

Development and validation of a three-marker urine DNA methylation assay complementary to mpMRI for pre-biopsy risk stratification of clinically significant prostate cancer

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Abstract

Background

Current prostate cancer (PCa) diagnostic strategies remain limited in accurately identifying clinically significant disease while avoiding unnecessary biopsy. This study aimed to develop and independently validate a non-invasive urine DNA methylation assay using naturally voided, non-digital rectal examination (non-DRE) urine and assess its complementary value with multiparametric magnetic resonance imaging (mpMRI) for detecting clinically significant prostate cancer (csPCa; Grade Group ≥ 2).

Methods

The biomarker discovery phase was initiated using microarray-based DNA methylation profiling to screen for tumor-specific alterations, and the candidate regions were subsequently validated by bisulfite-pyrosequencing in the same tissue specimens. Three prioritized markers—*CDYL*, *EMX2*, and *LRRC4*—were incorporated into a linear target enrichment quantitative methylation-specific PCR assay. Marker-specific thresholds optimized in a training cohort ($n = 33$) were locked and evaluated in an independent validation cohort of men undergoing biopsy for suspected PCa ($n = 97$). All participants underwent pre-biopsy mpMRI and standard prostate biopsy. Of the 97 validation patients, 89 were evaluable for combined-modality analysis, while 8 were excluded due to missing mpMRI scans.

Results

In the training cohort, the assay achieved 100% sensitivity and 31.6% specificity for csPCa. In the independent validation cohort, standalone assay performance yielded 75.9% sensitivity and 44.2% specificity. Among the 89 patients evaluable for both modalities (49 csPCa, 40 no-csPCa), mpMRI alone (PI-RADS ≥ 4) showed a sensitivity of 69.4%. Combining the urine assay and mpMRI using an either-positive strategy significantly increased diagnostic sensitivity to 91.8% ($P < 0.01$ vs. mpMRI alone), with a negative predictive value of 78.9%. In an exploratory analysis of the indeterminate PI-RADS 3 subgroup, the urine assay correctly identified all 4 biopsy-confirmed cases of csPCa (100% sensitivity).

Conclusions

The three-marker non-DRE urine DNA methylation assay demonstrates independent diagnostic performance and complementarity to mpMRI. Integrating this molecular assay with anatomical imaging significantly enhances csPCa detection compared with mpMRI alone. These findings support prospective evaluation of predefined, integrated MRI-urine methylation algorithms to refine pre-biopsy risk stratification and optimize biopsy decision-making.

Background

Prostate cancer (PCa) is one of the most commonly diagnosed malignancies in men worldwide and remains a major cause of cancer-related mortality [1, 2]. The clinical challenge in PCa detection is not simply to identify the presence of cancer, but to distinguish clinically significant PCa (csPCa), generally defined as Grade Group (GG) ≥ 2 , from indolent disease that may not require immediate intervention [3, 4]. Accordingly, contemporary diagnostic strategies increasingly aim to improve the detection of csPCa while minimizing unnecessary prostate biopsies and the overdiagnosis of low-risk disease.

Serum prostate-specific antigen (PSA) testing remains an important initial tool for identifying men who may require further evaluation. However, elevated PSA is not specific for malignancy and may occur in benign prostatic conditions, resulting in a substantial number of men undergoing biopsy without a diagnosis of clinically significant disease [5–7]. Multiparametric magnetic resonance imaging (mpMRI) has therefore become an important component of the pre-biopsy diagnostic pathway, improving lesion localization, risk stratification, and informing decisions regarding prostate biopsy. Nevertheless, mpMRI does not completely resolve diagnostic uncertainty. Clinically significant cancers may occur despite non-suspicious imaging, while equivocal PI-RADS 3 lesions remain particularly challenging for biopsy decision-making [8, 9]. These limitations support the need for complementary biomarkers that provide biological information independent of anatomical imaging.

Urine represents an attractive source of tumor-derived molecular material because of its anatomical relationship with the prostate and the potential for completely non-invasive sample collection [10, 11]. Among molecular alterations detectable in urine, aberrant DNA methylation is particularly promising because epigenetic changes occur during prostate carcinogenesis and can provide stable, amplifiable targets for molecular detection [12–16]. Several urinary molecular assays have consequently been investigated for PCa risk assessment. However, many previously developed urine-based approaches use specimens collected following digital rectal examination (DRE) to increase the release of prostate-derived cells and nucleic acids into urine [17–20]. Although such enrichment can improve target availability, dependence on DRE introduces an additional procedure and may complicate sample standardization and broad implementation. A sensitive assay capable of detecting PCa-associated methylation signals in naturally voided urine without prior DRE could therefore offer practical advantages for routine clinical use.

Importantly, the potential clinical value of such a molecular assay should be considered within the existing pre-biopsy diagnostic pathway rather than solely as a standalone test. Men with elevated PSA or other clinical risk factors commonly undergo mpMRI, and biopsy decisions are subsequently made by integrating imaging findings with PSA levels, clinical characteristics, and individual risk [21, 22]. Because molecular biomarkers and mpMRI interrogate fundamentally different aspects of disease—tumor-associated molecular alterations and imaging-detectable anatomical abnormalities, respectively—their combination may provide complementary information for csPCa risk stratification [8, 9]. A molecular assay may identify biological evidence of malignancy in some cancers that are not clearly detected by

imaging, whereas concordant molecular and imaging findings may provide greater confidence regarding the underlying disease risk. Establishing such complementarity is an important prerequisite before a combined biomarker-imaging strategy can be prospectively evaluated for its ability to guide biopsy decisions.

In the present study, we performed genome-wide DNA methylation profiling of PCa tissues using microarray analysis to systematically discover novel epigenetic biomarkers. Through this discovery pipeline, we developed a three-marker urine DNA methylation assay targeting *CDYL*, *EMX2*, and *LRRC4* using linear target enrichment quantitative methylation-specific PCR (LTE-qMSP). The assay was developed using naturally voided, non-DRE urine, with biomarker thresholds established in a developmental training cohort and subsequently evaluated without modification in an independent validation cohort of men undergoing biopsy for suspected PCa. We evaluated the diagnostic performance of the three-marker assay and investigated whether urine methylation provides complementary information to mpMRI for pre-biopsy risk stratification of csPCa.

