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Translational Research for Identifying Potential Early-stage Prostate Cancer Biomarkers

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Abstract. *Background/Aim: Prostate cancer (PCa) is one of the most common types of cancer in men. Prostate-specific antigen (PSA) is currently the only biomarker used to screen for the risk of developing PCa. Because PSA tests may show false positives, identifying novel PCa-specific biomarkers would improve prediction and diagnosis at an early stage. Previously, we identified a number of genes/microRNAs (miRNAs) in prostate tissue as potential biomarkers of chronic prostatitis in a rat model of chemical-induced prostatitis. The current study aimed to evaluate their potential for use as translational, diagnostic markers in humans. Materials and Methods: We performed quantitative polymerase chain reaction analysis using pathologically clear (normal) or confirmed PCa tissue samples from the same patients (N=18 per group). Results: Levels (relative fold changes) of bone morphogenetic protein 7 (BMP7) transcripts were significantly lower in PCa tissues, compared with clear tissues, in a paired t-test (p=0.0075). Although neural cell adhesion molecule 1 (NCAM1) transcripts tended to be altered in PCa tissues, statistically insignificant differences were observed (p=0.0521). No statistically significant differences were observed for the other genes/miRNAs analyzed in PCa tissues due to a high degree of individual variance in expression. Conclusion: Similar to the results previously observed in rats, changes in the levels of BMP7 and NCAM1 transcripts were evident in human PCa tissues, suggesting that these genes may serve as potential diagnostic biomarkers during the early stages of PCa. Further studies are needed to determine the potential use of these molecules as biomarkers.*

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Key Words: Humans, chronic prostatitis, genes, prostatitis, rats, prostate cancer tissues.

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Prostate cancer (PCa) is one of the most common types of cancer in men. Nonmetastatic PCa has an approximately 100% five-year survival rate, while the survival rate for metastatic PCa is only 30% (1). Therefore, early detection is crucial for patient survival. There is a compelling clinical need to identify novel biomarkers in the blood and/or urine for diagnosing diseases, monitoring drug treatments, and/or predicting the risk of developing prostate diseases. Currently, serum prostate-specific antigen (PSA) is the standard PCa screening test. However, some studies indicate that 70%-80% of men without PCa have elevated levels of PSA (false-positive) (2-5). As a result, a prostate tissue biopsy is necessary to accurately diagnose PCa. Affected patients will experience pain and bleeding. Several biomarker-based methods, [*i.e.*, prostate health index (PHI), 4Kscore test (total PSA, free PSA, intact PSA, and human kallikrein 2 (hk2)), Michigan Prostate Score (MiPS), *etc.*], which were developed to enhance PSA test results for diagnosis of PCa and/or metastasized PCa have been reviewed (6). However, none of these methods have been found to be suitable as predictive/prognostic markers for diagnosis and monitoring.

Identifying sensitive biomarkers would improve the prediction and diagnosis of PCa at an early stage. Several studies have conducted gene and microRNA (miRNA) expression profiling using patient samples to identify potential

biomarkers for prostate diseases (including cancer) (3, 4, 7-12). However, their use in PCa screening remains to be established. Our previous studies (13, 14) identified potential biomarkers for detecting the onset of prostate diseases using the rat model described by Ho *et al*. (15). This model was dosed with endocrine-disrupting chemicals and sex hormones to induce chronic inflammation of the prostate. Because chronic inflammation is thought to contribute to the development of PCa (16, 17), the genes/miRNAs we identified in the rat model have the potential to be used for screening during the early stages of PCa development in humans.

The purpose of the current study was to evaluate genes and miRNAs identified in the rat prostate model for their potential use as diagnostic markers in humans. As a preliminary experiment, this study verified the transcriptional profiles of the selected RNAs through quantitative polymerase chain reaction (qPCR) analysis of human prostate tissue. Due to the difficulties in obtaining prostate tissues from healthy men, this study utilized pathologically clear (normal) or confirmed PCa tissue samples from the tissue bank at the University of Arkansas for Medical Sciences (UAMS).

