Biomarkers, Genomics, Proteomics, and Gene Regulation

Novel Biomarkers for Prostate Cancer Including Noncoding Transcripts

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Levels of 27 transcripts were investigated as potential novel markers for prostate cancer, including genes encoding plasma membrane proteins (*ADAM2*, *ELOVL5*, *MARCKSL1*, *RAMP1*, *TMEM30A*, and *TMEM66*); secreted proteins (*SPON2*, *TMEM30A*, *TMEM66*, and truncated *TMEFF2* (called POP4)); intracellular proteins (*CAMK2N1*, *DHCR24*, *GLO1*, *NGFRAP1*, *PGK1*, *PSMA7*, *SDBS*, and *YWHAQ*); and noncoding transcripts (POP1 (100 kb) from mRNA AK000023), POP2 (4 kb from mRNA AL832227), POP3 (50 kb from EST CFI40309), POP5 (intron of *NCAM2*, accession DO668384), POP6 (intron of *FHIT*), POP7 (intron of *TNFAIP8*), POP8 (intron of *EFNA5*), POP9 (intron of *DSTN*), POP10 (intron of *ADAM2*, accession DO668396), POP11 (87kb from EST BG194644), and POP12 (intron of EST BQ226050)). Expression of POP3 was prostate specific, whereas *ADAM2*, POP1, POP4, POP10, *ELOVL5*, *RAMP1*, and *SPON2* had limited tissue expression. *ELOVL5*, *MARCKSL1*, *NGFRAP1*, *PGK1*, POP2, POP5, POP8, *PSMA7*, *RAMP1*, and *SPON2* were significantly differentially expressed between laser microdissected malignant versus benign clinical samples of prostate tissue. *PGK1*, POP2, and POP12 correlated to clinical parameters. Levels of *CAMK2N1*, *GLO1*, *SDBS*, and *TMEM30A* transcripts tended to be increased in primary prostate cancer from patients who later had biochemical failure. Expression of *GLO1*, *DHCR24*, *NGFRAP1*, *KLK3*, and *RAMP1* were significantly decreased in metastatic castration-recurrent disease compared with androgen-dependent primary prostate cancer. These novel potential biomarkers may therefore be useful in the diagnosis/prognosis of

prostate cancer. (*Am J Pathol* 2009, 175:2264–2276; DOI: 10.2353/ajpath.2009.080868)

Prostate-specific antigen (PSA) has been used as a serum biomarker to monitor and screen for prostate cancer since 1986 and 1994, respectively.¹ A recommendation for biopsy is set at an arbitrary serum PSA level of 4 ng/ml. At this threshold, PSA displays 93% specificity and a poor sensitivity of 24% for the detection of prostate cancer.² In addition to carcinoma of the prostate, PSA is expressed in normal prostate tissue, prostatitis, and benign prostatic hyperplasia.³ Furthermore, 27% of men with borderline serum PSA levels (3.1 to 4 ng/ml) have detectable prostate cancer by biopsy.⁴

Serum PSA levels correlate with the degree of dissemination^{5,6} and aggressiveness⁶ of prostate cancer. For example, serum PSA levels > 10 ng/ml are associated with a high pathological stage (odds ratio (OR) 1.7) and high Gleason sum (i.e., 7 to 10; OR 1.9), respectively, compared with PSA levels < 4 ng/ml.⁶ Following radical prostatectomy or brachytherapy, 7 to 15% of prostate cancers will exhibit biochemical recurrence at 8 years of follow-up as defined by rising PSA levels.^{7,8} Approximately 1% of prostate cancer patients will develop metastases following first-line therapy concomitant with serum PSA levels ≤ 2 ng/ml.⁹ Thus, measurement of serum PSA levels is inadequate for monitoring progression for a small subset of patients.

Patients receiving androgen-deprivation therapy for disseminated disease will relapse and their disease will progress to the terminal, castration-recurrent prostate cancer for which there is no effective treatment.^{10–12} Initial response to androgen-deprivation therapy is measured by PSA nadir. PSA nadir is prognostic of the time it takes to reach castration-recurrent prostate cancer and

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death.¹³ However, it is unknown whether pretreatment serum PSA levels can predict response to androgen-deprivation therapy.

These limitations of PSA emphasize the need for new biomarkers to accurately detect, monitor, and predict the aggressiveness of prostate cancer. In particular, biomarkers that are prognostic and/or signify the propensity to rapidly develop advanced disease are required. Such biomarkers may stem from gene expression studies using *in vivo* models of advanced prostate cancer. Here, we characterize the expression of genes and novel non-coding transcripts that were previously identified by Long Serial Analysis of Gene Expression (LongSAGE) (unpublished data) and Subtractive Hybridization¹⁴ technologies using samples from the *in vivo* LNCaP Hollow Fiber model.¹⁵ Both technologies can be used to discover

unannotated transcripts, and Subtractive Hybridization is particularly well suited for the identification of differentially expressed low abundance transcripts.¹⁶

Novel transcripts identified by Subtractive Hybridization are referred to as POP 1 through 12: POP1, transcript 100 kb from mRNA AK000023; POP2, transcript 4 kb from mRNA AL832227; POP3, transcript 50 kb from EST CFI40309; POP4, transcript from the intron of transmembrane protein with epidermal growth factor-like and two follistatin-like domains 2 (*TMEFF2*); POP5, transcript from the intron of neural cell adhesion molecule 2 (*NCAM2*; accession DO668384); POP6, transcript from the intron of fragile histidine triad gene (*FHIT*); POP7, transcript from the intron of tumor necrosis factor, α -induced protein 8 (*TNFAIP8*); POP8, transcript from the intron of ephrin-A5 (*EFNA5*); POP9, transcript from the intron of actin depo-

Table 1. Demographics, PSA, Stage, Gleason grades, and Sum of the Prostate Cancer Patients Used in This Study (Laser Capture-Microdissected Samples)

