Evidence for Prostate Cancer-Associated Diagnostic Marker-1: Immunohistochemistry and *in Situ* Hybridization Studies

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ABSTRACT

Purpose: **The purpose of this study was to characterize a novel gene/protein associated with prostate cancer, termed prostate cancer-associated diagnostic marker-1 [PCADM-1 (Hu Y, Wang M, Garcia FU, Aoyaki K, Stearns ME. Identification of PCADM-1 as a novel diagnostic marker for prostate cancer, submitted for publication)].**

Experimental Design and Results: **Immunological studies revealed that rabbit polyclonal antibodies generated against recombinant PCADM-1 specifically recognize the protein in crude protein extracts from a variety of prostate cancer cell lines (*i.e.*, PC-3 ML, LNCaP, DU145, and CPTX-1532) and prostate cancer tissue. Combined immunolabeling and *in situ* hybridization studies demonstrated that PCADM-1 mRNA was expressed by the luminal epithelial cells of prostate cancer glands and was not expressed by high-grade prostatic intraepithelial neoplasia or HPV-MLC7 cells. Immunolabeling studies of tissue arrays from biopsies of archival material ($n = 200$ samples) confirmed that PCADM-1 was expressed by the luminal epithelial cells of prostate cancer.**

Conclusions: **Taken together, the data suggest that PCADM-1 is a specific marker for human prostate cancer.**

INTRODUCTION

Currently, there are >400 tentative marker genes identified for prostate cancer (1). Prostate-specific antigen is the only serum marker developed that is used to screen for prostate cancer, but the sensitivity and specificity range from about 43–65%, respectively, depending on the nature of the study (1). The widespread belief is that other prostate cancer-specific genes might be identified that provide a more accurate diagnostic marker for the cancer. Several tentative examples of candidate markers are reviewed here. Prostate specific membrane antigen, a membrane-bound prostate-specific antigen, has been identified as specific for prostate cancer and may eventually improve the prostate-specific antigen assay (1). Su *et al.* (2) reported that the product of a gene known as prostate carcinoma tumor antigen-1 was expressed in malignant cells, but not by normal or benign prostatic hyperplastic tissue. ELISAs of patient serum indicated the antigen was diagnostic for prostate cancer. Similarly, another gene, *HPCA1*,was reported to have a high linkage with prostate cancer when compared with a reference population from the National Cancer Database (3). We and others believe that further attempts to identify prostate cancer-specific marker genes of prognostic or diagnostic utility are needed.

In a preceding paper,[3] we cloned and partially characterized a gene encoding prostate cancer-associated diagnostic marker-1 (PCADM-1). Sequencing indicated that PCADM-1 was ~99% homologous to human S2 ribosomal protein and chromosomal protein *LLRep3*. PCADM-1 exhibited six specific point mutations, and the deduced amino acid sequence indicated five specific amino acid base substitutions distinguishing mutant PCADM-1 from the human S2 ribosomal protein. In this paper, immunohistochemistry and *in situ* hybridization studies revealed that PCADM-1 was expressed by various prostate cancer cell lines and specifically expressed by human prostate cancer luminal epithelial cells with a 100% sensitivity.

MATERIALS AND METHODS

Cell Lines. Malignant CPTX-1532 and normal NPTX-1532 human prostate cell lines were derived from the same human prostate tissue (Ref. 4; generously provided by Drs. Robert Bright and Susan Topalian; National Cancer Institute, NIH, Bethesda, MD). These cells were immortalized with E6 and E7 transforming proteins of human papilloma virus serotype 16 and maintained in medium 154 (Cascade Biologics, Inc., Portland, OR) containing 1× human keratinocyte growth supplement 100X (Cascade Biologics, Inc.), 1× antibiotic-antimycotic 100X (Life Technologies, Inc., Grand Island, NY), and 5% fetal bovine serum (Biofluids, Rockville, MD). Human prostate cancer PC-3, LNCaP, and DU145 cell lines were obtained from American Type Culture Collection (Manassas, VA). The bone-metastasizing PC-3 ML subclones were selected from the parent PC-3 cell line by the Stearns laboratory (5). All these cell lines were cultured according to the directions of American Type Culture Collection.

