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Expression of Voltage-Gated Sodium Channel $Na_v1.8$ in Human Prostate Cancer is Associated with High Histological Grade

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Abstract

Voltage-gated sodium (Na_v) channels are required for impulse conductance in excitable tissues. Na_vs have been linked to human cancers, including prostate. The expression and distribution of Na_v isoforms ($Na_v1.1$-$Na_v1.9$) in human prostate cancer are not well established. Here, we evaluated the expression of these isoforms and investigated the expression of $Na_v1.8$ in human prostate cancer tissues. $Na_v1.8$ was highly expressed in all examined cells. Expression of $Na_v1.1$, $Na_v1.2$, and $Na_v1.9$ were high in DU-145, PC-3 and PC-3M cells compared to LNCaP (hormone-dependent), C4-2, C4-2B, and CWR22Rv-1 cells. $Na_v1.5$ and $Na_v1.6$ were expressed in all cells examined. $Na_v1.7$ expression was absent in PC-3M and CWR22Rv-1, but expressed in the other cells examined. Immunohistochemistry revealed intensive $Na_v1.8$ staining correlated with more advanced pathologic stage of disease. Increased intensity of nuclear $Na_v1.8$ correlated with increased Gleason grade. Our results revealed that $Na_v1.8$ is universally expressed in human prostate cancer cells. $Na_v1.8$ expression statistically correlated with pathologic stage (P=0.04) and Gleason score (P=0.01) of human prostate tissue specimens. The aberrant nuclear localization of $Na_v1.8$ with advanced prostate cancer tissues warrant further investigation into use of $Na_v1.8$ as a potential biomarker to differentiate between early and advanced disease.

Keywords

Voltage-gated sodium channel; Prostate cancer; Prostate biomarker; Gleason score

Introduction

Prostate cancer is the most common cancer diagnosed in men and the second leading cause of cancer death among men in the United States [1]. A major challenge encountered in the

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treatment of prostate cancer is that a significant portion of the tumors will recur. The genomic/proteomic alterations underlying prostate cancer progression and therapy resistance are poorly understood. Hence, the discovery of novel biomarkers remains central to earlier and improved accuracy of detection and diagnosis of human prostate cancer disease.

Recently, Na_v channels have been linked to human prostate cancer cell proliferation [2], invasion [3] and metastasis [4]. Na_vs are members of a single gene family with at least nine isotypes (Na_v1.1-1.9) which are commonly found in excitable cells including heart (Na_v1.5), skeletal muscles (Na_v1.4) and neurons (CNS, Na_v1.1–Na_v1.2, Na_v1.6; PNS, Na_v1.7–Na_v1.9) [5]. Na_vs mediate sodium influx to generation action potentials which are critical for normal cellular functions [5].

Na_v is a pore forming trans-membrane protein composed of a large α subunit (>200 kDa) and at least two associated β subunits (∼30-40 kDa) [5]. The large pore forming alpha subunit consists of four internal homologous transmembrane domains (I-IV) linked by three interdomain cytoplasmic loops which contain a number of phosphorylation sites that participate in intracellular signaling [6]. Protein kinases (PKA, PKC, receptor tyrosine kinase (RTK), Ca^{2+}/calmodulin-dependent protein kinase (CaMK), p38MAPK, and p42/44 MAPK) have been shown to modulate Na_v current by promoting protein-protein interactions, stimulating channel trafficking and insertion to the membrane, and rapid translation of Na_v channels [6,7]. Expression of the α subunit alone is sufficient for cellular function [5].

Na_vs are classified into two groups based on their sensitivity to a sodium channel specific toxin, tetrodotoxin, (TTX). Na_v1.5, 1.8, and 1.9 are resistant to TTX (IC_{50}, μM) while the other six isotypes are highly sensitive to TTX (IC_{50}, nM) [5]. Various Na_v mRNA transcripts have been detected in a variety of human cancers [4,8-11]. Abdul and Hoosein have provided evidence that Na_vs were highly expressed in human prostate cancer tissues using a pan-Na_v antibody, which recognizes the conserved DIII-DIV linker of all Na_v isotypes [12]. However, the expression of Na_v specific protein isotypes and its subcellular localization in human prostate cancer remain elusive. In this present study, we have examined the protein expression of Na_v specific α subunits (Na_v1.1-1.9) in hormone-dependent and hormone-independent human prostate cancer cell lines and explored the clinical relevance of a specific voltage gated sodium channel Na_v1.8 in human prostate cancer disease progression.

