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Epigenome-wide Tumor DNA Methylation Profiling Identifies Novel Prognostic Biomarkers of Metastatic-lethal Progression in Men with Clinically Localized Prostate Cancer

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Abstract

Purpose—Aside from Gleason sum few factors accurately identify the subset of prostate cancer (PCa) patients at high risk for metastatic progression. We hypothesized that epigenetic alterations could distinguish prostate tumors with life-threatening potential.

Experimental Design—Epigenome-wide DNA methylation profiling was performed in surgically resected primary tumor tissues from a population-based (n = 430) and a replication (n = 80) cohort of PCa patients followed prospectively for at least five years. Metastasis was confirmed by positive bone scan, MRI, CT or biopsy, and death certificates confirmed cause of death. AUC, partial AUC (pAUC, 95% specificity), and P-value criteria were used to select differentially methylated CpG sites that robustly stratify patients with metastatic-lethal from non-recurrent tumors, and which were complementary to Gleason sum.

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Results—Forty-two biomarkers stratified patients with metastatic-lethal versus non-recurrent PCa in the discovery cohort, and eight of these CpGs replicated in the validation cohort based on a significant (P <0.05) AUC (range: 0.66-0.75) or pAUC (range: 0.007-0.009). The biomarkers that improved discrimination of patients with metastatic-lethal PCa include CpGs in five genes (*ALKBH5*, *ATP11A*, *FHAD1*, *KLHL8*, and *PI15)* and three intergenic regions. In the validation dataset the AUC for Gleason sum alone (0.82) significantly increased with the addition of four individual CpGs (range: 0.86-0.89; all P <0.05).

Conclusion—Eight differentially methylated CpGs that distinguish patients with metastatic-lethal from non-recurrent tumors were validated. These novel epigenetic biomarkers warrant further investigation as they may improve prognostic classification of patients with clinically localized PCa and provide new insights on tumor aggressiveness.

Introduction

Prostate cancer (PCa) is a biologically and clinically heterogeneous disease with 180,890 new cases and 26,120 cancer-specific deaths expected in the U.S. for 2016 and over 300,000 deaths worldwide each year (1, 2). Prostate cancer most often has an indolent course, but a subset of patients progress to metastasis and eventually die from PCa (3, 4). The single most important predictor of PCa prognosis is Gleason sum,however, Gleason grading is frequently inaccurate (5). By comparing the Gleason sum of the diagnostic biopsy to subsequent prostatectomy, upgrading and downgrading occurs in 14% to 51% and 9%, respectively (5-7). Furthermore, while a tumor with Gleason sum ≤ 6 is low-risk and Gleason sum ≥8 is high-risk, tumors that are Gleason sum = 7 (ie, grades 3+4 or 4+3) are heterogeneous and comprise the majority of tumors (8). Thus, better prognostic biomarkers that can improve upon Gleason sum for stratification of higher risk patients most likely to benefit from targeted therapies are needed (4, 9-11).

Several recent biomarker studies of altered gene expression have led to development of mRNA signatures of tumor aggressiveness (9, 11, 12). Epigenetic alterations in tumor DNA may also provide valuable prognostic information (13, 14), and because DNA is 100-fold more stable than RNA it may be a more reliable biological material to use for tissue-based biomarkers. The most widely studied epigenetic alteration is DNA methylation, which occurs at CpG sites across the genome and regulates gene expression (15, 16). To date, most studies of DNA methylation and PCa progression have been limited to small sets of candidate genes in relation to biochemical (ie, PSA) recurrence (14, 17-19). Although patients with biochemical recurrence are at higher risk of cancer-specific death, most will not die from their PCa. Studies of patients with biochemical recurrence after radical prostatectomy found that only 17% to 22% died of PCa after a median follow-up of 10 years (20, 21). Therefore rather than focusing on PSA recurrence alone, biomarker studies of more serious clinical endpoints indicating metastatic progression and lethal PCa are needed.

We investigated epigenome-wide DNA methylation profiles in primary prostate cancers. The study includes patients derived from a population-based (multi-institution) radical prostatectomy cohort with long-term follow-up for metastatic progression and cancer-specific survival. The goal of this study was to identify differentially methylated biomarkers

that could distinguish patients with metastatic-lethal PCa from those with less aggressive tumors. The most robust methylation biomarkers identified were then tested in an independent validation cohort.

