Overexpression of Na\textsubscript{V}1.6 channels is associated with the invasion capacity of human cervical cancer

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Cervical cancer (CaC) is the third most common female tumor worldwide and the second in developing countries, with an estimated annual incidence of 452,000 cases.\textsuperscript{1} In view of the finding that carcinogenic human papillomavirus (HPV) infections cause virtually all CaC cases, recently a new approach for CaC prevention has emerged with the HPV vaccination of younger women (aged ≤18 years).\textsuperscript{2} Despite the highly significant advance that the vaccine itself represents, a mathematical model has predicted an increase in CaC incidence if vaccine is not followed by a continuous Pap smear screening program.\textsuperscript{3} Therefore, the finding of effective diagnosis and therapeutic strategies for CaC still remains as a priority.

Lately, there has been an increasing amount of evidences that correlate the function of ion channels with several aspects of cancer progression.\textsuperscript{4,5} In particular, voltage-gated sodium channels (VGSC) have been clearly associated to invasion and metastasis behaviors in several types of cancer, including breast, colon, lung, ovary and prostate.\textsuperscript{6–10} Sodium channels are protein complexes formed by a large \(\alpha\)-subunit and smaller auxiliary \(\beta\)-subunits. The \(\alpha\)-subunit alone is sufficient to form a functional channel, but its biophysical properties, trafficking and anchoring to the cell membrane are modulated by \(\beta\)-subunits.\textsuperscript{11} The VGSC family is composed by nine different \(\alpha\)-subunits (Na\textsubscript{V}1.1–Na\textsubscript{V}1.9) and four \(\beta\)-subunits (Na\textsubscript{V}\(\beta\)1–Na\textsubscript{V}\(\beta\)4); all of them have been cloned, functionally expressed and characterized.\textsuperscript{12} A useful tool to discriminate among VGSC is their sensitivity to tetrodotoxin (TTX); Na\textsubscript{V}1.1–Na\textsubscript{V}1.4, Na\textsubscript{V}1.6 and Na\textsubscript{V}1.7 are blocked by nanomolar concentrations of TTX, whereas Na\textsubscript{V}1.5, Na\textsubscript{V}1.8

Key words: cervical cancer, Na\textsubscript{V}1.6, invasiveness, molecular marker, Na\textsubscript{V}1.7

Abbreviations: CaC: cervical cancer; Cn2: B-class toxin from the scorpion Centruroides noxius; HPRT1: hypoxanthine phosphoribosyltransferase 1; HPV: human papillomavirus; Na\textsubscript{V}1.6: voltage-gated sodium channel \(\alpha\)-subunit encoded by the SCN8A gene; Na\textsubscript{V}\(\beta\)1: voltage-gated sodium channel \(\beta\)-subunit encoded by the SCN1B gene; NCC: noncancerous cervix; TTX: tetrodotoxin; VGSC: voltage-gated sodium channels

Additional Supporting Information may be found in the online version of this article

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Functional activity of voltage-gated sodium channels (VGSC) has been associated to the invasion and metastasis behaviors of prostate, breast and some other types of cancer. We previously reported the functional expression of VGSC in primary cultures and biopsies derived from cervical cancer (CaC). Here, we investigate the relative expression levels of VGSC subunits and its possible role in CaC. Quantitative real-time PCR revealed that mRNA levels of Na\textsubscript{V}1.6 \(\alpha\)-subunit in CaC samples were ~40-fold higher than in noncancerous cervical (NCC) biopsies. A Na\textsubscript{V}1.7 \(\alpha\)-subunit variant also showed increased mRNA levels in CaC (~20-fold). All four \(\alpha\)-subunits were also detected in CaC samples, being Na\textsubscript{V}\(\beta\)1 the most abundant. Proteins of Na\textsubscript{V}1.6 and Na\textsubscript{V}1.7 \(\alpha\)-subunits were immunolocalized in both NCC and CaC biopsies and in CaC primary cultures as well; however, although in NCC sections proteins were mainly relegated to the plasma membrane, in CaC biopsies and primary cultures the respective signal was stronger and widely distributed in both cytoplasm and plasma membrane. Functional activity of Na\textsubscript{V}1.6 channels in the plasma membrane of CaC cells was confirmed by whole-cell patch-clamp experiments using Cn2, a Na\textsubscript{V}1.6-specific toxin, which blocked ~20% of the total sodium current. Blocking of sodium channels VGSC with tetrodotoxin and Cn2 did not affect proliferation neither migration, but reduced by ~20% the invasiveness of CaC primary culture cells in vitro assays. We conclude that Na\textsubscript{V}1.6 is upregulated in CaC and could serve as a novel molecular marker for the metastatic behavior of this carcinoma.
Prostate cancer cells have been reported to specifically overexpress Na\textsubscript{v}1.7 subunits, whereas in breast, colon and ovary the Na\textsubscript{v}1.5 subunit is mostly overexpressed; in addition, substantial evidence supports the contribution of such VGSC in the \textit{in vitro} invasiveness of cell lines derived from these carcinomas.\textsuperscript{6,7,10,13} We recently reported the presence of functional VGSC in primary cultures of CaC and the overexpression of several VGSC \(\alpha\)-subunits in CaC biopsies and primary cultures, in comparison with noncancerous cervix (NCC) biopsies.\textsuperscript{14} Now, the goal of our study was to establish whether a particular VGSC \(\alpha\)-subunit is specifically overexpressed in CaC biopsies and primary cultures, and if they have any contribution to the metastasis behavior of this carcinoma. Importantly, we have used primary cultures, not cell lines, derived from CaC, and for the first time we have demonstrated a role for VGSC, particularly Na\textsubscript{v}1.6 channels, in invasiveness of a non-cell line carcinoma.