Materials and Methods

Materials. All reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

Human samples. This study using human samples was reviewed and approved by the National Center for Toxicological Research/U.S. Food and Drug Administration (NCTR/FDA) and the UAMS Institutional Review Boards (IRBs) (FDA, non-human subjects; UAMS, Human Subjects Research Determination (HSRD), IRB #261804). Human prostate tissues were obtained from the Tissue Biorepository and Procurement Service (TBAPS) at UAMS, Little Rock, AR, USA. All tissue bank samples were obtained with the patients' consent using a UAMS IRB-approved protocol (IRB #35954). Prostate tissues were obtained from patients by surgery and embedded into optimal cutting temperature (OCT) compounds for histological analyses. Pathologically clear prostate samples and PCa tissues from the same patients (*N*=18 per group) were used for the study. All subjects were over 45 years of age. Patient characteristics are shown in Table I.

RNA extraction and cDNA synthesis. RNA and miRNA were extracted from OCT-embedded frozen prostate tissues using the miRNeasy Kit (Qiagen, Valencia, CA, USA). After extraction, the concentration of each RNA sample was determined using a DS-11 spectrophotometer (DeNovix, Inc., Wilmington, DE, USA). Next, the cDNA for qPCR of gene expression changes was synthesized with a Super Script IV VILO Master Mix (Thermo Fisher Scientific Inc., Carlsbad, CA, USA) using 1 μg of RNA obtained from prostate tissues.

Gene expression profiling (RT-qPCR). We measured transcript levels of the predicted target genes using qPCR. Analysis was performed using an ABI ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific Inc.), according to the manufacturer's instructions. The synthesized cDNA served as a template in 10 μl reaction mixtures. Reaction conditions were as follows: initial denaturation at 50˚C for 2 min and 95˚C for 2 min, followed by 40-45 amplification cycles (95˚C for 15 s, 60˚C for 1 min) and a dissociation step (95˚C for 15 s, 60˚C for 1 min, 95˚C for 15 s). Relative-steady state transcript levels were calculated from threshold cycle (Ct) values using the following equation: relative quantity=$2^{-\Delta\Delta Ct}$ (18). Expression levels were normalized using the housekeeping gene *GAPDH* as an internal control for each sample. Relative ratios of transcript levels in each sample were calculated by setting the values for clear prostate tissues in each patient to 1. Reactions were performed in triplicate (*N*=18 per group). Specific primer pairs used in this study are shown in Table II.

Table I. *Characteristics of the patient cohort*.

Median age, years	66.5 (50-72)
Median Gleason score	7 (7-9)
Median PSA (ng/ml)	5.6 (1.79-44)

miRNA cDNA synthesis. Synthesis of cDNAs from samples (*N*=18/group) was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) using 10 ng total RNA and purified miRNA from human prostate samples.

miRNA qPCR. Analysis was performed using the ViiA7 Real-Time PCR System (Applied Biosystems) with TaqMan Universal Master Mix II and TaqMan Small RNA Assays (Applied Biosystems), according to the manufacturer's protocols. The TaqMan Small RNA assays used in this study are shown in Table III. Reactions (10 μl) used cDNA (synthesized as above) as a template. The reaction conditions for TaqMan Universal Master Mix were as follows: initial denaturation at 50˚C for 2 min and 95˚C for 10 min, followed by 40 amplification cycles (95˚C for 15 s, 60˚C for 1 min). Relative fold changes were calculated using Ct values, as described above and referenced elsewhere (18). As internal controls, expression levels were normalized using hsa-miR-26b and hsa-miR-92 (19). The relative ratios of transcripts in each sample were calculated by setting the values for clear tissues to 1.0; reactions were performed in triplicate (*N*=18 per group).

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analysis of qPCR data was performed on normalized ΔCt values for each gene and miRNA using a two-sided paired *t*-test and Wilcoxon signed rank test, respectively. All paired *t*-tests and Wilcoxon signed rank tests had 80% or greater statistical power. The paired *t*-test was selected for the comparison of mean differences between the two gene groups due to dependent/paired data from the same patients. In addition, the differences in ΔCt values for each gene were found to be normally distributed via the Shapiro-Wilk test. As the differences in the miRNA data were not normally distributed, the nonparametric Wilcoxon signed rank test was used to examine these differences. In addition, the non-parametric Spearman rank-order correlation test was used for

