ELSEVIER

Urologic Oncology: Seminars and Original Investigations 37 (2019) 297.e9–297.e17

UROLOGIC ONCOLOGY

Laboratory-Prostate cancer

Combining urinary DNA methylation and cell-free microRNA biomarkers for improved monitoring of prostate cancer patients on active surveillance

Fang Zhao, M.Sc.[a,b,1], Danny Vesprini, M.D., M.Sc., F.R.C.P.C.[c,1], Richard S.C. Liu, M.Sc.[a,b], Ekaterina Olkhov-Mitsel, Ph.D.[a,b], Laurence H. Klotz, M.D., F.R.C.S.C.[c], Andrew Loblaw, M.D., F.R.C.P.C.[c], Stanley K. Liu, Ph.D., M.D., F.R.C.P.C.[c], Bharati Bapat, Ph.D.[a,b,*]

[a] *Lunenfeld-Tanenbaum Research Institute, Sinai Health System, Toronto, Ontario, Canada*
[b] *Laboratory Medicine & Pathobiology, University of Toronto, Toronto, Ontario, Canada*
[c] *Odette Cancer Research Program, Sunnybrook Research Institute, Toronto, Ontario, Canada*

Received 27 August 2018; received in revised form 18 December 2018; accepted 31 January 2019

Abstract

Purpose: Prostate cancer (CaP) patients with low-grade tumors are enrolled in active surveillance (AS) programs and monitored with digital rectal exams (DREs), prostate-specific antigen (PSA) tests, and periodic invasive biopsies. Patients are "reclassified" with higher-risk disease if they show signs of disease progression. However, AS patients who will reclassify cannot be easily identified upfront and suffer morbidities associated with biopsy. Biomarkers derived from noninvasively obtained specimens such as serum or urine samples are promising alternatives to monitor patients with clinically insignificant cancer. Previously, we have characterized and validated a urinary DNA methylation panel and a serum miRNA panel for the prediction of patient reclassification in 2 independent AS cohorts. In this exploratory study, we have investigated cell-free miRNAs in the urinary supernatant combined with urinary DNA methylation markers to form an integrative panel for prediction of AS patient reclassification.

Methods: Post-DRE urine was collected from 103 CaP patients on active surveillance. Urinary sediment DNA methylation levels of selected genes were previously analyzed using qPCR-based MethyLight assay. Using qRT-PCR, we analyzed the urinary supernatants for relative quantities of 10 miRNAs previously shown to be associated with AS reclassification. Logistic regression and Receiver Operating Characteristics curve analyses were performed to assess the predictive ability of miRNAs and DNA methylation biomarkers.

Results: We identified a 3-marker panel, consisting of miR-24, miR-30c and *CRIP3* methylation, that was significant for prediction of patient reclassification (Odds ratio = 2.166, 95% confidence interval = 1.22–3.847) with a negative predictive value of 90.9%. Our 3-marker panel also demonstrated additive value to PSA for prediction of patient reclassification (c-statistic = 0.717, ROC bootstrapped 1000 iteration $P = 0.041$).

Conclusion: A urinary integrated panel of methylation and miRNA markers is a promising approach to identify AS patients at risk for reclassification. Our 3-marker panel, with its high negative predictive value, would be beneficial to identify and preclude AS patients with truly indolent cancer and to personalize monitoring strategies for AS patients. © 2019 Elsevier Inc. All rights reserved.

Keywords: Prostate cancer; Biomarkers; DNA methylation; miRNA; Active surveillance; Urinary biomarkers

The authors have no conflict of interest to declare.

This work was funded by the Ontario Institute of Cancer ResearchPersonalized Medicine Research Fund No. 10Nov-412, Prostate Cancer Canada No. 2011-700, and PCC Movember TAG No. 2014-01 (BB). The Ontario Student Opportunity Trust Funds Awards (FZ), Ontario Graduate Scholarships (FZ, RSCL, and EO-M), and University of Toronto Fellowships (FZ, RSCL).

*Corresponding author. Tel.: +1 4165864800, ext: 5175
E-mail address: bapat@lunenfeld.ca (B. Bapat).

[1]These authors contributed equally to this work

https://doi.org/10.1016/j.urolonc.2019.01.031
1078-1439/© 2019 Elsevier Inc. All rights reserved.

1. Introduction

Prostate cancer (CaP) is the most common malignancy diagnosed in men [1]. Most CaP patients are diagnosed with low-grade, localized tumors with good prognosis [2]. However, a subset of these patients will experience disease progression and/or harbor occult aggressive tumors that may become life threatening.

Current standards for diagnosis and prognosis include palpating the prostate tumor through digital rectal exams (DRE), measuring circulating prostate specific antigen (PSA), histological assessment of prostate tissue biopsies with the Gleason grading system, and multiparametric (mp) MRI where available. DRE is routinely used for detection of CaP in men over 50 but can only detect large tumors in the posterior of the prostate, and as such cannot identify aggressive tumors before they have grown to be palpable. Due to its high sensitivity, measurement of circulating PSA has led to >50% increase in the diagnosis of CaP [3]. However, PSA cannot reliably differentiate between indolent, low-risk CaP and aggressive, high-risk tumors. Consequently, many patients with elevated PSA but without CaP or with clinically insignificant, low-risk CaP undergo unnecessary invasive biopsies. Prostate biopsies are stratified by the Gleason Grade Groups (GG), which are based on the Gleason patterns ranging from 1 to 5. GG1 (pattern 3+3 or lower) tumors are considered low-risk, with a small chance of metastasis. GG2&3 (pattern 3 + 4 and 4 + 3 respectively) tumors are intermediate risk with the latter being at higher risk, and GG4&5 (patterns 4 + 4 and above) are high-risk tumors. Although histologic assessment remains the gold standard for CaP diagnosis and prognostication, the biopsy procedure is highly invasive and has many associated morbidities such as pain, bleeding, infections and even hospitalizations [4,5]. Additionally, biopsies sample less than 1% [6] of the prostate by volume and may miss occult high-grade tumors or CaP altogether. MpMRI has been shown to effectively reduce the number of prostate biopsies through improved detection of patients with GG2&3 disease and reduction in the number of GG1 diagnoses [7]. However, MRI availability and cost are prohibitive, and consensus regarding the effectiveness of monitoring low-risk patients using mpMRI remains inconclusive [8]. To avoid overtreatment, CaP patients diagnosed with low-risk, localized tumors are often enrolled in an Active Surveillance (AS) program instead of being subjected to definitive treatment such as radical prostatectomy or radiation. AS patients are monitored with repeated PSA measurements, DREs, multiparametric magnetic resonance imaging (mpMRI) if available, and periodic prostate biopsies [9,10]. If signs of disease progression are found, including rapid increase in PSA, GG increase with repeat biopsy, and/or detection of a significant tumor by mpMRI, patients are considered to have "reclassified" with higher-risk disease and are offered definitive treatment [11]. Around 20% to 30% of AS patients will eventually reclassify with higher-risk disease after 5 years. [11–13].

