



# A novel urinary mRNA signature using the droplet digital polymerase chain reaction platform improves discrimination between prostate cancer and benign prostatic hyperplasia within the prostate-specific antigen gray zone

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**Purpose:** The aim of this study was to identify a noninvasive urinary marker for prostate cancer (PCa) diagnosis and to validate the clinical performance of this novel urinary mRNA signature using the droplet digital polymerase chain reaction (ddPCR) approach.

**Materials and Methods:** A gene expression microarray (HT-12, Illumina Inc., USA) was used to identify genes differentially expressed between 16 PCa and 8 benign prostatic hyperplasia (BPH) tissues; ddPCR (QX200; Bio-Rad Laboratories, USA) was carried out to quantify the expression of selected genes in urine. The urinary molecular PCa risk score (UMPCaRS) was calculated by using the sum of three upregulated genes as the numerator and the sum of three downregulated genes as the denominator. The diagnostic utility of the UMPCaRS was validated by using a screening set (10 PCa and 10 BPH samples) and a validation set (131 PCa and 105 BPH samples).

**Results:** Three upregulated genes (*PDLIM5*, *GDF-15*, *THBS4*) and three downregulated genes (*UPK1A*, *SSTR3*, *NPFFR2*) were selected from the microarray and subjected to ddPCR. The UMPCaRS for PCa in the screening and validation sets was significantly higher than that for BPH. For the validation set, the diagnostic accuracy of the UMPCaRS was comparable with that of prostate-specific antigen (PSA). Importantly, in the "PSA gray zone" (3–10 ng/mL), the AUC for the UMPCaRS was 0.843 and that for PSA was 0.628 ( $p < 0.001$ ).

**Conclusions:** The data demonstrate that the UMPCaRS is useful for discriminating between PCa and BPH in the "PSA gray zone".

**Keywords:** Diagnosis; Microarray analysis; Prostatic neoplasms; Urine

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## INTRODUCTION

Although the prostate-specific antigen (PSA) test has undoubtedly increased detection rates for prostate cancer (PCa), one of its main drawbacks is its lack of specificity, which results in a high rate of negative biopsies [1,2]. The large population of men with elevated serum PSA levels but negative prostate biopsy findings presents a dilemma owing to the lack of an accurate diagnostic test [3,4]. Therefore, more reliable tests are needed that can identify patients at high risk of developing PCa and for whom repeat prostate biopsies are mandatory [1,5,6].

There is a growing trend toward exploring the use of minimally invasive “liquid biopsies” to identify biomarkers of PCa [7]. In particular, urine-based biomarkers are the subject of ongoing research because they represent a promising alternative or adjunct to serum-based biomarkers. This is because urine can be obtained easily in a convenient and noninvasive manner, and the fact that circulating cell-free DNAs, circulating RNAs (microRNAs, long noncoding RNAs, and mRNAs), cell-free proteins, and exosomes originating from tumor-derived necrosis or apoptosis are released into the urethra through the prostatic ducts [8-11]. Analysis of nucleic acid in urine samples is challenging for technical reasons, however. Assays must be highly sensitive because these molecules are present in urine at low levels [12]. When using quantitative real-time polymerase chain reaction (qPCR), the relative quantification method is used most commonly to identify urinary markers of PCa; however, there is a lack of reliable endogenous reference genes in urine [13,14]. In addition, the PCR efficiency of pure synthetic standards may differ from that of serum samples, which may contain factors that inhibit the PCR reaction [15]. In this context, digital droplet PCR (ddPCR) is a new high-throughput PCR platform that enables quantification of low concentrations of urinary nucleic acids [16,17]. The ddPCR platforms allow direct quantification without the need for a standard curve [12].

We aimed to identify a more practical and noninvasive urinary biomarker for PCa diagnosis. Using a microarray, we compared differentially up- or downregulated genes in PCa tissue with those in benign prostatic hyperplasia (BPH) tissue. We then quantified the selected genes in urine using ddPCR and evaluated the clinical performance of the candidate urinary mRNAs using expression ratio analysis rather than normalization against an internal control.