Methods

Study population and clinical specimens

Paired prostate tumor and adjacent non-tumor tissues ($n = 13$ pairs) were obtained from radical prostatectomy specimens of patients with csPCa ($GG \geq 2$). These tissue specimens, consisting of four GG2 and nine $GG \geq 3$ cases, were provided by the Biobank of Chonnam National University Hwasun Hospital (Hwasun, Republic of Korea), a member of the Korea Biobank Network.

For clinical validation of the urinary biomarker assay, urine samples were prospectively collected from men aged ≥ 40 years undergoing an initial prostate biopsy due to suspected PCa, defined by a serum PSA level ≥ 4.0 ng/mL. All urine specimens were collected at Chungnam National University Hospital (Daejeon, Republic of Korea).

Pre-biopsy mpMRI was performed as part of routine clinical assessment when clinically available, followed by standard transrectal ultrasound (TRUS)-guided prostate biopsy according to institutional practice. Biopsy was performed as part of the clinical diagnostic evaluation and independently of the investigational urine methylation assay results. Biopsy histopathology was therefore used as the reference standard for evaluating the diagnostic performance of the urine methylation assay and its relationship with mpMRI findings.

To evaluate the feasibility of a fully non-invasive urine-based assay without prostatic manipulation, all urine samples were naturally voided and collected before biopsy without prior DRE. A total of 20 mL of urine was immediately mixed with an equal volume of proprietary stabilization buffer (Genomictree Inc., Daejeon, Republic of Korea) and stored at 4°C until DNA extraction.

Histopathological diagnoses for all tissue and biopsy specimens were assigned according to the 2014 International Society of Urological Pathology (ISUP) consensus grading system [23]. csPCa was defined as GG $\geq$ 2, whereas GG1 PCa was considered clinically non-significant disease and biopsy-negative participants were classified as the no-PCa group. For analyses of diagnostic performance for csPCa, GG1 and no-PCa cases were classified as no-csPCa. Urine samples were collected between August 2023 and May 2025. Baseline clinicopathological characteristics of the tissue and urine study cohorts are summarized in Table 1.

Table 1
Baseline clinicopathological characteristics of the developmental training and independent validation cohorts

Characteristic	Group	Developmental training cohort	Independent validation cohort
No. of participants	Biopsy-negative	18	40
Age, years, median (range)		70.5 (56–88)	69.5 (53–82)
PSA, ng/mL, median (range)		5.9 (4.1–14.4)	6.1 (4.0–44.6)
mpMRI, n (%)	PI-RADS 1–2	5 (27.8)	22 (55.0)
	PI-RADS 3	5 (27.8)	7 (17.5)
	PI-RADS 4	7 (38.9)	6 (15.0)
	PI-RADS 5	1 (5.6)	2 (5.0)
	Unknown	–	3 (7.5)
No. of participants	PCa	15	57
Age, years, median (range)		78.0 (59–85)	69.0 (58–88)
PSA, ng/mL, median (range)		7.8 (4.7–744)	9.1 (4.2–1,420)
mpMRI, n (%)	PI-RADS 1–2	0 (0.0)	16 (28.1)
	PI-RADS 3	3 (20.0)	1 (1.8)
	PI-RADS 4	4 (26.7)	21 (36.8)
	PI-RADS 5	8 (53.3)	14 (24.6)
	Unknown	–	5 (8.8)
ISUP Grade Group, n (%)	1	1 (6.7)	3 (5.3)
	2	2 (13.3)	17 (29.8)
	≥ 3	12 (80.0)	37 (64.9)
D’Amico risk group, n (%) ^a	Low	1 (6.7)	3 (5.7)
^a D’Amico risk classification was applicable to 53 patients with non-metastatic PCa in the independent validation cohort.			
^b Regional lymph node metastasis (cN1) or distant metastasis (cM1).			
ISUP, International Society of Urological Pathology; mpMRI, multiparametric magnetic resonance imaging; PCa, prostate cancer; PI-RADS, Prostate Imaging Reporting and Data System; PSA, prostate-specific antigen.			

Characteristic	Group	Developmental training cohort	Independent validation cohort
	Intermediate	3 (20.0)	24 (45.3)
	High	11 (73.3)	26 (49.1)
Regional or distant metastasis ^a , n (%)		–	4 (7.0)
^a D’Amico risk classification was applicable to 53 patients with non-metastatic PCa in the independent validation cohort.			
^b Regional lymph node metastasis (cN1) or distant metastasis (cM1).			
ISUP, International Society of Urological Pathology; mpMRI, multiparametric magnetic resonance imaging; PCa, prostate cancer; PI-RADS, Prostate Imaging Reporting and Data System; PSA, prostate-specific antigen.			

The study protocol was approved by the Institutional Review Board of Chungnam National University Hospital, and written informed consent was obtained from all participants.

Identification and tissue validation of candidate DNA methylation biomarkers

Genome-wide CpG DNA methylation profiling was performed using 13 paired PCa and adjacent non-tumor tissues to identify candidate loci showing tumor-associated differential methylation, as previously described [24]. Specifically, Human CpG Island 244k microarrays (Agilent Technologies, CA, USA) were used to profile DNA methylation. Raw microarray data were processed using Feature Extraction software v9.3.2.1 and GeneSpring software v7.3.1 (Agilent Technologies).

Tumor-associated differential methylation was assessed using analysis of variance (ANOVA) with Benjamini–Hochberg correction for multiple testing, followed by fold-change analysis. Candidate loci were prioritized when at least two adjacent CpG probes, allowing one intervening probe, demonstrated concordant tumor-specific hypermethylation within a CpG island.

Candidate hypermethylated genes identified through CpG microarray profiling were subsequently validated using bisulfite-pyrosequencing [25]. Candidate gene methylation was quantified at CpG islands within the promoter or 5' untranslated region (5' UTR). PCR and sequencing primers were designed using PSQ Assay Design software (Qiagen, Hilden, Germany), and the primer sequences are provided in Table S1.