### Material and Methods

#### Biological samples

Biopsies were obtained from patients with invasive CaC diagnosed at the Oncology Unit of the Hospital General in Mexico City. For our study, only HPV-16-positive biopsies from patients who had not received any anticancer therapy were used. NCC biopsies were obtained by hysterectomy from patients of the same hospital, all of them with pap smear test negative to cancer. Our study was approved by the Institutional Ethics Committee on Human Research, and informed written consent was obtained from all patients.

#### Primary cultures

CaC cultures were obtained by thawing cells from previously established primary cultures; these cells were formerly characterized to express cytokeratins and the E7 gene from HPV-16,\textsuperscript{15} supporting the cancer epithelial nature of the cells. Herein, CaC cells from these cultures will be called 085, 354 and JP cells. Cells were grown in DMEM with 10% fetal bovine serum (FBS) and kept in a \(CO_2\) incubator at 37 and \(JP\) cells. Cells were grown in DMEM with 10% fetal bovine serum (FBS) and kept in a \(CO_2\) incubator at 37 and \(JP\) cells. Cells were grown in DMEM with 10% fetal bovine serum (FBS) and kept in a \(CO_2\) incubator at 37°C. Every 6–7 days the maintenance cultures were regenerated by replating cells at tenfold lower density. For recording purposes, an aliquot of cells (300–500 \(\mu\)l) was seeded on coverslips and placed in 35-mm culture dishes. Electrophysiological experiments were carried out on those cells, usually 2–24 hr after seeding. We used only cultures up to five passages to prevent additional changes due to \textit{in vitro} culture conditions.

#### RNA isolation

Total RNA from CaC primary cultures was isolated with the NucleoSpin RNA II kit (Clontech). Total RNA was also isolated directly from CaC and NCC biopsies with Trizol reagent (Invitrogen). CaC biopsies used for standard and \textit{in vivo} RT-PCR included three IB1, two IIB and one IIIB, according to FIGO system classification. Commercial total RNA from human brain and dorsal root ganglia (Clontech) served as positive controls. Total RNA samples were stored at \(-70^\circ\)C until analyzed by PCR experiments.