Materials and Methods

Study populations

Fred Hutchinson (FH) Cancer Research Center cohort—The FH cohort includes 430 European-American PCa patients who underwent radical prostatectomy as primary therapy for clinically localized adenocarcinoma of the prostate. These patients were previously enrolled in population-based (multi-institutional) studies (22, 23), and their clinical characteristics (eg, age at diagnosis, Gleason sum, stage, PSA level) are similar to the larger group of European-American patients interviewed for the prior studies and who were treated surgically. The first study included men ages 40-64 years who were diagnosed between January 1993 and December 1996, and in the second study, men were ages 35-74 years and were diagnosed between January 2002 and December 2005. Gleason grade (primary and secondary patterns) and sum, diagnostic PSA, and pathological tumor stage were collected and centrally coded by the Seattle-Puget Sound Surveillance, Epidemiology, and End Results Program cancer registry. Vital status and underlying cause of death were also obtained from the cancer registry, and cause of death was confirmed by centralized review of death certificates. Prostate cancer-specific deaths included those with underlying cause of death attributed to ICD-9 code 180.0 or ICD-10 code C61.9. Prostate cancer recurrence status was determined from prospectively collected information from follow-up surveys that were completed by patients in 2004-2005 and in 2010-2011, review of medical records, and/or physician follow-up as needed. Metastatic progression was confirmed by positive bone scan, MRI, CT or biopsy. Patients who developed metastases or died from PCa were combined in a metastatic-lethal phenotype category. Over the follow-up period, 317 patients had no evidence of recurrence and 113 had recurred, including 86 PSA recurrences and 27 metastatic-lethal events. For the present analysis, patients with the metastatic-lethal phenotype were compared to patients who had not recurred. The FH Institutional Review Board approved the study and all participants signed informed consent statements.

Eastern Virginia (EV) Medical School cohort—The validation dataset includes 80 patients diagnosed with localized stage PCa who underwent radical prostatectomy at EV Medical School. The study population includes men who experienced disease progression to metastatic or lethal PCa ($n = 31$) and a similar number of patients ($n = 49$) selected on the basis of having no evidence of recurrence during five or more years after diagnosis (nested case-control design). Metastatic-lethal events were identified as described in the FH cohort, and all patients were European-Americans. The patients in the EV cohort were diagnosed in 1992-2009.

Tumor tissue sample preparation and DNA methylation profiling

Formalin-fixed paraffin-embedded prostate tumor tissue blocks were obtained from radical prostatectomy specimens for both cohorts and used to make hematoxalin and eosin stained slides, which were reviewed by pathologists to confirm the presence and location of

adenocarcinoma. For each patient two 1-mm tumor tissue cores from the dominant lesion that were enriched with ≥75% tumor cells were taken for DNA and two cores for RNA purification. The RecoverAll Total Nucleic Acid Isolation Kit (Ambion/Applied Biosciences, Austin, TX) was used to extract DNA, which was then quantified (PicoGreen), aliquoted onto 96-well plates and shipped to Illumina (Illumina, Inc., San Diego, CA) for DNA methylation profiling.

The EZ DNA Methylation Kit (Zymo Research, Irvine, CA) was used to bisulfite convert tumor DNA samples. Controls on the array were used to track the bisulfite conversion efficiency. The Infinium® HumanMethylation450 BeadChip (Illumina) was used to measure genome-wide methylation using beads with target-specific probes designed to interrogate individual CpG sites (>485,000) (24). Samples from the FH cohort were assayed as one batch (7 plates) and the EV samples were assayed as a second batch (2 plates). Across the 96-well plates, we incorporated blind duplicate (FH, $n = 16$; EV, $n = 7$) and replicate (FH, $n = 2$; EV, $n = 3$) samples for each cohort. All plates also contained Illumina controls and two negative controls. PCa outcome events were randomly distributed across plates, and laboratory personnel were blinded to the location of duplicate and replicate samples.

Failed samples were identified by using the detection *P*-value metric (probability of a CpG being detected above the background level defined by negative control probes) according to Illumina protocols. A sample was excluded if less than 95% of the CpG sites for that sample on the array were detected with a detection *P*-value <0.05, resulting in removal of 17 FH and 15 EV samples. The final number of patients in the FH cohort and EV cohort was 327 (303 non-recurrent, 24 metastatic-lethal) and 65 (41 non-recurrent, 24 metastatic-lethal), respectively. Further, CpG sites with a detection *P*-value of >0.01 were excluded. After data filtering, 478,998 CpGs were available in the FH cohort and 479,103 in the EV cohort (477,460 overlapped). Correlation coefficients for duplicate samples in the FH and EV cohorts were 0.96-0.99 and 0.99, respectively. The correlation coefficients for replicate samples in FH and EV were 0.99 and 0.98.

The same FH and EV patients' tumor samples used for DNA methylation profiling were also utilized for mRNA expression profiling using the Whole-Genome DASL® HT Assay (Illumina). Transcript correlations between duplicated samples (19 pairs) ranged from 0.96-0.99. In addition, replicate tumor RNA samples (6 pairs) were included, and the transcript correlations across plates were 0.95-0.99. There were 353 patients (FH: $n = 288$; EV: $n = 65$) with both tumor DNA methylation and mRNA expression data.