Although AS is the preferred option to monitor low-risk CaP patients, it still relies on invasive prostate biopsies. Conventional predictors for aggressive CaP at diagnosis, including PSA or the percent of biopsy cores positive (%core) cannot differentiate between patients whose disease will reclassify and whose tumors will remain indolent. Noninvasive detection of informative biomarkers that can identify the subset of patients at risk of reclassification early or preclude patients with truly indolent tumors and unlikely to progress would be ideal for guiding patient management and reducing morbidity.

Epigenetic alterations, including DNA methylation and microRNAs (miRNAs), are frequently dysregulated in CaP [14]. These changes are stable and can be detected using small quantities of patient specimens ranging from prostate needle biopsies to liquid biopsy samples such as serum [15], semen [16], and urine [17].

Previously our group has identified and validated 2 epigenetic biomarker panels in independent AS patient cohorts for the prediction of reclassification: a 4-gene urinary DNA methylation Classifier Panel [17,18] consisting of methylation of *APC*, *CRIP3*, *GSTP1*, and *HOXD8* genes, as well as a 3-miR score miRNA panel detected in serum samples [15] consisting of circulating miR-24, miR-223, and miR-375. Additionally, these biomarkers were previously shown to be prognostic for aggressive CaP and/or adverse patient outcomes in multiple independent studies [14,19–29]. We sought to investigate if different epigenetic biomarkers from a single biospecimen source can be optimally combined to develop an integrated biomarker panel. In this regard, urine samples are easily obtained and constitute the least invasive strategy for biomarker assessment. Therefore, our study examined the combination of cell-free urinary miRNA and urinary sediment DNA methylation to develop a multiparametric model for predicting AS patients' risk reclassification.

2. Methods

2.1. Patient cohort

Treatment naïve patients diagnosed with GG1 tumors were prospectively recruited into the AS program at the Odette Cancer Centre, Sunnybrook Health Sciences Centre, Toronto, Ontario, Canada [12]. AS patients were monitored with PSA measurements and DREs every 3 to 12 months, biopsies every 1 to 3 years and mpMRI every 1 to 3 years. Patients recruited in this study constitute a subset of the AS cohort described in our previous studies [15,17].

Following rigorous prostate DRE (3 strokes per lobe), first catch urine samples were collected from 103 AS patients from February 2012 to May 2015. The median time on AS was 45 months (range 0.6–79.4). Patients who did not have urinary sediment DNA methylation profile,

serum miRNA profile, available urine supernatant, or who were diagnosed through Transurethral resection of the prostate (TURP) were excluded. Informed consent was obtained following protocols approved by the research ethics board of Sunnybrook Health Sciences Centre and Sinai Health System, Toronto, Canada.

2.2. Patient reclassification

Patients were considered reclassified if they experienced GG increase on repeat biopsy. Patients who opted for treatment instead of biopsy after experiencing signs of disease progression, including rapidly increasing PSA (measured as PSA doubling time <3 years [PSA-DT]) according to the Sunnybrook ASURE PSA kinetics calculator (www.asure.ca) [30] and/or MRI progression (Prostate Imaging Reporting and Data System [PI-RADS v2] score 4-5) [31] were considered reclassified given the high likelihood of clinically significant disease. Overall, 23/103 patients (22.3%) experienced risk-reclassification. Of these, 8 patients had experienced risk-reclassification after the completion of our study published in 2017 [17]. Among patients enrolled in this study, 59.2% had mpMRI. Some patients reclassified due to increase in GG also experienced PSA-DT and/or MRI progression.

2.3. Sample collection, processing, and nucleic acid isolation

Post-DRE first catch urine samples (20–80 ml) were collected and processed within 5 hours of collection. Samples were stored at room temperature until processing. Samples were centrifuged at 1800 x*g* for 10 minutes. Urinary supernatant (up to 45 ml) was separated from the sediment and stored at −80˚C. Urinary sediment was washed with 5 ml of cold 1 × PBS and resuspended in 200 μl of 1 × PBS and stored at −80˚C.

DNA was isolated from the urinary sediment using the QiaAMP DNA micro kit (QIAGEN) according to manufacturer's protocol with overnight protease K digestion. DNA quantity and quality (260/280 0.4–3.0; 260/230 0.4–3.2) [17] were measured by Nanodrop 8000 (Thermo Scientific). For each sample, 100 ng of DNA was bisulfite converted using the EZ DNA Methylation-Gold kit (Zymo Research) according to manufacturer's protocol and eluted to a final concentration of 5 ng/μl.

Total RNA was extracted from 200 μl of urinary supernatant with miRNeasy Serum/Plasma kit (QIAGEN) per manufacturer's protocol with minor modifications as previously described [15]. MaXtract gel phase lock tubes (QIAGEN) were used during phenol-chloroform extraction.

