Epigenetic signature of Gleason score and prostate cancer recurrence after radical prostatectomy

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Abstract

Background: Identifying the subset of patients with clinically localized prostate cancer (PCa) at the highest risk of recurrence remains challenging, and better prognostic markers are needed. Gleason score is the best predictor of PCa aggressiveness and prognosis. In the present study, we generated an epigenetic signature based on high versus low Gleason score tumors and evaluated its ability to predict recurrence after radical prostatectomy.

Methods: Genome-wide DNA methylation data from The Cancer Genome Atlas (TCGA; no. of patients = 333) and the elastic net method were used to generate an epigenetic signature by contrasting patients with high (8–10) versus low (≤6) Gleason score tumors. The signature was then tested in a cohort of 523 patients with clinically localized disease who had radical prostatectomy. Samples taken from the primary tumor were used for DNA methylation and mRNA expression profiling. Patients were followed for PCa recurrence on average for 8 years after diagnosis.

Results: The epigenetic signature includes 52 differentially methylated CpG sites. In the testing cohort, the signature was associated with poorer recurrence-free survival (hazard ratio per 25% increase = 1.78; 95% confidence interval 1.48, 2.16). The signature significantly improved the area under the curve (AUC) for PCa recurrence compared to clinical-pathological parameters alone, particularly among patients diagnosed with Gleason score 7 tumors (0.64 vs. 0.76, \( P = 1.34 \times 10^{-4} \)). Results were comparable for patients with Gleason 3 + 4 and those with 4 + 3 tumors. Gene Set Enrichment Analysis showed that higher levels of the signature were associated with increased expression of genes related to cell cycle proliferation and decreased expression of androgen-responsive genes.

Conclusions: This report shows evidence that DNA methylation patterns measured in prostate tumor cells are predictive of PCa aggressiveness. The epigenetic signature may have clinical utility to improve prognostication particularly in patients with intermediate Gleason score 7 tumors.

Keywords: Clinically localized prostate cancer, Tumor tissue, DNA methylation, Gene expression, Risk prediction for prognosis, Genome-wide profiling, Elastic net regularization

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Background

Prostate cancer (PCa) is the most common solid tumor in men [1]. While many PCa patients are diagnosed with indolent disease that is unlikely to progress even if left untreated, other patients will have aggressive tumors that may become life-threatening [2–4]. Although current clinical and pathological measures such as Gleason score (tumor grade), disease stage, and prostate-specific antigen (PSA) level provide important prognostic information, they do not accurately predict an individual patient’s risk of progression, and better markers to aid prognostication are needed [4, 5].

DNA methylation is an epigenetic alteration that occurs at CG dinucleotides (CpG sites) [6]. The human DNA methylome is generated in a programmed manner during normal development and methylation patterns change as a result of aging [7]. Patterns of DNA methylation record a remarkable breadth of information about cells, including their chronological age, developmental history, and differentiation potential [8, 9]. In PCa, there is substantial heterogeneity in tumor DNA methylation profiles [10], and these epigenetic changes may also predict PCa aggressiveness. Some previous studies showed that differentially methylated CpG sites in specific genes are associated with more aggressive and advanced PCa, but most of these investigations focused on selected candidate genes and findings require validation [11–18].

In this study, we generated an epigenetic (DNA methylation) signature for use as a prognostic classifier in PCa. Because Gleason score is the best predictor of PCa prognosis [19], we generated the signature by contrasting patients with high (8–10) versus low (≤6) Gleason score tumors. The signature was then tested for its ability to predict recurrence in a validation cohort of patients with clinically localized PCa who had radical prostatectomy.

Methods

Study population

The Fred Hutchinson (FH) Cancer Research Center cohort includes 565 PCa patients who underwent radical prostatectomy as primary therapy for clinically localized adenocarcinoma of the prostate. These patients were previously enrolled in population-based studies of PCa (diagnosed in 1993–1996 or 2002–2005) [20, 21]. Clinical information and vital status were collected from the Seattle-Puget Sound Surveillance, Epidemiology, and End Results (SEER) Program cancer registry. Prostate cancer recurrence status was determined from two detailed follow-up surveys that were completed by patients in 2004–2005 and in 2010–2011, with review of medical records or physician follow-up as needed. A patient was considered to have disease recurrence based on (1) a postsurgery PSA value of 0.2 ng/mL or greater; (2) metastatic progression on a bone scan, MRI, CT, or biopsy; and (3) PCa-specific death. The mean follow-up time for recurrence was 8 years. The Institutional Review Board of the Fred Hutchinson Cancer Research Center approved the study, and all participants signed informed consent statements.