Statistical analysis

The methylation data were normalized using subset-quantile within array normalization (25) and batch effects were removed using Combat (26). Methylation β- and M-values were calculated, where β-values represent the percentage of DNA methylation at a CpG site. Methylation M-values are the logit transformed β-values that are approximately normally distributed. M-values were used for statistical testing and β-values to represent methylation differences between patient groups. Genome annotation of the CpGs was based on the Illumina protocol (27).

DNA methylation biomarkers for prognosis were identified using the FH cohort, following an *a priori* decision to select the top-ranked 5% of the CpGs based on their classification performance. First, for all individual CpG sites, the AUC and partial AUC (pAUC) for predicating metastatic-lethal versus non-recurrent PCa outcomes were calculated. The pAUC evaluates performance at a fixed high (95%) specificity as we aimed to select biomarkers with a low false-positive rate, providing more confidence that patients classified as high-risk by the biomarker indeed have high-risk tumors. Accordingly, we selected the top-ranked 4% of biomarkers based on pAUC and the top-ranked 1% based on AUC, yielding 22,290 CpGs for further analysis.

Next, we identified the subset of the 22,290 biomarkers that showed the greatest improvement in predicting metastatic-lethal PCa compared to Gleason sum alone. Because Gleason sum is the most widely used measure of tumor aggressiveness, we aimed to identify CpGs that could improve the prognostic discrimination of patients beyond that provided by Gleason sum alone. Other potential prognostic classifiers were also considered in models, including age at diagnosis, diagnostic PSA level, and pathologic tumor stage (local = pT2, N0/NX, M0; regional = pT3/T4 and/or N1, M0), but these factors did not improve the prediction of metastatic-lethal PCa compared to models with Gleason sum only ($P > 0.05$), and were therefore not considered in further analyses.

A logistic regression model for discriminating patients with metastatic-lethal versus non-recurrent PCa was fit containing Gleason sum as the only predictor. Based on that model, forward model selection was done using three selection criteria: AUC, pAUC (95% specificity), and *P*-value (Wald test). For each criterion, we identified the CpG that showed the greatest improvement, ie, was the most significant in predicting metastatic-lethal PCa, compared to the base model with Gleason sum alone; the identified biomarker was then added to the model with Gleason. Forward selection was continued, each time selecting one additional CpG to be included in the model, until a pre-specified stopping criterion was met: for AUC this was an increment of AUC <0.005; for pAUC this was an increment of pAUC <0.0005; and for *P*-value this was >0.05. This entire process was repeated 100 times with bootstrap samples. The biomarkers that were selected multiple times in the different bootstrap cohorts (≥3 when considering AUC; ≥3 when considering pAUC; ≥4 when considering *P*-value) were chosen for further evaluation.

The methylation biomarkers that were most predictive for metastatic-lethal disease in the FH cohort were then tested in the EV cohort for validation. For each biomarker we calculated the AUC and pAUC (95% specificity) for distinguishing patients with metastatic-lethal versus non-recurrent PCa. *P*-values for AUC and pAUC were computed using 10,000 permutations, and 95% confidence intervals for AUC and pAUC were calculated using 2,000 stratified bootstrap replicates. Likelihood ratio tests were also computed to compare models fit with Gleason sum and a CpG biomarker compared to a model with Gleason sum only. All statistical analyses were conducted using R.

Results

There was no difference in mean age between patients with metastatic-lethal PCa compared to those who did not recur in either cohort (Table 1). In both cohorts, Gleason sum, pathological stage, and PSA level at diagnosis were higher in men with the metastatic-lethal phenotype relative to men with no evidence of recurrence (all *P*-values <0.01). The FH cohort had a mean follow-up time of 8.1 years for recurrence and 12.2 years for survival. The EV cohort was followed for outcomes on average for 9.0 years.

Table 2 shows the 42 DNA methylation biomarkers that were most predictive for metastatic-lethal PCa in the FH cohort. These CpGs were identified based on their ability to improve the prognostic discrimination beyond Gleason sum alone (Supplemental Table S1). Half of the 42 biomarkers showed higher methylation in patients with metastatic-lethal PCa compared to those with non-recurrent disease (Table 2). The 42 biomarkers had a mean methylation difference between patient groups (metastatic-lethal vs. non-recurrent) that ranged from 1% to 22% (average = 6.1%), and pAUC and AUC values for metastatic-lethal PCa ranged from 0.006 to 0.018 and 0.54 to 0.84, respectively. DNA methylation levels of the 42 biomarkers were not strongly correlated (all pairwise r^2 <0.5).