2.4. Biomarker assessment

2.4.1. Multiplex MethyLight assay

DNA extracted from the urinary sediment was analyzed using Multiplex MethyLight, previously developed by our group [32]. *ALU-C4* (*ALU*) repeats were used as the methylation independent, bisulfite conversion dependent normalization control. Primers and probe sequences were as previously reported [17]. Briefly, the fluorophores used for probes of each gene of interest (GOI) are as follows: *ALU* (FAM), *APC* (HEX), *GSTP1* (HEX), *CRIP3* (Cy5), *HOXD8* (HEX). *ALU* was used in multiplex with all GOIs, *HOXD8* and *CRIP3* were multiplexed in the same reaction.

Each sample was analyzed in duplicate. Reactions consisted of bisulfite converted DNA with 100 μM DNTPs, 1 mM $MgCl_2$, and 15 μl TaqMan Universal Master Mix II, No UNG. Assays were performed using the ABI 7500 Real-Time PCR system as previously described [17].

Methylation of genes was expressed as percent of methylated reference (PMR) by calculating the ratios of GOI and ALU according to Eads et al. [33] CpGenome universal methylated DNA (EMD Millipore) was used as the positive control and to generate standard curves (GOI standard curve R2 >0.95, ALU R2 >0.99, slope range −2.9 to −4). Any sample with ALU cycling threshold (Ct) above the least concentrated standard curve point for which all GOIs amplified was excluded from analysis (inadequate DNA quality). Analysis was repeated for duplicate reactions with a >10 PMR difference.

2.4.2. miRNA detection and analysis

MicroRNAs were assessed using the miRCURY LNA miRNA SYBR Green PCR system (QIAGEN, Exiqon). Total urinary supernatant RNA was reverse transcribed using the Universal cDNA Synthesis Kit (QIAGEN, Exiqon). ExiLENT SYBR Green assay (QIAGEN, Exiqon) was used to analyze cDNA. Quantitative RT-PCR was performed on a QuantStudio6Flex (ABI) Real-Time PCR system. Assays were run in single reactions. Samples with Ct >35 were deemed to be nonspecific amplification. Each miRNA was run in duplicate reactions for 10% of randomly selected samples to ensure reproducibility and/or assess batch effects. Duplicate samples showed high technical reproducibility with a correlation coefficient of 0.877 (Spearman ρ $P = 2.35E\text{-}13$). Relative quantities of candidate miRNAs were calculated by normalization to cel-miR-39 spike-in control RNA using the formula, $2\text{^}(Ct_{cel\text{-}miR\text{-}39} - Ct_{miRNA})$ as previously described [15].

Data acquisition and analysis were performed in accordance with MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [34].

2.5. Statistical analysis

Spearman's ρ ranked test was used to assess correlations between biomarkers and clinical variables.

Backward step-wise logistic regression was used to construct multivariable models.

Mann-Whitney U test was used to assess individual markers, clinical characteristics and the 3-marker panel's association with patient reclassification.

Receiver Operating Characteristics curve analysis was used to calculate Area Under Curve (AUC), sensitivity, and specificity at every value of the 3-marker panel. The 3-marker panel value with the highest combined sensitivity and specificity was selected as the cut-off threshold. AUC of ROC, or c-statistics was used to estimate additive value of the 3-marker panel to clinical characteristics.

Univariable and multivariable logistic regression were used to assess the predictively ability of biomarkers and clinical characteristics for patient risk-reclassification. Multivariable logistic regression was used to combine the 3-marker panel with clinical characteristics for estimation of additive value.

All statistical analyses were performed with IBM SPSS, version 22.0. REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) guidelines were followed [35].

3. Results

3.1. Patient cohort description

This prospective study included 103 treatment naïve patients on AS and is a subset of AS patient cohort investigated in our previous studies [15,17]. Table 1 summarizes patients' clinicopathological characteristics. All patients enrolled were diagnosed with GG1, localized (cT1-T2) tumors. Median patient age was 70 (range 53−83). Median PSA was 5.38 ng/ml (range 0.89−11.95) and median %core was 12.5% (range 3−83%). Median follow up time was 45.7 months (range 0.6−79.4) with 23 patients reclassified (22.3%) at the time of data analysis. Among these, 8 patients were recently reclassified following completion of our 2017 study [17].

Table 1
Clinical characteristics of patients in the cohort.

Total	103
Gleason grade group	No. of patients (%)
1	103 (100)
Clinical T stage by DRE	No. of patients (%)
T1	95 (92.2)
T2	5 (4.9)
Not available (N/A)	3
Biopsy cores with CaP at diagnosis	
Average	18.50%
Median	12.50%
Range	3-83%
N/A	1
Age	
Average	70.24
Median	70
Range	53-83
PSA at diagnosis (ng/ml)	
Average	5.7
Median	5.38
Range	0.89-11.95
Reclassification	No. of patients (%)
Patients reclassified	23 (22.3)

3.2. Correlation of urine miRNAs, DNA methylation markers, and clinical variables

Ten circulating serum miRNAs (miR-375, miR-30c, miR-30e, miR-223, miR-24, miR-21, miR-145, miR-141, miR-26b, and let-71) and four urinary DNA methylation biomarkers (*APC*, *CRIP3*, *GSTP1*, and *HOXD8*) were previously investigated in AS patients by our group [15,17,18]. All miRNAs could be detected in the urinary supernatant of AS CaP patients. Spearman's ρ rank test was used to assess correlations between individual miRNAs, DNA methylation markers, and clinical variables. We found that all urinary miRNAs, except for miR-145 with miR-26b or miR-30c, were significantly correlated with each other. DNA methylation markers were not correlated with miRNAs, *CRIP3* methylation was correlated with *HOXD8* methylation. Age was correlated with miR-141 and miR-375. (Table 2)

3.3. Association with patient reclassification

Mann-Whitney U test was used to determine association of individual miRNAs, DNA methylation markers, clinical characteristics and the 3-marker panel with patient reclassification. Only *CRIP3* and the 3-marker panel were significantly associated with reclassification (Table 3).