## MATERIALS AND METHODS

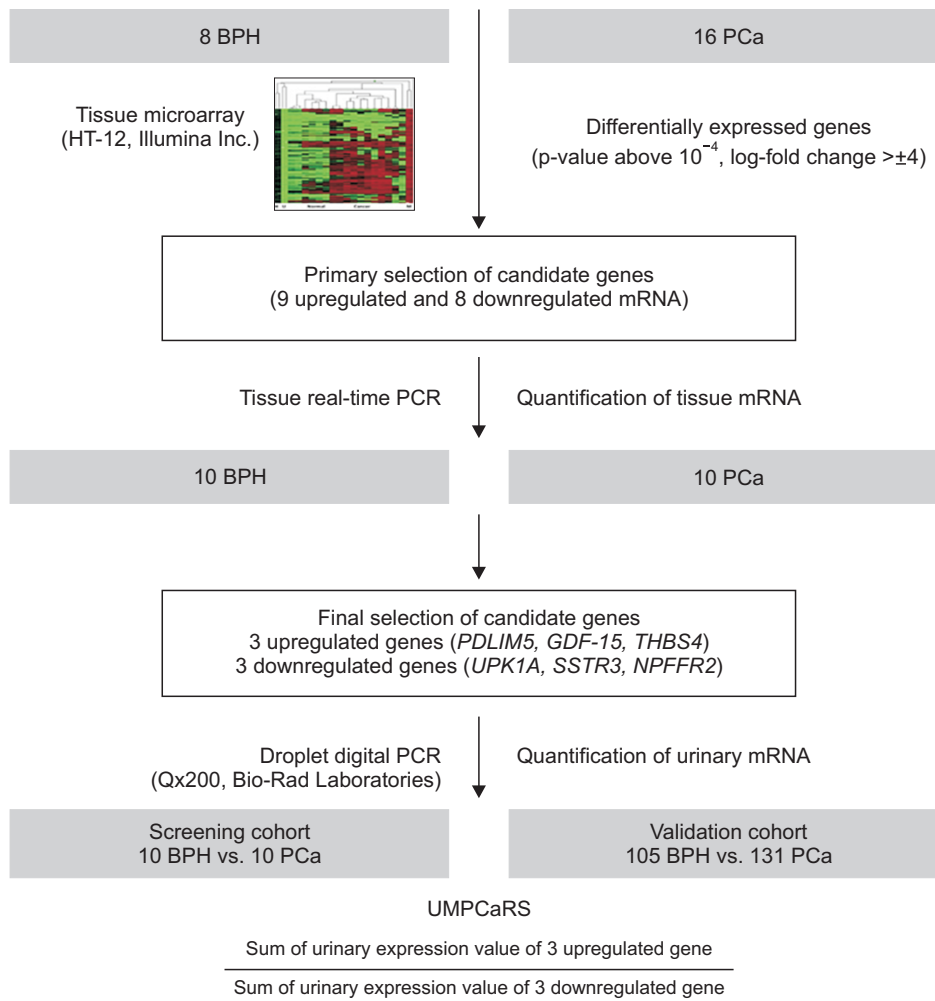
### 1. Patients and samples

The study complied with the applicable laws and regulations, good clinical practice, and the ethical principles described in the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Chungbuk National University (approval number: GR2010-12-010). All patients provided written informed consent before enrollment in the study. Sample collection and analysis procedures were approved by the Institutional Review Board of Chungbuk National University.

The study design and validation strategies are outlined in Fig. 1. Human prostate tissues from 8 individuals with BPH and 16 patients with PCa were used for the gene expression microarray (array set; Table 1). Further candidate genes were selected from 10 PCa and 10 BPH tissues. All tissues from patients with PCa were harvested during radical prostatectomy or palliative transurethral resection (TUR). BPH tissues were obtained from individuals who underwent TUR. Control patients with BPH with serum PSA levels  $\geq 3$  ng/mL underwent transrectal prostate biopsy before TUR to rule out the presence of cancer. Tissue samples were macrodissected within 15 minutes of surgical resection. Each prostate specimen was confirmed by analysis of fresh-frozen sections, and the remaining tissue was frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use. All tissue specimens were examined by an experienced senior pathologist. Expression of urinary mRNAs encoding candidate genes was measured by using a screening set (10 PCa and 10 BPH urine samples) and a validation set (131 PCa and 105 BPH urine samples) (Table 2). First morning-voided urine samples were collected before surgery. Urine samples were centrifuged at 2,500 rpm for 15 minutes and the supernatant was stored at  $-80^{\circ}\text{C}$  until use.