We next evaluated the 42 top-ranked biomarkers in the EV replication cohort. For 30 of the CpGs, the difference in methylation level between patients with metastatic-lethal vs. non-recurrent PCa was in the same direction in the EV as in the FH cohort. Eight of these biomarkers demonstrated a significant AUC or pAUC in the EV cohort (all *P*-values <0.05; Table 3). One of the biomarkers had both a significant AUC and pAUC (*ATP11A* cg21513610). The CpG with the largest mean methylation difference was cg01135464 (*P* = 0.008). The biomarker with the highest AUC was *KLHL8* cg16713292 (0.75), and the largest pAUC was for *ATP11A* cg21513610 (0.009). We next investigated whether methylation levels of these CpGs were correlated with methylation levels of adjacent CpGs in the same gene or intergenic region. For five of the CpGs the methylation levels were correlated (pairwise r^2 >0.5) with methylation levels of nearby CpG sites (79 of 347 CpGs in *ATP11A*; 1 of 33 CpGs in *FHAD1*; 3 of 6 CpGs in *PI15*; 2 of 2 CpGs near cg01135464 [Chr. 17, OpenSea]; and 1 of 2 CpGs near cg22501793 [Chr. 1, S_Shore]).

We then evaluated the performance of the eight validated biomarkers for classifying patients with metastatic-lethal PCa when combined with Gleason sum (Table 4). Figure 1 shows the ROC curves for Gleason sum alone, the eight individual CpGs, and each CpG plus Gleason sum. The AUC for Gleason sum alone in the EV cohort was 0.82. This is higher than what has been reported in other studies and likely reflects our nested case-control study design, which involved selecting patients with metastatic-lethal PCa and a similar number of patients without evidence of recurrence. For comparison, in the FH cohort that is unselected for patient outcomes, Gleason sum alone has an AUC of 0.75 for metastatic-lethal PCa. Gleason sum had a pAUC for predicting metastatic-lethal PCa of 0.010 in the EV dataset. Likelihood ratio tests were then performed comparing the model with Gleason sum only to a model that included both Gleason sum and one of the eight CpGs. This test was significant for four of the CpGs (*P* <0.05): *ALKBH5* cg07166550, *FHAD1* cg02394978, *KLHL8* cg16713292, and *PI15* cg24349665, providing further evidence that these biomarkers are

complementary to Gleason sum for the prognostic discrimination of high-risk patients. Further adjustment for pathological stage (in addition to Gleason sum) increased the level of significance based on the likelihood ratio test for the three CpGs with the highest AUC values (AUC = 0.89), including the intergenic CpG on chr. 17 ($P = 0.023$) and the CpGs in *KLHL8* ($P = 0.0015$) and *PI15* ($P = 0.004$).

In a final analysis we evaluated tumor mRNA expression in the same FH and EV patients' tumor tissues that were used for methylation profiling. For two of the five genes that encompassed a validated CpG biomarker, DNA methylation levels were significantly correlated with transcript levels: *ATP11A* (Pearson $r^2 = -0.29$, $P = 2.78E\text{-}18$) and *PI15* (Pearson $r^2 = -0.28$, $P = 5.77E\text{-}08$). *ATP11A* cg21513610 is located in the gene body, whereas cg24349665 is in the promoter region of *PI15*.

Discussion

Our results demonstrate that DNA methylation biomarkers measured in primary tumor tissue can distinguish patients with metastatic-lethal PCa from those men at least five years post-radical prostatectomy without disease recurrence. Of the 42 top-ranked differentially methylated CpG sites that stratified patients with aggressive tumors in our discovery cohort, and improved the prognostic discrimination beyond that provided by Gleason sum alone, eight were subsequently validated to predict metastatic-lethal outcomes in an independent patient cohort.

Prior studies of tumor DNA methylation in PCa mainly used biochemical recurrence as the outcome event (14, 18, 28, 29). Hypermethylation of CpGs in the promoter region of two genes, *PITX2* and *GSTP1,* was previously associated with PSA recurrence (14, 29, 30). However, most men who have a rising PSA after surgery will not develop life-threatening disease (31), making biochemical recurrence less relevant than metastatic-lethal PCa outcomes as a study endpoint. A few prior candidate gene studies did assess lethal PCa (32-37), although the analyses were limited by both small sample sizes and short durations of follow-up for survival. Those studies highlighted a few differentially methylated genes (eg, *APC, PITX2*), but our results do not provide further support for aberrant methylation of these genes being biomarkers for progression to metastatic-lethal PCa.