### Real-time RT-PCR

Quantitative real-time PCR (qRT-PCR) was carried out in a 7300 Real-Time PCR system with the use of Custom TaqMan Gene Expression Assays (Applied Biosystems) for VGSC \(\alpha\)-and \(\beta\)-subunits. Primer and probe sequences, the GenBank Accession Numbers of target cDNAs as well as the amplification reaction information are shown in Table 1. Because we have shown that the Na\textsubscript{v}1.4 \(\alpha\)-subunit is equally expressed in CaC and NCC samples,\textsuperscript{14} this was not included in our study. Treated RNA (3 \(\mu\)g) was reverse transcribed using the High Capacity kit (Applied Biosystems) in 20 \(\mu\)l of final volume, according to the manufacturer’s specifications. Amplification efficiency of each primer pair was evaluated by the standard curve method using serial dilutions of pooled cDNA. Amplification of specific PCR products was detected simultaneously by triplicate in one-assay run, and the Ct (threshold cycle) value for each experimental group was determined. When standard variation was larger than 0.4 among triplicates the assay was performed again. Data normalization was performed by using the Ct from human HPRT1 (\(Ct_{\text{HPRT1}} = Ct_{\text{NCC}} - \Delta Ct\)). \(\Delta Ct\) values for CaC samples were then normalized to NCC samples (\(\Delta Ct \text{ CaC} - \Delta Ct \text{ NCC} = \Delta \Delta Ct\)). Finally, \(\Delta \Delta Ct\) values were converted to \(2^{-\Delta \Delta Ct}\) to calculate the relative expression levels of the target gene.\textsuperscript{16} Therefore, data were expressed as an \(n\)-fold change in gene expression normalized to a reference gene (HPRT1) and relative to NCC samples. Primers and probe for HPRT1 were obtained from TaqMan Endogenous Control from Applied Biosystems, and the amplification efficiency was of 100%. Amplified qPCR fragments were subcloned into the pcR8 vector contained in the GW/TOPO TA Cloning kit (Invitrogen) to further confirm their molecular identity by automated sequencing.

### Immunocytochemistry and immunohistochemistry

The immunochemical analysis was performed on the three CaC primary cultures (085, 354 and JP), nine cancerous (four IB1, two IB2, one IIA, one IIB and one IIIB stage) and six NCC biopsies using anti-Na\textsubscript{v}1.6 and anti-Na\textsubscript{v}1.7 polyclonal rabbit antibodies (Alomone Labs). Primary culture cells were fixed with absolute ethanol, whereas sample biopsies were fixed in a buffered 4% formaldehyde solution, processed as standardized for histology and embedded in paraffin, as reported elsewhere.\textsuperscript{17} Briefly, serial sections of 4-\(\mu\)m thick were mounted on Kling-On charged glass slides (Biocare Medical), deparaffinized using xylene and decreasing series of ethanol. Antigen recuperation was hot induced by immersing slides in 10 mM EDTA solution (pH 8.0) and boiled for 30 min. After blocking endogenous peroxidase activity and nonspecific proteins, slides were incubated with primary antibodies (anti-Na\textsubscript{v}1.6 1:150 or anti-Na\textsubscript{v}1.7 1:25) during 60 min, followed by 10 min with the secondary antibody (MACH 4 mouse probe) and finally 10 min
with the MACH 4 HRP-polymer. Peroxidase activity was revealed by incubation with 3,3-diaminobenzidine/H₂O₂ (all from Biocare Medical) for 4 min. Sections were contrasted by incubation with hematoxylin (Dako) for 5 min. Immunohistochemical controls were prepared as described above but omitting primary antibodies. Finally, samples were mounted with Entellan permanent resin (Merck) and visualized in a Leica DM750 microscope. Images were taken with the software ImagePro plus v 7.0.1 or the LAS EZ v.1.8.0 from Leica Microsystems.

**Migration and invasion in vitro assays**

Migration assays of CaC cells were performed using transwell migration chambers with 8-µm pore size polycarbonate membrane cell culture inserts (BD Biosciences). The upper compartment (insert) was seeded with 5 × 10⁴ cells in culture medium plus 5% FBS. The insert was submerged in the lower compartment filled with culture medium enriched with 15% FBS as a chemoattractant. After 72 hr at 37°C, the number of cells in the lower compartment was estimated according to kit’s guidelines. Briefly, the noninvading cells that remained in the upper compartment were removed carefully with a swab; cells in the bottom were stained and observed in the microscope. Then, cells were lysed and measured by densitometry at 570 nm with an ELX800 Universal Microplate Reader (Bio-Tek Instruments). To investigate the in vitro invasion capacity of CaC cells, we used the QCM™ 24-well collagen-based cell invasion assay with 8-µm pore size (Millipore). To evaluate the effect of VGSC blockers on the