Table II. *Primer pairs for qPCR.*

Genes	Primer sequences	Amplified size (bp)	GenBank Accession/reference
hESRRG	For: 5'-CCT ACG CTA ACA CTG TCG CA-3'		
	Rev: 5'-GCT GGA AGG TTC CGT CTT GA-3'	108	NM_001134285.3
hNCAM1	For: 5'-GCA GCG AAG AAA AGA CTC TGG-3'		
	Rev: 5'-GCA GAT GTA CTC TCC GGC AT-3'	110	NM_000615.7
hITGA4	For: 5'-TCC ATG CTT CCT CCA TAA AGA CT-3'		
	Rev: 5'-TGT ATT TGG CAT TGG CAT TGT GT-3'	133	NM_000885.6
hNCF1	For: 5'-AGT ACC GCG ACA GAC ATC AC-3'		
	Rev: 5'-CGC TCT CGC TCT TCT CTA CG-3'	136	NM_000265.7
hBMPR2	For: 5'-TGG CAG CAG TAT ACA GAG TGA G-3'		
	Rev: 5'-TTG ACT TCA CAG TCC AGC GA-3'	150	XM_011511687.1
hBMP7	For: 5'-GGG TAG CGC GTA GAG CC-3'		
	Rev: 5'-GTT GTC CAG GCT GAA GTC GG-3'	128	NM_001719.3
hNOS2	For: 5'-AGG TCC AAA TCT TGC CTG GG-3'		
	Rev: 5'-ATC TGG AGG GGT AGG CTT GT-3'	81	NM_000625.4
hNFKB1	For: 5'-CTT AGG AGG GAG AGC CCA C-3'		
	Rev: 5'-ACA TTT GTT CAG GCC TTC CC ´-3'	105	NM_01165412.1
hCXCR6	For: 5'-ACC AAT GCC TTG CCA ACA AC-3'		
	Rev: 5'-GTT GGC CTG CTC TCC TTA CC-3'	95	XM_011533290.2
hCXCL13	For: 5'-TCC AAG GTG TTC TGG AGG TC-3'		
	Rev: 5'-TCT TGG ACA ACC ATT CCC ACG-3'	128	NM_001371558.1
hCCL2	For: 5'-AGA TCT GTG CTG ACC CCA AG-3'		
	Rev: 5'-GGA GTT TGG GTT TGC TTG TCC-3'	73	NM_002982.4
hGAPDH	For: 5'-AAG ACG GGC GGA GAG AAA CC-3'		
	Rev: 5'-CGT TGA CTC CGA CCT TCA CC 3'	140	NM_001289745.3

Table III. *TaqMan microRNA assay.*

Assay name	miRbase Accession #*	ID#	Mature miRNA	miRbase Accession #*
hsa-miR-329	MI0001725	001101	hsa-miR-329-3p; -5p	MIMAT0001629, MIMAT0026555
hsa-miR-26b	MI0000084	000407	hsa-miR-26b-5p	MIMAT0000083
hsa-miR-92	MI0000719	000430	mmu-miR-92a-3p	MIMAT0000539
U6 snRNA	NR_004394**	001973	U6 snRNA	

*miRbase version: version 22.1; **NCBI Accession number.

correlation analysis. Statistical analysis was performed using version 9.4 of SAS software (PASS, 2008; IBM, Armonk, NY, USA). *p*-Values of ≤0.05 were considered statistically significant.

Results

qPCR validation/identification of selected genes/miRNA in human PCa tissues. We selected the following genes and miRNA, which were shown to be associated with chronic inflammation in our previous study carried out in rats (12, 13): chemokines [CXC motif chemokine ligand 13 (*CXCL13*) and CC motif chemokine ligand 2 (*CCL2*), interleukin 7 receptor (*IL7R*)]; signaling pathway molecules [bone morphogenetic protein 7 (*BMP7*), bone morphogenetic protein receptor type 2 (*BMPR2*), nuclear factor kappa B subunit 1 (*NFKB1*), and nitric oxide synthase 2 (*NOS2*)]; cell adhesion molecules [integrin subunit alpha 4 (*ITGA4*) and neural cell adhesion molecule 1 (*NCAM1*)]; and miRNA hsa-miR-329 and its target gene, estrogen-related receptor gamma (*ESRRG*).