DNA and RNA isolation

Formalin-fixed paraffin-embedded (FFPE) prostate tumor tissue blocks were obtained from radical prostatectomy specimens and used to make hematoxylin and eosin-stained slides, which were reviewed by a PCa pathologist to confirm the presence and location of prostate 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 RNA purification. The RecoverAll Total Nucleic Acid Isolation Kit (Ambion, Applied Biosciences, Austin, TX) was used to extract DNA. The RNeasy FFPE Kit (Qiagen Inc., Valencia, CA) was used to isolate RNA. DNA and RNA samples were shipped to Illumina (Illumina, Inc., San Diego, CA) for DNA methylation and mRNA expression profiling.

Molecular profiling and data preprocessing

Tumor DNA was bisulfite converted. The Infinium HumanMethylation450 BeadChip array (Illumina) was used for methylation profiling. Methylation data were normalized using subset-quantile within array normalization (minfi in Bioconductor) [22], and batch effects were removed using ComBat [23]. Methylation β values were calculated, which represent the percentage of DNA methylation at a CpG site. Genome annotation was based on the Illumina Manifest, and a gene promoter region was defined as TSS1500, TSS200, 5’ UTR, or 1stExon. Across the 96-well plates, we incorporated blind duplicate (n = 16) and replicate (n = 2) samples. 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 (probability of a CpG being detected above the background level defined by negative control probes) of <0.05, and 42 samples were excluded. Further, CpG sites with a detection P value of >0.01 were excluded. Correlation coefficients for duplicate samples were 0.96–0.99. The correlation coefficient for the replicate samples was 0.99. There were 523 patients with DNA methylation data.

The HumanHT-12 v4.0 Whole-Genome DASL HT Assay (Illumina) was used for gene expression profiling. Low-quality probes were filtered out (illuminaHumanWGDSASLv4.db package in Bioconductor). Gene expression data were quantile normalized and log2 transformed. Batch effects were removed using ComBat [23]. Genome annotation was based on the Illumina protocol. Blind duplicate samples from 11 patients had correlations ranging from 0.98 to 0.99,
and replicate samples from two patients had mean correlations of 0.99. There were 469 patients with mRNA expression data, and these patients also had DNA methylation data.

The Cancer Genome Atlas DNA methylation data

Level 1 Infinium HumanMethylation450 data were downloaded from The Cancer Genome Atlas (TCGA) data portal (https://tcga-data.nci.nih.gov/tcga/). The TCGA dataset includes 333 PCa patients who received radical prostatectomy [10]. The number of patients with Gleason ≤6, 7(3 + 4), 7(4 + 3), and 8–10 tumors is 65, 102, 78, and 88, respectively. Data on long-term patient outcomes are not available in TCGA [10]. Gene expression data from TCGA were downloaded from the Cancer Browser (https://genome-cancer.ucsc.edu/).

Statistical data analysis

Genome-wide DNA methylation data from TCGA were used to generate an epigenetic signature of Gleason score by contrasting patients with high (8–10) versus low (≤6) Gleason score tumors. The signature was built using the elastic net method (glmnet in R), which is a regularization and variable selection method for high-dimensional data [24]. This approach is a combination of traditional LASSO and ridge regression methods, emphasizing model sparsity while appropriately balancing the contributions of correlated variables [25]. All CpG sites were used as input for the analysis. Fivefold cross-validation and the area under the curve (AUC) criterion were used to determine the optimal tuning parameter λ for classification. After variable selection using elastic net, the signature was calculated as follows: signature

\[ \text{signature}_i = \beta_{g} \times X_{gi} \]

where \( g \) is the marker (i.e., CpG site); \( n \) is the number of markers; \( \beta_{g} \) is the elastic net coefficient for marker \( g \); and \( X_{gi} \) is the methylation value for marker \( g \) and patient \( i \).

The epigenetic signature was next evaluated for its ability to predict PCa recurrence in the FH cohort. Kaplan-Maier analysis and Cox regression models were used to examine the association between quartiles of the signature and recurrence-free survival. Hazard ratios (HRs) and 95 % confidence intervals (CIs) were calculated. A receiver operating characteristic (ROC) analysis was performed to evaluate the ability of the signature to distinguish patients with no evidence of recurrence from those who developed recurrence. A likelihood ratio test was used to compare a model that included the standard clinical-pathological parameters Gleason score (≤6, 7(3 + 4), 7(4 + 3), and 8–10), pathological stage (local: pT2, N0/NX, M0; regional: pT3-T4 and/or N1, M0), and diagnostic PSA level (1-unit increase), with a model that included these clinical-pathological variables and the epigenetic signature.

