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Original Article

Epigenetic field alterations in non-tumor prostate tissues detect prostate cancer in urine

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Abstract: Prostate cancer (PC) development involves epigenetic DNA methylation changes that occur in the tumor. However, distinct DNA methylation changes have been previously found to encompass a widespread cancer field defect involving normal prostate tissue. In the current study, we analyzed a series of DNA methylation field markers to determine if they predict the presence of PC in urine. Urine samples were collected from patients undergoing prostate biopsy with biopsy-proven PC (90), and without PC (77). From the urine pellet, methylated DNA was quantified across several previously identified CpG island regions near the *caveolin 1 (CAV1), even-skipped homeobox 1 (EVX1), fibroblast growth factor 1 (FGF1), natural cytotoxicity triggering receptor 2 (NCR2) and phospholipase A and acyltransferase 3 (PLA2G16)* genes using bisulfite pyrosequencing. Univariate and multivariate analyses were performed. Urine cell pellets show significant increases in methylation in four of the markers from patients with PC compared to those without PC including *EVX1* 12.2 vs. 7.7%, *CAV1* 15.7 vs. 10.36%, *FGF1* 12.0 vs. 7.1%, and *PLA2G16* 12.2 vs. 8.3% [all P<0.01]. Area under the ROC Curve (AUCs) were generated for *EXV1* (0.74, Odds ratios (OR) 1.09; 95% confidence intervals (CI) 0.94-1.25, *CAV1* (0.72, OR 1.18; 95% CI 1.09-1.28) and *PLA2G16* (0.76, OR 1.35; 95% CI 1.199-1.51). In combination, a two-marker assay performs better than prostate specific antigen (PSA), AUC 0.77 vs. PSA AUC of 0.6 (P = 0.01) with the lowest error. In addition, *FGF1* distinguished between grade group 1 (GG1) and higher grade cancers (P<0.03). In conclusion, applying methylation of field defect loci to urine samples provides a novel approach to distinguish patients with and without cancer.

Keywords: Prostate cancer, DNA methylation, urine

Introduction

Prostate cancer (PC) is the most commonly diagnosed cancer in US males, but its detection remains a challenge [1]. The predominant screening tool for PC is prostate specific antigen (PSA) measurement, but this test is limited by its low specificity and inability to distinguish higher grade prostate cancer from lower grade disease [2]. Magnetic Resonance Imaging (MRI) has improved the ability to detect higher volume cancers, but it can miss up to 30% of significant tumors [3]. Up to 70% of men presenting with increased PSA value between 4-10 ng/dl will have negative biopsy subjecting them to the potential risks of infection and bleeding associated with a biopsy [4]. Urine has potential as a biospecimen source to help identify cancer avoiding invasive procedures.

Histologically, PC has been shown to be present in multiple foci within the prostate with an overall incidence of multifocal PC detected by whole-mount examination of the prostate of 60-90% [5]. This multifocality suggests that preneoplastic molecular alterations may exist even in histologically normal prostate tissue supporting the concept of field defect hypothesis associated with prostate cancer tumorigenesis [6]. Furthermore, PC development and progression is driven by the interplay of genetic and epigenetic changes that include DNA methylation. Methylation of clusters of CpG dinucleotides (termed CpG islands or CGIs) near the promoter region of genes is the most thoroughly studied epigenetic alteration [7]. These epigenetic DNA methylation alterations are a superb source of biomarkers that can be detected by polymerase chain reaction (PCR)

based assays, and likely occur early and persist throughout tumorigenesis [8]. We previously shown that using unbiased methylation microarray approach, CpG regions in the caveolin 1 *(CAV1),* even-skipped homeobox 1 *(EVX1),* fibroblast growth factor 1 *(FGF1),* natural cytotoxicity triggering receptor 2 *(NCR2)* and phospholipase A and acyltransferase 3 *(PLA2G16)* genes showed aberrant DNA methylation changes in histologically normal tumor-associated prostate tissues. These widespread epigenetic field defect changes can be used to detect PC in patients using histologically negative biopsies [9, 10].