3.4. Prediction of patient reclassification

Univariate logistic regression was performed to estimate the odds ratio (OR) and 95% confidence interval (CI) of individual biomarkers and clinical variables for the prediction of patient reclassification. Of the individual markers and clinical characteristics, only *CRIP3* methylation (OR = 1.079, 95% CI = 1.013−1.15) and %core (OR = 1.038, 95% CI = 1.004−1.073) were found to be significant predictors. Using backward logistic regression, a 3-marker panel was built consisting of *CRIP3* methylation, miR-24, and miR-30c. The 3-marker panel was a significant predictor for patient reclassification (OR = 2.166, 95% CI = 1.22−3.847) (Table 4A).

In multivariable logistic regression including the only significant clinical predictor, %core, the 3-marker panel was found to be an independently significant predictor, with a more robust OR (OR = 1.992, 95% CI = 1.131−3.510) than %core (OR = 1.029, 95% CI = 0.99−1.069) which was not independently significant (Table 4B).

Table 2
Spearman's ρ rank test comparing correlations for individual urine cell-free miRNAs, DNA methylation biomarkers and clinical variables. Correlation coefficients are listed. (Spearman's ρ $^*P < 0.05$ $^{**}P < 0.01$)

Spearman's ρ rank test	miR-141	miR-145	miR-21	miR-223	miR-24	miR-26b	miR-30c	miR-30e	miR-375	*APC*	*GSTP1*	*CRIP3*	*HOXD8*	%core	Age	PSA
Let-7a	0.631**	0.248*	0.521**	0.259**	0.361**	0.474**	0.406**	0.352**	0.555**	−0.037	0.026	0.104	0.017	−0.050	−0.119	0.057
miR-141		0.301**	0.542**	0.262**	0.323**	0.538**	0.326**	0.302**	0.499**	−0.021	−0.012	0.105	0.019	0.108	−0.211*	0.011
miR-145			0.291**	0.438**	0.230*	0.1357	0.0723	0.235*	0.195*	−0.041	0.005	−0.038	−0.081	−0.053	−0.163	−0.012
miR-21				0.589**	0.723**	0.551**	0.593**	0.523**	0.511**	−0.080	−0.062	0.062	0.051	−0.035	−0.065	−0.114
miR-223					0.735**	0.472**	0.530**	0.560**	0.458**	−0.103	−0.051	0.042	0.021	−0.043	−0.040	−0.067
miR-24						0.687**	0.831**	0.786**	0.671**	−0.067	−0.010	0.081	0.078	−0.117	−0.035	−0.168
miR-26b							0.721**	0.635**	0.778**	−0.060	−0.041	0.138	0.066	0.030	−0.083	−0.082
miR-30c								0.786**	0.717**	−0.069	0.047	0.111	0.002	0.015	−0.082	−0.135
miR-30e									0.654**	−0.123	0.032	0.122	−0.024	−0.104	−0.176	−0.091
miR-375										−0.063	−0.033	0.100	0.033	−0.028	−0.249*	−0.155
APC											0.061	−0.008	0.161	−0.075	0.077	−0.075
GSTP1												−0.054	−0.036	0.001	−0.083	−0.010
CRIP3													0.455**	0.092	0.142	0.158
HOXD8														0.009	0.121	0.070
%core															0.113	0.068
Age																0.173

Table 3
Mann-Whitney U test for individual variables, clinical characteristics, and the 3-marker panel's association with patient reclassification. Mann Whitney U $^*P < 0.01$

Mann-Whitney U	
Variables	*P* value
Let-7a	0.9648
miR-141	0.9584
miR-145	0.0644
miR-21	0.8382
miR-223	0.2472
miR-24	0.6366
miR-26b	0.9425
miR-30c	0.7701
miR-30e	0.2376
miR-375	0.9776
APC	0.7387
GSTP1	0.1529
CRIP3	0.0089*
HOXD8	0.2567
Age	0.5713
PSA	0.2850
%core	0.0813
3-marker panel	0.0042*

Table 4
Univariable (A) and multivariable (B) logistic regression analysis for individual markers, the 3-marker panel and clinical variables. Multivariable regression was only conducted with significant variables (3-marker panel [which includes *CRIP3*] and %core]. (Logistic regression $^*P < 0.05$, $^{**}P < 0.01$)

A	Univariable	O.R.	95% C.I.		*P* value
	Let-7a	0.978	0.829	1.153	0.791
	miR-141	0.968	0.803	1.167	0.736
	miR-145	–	0.002	–	0.161
	miR-21	1.001	0.956	1.048	0.953
	miR-223	1.006	0.987	1.026	0.519
	miR-24	1.028	0.980	1.078	0.254
	miR-26b	4.496	0.223	90.761	0.327
	miR-30c	1.006	0.993	1.020	0.345
	miR-30e	1.196	0.853	1.678	0.299
	miR-375	1.003	0.997	1.009	0.356
	APC	0.921	0.637	1.330	0.660
	GSTP1	3.254	0.029	367.296	0.625
	CRIP3	1.079	1.013	1.150	0.017*
	HOXD8	1.016	0.935	1.103	0.714
	3-marker	2.166	1.220	3.847	0.008**
	%core	1.038	1.004	1.073	0.026*
	PSA	1.079	0.901	1.293	0.407
	Age	0.984	0.954	1.014	0.293
B	Multivariable	O.R.	95% C.I.		p-value
	%core	1.029	0.990	1.069	0.147
	3-marker	1.992	1.131	3.510	0.017*

3.5. Determining an optimal cut-off threshold

Using ROC curve analysis, we found that the 3-marker panel had an AUC of 0.708 for patient reclassification (95% CI = 0.586–0.83) (Fig. 1). An optimal threshold with maximized combined sensitivity (81%) and specificity (59.7%) was chosen to separate the 3-marker panel into high and low scores. We found that the 3-marker panel threshold can significantly separate nonreclassified and reclassified patients (χ^2 $P = 0.0011$) with a negative predictive value (NPV) of 90.9%. The dichotomized 3-marker panel was able to correctly identify over 80% of reclassified patients (Fig. 2, Table 5).