HPV-MLC7 cells (kindly provided by Dr. Donna Peehl; Department of Urology, Stanford University) are a benign prostatic hyperplasia (BPH) culture immortalized by human papilloma virus serotype 18 (6). A high-grade prostatic intraepithelial

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neoplasia (HGPIN) cell line was established in our laboratory from human prostate tissue showing histological evidence of HGPIN. The HGPIN cells were immortalized by human papilloma virus serotype 18 transfection. Both cell lines were maintained in keratinocyte serum free medium (Life Technologies, Inc.) containing 5 ng/ml epidermal growth factor, 50 μg/ml bovine pituitary extract, 5% fetal bovine serum (Biofluids), 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate.

All cultures were routinely passaged by trypsin-EDTA (Life Technologies, Inc.) detachment and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Preparation of Prostate Tissue. Fresh prostates were obtained immediately after radical prostatectomy, and pieces were removed from sagittal dissections. Pieces of approximately 1.5 cm^2 were dissected out and prepared for frozen sections. The sections were stained with H&E to identify regions containing primarily stoma tissue, seminal vesicle (SV), normal prostate, BPH, HGPIN, or prostate cancer adenocarcinoma (PCA).

Tissue array blocks were prepared with biopsy cores from archival specimens. Each block contained cores from SV, stroma, HGPIN, BPH, or PCA]Gleason score (GS) 4–8[plus localized neuronal metastases (n = ~200 cores/block).

Immunohistochemistry. The protocol for immunolabeling frozen sections was a modification of the technique of Aoyagi *et al.* (7) and Tokunaga *et al.* (8). In brief, tissue was frozen with liquid nitrogen, embedded in Tissue-Tek (Sakura Finetek USA, Torrance, CA), cut with a cryostat at 6 μm, and placed on Superfrost/Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA). Frozen tissue sections (~10-μm thick) were incubated for 20 min with 0.9% hydrogen peroxide in 100% methanol to inactivate any endogenous peroxidase. Sections were rinsed twice in 0.1 M PBS and incubated with 1.5% normal goat serum for 60 min followed by rabbit polyclonal PCADM-1 antibody (1:20 dilution with 0.1 M PBS containing 1.5% normal goat serum) in a humidified chamber for 72 h at 4°C. Specimens were washed three times in 0.1 M PBS and incubated in biotinylated goat antirabbit IgG (diluted 1:200 in PBS containing 1.5% normal goat serum) for 30 min at room temperature. After washing one time with PBS, the sections were developed using the avidin-biotin complex staining system (Santa Cruz Biotechnology, San Diego, CA). The sections were counterstained with H&E. Control sections were immunolabeled with β-actin antibodies (Sigma, St. Louis, MO).

Tissue array sections were processed and immunolabeled according to methods described previously by our laboratory (9). The sections were routinely steamed for 30 min in a 10 mM citrate buffer (pH 8.0) to improve antigen retrieval and then labeled with primary antibodies (1:20 dilution for 1 h) followed by biotinylated goat antirabbit IgG antibodies according to the protocol described for frozen sections.