Liquid biopsy approaches using urine for PC commonly utilize biomarkers that rely heavily on the shedding of PC cells and/or their constituents in urine [11, 12]. However, the detection of cancer cells in urine has been hindered by their infrequent shedding leading to low sensitivity [13]. In contrast, non-tumor prostate cells are found more frequently in urine (14-20%) [14]. In this study, we examined whether DNA from tumor-associated benign prostate cells harboring epigenetic methylation changes can be detected in urine and whether these methylation markers could improve the detection of PC.

Materials and methods

Subjects

The University of Wisconsin Institutional Review Board approved utilization all the urine samples in this study, and written and informed consents were obtained from all patients, (IRB #: 2017-0329-CR001). Urine samples were collected at the time of prostate biopsy procedure from 2014-2016. Ninety urine samples were collected from patients with confirmed PC, termed tumor associated (TA, media age 65 yrs, Grade Group GG ≥1), and 77 were from patients without PC on their biopsy and termed non-tumor associated (NTA, media age 64.0 yrs). NTA patients selected for the study all had a previous negative biopsy within the previous three years to help confirm the absence of cancer. MRI was not required for participation in the study. For each urine sample, samples were spun and pelleted, flash frozen, and purified genomic DNA generated (IBI, Valley Park, MO). Chemotropic salt was used to lyse cells (urine pellet) and allow DNA to bind to the genomic DNA spin column. Contaminants were effectively removed using wash buffers, followed by pure genomic DNA was eluted. All procedures were performed according to manufacturer's instructions.

Prostate cell counts and PSA staining

To determine the presence of prostate epithelial cells in the voided urine samples, we randomly selected 5 urine samples for further examination to examine the percentage of prostate cells using PSA immunohistochemistry (IHC) staining. Urine pellets were re-suspended in to ~200 ul of saline and cytospun on slides after being fixed and permeabilized. The urine cells were stained with anti-human PSA antibody (10 μg/ml) and detected with fluorescence-conjugated 2nd antibody. Total cell count was first done under bright field microscopy and then prostate cells were counted as fluorescence-positive cells. Five areas were analyzed for each sample, magnification (10× & 20×), and the prostate cell number was presented as percentage.

DNA methylation assay

DNA bisulfite modification was performed using the EpiTect Plus FFPE Bisulfite Kit (Qiagen, MD). We studied the methylation patterns at CpG shores regionally associated with the following genes: *CAV1, EVX1, FGF1, NCR2,* and *PLA2G16* using a methylation sequencing technique previously described [15]. Bisulfite-modified DNA was amplified using PCR with one primer-biotinylated. The PCR products were confirmed with 2% agarose gel. The biotinylated PCR products were captured with streptavidin sepharose beads, denatured to single strand, and annealed to the sequencing primer for the pyrosequencing assay. Human Premixed Calibration Standard with different percentages of methylation was used as controls in each run. Methylation was quantified with the PyroMark MD Pyrosequencing System within the linear range of the assay.

Statistical analysis

The methylation at each CpG site was expressed as a percentage. All samples were run in duplicate (three independent experiments) and the two values were averaged. A two-tailed t-test was performed to analyze the significant

Table 1. Clinical and pathological characteristics of the study cohort involving urine from patients with cancer (TA) and non-tumor associated (NTA) patients