| Table 1. Real-time PCR primer sequences and amplification summary |
|---------------------------------------------------------------|
| **Subunit** | **GenBank accession No.** | **Primer and probe sequences (5' → 3')** | **AmpliCon size (bp)** | **Amplification efficiency (%)** |
| NaV1.1 | AB093548 | F: GAGCAATGAGTATGCGACCATTCTTA | 81 | 95 |
| | | R: ACCAACAGGGTGCCATTT | | |
| | | P: CTGGCTGATTCTCTCA | | |
| NaV1.2 | M94055 | F: TGCGCATTTTCTTGCGCTATTCT | 83 | 85 |
| | | R: GGCTGATTCTGTCCCTCATTAGG | | |
| | | P: CCACAGCACCATA | | |
| NaV1.3 | NM_006922 | F: AGAGCGAATAGCACAACAGCATTT | 76 | 92 |
| | | R: CAGAAAGCATTCTTCGATCACCACA | | |
| | | P: CCGACCCTGAAAACGT | | |
| NaV1.6 | AY682081 | F: GGATCTCATGAACTGCGGATATT | 109 | 92 |
| | | R: GCTGGAGAACCTGATGTT | | |
| | | P: CCCAGGTCCCAACT | | |
| NaV1.7a | X82835 | F: GCAATGTTTCAGCCTCCTGAACTT | 74 | 91 |
| | | R: CTCAGCGCTGGATACACAAATA | | |
| | | P: TTTCAGGCTCCTCAATACT | | |
| NaV1.7b | JF437648 | F: GGCGAATGCTCTCACGCTTTGA | 83 | 92 |
| | | R: CGATGCTCTTTTAGTCCGGAATGAC | | |
| | | P: ATGCCTGGGAAACT | | |
| NaVβ1 | NM_001037 | F: GGAGGATGACGGCCTCTGCA | 70 | 97 |
| | | R: CAGATCTGCAAGTCTTTCGTT | | |
| | | P: CCCCCGGTCGACT | | |
| NaVβ2 | NM_004588 | F: TGCAGCCGAGGAGATGAG | 92 | 100 |
| | | R: GAGGACCTGAGATGATGTT | | |
| | | P: CCCCTGACCGCCCG | | |
| NaVβ3 | NM_018400 | F: CGCCAGCCCCAGAAGAT | 90 | 96 |
| | | R: CACAGGGAAGCAAGACACTGA | | |
| | | P: TTTCCCCGTGCTTCT | | |
| NaVβ4 | NM_001142349 | F: AAGAATGTGGCAACAACAGTGCACA | 93 | 100 |
| | | R: TGAGTCTTCTACTGACGGATGAG | | |
| | | P: ACCCGCCCGCACGAG | | |

F: forward primer; R: reverse primer; P: TaqMan probe. Most probe sequences (except for NaVβ2 and NaVβ3) were complementary to antisense DNA strand. JF437648: new sequence reported in our work (see Supporting Information Fig. S2).
invasion capacity of the cells, the toxins (TTX or Cn2) were added to the upper compartments seeded with $5 \times 10^4$ cells. After 72 hr of incubation, the amount of cells in the lower compartment was estimated as mentioned above for migration experiments. Four independent experiments were made in duplicate, and results were normalized to the control condition (i.e., without toxins). To have an estimation of the number on cells that actually migrated or invaded in our assays, we built up standard calibration curves with known number of cells in the same 24-well plates used for the assays. There was a linear relationship between absorbance and number of cells within the range of 1,500–25,000 for 085 and 354 primary culture cells (Supporting Information Fig. S1).

Electrophysiology

General methods for whole-cell patch-clamp recordings of single CaC cells were as specified by Diaz et al.\textsuperscript{14} and are only summarized here. Recordings were obtained at 21–23°C using the following solutions (in mM): external: 158 NaCl, 2 CaCl$_2$, 2 MgCl$_2$ and 10 HEPES-NaOH (pH 7.4); internal: 106 CsCl, 30 NaCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 EGTA and 10 HEPES-CsOH (pH 7.3). Because of the low availability of the Cn2, recordings to test its blocking effect on sodium current were performed by local application of the toxin to the recording chamber.

Results

Expression levels of TTX-sensitive VGSC $\alpha$-subunits in CaC and NCC samples

Automated sequencing of the standard RT-PCR NaV1.7 transcript (748 bp) from our previous work\textsuperscript{14} reveals the presence of two different sequences for this subunit. A BLAST analysis of both sequences showed 100% identity for the first one (named here NaV1.7a) and 87% identity for the second one (NaV1.7b, from now on; sequence data were deposited with GenBank under Accession No. JF437648) with the human NaV1.7 reported sequence (GenBank X82835) (for details of NaV1.7b sequence, see Supporting Information Fig. S2). NaV1.7b also shares 90% identity with a splice variant reported in dorsal ganglia (GenBank AY682084) by Raymond et al.\textsuperscript{18} and 96% with the rat NaV1.7 sequence (GenBank NM_133289). To determine the relative mRNA levels of each transcript in CaC and NCC biopsies, we designed a specific TaqMan probe for each of them (Table 1), as for NaV1.1, NaV1.2, NaV1.3 and NaV1.6 subunits.