The eight novel differentially methylated CpG sites validated in our study for the metastatic-lethal phenotype are located in five genes (*ALKBH5*, *ATP11A*, *FHAD1, KLHL8*, and *PI15)* and three intergenic regions (Chr. 1, 16, and 17). The five genes are involved in regulatory functions, response to hypoxia, protein-binding, developmental processes, and ion transport (38-42). The oxidative DNA demethylase *ALKBH5*, which is upregulated under hypoxia and also plays a role in spermatogenesis, belongs to the same gene family as *ALKBH3* (*Prostate Cancer Antigen 1*), which is highly expressed in prostate tumors and is a potential therapeutic target for PCa (43). In a previous study expression of *ATP11A*, which belongs to an extended family of adenosine triphosphate-binding cassette transporters, was associated with colorectal cancer mortality (41). A small study found that *PI15* (*peptidase inhibitor 15*) was amplified and overexpressed in 11% of advanced prostate tumors (44). The *PI15* gene was also identified as a candidate oncogene in colorectal cancer (45), and has been

implicated in regulating drug resistance in ovarian cancer (46). Interestingly, we also found that the DNA methylation alterations in *PI15* and *ATP11A* were significantly correlated with mRNA expression of these genes in the same patients' tumors. For *PI15*, the correlation was in the expected direction (ie, promoter hypermethylation and reduced expression). Specific molecular mechanisms whereby differential methylation of CpG sites in these five genes and three intergenic regions may enhance metastatic progression are unclear. There is biological plausibility for several of these genes contributing to more aggressive tumor biology, however further studies are needed to elucidate potential mechanisms.

Strengths of the current study include its relatively large sample size, the genome-wide approach for biomarker discovery, the population-based nature of the discovery cohort, with long-term prospective follow-up of patients diagnosed with clinically localized PCa, and a focus on the most serious clinical endpoint of metastatic-lethal disease. Validation of the DNA methylation biomarkers in an independent patient cohort is also critical, and confirms that these CpGs have added value to Gleason sum for predicting adverse patient outcomes. A potential weakness of our study is the limited number of patients with metastatic-lethal events, but these are not frequent outcomes in men diagnosed with clinically localized tumors that are treated surgically. Use of adjuvant therapy or salvage therapy may improve prognosis. In the FH cohort, adjuvant therapy use after RP was not frequent in non-recurrent (7.9%) or metastatic-lethal (16.7%) patients, making it unlikely that such therapies had a major impact on outcomes. As expected, most patients (94%) with metastatic progression received salvage therapy. Neither use of adjuvant nor salvage therapy, however, would affect methylation profiles in primary tumor tissue obtained at the time of surgery.

PCa is a heterogeneous disease and a combination of biomarkers may perform better than individual CpGs for prognostic classification. However, we did not intend to validate the combination of CpG biomarkers in our replication dataset due to the desire to avoid over fitting the data. Additional independent patient cohorts will be needed to build and test whether the combination of all or a subset of the eight CpG sites can further improve the prognostication for patients with more aggressive tumors. Further, additional investigation is needed to see if these biomarkers are predictive of patient outcomes in men not choosing radical prostatectomy as primary therapy.

In conclusion, we identified and then validated a novel panel of DNA methylation biomarkers in primary prostate tumor tissue that provide prognostic information, which improves upon Gleason sum for predicting metastatic-lethal patient outcomes. The methylation biomarkers replicated in this study have potential for improving clinical decision making by identifying patients likely to have a more aggressive cancer and who thereby are good candidates for adjuvant therapy or novel therapeutic clinical trials and who should be monitored more closely for metastatic progression. Future studies are needed to further evaluate the performance of our panel of prognostic DNA methylation biomarkers and to investigate if combining these epigenetic biomarkers may further improve their prognostic discrimination in early stage PCa patients. Investigations to elucidate the underlying molecular mechanisms through which these alterations in DNA methylation may enhance tumor aggressiveness are also needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Prostate cancer (PCa) is a clinically heterogeneous disease and it is challenging to accurately predict which patients with localized stage disease harbor tumors with life-threatening potential. DNA methylation alterations may mediate tumor aggressiveness and could be informative for prognostication. We comprehensively profiled primary tumor DNA methylation (>485K CpGs) in two independent PCa cohorts followed prospectively for >5 years after radical prostatectomy to assess outcomes. An initial panel of 42 differentially methylated CpGs robustly distinguished patients with metastatic-lethal compared to non-recurrent tumors in the discovery cohort, and eight of these biomarkers were subsequently confirmed to predict metastatic-lethal events in the validation cohort, including CpGs in five genes (*ALKBH5*, *ATP11A*, *FHAD1*, *KLHL8*, and *PI15)* and three intergenic regions. These eight differentially methylated CpG sites warrant further investigation as novel prognostic biomarkers for distinguishing PCa patients who need closer monitoring for metastatic progression and who may benefit most from adjuvant therapy.