Materials and Methods

Cell culture

LNCaP, C4-2, and C4-2B (gif from Dr. Robert Sikes, University of Delaware, Department of Biological Sciences, Newark, DE) and CWR22Rv-1, DU-145, PC-3, and PC-3M cell lines (ATCC, Manassas, VA) were cultured in RPMI-1640 with L-glutamine (Mediatech, Inc., Herdon, VA) containing 5% fetal bovine serum (FBS), 2.5 mM L-glutamine at 37°C with 5% CO_2. LNCaP cells were cultured in the presence of 0.5 nM dihydrotestosterone (5α-androstan-17β-ol-3-one) (Sigma-Aldrich, St. Louis, MO).

Western blot analysis

Western protocols were adapted from Collins et al. [13]. Briefly, prostate cancer cells were lysed in the radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl, pH 7.6, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 0.01% SDS, 0.5% sodium deoxycholate). Protease inhibitor cocktails (Sigma-Aldrich) were added to RIPA buffer prior to use. The protein samples were separated by 4% Tris-Glycine SDS-PAGE or 4-12% Bis-Tris SDS-PAGE

(Invitrogen, Carlsbad, CA) and transferred into immuno-Blot PVDF membranes (Biorad Laboratories, Hercules, CA). The membranes were blocked with blocking buffer (50 mM Tris-Cl, 150 mM NaCl, 10 g/L BSA) and probed with the following antibodies: anti-Na_v1.1, anti-Na_v1.2, anti-Na_v1.5, anti-Na_v1.6, anti-Na_v1.7, anti-Na_v1.8, anti-Na_v1.9 (Upstate/Millipore, Billerica, MA); anti-EGFR, anti-PARP (Cell Signaling Technology, Danvers, MA); and anti-alpha tubulin (Sigma-Aldrich). Chemiluminescent detection was performed using ECL reagents according to the vendor's instructions (Pierce, Rockford, IL).

Subcellular fractionation

C4-2 and PC-3 cells were fractionated using the Fraction PREP Cell Fractionation system (Biovision, Mountain View, CA) according to manufacturer's instructions. Fractionation was accomplished on cells (10^7) treated with a series of extraction buffers, followed by sequential centrifugation to separate the cytoplasmic, the plasma membrane, and nuclear enriched fractions. Western blot was performed on the fractions using antibodies to Na_v1.1, 1.7 and 1.8. The purity of the enriched fraction was determined using antibodies to PARP (nucleus), EGFR (plasma membrane) and α-tubulin (cytosol).

Immunohistochemical detection of Na_v1.8 in human prostate specimens

Paraffin-embedded cells or arrayed prostate cancer specimens (US Biomax, Inc, Rockville, VA) containing normal (17) and malignant (160) prostate tissues were deparaffinized, rehydrated, boiled with citrate buffer (pH 6), treated with 0.3% H_2O_2, and preincubated in blocking solution (10% normal goat serum). The primary antibody, anti-Na_v1.8, was incubated with the specimens at a concentration of 1:50 for one hour at room temperature. Antigen-antibody complexes were detected using a horseradish-peroxidase complexed anti-rabbit secondary antibody (Dako Envision-Plus) (Dako North America, Inc., Carpinteria, CA). 3,3'-diaminobenzidine (Dako) was used as chromogen and hematoxylin as counterstain. A subtype-specific IgG was used as a negative control. Sprague-Dawley rat sciatic nerve dorsal root ganglion (DRG) was used as a positive control tissue. Samples were imaged with Olympus BX61 camera/DP-70 inverted microscope (Center Valley, PA) using provided DP Controller software. Individual prostate samples were scored (TH and SS) using semi-quantitative steps of increasing staining intensity, where 0 was undetectable, low immunostaining, 1+; intermediate immunostaining, 2+; and high immunostaining, 3+ as previously described [14]. Fisher's exact test was used to compare presence of staining (negative stain vs. positive stain) and localization in each site among the clinical features. The exact Jonckheere-Terpstra test was used to determine if staining intensity was associated with the clinical features. The association between two sites of localization controlling for clinical factors were compared using the exact Cochran-Mantel-Haenszel test. All statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC).