Bisulfite-PCR was performed using 20 ng of bisulfite-converted DNA, followed by pyrosequencing on the PyroMark Q48 ID platform (Qiagen). The methylation index (Mtl) was calculated as the mean proportion of methylated cytosine across all analyzed CpG sites [mC/(mC + C)]. Tumor-associated methylation was

assessed by comparison with the corresponding matched adjacent non-tumor tissue, and negative (no-template) controls were included in each experiment.

Developmental training and independent validation cohorts

The clinical study population was divided into two sequential phases: a developmental training cohort and a subsequent independent validation cohort. The developmental training cohort (n = 33) comprised 14 men with biopsy-confirmed csPCa (GG $\geq$ 2), one man with GG1 PCa, and 18 no-PCa cases. This cohort was used to evaluate individual biomarker performance and to optimize biomarker-specific clinical cut-offs for the final three-marker panel.

The independent validation cohort (n = 97) comprised 54 men with csPCa (GG $\geq$ 2), three men with GG1 PCa, and 40 participants in the no-PCa group. To further assess assay specificity outside the biopsy-indicated clinical population, additional urine samples were collected from healthy volunteers (n = 16) and patients with benign urological conditions (n = 19), including renal cysts, urinary stone disease, and hydrocele.

DNA extraction and bisulfite conversion

Genomic DNA was extracted from tissue specimens using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). For urine specimens, DNA was isolated from 4.0 mL of stabilized urine using the GT NUCLEIC ACID PREP magnetic bead-based extraction kit (Genomictree Inc.). Bisulfite conversion of extracted DNA was performed using the EZ DNA Methylation Kit (Zymo Research, CA, USA) according to the manufacturer's instructions.

Three-marker urine DNA methylation analysis by LTE-qMSP

The final urine methylation assay consisted of *CDYL*, *EMX2*, and *LRRC4* and was performed using LTE-qMSP assays adapted from Bang et al [26]. *CDYL* and *EMX2* were simultaneously amplified with the reference gene *COL2A1* in a triplex reaction, whereas *LRRC4* was amplified with *COL2A1* in a separate duplex reaction. Bisulfite-converted DNA (18 μ L) was analyzed in a 25 μ L reaction containing target-specific forward primers, reverse primers carrying a universal tag sequence (UTS) at the 5' end, fluorescent hydrolysis probes, a UTS reverse primer, and *COL2A1* primers and probe. Primer and probe sequences and their concentrations are provided in Table S1.

LTE-qMSP reactions were performed using Fast qPCR PreMIX TaqMan Probe (Enzynomics, Daejeon, Republic of Korea) on the QuantStudio 5Dx platform (Thermo Fisher Scientific). Cycling conditions were as follows: 95°C for 5 min; 25 cycles of 95°C for 15 s and 70°C for 45 s; followed by 35 cycles of 95°C for 15 s and 60°C for 45 s. Each sample was analyzed using one triplex and one duplex LTE-qMSP reaction.

Relative methylation levels were expressed as $35 - \Delta C_T$, where ΔC_T was calculated as C_T (target) – C_T (*COL2A1*), with higher values indicating greater target methylation [26]. Undetected targets were assigned a value of 15, and laboratory personnel performing the methylation analyses were blinded to clinical and histopathological information.

Cut-off determination and optimization of the three-marker panel

Receiver operating characteristic (ROC) curve analysis was performed for each biomarker in the developmental training cohort to assess its ability to discriminate biopsy-confirmed csPCa from no-csPCa. Initial biomarker-specific cut-offs were determined using the Youden index and served as starting thresholds for subsequent panel optimization rather than as final clinical decision thresholds.

Given the intended clinical application of the assay for pre-biopsy risk stratification, these thresholds were subsequently refined in the developmental training cohort through a predefined iterative optimization process. Biomarker-specific cut-offs were adjusted and panel performance was re-evaluated to maximize sensitivity for csPCa detection while maintaining a prespecified specificity of $\geq 30.0\%$. Once this optimization criterion was achieved, the final biomarker-specific cut-offs were fixed and applied without modification to the independent validation cohort.

mpMRI interpretation

Pre-biopsy mpMRI images were independently reviewed by two experienced abdominal radiologists blinded to the urine methylation results. Target lesions were scored according to Prostate Imaging Reporting and Data System (PI-RADS) version 2.1 [27].

For the combination analysis, PI-RADS scores ≥ 4 were considered mpMRI-positive, whereas PI-RADS 3 findings were considered equivocal and were further examined to explore the potential contribution of the urine methylation assay in this subgroup.

Combined analysis of urine methylation and mpMRI

The diagnostic performance of the urine methylation assay, mpMRI, and their combinations were evaluated against biopsy histopathology. Two predefined combination strategies were evaluated. In the either-positive strategy, participants were classified as positive if either the three-marker urine methylation assay or mpMRI (PI-RADS ≥ 4) was positive. In the concordant-positive strategy, participants were classified as positive only if both the three-marker urine methylation assay and mpMRI were positive.

Statistical analysis

Statistical analyses were performed using MedCalc v20.115 (MedCalc Software, Belgium). Differences in tissue methylation levels between paired tumor and adjacent non-tumor tissues were analyzed using paired *t*-tests.

ROC curve analysis was performed to assess the discriminatory performance of individual urinary methylation biomarkers, and areas under the curve (AUC) with 95% confidence intervals (CIs) were calculated. The diagnostic performance of the urinary methylation assay alone, mpMRI alone, and the two predefined combination strategies was evaluated using biopsy histopathology as the reference standard. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), with corresponding 95% CIs, were calculated. Paired sensitivities between the urine methylation assay, mpMRI, and combined strategies were compared using McNemar's test, where appropriate.

For the combined strategies, the proportions of biopsies that could potentially be avoided and csPCa cases that would be missed were estimated to explore the trade-off between biopsy reduction and csPCa detection. All statistical tests were two-sided, with 95% CIs calculated where appropriate. A $P < 0.05$ was considered statistically significant.

