Clinical Cancer Research

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

Shanshan Zhao[1], Milan S. Geybels[2,3], Amy Leonardson[2], Rohina Rubicz[2], Suzanne Kolb[2], Qingxiang Yan[4], Brandy Klotzle[5], Marina Bibikova[5], Antonio Hurtado-Coll[6], Dean Troyer[7], Raymond Lance[8], Daniel W. Lin[2,9], Jonathan L. Wright[2,9], Elaine A. Ostrander[10], Jian-Bing Fan[5], Ziding Feng[4], and Janet L. Stanford[2,11]

Abstract

Purpose: Aside from Gleason sum, few factors accurately identify the subset of prostate cancer 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 prostate cancer patients followed prospectively for at least 5 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 nonrecurrent tumors, and which were complementary to Gleason sum.

Results: Forty-two CpG biomarkers stratified patients with metastatic-lethal versus nonrecurrent prostate cancer 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 prostate cancer 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 $P < 0.05$).

Conclusions: Eight differentially methylated CpGs that distinguish patients with metastatic-lethal from nonrecurrent tumors were validated. These novel epigenetic biomarkers warrant further investigation as they may improve prognostic classification of patients with clinically localized prostate cancer and provide new insights on tumor aggressiveness. *Clin Cancer Res; 23(1); 311–9. ©2016 AACR.*

[1]National Institute of Environmental Health Sciences, Biostatistics and Computational Biology Branch, Research Triangle Park, Durham, North Carolina. [2]Division of Public Health Sciences, Fred Hutchison Cancer Research Center, Seattle, Washington. [3]Department of Epidemiology, GROW School for Oncology and Developmental Biology, Maastricht University, Maastricht, the Netherlands. [4]MD Anderson Cancer Center, Department of Biostatistics, Houston, Texas. [5]Illumina, Inc., San Diego, California. [6]Department of Urologic Sciences, University of British Columbia, and the Prostate Center, Vancouver General Hospital, Vancouver, Canada. [7]Departments of Pathology, Microbiology, and Molecular Cell Biology, Eastern Virginia Medical School, Norfolk, Virginia. [8]Department of Urology, Eastern Virginia Medical School, Norfolk, Virginia. [9]Department of Urology, University of Washington School of Medicine, Seattle, Washington. [10]Cancer Genetics and Comparative Genomics Branch, National Human Genome Research Institute, NIH, Bethesda, Maryland. [11]Department of Epidemiology, University of Washington School of Public Health, Seattle, Washington.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

S. Zhao and M.S. Geybels share first authorship.

J.-B. Fan, Z. Feng, and J.L. Stanford share senior authorship.

Current address for J.-B. Fan: AnchorDx Corp., Guangzhou 510300, P.R. China.

Corresponding Author: Janet L. Stanford, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N., M4-B874, Seattle, WA 98109-1024. Phone: 206-667-2715; Fax 206-667-2717; E-mail: jstanfor@fredhutch.org

doi: 10.1158/1078-0432.CCR-16-0549

Introduction

Prostate cancer is a biologically and clinically heterogeneous disease with 180,890 new cases and 26,120 cancer-specific deaths expected in the United States 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 prostate cancer (3, 4). The single most important predictor of prostate cancer 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, although a tumor with Gleason sum ≤ 6 is low risk and Gleason sum ≥ 8 is high risk, tumors that are Gleason sum = 7 (i.e., 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

Translational Relevance

Prostate cancer 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 prostate cancer 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 with nonrecurrent tumors in the discovery cohort, and 8 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 prostate cancer patients who need closer monitoring for metastatic progression and who may benefit most from adjuvant therapy.

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 prostate cancer progression have been limited to small sets of candidate genes in relation to biochemical (i.e., PSA) recurrence (14, 17–19). Although patients with biochemical recurrence are at higher risk of cancer-specific death, most will not die from their prostate cancer. Studies of patients with biochemical recurrence after radical prostatectomy found that only 17% to 22% died of prostate cancer 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 prostate cancer are needed.

We investigated epigenome-wide DNA methylation profiles in primary prostate cancers. The study includes patients derived from a population-based 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 prostate cancer 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 Cancer Research Center cohort. The Fred Hutchinson (FH) cohort includes 430 European-American prostate cancer 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 (e.g., 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 to 64 years who were diagnosed between January 1993 and December 1996, and in the second study, men were ages 35 to 74 years and were diagnosed between January 2002 and December 2005. Gleason grade (primary and secondary patterns) and sum, diagnostic PSA, and pathologic 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 prostate cancer 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 with patients who had not recurred. The FH Institutional Review Board approved the study, and all participants signed informed consent statements.