### 2. Tissue RNA isolation and microarray analysis

Total RNA was isolated from tissue by using TRIzol reagent (Life Technologies, Grand Island, NY, USA), according to the manufacturer's protocol. The quality and integrity of the RNA was confirmed by agarose gel electrophoresis and ethidium bromide staining, followed by visual examination under ultraviolet light. Biotin-labeled cRNA samples used for hybridization on an Illumina HumanHT-12 v4 Expression BeadChip (Illumina Inc, San Diego, CA, USA) were prepared according to Illumina's recommended sample labeling procedure. In brief, 250 ng of total RNA was used for complementary DNA (cDNA) synthesis, followed by an amplification/labeling step (*in vitro* transcription) to synthesize biotin-



**Fig. 1.** Study design and validation strategies. BPH, benign prostatic hyperplasia; PCa, prostate cancer; PCR, polymerase chain reaction; UMPCaRS, urinary molecular PCa risk score.

**Table 1.** Clinicopathologic characteristics of the array cohort

Variable	BPH	PCa	p-value
Number of patients	8	16	
Mean age (y)	72.6±7.5	70.6±8.3	0.571
PSA (ng/mL)	1.26±0.59	14.71±12.90	0.001
Operation			
TURP	8 (100.0)		
Radical prostatectomy		16 (100.0)	

Values are presented as number only, mean±standard deviation, or number (%).

BPH, benign prostatic hyperplasia; PCa, prostate cancer; PSA, prostate-specific antigen; TURP, transurethral resection of the prostate.

labeled cRNA, as described in the protocol for the Message-Amp II aRNA Amplification kit (Ambion Inc., Austin, TX, USA). The quality of cRNA was monitored by using the RNA Nano Chip Assay on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Microarray scanning was performed with a Beadstation array scanner (Illumina Inc.), with the settings adjusted to a scaling factor of 1 and

photomultiplier tube settings of 430. Data extraction was performed for all beads individually, and outliers showing MAD (median absolute deviation) >25 were removed. All remaining data points were used to calculate the mean average signal for a given probe and the standard deviation for each probe was calculated. All arrays were quantile normalized using Illumina BeadStudio software without background subtraction.

### 3. Tissue real-time PCR

To quantify the expression of each candidate gene, 10 PCa and 10 BPH tissue samples were subjected to RT-qPCR using a Rotor Gene 6000 PCR system (Corbett Life Science; Qiagen Inc., Valencia, CA, USA). qPCR reactions containing primers and SYBR Premix EX Taq (Takara Biotechnology Co., Ltd., Dalian, China) were performed in micro-reaction tubes (Corbett Life Science). Spectral data were captured and analyzed using Rotor-Gene Real-Time Analysis Software 6.0 Build 14 (Corbett Life Science). All samples were run in triplicate. Gene expression was normalized to the expression of *GAPDH*.

**Table 2.** Clinicopathologic characteristics of the screening and validation cohorts

Variable	Screening cohort			Validation cohort		
	BPH	PCa	p-value	BPH	PCa	p-value
Number of patients	10	10		105	131	
Mean age (y)	70.1±10.0	70.4±3.9	0.931	71.0±7.3	70.2±7.2	0.369
PSA (ng/mL)	2.07±1.01	19.75±5.99	0.016	4.14±3.81	54.92±135.63	<0.001
PSA range (ng/mL)						
≤10	10	3		97 (92.4)	60 (45.8)	
>10	0	7		8 (7.6)	71 (54.2)	
Operation						
TURP	10 (100.0)			105 (100.0)	53 (40.5)	
Radical prostatectomy		10 (100.0)			78 (59.5)	
UMPCaRS	0.25±0.30	3.27±1.28	<0.001	1.55±4.26	9.83±16.84	<0.001

Values are presented as number only, mean±standard deviation, or number (%).

BPH, benign prostatic hyperplasia; PCa, prostate cancer; PSA, prostate-specific antigen; TURP, transurethral resection of the prostate; UMPCaRS, urinary molecular PCa risk score.