Sample no.*	Patient ID [†]	Age (years)	PSA [‡] (ng/ml)	Stage [§]		Normal or tumor [¶]	Gleason grade	Gleason sum ^{**}
				Clinical	Pathological			
1	A	59	9.7	NA	2b	N	NA	NA
2	A	59	9.7	NA	2b	T	3 + 4	7
3	B	64	19.0	1c	3b	N	NA	NA
4	B	64	19.0	1c	3b	T	4 + 4	8
5	C	71	24.2	2b	2b	N	NA	NA
6	C	71	24.2	2b	2b	T	3 + 4	7
7	D	68	9.5	2a	3b	N	NA	NA
8	D	68	9.5	2a	3b	T	5 + 4	9
9	E	64	19.1	2a	3b	T	3 + 4	7
10	F	71	5.5	2b	2b	N	NA	NA
11	G	69	25.1	1c	NA	N	NA	NA
12	G	69	25.1	1c	NA	N	NA	NA
13	G	69	25.1	1c	NA	T	4 + 3	7
14	H	67	6.4	2a	2a	N	NA	NA
15	H	67	6.4	2a	2a	T	4 + 4	8
16	I	64	7.7	2a	2a	N	NA	NA
17	J	70	29.9	2b	3b	T	3 + 3	6
18	K	62	10.0	2a	2b	N	NA	NA
19	L	63	15.6	2a	2b	T	3 + 4	7
20	M	74	5.2	2b	2b	N	NA	NA
21	N	70	14.1	2b	3b	T	4 + 3	7
22	O	NA	NA	NA	NA	T	3 + 4	7
23	O	NA	NA	NA	NA	N	NA	NA
24	O	NA	NA	NA	NA	T	3 + 5	8
25	P	74	5.7	2a	2a	N	NA	NA
26	Q	69	8.0	2b	3b	N	NA	NA
27	R	68	22.2	2a	3a	N	NA	NA
28	S	73	20.3	3a	3b	T	4 + 4	8
29	T	63	30.8	3a	3a	T	4 + 3	7
30	U	75	8.4	1c	3a	T	3 + 3	6
31	V	73	10.2	2b	2b	T	5 + 3	8
32	W	62	12.1	2b	3a	T	3 + 4	7
33	X	62	21.9	3a	3b	T	4 + 4	8
34	Y	71	25.9	3a	3b	T	3 + 3	6
35	Z	67	11.0	2a	3b	T	4 + 4	8
36	AA	64	13.7	3a	2b	T	4 + 4	8
37	AB	68	109.0	2b	2b	T	3 + 4	7
38	AC	67	18.4	2a	2b	T	5 + 4	9

NA, not applicable or not available.

*Sample no., sample number labeled "1" to "38".

[†]Patient ID, patient identification labeled "A" to "AC".

[‡]PSA serum levels upon diagnosis.

[§]Stage, tumor node metastasis (TNM) staging system.

[¶]N (normal), normal/benign prostate tissue; T (Tumor), malignant prostate tissue.

^{||}Gleason grade, grading system to describe degree of differentiation of tumor tissue cells. Gleason grading was applied to the slide of tissue used for laser microdissection by a trained pathologist.

^{**}Gleason sum, cumulative score of the two most prominent Gleason grades present on the slide of tissue.

lymerizing factor destrin (*DSTN*); POP10, transcript from the intron of *ADAM2* (accession DO668396); POP11, transcript 87 kb from EST BG194644; and POP12, transcript from the intron of EST BQ226050.¹⁴ Genes previously identified by LongSAGE and examined here were the known genes *ADAM2*, *CAMK2N1*, *ELOVL5*, *GLO1*, *MARCKSL1*, *NGFRAP1*, *PGK1*, *PSMA7*, *RAMP1*, *SBDS*, *SPON2*, *TMEM30A*, *TMEM66*, and *YWHAQ*. *DHCR24* is correlated with a higher incidence of metastases^{17–19} and was included here as a reference gene for comparison. The expression of these transcripts was measured in a variety of cell types and tissues, including clinical samples of androgen-dependent primary prostate cancer and metastasis of castration-recurrent prostate cancer. Here, we examine tissue specificity, androgen regulation, and feasibility of these transcripts in the prognosis of prostate cancer.

Materials and Methods

Cell Culture

Cell lines were maintained in RPMI 1640 media (LNCaP, 22Rv1, and COS1), Dulbecco's modified Eagle's medium (PC-3, DU145, and RKO), BRFF-HPC1 medium (MDA PCa 2b), or minimal essential medium (MG63, CV1, HEPG, and MCF7). All media (Stem Cell Technologies, Vancouver, BC, Canada) was supplemented with 100 U/ml penicillin and 100 units/ml streptomycin (Invitrogen, Burlington, ON, Canada) and fetal bovine serum (HyClone, Logan, UT). For androgen treatments, LNCaP cells were serum-starved for 48 hours and then treated for 16 hours in serum-free medium with 10 nmol/L synthetic androgen R1881 (PerkinElmer, Woodbridge, ON, Canada) or vehicle control (ethanol, final concentration $2.85 \times 10^{-4}\%$). Total RNA from cell lines was harvested using TRIzol Reagent (Invitrogen), and RNA from benign human tissue was obtained commercially (BD Clontech, Mountain View, CA).

Prostate Cancer Tissue Samples

Informed consent was obtained from each patient participating in the study according to guidelines set forth by the University of British Columbia/British Columbia Cancer Agency Research Ethics Board. Frozen primary androgen-dependent prostate specimens from patients in Japan who had undergone radical prostatectomies were obtained through coauthor T. Ueda and were embedded in Optimal Cutting Temperature™ (OCT) compound (Tissue-Tek, Torrance, CA). Prostatectomy specimens were accompanied by information, including the age of the patient, prior treatment history, serum PSA levels before surgery, and Tumor-Node-Metastasis (TNM) clinical and pathological stage (Table 1). Any patient who had received presurgical hormone ablation treatment was excluded. Malignant or benign epithelial cells were stringently obtained by laser microdissection using the μ Cut MMI AG Microscope (MMI Molecular Machines & Industries, Glattbrugg, Switzerland) (Figure 1, A–F). Total RNA was isolated and purified with the RNA Easy Micro kit

Figure 1. Laser microdissection of normal and tumor prostate tissue. Selected prostate epithelial cells were cut at $\times 20$ magnification using laser power and collected onto adhesive caps. Images show tissue before cutting (A and D), postcutting (location of cut is highlighted yellow; B and E), and remaining post capture on an adhesive cap (C and F). A–C represent normal tissue, whereas images D–F represent tumor tissue.

(Qiagen, Mississauga, ON, Canada). Contaminating genomic DNA was removed from RNA samples by TURBO DNA-free (Ambion, Austin, TX) or DNase I from the RNA Easy Micro Kit. RNA quality and quantity were assessed using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada) with RNA 6000 Nano LabChip kit (Caliper Technologies, Hopkinton, MA). RNA of poor quality (RNA integrity number < 2.8) and insufficient quantity (< 531 ng) was not used in this study. SuperScript III First-Strand Synthesis System (Invitrogen) with Oligo(dT) was used to reverse transcribe an input of 2.5 ng of RNA per quantitative (q)PCR.