Transcriptional levels of the six VGSC $\alpha$-subunits and the reference gene (HPRT1) were determined by qRT-PCR in the three primary cultures of CaC and in six CaC and five NCC biopsies. For comparison of gene transcription levels, Ct values were plotted directly, assuming the same threshold for all genes evaluated. The Ct value is inversely proportional to the amount of RNA template present in the PCR reaction (i.e., the smaller the Ct value the larger the amount of mRNA). Most of analyzed genes in CaC and NCC samples showed Ct values that fall in the moderate expression level category (26–30 cycles), including the reference gene HPRT1 (Fig. 1a). It should be noted that even HPRT1 Ct values varied about three cycles (from 28 to 25) between NCC and CaC samples. β-Actin, another reference gene commonly used, showed a larger variation (12 cycles; data not shown), meaning a 1,000-fold increase in CaC samples mRNA levels; therefore, we did not use β-actin as a reference gene. On the other hand, three genes, NaV1.1, NaV1.6 and NaV1.7b, in NCC samples display Ct values between 34 and 38, indicative of very low expressed mRNAs; interestingly, the latter two genes increased importantly their expression in CaC samples. This was not true for NaV1.1, however.

The mRNA expression of NaV1.6 and NaV1.7b is upregulated in CaC

Real-time PCR experiments showed that all VGSC $\alpha$-subunits investigated are expressed in both NCC and CaC samples.
The quantitative determinations of mRNA expression showed no substantial changes for NaV1.1, NaV1.2, NaV1.3 and NaV1.7a between CaC and NCC samples (Fig. 1b). On the contrary, NaV1.6 and NaV1.7b mRNAs were specifically overexpressed in CaC samples. Even though there were some differences in Ct values between CaC primary cultures and CaC biopsies, both set of samples followed the tendency to increase the expression levels of NaV1.6 and NaV1.7b in CaC samples. On an average, qRT-PCR determinations revealed that relative mRNA expression of NaV1.6 and NaV1.7b in CaC samples was 40- and 22-fold higher than in NCC samples, respectively (Fig. 1b). Similar results were obtained when NaV1.2 or NaV1.3 subunits were used as reference gene (data not shown).

**Differential expression of NaVβ subunits in primary cultures and biopsies from CaC**

Because NaVβ subunits have been implicated in process length and cell adhesion in neurons and cancer cells, we investigated the mRNA levels of these subunits in cancerous and NCC cells. In contrast with the mRNA levels observed for α-subunits, accessory NaVβ subunits showed significantly different tendencies between primary cultures and CaC biopsies, although β1 was always the most abundant NaVβ subunit (Fig. 2a). On the other hand, NaVβ2 and NaVβ4 displayed very low levels in CaC primary cultures (Ct ~34), but mRNA levels were lower in NCC samples (Ct ~30) than in CaC biopsies (Ct ~28) (Fig. 2a). In other words, compared with NCC biopsies, the mRNA levels of NaVβ2 and NaVβ4 were higher in CaC biopsies but they decreased in CaC primary cultures. After normalizing the values with HPRT1 gene, the $2^{-\Delta\Delta Ct}$ values indicated that only NaVβ3 is not overexpressed in CaC samples (Fig. 2b). The comparison between CaC primary cultures and NCC biopsies confirmed the strong downregulation of NaVβ2 and NaVβ4 in CaC (Fig. 2c). On the contrary, between CaC and NCC biopsies there were no substantial changes in expression, if any, a small increase (less than 100%) in NaVβ3 and ~50% decrease in NaVβ1 (Fig. 2d). A likely explanation for this difference between primary cultures and biopsies from CaC could be...
related to the assigned adhesion role of NaVβ subunits, a cell property that might be quite different in biopsies and primary cultures.