Eastern Virginia Medical School cohort. The validation dataset includes 80 patients diagnosed with localized stage prostate cancer who underwent radical prostatectomy at Eastern Virginia (EV) Medical School. The study population includes men who experienced disease progression to metastatic or lethal prostate cancer ($n = 31$) and a similar number of patients ($n = 49$) selected on the basis of having no evidence of recurrence during 5 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 hematoxylin 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 $\geq$75% tumor cells were taken for DNA and two cores for RNA purification. The RecoverAll Total Nucleic Acid Isolation Kit (Ambion/Applied Biosciences) was used to extract DNA, which was then quantified (PicoGreen), aliquoted onto 96-well plates, and shipped to Illumina (Illumina, Inc.) for DNA methylation profiling.

The EZ DNA Methylation Kit (Zymo Research) 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 epigenome-wide methylation using beads with target-specific probes designed to interrogate individual CpG sites (>485,000; ref. 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. Prostate cancer 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 nonrecurrent, 24 metastatic-lethal) and 65 (41 nonrecurrent, 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, respectively.

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), with the HumanHT-12 v4 BeadChip. 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 nonrecurrent prostate cancer 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 prostate cancer compared with 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 prostate cancer compared with 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 nonrecurrent prostate cancer 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, i.e., was the most significant in predicting metastatic-lethal prostate cancer, compared with 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 prespecified 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 ($\geq$3 when considering AUC; $\geq$3 when considering pAUC; $\geq$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 nonrecurrent prostate cancer. *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 with 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 prostate cancer compared with those who did not recur in either cohort (Table 1). In both cohorts, Gleason sum, pathologic 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 prostate cancer in the FH cohort. These CpGs were identified based on their ability to improve the prognostic discrimination beyond Gleason sum alone (Supplementary Table S1). Half of the 42 biomarkers showed higher methylation in patients with metastatic-lethal prostate cancer compared with those with nonrecurrent disease (Table 2). The 42 biomarkers had a mean methylation difference between patient groups (metastatic-lethal vs. nonrecurrent) that ranged from 1% to 22% (average = 6.1%), and pAUC and AUC

Table 1. Characteristics of the prostate cancer patient populations

	FH patients (Discovery cohort)							EV patients (Validation dataset)						
	Nonrecurrence ($n = 303$)			Metastatic-lethal PCa ($n = 24$)				Nonrecurrence ($n = 41$)			Metastatic-lethal PCa ($n = 24$)			
Characteristic	***N***	**%**	**Mean (SD)**	***N***	**%**	**Mean (SD)**	***P* value[a]**	***N***	**%**	**Mean (SD)**	***N***	**%**	**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		
Pathologic 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[c]							<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		

Abbreviation: PCa, prostate cancer.
[a]A *t* test (age) or χ^2 test was used (all categorical variables).
[b]Local stage = pT2, N0/NX, M0; Regional stage = pT3/T4 and/or N1, M0.
[c]Percentages may not add to 100% due to missing PSA data.

values for metastatic-lethal prostate cancer 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 versus nonrecurrent prostate cancer 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 prostate cancer 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 prostate cancer 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 prostate cancer. Gleason sum had a pAUC for predicting metastatic-lethal prostate cancer of 0.010 in the EV dataset. Likelihood ratio tests were then performed comparing the model with Gleason sum only with 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 pathologic 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's r = –0.29, P = 2.78E–18) and *PI15* (Pearson's r = –0.28, P = 5.77E–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 prostate cancer from those men at least 5 years after 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 prostate cancer 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

Table 2. Top-ranked 42 DNA methylation biomarkers for distinguishing patients with metastatic-lethal versus nonrecurrent prostate cancer in the FH discovery cohort

CpG ID	Gene	Chr.	Genetic location	Epigenetic location	Mean β nonrecurrence	Mean β metastatic-lethal	Mean β difference	AUC	pAUC	*P* value[a]
Higher DNA methylation level[b]										
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[b]										
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
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: CpG biomarkers highlighted in boldface were validated in the EV replication cohort.

Abbreviations: TSS, transcription start site; UTR, untranslated region.