3.6. Additive value to clinical characteristics

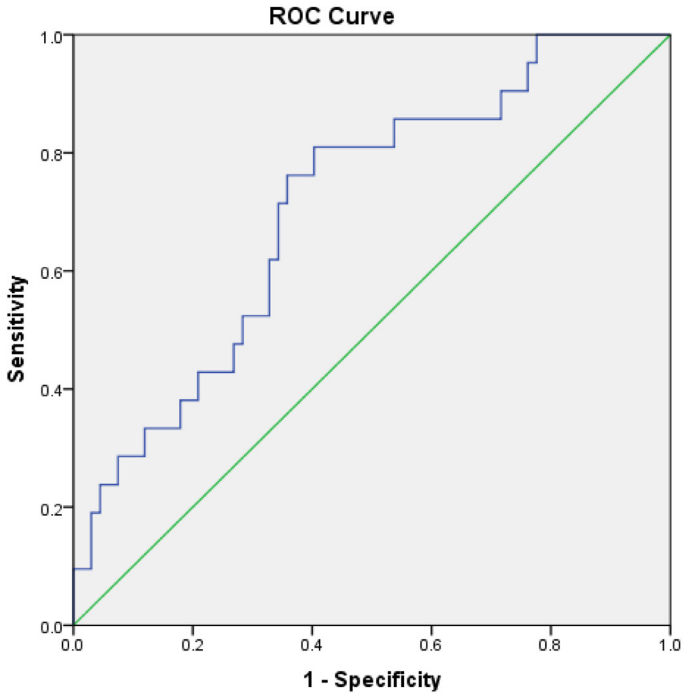
Using concordance statistics (bootstrapped 1000 iterations), we found the c-statistics for PSA was 0.56. When combined with our biomarker panel, the c-index improves to 0.717 ($P = 0.041$), indicating that our 3-marker panel has additive value to PSA for prediction of patient reclassification.

4. Discussion

Although patients on AS are usually diagnosed with low-risk, localized tumors, they are monitored with periodic invasive prostate biopsies, leading to unnecessary healthcare costs and increased morbidity. Non-invasive tests which can reduce or replace prostate biopsies would be ideal to improve management of these low-risk patients.

To our knowledge, this is the first exploratory study to assess combination of urinary cell-free miRNA and methylation markers for prognostication of low-risk CaP patients monitored by AS. Our integrated urinary 3-marker panel composed of miR-24, miR-30c, and methylation of *CRIP3* was able to significantly predict AS patient reclassification. Specifically, our 3-marker panel correctly identified over

A



B

	AUC	95% CI		p-value
3-marker panel	0.708	0.586	0.830	0.0042

Fig. 1. Receiver Operating Characteristics Curve analysis. ROC curve of the 3-marker panel for patient reclassification (A). AUC, 95% CI and *P* value are shown in panel B.

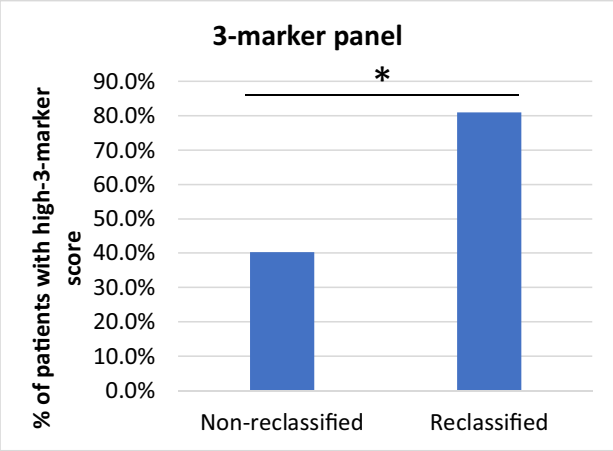


Fig. 2. Prediction of reclassification with high 3-marker score. Patients were separated into high- and low-3-marker scores based on threshold derived using ROC curve analysis. Patients who showed high-3-marker score were significantly more likely to experience risk-reclassification compared to patients with low-3-marker score (χ^2 *$P = 0.0011$).

80% of AS patients who will experience reclassification with an NPV of 91%. Our current panel has the advantage of detecting multiple biomarker types from a single biospecimen source. These promising results, once validated, would be a valuable addition to the current AS protocol for monitoring low-risk patients. Patients who fall below the threshold for the 3-marker panel could be monitored less frequently or less intensely including reduced number of invasive biopsies.

Previously we derived and validated, in 2 independent AS patient cohorts, a 3-miR score composed of serum miR-223, miR-375, and miR-24, the last of which is part of our 3-marker urinary panel [15]. The serum-based 3-miR score was an independent significant predictor of AS patient reclassification (validation cohort OR = 3.7, 95% CI = 1.29−10.6). The 3-miR score combined with PSA had a sensitivity of 66% and specificity of 72% for prediction of patient reclassification in the training cohort. Additionally, we identified and validated a 4-gene methylation classifier panel in the urinary sediment of AS patients [17,18]. Our urinary methylation classifier panel was an independent predictor of risk-reclassification (OR = 2.559, 95% CI = 1.257−5.212) and superior to clinical predictors such as PSA and %core with a sensitivity of 71% and specificity of 58% for prediction of patient reclassification in the training cohort. Both panels were able to significantly predict AS patient reclassification with a high NPV (DNA methylation classifier panel NPV = 86%, 3-miR score NPV = 89%). These 2 panels showed comparable performance for sensitivity (81%) and specificity (59.7%) with the current integrated 3-marker panel, however it should be noted that both previously published panels had been validated in an independent AS cohort. The 3-marker panel identified in this exploratory study is aimed at combining different types of epigenetic biomarkers from noninvasively collected urine samples and requires additional validation in independent AS cohorts.