***In Situ* Hybridization Studies.** The *in situ* hybridization protocol was a modification of the nonradioactive digoxigenin (DIG) technique of Wood *et al.* (10) and Miyajima *et al.* (11). In brief, a synthetic oligonucleotide probe with a sequence complementary to a coded region of human PCADM-1 mRNA (5′-CATCGGCAAGGCCCACACTGTCCG-3′) was synthesized and high-performance liquid chromatography purified by BioSource International (Foster City, CA). Freshly cut frozen sections (~10-μm thick) were fixed overnight in 4% paraformaldehyde in 0.1 M PBS, immersed in 20% sucrose at 4°C in PBS, and placed on Superfrost/Plus Microscope Slides (Fisher Scientific). The sections were washed three times in 0.1 M PBS, incubated with 0.2 M hydrochloric acid for 20 min at room temperature to inactivate endogenous alkaline phosphate, treated with 10 μg/ml proteinase K (Roche Molecular Biochemicals, Indianapolis, IN) in 0.1 M PBS for 15 min at room temperature, washed in PBS, and then fixed in 4% paraformaldehyde in 0.1 M PBS. Fixed sections were treated with 2 mg/ml glycine (Sigma) in PBS to neutralize aldehydes. The sections were equilibrated in prehybridization buffer (50% deionized formamide in 2× SSC). Hybridization was performed with probes diluted in hybridization buffer [50% deionized formamide, 10 mmol of Tris-HCl (pH 7.6), 200 μg/ml yeast transfer RNA (Sigma), 1 mmol of EDTA, 600 mmol of sodium chloride, 1× Denhardt's solution (Sigma), 10% dextran sulfate (Sigma), 0.25% SDS, and 20 μg/ml salmon sperm DNA]. A DIG-labeled PCADM-1 probe [3′-tailed with DIG dUTP (Roche Molecular Biochemicals, Princeton, NJ; 10 ng probe/100 μl)] was incubated with the tissue sections in hybridization buffer containing 50% formamide. Sections were incubated overnight in a humidified chamber at 40°C and washed twice with 5× SSC at 40°C. Then sections were washed twice for 15 min at 40°C in 50% formamide in 2× SSC. Sections were placed in DIG 1 buffer [100 mmol Tris-HCl (pH 7.5)] and then placed in DIG 2 buffer (1.5% blocking reagent in DIG 1) for 5 and 60 min, respectively, at room temperature. Sections were then incubated in anti-DIG antibody AP (Roche Molecular Biochemicals) in DIG 1 buffer for 60 min at 37°C. Sections were washed in DIG 1 buffer for 15 min and rinsed in DIG 3 buffer [100 mM Tris-HCl (pH 9.5), 100 mM sodium chloride, and 50 mM magnesium chloride] for 5 min at room temperature. Immunodetection of the DIG oligonucleotide was carried out using an antibody kit (Roche Molecular Biochemicals). The reaction was stopped in DIG 4 buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA], and the sections were counterstained with methyl green.

Computer-Assisted Analysis System (CASA). A computer-assisted analysis system (CASA) was used in our laboratory to measure antigen expression and compare the relative intensity of PCADM-1 expression in specific glandular regions of whole mount sections and tissue array cores according to the methods of Fudge *et al.* (12). The intensity of *in situ* hybridization and immunohistochemical labeling was assigned a value of 0, +1, +2, or +3, with the overall intensity of labeling defined as follows: 0, zero; +1, 10–25%; +2, 26–49%; and +3, 50–100%.

Statistical Analysis. The CASA data for each GS were compared and evaluated relative to BPH by either Student's t test or Fisher's exact test with a level of significance set at $P < 0.05$.

RESULTS

Western Blots. Western blots of crude whole cell extracts showed that the PCADM-1 antigen was expressed by a variety of human prostate tumor lines (PC-3 ML, LNCaP, DU145, and CPTX-1532), but not by BPH or HGPIN cells (Fig. 1, *A* and *B*). In Fig. 1*A*, *Lanes 3* and *4*, the antibodies were preabsorbed with excess recombinant PCADM-1 and PC-3 ML