Tumor mRNA expression data were then used to study correlations between methylation levels of individual CpG sites in the signature and expression levels of corresponding genes. Pearson correlation coefficients were used. Next, Gene Set Enrichment Analysis (GSEA) was performed [26]. First, correlations between the signature and genome-wide gene expression levels were evaluated. The genes were then pre-ranked according to Pearson correlation and false discovery rate (FDR) Q value. Genes with a Q value of <0.05 were used as input for GSEA. For those genes with multiple transcripts, the average mean correlation across transcripts was calculated. GSEA was run with 1000 gene set permutations to calculate FDR Q values. We tested for the enrichment of “hallmark” gene sets, which have been shown to reduce variation and redundancy thereby providing more refined and concise inputs for GSEA [27]. The GSEA results were shown using normalized enrichment scores (NES), which is a value assigned to each gene set after normalization across all analyzed gene sets. NES is calculated by the following formula: NES = actual ES/mean (ESs against all permutations of the dataset) [26, 28]. All statistical analyses were done using R.

Results

The epigenetic signature of Gleason score generated in the TCGA cohort includes 52 differentially methylated CpG sites (Fig. 1a, b). Patients with Gleason score 7 tumors had intermediate levels of the signature compared to Gleason ≤6 and 8–10 tumors (Fig. 1c). Twenty-one CpGs in the signature had higher methylation levels in Gleason 8–10 tumors (Table 1). Thirty-six CpGs are in genes (32 unique genes), and 18 are in gene promoter regions. In the FH cohort (Table 2), higher levels of the signature correlated with increasing Gleason score (correlation = 0.33; \( P = 2.11\text{E}^{-14} \)) (Additional file 1: Figure S1).

The epigenetic signature was then tested for its ability to predict recurrence in the FH cohort. Higher levels of the signature were associated with poorer recurrence-free survival (Fig. 2a). The 5- and 10-year risk of recurrence increased with increasing quartiles of the signature: 8, 9, 14, and 27 % and 11, 20, 32, and 44 %, respectively. The HR of recurrence for each 25 % increment in the signature was 1.78 (95 % CI 1.48, 2.16), which remained significant after adjusting for Gleason score, pathological stage, and diagnostic PSA level (Table 3). Adding the signature to a model that included these prognostic parameters significantly improved the AUC for recurrence (0.73 vs. 0.78, \( P = 2.72\text{E}^{-5} \); Fig 2b). In this study, 24 patients with no recurrence and 17 patients with recurrence had adjuvant therapy after radical prostatectomy. Excluding these 41 patients did not substantially change the signature’s association with recurrence (HR per 25 % increase 1.81; 95 % CI 1.49, 2.20).
Fig. 1 Epigenetic signature of Gleason score in The Cancer Genome Atlas. a Variable selection using elastic net. Each curve in the figure corresponds to a single CpG site. It shows the path of its coefficient (y-axis) against the log(lambda) or tuning parameter (x-axis). The vertical dashed line represents the optimal log(lambda) for classifying high (8−10) versus low (≤6) Gleason score tumors, which was identified using cross-validation. Based on an optimal log(lambda) of −1.7061, 52 CpG sites were selected. These 52 CpGs and their elastic net coefficients were then used to calculate the epigenetic signature as described in the “Methods” section. b Heatmap of the 52 CpG sites that were selected using elastic net. The rows of the heatmap are the CpG sites, and the columns are the tumor samples. The samples were grouped by Gleason score. Methylation β values (range 0−1) were used, and the highest methylation levels are shown in red. The number of patients with Gleason ≤6, 7(3+4), 7(4+3), and 8−10 tumors is 65, 102, 78, and 88, respectively. The rows were clustered based on Euclidean distance. c Box plots of the epigenetic signature (y-axis) for patients with different Gleason scores. The signature is presented as a proportion. Higher Gleason scores were associated with higher levels of the signature. The same patients as in Fig. 1b were used.
Table 1  Fifty-two CpG sites included in the epigenetic signature