Table 5
Positive- and negative-predictive values and sensitivity, specificity of the high-3-marker score

	NPV	PPV	Sensitivity	Specificity
3-marker panel	90.9%	38.6%	81.0%	59.7%

Urinary biomarkers are noninvasive, even compared to serum-based markers, and are ideal for frequent monitoring of low-risk patients such as those on AS. Previous studies have investigated urinary cell-free miRNAs for prognostication of CaP [36,37]. Although not in the context of AS, one such study by Fredsoe et al. [38] identified and validated a three-miRNA model in cell-free urine that could significantly distinguish BPH and CaP patients with an AUC of 0.89 to 0.95. Interestingly, 2 miRNAs from this model (miR-24 and miR-30c) are also part of our panel for low-risk AS patients, which further underscore the diagnostic and prognostic potential of our urinary 3-marker panel. Dysregulation of both these microRNAs is observed in CaP and is shown to contribute to prostate carcinogenesis through several functional mechanisms. MiR-24 has been previously shown to be down-regulated in CaP. miR-24 can regulate CDKN1B/p27 expression [39] and inhibit proliferation, migration, and invasion of CaP cell lines [40]. Similarly, miR-30c expression is also significantly down-regulated in CaP [41] and can regulate proliferation, migration, and invasion through the KRAS pathway [42].

There are several FDA or CLIA certified noninvasive prediagnostic biomarker tests for CaP patients, including the Prostate Health Index (PHI) [43], the 4Kscore [44], SelectMDx [45], and ExoDx Prostate IntelliScore [46]. These prediagnostic tests focus on detection of CaP tumors through noninvasive collection of patient samples to guide decisions for whether a confirmatory biopsy is needed to diagnose CaP. Although these tests have shown great promise in CaP diagnosis and some ability for prognostication of high-grade CaP, they require additional validation for use in the context of CaP AS cohorts. Currently, none can reliably identify AS patients at risk for reclassification.

PHI measures serum levels of total PSA, free PSA, and [−2]proPSA. In the context of CaP AS, a recent multicenter study by Heidegger et al. [47] found that PHI can predict GG increase of GG1 patients with a modest OR of 1.039 compared to our 3-marker panel at OR = 2.166. The 4Kscore measures plasma total PSA, free PSA, intact PSA, and kallikrein 2. The 4Kscore has been previously found to be able to predict GG increase in AS patients with modest OR (OR = 1.54, 95% CI = 1.31−1.81) [48]. The urinary assay SelectMDx measures mRNA expression of 2 genes (*HOXC6* and *DLX1*) for the detection of clinically significant CaP (GG ≥ 2). SelectMDx has a higher OR (up to 1.96) and very good AUC (0.9) for prediction of GG ≥

2CaP [49]. However, over 34% of GG≥2 cases were GG4+ which is less representative of an AS setting. A low SelectMDx score has an NPV of 90% for any CaP and an NPV of 98% for high-risk CaP [45] comparable to our 3-marker panel which has a 91% NPV for patient reclassification. SelectMDx's efficacy for prediction of AS patient reclassification is not yet known. Lastly, ExoDx measures RNA of PCA3, ERG with normalization control SPDEF in urinary exosomes. ExoDx has an AUC of 0.74 for identifying high-grade (GG≥2) CaP and an NPV of 96%.

Although all the aforementioned tests are promising, they need to be assessed in AS patients. In addition, a direct comparison between these biomarker panels in the same patient cohorts may aid in deciphering which assay is optimal for AS patient management. As proof of principle, we have shown that biomarkers from different components of noninvasive liquid biopsy samples from patients may be combined. In the future, our 3-marker panel may be combined with one or several of available biomarker tests to form a more comprehensive panel to improve prediction of AS patient risk-reclassification. There is enormous potential for this strategy to obtain various combinations of biomarkers for prognostication of different endpoints, including diagnosis and AS patient reclassification.

There are several limitations to the current study. The sample size is modest due to the requirement that patient must have post-DRE urine supernatant available since we did not have sufficient quantities of urinary cell sediment available for miRNA analysis. Although the 3-marker panel requires additional validation, the main purpose of this study is the exploration of combining urinary epigenetic biomarkers for prediction of reclassification. We have successfully shown that assessing 2 different epigenetic markers from post-DRE urine are possible and predictive of CaP progression. However, further investigation is required for validation of these findings. Also, there is no consensus on an endogenous control for urinary miRNAs. Therefore, we opted to use a spike-in RNA as previously described to control for technical variability among samples during processing and analysis. This may not account for endogenous expression differences among patients. Lastly, as AS is a continuing process, we do not know if patients with high 3-marker panel score who were not reclassified were truly indolent. However, ongoing follow-up for these patients will address this issue. It is possible that some or all these patients may experience reclassification in the future.

5. Conclusions

We have shown that it is possible to detect miRNAs in the urinary supernatant and that combining urinary cell-free miRNA and sediment DNA methylation is a valid approach to identify AS patients at increased risk for reclassification. Once validated, our 3-marker panel, with its high NPV, could be used to identify AS patients who are unlikely to reclassify so that they may be monitored less intensely, decreasing morbidity and costs, but further prospective confirmation will be required.