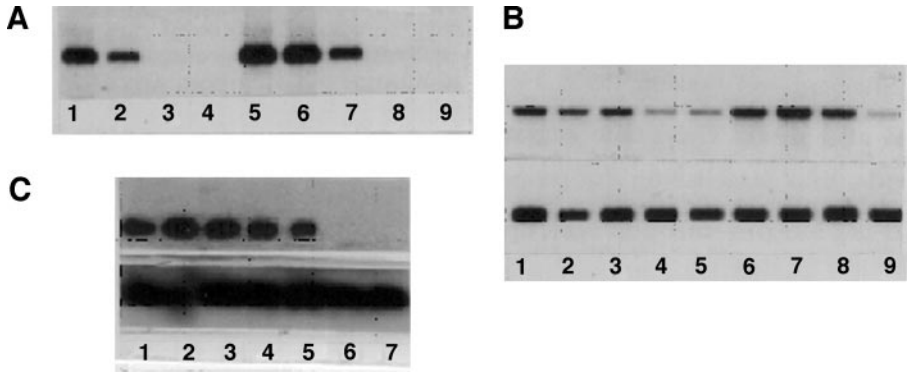

Fig. 1 *A–C,* Western blots with prostate cancer-associated diagnostic marker-1 (PCADM-1) antibodies (1:20 dilution at 0.01 μg/ml). Lanes were loaded with 10 μg protein/lane. *A,* blots with PCADM-1LG09 antibodies of crude protein extracts from the following: *Lane 1,* recombinant clones expressing PCADM-1 protein; *Lanes 2–4,* PC-3 ML; *Lane 5,* DU145; *Lane 6,* LNCaP; *Lane 7,* CPTX-1532; *Lane 8,* benign prostatic hyperplasia; and *Lane 9,* high-grade prostatic intraepithelial neoplasia cells. In *Lanes 3* and *4,* the antibodies were preabsorbed with excess recombinant PCADM-1 and PC-3 ML protein extracts, respectively. *B,* blots of extracts from the following: *Lanes 1–3,* Gleason score (GS) 6; *Lanes 4, 5,* and *9,* GS 4; and *Lanes 6–8,* GS 8 tumor tissues. *Bottom panel* in Fig. 1, *B* and *C,* shows β-actin antibody blots. *C,* blots of extracts from the following: *Lanes 1–5,* different regions of a GS 7 tumor; *Lane 6,* matching high-grade prostatic intraepithelial neoplasia; and *Lane 7,* benign prostatic hyperplasia tissue of the same prostate.

Table 1 Summary of immunolabeling results with cell lines and prostate tissue

Cells and tissues were either (+) positive or (−) negative for PCADM-1.

PCADM-1 (+)[a]
PC-3 ML
LNCaP
DU145
CPTX-1532
HPCA-10a
HPCA-10c
PCADM-1 (+)
PCA: GS 4–10
PCADM-1 (−)
BPH
HGPIN-1
HGPIN-2
NPTX-1532
WI38-fibroblasts
PCA-fibroblasts
PCADM-1 (−)
Stroma
HGPIN
Seminal vesicle

[a] PCADM-1, prostate cancer-associated diagnostic marker-1; BPH, benign prostatic hyperplasia; HGPIN, high-grade prostatic intraepithelial neoplasia; PCA, prostate cancer adenocarcinoma; GS, Gleason score.

protein, respectively, showing that the antibody specifically recognizes the M_r 33,000 PCADM-1 protein. Fig. 1*B* shows that PCADM-1 is expressed in prostate tumors of GS 4, GS 5, and GS 8. Fig. 1*C* further shows that extracts from different regions of a GS 7 tumor consistently expressed elevated levels of PCADM-1 (Fig. 1*C, Lanes 1–5*), whereas the matching HGPIN and BPH tissue of the same prostate failed to express any detectable PCADM-1 antigen. Table 1 summarizes the observations from Western blotting of crude extracts from a variety of prostate tumor cell lines and nonmalignant prostate cell lines, plus malignant and nonmalignant prostate tissues. Basically, PCADM-1 was expressed by malignant cells and tumors, but not by benign, normal, HGPIN, or fibroblast cells or by benign prostate tissue, seminal vesicle, and stroma.

Immunolabeling Studies. In Table 2, the data showed that the relative intensity of immunolabeling ranged from 0 to +3. The data showed that immunostaining was nonexistent in normal prostate tissue specimens ($n = 5$) and very low or nonexistent (*i.e.*, ranging from 0 to +1) in BPH ($n = 27$), SV ($n = 17$), and HGPIN ($n = 12$). Note that one HGPIN specimen showed positive labeling for PCADM-1 in a few glandular

Table 2 Summary of immunolabeling and *in situ* hybridization labeling studies with human prostate tissue specimens (n = 149 total)

Note that the immunolabeling and *in situ* labeling were carried out on adjacent sections from each specimen.

No. of prostates assayed	Pathology	Gleason score	Immunolabeling	ISH[a]
5	Normal	0	0	0
15	SV	0	0	0
2	SV	0	+1	0
20	BPH	0	0	0
7	BPH	0	+1	0
11	HGPIN		+1	+1
1	HGPIN		+3	+3
1	PCA	6	+1	+2
2	PCA	6	+2	+2
23	PCA	6	+3	+2
1	PCA	7	+1	+2
2	PCA	7	+2	+3
18	PCA	7	+3	+2
1	PCA	8	+1	+3
23	PCA	8	+2	+2
7	PCA	9	+3	+3
10	PCA	10	+3	+3

[a] ISH, *in situ* hybridization; SV, seminal vesicle; BPH, benign prostatic hyperplasia; HGPIN, high-grade prostatic intraepithelial neoplasia; PCA, prostate cancer adenocarcinoma.