	NTA	TA	All	*P*-value
Patients	77	90	167	
Age [yr]	64.0 [60.0, 67.5]	65.0 [58.8, 68.3]	64.0 [60.0, 68.0]	0.27
PSA [ng/mL]	6.8 [5.0, 9.8]	7.8 [5.0, 11.5]	7.5 [5.0, 10.8]	0.024
Prostate Size [g]	50.0 [35.0, 67.0]	38.0 [27.0, 54.0]	46.0 [29.8, 59.3]	0.010
PSA Density [ng/mL/g]	0.14 [0.09, 0.22]	0.18 [0.13, 0.34]	0.16 [0.10, 0.25]	<0.001
Number of Cores Involved	---	3 [2, 5]		
Max Core Involvement [%]	---	50 [15, 80]		
Ethnicity				
Caucasian	88% [68/77]	98% [88/90]	93% [156/167]	
Abnormal Digital Rectal Exam [%]	13% [10/77]	16% [14/90]	14% [24/167]	
Grade Group (GG)				
GG 1	---	31		
GG 2	---	24		
GG 3	---	16		
GG 4	---	14		
GG 5	---	5		

Data shown as median and interquartile range.

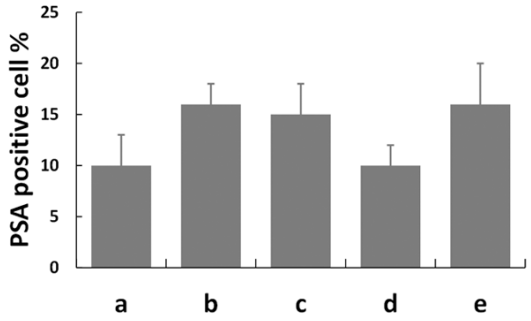

Figure 1. Cells (%) in urine immunostaining for PSA protein indicating prostate origin. Urine samples from 5 patients (a through e) were cytospun and immunostained with anti-human PSA Antibody. The average number of epithelial cells per 5 high power field (HPF) is demonstrated.

differences between NTA and TA groups. All CGs which significantly differentiated NTA from TA ($P<0.05$) were entered into a univariate logistic regression model to test their ability to predict the presence of cancer. Odds ratios (OR), 95% confidence intervals (CI), area under the ROC curve (AUC) and *P*-values were calculated. We then selected for each significant gene the CpG site with the highest AUC value and performed a Pearson correlation analysis to rule out significantly correlated variables. Multivariate analyses with combinations from one to three remaining markers were then performed. The models were also compared using Akaike's information criterion (AIC) which is an estimator of prediction error. The highest AUC and lowest AIC was compared its predictive ability to detect PC in our patient cohort vs. PSA or single marker alone.

Results

Urine samples were sequentially collected from 167 men who presented to our clinic with an elevated PSA and were scheduled for prostate biopsy. **Table 1** summarizes the patients' clinicopathologic characteristics. PSA values were higher in the tumor associated (TA) group than in the non-tumor associated (NTA), *P* value = 0.02, while prostate volume was greater in the NTA group (*P* value = 0.01). PSA density was higher in the TA group vs. NTA (*P* value <0.001).

We initially examined the frequency of cells of prostate epithelial origin in the urine samples by evaluating the number of PSA positive cells as described. Immunohistochemistry staining with anti-human PSA antibody to detect cells of prostate origin was performed on five urine samples. The median percentage of shed prostate cells in the urine staining positive for PSA was 15 with an interquartile range between 10-16% (**Figure 1**). Thus, the cells shed into the