Results

Tissue-based discovery and orthogonal validation of candidate DNA methylation markers

Genome-wide CpG DNA methylation profiling was initially performed using paired PCa and adjacent non-tumor tissues from 13 patients with csPCa. Candidate loci were selected based on preferential hypermethylation in PCa tissue, using a fold change (≥ 3.0) and statistical analysis ($P < 0.01$). This discovery analysis identified eight candidate DNA methylation markers (*CDYL*, *DAB1*, *DGKG*, *EMX2*, *FEZF2*, *FOXB1*, *LHX1*, and *LRRC4*) for further evaluation (Fig. S1). These genes met the predefined discovery criteria and had not previously been reported as DNA methylation biomarkers for PCa.

To confirm the differential methylation signals identified by microarray profiling, the candidate regions were subsequently evaluated using bisulfite-pyrosequencing in the same paired tissue specimens (Fig. 1). This orthogonal methylation analysis confirmed preferential hypermethylation of all selected candidate regions in PCa tissues relative to adjacent non-tumor tissues (all $P < 0.01$), supporting their further evaluation as potential urine-based biomarkers. MtIs ranged from 27.1% to 61.9% in tumor tissues compared with 5.6% to 28.7% in adjacent non-tumor tissues (Table S2). Among the eight candidates, six genes (*CDYL*, *DAB1*, *DGKG*, *EMX2*, *LHX1*, and *LRRC4*) showed tumor-associated methylation in all 13 tumor specimens (100%) and were therefore advanced for evaluation as potential urine-based biomarkers.

The six tissue-validated candidates were subsequently assessed for their suitability for non-invasive detection using the LTE-qMSP platform in naturally voided, non-DRE urine specimens. Among the six

candidates, *DAB1*, *DGKG*, and *LHX1* showed limited discriminatory performance or less favorable assay characteristics in the urine matrix and were not advanced further (data not shown). In contrast, *CDYL*, *EMX2*, and *LRR4* demonstrated more favorable urine-based discrimination and assay performance. Accordingly, these three markers were selected to constitute the final three-marker urine DNA methylation panel for subsequent cut-off optimization and independent clinical validation.

Study population

A total of 42 and 106 men were prospectively enrolled into the developmental training and independent validation cohorts, respectively. After exclusion for ineligibility or technical failure, 33 (78.6%) and 97 (91.5%) participants in the developmental training and independent validation cohorts, respectively, were included in the final analysis.

The developmental training cohort included 14 participants with csPCa, one with GG1 PCa, and 18 no-PCa participants, whereas the independent validation cohort comprised 54 participants with csPCa, three with GG1 PCa, and 40 no-PCa cases. Pre-biopsy mpMRI was available for all 33 participants in the developmental training cohort and 89 of 97 (91.8%) participants in the independent validation cohort.

Median age was comparable between the PCa and no-PCa groups, whereas serum PSA levels were higher in participants with PCa. PI-RADS 4–5 findings were more frequent among participants with PCa, and most cancers were classified as GG $\geq$ 3, whereas PI-RADS 1–3 findings predominated among no-PCa participants. Baseline demographic and clinicopathological characteristics of the study cohorts are summarized in Table 1. In addition, 19 participants with benign urological conditions and 16 healthy controls were evaluated as supplementary non-malignant control groups to assess assay specificity outside the biopsy-indicated clinical population; their characteristics are provided in Table S3.

Threshold development and locking in the training cohort

The three-marker panel was initially evaluated in the developmental training cohort for cut-off optimization and assessment of diagnostic performance. The distributions of marker-specific methylation levels across biopsy outcome groups in the developmental training cohort are shown in Fig. 2A.

Marker-specific thresholds were established using a predefined optimization procedure designed to maximize sensitivity for csPCa while maintaining an exploratory specificity of at least 30%. The resulting final cut-off values were 30.5 for *CDYL*, 38.5 for *EMX2*, and 40.0 for *LRR4*. Using these thresholds, the three-marker assay detected all 14 csPCa cases, corresponding to a sensitivity of 100% (14/14; 95% CI, 76.8–100.0%), with a specificity of 31.6% (6/19; 95% CI, 12.6–56.6%). The corresponding PPV and NPV were 51.9% (95% CI, 44.2–59.4%) and 100% (95% CI, 54.1–100%), respectively (Table 2).

Table 2

Diagnostic performance of the three-marker urine methylation assay for detecting clinically significant prostate cancer

Cohort	Sensitivity, % (n/N) (95% CI)	Specificity, % (n/N) (95% CI)	PPV, % ^a (95% CI)	NPV, % ^a (95% CI)	AUC (95% CI)
Developmental training (n = 33)	100.0 (14/14) (76.8–100.0)	31.6 (6/19) (12.6–56.6)	51.9 (44.2–59.4)	100.0 (54.1–100.0)	0.658 (0.473–0.813)
Independent validation (n = 97)	75.9 (41/54) (62.4–86.5)	44.2 (19/43) (29.1–60.1)	63.1 (55.7–69.9)	59.4 (45.0–72.3)	0.601 (0.496–0.699)
^a PPV and NPV reflect the observed csPCa prevalence in each cohort.					
AUC, area under the receiver operating characteristic curve; CI, confidence interval; csPCa, clinically significant prostate cancer; NPV, negative predictive value; PPV, positive predictive value.					

Following completion of the developmental analysis, the marker-specific thresholds and panel classification criteria were locked and subsequently applied without modification to the independent validation cohort.

Independent clinical validation of the three-marker urine assay

The three-marker assay was subsequently evaluated in an independent validation cohort of 97 men undergoing biopsy for suspected PCa. The distributions of marker-specific methylation levels according to biopsy outcome, together with those observed in the additional non-malignant control groups, are shown in Fig. 2B. The urine methylation assay identified 41 of 54 csPCa cases, corresponding to a sensitivity of 75.9% (95% CI, 62.4%–86.5%). Among the 43 participants without csPCa, 19 were classified as test-negative, yielding a specificity of 44.2% (95% CI, 29.1%–60.1%). The corresponding PPV and NPV were 63.1% (95% CI, 55.7–69.9%) and 59.4% (95% CI, 45.0–72.3%), respectively (Table 2).