| CpG ID   | Chr. | Gene name | Genetic location | Epigenetic location | Mean β Gleason ≤6 | Mean β Gleason 8–10 | Mean β difference Elastic Net coefficient |
|----------|------|-----------|------------------|--------------------|-------------------|---------------------|------------------------------------------|
| cg10145000 | 1    |           |                  |                    | 0.44              | 0.31                | 0.12                                     | --0.0043                                |
| cg00043524 | 1    |           |                  |                    | 0.87              | 0.90                | 0.03                                     | 0.2022                                  |
| cg00506866 | 2    | RRM2      | Body             | Island             | 0.16              | 0.11                | 0.04                                     | --0.2591                                |
| cg02601249 | 2    |           |                  | S_Shore            | 0.80              | 0.62                | 0.17                                     | --0.1426                                |
| cg15454811 | 2    |           |                  | N_Shore            | 0.82              | 0.65                | 0.17                                     | --0.1904                                |
| cg09741917 | 2    | WA3B      | TSS1500          | N_Shore            | 0.49              | 0.63                | 0.14                                     | 0.3933                                  |
| cg13607230 | 2    | MFS09     | Body             | N_Shore            | 0.43              | 0.56                | 0.13                                     | 0.5376                                  |
| cg17353895 | 2    | ANO7      | 3'UTR;Body       |                    | 0.65              | 0.76                | 0.10                                     | 0.5339                                  |
| cg05287437 | 3    | GALNTL2   | TSS1500          |                    | 0.77              | 0.63                | 0.14                                     | --1.5620                                |
| cg13333267 | 3    | SEMA3F    | 5'UTR            | S_Shelf            | 0.48              | 0.59                | 0.11                                     | 0.0180                                  |
| cg13320202 | 3    | ATXN7     | Body             | S_Shore            | 0.48              | 0.36                | 0.12                                     | --0.0575                                |
| cg10523671 | 3    | SLC15A2   | TSS1500          |                    | 0.45              | 0.62                | 0.17                                     | 0.3300                                  |
| cg02034887 | 3    | SLC15A2   | TSS200           |                    | 0.27              | 0.40                | 0.14                                     | 0.0982                                  |
| cg05962239 | 3    | MME       | 5'UTR;1stExon    | S_Shore            | 0.24              | 0.14                | 0.10                                     | --0.2355                                |
| cg27473997 | 4    | USP17     | TSS200           |                    | 0.71              | 0.55                | 0.17                                     | --0.2529                                |
| cg13432241 | 4    | KIAA0922  | Body             | S_Shore            | 0.61              | 0.44                | 0.17                                     | --0.4088                                |
| cg08415137 | 5    |           |                  |                    | 0.13              | 0.09                | 0.04                                     | --0.0529                                |
| cg11010614 | 5    | FOX1      | TSS1500          | N_Shore            | 0.41              | 0.52                | 0.11                                     | 0.0330                                  |
| cg20997710 | 7    |           | 5'UTR            | S_Shelf            | 0.47              | 0.30                | 0.17                                     | --0.2326                                |
| cg03490567 | 7    | URGCP     | 5'UTR;Body       | N_Shore            | 0.23              | 0.34                | 0.10                                     | 0.0660                                  |
| cg10218605 | 7    | PTPRN2    | Body             | N_Shore            | 0.30              | 0.50                | 0.20                                     | 0.0037                                  |
| cg00201595 | 8    |           | 5'UTR            | S_Shore            | 0.82              | 0.78                | 0.04                                     | --0.3187                                |
| cg08092111 | 8    | RPL1      | Body             | S_Shore            | 0.72              | 0.57                | 0.16                                     | --0.0906                                |
| cg26598831 | 8    |           |                  |                    | 0.63              | 0.42                | 0.21                                     | --0.1777                                |
| cg06728098 | 8    | MRP528    | Body             | N_Shore            | 0.12              | 0.10                | 0.02                                     | --0.4359                                |
| cg17929627 | 10   | MK67      | 5'UTR            | Island             | 0.17              | 0.11                | 0.06                                     | --2.2937                                |
| cg23656300 | 10   |           |                  |                    | 0.71              | 0.55                | 0.16                                     | --0.0168                                |
| cg20809737 | 11   | CPT1A     | Body             |                    | 0.78              | 0.70                | 0.09                                     | --0.7679                                |
| cg05280814 | 11   |           | N_Shelf          |                    | 0.55              | 0.73                | 0.18                                     | 0.8970                                  |
| cg15401862 | 12   | KCNMB4    | 5'UTR            |                    | 0.87              | 0.80                | 0.07                                     | --0.2301                                |
| cg02767665 | 12   | TMEM132D  | Body             | S_Shore            | 0.63              | 0.45                | 0.18                                     | --0.3994                                |
| cg22795345 | 13   | ARHGEF7   | Body             | Island             | 0.35              | 0.64                | 0.29                                     | 0.2448                                  |
| cg14270002 | 13   | ARHGEF7   | Body             | Island             | 0.28              | 0.57                | 0.29                                     | 0.2159                                  |
| cg24743156 | 14   | C7AGES    | TSS1500          | N_Shore            | 0.32              | 0.43                | 0.11                                     | 0.0097                                  |
| cg25407064 | 15   | EIF2AK4   | TSS200           | Island             | 0.04              | 0.03                | 0.01                                     | --1.5429                                |
| cg12921171 | 15   |           |                  |                    | 0.53              | 0.65                | 0.12                                     | 0.5505                                  |
| cg18054026 | 15   | C15orf26  | Body             | S_Shore            | 0.42              | 0.53                | 0.12                                     | 0.1548                                  |
| cg11470399 | 16   | PLK1      | 1stExon          | Island             | 0.14              | 0.11                | 0.03                                     | --1.5771                                |
| cg04138181 | 16   | PLK1      | Body             | S_Shore            | 0.25              | 0.15                | 0.10                                     | --1.1582                                |
| cg27106909 | 16   | YPEL3     | 1stExon;5'UTR    | N_Shore            | 0.20              | 0.37                | 0.17                                     | 0.2890                                  |
| cg06285575 | 16   | ZNF267    | Body             | S_Shelf            | 0.21              | 0.33                | 0.12                                     | 0.0053                                  |
| cg09848947 | 16   | FAM38A    | Body             |                    | 0.77              | 0.66                | 0.11                                     | --0.4029                                |
| cg06751612 | 16   | FAM38A    | Body             |                    | 0.86              | 0.76                | 0.11                                     | --0.5474                                |
Patients diagnosed with Gleason score 7 tumors represent a large and clinically heterogeneous subgroup of patients with a variable prognosis [19, 29]. In this study, Gleason 7 patients with the highest levels of the signature had lower recurrence-free survival rates (Fig. 2c; Table 3). Adding the signature to a model with traditional clinical-pathological parameters improved the AUC for recurrence (0.64 vs. 0.76, \( P = 1.34 \times 10^{-4} \); Fig. 2d).