#### 4. Urine RNA isolation and reverse transcription

Urine (0.5 mL) was obtained and total RNA was isolated using NucleoSpin® RNA virus F columns (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The integrity of isolated total RNA was checked using an RNA 6000 nano chip and a 2100 Bioanalyzer system. The RNA concentration was measured in a Quant-iT™ Ribogreen™ RNA Assay kit (Invitrogen, Carlsbad, CA, USA). Isolated RNA was converted to first-strand cDNA using the SuperScript™ III First-Strand Synthesis System (Invitrogen). Total RNA (500 ng) was used for reverse transcription in a final reaction volume of 20 µL. To increase the efficiency of cDNA synthesis, the reverse transcription reaction included a target gene-specific primer (this was the same primer used as the reverse primer for each target gene in the ddPCR reaction). The primers used in the amplification of each gene are shown in Supplementary Table 1.

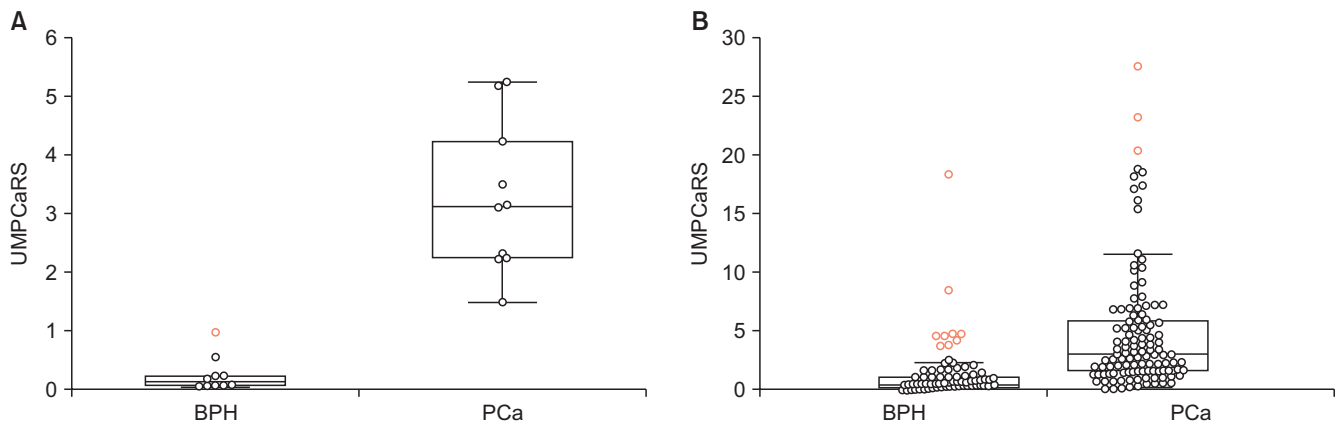
#### 5. Urinary gene expression by ddPCR

The ddPCR was performed in a total volume of 20 µL, containing 10 µL EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA), each primer set (final concentration, 150 nM), DNase/RNase-free sterile water, and 5 µL of the cDNA reaction mixture. The sequences of each primer are listed in Supplementary Table 1. Each ddPCR mixture plus 70 µL of generation oil for EvaGreen was loaded into each sample well of the DG8 droplet generator cartridge (Bio-Rad Laboratories). The cartridge was placed inside a QX200 droplet generator (Bio-Rad Laboratories). Droplets from each droplet well were transferred to a 96-well PCR plate. The PCR plate was placed in a C1000 thermal cycler with a deep well reaction module (Bio-Rad Laboratories) for amplification. Thermal cycling conditions were as follows: 5 minutes at 95°C,

followed by 40 cycles of denaturation for 30 seconds at 95°C, annealing/extension for 1 minute at 58°C, and three steps at 4°C for 5 minutes, 90°C for 5 minutes, and a 4°C infinite hold. After thermal cycling, the PCR plate containing the droplets was loaded onto the QX200 droplet reader (Bio-Rad Laboratories) to measure the fluorescence intensity of the EvaGreen fluorophore within each droplet using a multipixel photon counter. The detector reads the droplets and identifies those that contain a target gene (+) and those that do not (-). It then plots the fluorescence droplet-by-droplet. The QuantaSoft software (version 1.7.4, Bio-Rad Laboratories) was used to calculate the concentration (copies/µL) of the target genes.

#### 6. Statistical analysis

The urinary molecular PCa risk score (UMPCaRS) was calculated as the ratio of candidate genes with sum of three upregulated genes as the numerator and sum of three downregulated gene as the denominator. The Mann-Whitney U-test was used to compare the risk scores between the BPH and PCa groups. The diagnostic performance of the UMPCaRS was evaluated by plotting receiver operating characteristic (ROC) curves and calculating the area under the curve (AUC) to determine diagnostic accuracy. Differences were considered significant at  $p < 0.05$  and all reported p-values are two-sided. All analyses were performed using IBM SPSS 21.0 Statistics software (IBM Corp, Armonk, NY, USA).