[a]Based on a *t* test comparing mean methylation level between patients with metastatic-lethal versus nonrecurrent prostate cancer.

[b]Higher or lower DNA methylation level in tumor tissue of patients with metastatic-lethal versus nonrecurrent prostate cancer.

metastatic-lethal prostate cancer outcomes as a study endpoint. A few prior candidate gene studies did assess lethal prostate cancer (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 (e.g., *APC*, *PITX2*), but our results do not provide further support for aberrant methylation of these genes being biomarkers for progression to metastatic-lethal prostate cancer.

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 prostate cancer (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

Table 3. Eight validated DNA methylation biomarkers for distinguishing patients with metastatic-lethal versus nonrecurrent 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**

Abbreviation: 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 versus nonrecurrent prostate cancer.

[c]Based on a *t* test comparing mean methylation levels in patients with metastatic-lethal versus nonrecurrent prostate cancer.

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 (i.e., 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 epigenome-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 prostate cancer, 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 radical prostatectomy was not frequent in nonrecurrent (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.

Prostate cancer 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 overfitting 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

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 + Gleason		
CpG ID	Gene or region	AUC	pAUC	*P* value[a]
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: Significant *P* values are highlighted in boldface.

Abbreviation: 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.

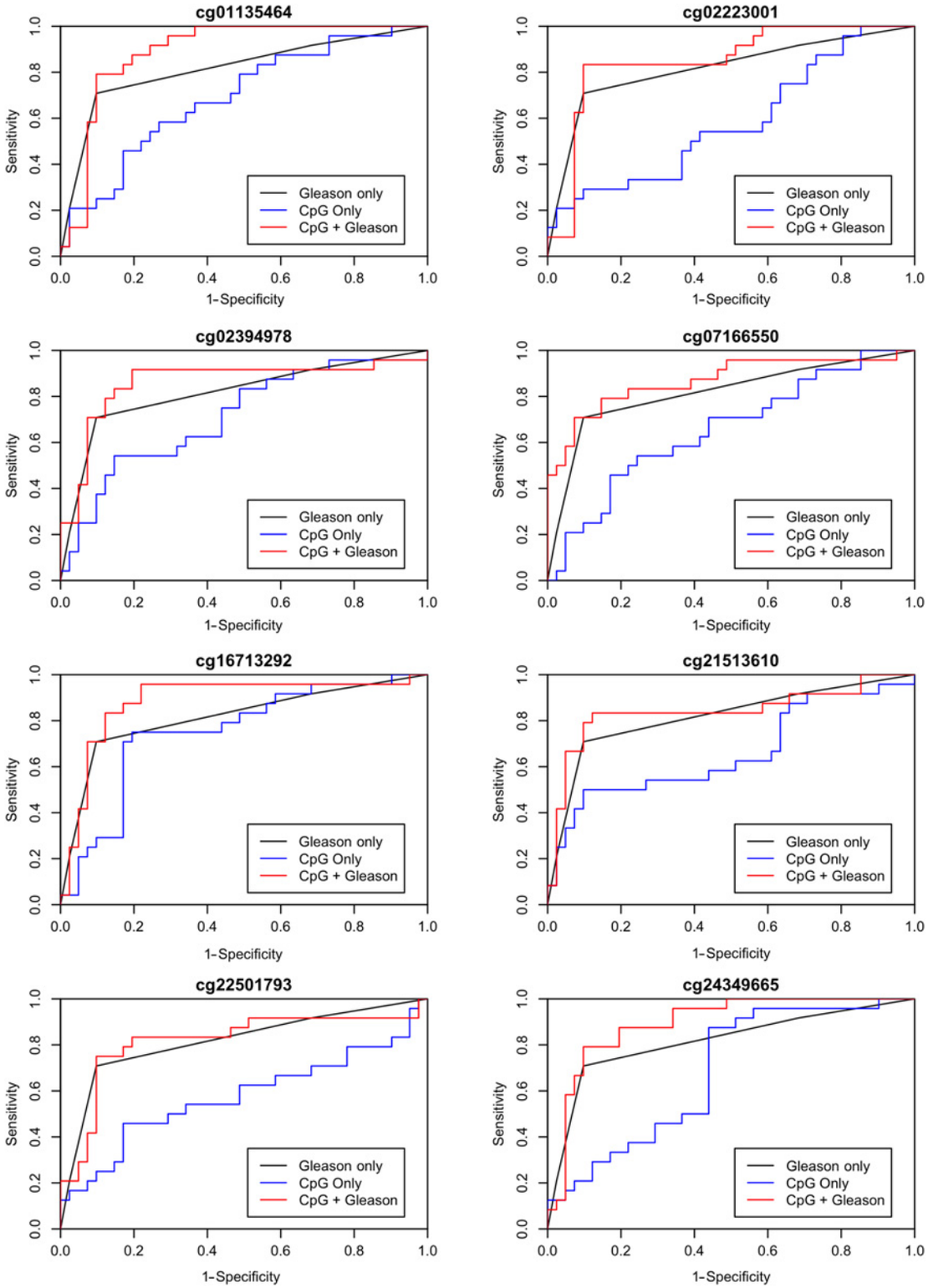

Figure 1.

ROC curves for predicting metastatic-lethal versus nonrecurrent 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.

these epigenetic biomarkers may further improve their prognostic discrimination in early stage prostate cancer patients. Investigations to elucidate the underlying molecular mechanisms through which these alterations in DNA methylation may enhance tumor aggressiveness are also needed.

Disclosure of Potential Conflicts of Interest

R. Lance reports receiving speakers bureau honoraria from Medivation. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: J.L. Wright, J.-B. Fan, Z. Feng, J.L. Stanford
Development of methodology: S. Zhao, M.S. Geybels, M. Bibikova, A. Hurtado-Coll, J.-B. Fan, Z. Feng, J.L. Stanford
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Kolb, B. Klotzle, M. Bibikova, A. Hurtado-Coll, D. Troyer, R. Lance, D.W. Lin, J.L. Wright, E.A. Ostrander, J.-B. Fan, J.L. Stanford
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Zhao, M.S. Geybels, A. Leonardson, R. Rubicz, Q. Yan, B. Klotzle, J.L. Wright, Z. Feng, J.L. Stanford
Writing, review, and/or revision of the manuscript: S. Zhao, M.S. Geybels, A. Leonardson, R. Rubicz, D. Troyer, R. Lance, D.W. Lin, J.L. Wright, E.A. Ostrander, Z. Feng, J.L. Stanford
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.L. Stanford
Study supervision: J.-B. Fan, Z. Feng, J.L. Stanford

Acknowledgments

The authors thank Drs. Beatrice Knudson and Xiaotun Zhou for their assistance with the pathology, and the GU Tumor Biorepository, Eastern Virginia Medical School. They also thank all the men who participated in these studies.