The majority of patients with Gleason score 7 tumors had Gleason 3 + 4, which is considered less aggressive compared to Gleason 4 + 3 [19]. In the subset of patients with Gleason 7(3 + 4) tumors, the signature was associated with a higher risk of recurrence (Fig. 2e; Table 3) and significantly improved the AUC for recurrence (0.59 vs. 0.76, \( P = 2.12 \times 10^{-4} \); Fig 2f). Although there were few patients with Gleason 7(4 + 3) tumors in the testing dataset, the signature also improved the AUC for recurrence in this subgroup (0.62 vs. 0.70; \( P = 0.14 \)).

For 14 of the genes that encompass CpGs in the signature, DNA methylation levels were associated with mRNA expression levels of the corresponding genes in the FH cohort (\( P \) < 0.05; Additional file 2: Table S1). For three genes, there was a (modest) inverse correlation between methylation levels of CpGs in gene promoter regions and mRNA expression levels. Three other genes had stronger correlations of higher methylation and lower expression levels (correlation < -0.2): ANO7, ARHGEF7, and PTPRN2. The CpG sites in these genes were in the gene body or 3’UTR region. Analysis of TCGA data provided confirmatory evidence for these findings (Additional file 2: Table S1).

Correlations between the signature and genome-wide gene expression levels were then evaluated in the FH cohort. We identified 1266 significantly upregulated transcripts (FDR Q value < 0.05), which involved 1108 unique genes, and 1673 downregulated transcripts, which involved 1357 unique genes (Additional file 3: Table S2). The genes were used as input for GSEA (Table 4). The analysis showed that the upregulated genes in our study (positive NES) were enriched for gene sets related to cell cycle proliferation (i.e., HALLMARK_E2F_TARGETS, HALLMARK_G2M_CHECKPOINT, HALLMARK_MITO-TIC_SPINDLE, and HALLMARK_MYC_TARGETS_V1). Comparing our list of genes to KEGG pathways identified the gene set KEGG_CELL_CYCLE as the top-ranked gene set (NES = 2.36). Finally, we evaluated a 31-gene expression signature of cell cycle proliferation (CCP) that was

| Table 1 | Fifty-two CpG sites included in the epigenetic signature (Continued) |
|---|---|
| cg10576459 | 17 | 0.89 | 0.79 | 0.10 | -0.0067 |
| cg01135464 | 17 | 0.33 | 0.57 | 0.24 | 0.7303 |
| cg22438006 | 18 | N_Shelf | 0.66 | 0.49 | 0.18 | -0.0625 |
| cg07164161 | 18 | KCNG2 | 0.53 | 0.42 | 0.11 | -0.0296 |
| cg09960641 | 19 | LOC100128675 | 0.31 | 0.46 | 0.14 | 0.2864 |
| cg25286393 | 19 | NAPSA | 0.70 | 0.59 | 0.11 | -0.0201 |
| cg12551567 | 20 | CDC25B | 0.10 | 0.08 | 0.02 | -0.4047 |
| cg07944494 | 21 | Island | 0.94 | 0.96 | 0.02 | 0.1070 |
| cg07260325 | X | 0.74 | 0.66 | 0.08 | -0.1695 |