Clinical samples of primary prostate cancer from men who later did, and did not, develop biochemical failure within 5 years after radical prostatectomy as well as lymph node metastasis from men with castration-recurrent metastatic disease (Prostate Cancer Rapid Autopsy Program) were obtained from the University of Washington through coauthor R. L. Vessella. Total RNA was extracted from tissue with RNA STAT-60 kit (Teltest, Friendswood, TX), and cDNA was synthesized with Oligo dT using Advantage RT for PCR kit (BD Clontech).

Relative Quantitation of Gene Expression

Input RNA was reverse transcribed with SuperScript III First Strand Synthesis kit (Invitrogen). For most RNA samples, a quantity of $0.5 \mu\text{g}$ was used in the RT reaction, but for limited sample quantities, such as those from the laser microdissected prostate tissue, 0.1 or $0.05 \mu\text{g}$ of RNA was used. A $10\text{-}\mu\text{l}$ quantitative (q)RT-PCR consisted of $1 \mu\text{l}$ of template cDNA, $1\times$ TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and $0.9 \mu\text{mol/L}$ each of forward and reverse primers and $0.25 \mu\text{mol/L}$ of TaqMan probe (FAM-BHQ-1 or TET-BHQ-1; Integrated DNA Technologies, San Diego, CA) that produce specific PCR products ranging in size between 85 and 235 bp (see Table 2 for primer and probe sequences). qRT-PCR reactions were cycled as follows in a 7900HT Sequence Detection System (Applied Biosystems): 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 0.25 minutes, followed by 60°C for 1 minute. All qRT-PCRs were performed using technical triplicates. cDNAs (from different conditions/patients)

Table 2. Primer and Probe Sequences for qRT-PCR of Candidate Transcripts

Gene	Forward primer	Probe	Reverse primer
<i>ADAM2</i>	5'-TGGTGAAAGTTAATTCCAAAGG-3'	5'-ATTCAAGCGATGAGCAACCT-3'	5'-TCATGGCATCTCTGTTGTCC-3'
<i>CAMK2N1</i>	5'-TGCAGGACACCAACAATTC-3'	5'-AGCAAGCGGGTGTATTGA-3'	5'-GCACGTATCATCTCTATCATC-3'
<i>DHCR24</i>	5'-GAGGCGCTGGAGAAGTTTG-3'	5'-TGCTGTATGCCGACTGCTAC-3'	5'-CTTGTGGTACAAGGAGCCATC-3'
<i>ELOVL5</i>	5'-GTTTGTCTGCTCAGTCCCTTCC-3'	5'-CGTCCATACCTCTGGTGGAA-3'	5'-TGGTCTGGATGATTTGTCAGC-3'
<i>GAPDH</i>	5'-CTGACTTCAACAGCGACACC-3'	5'-CGACCACTTTGTCAAGCTCA-3'	5'-TGCTGTAGCCAAATTCGTTG-3'
<i>GLO1</i>	5'-AAGGTTTGAAGAACTGGGAGTC-3'	5'-AAGGCTTGGCATTATTCAT-3'	5'-TTCAATCCAGTAGCCATCAGG-3'
<i>MARCKSL1</i>	5'-GCAGCCAGAGCTCCAAAGG-3'	5'-CCAACGGCCAGGAGAATG-3'	5'-AAGTCTCCATTGCTTTTCACG-3'
<i>NGFRAP1</i>	5'-GTCACCTCGCTCTGGCTAC-3'	5'-AAAGCGGAGCAGGTCTGC-3'	5'-GCCGCGAGACACTTACG-3'
<i>PGK1</i>	5'-GAAGGGAAGGGAAGATGC-3'	5'-CGAGCCAGCCAAATAGAAG-3'	5'-GACATCCCTTAGCTTGGAAAG-3'
<i>POP1*</i>	5'-AAGCTCTTGCTAGGCATGTAGG-3'	5'-CCTGGACAGCCATTCTTTA-3'	5'-TTTGGGTAGACATTTCCCC-3'
<i>POP2</i>	5'-GGAGGATCAACAGCAGCATT-3'	5'-CAACTGTGTCTCATTTGACGT-3'	5'-GGTATCATTTAGGGCTGGGTG-3'
<i>POP3</i>	5'-TATGGTGTGCCATTCTCGGA-3'	5'-CGTGTTCATCTCTGAGTGA-3'	5'-GTGGAACAAAATCCCCCTCT-3'
<i>POP4</i>	5'-CCCTTGTGCAATGGGTTA-3'	5'-TCATTTATGATGCCACACATGA-3'	5'-TTGTTCCTTTCATCTTTTGTTC-3'
<i>POP5</i>	5'-TTTGAAAGGTGAGCCTCTG-3'	5'-CATTGTTTGGGCAGGAGAGT-3'	5'-AAAGAAGTGGACGTGGCAC-3'
<i>POP6</i>	5'-TTTAAGTGGTTTCAGCACAAAAC-3'	5'-CAAAAGGATGACCTTGGGAA-3'	5'-TGATGACTTCTTGTGTTTAAACAA-3'
<i>POP7</i>	5'-TTGGTTTCTGGACCCCTTTTG-3'	5'-AAAGCTTGAGGGTGGTGTAG-3'	5'-CAGAAGAGCAGGGTGGGTAG-3'
<i>POP8</i>	5'-TTTCGGTTCCTTTCTCTTC-3'	5'-CCCACATTCCATTTCACAA-3'	5'-ATTCCTTTATGGCTTGAAGGGT-3'
<i>POP9</i>	5'-CCTGTTTCCAGTCACACCT-3'	5'-TTAACAATTCCCAAGCACCC-3'	5'-ATTTGTCTTCCACCAAGGC-3'
<i>POP10</i>	5'-TTGCTAGGGAAGAGCAGCAT-3'	5'-TTCTTACCAAACTCTTAAACAGA-3'	5'-GAATCATTAAGGCAGCCCTCCTT-3'
<i>POP11</i>	5'-GTTCGCTCTTGCTTTGAAC-3'	5'-TTCCCTGTCCCTTAACCTCT-3'	5'-TTTGCCCTTTTGCAGAAATGTG-3'
<i>POP12</i>	5'-TGTGACAAATGGGAGGACA-3'	5'-GCTTGTGTGAGTTGCAAGCA-3'	5'-CAGAAAAGTGTATGGCAGGGA-3'
<i>PSMA7</i>	5'-CGTCAAGAAGGGCTCGAC-3'	5'-AAGAAGTTCAGTGGCCAACTG-3'	5'-CGCATGTTCTTTCATCCTG-3'
<i>RAMP1</i>	5'-CCTCACCAGTTCAGGTCAG-3'	5'-CAGGACCATCAGGAGCTACA-3'	5'-CATGTGCCAGGTGCAGTC-3'
<i>SBDS</i>	5'-CGCCTGCTACAAAACAAGG-3'	5'-CGTGGAAAAAGACCTCGAT-3'	5'-CAACACTGAGTGGGTCTGC-3'
<i>SDHA</i>	5'-ACCAGGTCACACACTGTTGC-3'	5'-ACATGGAGGAGGACAACCTG-3'	5'-CCTGTGGTCTGCTAGAAATGC-3'
<i>SPON2</i>	5'-CCCAGCAGGGAACAATGAG-3'	5'-TGTAGACAGCGCTCAGTTC-3'	5'-CACAGTCCCAGGACGAC-3'
<i>TMEM30A</i>	5'-GGATGTGACACCTTGCTTTTG-3'	5'-CCATTAACTTCACACTGGAAAAG-3'	5'-ACGTAAACGACGATGGTTTGTG-3'
<i>TMEM66</i>	5'-GGGCAGCTATTTCGTATGTTTC-3'	5'-CGAAAACAGAACTGCATCA-3'	5'-TGATCCAGTGTGTTGACTCC-3'
<i>YWHAQ</i>	5'-CTGAGATCCATCTGCACCAC-3'	5'-AGCCAATGCAACTAATCCAGA-3'	5'-ACCGGAAGTAATCACCCTTC-3'