The qPCR data are shown in Figure 1. Transcript levels for *BMP7* were down-regulated in PCa tissues, compared with clear tissues; the difference was statistically significant (p=0.0075). The transcript levels of *NCAM1* gene were consistently altered in PCa tissues when compared with clear tissues. However, the *p*-value for the difference was greater than 0.05 (p=0.0521). No other significant differences (p≤0.05) in the expression of the remaining genes or miRNA

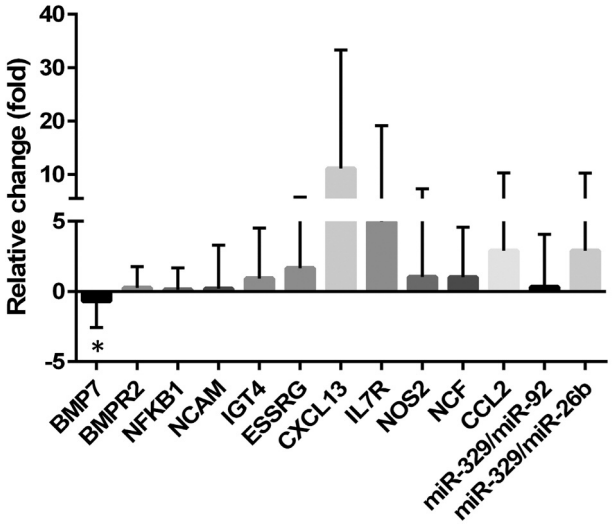

Figure 1. *Relative transcript levels of BMP7, BMPR2, NFKB1, IL7R, NCF, CXCL13, NOS2, CCL2, NCAM1, ITGA4, ESRRG, hsa-miR329/miR-92, and hsa-miR-329/miR-26b, in the PCa tissues Data are expressed as mean fold change ± standard error of the mean (N=18/group). *p≤0.05 compared to clean tissues.*

were observed. *CXCL13, IL7R,* and *CCL2* genes may reflect the high level of individual variance in expression (Figure 1).

We analyzed the correlation between PSA levels (currently PCa screening biomarker) and the gene expression profile to determine if the alteration of genes/miRNA expression found in this study was associated with PSA levels and if they can be used as potential biomarkers. A significant correlation between PSA and fold change was identified for *BMP7* (p=0.045); however, no correlations were observed for the other genes or miRNA analyzed in the PCa tissue samples (Table IV).

Discussion

This study evaluated the expression of several genes and miRNAs in human PCa tissues that were previously identified as having altered expression in rats with chronic prostatitis. These molecules were also associated with human PCa in other studies (Table V). We identified altered expression of *BMP7* and *NCAM1* genes in human PCa tissues, similar to our findings in rats (13, 14); however, the differential expression of the *NCAM1* gene in the PCa tissues was not statistically significant when compared to the clear tissues.

Roles have been suggested for both BMP7 and NCAM1 in PCa development. BMP7 has been shown to play a role in regulating epithelial-mesenchymal transition (in normal prostate tissues) and in inhibiting tumor growth (20). Levels of *BMP7* transcripts have also been observed to be down-regulated in human PCa cell lines and bone metastases (20-22). Furthermore, NCAM1 protein is known to be up-regulated in perineural invasion in PCa (23), while *NCAM1* gene expression has been shown to be related to perineural invasion of cancers in general, and PCa in particular (24). In addition, NCAM1 has been proposed as a potential biomarker and therapeutic target for such cancers as myeloid leukemia and breast cancer (25, 26). Gong *et al*. (27) validated NCAM1 was one of serum potential biomarkers for early diagnosis of PCa. NCAM was reported to be a target gene of miR-210, the levels of miR-210 were elevated in hypoxia induced by PCa, and there was a negative correlation between the expression of miR-210 and NCAM (28). Based on other publications (25-27), NCAM1 is a possible candidate for the biomarker of early diagnosis of PCa. As this study did not examine miR-210 expression levels in PCa tissues and did not obtain obviously statistical significances between the clear and PCa tissues, further studies are necessary to confirm this.