**Table 2** Selected characteristics of patients in the Fred Hutchinson prostate cancer patient cohort

| Variables | Patients (n = 523) |
|---|---|
| Age at diagnosis (years) | 58.1 (7.1) |
| Race |  |
| African-American | 44 | 8.4 |
| European-American | 479 | 91.6 |
| Body mass index at diagnosis (kg/m²) | 269 (3.7) |
| Pathological stage[a] |  |
| Local | 360 | 68.8 |
| Regional | 163 | 31.2 |
| Gleason score |  |
| ≤6 | 252 | 48.2 |
| 7(3 + 4) | 188 | 35.9 |
| 7(4 + 3) | 45 | 8.6 |
| ≥8–10 | 38 | 7.3 |
| PSA at diagnosis (ng/mL)[b] |  |
| 0–3.9 | 80 | 16.2 |
| 4–9.9 | 313 | 63.5 |
| 10–19.9 | 67 | 13.6 |
| ≥20 | 33 | 6.7 |
| Recurrence[b] |  |
| No recurrence | 323 | 74.9 |
| Recurrence | 108 | 25.1 |
| Follow-up time (years)[b] | 8.0 (4.2) |

**Table 3**

| Patients diagnosed with Gleason score 7 tumors represent a large and clinically heterogeneous subgroup of patients with a variable prognosis [19, 29]. In this study, Gleason 7 patients with the highest levels of the signature had lower recurrence-free survival rates (Fig. 2c; Table 3). Adding the signature to a model with traditional clinical-pathological parameters improved the AUC for recurrence (0.64 vs. 0.76, \( P = 1.34 \times 10^{-4} \); Fig. 2d).

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PSA prostate-specific antigen

[a]Local stage is pT2, N0/NX, M0. Regional stage is pT3-T4 and/or N1, M0
[b]Twenty-six patients had missing data on diagnostic PSA levels, and 92 patients had missing data on recurrence. Three additional patients had missing follow-up data.
previously shown to be associated with PCa mortality when assessed in primary tumors [30, 31]. In our study, expression levels of 25 genes in the CCP score significantly increased with higher levels of the epigenetic signature (FDR Q value < 0.05).

**Discussion**

In the present study, an epigenetic signature of Gleason score was generated. The study showed that the signature predicted recurrence-free survival after radical prostatectomy.
Gleason score, or grade of the tumor, is the best predictor of PCa prognosis in patients with localized disease [19]. While patients diagnosed with Gleason ≤6 tumors typically have a favorable prognosis, patients with Gleason 8–10 tumors are most likely to experience disease recurrence and progression [19, 32]. We therefore

| Patients                        | Analysis model | Variables                          | Recurrence |
|---------------------------------|----------------|------------------------------------|------------|
|                                 |                |                                    | HR         | (95 % CI)       | P value    |
| All                             | Univariate     | Signature (per 25 % increase)      | 1.78       | (1.48, 2.16)    | 2.05E−09  |
|                                 | Multivariate   | Signature (per 25 % increase)      | 1.48       | (1.21, 1.81)    | 1.38E−04  |
|                                 |                | Age (per 1-year increase)          | 0.98       | (0.95, 1.01)    | 0.21      |
|                                 |                | Gleason score ≤6                   | 1.00       |                  |           |
|                                 |                | 7(3 + 4)                           | 2.25       | (1.32, 3.48)    | 2.89E−03  |
|                                 |                | 7(4 + 3)                           | 5.04       | (2.64, 9.65)    | 1.02E−06  |
|                                 |                | 8–10                               | 4.06       | (2.00, 8.26)    | 1.08E−04  |
|                                 |                | Pathological stage a               |            |                  |           |
|                                 |                | Local                              | 1.00       |                  |           |
|                                 |                | Regional                           | 2.04       | (1.33, 3.14)    | 1.13E−03  |
|                                 |                | Diagnostic PSA level (per 1-unit increase) | 1.00       | (1.00, 1.00)    | 0.92      |
| Patients with Gleason 7 tumors  | Univariate     | Signature (per 25 % increase)      | 1.81       | (1.42, 2.31)    | 1.38E−06  |
|                                 | Multivariate   | Signature (per 25 % increase)      | 1.59       | (1.24, 2.05)    | 3.21E−04  |
|                                 |                | Age (per 1-year increase)          | 0.99       | (0.95, 1.03)    | 0.56      |
|                                 |                | Gleason score ≤7(3 + 4)            | 1.00       |                  |           |
|                                 |                | 7(4 + 3)                           | 2.08       | (1.18, 3.65)    | 1.09E−02  |
|                                 |                | Pathological stage a               |            |                  |           |
|                                 |                | Local                              | 1.00       |                  |           |
|                                 |                | Regional                           | 1.82       | (1.06, 3.11)    | 2.94E−02  |
|                                 |                | Diagnostic PSA level (per 1-unit increase) | 1.00       | (0.99, 1.01)    | 0.82      |
| Patients with Gleason 7(3 + 4)  | Univariate     | Signature (per 25 % increase)      | 1.83       | (1.36, 2.45)    | 5.64E−05  |
| tumors                          | Multivariate   | Signature (per 25 % increase)      | 1.65       | (1.21, 2.25)    | 1.54E−03  |
|                                 |                | Age (per 1-year increase)          | 1.00       | (0.95, 1.05)    | 0.86      |
|                                 |                | Pathological stage a               |            |                  |           |
|                                 |                | Local                              | 1.00       |                  |           |
|                                 |                | Regional                           | 1.83       | (0.97, 3.45)    | 0.06      |
|                                 |                | Diagnostic PSA level (per 1-unit increase) | 1.00       | (0.99, 1.01)    | 0.80      |