*Genes were represented by Human Genome Organisation Gene Nomenclature Committee-approved nomenclature when available. Non-Human Genome Organisation Gene Nomenclature Committee gene names were not italicized.

and genes (target and reference) to be directly compared were assayed in the same instrument run. Glyceraldehyde-3-phosphate (*GAPDH*) was used as a reference gene for all experiments, except the androgen regulation experiment using LNCaP cells in which succinate dehydrogenase complex, subunit A, flavoprotein was used due to its consistent levels of expression despite androgen stimulation. These reference genes were carefully assessed for consistent levels of expression across all conditions. Reactions without template were run for each gene to ensure that DNA had not contaminated the qRT-PCR reactions. Efficiency checks were performed for each primer pair. Relative expression was determined using the Pfaffl method, which takes into consideration the efficiency of the reaction for both the target and reference genes.

Western Blot Analyses

LNCaP cells (2×10^6) were cultured in 15-cm plates in RPMI 1640 medium containing 5% fetal bovine serum for 24 hours. Medium was changed to serum-free and phenol red-free RPMI 1640 medium. After at least 24 hours of serum starvation, cells were treated with the indicated concentrations of R1881 or ethanol (vehicle) and were further incubated for 24 or 48 hours. Cells were harvested, and total cell lysates were analyzed by Western blot analysis with the respective antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Analysis

To identify significant changes in gene expression in response to androgen, we used the two-sample Student's *t*-test for unequal variance. Nonparametric methods were used with data that were sampled from nonnor-

mal distributions. For gene expression analysis on RNA from laser microdissected prostatic tissue, the Spearman's correlation test was used to identify associations to patient age or PSA levels, and the Kruskal-Wallis test was used to identify significant differences between mean gene expression in normal (benign) and tumor tissue, TNM stages of cancer, Gleason scores, or biochemical recurrence and terminal castration-recurrent metastases.

Figure 2. Tissue specificity of gene expression. **A:** Expression in cell lines. LNCaP, MDAPCa-2B, 22Rv1, PC-3, DU145, MG63, RKO, HEPG, MCF7, COS1, and CV1 cells were maintained in tissue culture under individualized conditions for optimal growth to gauge constitutive levels of gene expression measured using total RNA and analyzed by qRT-PCR. The heat map indicates the average degree of fold-change in gene expression relative to LNCaP cells of three biological replicates. **B:** Specificity of gene expression in benign tissue. RNA was obtained commercially from benign human tissues and analyzed by qRT-PCR. The heat map indicates the degree of fold-change in gene expression relative to prostate tissue.

Results

Tissue Specificity of Gene Expression

Tissue-specific expression of transcripts in cell lines were measured by qRT-PCR using RNA isolated from five human prostate cancer cells (LNCaP, MDA-PCa-2B, 22Rv1, PC-3, and DU145) and nonprostate human cancer cell lines that included the following: MG63, osteosarcoma cells; RKO, colon carcinoma cells; HEPG, hepatocellular carcinoma cells; MCF7, mammary adenocarcinoma cells; and large T-antigen transformed and normal monkey kidney cells (COS1 and CV1, respectively). Expression of genes *ADAM2* and *POP11* were relatively specific for LNCaP cells (Figure 2A). *ADAM2* and *POP10* (intron of *ADAM2*) showed differences in expression in HEPG cells suggesting tissue-specific expression of splice variants of *ADAM2*. *MARCKSL1*, *POP1*, *POP2*, *POP3*, *POP4*, *POP5*, *POP12*, and *SPON2* were enriched in human prostate cancer cell lines compared with all other human cancer cell lines tested.

To address tissue-specific expression in benign tissues, levels of transcripts in 20 human tissue samples were measured. Gene expression was displayed relative to the levels in benign human prostate tissue. *POP3* was the only transcript to exhibit exclusive expression in benign prostate tissue versus other benign human tissues tested (Figure 2B), suggesting it is prostate specific. This was consistent with expression of *POP3* predominantly in LNCaP, MDA-PCa-2B, and 22Rv1 cells that express androgen receptor and low expression in all other cell lines examined (Figure 2A). Some genes were expressed at a level on par with that of the benign prostate in the adrenal gland (*ELOVL5*) and testis (*ELOVL5* and *POP1*; Figure 2B). Both adrenal glands and testes produce androgens that are essential for regulating the growth of the prostate.²⁰ *POP1* had relatively specific expression in prostate cancer cell lines with similar expression patterns to *POP3*, whereas *ELOVL5* had broad expression across most cell lines (Figure 2A). *ADAM2* and *POP10* (intron of *ADAM2*) showed similar expression patterns in prostate, placenta, and testis with the exception of expression of only *ADAM2* in thymus tissue (Figure 2B). These data support the tissue-specific expression of splice variants of *ADAM2*. *POP4* (splice variant of *TMEFF2*) was expressed in the prostate, brain, and prostate cancer cells only expressing the androgen receptor. Both *RAMP1* and *SPON2* had relatively restricted expression in prostate and uterine tissues (Figure 2B). *SPON2* had expression generally specific for prostate cancer cell lines (LNCaP and MDA-PCa-2B), while *RAMP1* was also highly expressed in MG63 osteosarcoma cells (Figure 2A). Taken together, these data suggest that *ADAM2*, *ELOVL5*, *POP1*, *POP3*, *POP4*, *POP10*, *RAMP1*, and *SPON2* have relatively restricted expression patterns in the prostate when comparing benign tissues (Figure 2B).