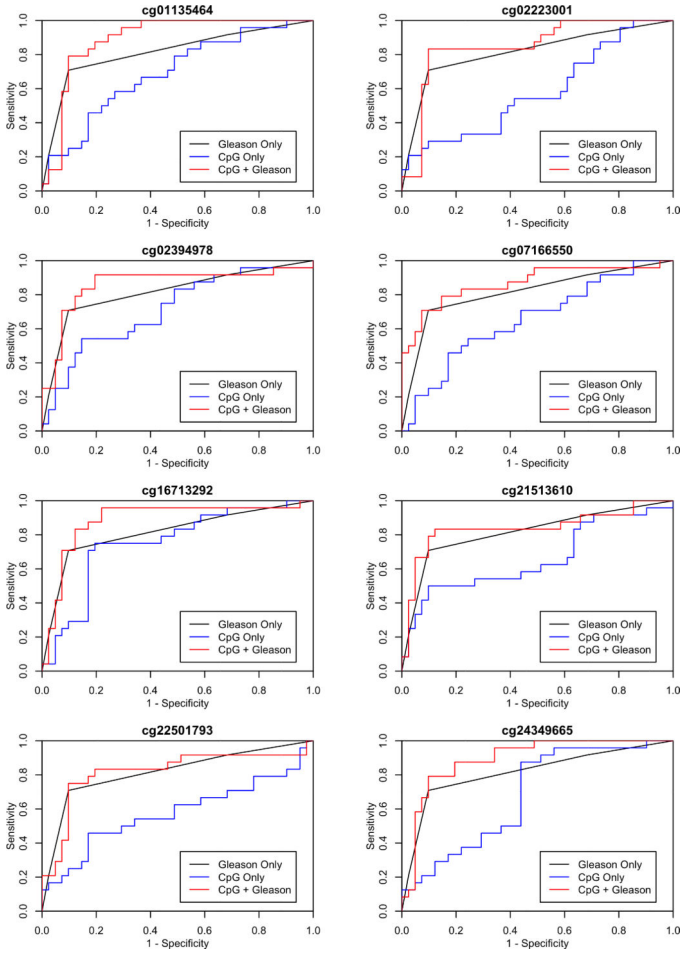

Fig 1.

ROC curves for predicting metastatic-lethal vs. non-recurrent prostate cancer for eight validated DNA methylation biomarkers. Curves are shown for each CpG biomarker alone, Gleason sum alone, and the biomarker plus Gleason sum.

Table 1

Characteristics of the prostate cancer patient populations

	FH patients (Discovery cohort)							EV patients (Validation dataset)						
	Non-recurrence (*n* = 303)			Metastatic-lethal PCa (*n* = 24)				Non-recurrence (*n* = 41)			Metastatic-lethal PCa (*n* = 24)			
Characteristic	**No.**		**Mean (SD)**	**No.**	**%**	**Mean (SD)**	***P*-value[a]**	**No.**	**%**	**Mean (SD)**	**No.**	**%**	**Mean (SD)**	***P*-value[a]**
Age at diagnosis (years)			58.2 (7.1)			58.6 (7.3)	0.76			60.2 (6.0)			60.3 (5.7)	0.97
Gleason sum							<0.01							<0.01
≤ 6	173	57.1		5	20.8			13	31.7		2	8.3		
7 (3+4)	101	33.3		8	33.3			24	58.5		5	20.8		
7 (4+3)	16	5.3		5	20.8			1	2.4		5	20.8		
8–10	13	4.3		6	25.0			3	7.3		12	50.0		
Pathological stage[b]							<0.01							<0.01
Local	235	77.6		11	45.8			22	53.7		0	0.0		
Regional	68	22.4		13	54.2			19	46.3		24	100.0		
PSA (ng/mL) at diagnosis							<0.01							<0.01
< 4.0	54	17.8		2	8.3			10	24.4		3	12.5		
4.0–4.9	189	62.4		7	29.2			27	65.9		14	58.3		
10.0–19.9	28	9.2		5	20.8			2	4.9		5	20.8		
≥ 20	14	4.6		7	29.2			1	2.4		2	8.3		

Note: PCa = prostate cancer; FH = Fred Hutchinson; EV = Eastern Virginia

[a] A t-test (age) or chi-square test was used (all categorical variables).

[b] Local stage = pT2, N0/NX, M0; Regional stage = pT3/T4 and/or N1, M0.