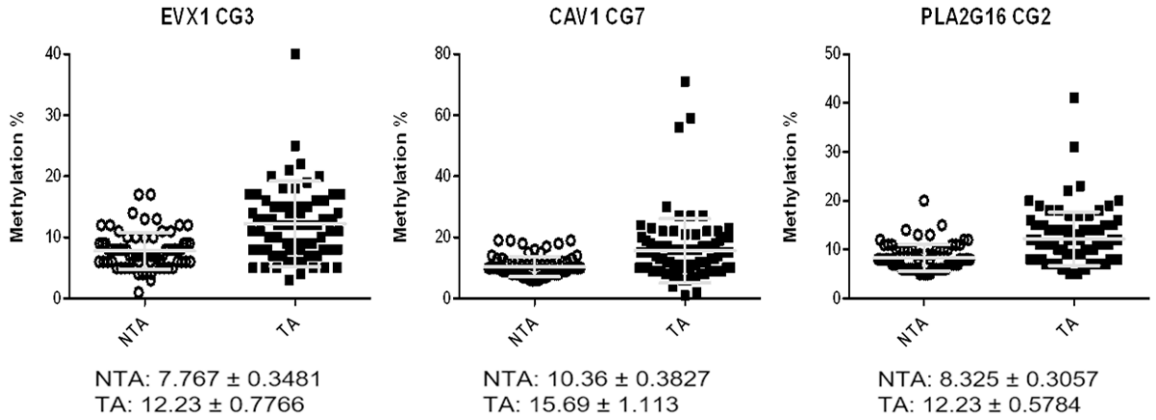

Figure 2. Methylation of each locus in non-tumor associated (NTA) and tumor associated (TA) urine samples. Methylation was analyzed using quantitative bisulfite sequencing performed in duplicate for 3 separate experiments, shown as Mean ± SEM, all are statistically significant, P<0.01.

urine of prostate origin represent a minority of overall cells.

We have previously validated that using an unbiased methylation microarray approach that CpG regions in the *EVX1, FGF1, NCR2, CAV1* and *PLA2G16* genes showed aberrant DNA methylation changes in histologically normal tumor-associated prostate tissues [10, 16]. We sought to determine whether the increase in DNA methylation found in non-tumor biopsy samples from patients with associated cancer could be detected in urine using quantitative bisulfite sequencing. Urine cell pellets were analyzed from 167 patients obtained at the time of prostate biopsy. TA samples (90) show significantly increased methylation at CpG island shores associated with *CAV1, EVX1, FGF1* and *PLA2G16* compared to patients without PC (**Figure 2**). Methylation results for all tested loci are listed in **Table 2**. Methylation changes at CpG sites associated with *NCR2* were not predictive for PC presence in urine samples.

Methylation at all loci were then individually examined for their association with grade specifically comparing methylation in indolent Grade Group 1 (GG1) tumors (n = 31) compared to higher grade cancers (n = 59). At *FGF1* CG1 and CG3 methylation correlated with grade (p=0.02) while *CAV1* approached significance at several sites (**Table 3**).

Univariate logistic regression analyses were then performed on specific CG sites within these genes (**Table 4**) to predict the presence of cancer. Receiver operating characteristic curve (ROC) curves were generated for those loci showing the greatest ability to detect cancer including *EXV1* (0.74, OR 1.09; 95% CI 0.94-1.25), *CAV1* (0.72, OR 1.18; 95% CI 1.09-1.28) and *PLA2G16* (0.76, OR 1.35; 95% CI 1.199-1.51) (**Figure 3A-D**).

Selecting the locus with the highest AUC for each gene regions, we then performed a correlation analysis and found that *EVX1* CG3 and *PLA2G16* CG2 had a high correlation with a value of 0.77. In a multivariate analysis we examined combinations of one to three markers and found the combination of *EVX1* CG3 and *PLA2G16* CG2 had the highest AUC with 0.77 (**Table 4**). Akaike's information criterion (AIC) which is an estimator of prediction error was lower (AIC = 179) for the *EVX1* CG3 and *PLA2G16* CG2 combination performing better than any single marker-*PLA2G16* CG2 alone (AIC = 196). Using this combined two-marker assay also performed better than PSA alone with AUC of 0.77 versus PSA AUC of 0.61 (P = 0.01) (**Figure 3E**). These data indicate that markers associated with an epigenetic field defect can detect PC in the urine of patients with elevated PSAs.