Androgen Regulation of Gene Expression

The androgen signaling axis plays an important role in the growth, survival, and differentiation of the pros-

tate.^{21–23} Treatment for locally advanced and metastatic prostate cancer includes androgen-deprivation therapy. Thus, it is essential to determine whether levels of expression of any of the 27 transcripts were altered by andro-

Figure 3. Regulation of gene expression by androgen. **A:** Expression of genes and noncoding transcripts increased (**A**) or decreased (**B**) by androgen. RNA was isolated from LNCaP cells that were treated with R1881 and analyzed by qRT-PCR. Fold change was calculated by normalizing the mean normalized expression of transcripts in R1881-treated cells to the mock vehicle-treated cells. In doing this, the vehicle treatment fold change became one and SD zero. Error bars represent $\pm$ SD for six biological replicates. Two-sample Student's *t*-test for unequal variance ($*P \leq 0.05$). **C:** Western blot analyses of whole-cell lysates of LNCaP cells treated with different concentrations of androgen (R1881) for 24 or 48 hours. Representative blots of three (**left panel**) or two (**right panel**) experiments are shown.

gen. To do this, levels of expression of these genes were assessed in prostate cancer cells with androgen receptor (LNCaP, MDA-PCa-2B, and 22Rv1) and without a functional androgen receptor (PC-3 and DU145).^{24–28} Expression of ADAM2, CAMK2N1, DHCR24, MARCKSL1, NGFRAP1, POP1, POP3, POP4, POP5, POP7, POP8, POP10, POP11, SPON2, and TMEM66 transcripts were enriched in prostate cancer cell lines with a functional androgen axis (compare levels in LNCaP, MDA-PCa-2B, and 22Rv1 to PC3 and DU145 cells in Figure 2A). Although MCF7 mammary carcinoma cells express the androgen receptor at low levels,²⁹ activation of the endogenous androgen signaling axis has not been documented.³⁰ With this potential lack of androgen signaling, expression of only ADAM2, MARCKSL1, POP1, POP3, POP4, POP5, POP10, POP11, and SPON2 was not obviously elevated in MCF7 cells (Figure 2A). Of these genes, expression of ADAM2, POP1, POP3, POP10, and SPON2 were generally restricted to the prostate.

Differential expression of these transcripts in response to androgen was also measured in LNCaP cells treated with androgen. *KLK3* (PSA) was included as a positive control and was strongly induced by androgen as expected. Expression of 11 other genes (*DHCR24*, *ELOVL5*, *GLO1*, *PGK1*, POP4, POP6, POP7, POP8, SPON2,

TMEM66, and *YWHAQ*) increased (Figure 3A), whereas significant decreases in expression of 5 genes (*ADAM2*, *CAMK2N1*, POP5, POP10, and POP11) were detected (Figure 3B). Consistent changes in levels of POP10 and ADAM2 in response to androgen suggest that these two variant transcripts may share common androgen-response element(s) in the regulatory regions to modulate transcription. Androgen regulation of genes *MARCKSL1*, *NGFRAP1*, POP1, POP2, POP3, POP9, POP12, *PSMA7*, *RAMP1*, *SBDS*, and *TMEM30A* were not detected (data not shown). A few genes were randomly selected for analysis that changes in RNA correlated to protein. PSA (*KLK3*) was included as a positive control and was robustly induced as expected (Figure 3c). *ELOVL5* protein increased in a dose-dependent manner in response to increasing concentrations of androgen with up to eightfold at 10 nmol/L concentration when normalized to levels of β -actin. SPON2 and PGK1 proteins increased 1.7- and 1.8-fold, respectively, which was consistent with the less than twofold increase in mRNA. A weak band for NGFRAP protein could be detected with no apparent increase in response to 10 nmol/L androgen in agreement with mRNA levels. Although levels of MARCKSL1, NGFRAP1, POP1, and POP3 transcripts were elevated in prostate cancer cells with endogenous androgen recep-

Figure 4. Candidate biomarkers differentially expressed between benign and malignant prostate epithelial cells. RNA was isolated from laser-microdissected benign epithelial cells from 15 patients and malignant epithelial cells from 13 or 23 patients and analyzed by qRT-PCR. y axis is the mean normalized expression (MNE) of candidate genes. Kruskal Wallis test, $P \leq 0.05$. Box-and-whisker plots display the median MNE (bold line), the first and third quartile range (the box), minimum and maximum nonoutlier values (whiskers), and outliers (○).

tor compared with those cells without a functional receptor, no direct evidence supports that these genes are regulated by androgen. Enhanced expression of *ELOVL5*, *GLO1*, *PGK1*, *POP6*, and *YWHAQ* in response to androgen, although lacking enrichment in prostate cancer cells with androgen receptor compared with those without a functional receptor, suggests that these genes may be regulated by secondary downstream effects of androgens such as changes in proliferation, metabolism, and/or differentiation.

Characterization of Gene Expression in Prostate Cancer

To determine whether levels of any of the transcripts were altered in prostate cancer compared with benign prostate epithelial cells, total RNA was isolated from laser microdissected samples of prostate obtained by radical prostatectomy from prostate cancer patients (Table 1). These studies revealed that levels of expression of *ELOVL5*, *NGFRAP1*, *PGK1*, *POP5*, *POP8*, and *PSMA7* were significantly reduced, whereas levels of expression of *MARCKSL1*, *POP2*, *RAMP1*, and *SPON2* were significantly increased in malignant compared with benign epithelial prostate cells (Kruskal-Wallis test, $P \leq 0.05$; Figure 4). *POP3* and *POP10* were borderline significantly increased ($P < 0.1$). Borderline significant changes in expression of genes indicate that analysis using a larger sample size may be required to achieve statistical significance.

To determine whether the levels of expression of genes in tumor tissue samples correlated to patient age, serum PSA, Gleason grades, or stage of the disease (TNM), only levels of each transcript in the tumor samples were used in statistical analyses. Negative association between the expression of *POP2* ($P = 0.044$, $\rho = -0.44$) and the age of the patient was detected using Spearman's correlation (Figure 5A). Some pathways that play a role in prostate cancer that are known to be affected by aging include reduced levels of testosterone, changes in the expression of specific G-protein isoforms, and levels of cAMP and melatonin. The mean expression of *POP12* was significantly higher in Gleason scores 8 and 9 compared with 6 and 7 ($P = 0.039$) as assessed with the Kruskal-Wallis test (Figure 5B), suggesting a possible function in regulating differentiation. Expression of *PGK1* ($P = 0.022$) was positively correlated with high serum PSA levels using Spearman's correlation (Figure 5C). Borderline significance for expression of *RAMP1* ($P = 0.07$) with both serum PSA and TNM stage of prostate cancer was detected (data not shown).