Table 2

Top-ranked 42 DNA methylation biomarkers for distinguishing patients with metastatic-lethal versus non-recurrent prostate cancer in the FH discovery cohort

CpG ID	Gene	Chr.	Genetic location	Epigenetic location	Mean β non-recurrence	Mean β metastatic-lethal	Mean β difference	AUC	pAUC	*P*-value[b]
Higher DNA methylation level[a]										
cg00022858	*PDGFRA*	4	5′UTR	Island	0.09	0.11	0.02	0.58	0.008	8.51E-02
cg00107241	–	1	Intergenic	Island	0.85	0.89	0.05	0.76	0.010	1.82E-04
cg00750074	*SPG7*	16	Body	Island	0.91	0.93	0.02	0.75	0.007	8.98E-05
cg01135464	–	17	Intergenic	OpenSea	0.41	0.63	0.22	0.84	0.018	1.25E-06
cg02223001	–	16	Intergenic	Island	0.08	0.10	0.02	0.63	0.010	3.62E-02
cg04670359	*NXT1*	20	TSS200	Island	0.08	0.10	0.02	0.77	0.009	7.61E-04
cg07065941	*KLF10*	8	Body	Island	0.09	0.11	0.02	0.75	0.006	9.18E-04
cg08092830	*SOHLH1*	9	TSS200	Island	0.69	0.74	0.04	0.74	0.012	4.90E-03
cg09734394	–	7	Intergenic	S_Shore	0.69	0.78	0.09	0.71	0.011	1.00E-02
cg11084729	–	15	Intergenic	OpenSea	0.94	0.95	0.01	0.56	0.008	1.85E-01
cg12300288	*MUC4*	3	Body	S_Shore	0.81	0.85	0.05	0.70	0.013	1.70E-03
cg13371199	*NDUFAF4*	6	Body	Island	0.06	0.08	0.01	0.66	0.012	1.47E-02
cg15850155	*IGF2R*	6	Body	Island	0.90	0.92	0.02	0.63	0.008	3.37E-02
cg15965055	*ILDR2*	1	TSS1500	Island	0.11	0.14	0.03	0.69	0.010	8.70E-03
cg15996882	*SREBF1*	17	3′UTR	Island	0.19	0.24	0.05	0.73	0.014	8.17E-04
cg16696648	*SDHB*	1	TSS1500	S_Shore	0.67	0.73	0.06	0.73	0.008	1.40E-03
cg18771570	–	2	Intergenic	OpenSea	0.51	0.61	0.10	0.69	0.010	1.23E-02
cg19104976	*PHF15*	5	Body	OpenSea	0.93	0.94	0.01	0.60	0.009	1.18E-01
cg22501793	–	1	Intergenic	S_Shore	0.12	0.20	0.09	0.71	0.010	2.80E-03
cg24349665	*PI15*	8	TSS200	OpenSea	0.23	0.41	0.18	0.81	0.012	9.03E-06
cg24867247	–	13	Intergenic	S_Shelf	0.24	0.33	0.10	0.71	0.011	2.20E-03
Lower DNA methylation level[a]										
cg00837987	–	8	Intergenic	OpenSea	0.72	0.56	0.16	0.74	0.008	9.69E-04
cg01166180	*POLR2I;TBCB*	19	TSS1500;Exon 1	Island	0.08	0.07	0.01	0.69	0.008	4.90E-03
cg02067030	*EDIL3*	5	Body	OpenSea	0.66	0.49	0.17	0.84	0.009	7.09E-07
cg02394978	*FHAD1*	1	TSS1500	N_Shore	0.82	0.73	0.09	0.71	0.011	1.70E-03

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CpG ID	Gene	Chr.	Genetic location	Epigenetic location	Mean β non-recurrence	Mean β metastatic-lethal	Mean β difference	AUC	pAUC	*P*-value[b]
cg03960699	*USP34*	2	Body	OpenSea	0.04	0.04	0.01	0.64	0.009	1.18E-02
cg04086197	*PPP1R13B*	14	Body	Island	0.86	0.80	0.06	0.69	0.012	4.80E-03
cg07166550	*ALKBH5*	17	Body	S_Shore	0.80	0.74	0.06	0.60	0.012	1.44E-01
cg07466320	*SRRM2*	16	Body	Island	0.80	0.75	0.05	0.72	0.009	2.00E-03
cg10462356	*FBXO21*	12	Body	Island	0.87	0.80	0.07	0.69	0.009	1.52E-02
cg12629515	*HIST1H3J;HIST1H2BO*	6	TSS1500	N_Shore	0.77	0.70	0.07	0.60	0.012	1.21E-01
cg12817908	*NMNAT3*	3	TSS1500	S_Shore	0.46	0.38	0.09	0.64	0.012	2.52E-02
cg14162120	*VSX2*	14	Body	Island	0.09	0.08	0.01	0.67	0.009	9.50E-03
cg14419310	*RASEF*	9	Body	Island	0.06	0.05	0.01	0.54	0.008	1.82E-01
cg14769589	–	17	Intergenic	N_Shore	0.32	0.24	0.08	0.83	0.015	1.06E-05
cg16713292	*KLHL8*	4	Body	OpenSea	0.86	0.76	0.11	0.72	0.015	1.40E-03
cg17603271	–	1	Intergenic	OpenSea	0.89	0.87	0.02	0.72	0.012	9.43E-04
cg20411049	*RSF1*	11	Body	N_Shore	0.08	0.07	0.01	0.66	0.010	1.58E-02
cg21513610	*ATP11A*	13	Body	S_Shore	0.90	0.86	0.04	0.73	0.010	9.90E-04
cg22282498	–	7	Intergenic	N_Shelf	0.66	0.54	0.12	0.77	0.010	1.20E-03
cg25541259	*DNER*	2	Body	N_Shore	0.47	0.38	0.09	0.76	0.007	5.31E-04
cg26756208	–	10	Intergenic	Island	0.14	0.10	0.04	0.73	0.013	8.80E-03

