenic pathways and G protein-coupled receptor (GPCR) signalling are indicated in bold.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Cytoband</th>
<th>Pearson score</th>
<th>Signalling pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td>WNT11</td>
<td>11q13.5</td>
<td>0.95</td>
<td>Calcium modulating pathway; <strong>GPCR; WNT</strong></td>
</tr>
<tr>
<td>SMS</td>
<td>Xp22.11</td>
<td>0.94</td>
<td>Sperm biosynthesis; metabolism</td>
</tr>
<tr>
<td>ADAM7</td>
<td>8p21.2</td>
<td>0.94</td>
<td>Integral component of membranes; <strong>NOTCH</strong>; melanoma cells; mutations may play a role in melanoma progression and metastasis</td>
</tr>
<tr>
<td>OMP</td>
<td>11q13.5</td>
<td>0.94</td>
<td>Metabolism; mature olfactory receptor neurons</td>
</tr>
<tr>
<td><strong>GPR85</strong></td>
<td>7q31.1</td>
<td>0.94</td>
<td>Class A. Rhodopsin family; <strong>GPCR</strong>; highly expressed in brain and testis</td>
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<tr>
<td>RN7SL453P</td>
<td>10q11.22</td>
<td>0.93</td>
<td>Unknown</td>
</tr>
<tr>
<td>ADRA2A</td>
<td>10q25.2</td>
<td>0.93</td>
<td>Calcium regulation; metabolism; <strong>GPCR; MAPK</strong>; adrenergic receptor; wound healing (platelets)</td>
</tr>
<tr>
<td>MFSD4</td>
<td>1q32.1</td>
<td>0.91</td>
<td>Glucose transmembrane transport</td>
</tr>
<tr>
<td>CAPN5</td>
<td>11q13.5</td>
<td>0.90</td>
<td>Calcium-dependent cysteine-type endopeptidase activity; proteolysis; apoptosis; retinal degradation</td>
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<td>LRC7</td>
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<td>Innate immune system (neutrophil degranulation); neuron development; brain-specific; somatic mutation in breast cancer</td>
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<td>IL12B</td>
<td>5q33.3</td>
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<td>Immune system; cytokines</td>
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<td><strong>ARHGEF9</strong></td>
<td>Xq11.1</td>
<td>0.88</td>
<td>Neurotransmitter; <strong>GPCR</strong>; apoptosis; <strong>JNK</strong></td>
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<tr>
<td>RN7SL248P</td>
<td>10q11.22</td>
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<td>ADAMTSL3</td>
<td>15q25.2</td>
<td>0.84</td>
<td>Peptidase; glycosylation; colon, breast, prostate, renal and skin tumors</td>
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<td>CRYBA4</td>
<td>22q12.1</td>
<td>0.82</td>
<td>Structural constituent of eye lens; cataract</td>
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<td>RNA5SP521</td>
<td>Yq17.559</td>
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<td>FAM224A</td>
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<td><strong>MCS5</strong></td>
<td>18p11.21</td>
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<td>Neuroactive ligand-receptor interaction; <strong>GPCR</strong>; hormone and melanocortin (immune system); expressed in the brain</td>
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<td>SMIM21</td>
<td>18q23</td>
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<td>IL1RAPL2</td>
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<td>CRISP1</td>
<td>6p12.3</td>
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<td>EAF2</td>
<td>3q13.33</td>
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<td>LINC00308</td>
<td>21q21.1</td>
<td>0.70</td>
<td>Unknown; ncRNA</td>
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**Supplementary Table I:** copy number variants (CNVs) in chromosomal regions of CACNAs genes. For genes have been chosen within chromosome 16 (CACNA1H) 17 (CACNA1G) and 22 (CACNA1I) (10Mb from each other along the chromosome) and analyzed for CNVs in patients affected by hormone naïve, castration resistant and neuroendocrine prostate cancer, according to cBioPortal datasets Broad/Cornell, Nat. Genet. 2012 and Trento/Cornell/Broad 2016.

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<tr>
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**Supplementary Table II**: complete results of the MTT-assay performed on PC-3 cells treated with increased concentrations of mibefradil or ethosuximide. Table shows the relative survival for each concentration along with the standard deviation and the p-value using the viability at the lower concentration as a reference.

<table>
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<th>Concentration (μM)</th>
<th>Relative Survival</th>
<th>St. Dev</th>
<th>p-value</th>
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