Results

Voltage-gated sodium channel isoforms are differentially expressed in human prostate cancer cell lines

We examined the expression of specific Na_v isotypes in seven human prostate cancer cells, including hormone-dependent (LNCaP) and hormone-independent (C4-2, C4-2B, CWR22Rv-1, DU145, PC-3, and PC-3M) cells. Antibodies against unique epitopes of the α-subunit of sodium channel isoforms were used to detect the neuronal (Na_v1.1–Na_v1.2, Na_v1.6), cardiac (Na_v1.5), and peripheral neurons (Na_v1.7–Na_v1.9) isoforms. The specificity of the antibodies was verified by peptide competition studies (data not shown). The isotype-specific antibodies detected band of appropriate molecular weight by Western blot analysis (Figure 1).

As shown in Figure 1, Na_v1.8 demonstrated ubiquitous expression in both hormone-dependent and independent human prostate cancer cells. In contrast, the expression of Na_v isoforms, 1.1, 1.2 and 1.9, were higher in DU-145, PC-3, and PC-3M cells as compared to LNCaP and its two lineage cell lines, C4-2 and C4-2B, and CWR22Rv-1. Na_v1.5 and Na_v1.6 were expressed in all prostate cancer cells examined. The expression of Na_v1.7 was absent in PC-3M and CWR22Rv-1, but expressed in the other prostate cancer cells examined. The levels of Na_v1.3 and Na_v 1.4 were undetectable (data not shown). This could be due to the quality of the antibodies and/or the lack of expression of these isoforms.

Na_v1.8 expression in human prostate cancer tissues

Because Na_v1.8 was highly expressed in all the human prostate cancer cell lines examined, we asked whether the expression of Na_v1.8 increased in prostate cancer tissues with advancing Gleason score. For optimization of Na_v1.8 tissue staining, we initially performed an immunocytochemical analysis of Na_v1.8 in both hormone-dependent and independent cells. The specificity of Na_v1.8 antibody was verified by peptide inhibition (negative control) and rat DRG staining (positive control) (Figure 2). Human prostate tissue specimens were then immunostained and sections were scored with a semi-quantitative scoring method (0-3+) based on the intensity of Na_v1.8 staining. Normal prostate epithelium was either absent or weakly stained (intensity score of 0 to 1+) for Na_v1.8 (A representative sample in Figure 3A). The malignant prostate tissues with GS 4 or less were either absent or weak Na_v1.8 stained (A representative sample in Figure 3B, GS 4). A moderate (A representative sample in Figures 3C and 3D; GS 6, GS 7) to strong (A representative sample in Figures 3E and 3F; GS 8, GS 10) Na_v1.8 staining was observed in more advanced prostate tissue specimens. Figure 4 shows the distribution of Na_v1.8 staining intensity (0-3) and Gleason score. The percentage of prostate cancer specimens with high Na_v1.8 immunoreactivity (2+ and 3+) increased as GS increased. In contrast, the percentage of prostate cancer specimens with little or low Na_v1.8 immunoreactivity decreased as GS increased.

Table 1 provides a history of the patient specimens and statistical correlations. The observed difference in staining intensity between normal and malignant tissues was statistically significant ($P<0.0001$). When Na_v1.8 expression was compared with PSA secretion, pathologic stage, Gleason score, and lymph node involvement in malignant prostate cancer tissues, statistically significant correlations were observed in all clinical features (p-values for pathological stage, $p=0.04$; Gleason Score, $p= 0.01$; pathologic lymph node stage, $p<0.001$). These findings were not observed with PSA secretion and Na_v1.8 staining ($p=0.15$).