To further assess assay specificity outside the biopsy-indicated study population, the locked assay was also evaluated in the additional non-malignant control groups included in Fig. 2B. Specificity was 78.9% (15/19; 95% CI, 54.5–93.9%) among participants with benign urological conditions and 93.8% (15/16; 95% CI, 69.4–99.8%) among healthy controls.

Diagnostic performance and complementarity of urine methylation and mpMRI.

Among the 89 participants evaluable for both modalities, 49 had biopsy-confirmed csPCa and 40 did not have csPCa. The urine methylation assay alone showed a sensitivity of 73.5% (36/49; 95% CI, 58.9–

85.1%) and specificity of 42.5% (17/40; 95% CI, 27.0–59.1%), whereas mpMRI alone showed a sensitivity of 69.4% (34/49; 95% CI, 54.6–81.7%) and specificity of 77.5% (31/40; 95% CI, 61.5–89.2%) (Table 3).

Table 3

Diagnostic performance of the urine methylation assay, mpMRI, and the either-positive strategy in the independent validation cohort

Test	Decision criterion	Sensitivity, % (n/N) (95% CI)	Specificity, % (n/N) (95% CI)	PPV, % ^a (95% CI)	NPV, % ^a (95% CI)
Three-marker urine methylation assay	At least one methylation marker positive ^b	73.5 (36/49) (58.9–85.1)	42.5 (17/40) (27.0–59.1)	61.0 (53.3–68.2)	56.7 (42.0–70.2)
mpMRI	PI-RADS $\geq$ 4	69.4 (34/49) (54.6–81.7)	77.5 (31/40) (61.5–89.2)	79.1 (67.4–87.4)	67.4 (56.8–76.5)
Either-positive strategy ^c	Urine assay positive or PI-RADS $\geq$ 4	91.8 (45/49) (80.4–97.7)	37.5 (15/40) (22.7–54.2)	64.3 (58.3–69.9)	78.9 (57.5–91.2)
The non-csPCa group comprised patients with GG1 PCa or a negative biopsy.					
^a PPV and NPV reflect the observed csPCa prevalence in the mpMRI-evaluable validation cohort.					
^b The urine methylation assay was considered positive when at least one of the three methylation markers was positive.					
^c The either-positive strategy classified a participant as positive if either the urine methylation assay or mpMRI (PI-RADS $\geq$ 4) was positive.					
CI, confidence interval; csPCa, clinically significant prostate cancer; GG, Grade Group; mpMRI, multiparametric magnetic resonance imaging; NPV, negative predictive value; PCa, prostate cancer; PI-RADS, Prostate Imaging Reporting and Data System; PPV, positive predictive value.					

When the predefined either-positive strategy was applied, 45 of 49 csPCa cases were identified, increasing sensitivity to 91.8% (95% CI, 80.4–97.7%) ($P < 0.01$ vs. mpMRI alone, McNemar test), with a specificity of 37.5% (15/40; 95% CI, 22.7–54.2%) and an NPV of 78.9% (95% CI, 57.5–91.2%) (Table 3). Compared with mpMRI alone, this strategy identified 11 additional csPCa cases (45 vs. 34 of 49), while four cases remained negative by both modalities.

The projected clinical consequences of the predefined combination strategies are summarized in Table 4. Under the either-positive strategy, 19 of 89 biopsies (21.3%) were projected to be deferred, with four of 49 csPCa cases (8.2%) missed. In contrast, the concordant-positive strategy increased the projected proportion of deferred biopsies to 64.0% (57/89), while detecting 25 of 49 csPCa cases (51.0%) and missing 24 (49.0%).

Table 4
Projected clinical consequences of mpMRI- and urine methylation-guided biopsy strategies

Strategy	Sensitivity, % (n/N)	Specificity, % (n/N)	csPCa missed, % (n)	Projected biopsies deferred, % (n)
Biopsy all	–	–	0 (0)	0 (0)
mpMRI alone (PI-RADS ≥ 4)	69.4 (34/49)	77.5 (31/40)	30.6 (15)	51.7 (46)
Urine methylation alone	73.5 (36/49)	42.5 (17/40)	26.5 (13)	33.7 (30)
Either-positive (mpMRI or urine)	91.8 (45/49)	37.5 (15/40)	8.2 (4)	21.3 (19)
Concordant-positive (mpMRI and urine)	51.0 (25/49)	82.5 (33/40)	49.0 (24)	64.0 (57)
Analyses included 89 participants with available mpMRI: 49 with csPCa and 40 without csPCa. Projected biopsy deferral was estimated retrospectively and was not prospectively observed. Either-positive was defined as positivity on either test; concordant-positive required both tests to be positive.				
csPCa, clinically significant prostate cancer; mpMRI, multiparametric magnetic resonance imaging; PI-RADS, Prostate Imaging Reporting and Data System.				

The potential contribution of urine methylation in patients not classified as positive by mpMRI was further explored using pooled data from the developmental training and independent validation cohorts. Among 18 patients with biopsy-confirmed csPCa and PI-RADS ≤ 3 , 14 (77.8%) were positive by the urine assay. All four patients with csPCa and equivocal PI-RADS 3 findings were urine methylation-positive (Table S4). Given the small number of patients, particularly in the PI-RADS 3 subgroup, these findings should be considered exploratory.

Discussion

In this study, we developed and independently evaluated a three-marker urine DNA methylation assay targeting *CDYL*, *EMX2*, and *LRRC4* for the detection of csPCa using naturally voided urine collected without prior DRE. The principal finding was that the urine methylation assay provided molecular information complementary to mpMRI in men undergoing evaluation for suspected PCa. Although the standalone performance of the urine assay was moderate in the independent validation cohort, integration of urine methylation status with mpMRI substantially increased sensitivity for csPCa compared with mpMRI alone. Importantly, all participants underwent prostate biopsy irrespective of their urine methylation or mpMRI findings. Thus, the present study does not demonstrate that biopsy can be safely omitted on the basis of the combined test. Rather, histopathological verification across the study population allowed evaluation of the complementarity between molecular and imaging information and provided a rationale for prospective testing of an integrated MRI–urine methylation strategy for biopsy decision-making.