Discussion

Virtually all urine-based screening tests for PC developed to date utilize biomarkers that rely heavily on the shedding of PC cells and/or their constituents in urine. However, attempts at

Table 2. Methylation quantification at individual CpG sites using bisulfite pyrosequencing for nontumor associated (NTA) and cancer associated (TA) Urine Samples

	Status	CG1	CG2	CG3	CG4	CG5	CG6	CG7	CG8	CG9	CG10
CAV1	NTA	2.5 (1.2)	5.8 (2.8)	8.1 (3.4)	4.9 (2.6)	6.2 (3.4)	5.4 (2.9)	10.3 (3.3)	3.5 (2.8)	11.0 (3.4)	5.7 (2.6)
	TA	3.2 (1.8)**	8.3 (7.5)**	11.8 (9.5)**	7.4 (8.9)*	8.7 (8.9)*	8.4 (10.6)*	15.6 (10.5)**	5.6 (8.0)*	14.2 (8.9)**	8.5 (8.2)**
EVX1	NTA	8.3 (3.5)	5.3 (2.8)	7.7 (2.9)	13.3 (5.6)	10.4 (4.5)	6.3 (4.2)				
	TA	10.4 (7.2)*	7.5 (6.9)*	12.2 (7.0)**	20.2 (11.0)**	14.6 (8.6)**	8.3 (6.9)*				
FGF1	NTA	90.6 (6.9)	83.5 (8.2)	84.8 (6.9)	92.1 (7.1)	64.4 (9.4)					
	TA	87.0 (11.3)*	79.7 (12.2)*	79.5 (11.9)**	88.2 (12.0)*	63.7 (10.0)					
NCR2	NTA	80.6 (5.6)	61.8 (9.9)	83.4 (5.2)							
	TA	78.9 (7.8)	58.9 (11.1)	81.3 (7.4)							
PLA2G16	NTA	16.2 (5.4)	8.1 (2.7)	7.3 (3.2)	26.0 (8.8)	11.5 (5.4)	12.3 (6.3)				
	TA	22.3 (8.9)**	12.0 (5.4)**	10.1 (6.0)**	33.9 (11.3)**	14.8 (6.3)**	16.1 (8.0)**				

*T-TEST P<0.05; **P<0.01, data shown as Mean methylation values (%) with SD.

Table 3. Statistical comparison (*P values*) of methylation in cancer (TA) urine samples comparing low grade group (GG1) vs. higher grade groups (GG2 and above)

	CG1	CG2	CG3	CG4	CG5	CG6	CG7	CG8	CG9	CG10
EVX1	0.75	0.33	0.55	0.49	0.43	0.93				
CAV1	0.12	0.22	0.43	0.19	0.28	0.47	0.48	0.79	0.06	0.07
FGF1	0.03	0.19	0.04	0.08	0.33					
NCR2	0.83	0.97	0.68							

Parametric T-Test for methylation between GG 1 (n = 31) vs. GG2 and above (n = 59). Data shown are the *P*-value of TTEST.

Table 4. Univariate and multivariate analyses to evaluate the ability of each loci to predict the presence of prostate cancer in urine