Localization of NaV1.6 and NaV1.7 channel protein in cervical cancer

Immunohistochemical staining using specific antibodies against NaV1.6 and NaV1.7 demonstrated channel protein expression in NCC sections (brown staining: a' and b'); proteins immunoreactivity in basal layer’s (BL) cells was weak. On the contrary, the signal for NaV1.6 channels was widely distributed in the plasma membrane, cytoplasm and nucleus of most cells in CaC biopsies sections from IB (c') and IIIB (e') FIGO stages. The immunolocalization of NaV1.7 showed a similar pattern than NaV1.6, although the nucleus staining was not consistent in all sections (d' and f'). No immunoreactivity was observed when primary antibodies were omitted from the experiments (g' and h'). Images are representative of nine CaC and six NCC biopsies. (b) Immunocytochemistry of NaV1.6 and NaV1.7 proteins expression in CaC cell cultures. Primary cultures (085, 354 and JP) derived from CaC biopsies displayed immunoreactivity for NaV1.6 (a', c' and e') and NaV1.7 (b', d' and f') channels. Again negative controls in the absence of primary antibodies lead to only the hematoxylin counterstain of the cell nucleus (g' and h'). Bar scale for all images, 50 µm.

Contribution of NaV1.6 channels to the whole-cell sodium current of CaC cells

To investigate if the upregulation of the mRNA that encodes NaV1.6 channels and the strong immunoreactivity detected in CaC biopsies have in fact any functional contribution to the total sodium current of these cells, we performed whole-cell patch-clamp experiments in CaC primary culture cells that we have previously reported for displaying a robust sodium current, the 085 cells.14 As a pharmacological tool to
discriminate the participation of NaV1.6 channels in the total sodium current, we use the Cn2 toxin, which has been recently reported as a specific blocker of NaV1.6 channels.22,23 Figure 4 shows the block of whole-cell sodium currents recorded at 0 mV from a 085 cell by 350 and 1,000 nM of Cn2 toxin. On an average, 350 nM Cn2 decreased the sodium current amplitude by 30.3% (5.0% (n = 8; Fig. 4b)). By increasing the Cn2 concentration to 1 μM, an additional 18% of VGSC activity was blocked (48.3% ± 6.9%; n = 6; Fig. 4b). We did not use higher Cn2 toxin concentrations to prevent further nonspecific block of sodium channels. When tested, the remaining sodium current was totally blocked with 1 μM TTX (Fig. 5a), confirming our previous reports about the TTX sensitivity nature of the VGSC expressed in these cells.14 Similar results were observed in 354 cells (data not shown).

Participation of VGSC activity in metastatic cell behaviors

To explore the possibility that the VGSC activity, in general, and NaV1.6 channels, in particular, could be involved in metastatic behaviors of CaC cells such as migration and invasion, we performed in vitro assays in the presence and absence of 6 μM TTX24 or 1 μM Cn2.23 Proliferation of 085, 354 and JP CaC primary culture cells was not affected after exposure to 6 μM TTX during 72 hr, also that toxin concentration was not toxic for the cells (data not shown). Migration and invasion assays were performed only with two primary cultures, 085 and 354, where the cells of the first generate almost the double of sodium current density than the second (20.4 ± 4.3, 9.8 ± 2.0 and 11.9 ± 1.6 pA/pF for 085, 354 and JP cells; data from Ref. 14). As shown in Figure 5a, migration was not significantly modified by the presence of TTX or Cn2 in the culture medium in 085 and 354 cells. By contrast, relative invasiveness of these cells was sensitive to both toxins (Fig. 5b). After 72 hr of incubation with 6 μM TTX, relative invasion of 085 cells showed a significant decrease when compared to control condition (no toxin; normalized invasion decreased to 81.5% ± 5.6%). More interestingly, Cn2 toxin (1 μM) had a similar effect on this cell behavior. The same tendency was observed in 354 cells, normalized invasion decreased to 79.5% ± 7.6% and 78.3% ± 7.6% with TTX and Cn2, respectively. These results suggest that blocking of sodium channels, and particularly NaV1.6 channels, reduces the relative invasiveness of CaC primary culture cells.

Discussion

Our study shows that mRNA levels of two VGSC α-subunits, NaV1.6 and NaV1.7b, were strongly upregulated in biopsies and primary cultures from CaC. Functional expression of both channel proteins was also confirmed, in particular NaV1.6 subunit contributed to approximately one-third of the sodium current recorded in CaC cells. Specific block of NaV1.6 channels reduced the in vitro invasiveness of these cells. The main implications of such results are that NaV1.6
could be considered as a novel marker for CaC and also as a potential target for the development of new therapeutic strategies.