The three methylation markers were identified through a sequential discovery and assay-development process beginning with genome-wide methylation profiling of PCa tissues. Aberrant DNA methylation represents a biologically plausible source of PCa biomarkers because epigenetic alterations occur during tumorigenesis and can generate stable molecular signals suitable for PCR-based detection [12–15]. *CDYL*, *EMX2*, and *LRRC4* have been implicated in chromatin regulation, cellular differentiation, and growth-associated signaling, respectively [28–30], providing biological context for their potential relevance to tumor-associated epigenetic alterations. Nevertheless, the present study was designed to establish their diagnostic utility as methylation biomarkers rather than to demonstrate a causal role for methylation-mediated silencing of these genes in prostate carcinogenesis. Further functional studies are needed to determine the mechanistic significance of these alterations in PCa.

An important practical feature of the assay is the use of naturally voided urine without prior DRE. Many urinary PCa biomarker approaches have used post-DRE urine to increase the release of prostate-derived cells and nucleic acids into the urinary tract. For example, urine-based assays such as PCA3-based tests, SelectMDx, and MyProstateScore 2.0 have generally relied on post-DRE urine collection, whereas ExoDx Prostate IntelliScore is designed for non-DRE urine collection [10, 31]. Avoiding DRE simplifies specimen collection and may facilitate standardized implementation in routine diagnostic workflows. This convenience, however, presents an analytical challenge because the abundance of prostate-derived tumor DNA in naturally voided urine may be relatively low. The LTE-qMSP platform was used to enhance detection of low-abundance methylated targets. The independent validation results therefore demonstrate the feasibility of detecting PCa-associated methylation signals from non-DRE urine, while the moderate standalone sensitivity and specificity also indicate that the assay should not presently be considered a replacement for established clinical assessment or imaging.

The distinction between developmental and independent validation performance is important. In the training cohort, thresholds were optimized to maximize csPCa sensitivity while maintaining a predefined minimum specificity, resulting in a sensitivity of 100% and specificity of 31.6%. When these thresholds were applied without modification to the independent validation cohort, sensitivity was 75.9% and specificity was 44.2%. This reduction in sensitivity is not unexpected when predefined thresholds are applied to an independent population and underscores the importance of reporting the validation results as the more reliable estimate of standalone diagnostic performance. Maintaining the prespecified thresholds preserves the independence of the evaluation and provides a more appropriate basis for subsequent prospective validation.

An important finding was the complementary performance of urine methylation and mpMRI. Among patients evaluable for both modalities, mpMRI alone showed a sensitivity of 69.4% for csPCa. When a positive result from either mpMRI or the urine methylation assay was considered test-positive, sensitivity increased to 91.8%, although specificity decreased to 37.5%. These findings demonstrate the expected trade-off between greater detection of clinically significant disease and lower specificity. More importantly, they suggest that the molecular assay may identify a subset of csPCa not clearly identified by imaging alone. This biological complementarity is plausible because mpMRI primarily identifies

structural and tissue-level abnormalities, whereas urinary DNA methylation reflects tumor-associated molecular alterations. The two modalities therefore need not identify the same cancers to provide clinical value.

Because every participant underwent biopsy, biopsy avoidance was not an observed clinical outcome. Any estimated reduction in biopsy based on retrospective application of MRI–urine decision rules should therefore be regarded as a projected clinical consequence rather than demonstrated biopsy avoidance. A rule requiring concordant positivity of both mpMRI and urine methylation would substantially reduce the projected number of biopsies but would also markedly reduce sensitivity for csPCa. Conversely, an either-positive rule increased csPCa sensitivity but reduced the proportion of biopsies that could potentially be deferred. These trade-offs highlight the need for an acceptable balance between avoiding unnecessary procedures and maintaining sufficiently high sensitivity for clinically significant disease.

The potential contribution of urine methylation may be particularly relevant in patients with indeterminate imaging findings. The urine assay was positive in all four biopsy-confirmed csPCa cases with PI-RADS 3 findings. Although the very small number of cases precludes definitive conclusions, this finding should be regarded as exploratory. PI-RADS 3 represents an important area of diagnostic uncertainty in which additional clinical, imaging, and molecular risk information may influence biopsy decisions. A larger prospective cohort enriched for patients with equivocal or non-highly suspicious MRI findings will therefore be required to determine whether urine methylation can meaningfully refine risk within this subgroup.

Importantly, the potential clinical value of such a molecular assay should be considered within the contemporary pre-biopsy diagnostic pathway rather than solely as a standalone test. In men with suspected PCa, biopsy decisions increasingly incorporate mpMRI findings together with PSA-based measures, clinical characteristics, and individual risk. Current European Association of Urology and American Urological Association/Society of Urologic Oncology guidelines recognize validated biomarkers as potential adjuncts to risk assessment and MRI-based biopsy decision-making [21, 22]. Within this context, a non-invasive urine DNA methylation assay may be most useful as an additional source of molecular risk information, particularly when imaging findings are equivocal or discordant with other clinical risk indicators.

Several limitations should be considered. First, the independent validation cohort was derived from a single tertiary center and was relatively small, with a high prevalence of csPCa compared with that expected in broader diagnostic populations. Consequently, predictive values and projected biopsy effects should not be directly extrapolated to population-based screening or lower-risk clinical settings. Second, the standalone specificity of the three-marker assay was modest, and increasing sensitivity through combination with mpMRI further reduced specificity. Optimization of the molecular panel or integration with additional clinical variables may therefore be required to improve the overall risk-benefit balance. Third, subgroup analyses, particularly those involving PI-RADS 3 lesions, included small numbers and required independent confirmation. Finally, the combination strategies were applied

analytically to patients who had already undergone biopsy; they were not prospectively used to determine whether biopsy should be performed.