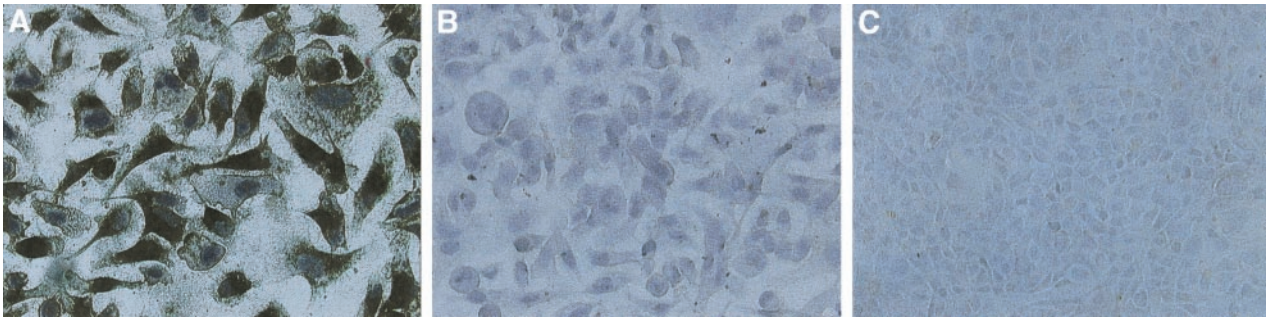

Fig. 2 Immunolabeling with prostate cancer-associated diagnostic marker-1 protein antibodies of the following cells: *A*, PC-3 ML; *B*, HPV-MLC7; *C*, NPTX-1532 normal prostate cells.

regions where the luminal epithelial cells exhibited abnormal nuclei and appeared malignant and invasive. Note that this would be considered intraductal carcinoma by some pathologists, which represents the spread of preexisting adenocarcinoma within prostatic ducts, rather than a precursor lesion. In contrast to the BPH samples, the intensity of immunolabeling was higher in PCA specimens ($n = 88$; *i.e.*, intensity ranged from +1 to +3; $P < 0.05$) with an increased intensity of labeling as a function of the GS. Among tumor samples of GS 6 ($n = 26$), the vast majority ($n = 23$; $P < 0.05$) were intensely positive (+3). Likewise, in GS 7 ($n = 21$; $P < 0.05$), GS 8 ($n = 24$; $P < 0.05$), and GS 9–10 ($n = 17$; $P < 0.05$) samples, all of the samples were intensely positive (+3), with the exception of a very few that were negative for PCADM-1. Note that all of the cells in all of the glands were immunolabeled in the prostates examined. Control studies showed that all of the specimens were uniformly immunolabeled with β-actin antibodies.

The *in situ* hybridization results closely correlated with the immunolabeling data and revealed that PCADM-1 mRNA was not expressed in normal prostate, seminal vesicle, or BPH tissue but was weakly expressed in some HGPIN glands. In contrast to the BPH samples, PCADM-1 mRNA was intensely expressed in the luminal epithelial cells of the PCA specimens, with an intensity ranging from +2 to +3 ($P < 0.05$). The intensity of labeling tended to increase with the GS, and GS 9–10 specimens strongly expressed PCADM-1 mRNA (*i.e.*, +3; $P < 0.05$).

Figs. 2–4 provide examples of the immunolabeling and *in situ* hybridization results observed for several human prostate cell lines and human prostate tissue. Fig. 2*A* shows that the cytoplasm of PC-3 ML tumor cells was intensely labeled with the PCADM-1 antibody. Fig. 2, *B* and *C*, shows that BPH and normal human prostate cells (NPTX-1532) were not labeled with the PCADM-1 antibody. Control studies with preimmune serum or antibody preabsorbed with excess recombinant antigen failed to label the PC-3 ML cells (data not shown). Fig. 3, *A–C*, shows that the PCADM-1 antibody failed to immunolabel the glands or stromal tissue in regions containing SV, BPH, and HGPIN, respectively. Note that occasional foci of positive labeling were sometimes observed in the HGPIN glands (Fig. 3*C*). Fig. 3*D* shows that all of the luminal epithelial cells in glands of a high-grade cancer specimen were intensely labeled with PCADM-1 antibodies, but the antibody did not label the stroma compartment. Note that the preimmune serum did not label the tissue. Comparative studies showed that the immunolabeling consistently coincided with the *in situ* hybridization results on adjacent sections (Fig. 4). Fig. 4 shows that SV and BPH tissues were not labeled with PCADM-1 antibodies or by *in situ* hybridization with ^{32}P-labeled antisense oligonucleotides (40-mer) specific for PCADM-1 mRNA. The HGPIN cells were immunolabeled in one region of the gland, but the *in situ* labeling was difficult to detect. In contrast, both the PCADM-1 antibodies and the *in situ* hybridization intensely labeled the luminal epithelial cells of the PCA GS 8 glands. Note that the stroma and blood vessel walls were uniformly negative in all of the labeling studies. Controls with the ^{32}P-labeled sense oligonucleotides (40-mer) failed to label the PCA glands.