Localization of Na_v1.8 in human prostate cancer tissues

While previous studies show Na_vs localize to the plasma membrane in excitable cells (such as neurons), the cellular localization of Na_vs in prostate cancer is not well established. Cellular localization of Na_v1.8 in human prostate tissue is shown in Figure 5. In normal human prostate tissue, the nuclei of the prostatic acinar basal cells showed either absent or weak Na_v1.8 staining (Figure 5A). In a case of GS 6 tumor, Na_v1.8 staining was observed in both the plasma membrane and cytosol of the prostate epithelial cells (Representative sample in Figure 5B). We noticed a disappearance of Na_v1.8 staining in the plasma membrane of many prostate cancer tissue specimens with higher Gleason grade. In epithelial cells of a case of GS7 tumor, moderate Na_v1.8 staining was seen in the nucleus (Representative sample in Figure 5C). Strong staining was observed in the cytosol (Figure 5D, GS10), or strong mixed cytosol and nucleus staining (Figure 5E) in advanced prostate cancer tissues. The observation that Na_v1.8 localized to the nucleus was unexpected. To verify that Na_v1.8 localized to the nucleus, subcellular enriched fractions of C4-2 and PC-3 cells were immunoblotted with antibody to Na_v1.8. Western blot analysis revealed detection of $Na_v1.8$ in the nuclear enriched fractions of PC-3 cells (Figure 6A) and C4-2 cells (Figure 6B). We also probed the subcellular enriched fractions of C4-2 for the presence of $Na_v1.1$ and $Na_v1.7$. As illustrated in Figure 6B, $Na_v1.1$ was detected in the cytosolic enriched fractions while $Na_v1.7$ was present in the membrane enriched fractions. These results further validate our observation of nuclear $Na_v1.8$ in human prostate cancer. The purity of the subcellular enriched fractions was probed using EGFR as a plasma membrane marker, cytosolic marker alpha-tubulin, and nuclear marker PARP (Figures 6A and 6B).

Discussion

Although a number of reports have linked voltage-gated sodium channels to human prostate cancer, little information is known regarding the expression of Na_v specific proteins and its cellular distribution [4,9,12,15-17]. In this study, we demonstrate for the first time that $Na_v1.8$, normally found in excitable cells like dorsal root ganglia, is highly expressed in human prostate cancer cells. Additionally, $Na_v1.8$ expression is associated with advanced histopathological grade of human prostate cancer disease. Our current observations are consistent with Abdul and Hoosein [12] studies in which the authors used a pan-Na_vs antibody and demonstrated that Na_v s immunoreactivity is greater in human prostate cancer tissues as compared to normal human prostate.

The molecular mechanisms for the upregulation of $Na_v1.8$ are unknown. It has been suggested that that increased $Na_v1.8$ expression by p38MAPK is regulated at the post-transcriptional level [18]. Our laboratory is currently exploring a possible connection between $Na_v1.8$ and p38 MAPK activation in human prostate cancer. Our Western blot data demonstrated that both LNCaP and PC3 cells have comparable of $Na_v1.7$ protein. This result did not agree with previous reports identifying $Na_v1.7$ as the major isoform found in PC-3 prostate cancer cells [9]. These discrepancies suggest that increased $Na_v1.7$ mRNA in prostate cancer may not be a reflective of functional expression of $Na_v1.7$.

Localization of $Na_v1.8$ in the nucleus is another intriguing finding from our current study. Recently the voltage-independent potassium ion channel Trek-1 was found to be localized to the nucleus of human prostate cancer cells [19]. Furthermore, the nuclear localization of a plasma membrane protein ErbB3 has been linked to poor prognosis in prostate cancer survival [20]. These studies strongly support that the translocation of membrane proteins to the nucleus of human prostate cancer cells.

The functional and mechanistic roles of $Na_v1.8$ in prostate cancer cells are not known. However, the altered cytoplasmic to nuclear ratio of $Na_v1.8$ in prostate cancer tissues may be useful in differentiating early and advanced stages of prostate cancer. Currently, the role of this isoform and other Na_v isoforms in human prostate cancer cells and tissues are under investigation by our laboratory. Finally, our study identified $Na_v1.8$, a voltage regulated ion channel protein, in human prostate cancer cells and tissues and strongly supports the further study of $Na_v1.8$ as a potential biomarker and a therapeutic target in this disease.