CI confidence interval, HR hazard ratio, PSA prostate-specific antigen
*Local stage is pT2, N0/NX, M0. Regional stage is pT3-T4 and/or N1, M0
generated a signature by contrasting patients with high (8–10) versus low (≤6) Gleason score tumors. Importantly, the study showed that the signature significantly improved the prediction of recurrence in patients diagnosed with Gleason score 7 tumors. Compared to standard clinical-pathological parameters, the signature improved the AUC for recurrence by 12% and for patients with 3 + 4 tumors, there was a 19% increase in AUC. Patients with Gleason score 7 tumors are clinically heterogeneous [29, 33]. These patients have a variable prognosis, and predicting PCa outcomes is often challenging. The methylation signature may therefore have potential to further improve the prognostication of these patients and might have clinical utility to help guide clinical decision-making (e.g., adjuvant therapy) after radical prostatectomy [34]. Further validation of the signature is however required.

The epigenetic signature, which was created using an agnostic method, includes 52 differentially methylated CpG sites. The genes that encompass CpGs in the signature have roles in different biological pathways including ion channel transport, Akt signaling, and cell cycle, all of which are important for PCa growth. Four genes in the signature encode cell cycle-related targets of E2F transcription factors (PLK1, CDC25B, MKI67, and RRM2) [27]. The E2F pathway has a crucial role in cell cycle proliferation and the progression of PCa [35]. We also showed that the methylation levels of several CpGs were associated with mRNA expression levels of the corresponding genes. While the strength of the correlation was modest for most genes, a few genes revealed stronger correlations: ANO7, ARHGEF7, and PTPRN2. The CpGs in these genes were in the gene body or 3’UTR, and higher methylation levels in higher Gleason score tumors were associated with lower mRNA expression of all three genes. Although the link between methylation in these regions of the gene and mRNA expression is not well understood, there is evidence from previous studies that intragenic (gene body) DNA methylation could reduce the efficiency of transcription elongation [36]. The role of ARHGEF7 and PTPRN2 in PCs is unknown, but ANO7 encodes a polytopic membrane protein that is prostate-specific [37], and the methylated CpG site in this gene might therefore be a promising biomarker for more aggressive PCs. The gene is also being studied as a potential target for PCa immunotherapy [38]. Further, a previous study showed that the expression of ANO7 and PTPRN2 is downregulated in metastatic prostate tumors [39].

Gene Set Enrichment Analysis showed that the epigenetic signature was associated with increased expression of genes related to cell cycle proliferation. Increased cell proliferation is a key feature of cancer that is required for further neoplastic progression [40]. Previously, Cuzick et al. identified a 31-gene expression score of cell cycle proliferation (CCP) for predicting PCa outcomes [30]. The score is associated with PCa-specific mortality when assessed in primary tumors [30, 31]. In our study, the majority of the genes in the CCP score (n = 25) were upregulated with higher levels