**Fig. 2.** The UMPCaRS between BPH and PCa in the (A) screening and (B) validation cohorts. UMPCaRS, urinary molecular PCa risk score; BPH, benign prostatic hyperplasia; PCa, prostate cancer.

**RESULTS**

**1. Selection of candidate urinary mRNAs from a microarray**

Genes differentially expressed between 16 PCa and 8 BPH tissues were identified in the gene expression microarray (Table 1). We chose 17 mRNAs (9 upregulated and 8 downregulated mRNAs) on the basis of p-values  $>10^{-4}$  and a log-fold change  $>\pm 4$ . Further candidate genes were selected from 10 PCa and 10 BPH tissues using real-time polymerase chain reaction (Supplementary Table 2, Supplementary Fig. 1). Subsequently, three upregulated genes (*PDLIM5*, *GDF15*, and *THBS4*) and three downregulated genes (*UPK1A*, *SSTR3*, and *NPPFR2*) were selected for digital PCR (Supplementary Figs. 2, 3).

**2. Expression of urinary mRNA encoding the six candidate genes**

Next, ddPCR (QX200) was carried out to quantify the selected genes in urine. After screening (10 PCa and 10 BPH samples) and validation (131 PCa and 105 BPH samples), the calculated UMPCaRS for PCa was significantly higher than that for BPH (each  $p < 0.05$ ; Table 2, Fig. 2).

**3. Diagnostic and prognostic performance of the UMPCaRS**

ROC curve analysis was performed to evaluate the diagnostic value of the UMPCaRS and PSA. The diagnostic accuracy of the UMPCaRS was comparable with that of PSA in the total validation cohort (n=236). The AUC for the validation cohort UMPCaRS was 0.872, whereas that for PSA was 0.840 (each  $p < 0.001$ ; comparison of ROC curve,  $p = 0.353$ ; Fig. 3A). Importantly, however, the AUC for the UMPCaRS was 0.843 and that for PSA was 0.628 for samples that fell

in the “PSA gray zone” (3–10 ng/mL total PSA, n=91; each  $p < 0.05$ ; comparison of ROC curve,  $p < 0.001$ ; Fig. 3B).

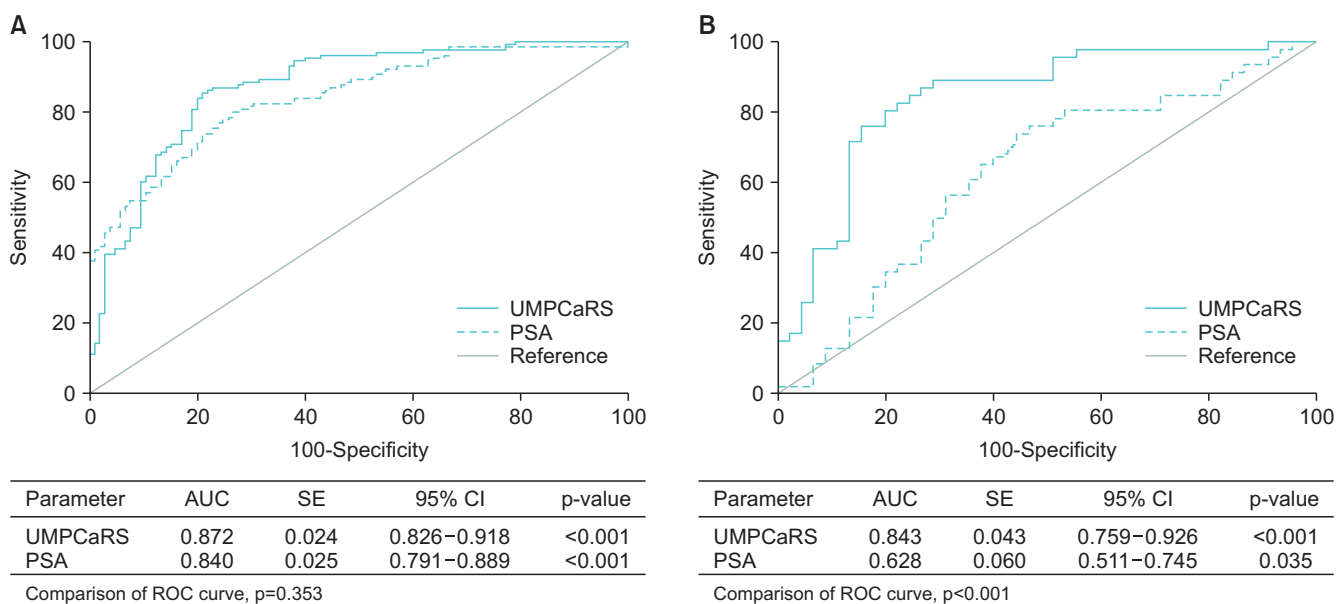
We also examined the association between the UMPCaRS and clinicopathologic characteristics of patients in the validation cohorts who underwent radical prostatectomy (n=78). There were no significant differences in clinicopathologic variables (preoperative serum PSA level, pathologic stage, or Gleason score) between the groups (Table 3).