Grant Support

This work was supported by grants from the NCI (R01 CA056678, R01 CA092579, K05 CA175147, and P50 CA097186), with additional support provided by the Fred Hutchinson Cancer Research Center, the Intramural Program of the National Human Genome Research Institute, the National Institute of Environmental Health Sciences, and the Prostate Cancer Foundation. Illumina, Inc. provided and performed the methylation arrays. M.S. Geybels is the recipient of a Dutch Cancer Society Fellowship (BUIT 2014–6645).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 4, 2016; revised June 8, 2016; accepted June 11, 2016; published OnlineFirst June 29, 2016.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016;66:7–30.
2. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, et al. GLOBOCAN 2012 v1.0, Cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet]. 2012. [cited 2015 Dec 7]. Available from: http://globocan.iarc.fr.
3. Attard G, Parker C, Eeles RA, Schroder F, Tomlins SA, Tannock I, et al. Prostate cancer. Lancet 2015;387:70–82.
4. Fraser M, Berlin A, Bristow RG, van der Kwast T. Genomic, pathological, and clinical heterogeneity as drivers of personalized medicine in prostate cancer. Urol Oncol 2015;33:85–94.
5. Porten SP, Whitson JM, Cowan JE, Cooperberg MR, Shinohara K, Perez N, et al. Changes in prostate cancer grade on serial biopsy in men undergoing active surveillance. J Clin Oncol 2011;29:2795–800.
6. Epstein JI, Feng Z, Trock BJ, Pierorazio PM. Upgrading and downgrading of prostate cancer from biopsy to radical prostatectomy: Incidence and predictive factors using the modified Gleason grading system and factoring in tertiary grades. Eur Urol 2012;61:1019–24.
7. Treurniet KM, Trudel D, Sykes J, Evans AJ, Finelli A, Van der Kwast TH. Downgrading of biopsy based Gleason score in prostatectomy specimens. J Clin Pathol 2014;67:313–8.
8. Wright JL, Salinas CA, Lin DW, Kolb S, Koopmeiners J, Feng Z, et al. Prostate cancer specific mortality and Gleason 7 disease differences in prostate cancer outcomes between cases with Gleason 4 + 3 and Gleason 3 + 4 tumors in a population based cohort. J Urol 2009;182: 2702–7.
9. Choudhury AD, Eeles R, Freedland SJ, Isaacs WB, Pomerantz MM, Schalken JA, et al. The role of genetic markers in the management of prostate cancer. Eur Urol 2012;62:577–87.
10. Loeb S, Bjurlin MA, Nicholson J, Tammela TL, Penson DF, Carter HB, et al. Overdiagnosis and overtreatment of prostate cancer. Eur Urol 2014;65: 1046–55.
11. Ross AE, D'Amico AV, Freedland SJ. Which, when and why? Rational use of tissue-based molecular testing in localized prostate cancer. Prostate Cancer and Prostatic Diseases 2016;19:1–6.
12. Bostrom PJ, Bjartell AS, Catto JWF, Eggener SE, Lilja H, Loeb S, et al. Genomic predictors of outcome in prostate cancer. Eur Urol 2015;68: 1033–44.
13. Issa JP. DNA methylation as a clinical marker in oncology. J Clin Oncol 2012;30:2566–68.
14. Chao C, Chi M, Preciado M, Black MH. Methylation markers for prostate cancer prognosis: A systematic review. Cancer Causes Control 2013;24: 1615–41.
15. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042–54.
16. Jeronimo C, Bastian PJ, Bjartell A, Carbone GM, Catto JW, Clark SJ, et al. Epigenetics in prostate cancer: Biologic and clinical relevance. Eur Urol 2011;60:753–66.
17. Ashour N, Angulo JC, Andres G, Alelu R, Gonzalez-Corpas A, Toledo MV, et al. A DNA hypermethylation profile reveals new potential biomarkers for prostate cancer diagnosis and prognosis. The Prostate 2014;74:1171–82.
18. Haldrup C, Mundbjerg K, Vestergaard EM, Lamy P, Wild P, Schulz WA, et al. DNA methylation signatures for prediction of biochemical recurrence after radical prostatectomy of clinically localized prostate cancer. J Clin Oncol 2013;31:3250–8.
19. Horning AM, Awe JA, Wang CM, Liu J, Lai Z, Wang VY, et al. DNA methylation screening of primary prostate tumors identifies *SRD5A2* and *CYP11A1* as candidate markers for assessing risk of biochemical recurrence. The Prostate 2015;75:1790–801.
20. Freedland SJ, Humphreys EB, Mangold LA, Eisenberger M, Dorey FJ, Walsh PC, et al. Risk of prostate cancer-specific mortality following biochemical recurrence after radical prostatectomy. JAMA 2005; 294:433–9.
21. Abdollah F, Boorjian SA, Cozzarini C, Suardi N, Sun M, Fiorino C, et al. Survival following biochemical recurrence after radical prostatectomy and adjuvant radiotherapy in patients with prostate cancer: The impact of competing causes of mortality and patient stratification. Eur Urol 2013;64: 557–64.
22. Agalliu I, Salinas CA, Hansten PD, Ostrander EA, Stanford JL. Statin use and risk of prostate cancer: Results from a population-based epidemiologic study. Am J Epidemiol 2008;168:250–60.
23. Stanford JL, Wicklund KG, McKnight B, Daling JR, Brawer MK. Vasectomy and risk of prostate cancer. Cancer Epidemiol Biomarkers Prev 1999; 8:881–6.
24. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le J, et al. High density DNA methylation array with single CpG site resolution. Genomics 2011;98: 288–95.
25. Maksimovic J, Gordon L, Oshlack A. SWAN: Subset-quantile within array normalization for Illumina Infinium HumanMethylation450 BeadChips. Genome Biology 2012;13:R44.
26. Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics 2007;8: 118–27.
27. Hansen K. Illumina HumanMethylation450kanno.ilmn12.hg19: Annotation for Illumina's 450k methylation arrays. R package version 0.2.1.