Note: FH = Fred Hutchinson; TSS = transcription start site; UTR = untranslated region; CpG biomarkers highlighted in boldface were validated in the Eastern Virginia replication cohort.

[a]Higher or lower DNA methylation level in tumor tissue of patients with metastatic-lethal vs. non-recurrent prostate cancer.

[b]Based on a t-test comparing mean methylation level between patients with metastatic-lethal vs. non-recurrent prostate cancer.

Table 3

Eight validated DNA methylation biomarkers for distinguishing patients with metastatic-lethal versus non-recurrent prostate cancer in the EV replication dataset[a]

CpG ID	Gene or region	Mean β difference[b]	AUC	95% CI AUC	*P*-value AUC	pAUC	95% CI pAUC	*P*-value pAUC	*P*-value t-test[c]
cg01135464	Intergenic (chr 17)	0.12	0.69	(0.56, 0.82)	**0.008**	0.006	(0, 0.018)	0.058	**0.008**
cg02223001	Intergenic (chr 16)	0.01	0.58	(0.44, 0.73)	0.279	0.008	(0.002, 0.019)	**0.024**	0.070
cg02394978	*FHAD1*	−0.06	0.71	(0.58, 0.83)	**0.003**	0.004	(0, 0.020)	0.159	**0.007**
cg07166550	*ALKBH5*	−0.05	0.66	(0.51, 0.79)	**0.035**	0.001	(0, 0.015)	0.566	**0.037**
cg16713292	*KLHL8*	−0.10	0.75	(0.63, 0.87)	**0.0004**	0.002	(0, 0.017)	0.359	**0.002**
cg21513610	*ATP11A*	−0.06	0.66	(0.51, 0.78)	**0.030**	0.009	(0.0004, 0.025)	**0.022**	**0.049**
cg22501793	Intergenic (chr 1)	0.03	0.58	(0.42, 0.73)	0.319	0.007	(0.002, 0.017)	**0.046**	0.151
cg24349665	*PI15*	0.07	0.68	(0.54, 0.81)	**0.014**	0.006	(0.0003, 0.015)	0.074	**0.029**

Note: EV = Eastern Virginia; chr = chromosome

[a] Biomarkers were considered validated when the AUC or pAUC (at 95% specificity) was significant (*P*-value <0.05). Significant *P*-values are highlighted in boldface.

[b] A positive value indicates a higher DNA methylation level (a negative value indicates a lower DNA methylation level) in patients with metastatic-lethal vs. non-recurrent prostate cancer.

[c] Based on a t-test comparing mean methylation levels in patients with metastatic-lethal vs. non-recurrent prostate cancer.

Table 4

Performance of the eight validated DNA methylation biomarkers combined with Gleason sum for predicting metastatic-lethal prostate cancer in the EV replication dataset

CpG ID	Gene or region	CpG + Gleason		*P*-value[a]
		AUC	pAUC	
cg01135464	Intergenic (chr 17)	0.89	0.004	0.073
cg02223001	Intergenic (chr 16)	0.85	0.004	0.219
cg02394978	*FHAD1*	0.86	0.013	**0.038**
cg07166550	*ALKBH5*	0.87	0.024	**0.030**
cg16713292	*KLHL8*	0.89	0.008	**0.014**
cg21513610	*ATP11A*	0.84	0.013	0.155
cg22501793	Intergenic (chr 1)	0.82	0.011	0.959
cg24349665	*PI15*	0.89	0.006	**0.026**

Note: EV = Eastern Virginia; chr = chromosome

[a] *P*-value for the likelihood ratio test comparing a model with Gleason sum alone (AUC = 0.816, pAUC = 0.010) to a model with Gleason sum and the CpG biomarker; significant *P*-values are highlighted in boldface.