Expression Levels in Primary Lesions from 5-Year Disease-Free Patients and Those That Developed Biochemical Recurrence Compared with Metastatic Castration-Recurrent Lesions

To assess whether expression of any of these transcripts could possibly aid in predicting which patients may de-

Figure 5. Tissue levels of transcripts correlated with clinical parameters. **A:** POP2 transcript in prostate cancer tissue is negatively correlated with the age of the patient. Spearman's correlation test ($P < 0.05$), $n = 13$. **B:** The mean levels of POP12 transcript is significantly increased in Gleason scores 8 and 9 (mean, 8.71×10^{-3}) compared with Gleason scores 6 and 7 (mean, 8.4×10^{-3}). Kruskal-Wallis test, $P \leq 0.05$, $n = 23$. **C:** Tissue levels of expression of PGK1 mRNA is positively associated with serum levels of PSA at the time of diagnosis. Spearman's correlation test ($P < 0.05$), $n = 23$.

velop aggressive disease, we compared their levels in clinical samples obtained up to 8 years ago by radical prostatectomy. Half of these samples were from the primary tumor tissue of patients who later developed recurrent disease (RP) measured as biochemical failure ($n = 6$), and the remaining samples ($n = 6$) were from the primary tumor tissue of patients who were still disease-free 5 years after primary treatment (nonrecurrent, NP) (range, 5.5 to 8.4 years). *CAMK2N1* ($P = 0.08$), *GLO1* ($P = 0.06$), *SBDS* ($P = 0.08$), and *TMEM30A* ($P = 0.06$) transcripts were all borderline significantly elevated in the tissue from patients who later had recurrence (Figure 6). The mean levels of *KLK3* (PSA) transcript were not significantly different between primary lesions from men with or without biochemical failure ($P = 0.87$). Comparison of levels of expression of transcripts in androgen-dependent primaries that later recurred (RP) versus castration-recurrent metastases (Met, $n = 6$) revealed that *GLO1* ($P = 0.01$) expression was significantly decreased in metastases, whereas *CAMK2N1* ($P = 0.08$), *NGFRAP1* ($P = 0.06$), and *KLK3* ($P = 0.06$) expression were borderline significantly decreased. Comparing both primaries that did (RP) and did not (NP) later recur that were androgen-dependent versus castration-recurrent metastases (Met) resulted in significance for *DHCR24* ($P = 0.04$), *KLK3* ($P = 0.03$), *NGFRAP1* ($P = 0.03$), and *RAMP1* ($P = 0.03$), whereas *GLO1* remained significant ($P = 0.03$). *CAMK2N1* ($P = 0.09$) and *PSMA7* ($P = 0.09$) were borderline significant. POP transcripts were not measured because these samples were not DNase-treated and the primers for the POPs cannot span introns.

Discussion

In the United States, 86 men die from prostate cancer each day. However, these numbers only represent the

Figure 6. Candidate biomarkers differentially expressed between androgen-dependent primary prostate cancer from patients who do and do not later have biochemical failure versus levels in castration recurrent metastatic disease. Total RNA isolated from castration-recurrent lymph node metastasis of prostate cancer (Met) ($n = 6$) or androgen-dependent primary prostate cancer samples from patients who did not have biochemical failure within 5 years (nonrecurrent primary; NP) ($n = 6$) or those who later developed biochemical failure (recurrent primary; RP) ($n = 6$) and analyzed by qRT-PCR. Kruskal-Wallis test, $P \leq 0.05$.

2.5 to 3% of men who die from the disease from the 10% of men over 50 years who will have clinical progression. Autopsy studies indicate that 30% of men over the age of 50 have malignant cells in their prostate.³¹ The European Study of Screening and the Prostate Cancer Prevention Trial indicate that screening for prostate cancer elevates the incidence rate³² with increases in the ratio of incidence to mortality from 2.5:1 to 17:1. This suggests that a substantial proportion of men with clinically insignificant disease are being overtreated. In other words, their disease will never cause morbidity or mortality. Current treatments for organ-confined malignancy include brachytherapy,³³ external beam radiation,³⁴ and radical prostatectomy.³⁵ These forms of therapy can produce significant morbidity such as incontinence and impotence and are not effective for disease that has spread

beyond the prostate. Only palliative therapy is available for disseminated disease, which requires reducing levels of androgen and/or using antiandrogens.^{36,37} Thus, there is an urgent need for selective intervention to spare those men from receiving unnecessary treatment but still provide radical curative treatment to those men who will develop clinically significant disease. Currently, there are no prognostic tools that can distinguish aggressive tumors from latent tumors.

To address this need, the expression of a number of genes and novel noncoding transcripts previously identified to be differentially expressed in an *in vivo* model of hormonal progression of prostate cancer were studied with the notion that levels of expression may be indicative of aggressive disease. This study revealed the following: 1) *GLO1*, *CAMK2N1*, *SBDS*, and *TMEM30A* may be as-