Our qRT-PCR results showed that NaV1.6 mRNA levels were upregulated by 40-fold in CaC samples compared to NCC samples (Fig. 1b). Interestingly, mRNA levels of NaV1.6 α-subunit were the lowest in NCC samples, together with NaV1.1 and NaV1.7b (Fig. 1a); however, only the latter and NaV1.6 were clearly upregulated in CaC samples (Fig. 1b). On the contrary, mRNA levels for NaV1.2, NaV1.3 and NaV1.7b were moderately expressed in NCC samples and did not show significant changes when compared to CaC samples (Figs. 1a and 1b). Notably, the sequences of NaV1.6 and NaV1.7b α-subunits identified in our study correspond to a specific splice isoform of NaV1.6 (Q9UQD0-2),25 and a NaV1.7 channel isoform does not correspond to any splice variant reported for human. Because of the observed upregulation of this NaV1.7 isoform in CaC, it will be important to clone the full sequence of this channel to confirm its functionality and to investigate its biophysical properties.

All the sequenced NaV1.6 transcripts indicated that it contains the exon 5A, but not the 5N, which have been shown to be developmentally regulated in NaV1.1 and NaV1.3 channels26,27 as well as in NaV1.5, whose fetal isoform is abundantly expressed by metastatic breast cancer cells.13 However, the 5A isoform (adult isoform) of NaV1.6 has been shown to be expressed in both fetal and adult brain.18,28 It should be noted that NaV1.1–1.3 and NaV1.7 are located in chromosome 2q23-24, whereas NaV1.6 is located alone in chromosome 12q13; therefore, we speculate that chromosomal instability would influence the transcription profile in the CaC cells. This hypothesis is supported by a previous study, where it was shown that the chromosomal abnormalities in prostate cancer cell line DU145 impact the gene expression profile.29

It has been shown that VGSCs are upregulated in several types of cancer cells, including breast, colon, lung, ovary and cancer, and that their functional activity has a relevant contribution in functions linked to invasion, secretion, adhesion and motility.7–10,30 Interestingly, NaV1.5 is the upregulated subunit in most cases, with the exception of prostate cancer (NaV1.7); however, in CaC the functional activity of NaV1.5 seems to be almost none, due to the fact that more than 97% of the sodium current was blocked with 1–2 μM TTX in CaC primary cultures,14 which indicates the participation of almost exclusively TTX-sensitive sodium channels (NaV1.5 does not belong to this category). In addition, qRT-PCR results demonstrated that mRNA of neonatal NaV1.5 isoform is significantly less expressed than the adult NaV1.5 isoform, and both showed decreased levels of mRNA in CaC samples compared to NCC biopsies (Supporting Information Figs. S3a and S3b). In addition, immunohistochemical experiments confirmed the absence of NaV1.5 protein in CaC biopsies (Supporting Information Fig. S3c). Together, these results strongly suggest that the NaV1.5 role in this type of cancer, if any, has not the same relevance as in others.

By contrast, we demonstrated in our study that NaV1.6 channel protein is substantially expressed in the plasma membrane and intracellular compartments of CaC primary culture cells as well as in fresh CaC biopsies (Fig. 3); furthermore, the functional activity of NaV1.6 channel proteins contributes to approximately one-third of the total sodium current recorded from the CaC cells (Fig. 4). It is very likely that the remaining two-thirds of the sodium current could be carried by other TTX-sensitive VGSCs whose mRNA was detected here (i.e., NaV1.2, NaV1.3 and NaV1.7). Thus, our qRT-PCR results showing increased mRNA levels for NaV1.6 are consistent with the channel protein expression and function observed with immunostaining and patch-clamp experiments. An interesting immunochemical result indicates that NaV1.6 (and also NaV1.7) channel protein is widely distributed in membrane and cytoplasmic compartments in CaC samples; on the contrary, in NCC samples the proteins signal is limited to the plasma membrane (Fig. 3). The intracellular expression of NaV1.6 has been observed in human macrophages, where it is colocalized with vesicles that are distributed throughout the cytoplasm and some of which are associated with the actin cytoskeleton,31 participating in the control of podosoma and invadopodia formation, thus suggesting a role for cellular invasion of macrophages.32 Interestingly, we found β-actin mRNA levels augmented around 1,000-fold in CaC samples (data not shown). Changes in cell distribution of NaV1.6 channels are better exemplified in multiple sclerosis (MS) human patients where the channel is expressed along demyelinated and degenerating axons in MS, instead of being confined to its normal localization in Ranvier’s nodes.33 Interestingly, it was also reported that colocalization of NaV1.6 channels and the sodium–calcium exchanger (NCX) occurs at sites of axonal injury in the spinal cord in experimental allergic encephalomyelitis.34 Such colocalization might be relevant for a likely mechanism involving NaV1.6 channels in the metastatic behavior of CaC cells (see below).