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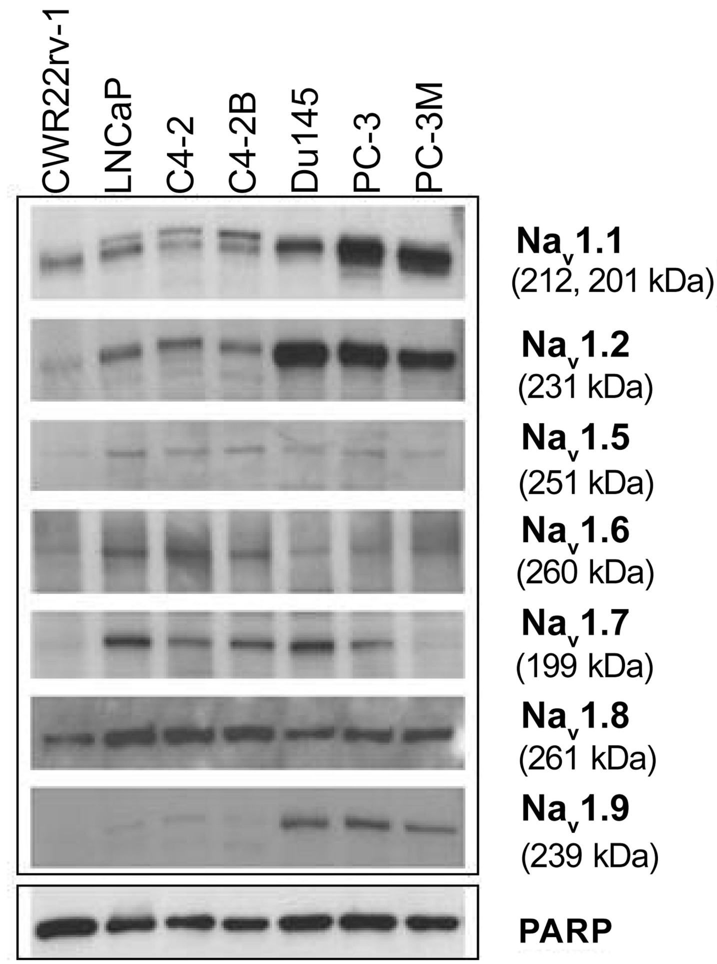

Figure 1. Expression of Na_V isoforms in human prostate cancer cells

Western blot analyses of extracts from human prostate cancer cell lines. Cell lysates were analyzed by immunoblotting with anti-Na_V1.1, Na_V1.2, Na_V1.5, Na_V1.6, Na_V1.7, Na_V1.8, and Na_V1.9. Blots were probed with anti-PARP-1 antibody to normalize for protein loading.

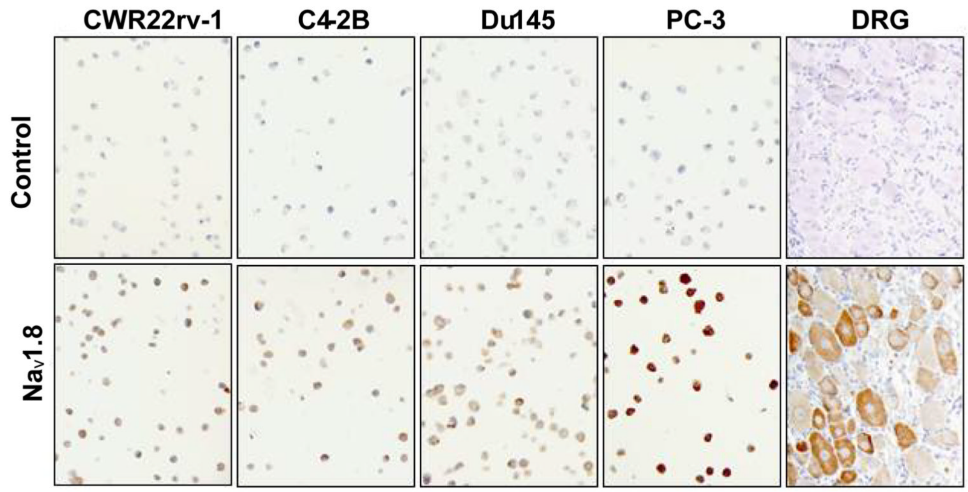

Figure 2. $Na_v1.8$ immunohistochemical analysis of human prostate cancer cell lines

DRG from rat tissue was used as a positive control for $Na_v1.8$. Negative control (top panels) represents neutralization of $Na_v1.8$ antibody with $Na_v1.8$ peptide.