Univariate analysis				
	OR	95% CI	AUC	*P*-value
EVX1_CG1	1.10	(1.01, 1.20)	0.61	0.03
EVX1_CG2	1.15	(1.03, 1.29)	0.63	0.01
EVX1_CG3	1.09	(0.94, 1.25)	0.74	<0.001
EVX1_CG4	1.14	(1.07, 1.21)	0.74	<0.001
EVX1_CG5	1.12	(1.05, 1.2)	0.67	0.001
EVX1_CG6	1.08	(1.003, 1.15)	0.62	0.04
CAV1_CG1	1.33	(1.07, 1.67)	0.64	0.01
CAV1_CG2	1.21	(1.08, 1.35)	0.69	0.001
CAV1_CG3	1.15	(1.06, 1.26)	0.68	0.001
CAV1_CG4	1.13	(1.02, 1.25)	0.63	0.02
CAV1_CG5	1.10	(1.01, 1.2)	0.63	0.03
CAV1_CG6	1.13	(1.02, 1.26)	0.64	0.02
CAV1_CG7	1.18	(1.09, 1.28)	0.72	<0.001
CAV1_CG8	1.12	(0.998, 1.26)	0.61	0.05
CAV1_CG9	1.12	(1.03, 1.21)	0.65	0.01
CAV1_CG10	1.21	(1.07, 1.38)	0.69	0.003
FGF1_CG1	0.95	(0.91, 0.99)	0.65	0.03
FGF1_CG2	0.96	(0.93, 0.996)	0.61	0.03
FGF1_CG3	0.93	(0.89, 0.98)	0.68	0.003
FGF1_CG4	0.96	(0.92, 0.99)	0.63	0.03
FGF1_CG5	0.99	(0.96, 1.02)	0.52	0.63
NCR2_CG1	0.96	(0.91, 1.01)	0.55	0.16
NCR2_CG2	0.97	(0.94, 1.01)	0.58	0.12
NCR2_CG3	0.95	(0.9, 1.01)	0.57	0.08
PLA2G16_CG1	1.13	(1.07, 1.2)	0.72	<0.001
PLA2G16_CG2	1.35	(1.199, 1.51)	0.76	<0.001
PLA2G16_CG3	1.21	(1.09, 1.35)	0.70	<0.001
PLA2G16_CG4	1.09	(1.05, 1.13)	0.72	<0.001
PLA2G16_CG5	1.12	(1.05, 1.2)	0.69	0.001
PLA2G16_CG6	1.09	(1.03, 1.15)	0.68	0.001
logPSA	3.02	(1.19, 7.65)	0.59	0.02
Multivariate analysis				
	OR	95% CI	AUC	*P*-value
EVX1_CG3	1.09	(0.94, 1.25)	0.76	0.256
CAV1_CG7	1.07	(0.96, 1.20)		0.229

detecting PC cells in voided urine by traditional cytology have been impeded by undesirably low sensitivities. In contrast, non-tumor prostate cells are found more frequently in urine. In the current study, we were able to use urine samples from patients undergoing prostate biopsy and detect DNA methylation alterations associated with a cancer field defect in normal prostate tissue [15]. These methylation alterations arise at the edges of CpG islands, so called CpG shores, outside the promoter transcription start area and are not clearly related to transcription of the nearby gene. Using this strategy of examining field effect methylation alterations we find they are able to differentiate between tumor associated and nontumor associated urine samples.

In our analysis of the three studied loci, *PLA2G16* methylation has the highest predictive accuracy for PC from TA vs. NTA urine samples (AUC 0.76). *PLA2G16* has been identified as a tumor suppressor gene in both breast cancer and nasopharyngeal carcinoma [17, 18]. Upstream hypermethylation of a promoter CpG island of *PLA2G16* was found in 17% of nasopharyngeal cancer patients [17]. The aberrantly hypermethylated locus studied in the current work is located at a CpG shore (<0.2 kb from *PLA2G16* promoter CpG island) which is spatially distinct from the promoter analyses used in previous cancer investigations. Hypermethylation of promoter CpG and their surrounding area, called shores, is considered a hallmark of cancer and is believed to be involved in gradual silencing of tumor suppressor genes. These changes may reflect subtle alterations in nuclear structure and have recently been associated with higher microsatellite instability in colorectal stem cells [19]. We previously showed that the

PLA2G16_CG2	1.19	(1.02, 1.38)		0.024
EVX1_CG3	1.16	(1.01, 1.33)	0.74	0.032
CAV1_CG7	1.10	(0.99, 1.23)		0.084
EVX1_CG3	1.16	(1.03, 1.30)	0.77	0.018
PLA2G16_CG2	1.21	(1.05, 1.40)		0.008
CAV1_CG7	1.10	(1.00, 1.20)	0.76	0.049
PLA2G16_CG2	1.24	(1.09, 1.41)		0.001

methylation of this locus near *PLA2G16* distinguishes between TA and NTA in histologically benign prostate tissues [15]. *EVX1* encodes a member of the even-skipped homeobox family and has previously been shown to be frequently hypermethylated in PC and is a marker of clinical outcomes [20]. Finally, *CAV1* encodes for a scaffolding protein which is the main component of the caveolae plasma membranes found in most cell types. Reportedly, *CAV1* overexpression was found in PC cells and is associated with disease progression [21]. Using an unbiased methylation microarray approach, these CpG regions in the *EVX1, FGF1, NCR2* and *PLA2G16* genes showed aberrant DNA methylation changes in histologically normal tumor-associated prostate tissues [10, 16].