References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015;65(2):87–108.
[2] Scosyrev E, Wu G, Mohile S, Messing EM. Prostate-specific antigen screening for prostate cancer and the risk of overt metastatic disease at presentation: analysis of trends over time. Cancer 2012;118 (23):5768–76.
[3] Howlader N NA, Krapcho M, Miller D, Bishop K, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). SEER cancer statistics review, 1975-2014, Bethesda, MD: National Cancer Institute; 2017. Available from: https://seer.cancer.gov/csr/1975_2014/.
[4] Womble PR, Dixon MW, Linsell SM, Ye Z, Montie JE, Lane BR, et al. Infection related hospitalizations after prostate biopsy in a statewide quality improvement collaborative. J Urol 2014;191 (6):1787–92.
[5] Nam RK, Saskin R, Lee Y, Liu Y, Law C, Klotz LH, et al. Increasing hospital admission rates for urological complications after transrectal ultrasound guided prostate biopsy. J Urol 2010;183(3):963–8.
[6] Ladjevardi S, Auer G, Castro J, Ericsson C, Zetterberg A, Haggman M, et al. Prostate biopsy sampling causes hematogenous dissemination of epithelial cellular material. Dis Markers 2014;2014:707529.
[7] Kasivisvanathan V, Rannikko AS, Borghi M, Panebianco V, Mynderse LA, Vaarala MH, et al. MRI-targeted or standard biopsy for prostate-cancer diagnosis. N Engl J Med 2018;378(19):1767–77.
[8] Briganti A, Fossati N, Catto JWF, Cornford P, Montorsi F, Mottet N, et al. Active surveillance for low-risk prostate cancer: the European Association of Urology Position in 2018. Eur Urol 2018;74(3): 357–68.
[9] Morash C, Tey R, Agbassi C, Klotz L, McGowan T, Srigley J, et al. Active surveillance for the management of localized prostate cancer: guideline recommendations. Can Urol Assoc J 2015;9(5-6):171–8.
[10] Hayes JH, Ollendorf DA, Pearson SD, Barry MJ, Kantoff PW, Stewart ST, et al. Active surveillance compared with initial treatment for men with low-risk prostate cancer: a decision analysis. JAMA 2010;304(21):2373–80.
[11] Klotz L. Active surveillance for low-risk prostate cancer. Curr Opin Urol 2017;27(3):225–30.
[12] Klotz L, Vesprini D, Sethukavalan P, Jethava V, Zhang L, Jain S, et al. Long-term follow-up of a large active surveillance cohort of patients with prostate cancer. J Clin Oncol 2015;33(3):272–7.
[13] Klotz L, Loblaw A, Sugar L, Moussa M, Berman DM, Van der Kwast T, et al. Active surveillance magnetic resonance imaging study (ASIST): results of a randomized multicenter prospective trial. Eur Urol 2019;75 (2):300–9.
[14] Kron KJ, Liu L, Pethe VV, Demetrashvili N, Nesbitt ME, Trachtenberg J, et al. DNA methylation of HOXD3 as a marker of prostate cancer progression. Lab Invest 2010;90(7):1060–7.
[15] Liu RSC, Olkhov-Mitsel E, Jeyapala R, Zhao F, Commisso K, Klotz L, et al. Assessment of serum microRNA biomarkers to predict reclassification of prostate cancer in patients on active surveillance. J Urol 2018;199(6):1475–81.
[16] Wu C, Ding X, Li H, Zhu C, Xiong C. Genome-wide promoter methylation profile of human testis and epididymis: identified from cell-free seminal DNA. BMC Genomics 2013;14:288.
[17] Zhao F, Olkhov-Mitsel E, van der Kwast T, Sykes J, Zdravic D, Venkateswaran V, et al. Urinary DNA methylation biomarkers for noninvasive prediction of aggressive disease in patients with prostate cancer on active surveillance. J Urol 2017;197(2):335–41.
[18] Zhao F, Jeyapala R, Olkhov-Mitsel E, Vesprini D, Fleshner NE, Bapat B. Re: Urinary DNA methylation biomarkers for noninvasive prediction of aggressive disease in patients with prostate cancer on

active surveillance: F. Zhao, E. Olkhov-Mitsel, T. van der Kwast, J. Sykes, D. Zdravic, V. Venkateswaran, A. R. Zlotta, A. Loblaw, N. E. Fleshner, L. Klotz, D. Vesprini and B. Bapat J Urol 2017;197: 335-341. J Urol 2018;199(5):1354−5.

[19] Liu L, Kron KJ, Pethe VV, Demetrashvili N, Nesbitt ME, Trachtenberg J, et al. Association of tissue promoter methylation levels of APC, TGFβ2, HOXD3 and RASSF1A with prostate cancer progression. Int J Cancer 2011;129(10):2454–62.

[20] Olkhov-Mitsel E, Van der Kwast T, Kron KJ, Ozcelik H, Briollais L, Massey C, et al. Quantitative DNA methylation analysis of genes coding for kallikrein-related peptidases 6 and 10 as biomarkers for prostate cancer. Epigenetics 2012;7(9):1037–45.

[21] Mihelich BL, Maranville JC, Nolley R, Peehl DM, Nonn L. Elevated serum microRNA levels associate with absence of high-grade prostate cancer in a retrospective cohort. PLoS One 2015;10(4): e0124245.

[22] Moltzahn F, Olshen AB, Baehner L, Peek A, Fong L, Stoppler H, et al. Microfluidic-based multiplex qRT-PCR identifies diagnostic and prognostic microRNA signatures in the sera of prostate cancer patients. Cancer Res 2011;71(2):550–60.

[23] Brase JC, Johannes M, Schlomm T, Falth M, Haese A, Steuber T, et al. Circulating miRNAs are correlated with tumor progression in prostate cancer. Int J Cancer 2011;128(3):608–16.

[24] Bryant RJ, Pawlowski T, Catto JW, Marsden G, Vessella RL, Rhees B, et al. Changes in circulating microRNA levels associated with prostate cancer. Br J Cancer 2012;106(4):768–74.