Besides their possible role at the intracellular level, NaV1.6 channels, expressed in the plasma membrane of CaC cells, participate in some metastatic behaviors of this carcinoma. Our observations regarding the block of one-third of the total sodium current in CaC primary cultures cells (Fig. 4), and the decrease in relative invasiveness (Fig. 5) by the action of the NaV1.6-specific Cn2 toxin, implies the direct participation of the ionic current through NaV1.6 α-subunit. The contribution of other VGSCs (i.e., NaV1.1, NaV1.2, NaV1.3 and NaV1.7) to the metastatic behavior seems to be less significant, due to the fact that TTX effect on invasiveness was not different than that observed with the Cn2 toxin (Fig. 5). A positive correlation between sodium current density and relative invasiveness has been observed in breast and prostate cancer, where highly metastatic cell lines express larger sodium currents, compared to poorly metastatic cell lines.13,35,36 However, our results in CaC cells were not current density dependent, as both types of CaC cells (085 and 354) were equally affected in their in vitro invasiveness behavior by...
TTX and Cn2 (Fig. 5). Nevertheless, our results show for the first time the relationship between sodium channel activity and metastatic behavior in primary cultures derived from a primary carcinoma, suggesting that NaV1.6 channels may play an important functional role in CaC progression.

There is no unanimously accepted mechanism to explain the role of VGSC in the metastatic behavior of some cancer cells, but at least two main roles should be considered: the Na⁺ influx through VGSC α-subunits and the role of VGSC β-subunits as adhesion molecules. The first one includes observations regarding tonic Na⁺ influx through VGSCs, inhibition of various basal metastatic cell behaviors by TTX and diminishing or enhancing of invasiveness by increased Na⁺ concentrations or by the agonist effect of veratridine on VGSCs. However, the major criticism of the Na⁺-influx hypothesis is that the transient current through VGSCs may have negligible effect on the global [Na⁺], in these cells and any downstream effect is not likely. Nonetheless, generation of local Na⁺ gradients cannot be ruled out. In addition, the implicated participation of NCX driven in reverse mode (Na⁺ export/Ca²⁺ import) observed when [Na⁺] is elevated, and in the ionic mechanism of anoxic injury in mammalian axons, with the well-known downstream effects of Ca²⁺, is in agreement with the specific colocalization of NaV1.6 channels and the NCX observed in experimental axonal injury.

Alternatively, the role of VGSCs in metastasis could involve a nonconducting function via direct interactions of VGSC β-subunits with other plasma membrane and/or intracellular proteins. For example, VGSC β-subunits (β1 and β2) can associate with tenasin-C and tenasin-R (thereby influencing cell migration) and participate in homophilic cell adhesion, resulting in cellular aggregation and ankyrin recruitment. Importantly, VGSC β-subunits (β1, β2 and β4) are expressed in breast cancer cell lines, where β1 mRNA and protein levels are significantly higher in the weakly metastatic MCF-7 cell line compared to the highly metastatic MDA-MB-231 cells, consistent with the higher adhesiveness of MCF-7 cells. Furthermore, stable overexpression of β1 in MDA-MB-231 cells increased the VGSC activity and cell adhesion, but reduced cellular motility, suggesting that the cell adhesive effects of β1 may be independent of changes in cellular excitability. Finally, a reciprocity of function between β1 and NaV1.6 was recently reported in cerebellar granule neurons, where β1-mediated neurite outgrowth requires NaV1.6-mediated Na⁺ influx, whereas NaV1.6 localization and high-frequency firing require β1. In our study, we observed that direct blocking of VGSC α-subunits induced a decrease (around 20%) in the invasiveness of CaC primary cultures, if VGSC β-subunits, in particular the high levels of NaV1β1 detected in CaC samples (Fig. 2a), play an additional role in the metastatic behavior of CaC, it remains to be further investigated. Additionally, the contrasting mRNA levels expressed for NaVβ2 and NaVβ4 in CaC primary cultures and CaC biopsies might be implying new roles for these subunits in cell properties related to tumor integrity maintenance.

In conclusion, functional overexpression of NaV1.6 channels and to a lesser extent NaV1.7 in CaC might represent the identification of novel molecular markers and potential targets for this carcinoma. Examination of the human ion channel set from a cancer perspective provides important insights into issues relevant to ion channel drug discovery. Thus, our work will provide an opportunity to assess the therapeutic potential of functionally intriguing channels such as VGSCs.

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