Currently, there are multiple proposed urine marker panels that can provide diagnostic information regarding the presence of prostate cancer. The urine has direct access to the prostate making it an attractive source for biomarkers [22]. However, to date these urine marker panels have focused on cancer-specific changes gene expression. The first US Food and Drug Administration (FDA)-approved urine marker for PC detection was the Prostate Cancer Antigen 3 (PCA3) assay, a noncoding messenger RNA (Progensa; Hologic) roughly 100 times higher in cancerous than benign tissue [23]. PCA3 has been combined with other genes in several other assays including the Transmembrane serine protease 2 (TMPRSS2): ETS (erythroblast transformation-specific)-related gene (ERG) fusion gene (Mi-Prostate Score, MiPS) [24], and more recently ERG (V-ets erythroblastosis virus E26 oncogene homologs) RNA [25, 26] with AUCs ranging from 0.68 to 0.77. In contrast, we focused on looking at epigenetic changes broadly altered in the field of cancer susceptibility that gives rise to and defines patients who have prostate cancer.

One of the concerns for using non-tumor prostate cells as a source for PC biomarkers development is the detection level. We find that PSA producing prostate cells of epithelial origin encompass 10-16% of the total cells in the urine specimen. These other cells are of urothelial or renal origin or may represent inflammatory cells. Our analysis agrees with previous studies (14-20%) that have analyzed prostate cells in the urine and detect them infrequently [14]. In the current work, the average differences in DNA methylation measured in the current study between tumor associated and non-cancer urine specimens ranged from 3-5% and represents dilution of these field methylation changes. Isolation of PSA producing cells prior to methylation analysis might increase the level of detection, but this strategy is difficult because of crystals, mucous and other constituents in the urine sample [27, 28].

This discovery-based study has several limitations. This was a single center study with a restricted number of patients included that made it difficult to differentiate PC grade. Second, all men included in this study presented with elevated PSA. While we did follow up the patients who had negative biopsies (median follow up of 2 years) and excluded those who had prostate cancer diagnosis on subsequent biopsy (N = 2), we cannot completely rule out the potential of unrecognized PC in this group.

Conclusion

Urine presents a promising source for prostate cancer screening as it is non-invasive and may be utilized to reduce the need for repeat biopsy, associated with unnecessary costs and complications. We find methylation of *PLA2G16 and EVX1* in the urine distinguishes between tumor associated and non-tumor associated prostate tissues marking a field of susceptibility associated with the development of PC. Genes methylated in this field defect can be detected in urine and may be utilized as a novel biomarker approach to detect PC with additional validation.