Table 3. Review of Expression Trends of Candidate Genes

Gene	Expressed in CRPC versus RAD*	Expressed in RAD versus AS†	Enriched in AR+ CaPcells‡	Reg. by A§	S or PM Protein¶	Enriched in human	Enriched in CaP Cell Lines**	Enriched in Normal Prostate††	Assoc. w/PSA‡‡	Assoc. w/Age§§	Expressed in Tumor versus Normal¶¶	Expressed in High GG versus Low GG	Expressed in NP versus RPversus Met***	Expressed in NP+RP versus Met†††
<i>ADAM2</i>	↑	NS	Y†††	↓	PM	Y	Y	Y	N	N	NS	NS	NS	NS
<i>CAMK2N1</i>	↑	↑	Y	↓	N	N	N	N	N	N	NS	NS	b	b
<i>DHCR24</i>	↑§§§	↓	Y	↑§§§	N	Y	N	N	N	N	NS	NS	NS	Y
<i>ELOVL5</i>	↑	↓	N	↑§§§	PM	N	N	Y	N	N	↓	NS	NS	NS
<i>GLO1</i>	↑	NS	N	↑	N	N	N	N	N	N	NS	NS	Y	Y
<i>MARCKSL1</i>	↓	NS	Y	N	PM	Y	Y	N	N	N	↑§§§	NS	NS	NS
<i>NGFRAP1</i>	↓	NS	Y	N	N	N	N	N	N	b-	↓	NS	b	Y
<i>PGK1</i> ¶¶¶	↑	↓	N	↑§§§	N	N	N	N	Y+	N	↓	NS	NS	NS
<i>POP1</i> ¶¶¶	↑	UD	Y	N	NA	Y	Y	Y	N	N	NS	NS	UD	UD
<i>POP2</i>	↑	UD	N	N	NA	Y	Y	N	N	Y-	↑	NS	UD	UD
<i>POP3</i>	↑	UD	Y	N	NA	Y	Y	Y	N	N	b↑	NS	UD	UD
<i>POP4</i>	↑	UD	Y	↑	NA	Y	Y	Y	N	N	NS	NS	UD	UD
<i>POP5</i>	↑	UD	Y	↓	NA	Y	Y	N	N	N	↓	NS	UD	UD
<i>POP6</i>	↑	UD	N	↑	NA	Y	N	N	N	N	NS	NS	UD	UD
<i>POP7</i>	↑	UD	Y	↑	NA	Y	N	N	N	N	NS	NS	UD	UD
<i>POP8</i>	↑	UD	Y	↑	NA	Y	N	N	N	N	↓	NS	UD	UD
<i>POP9</i>	↑	UD	N	N	NA	N	N	N	N	N	NS	NS	UD	UD
<i>POP10</i>	↑	UD	Y	↓	NA	Y	N	Y	N	N	b↑	NS	UD	UD
<i>POP11</i>	↑	UD	Y	↓	NA	Y	Y	N	N	N	NS	NS	UD	UD
<i>POP12</i>	↑	UD	N	N	NA	Y	Y	N	N	N	NS	↑	UD	UD
<i>PSMA7</i>	↑	NS	N	N	N	N	N	N	N	N	↓	NS	NS	b
<i>RAMP1</i>	↓	↑	N	N	PM	Y	N	Y	B	N	↑	NS	NS	Y
<i>SBDS</i>	↑	NS	N	N	N	Y	N	N	N	N	NS	NS	NS	NS
<i>SPON2</i>	↑	NS	Y	↑	S	Y	Y	Y§§§	N	N	↑	NS	NS	NS
<i>TMEM30A</i>	↑	NS	N	N	S & PM	Y	N	N	N	N	NS	NS	NS	NS
<i>TMEM66</i>	↑	NS	Y	↑	S & PM	N	N	N	N	N	NS	NS	NS	NS
<i>YWHAQ</i>	↓	NS	N	↑	N	N	N	N	N	N	NS	NS	NS	NS

*Expressed in CRPC versus RAD, Gene expression in castration-recurrent prostate cancer versus responsive to androgen deprivation in the LNCaP Hollow Fiber model.

†Expressed in RAD versus AS, Gene expression in the stage of prostate cancer that is responsive to androgen deprivation versus androgen sensitive in the LNCaP Hollow Fiber model.

‡Enriched in AR+ CaP cells, gene expression is enriched in androgen receptor positive prostate cancer cells versus androgen receptor negative prostate cancer cells.

§Reg. by A, expression of gene is regulated by androgen; arrow indicates direction of regulation with androgen.

¶S or PM Prot., gene product is a secreted (S) or plasma membrane (PM) protein.

||Enriched in Human, gene expression is enriched in human cell lines versus monkey kidney cells.

**Enriched in CaP cell lines, gene expression is enriched in prostate cancer cell lines versus cell lines of cancer from other organ sites.

††Enriched in normal prostate, gene expression is enriched in normal human prostate tissue versus other normal human tissues.

‡‡Assoc. w/PSA, gene expression in laser microdissected sample is associated with serum prostate-specific antigen levels measured from the patient at the time of diagnosis of prostate cancer; '+' indicates a positive association.

§§Assoc. w/age, Gene expression in laser microdissected sample is associated with age of the patient at the time of diagnosis of prostate cancer; '-' indicates a negative association.

¶¶Expressed in Tumor versus Normal, gene expression in tumor tissue versus normal prostate tissue.

|||Expressed in High GG versus Low GG, Gene expression in high Gleason Grade tumor tissue versus low Gleason grade tumor tissue.

***Expressed in NP versus RP versus Met, Gene expression in primary tumor tissue that did not recur versus primary tissue which later developed recurrent disease versus castration-resistant metastatic tumor.

†††Expression metastatic versus both NP and RP primary tumor tissues.

+++Y, yes; N, no; NS, no significant difference; NA, not applicable; UD, undetermined; ↑, higher expression; ↓, lower expression; b, borderline significance.

§§§Known.

¶¶¶¶Genes were represented by Human Genome Organisation Gene Nomenclature Committee approved nomenclature when available. Non-Human Genome Organisation Gene Nomenclature Committee gene names were not italicized.

sociated ($P < 0.1$) with increased expression in prostate cancer tissue from men that will go on to have biochemical failure; 2) *GLO1*, *RAMP1*, *PSMA7*, *DHCR24*, and possibly *NGFRAP1* and *CAMK2N1* were associated with decreased expression in metastatic castration-recurrent tissue compared with androgen-dependent primary lesions; 3) *PGK1*, *POP2*, and *POP12* levels of expression correlated to clinical parameters; 4) differential expression of *ELOVL5*, *RAMP1*, *SPON2*, *MARCKSL1*, *NGFRAP1*, *PGK1*, *PSMA7*, *POP2*, *POP5*, *POP8*, and possibly *POP3* and *POP10* between malignant and benign prostate epithelial cells; and 5) evidence for androgen regulation and tissue-specific expression of these transcripts. A summary of results is presented in Table 3.