The above-mentioned results were strongly supported by immunolabeling studies of tissue array sections containing 200 biopsy cores from a variety of prostate cancer tumors ($n = 108$), stroma ($n = 20$), BPH ($n = 50$), SV ($n = 10$), and neural metastases ($n = 12$). Evaluation of these specimens clearly showed that PCADM-1 was expressed solely by the glandular epithelia of cancer specimens at an intensity of +1 to +3 in GS 2, GS 3, and GS 4 glands and that PCADM-1 was not expressed by BPH, HGPIN, stroma, SV, or the other tissues or cells present in prostate tissue.

Finally, immunolabeling studies of a variety of other cancers and associated normal tissues (*i.e.*, colorectal, gastric, breast, ovarian, lung, bladder, liver, and kidney cancers and tissues) indicated that PCADM-1 was uniquely expressed by prostate cancer cells and not by other cancers.

DISCUSSION

In a preceding paper,[3] we identified a double-stranded CACGGATG sequence that binds a M_r 33,000 S2-like ribosomal protein present in nuclear extracts of prostate tumor cell lines and advanced human prostate cancer. The gene encoding the prostate cancer-specific binding protein was cloned. Sequencing and the deduced amino acid sequence revealed that the nucleic acid sequence was ~99% homologous to the human S2 ribosomal mRNA sequence and that the gene exhibited six specific bp substitutions in the open reading frame. The amino acid sequence was 99% homologous to ribosomal chromosome protein S2 and LLRep3 and exhibited five amino acid substitutions in the NH_2-terminal domain. Interestingly, four amino acid substitutions were observed in