We confirmed that the paired *t*-tests for the ΔCt values obtained in this study were appropriately powered for all genes (fold difference effect size=1, power=88.5%, Figure 2A). In addition, the Wilcoxon signed rank test for the miRNA values was appropriately powered (fold difference effect size=1, fold difference effect size=3.5, power=80%; Figure 2B). However, there was substantial variation in the expression of many genes between individual samples, such that any alteration in the other genes (*BMPR2*, *NFKB1*, *IL7R*, *CXCL13*, *NOS2*, *CCL2*, *ITGA4*, and *ESRRG*) and hsa-miR329 in PCa tissues were not statistically significant (Figure 1). Genes related to inflammation (*IL7R*, *CXCL13*, *NOS2*, and *CCL2*) showed a wide variation between patient samples. Relevant to this, Prins (29) and Bosland *et al*. (30) found that sites of inflammation were invaded by mononuclear cells in animal studies. Therefore, it is possible that the wide range of expression for these genes (*IL7R*, *CXCL13*, *NOS2*, and *CCL2*) in PCa tissues observed in our study may reflect differences in mononuclear cell invasion, or genetic differences may be related to these changes. Further studies using pathological data to clarify this point are required.

Although increased transcript levels for the genes examined in our study have been reported in PCa (Table IV) (23, 31-36), we observed decreased expression in a number of these genes in some patients. This may be due to the use of advanced PCa samples (showing prostate cell invasion and migration/invasion of the bone marrow) in other studies compared to our use of mainly Gleason score 7 (medium-grade) cancer samples (15 samples with a Gleason score of 7, two samples with a Gleason score of 9, Table I). We observed a statistical significance of *BMP7* gene expression in the PCa samples when analyzed using samples with a Gleason score of only 7 (Table VI). This would suggest that lower grades of cancer may result in different expressions of these genes. Further experiments will be necessary to investigate this using different grade samples (Gleason scores <6, 7, and 8-10).

Table IV. *The expression of gene/miRNA identified in our rat studies in human prostate cancer tissues.*

Genes/miRNAs identified in rat prostate tissues with chronic prostatitis in our previous studies		Grade of prostate cancer patients	Altered expression in human tissues	References
Chemokines	*Cxcl13*	Prostate cancer cell migration	Up-regulated	(35)
	Ccl2	Prostate cancer/bone marrow invasion	Up-regulated	(31)
	Il7r	Prostate cancer cell invasion and migration	Up-regulated	(31)
NFKB signaling	*Nfkb1*	Carcinogenesis	Up-regulated	(32, 33)
Cell adhesion molecules	*ITGA4**	Prostate cancer cell adhesion and metastasis	Up-regulated**	(36)
	Ncam1	Prostate cancer invasion	Up-regulated	(23)
NO synthetase	*Nos2*	Prostate cancer	Highly up-regulated	(34)
BMP signaling	*Bmp7*	Prostate cancer	Down-regulated	(20)
miRNA	miR-329-3p and its target gene: *Esrrg*	Prostate cancer	Down-regulated	(38, 39)

*Protein expression; **regulated by prostate stem cell in prostate cancer cells.

Table V. *Correlation between PSA vs. relative fold changes in the prostate cancer tissues.*

Gene	Spearman correlation coefficients (ϱ)	Pr > |r|
BMP7	–0.493	0.045
IL7R	0.071	0.786
NFKB1	–0.226	0.384
CCL2	–0.027	0.918
NCAM1	–0.034	0.896
ITGA4	–0.137	0.599
BMPR2	–0.233	0.369
ESSRG	0.034	0.369
CXCL13	0.324	0.205
NOS2	–0.233	0.369
miR-329/miR-92	–0.118	0.653
miR-329/miR-26b	–0.115	0.660

There are a few potential limitations. One limitation of this study is the lack of confirmation of the results from previous rat studies (13, 14) using prostate tissues obtained from chronic prostatitis patients. The genes we examined in this study were based on the findings from our previous rat models with chronic prostatitis. We should use prostate tissues from chronic prostatitis patients to validate the previous findings in rat models; however, there are no prostatitis tissues available in any of the tissue banks. If we had used prostatitis tissues, there would be more genes with statistically significant differences. Moreover, the second limitation is limited availabilities of various Gleason score samples. We used clear and PCa samples from the same patients in this study. Due to this constraint, it was difficult to obtain appropriate sample size of prostate tissues with various Gleason grades. Another limitation is that we used