Several of the protein-coding genes investigated in this study are poorly explored in cancer pathobiology with little to nothing known with regard to their expression or function in prostate cancer. For several of these

genes, their known general function is consistent with their expression pattern identified here (summarized in Table 4).^{38–55}

Of particular interest is *CAMK2N1*, which encodes a protein that interacts and inhibits the activity of Ca^{2+} /calmodulin-dependent protein kinase II (CAMK2). CAMK2 signaling plays a role in cell cycle progression by activating MEK/ERK to enhance phosphorylation of p27Kip1 (Thr¹⁸⁷), resulting in degradation and rapid entry into G₂-M phase.⁵⁶ Inhibitors of CAMK2 decrease the activities of CDK4 and CDK2 and enhance levels of p27Kip1 expression to cause cell cycle arrest in G₁.⁵⁷ CAMK2N1 deactivates MEK/ERK to decrease progression of S phase and stabilizes p27 to induce cell cycle arrest, thereby reducing tumor growth.⁵⁸ In prostate cancer cells, overexpression of CAMK2 decreases apoptosis and caspase activity,⁵⁹ whereas inhibition of CAMK2 activity reduces both proliferation and Matrigel invasion.⁶⁰

Table 4. Function and Expression of Potential Novel Biomarkers in Prostate Cancer or Other Diseases

Gene	General function	Previously known expression/function in prostate cancer	Role in other diseases
<i>GLO1</i>	Detoxifies methylglyoxal	Increased activity in prostate cancer; ^{39, 39} responsive to androgen ⁴⁰	Resistance apoptosis induced by epoposide and adriamycin in leukemia. ⁴¹
<i>SBDS</i>	Possibly involved in RNA processing ^{42,43} and cell survival (prosurvival gene) ⁴⁴		Mutations in <i>SBDS</i> associated with bone marrow failure, pancreatic dysfunction, and metaphyseal dysostosis. ^{45–47}
<i>TMEM30A</i>	Putative b-subunit or chaperone for the ATPase, ATP8B1, ⁴⁸ which translocates phosphatidylserine into the inner wall of cell membrane		High levels of phosphatidylserine on the outer wall of the membrane in cancer cells compared to normal cells suggesting possible dysfunction of the translocation machinery. ⁴⁹
<i>PSMA7</i>	Subunit of the 20S proteasome	Enhances the transcriptional activity of the androgen receptor measured using the MMTV-luciferase reporter in PC3 prostate cancer cells transfected with androgen receptor. ⁵⁰	Elevated levels of PSMA7 in colon cancer are generally associated with metastasis to the liver and reduced survival; ⁵¹ inhibitors of the proteasome are under clinical investigation
<i>DHCR24</i>	Cholesterol biosynthesis ⁵² and Ras-induced senescence ⁵³	DHCR24 is down-regulated in both high-grade lesions and lymph node metastases and correlated with a higher incidence of metastases ^{17–19}	.
<i>PGK1</i>	Glycolysis	<i>PGK1</i> locus located within Xq11-Xq13 and closely linked to the androgen receptor and implicated in familial prostate cancer; ⁵⁴ overexpressed in prostate cancer. ⁵⁵	Implicated in several cancer types.

Thus, reduced expression of *CAMK2N1* in castration-recurrent metastatic disease as shown here would lead to increased CAMK2 activity, which is associated with increased proliferation and invasion. CAMK2 activity is under androgen receptor control.⁵⁹ Here, we show that androgen decreased expression of *CAMK2N1*, which would theoretically increase CAMK2 activity to potentially contribute to increased proliferation. Contrary to predicted results, expression of *CAMK2N1* was elevated in tumors that go on to recur, which would theoretically reduce CAMK2 activity and proliferation. Such discrepancies have been previously reported for other potential markers such as hepsin and *PIM-1*, which are overrepresented in prostate cancer but surprisingly reduced or absent levels in cancers associated with increased risk of relapse after prostatectomy.⁶¹ These observations suggest that various markers may be required for more definitive prognosis of different stages of prostate cancer.

Levels of transcripts for several genes were altered in response to androgen. These findings are useful to begin to appreciate a role for these transcripts in prostate pathobiology and may also have implications for their use as biomarkers. Here, we showed that *RAMP1* had an expression profile that was prostate-specific in normal tissues for men. This tissue specificity combined with RAMP1 protein being localized on the plasma membrane indicates potential for RAMP1 to be used for targeting therapeutics or for imaging prostate cancer. No changes in expression of *RAMP1* were detected in response to androgen, suggesting that possibly its expression would not be compromised in patients receiving androgen-dep-

ri-
 vation therapies. An example of this can be drawn from prostate-specific membrane antigen that is expressed on the plasma membrane and used clinically for detection of recurrent prostate tumors.^{62–64}

Noncoding transcripts display a diverse array of functions, including the regulation of expression of other genes. Sense noncoding transcripts can silence gene expression by recruiting chromatin remodeling complexes that methylate and deacetylate histones of specific genomic sequences. Intergenic, noncoding transcripts may promote the expression of the surrounding gene by recruiting chromatin remodeling complexes that demethylate and acetylate histones in the wake of RNA polymerase II. Moreover, steric hindrance of sense transcription via antisense transcription and formation of RNA hybrids between noncoding transcripts and target transcripts may also lead to transcriptional suppression. Double-stranded RNAs may result in RNA interference, RNA masking, RNA hyperediting, and degradation.⁶⁵ These noncoding POP transcripts are not considered to be micro-RNAs, because their sequences range between 155 and 231 bp in length. POP2 (4 kb from mRNA AL832227) and POP12 (intron of EST BQ226050) are noncoding transcripts of unknown function¹⁴ with expression that correlated to clinical parameters, which suggests they may play a role in prostate pathology. POP1 (AK000023) and POP3 (50 kb from EST CFI40309), also noncoding transcripts,¹⁴ are of potential interest in prostate biology because of their restricted expression profiles, which may be useful for the identification that disseminated circulating/shed cells or metastatic lesions are of prostatic origin. Thus, further investigation of these

noncoding transcripts in prostate biology and pathology is warranted based on their prostate-specific expression, potential to regulate gene expression, and/or correlation to clinical parameters of prostate cancer.

Some of the most exciting biomarkers for prostate cancer are detected at the DNA or RNA levels. These include the following: micro-RNAs⁶⁶ in both tissue and blood; fusions between the untranslated region of the androgen-regulated gene *TMPRSS2* and the ETS gene family⁶⁷ in tissue; detection of the noncoding transcript PCA3 in urine^{68,69} and samples of circulating tumor cells from men with metastatic prostate cancer⁷⁰ by itself or in conjunction with PSA mRNA; and the enhancer of zeste homolog 2 (*EZH2*), which is associated with aggressive disease and poor survival for prostate cancer patients.⁷¹ The combination of gene expression profiles of *PCA3*, prostatein, and transient receptor potential cation channel subfamily M member 8, in concert with *EZH2*, was reported to provide additional prognostic power in a study of 106 patients with matched prostatectomy samples.⁷²

The present study has identified potentially promising novel biomarkers for the diagnosis/prognosis of prostate cancer and/or for the confirmation of prostatic origin of unknown metastatic cancer and/or disseminated/shed cells. Further development and confirmation of the usefulness of these markers in larger patient cohorts may potentially lead to clinical applications aimed at selective interventions with the goal to spare patients from unnecessary treatment but still provide radical curative treatment to those men who will develop clinically significant disease.

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