28. Chiam K, Ricciardelli C, Bianco-Miotto T. Epigenetic biomarkers in prostate cancer: Current and future uses. Cancer Letters 2014;342:248–56.
29. Valdes-Mora F, Clark SJ. Prostate cancer epigenetic biomarkers: Next-generation technologies. Oncogene 2015;34:1609–18.
30. Banez LL, Sun L, van Leenders GJ, Wheeler TM, Bangma CH, Freedland SJ, et al. Multicenter clinical validation of *PITX2* methylation as a prostate specific antigen recurrence predictor in patients with post-radical prostatectomy prostate cancer. J Urol 2010;184:149–56.
31. Uchio EM, Aslan M, Wells CK, Calderone J, Concato J. Impact of biochemical recurrence in prostate cancer among US veterans. Arch Intern Med 2010;170:1390–5.
32. Henrique R, Ribeiro FR, Fonseca D, Hoque MO, Carvalho AL, Costa VL, et al. High promoter methylation levels of *APC* predict poor prognosis in sextant biopsies from prostate cancer patients. Clin Cancer Res 2007; 13:6122–9.
33. Richiardi L, Fiano V, Vizzini L, De Marco L, Delsedime L, Akre O, et al. Promoter methylation in *APC*, *RUNX3*, and *GSTP1* and mortality in prostate cancer patients. J Clin Oncol 2009;27:3161–8.
34. Rosenbaum E, Hoque MO, Cohen Y, Zahurak M, Eisenberger MA, Epstein JI, et al. Promoter hypermethylation as an independent prognostic factor for relapse in patients with prostate cancer following radical prostatectomy. Clin Cancer Res 2005;11:8321–5.
35. Vasiljevic N, Ahmad AS, Beesley C, Thorat MA, Fisher G, Berney DM, et al. Association between DNA methylation of HSPB1 and death in low Gleason score prostate cancer. Prostate Cancer Prostatic Dis 2013;16: 35–40.
36. Vasiljevic N, Ahmad AS, Carter PD, Fisher G, Berney DM, Foster CS, et al. DNA methylation of *PITX2* predicts poor survival in men with prostate cancer. Biomark Med 2014;8:1143–50.
37. Vasiljevic N, Ahmad AS, Thorat MA, Fisher G, Berney DM, Moller H, et al. DNA methylation gene-based models indicating independent poor outcome in prostate cancer. BMC Cancer 2014;14:655.
38. Dhanoa BS, Cogliati T, Satish AG, Bruford EA, Friedman JS. Update on the Kelch-like (*KLHL*) gene family. Hum Genomics 2013;7:13.
39. Durocher D, Jackson SP. The FHA domain. FEBS Letters 2002;513:58–66.
40. Falak S, Schafer S, Baud A, Hummel O, Schulz H, Gauguier D, et al. Protease inhibitor 15, a candidate gene for abdominal aortic internal elastic lamina ruptures in the rat. Physiol Genomics 2014;46:418–28.
41. Miyoshi N, Ishii H, Mimori K, Tanaka F, Nagai K, Uemura M, et al. ATP11A is a novel predictive marker for metachronous metastasis of colorectal cancer. Oncol Rep 2010;23:505–10.
42. Thalhammer A, Bencokova Z, Poole R, Loenarz C, Adam J, O'Flaherty L, et al. Human AlkB homologue 5 is a nuclear 2-oxoglutarate dependent oxygenase and a direct target of hypoxia-inducible factor 1alpha (HIF-1alpha). PloS One 2011;6:e16210.
43. Koike K, Ueda Y, Hase H, Kitae K, Fusamae Y, Masai S, et al. Anti-tumor effect of AlkB homolog 3 knockdown in hormone- independent prostate cancer cells. Curr Cancer Drug Targets 2012;12:847–56.
44. Vainio P, Wolf M, Edgren H, He T, Kohonen P, Mpindi JP, et al. Integrative genomic, transcriptomic, and RNAi analysis indicates a potential oncogenic role for FAM110B in castration-resistant prostate cancer. The Prostate 2012;72:789–802.
45. Tuupanen S, Hanninen UA, Kondelin J, von Nandelstadh P, Cajuso T, Gylfe AE, et al. Identification of 33 candidate oncogenes by screening for base-specific mutations. Br J Cancer 2014;111:1657–62.
46. Zou J, Yin F, Wang Q, Zhang W, Li L. Analysis of microarray-identified genes and microRNAs associated with drug resistance in ovarian cancer. Int J Clin Exp Pathol 2015;8:6847–58.