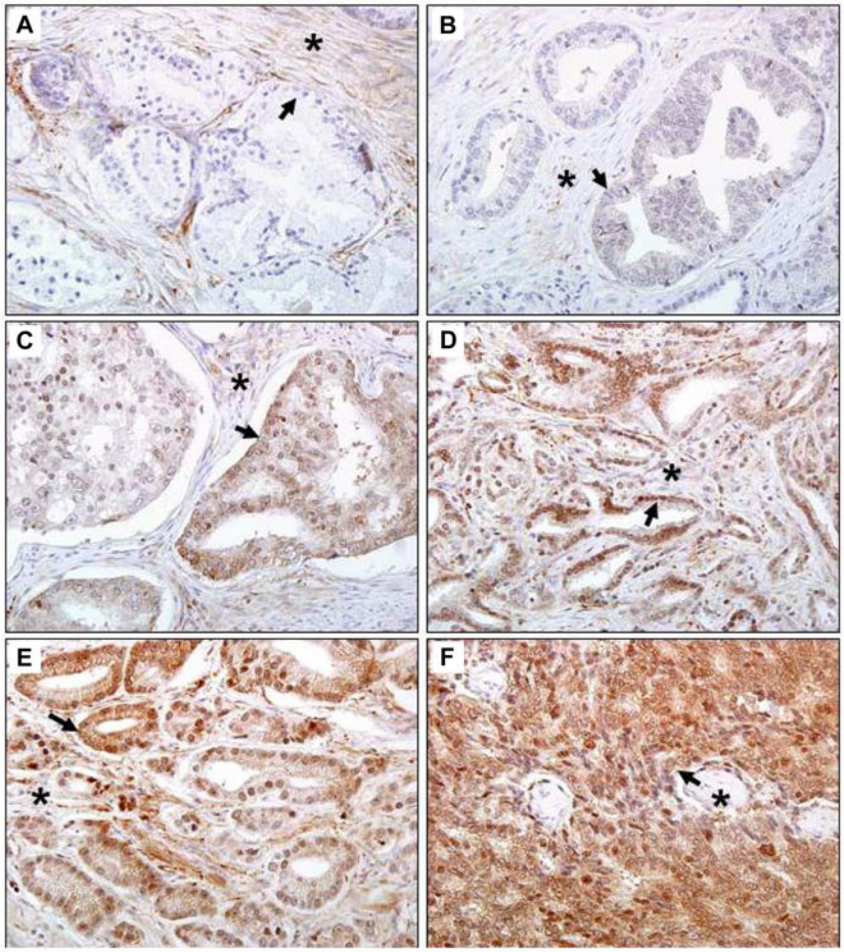

Figure 3. Expression of Na_v 1.8 in human prostate cancer tissues

Human prostate tissue specimens consist of normal (n=17) and malignant (n=160) were analyzed by immunohistochemical staining using a polyclonal anti- Na_v 1.8 antibody. Representative samples are shown. A. Normal human prostate specimen with very low Na_v1.8 staining. B. Prostatic adenocarcinoma specimen GS 4 with very low Na_v1.8 staining. Prostatic adenocarcinoma specimens with moderate Na_v1.8 staining, GS 6 (C) and GS 7(D). Prostatic adenocarcinoma specimens with strong Na_v1.8 staining, GS 8 (E) and GS10 (F). Images were taken at 40× (Olympus BX61 Camera/DP70 inverted microscope) using DP Controller Software. Arrows indicate prostate epithelium. Asterisks indicate stroma. GS, Gleason score.