**DISCUSSION**

PSA-based screening can reduce disease-specific mortality; however, unnecessary testing and over-diagnosis mean that further research is needed to identify specific biomarkers for PCa. Here, we used ddPCR to evaluate the clinical performance of urinary mRNA expression as a noninvasive urine-based marker for PCa diagnosis. We found that the ddPCR approach was useful for quantitating urinary mRNA. We used ddPCR to analyze the levels of six urinary mRNAs and used the results to calculate the UMPCaRS. This quantitative approach had accuracy comparable with that of the PSA test for PCa detection. Of particular clinical interest was the finding that the UMPCaRS was significantly better at discriminating between PCa and BPH within the diagnostic “gray zone” of total PSA (3–10 ng/mL).

Numerous promising biomarkers for PCa have been identified; these include genes specific for PCa (*AMACR* and *PCA3*) and recurrent gene fusions involving *TMPRSS2* and *ETS* family members (e.g., *TMPRSS2ERG*) [10,11,18]. The urinary prostate cancer gene 3 (*PCA3*) has also been used to select patients for repeat prostate biopsy [19]. A study that used different assay methods revealed that mRNA encoding *PCA3* can be quantitated in urine after prostatic massage or digital rectal examination [20]. Normalization to mRNA





**Fig. 3.** Receiver operating characteristic (ROC) curve for cancer detection in (A) the total validation cohort (n=236) and (B) patients with PSA levels between 3 and 10 ng/mL (n=91). UMPCaRS, urinary molecular PCa risk score; PCa, prostate cancer; PSA, prostate-specific antigen; AUC, area under the curve; SE, standard error; CI, confidence interval.

**Table 3.** Urinary molecular PCa risk score (UMPCaRS) according to PSA level, Gleason score, and pathologic stage in the validation cohorts who underwent radical prostatectomy (n=78)

Characteristic	Patient number	UMPCaRS	p-value
PSA (ng/mL)			0.166
≤10	49	11.68±19.77	
>10	29	6.92±10.20	
Pathologic Gleason score			0.094
≤7	57	7.18±11.44	
≥8	21	17.31±25.66	
Pathologic stage			0.675
T2	41	10.68±15.14	
T3–4	37	9.05±18.90	

Values are presented as number only or mean±standard deviation. PCa, prostate cancer; PSA, prostate-specific antigen. All p-values were calculated using Student's t-test.

encoding *PSA* allows calculation of a *PCA3* score, which has much greater specificity than serum PSA levels alone [21]. In addition, other RNA-based urinary biomarkers such as the *TMPRSS2-ERG* fusion gene, *GOLPH2* transcripts, and *SPINK1* (or a combination of these) have been the subject of many studies that show encouraging results [9,11,22]. Laxman et al. [18] used qPCR to examine the expression of seven multiplexing putative PCa biomarkers (*AMACR*, *ERG*, *GOLPH2*, *PCA3*, *SPINK1*, *TFF3*, and *TMPRSS2-ERG*) in urine and suggested that the multiplexed model may provide better specificity and sensitivity than *PCA3* alone. Those authors argue that single-marker tests, such as those