### Table 4 Gene Set Enrichment Analysis results

| Gene set name                        | Description                                                                 | NES   |
|--------------------------------------|-----------------------------------------------------------------------------|-------|
| HALLMARK_E2F_TARGETS                 | Genes encoding cell cycle-related targets of E2F transcription factors       | 3.86  |
| HALLMARK_G2M_CHECKPOINT              | Genes involved in the G2/M checkpoint, as in progression through the cell division cycle | 3.08  |
| HALLMARK_MITOTIC_SPINDLE             | Genes important for mitotic spindle assembly                                 | 2.19  |
| HALLMARK_EPITHelial_MESENCHYMAL_TRANSITION | Genes defining epithelial-mesenchymal transition, as in wound healing, fibrosis, and metastasis | 2.15  |
| HALLMARK_ALLOGRAFT_REJECTION         | Genes upregulated during transplant rejection                               | 1.96  |
| HALLMARK_MYC_TARGETS_V1              | A subgroup of genes regulated by MYC—version 1 (v1)                         | 1.87  |
| HALLMARK_ANDROGEN_RESPONSE           | Genes defining response to androgens                                        | −2.83 |
| HALLMARK_FATTY_ACID_METABOLISM       | Genes encoding proteins involved in metabolism of fatty acids               | −2.04 |
| HALLMARK_XENOBIOTIC_METABOLISM       | Genes encoding proteins involved in processing of drugs and other xenobiotics | −1.98 |

We identified genes that showed increased expression with higher levels of the epigenetic signature (FDR Q < 0.05; n = 1108) or decreased expression with higher levels of the signature (FDR Q < 0.05; n = 1357). These genes were pre-ranked according to Pearson correlation, and this pre-ranked list was used as input for GSEA.

The gene sets in the table have an FDR Q value < 0.05. A positive value for the NES indicates that higher levels of the signature were associated with increased expression of the genes in a gene set.

FDR false discovery rate. NES normalized enrichment score.
of the epigenetic signature. In addition, GSEA showed that the signature was associated with decreased expression of androgen-responsive genes. Androgens regulate vital aspects of prostate growth and function [41], and androgen receptor activity inversely correlates with cell cycle proliferation in advanced PCa [42]. Therefore, the epigenetic signature seems to capture important biological pathways and events related to prostate tumor progression.

A number of previous studies on DNA methylation biomarkers for predicting PCa outcomes in patients with localized disease have been conducted. Most of these previous investigations focused on specific candidate genes. Evidence from these studies suggests that hypermethylation of PITX2, GSTPI, and APC is associated with more aggressive PCa and disease prognosis [11, 12]. Some other prior studies have focused on larger sets of CpG sites across the genome and identified different panels of CpG biomarkers for distinguishing more from less aggressive prostate tumors [13–18]. For example, in a previous epigenome-wide analysis from our group, we identified a panel of methylation biomarkers for predicting metastatic-lethal PCa [18]. None of the differentially methylated CpG sites in the prior studies, however, are included in the epigenetic signature of Gleason score. It is important to note that several of the previous studies were limited by small sample size and a limited number of CpG markers evaluated. Additional large investigations of DNA methylation biomarkers for PCa recurrence are therefore needed, including studies to further validate our epigenetic signature and other previously identified prognostic CpG biomarkers.

The present study has important strengths including the relatively large number of patients and the agnostic, genome-wide approach used for building the prognostic epigenetic classifier in the TCGA discovery dataset. The testing cohort has a prospective design with long-term follow-up for patient outcomes. Other prognostic tools (e.g., CAPRA-S, Decipher) were not tested in this study, and additional comparative studies are therefore needed.

Conclusions
Better tools to identify at the time of diagnosis the subset of PCa patients at the highest risk of recurrence are urgently needed. Our study provides new evidence that DNA methylation profiling has the clinical potential to improve risk prediction for PCa outcomes in patients with clinically localized disease, particularly in patients with Gleason score 7 tumors, which have a variable clinical course and represent a substantial proportion of prostate cancer patients diagnosed worldwide each year.

Additional files

| Additional file 1: Figure S1. Heatmap of the CpG sites included in the epigenetic signature in the Fred Hutchinson cohort. (TIF 16594 kb) |
| Additional file 2: Table S1. Correlations between methylation levels of CpG sites included in the epigenetic signature and mRNA expression levels of corresponding genes. (XLSX 17 kb) |
| Additional file 3: Table S2. Correlations of gene expression levels and the epigenetic signature. (TXT 64 kb) |

Abbreviations
AUC: Area under the curve; CI: Confidence interval; FDR: False discovery rate; FH: Fred Hutchinson; GSEA: Gene Set Enrichment Analysis; HR: Hazard ratio; NES: Normalized enrichment score; PCa: Prostate cancer; PSA: Prostate-specific antigen; ROC: Receiver operating characteristic; TCGA: The Cancer Genome Atlas

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Availability of data and materials
Data used to create the epigenetic signature can be downloaded from: https://tcga-data.nci.nih.gov/tcga/.

Authors’ contributions
MSG performed the data analysis and drafted the manuscript. BK carried out the methylation assays for the FH study. JLS initiated the FH patient cohort study and helped draft the manuscript. All authors read the manuscript; revised it critically for important intellectual content, and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The Fred Hutchinson Cancer Research Center Institutional Review Board approved the study, and all participants signed informed consent statements.

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