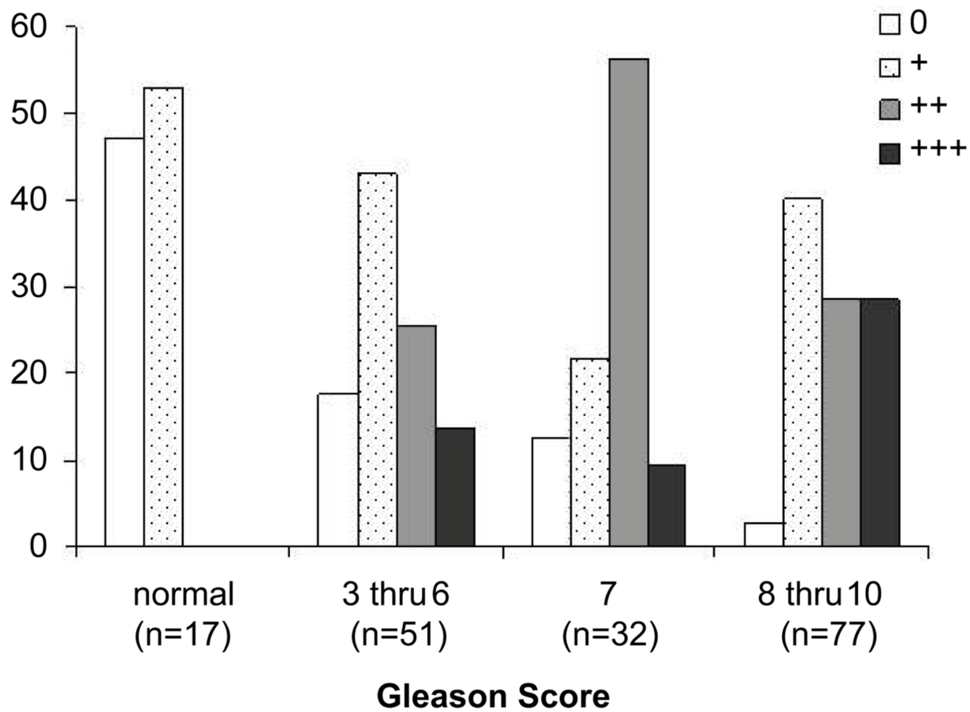

Figure 4. Relationship between Na_v 1.8 staining intensity and Gleason score

Human prostate tissue specimens consist of normal (n=17) and malignant (n=160) were analyzed by immunohistochemical staining using a polyclonal anti- Na_v 1.8 antibody. Prostate tissue specimens were divided into four groups (normal, GS 3-6, GS 7, and GS 8-10) and individually scored for Na_v 1.8 staining: 0 = no detectable, 1+ = low Na_v 1.8 staining, 2+ = moderate Na_v 1.8 staining, 3+ = strong Na_v 1.8 staining.

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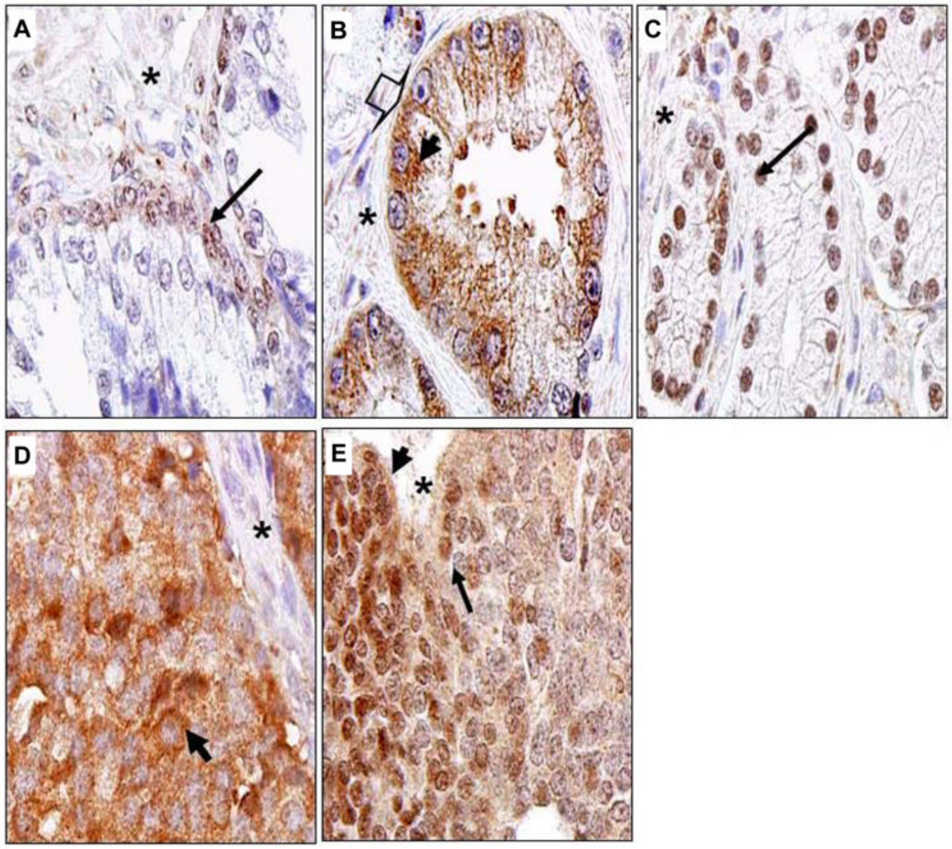