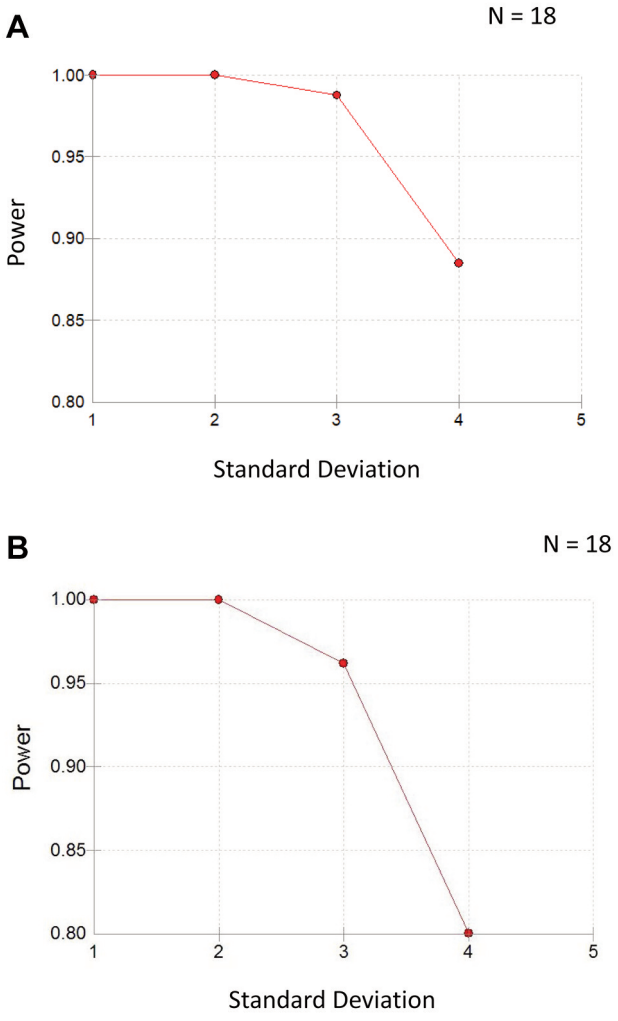

Figure 2. *Power versus standard deviation for a one-unit mean difference in a paired t-test (A) or a Wilcoxon signed rank test (B) with a sample size of 18, at a 0.05 level of significance.*

Table VI. *Statistical analysis of relative transcript levels of genes/miRNA in the prostate cancer tissues with Gleason Score 7 only.*

Gene/miRNA	*p*-Value
BMP7	0.032
IL7R	0.344
NFKB1	0.489
CCL2	0.650
NCF	0.590
NCAM1	0.156
ITGA4	0.863
BMPR2	0.549
ESSRG	0.616
CXCL13	0.368
NOS2	0.483
hsa-miR-329/miR-92	0.421
has-miR-329/miR-26b	0.720

whole prostate samples for RNA extraction and qPCR analysis since we have no laser-captured microdissection system. Luminal cells, one kind of epithelial cells of prostate, are considered to be origin for human PCa (37). If we would use specific cells and location, the results we could obtain would be clearer.

In conclusion, this study examined several genes and miRNAs previously identified as altered in rats with chronic prostatitis. We confirmed that the relative fold changes of *BMP7* (down-regulated) and *NCAM1* (further studies are required to verify the change in *NCAM1* gene) transcripts were similarly altered in human PCa tissues, compared with clear tissues from the same patients. Although we did not obtain samples from chronic prostatitis patients, current and previous findings suggest that these genes may serve as potential biomarkers for predicting prostatitis during early stages of PCa. No statistically significant differences were observed for the remaining RNAs, although other researchers have reported altered expression of these genes in PCa. Further studies are needed to determine the potential utility of these molecules as biomarkers and to evaluate whether the grade of PCa is associated with the level of gene expression. Together, such studies could enhance diagnosis of the severity of PCa.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

N.N. conceived and designed the experiments. N.N. performed the experiments (interpreting data and qPCR), analyzed the data, and wrote the manuscript. P.R. performed the statistical analysis. S.R.P. and M.E. provided human samples from the TBAPS. R.D. suggested ideas for this research proposal and interpreted data. N.N. finalized the manuscript, incorporating all co-authors' comments. P.R. edited the manuscripts. P.R., S.R.P., M.E., and R.D. provided feedback and critical comments. The views expressed are those of the Authors and do not necessarily reflect the views of the U.S. Food and Drug Administration.

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