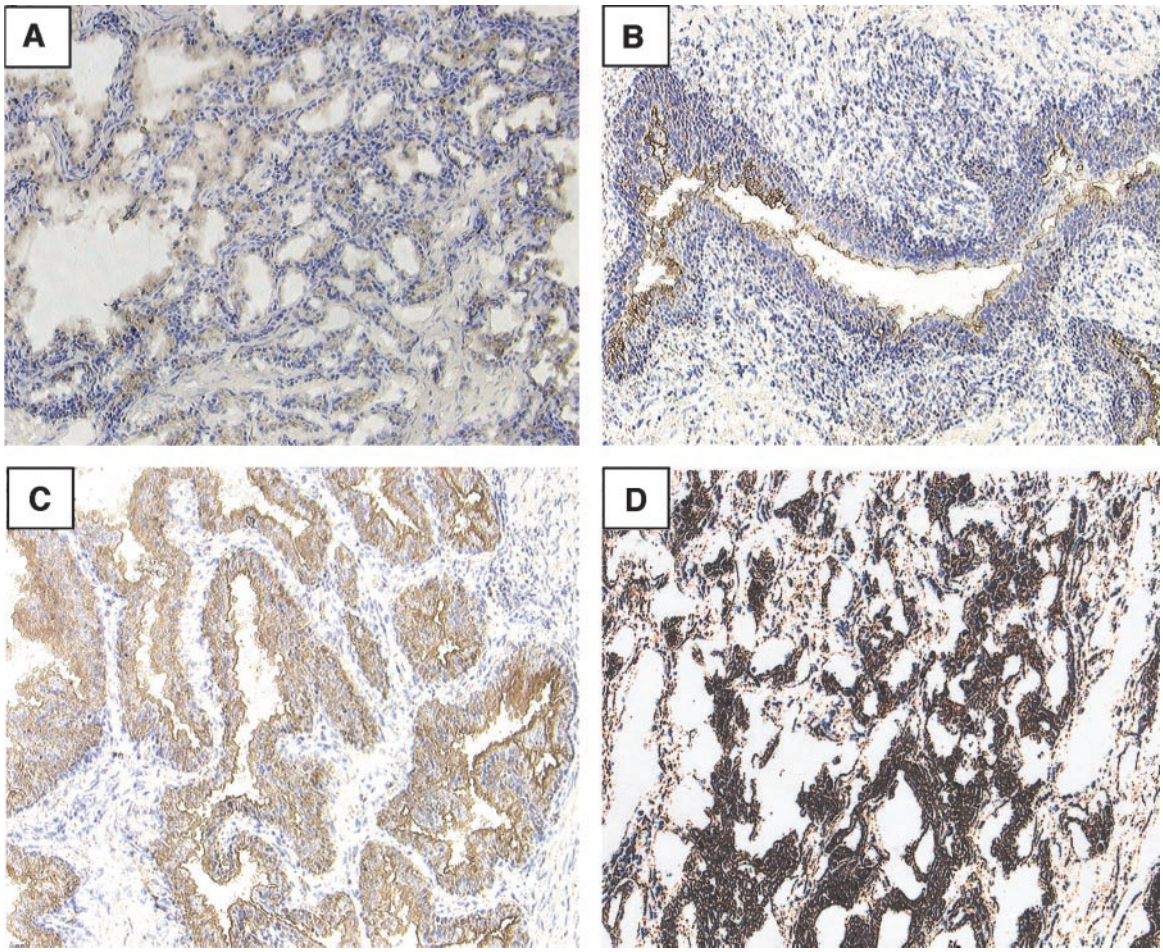

Fig. 3 Immunolabeling with prostate cancer-associated diagnostic marker-1 protein antibodies of the following tissues: *A*, seminal vesicle; *B*, benign prostatic hyperplasia, *C*, high-grade prostatic intraepithelial neoplasia; and *D*, Gleason score 9 prostate glands.

a putative DNA-binding heptamer region and the leucine zipper-like domain (*e.g.*, N***N****IGK****A****HTV****R****CKVTG****R****CGSVLVRLIPA-PRGTGIVSAPVPKKLLMMAGIDDCYTS*), suggesting that these amino acid substitutions (see amino acids underlined, bolded, and italicized) may account for a gain of function and the DNA binding properties of the mutated S2 protein.

In this paper, we have used immunohistochemistry and *in situ* hybridization assays to show that PCADM-1 was specifically expressed by malignant PC-3 ML prostate cancer cell lines and by the luminal epithelial cells of human prostate cancer tissue. In comparison, PCADM-1 was not expressed in primary epithelial cell lines derived from stromal tissue, normal prostate, HGPIN, or BPH glands. Combined immunolabeling and *in situ* hybridization studies of adjacent sections further demonstrated that PCADM-1 mRNA was expressed by the luminal epithelial cells of prostate cancer glands ($n = 28$) but was not expressed by HGPIN or BPH tissue or cells. Taken together, the data suggest that PCADM-1 is a specific marker for human prostate cancer.

New biological markers are being explored that might improve detection of cancer as well as the ability to detect patients at high risk of recurrence for different cancers. Among the antigens of potential benefit are the estrogen receptor (13), progesterone receptor (14), *c-erbB-2* (15), carcinoembryonic antigen (16), p53 (17), *MIB-1* antigen (18), bcl-2 (19), and metallopanstimulin (20–22). Unfortunately, in most instances, the biological properties of these markers and related importance as predictors of the disease state or behavior remain unclear (13–22). For example, recent work has identified metallopanstimulin, a multifunctional S27 ribosomal protein, as a marker for breast cancer as wells as numerous other human cancers, including prostate cancer (20–22). Metallopanstimulin-1 is expressed at low levels in normal cells (20–22). Likewise, the S2 ribosomal or chromosomal protein was found to be elevated in head and neck cancer but barely detectable in normal tissue (23). We therefore suggest that both the S2-like ribosomal protein and metallopanstimulin-1 or S27 ribosomal protein are multifunctional nuclear zinc finger proteins and each may be diagnostic for cancer.