Figure 5. Localization of Na_V 1.8 in human prostate cancer tissues

Human prostate tissue specimens consist of normal (n=17) and malignant (n=160) were analyzed by immunohistochemical staining using a polyclonal anti- Na_V 1.8 antibody. Representative samples are shown. A. Normal human prostate specimen, nuclei of prostatic acinar basal cells with very low Na_V1.8 staining. B. Prostatic adenocarcinoma specimen GS 6 with moderate Na_V1.8 staining in the plasma membrane (arrow head) and cytosol (bold thick arrow). C. Nuclear staining of a case of GS 7. D. Cytosolic staining of a case of GS10. E. A case of GS10 with mixed cytosolic and nuclear staining. Images were taken at 100× (Olympus BX61 Camera/DP70 inverted microscope) using DP Controller Software. Asterick represents stroma. Long thin arrows denote nuclei.

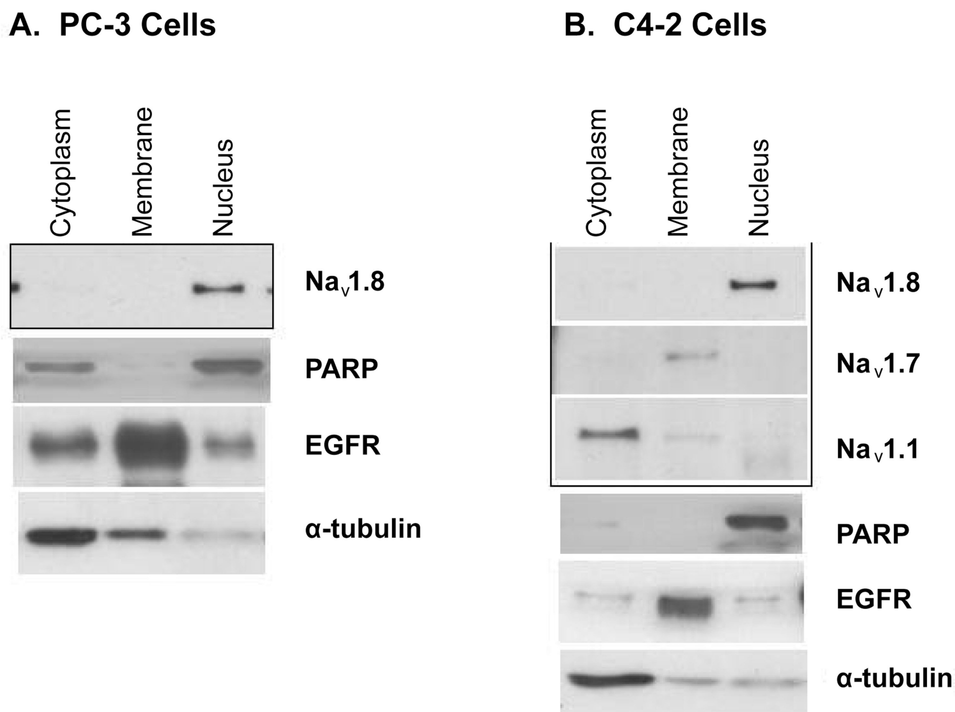

Figure 6. Na_V 1.8 is localized to the nucleus

Human prostate cancer cell lines were fractionated into plasma membrane, cytosolic and nuclear enriched fractions. Proteins were separated on a 4-12% SDS-PAGE gradient gel. A. Subcellular fractions of PC-3 cells immunoblotted with Na_V 1.8. B. Subcellular fractions of C4-2 cells immunoblotting with Na_V 1.1 and 1.7 and 1.8. Purity of the enriched fractions were shown using anti-α-tubulin for the cytoplasm, EGFR for the plasma membrane, and PARP for the nucleus.

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Table 1

Summary of Patient History and Correlations to $Na_v1.8$ Epithelial Staining.

		***P*-values**	
Characteristic			
	n	**Negative vs. Positive**[1]	**Intensity**[2]
All healthy tissues	17	<0.0001	<0.0001
All tumors	160		
PSA (ng/ml)		---	0.15
< 4	12		
4–10	10		
10–20	17		
> 20	24		
Pathologic Stage		0.05	0.04
pT_2	27		
pT_3	47		
pT_4	27		
Gleason Score		0.01	0.01
3–6	51		
7	32		
8–10	77		
Pathologic lymph node stage		0.0006	<0.0001
pN_0	61		
pN+	31		

[1] P-values computed using the fisher's exact test

[2] P-values computed using the exact Jonckheere-Terpstra test