These findings therefore support a clear next step. A prospective, multicenter clinical utility study should evaluate a predefined MRI–urine methylation decision algorithm before biopsy, with biopsy decisions and subsequent clinical follow-up incorporated into the study design. Such a trial should determine sensitivity and specificity for csPCa, the proportion of biopsies avoided, csPCa cases potentially missed or diagnosis delayed, and the clinical consequences of discordant MRI and urine methylation findings. Incorporation of established clinical variables such as PSA, PSA density, age, family history, and other relevant risk factors may further improve individualized biopsy decision-making.

Conclusions

In conclusion, the three-marker non-DRE urine DNA methylation assay demonstrated independent diagnostic activity for csPCa and provided information complementary to mpMRI. Combining molecular and imaging findings increased sensitivity for clinically significant disease compared with mpMRI alone, while highlighting the inevitable trade-off between cancer detection and potential biopsy reduction. The present results do not establish that biopsy can be safely omitted on the basis of the combined test; rather, they provide the diagnostic evidence and clinical rationale needed to prospectively evaluate whether an integrated MRI–urine methylation strategy can more accurately identify men who require biopsy and those in whom biopsy may safely be deferred.

Abbreviations

AUC: Area under the receiver operating characteristic curve; csPCa: Clinically significant prostate cancer; DRE: Digital rectal examination; GG: Grade Group; LTE-qMSP: Linear target enrichment quantitative methylation-specific polymerase chain reaction; mpMRI: Multiparametric magnetic resonance imaging; NPV: Negative predictive value; PI-RADS: Prostate Imaging–Reporting and Data System; PPV: Positive predictive value; PSA: Prostate-specific antigen; ROC: Receiver operating characteristic.

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Chungnam National University Hospital (IRB No. CNUH 2023-11-002-004). Written informed consent was obtained from all participants, and the study was conducted in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable

Availability of data and materials

The raw methylation microarray data generated during this study have been deposited in the Gene Expression Omnibus under accession number GSE306933. The clinical and urine methylation data supporting the findings of this study are available from the corresponding author upon reasonable request, subject to applicable ethical and privacy restrictions.

Competing interests

Tae Jeong Oh, JaeJin Lee, Min A Woo, Su Ji Kim, Yangyei Seo, and Sungwhan An are employees and shareholders of Genomictree, Inc. The other authors declare no competing interests.

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Authors' contributions

SA conceived and designed the study. JL, MAW, SJK, and YS acquired, analyzed, and interpreted the data. CLL, CY, and JHS analyzed and interpreted the patient data. TJO and CLL drafted the manuscript. SA, CLL, and JHS critically revised the manuscript for important intellectual content. TJO, JL, and YS performed the statistical analyses. TJO and YS provided administrative, technical, and material support. SA and CLL supervised the study. All authors read and approved the final manuscript.