In this respect, there are many reports showing a connection between overexpression of genes encoding ribosomal proteins and cancer (20–29). The implication is that these ribosomal proteins have additional functions distinct from their role as ribosomal proteins regulating protein synthesis (20–29). Inter-

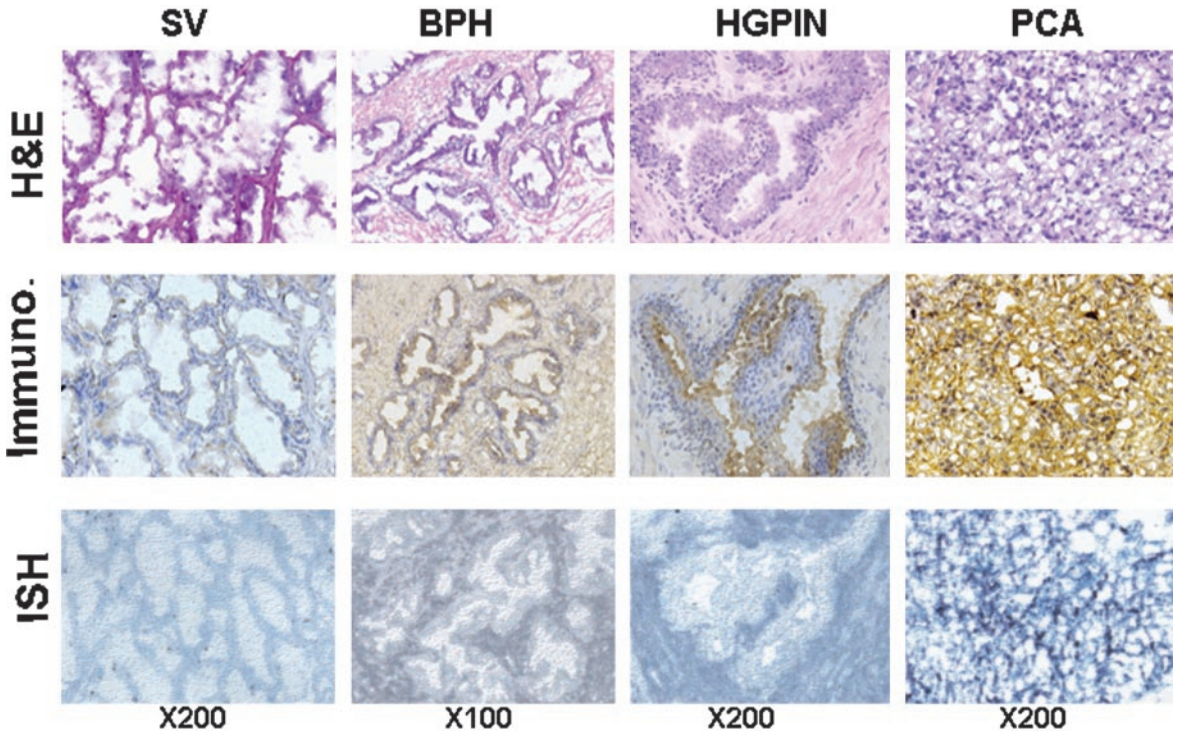

Fig. 4 Immunolabeling, *in situ* hybridization, and H&E labeling of adjacent sections from seminal vesicle, benign prostatic hyperplasia, high-grade prostatic intraepithelial neoplasia, and prostate cancer adenocarcinoma glands. The *X* axis shows the final magnification of the picture at ×100 or ×200.

estingly, specific leucine zipper sequence motifs are characteristic of numerous ribosomal proteins that allow binding to nucleic acids (22–29) and a possible role in regulating transcriptional and translational mechanisms. For example, the rat ribosomal protein S3a is identical to the product of the rat v-*fos* transformation effector gene (24). S3a is involved in initiation of protein synthesis and is also related to proteins involved in the regulation of growth and the cell cycle (24). Likewise, the rat ribosomal protein L10 is homologous to a DNA-binding protein and to a putative Wilms' tumor suppressor gene (25). The S2-like ribosomal protein characterized here binds a specific 8-mer sequence that is highly homologous to breakpoint cluster region consensus domains.[3] We therefore speculate that the five point mutations identified in the heptamer domain or leucine zipper region of the S2-like ribosomal protein might influence the nucleic acid binding affinities of the protein and change the functional role of the protein as well. For example, S2-like ribosomal protein binding to breakpoint cluster regions might be involved in chromosomal translocations.

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