based on *PCA3*, ignore the heterogeneity of cancer development and may capture only a proportion of cancers [18]. Despite the effectiveness of multiplex RNA-based platforms, the high cost of multiple RNA assays inhibits widespread clinical application [8]. Quantification of urinary nucleic acid is technically challenging, owing mainly to the low amounts present [12]. Also, selecting the optimal gene for normalization remains an issue because few are expressed in a stable manner [14]. On the other hand, ddPCR has been suggested to provide higher sensitivity and accuracy than qPCR with less variation among technical replicates. This method partitions a conventional qPCR into water-in-oil droplets numbering up to 20,000, which permits the amplification of a single-template molecule in each droplet [16]. PCR-positive and PCR-negative droplets are counted at the end of the amplification procedure, thereby providing direct and absolute quantification of target DNA in a digital format [15,23,24]. The fact that ddPCR does not require a reference or a standard calibrator curve for quantification is one of its major advantages over qPCR [15,25,26]. It is proposed that ddPCR is less sensitive to differences in sample quality and to the presence of PCR inhibitors, two factors known to affect the efficiency of qPCR amplification [15]. Thus, comparing scores obtained in different laboratories using ddPCR may be simple and reliable, which is not the case for qPCR results; this is because the latter relies on the use of calibrators or endogenous reference miRNAs (and currently no consensus exists about the best ones to use) [15,27]. In this context, the present study used ddPCR to evaluate the clinical performance of a

urinary mRNA signature and identified six genes that are consistently up- or downregulated in PCa compared with BPH tissue. The UMPCaRS was successfully validated in an independent cohort. It is interesting to note that the UMPCaRS was able to discriminate early PCa (the gray zone of 3–10 ng/mL total PSA) from BPH. This is, at least in part, because the specificity of PSA is unreliable in the range of 3–10 ng/mL, leading to almost 70% to 80% of all subsequent prostate biopsies in this group being negative.

Ideal biomarkers should offer not only diagnostic accuracy, but also represent an aggressive tumor phenotype and provide prognostic information that facilitates optimal therapeutic decision-making. Another major challenge with current PSA screening is detection of non-life-threatening disease, which often results in over-treatment [9,28]. Although our data reveal high diagnostic accuracy in the range of 3–10 ng/mL, we found no significant association between the UMPCaRS and the clinicopathologic characteristics of PCa, which means that our urinary mRNA biomarker has limited value for predicting aggressive PCa.

This study had both limitations and strengths. Limitations include the single-center retrospective design, which could lead to selection bias. The small sample numbers may also reduce statistical power. Thus, further collaborative studies are required to validate the diagnostic accuracy and clinical application of the UMPCaRS as a PCa screening tool. Also, there is limited information in the literature about the specific functions of the six candidate genes. Additional studies are needed to define the precise biological functions of these novel biomarkers with respect to PCa development. Despite the above limitations, the present study represents an important step toward the clinical use of ddPCR for quantification of urinary mRNAs markers for PCa diagnosis.

## CONCLUSIONS

The present study shows that the ddPCR approach is useful for quantification of urinary mRNAs and demonstrates that the UMPCaRS can discriminate between cancer and BPH in cases that fall into the gray zone of 3–10 ng/mL total PSA. The UMPCaRS could represent a promising alternative or adjunct to serum PSA tests for PCa diagnosis.

## CONFLICTS OF INTEREST

The authors have nothing to disclose.

## ACKNOWLEDGMENTS

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## AUTHORS' CONTRIBUTIONS

Research conception and design: Ho Won Kang and Seok Joong Yun. Data acquisition: Young Joon Byun, Pildu Jeong, Jin Sun Yoon, and Dong Ho Kim. Statistical analysis: Ho Won Kang and Won Tae Kim. Data analysis and interpretation: Ho Won Kang, Jin Sun Yoon, and Won Tae Kim. Drafting of the manuscript: Ho Won Kang and Hee Youn Lee. Critical revision of the manuscript: Yong-June Kim, Sang-Cheol Lee, and Wun-Jae Kim. Administrative, technical, or material support: Seok Joong Yun and Wun-Jae Kim. Supervision: Dong Ho Kim, Yong-June Kim, Sang-Cheol Lee, Seok Joong Yun, and Wun-Jae Kim. Approval of the final manuscript: all of the authors.

## SUPPLEMENTARY MATERIALS

Scan this QR code to see the supplementary materials, or visit <https://www.icurology.org/src/sm/icurology-61-411-s001.pdf>.



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