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Figures

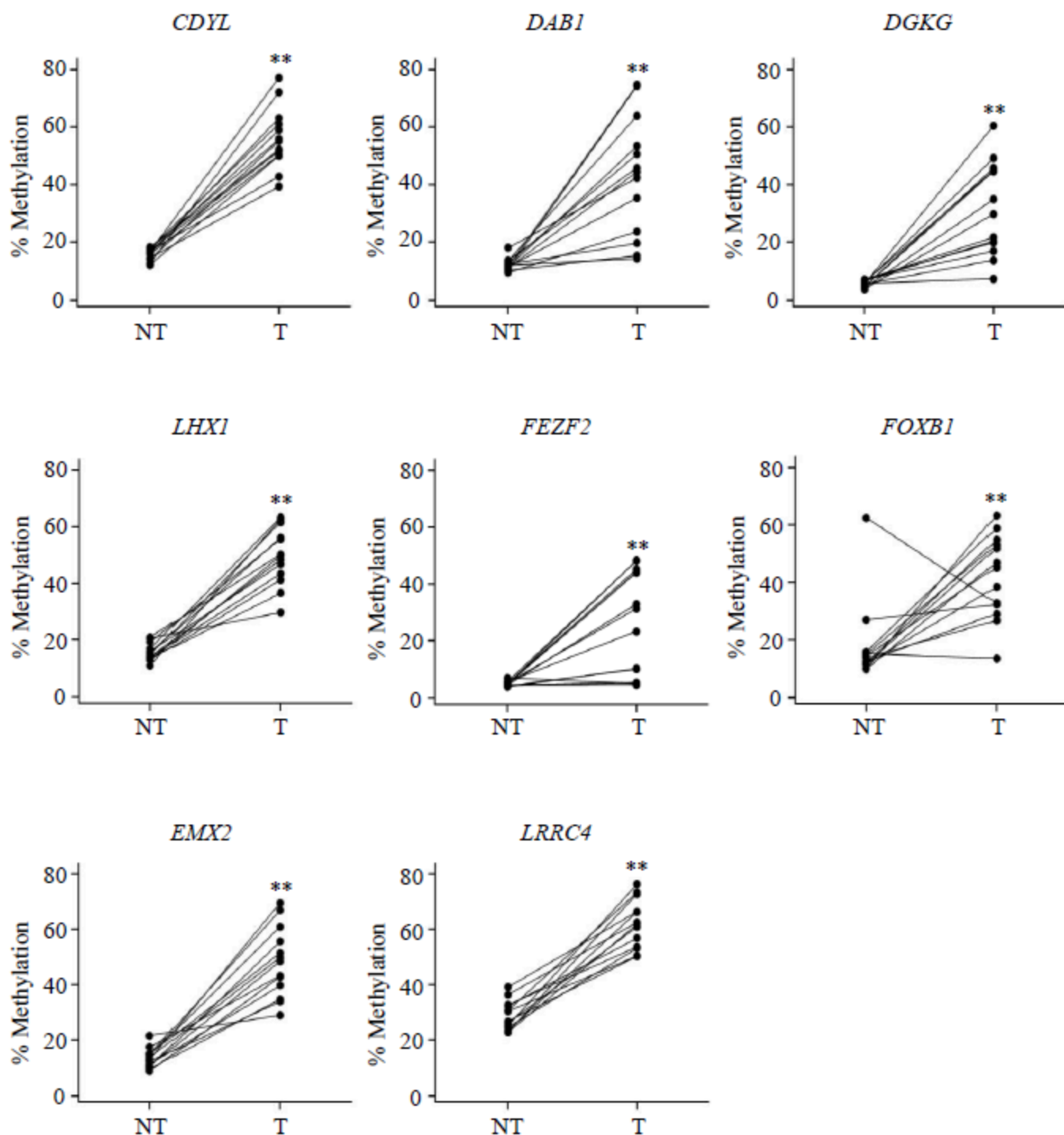
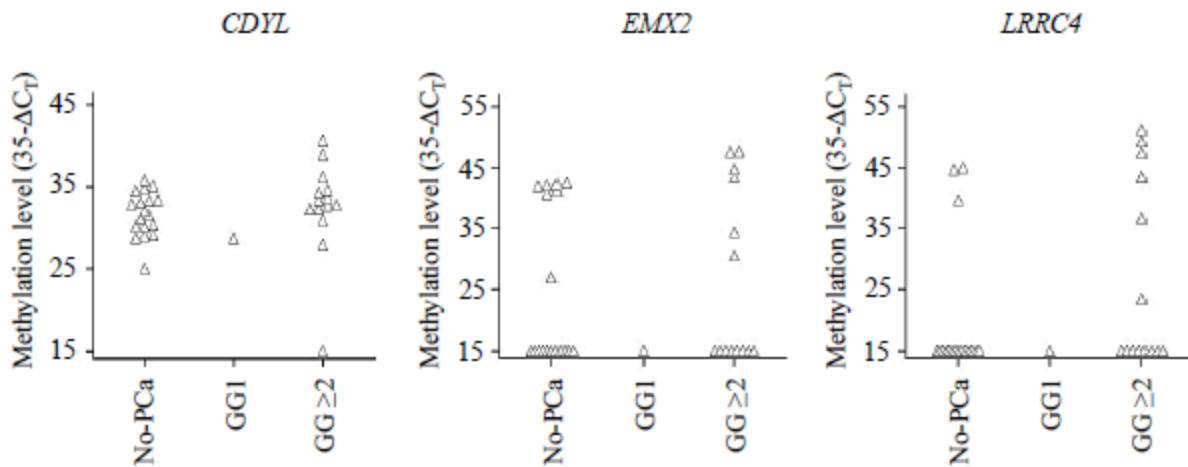
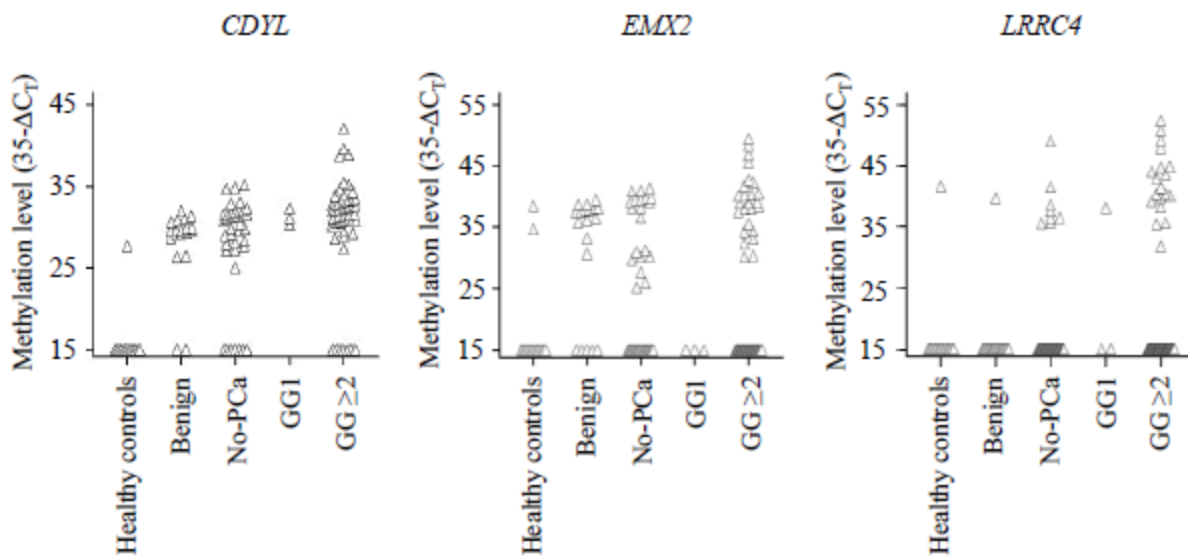


Figure 1

Validation of candidate methylation genes in prostate tissues. Methylation levels of eight candidate genes (*CDYL*, *DAB1*, *DGKG*, *LHX1*, *FEZF2*, *FOXB1*, *EMX2*, and *LRRC4*) were measured in paired prostate tumor (T) tissues and adjacent non-tumor (NT) tissues by bisulfite-pyrosequencing. Paired samples from the same patient are connected by lines. ** $P < 0.01$, paired t -test.

A**B****Figure 2**

Distribution of urine DNA methylation levels of the three biomarkers in the developmental training and independent validation cohorts. (A) Methylation levels of *CDYL*, *EMX2*, and *LRRC4* in the developmental training cohort according to biopsy outcome (no-PCa, GG1, and csPCa [GG ≥ 2]). (B) Methylation levels of the three biomarkers in the independent validation cohort according to biopsy outcome, with additional benign urological condition and healthy control groups shown for comparison. Methylation levels were measured by LTE-qMSP and expressed as $35-\Delta C_T$ values. Each symbol represents an individual urine

sample. csPCa, clinically significant prostate cancer; GG, Grade Group; LTE-qMSP, linear target enrichment quantitative methylation-specific PCR; no-PCa, no prostate cancer.

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