Acknowledgements

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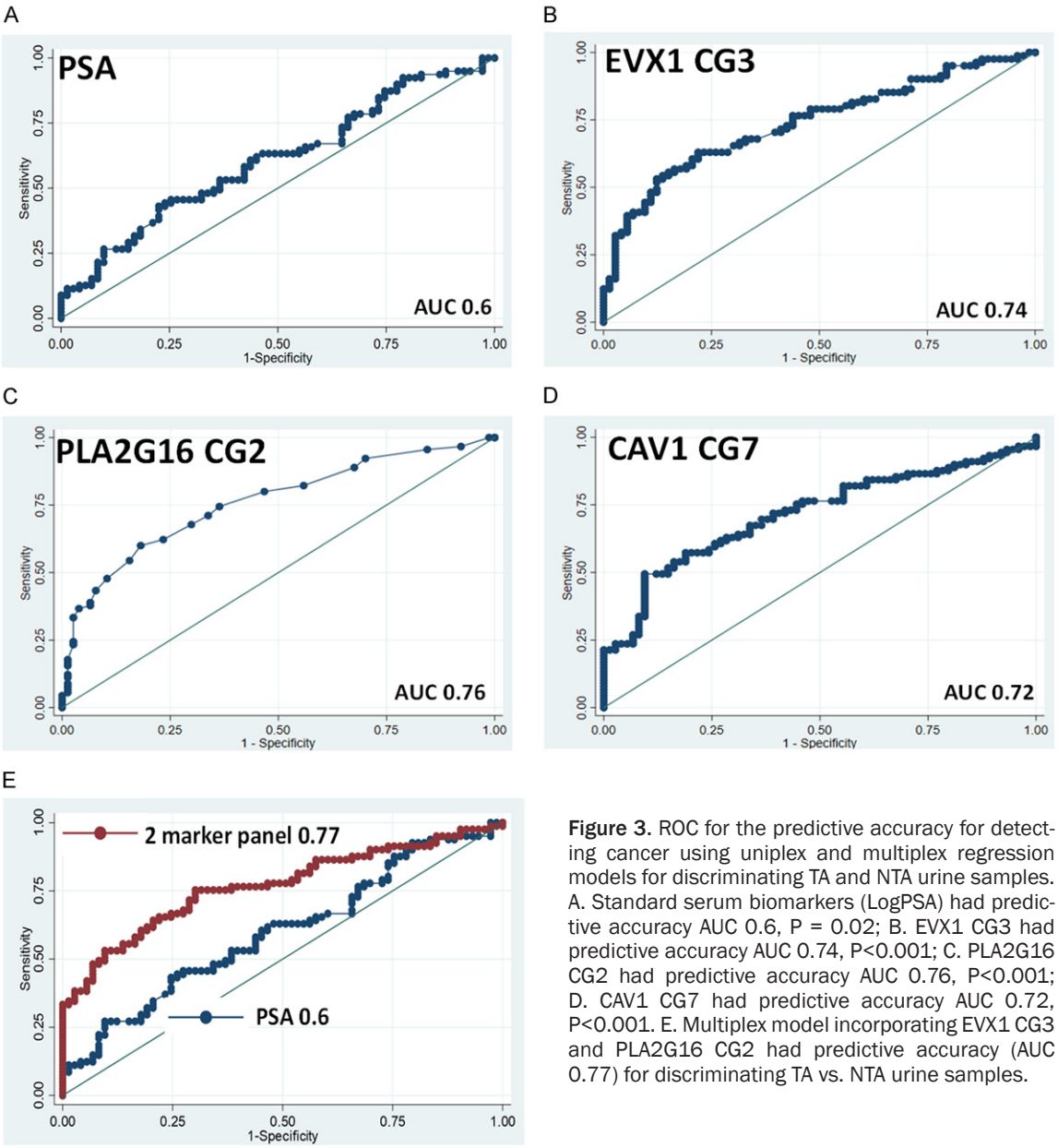

Figure 3. ROC for the predictive accuracy for detecting cancer using uniplex and multiplex regression models for discriminating TA and NTA urine samples. A. Standard serum biomarkers (LogPSA) had predictive accuracy AUC 0.6, P = 0.02; B. EVX1 CG3 had predictive accuracy AUC 0.74, P<0.001; C. PLA2G16 CG2 had predictive accuracy AUC 0.76, P<0.001; D. CAV1 CG7 had predictive accuracy AUC 0.72, P<0.001. E. Multiplex model incorporating EVX1 CG3 and PLA2G16 CG2 had predictive accuracy (AUC 0.77) for discriminating TA vs. NTA urine samples.

Disclosure of conflict of interest

A patent has been filed by the Wisconsin Alumni Research Foundation (WARF) regarding this technology.

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