[25] Cheng HH, Mitchell PS, Kroh EM, Dowell AE, Chery L, Siddiqui J, et al. Circulating microRNA profiling identifies a subset of metastatic prostate cancer patients with evidence of cancer-associated hypoxia. PLoS One 2013;8(7):e69239.

[26] Haldrup C, Kosaka N, Ochiya T, Borre M, Hoyer S, Orntoft TF, et al. Profiling of circulating microRNAs for prostate cancer biomarker discovery. Drug Deliv Transl Res 2014;4(1):19–30.

[27] Huang X, Yuan T, Liang M, Du M, Xia S, Dittmar R, et al. Exosomal miR-1290 and miR-375 as prognostic markers in castration-resistant prostate cancer. Eur Urol 2015;67(1):33–41.

[28] Nguyen HC, Xie W, Yang M, Hsieh CL, Drouin S, Lee GS, et al. Expression differences of circulating microRNAs in metastatic castration resistant prostate cancer and low-risk, localized prostate cancer. Prostate 2013;73(4):346–54.

[29] Watahiki A, Macfarlane RJ, Gleave ME, Crea F, Wang Y, Helgason CD, et al. Plasma miRNAs as biomarkers to identify patients with castration-resistant metastatic prostate cancer. Int J Mol Sci 2013;14 (4):7757–70.

[30] Zhang L, Loblaw A, Klotz L. Modeling prostate specific antigen kinetics in patients on active surveillance. J Urol 2006;176(4 Pt 1):1392–7;discussion 7-8.

[31] Barentsz JO, Richenberg J, Clements R, Choyke P, Verma S, Villeirs G, et al. ESUR prostate MR guidelines 2012. Eur Radiol 2012;22 (4):746–57.

[32] Olkhov-Mitsel E, Zdravic D, Kron K, van der Kwast T, Fleshner N, Bapat B. Novel multiplex MethyLight protocol for detection of DNA methylation in patient tissues and bodily fluids. Sci Rep 2014;4:4432.

[33] Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, et al. MethyLight: a high-throughput assay to measure DNA methylation. Nucl. Acids Res 2000;28(8):E32.

[34] Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55(4):611–22.

[35] McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, et al. Reporting recommendations for tumor marker prognostic studies. J Clin Oncol 2005;23(36):9067–72.

[36] Korzeniewski N, Tosev G, Pahernik S, Hadaschik B, Hohenfellner M, Duensing S. Identification of cell-free microRNAs in the urine of patients with prostate cancer. Urol Oncol 2015;33(1):16.e7−e22.

[37] Sapre N, Hong MK, Macintyre G, Lewis H, Kowalczyk A, Costello AJ, et al. Curated microRNAs in urine and blood fail to validate as predictive biomarkers for high-risk prostate cancer. PLoS One 2014;9 (4):e91729.

[38] Fredsoe J, Rasmussen AKI, Thomsen AR, Mouritzen P, Hoyer S, Borre M, et al. Diagnostic and prognostic MicroRNA biomarkers for prostate cancer in cell-free urine. Eur Urol Focus 2018;4(6):825–33.

[39] Lynch SM, McKenna MM, Walsh CP, McKenna DJ. miR-24 regulates CDKN1B/p27 expression in prostate cancer. Prostate 2016;76 (7):637–48.

[40] Kang H, Rho JG, Kim C, Tak H, Lee H, Ji E, et al. The miR-24-3p/p130Cas: a novel axis regulating the migration and invasion of cancer cells. Sci Rep 2017;7:44847.

[41] Zhu C, Hou X, Zhu J, Jiang C, Wei W. Expression of miR-30c and miR-29b in prostate cancer and its diagnostic significance. Oncol Lett 2018;16(3):3140–4.

[42] Zhang J, Wang X, Wang Y, Peng R, Lin Z, Wang Y, et al. Low expression of microRNA-30c promotes prostate cancer cells invasion involved in downregulation of KRAS protein. Oncol Lett 2017;14 (1):363–8.

[43] Catalona WJ, Partin AW, Sanda MG, Wei JT, Klee GG, Bangma CH, et al. A multicenter study of [-2]pro-prostate specific antigen combined with prostate specific antigen and free prostate specific antigen for prostate cancer detection in the 2.0 to 10.0 ng/ml prostate specific antigen range. J Urol 2011;185(5):1650–5.

[44] Parekh DJ, Punnen S, Sjoberg DD, Asroff SW, Bailen JL, Cochran JS, et al. A multi-institutional prospective trial in the USA confirms that the 4Kscore accurately identifies men with high-grade prostate cancer. Eur Urol 2015;68(3):464–70.

[45] Leyten GH, Hessels D, Smit FP, Jannink SA, de Jong H, Melchers WJ, et al. Identification of a candidate gene panel for the early diagnosis of prostate cancer. Clin Cancer Res 2015;21(13):3061–70.

[46] McKiernan J, Donovan MJ, O'Neill V, Bentink S, Noerholm M, Belzer S, et al. A novel urine exosome gene expression assay to predict high-grade prostate cancer at initial biopsy. JAMA Oncol 2016;2 (7):882–9.

[47] Heidegger I, Klocker H, Pichler R, Pircher A, Prokop W, Steiner E, et al. ProPSA and the Prostate Health Index as predictive markers for aggressiveness in low-risk prostate cancer-results from an international multicenter study. Prostate Cancer Prostatic Dis 2017;20(3):271–5.

[48] Lin DW, Newcomb LF, Brown MD, Sjoberg DD, Dong Y, Brooks JD, et al. Evaluating the four kallikrein panel of the 4kscore for prediction of high-grade prostate cancer in men in the canary prostate active surveillance study. Eur Urol 2017;72(3):448–54.

[49] Hendriks RJ, van der Leest MMG, Dijkstra S, Barentsz JO, Van Criekinge W, Hulsbergen-van de Kaa CA, et al. A urinary biomarker-based risk score correlates with multiparametric MRI for prostate cancer detection. Prostate 2017;77(14):1401–7.