

## Validation Study of Genes with Hypermethylated Promoter Regions Associated with Prostate Cancer Recurrence

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### Abstract

**Background:** One challenge in prostate cancer is distinguishing indolent from aggressive disease at diagnosis. DNA promoter hypermethylation is a frequent epigenetic event in prostate cancer, but few studies of DNA methylation in relation to features of more aggressive tumors or prostate cancer recurrence have been completed.

**Methods:** We used the Infinium HumanMethylation450 BeadChip to assess DNA methylation in tumor tissue from 407 patients with clinically localized prostate cancer who underwent radical prostatectomy. Recurrence status was determined by follow-up patient surveys, medical record review, and linkage with the Surveillance, Epidemiology, and End Results (SEER) registry. The methylation status of 14 genes for which promoter hypermethylation was previously correlated with advanced disease or biochemical recurrence was evaluated. Average methylation level for promoter region CpGs in patients who recurred compared with those with no evidence of recurrence was analyzed. For two genes with differential methylation, time to recurrence was examined.

**Results:** During an average follow-up of 11.7 years, 104 (26%) patients recurred. Significant promoter hypermethylation in at least 50% of CpG sites in two genes, *ABHD9* and *HOXD3*, was found in tumors from patients who recurred compared with those without recurrence. Evidence was strongest for *HOXD3* (lowest  $P = 9.46 \times 10^{-6}$ ), with higher average methylation across promoter region CpGs associated with reduced recurrence-free survival ( $P = 2 \times 10^{-4}$ ). DNA methylation profiles did not differ by recurrence status for the other genes.

**Conclusions:** These results validate the association between promoter hypermethylation of *ADHB9* and *HOXD3* and prostate cancer recurrence.

**Impact:** Tumor DNA methylation profiling may help to distinguish patients with prostate cancer at higher risk for disease recurrence. *Cancer Epidemiol Biomarkers Prev*; 23(7); 1331–9. ©2014 AACR.

### Introduction

Prostate cancer is one of the most common malignancies and the second leading cause of cancer-related death in American men, responsible for more than 29,000 deaths annually (1). The majority of prostate tumors are clinically

localized at diagnosis and although many are unlikely to cause harm if left untreated, most patients are treated definitively with surgery or radiation. Up to a third of patients treated with curative intent, however, will ultimately experience disease recurrence or relapse (2–4). On the other hand, many patients with indolent tumors are overtreated, potentially suffering adverse effects of therapy. Thus, a major clinical challenge is distinguishing indolent from aggressive disease at the time of prostate cancer diagnosis.

DNA methylation is a common, heritable epigenetic modification in cancer and involves transfer of a methyl group to the 5' position of the cytosine ring of CpG dinucleotides via DNA methyltransferases. Hypermethylation of CpG sites in gene promoter regions has been associated with carcinogenesis and is an important mechanism for inactivation of genes involved in tumor suppression, DNA repair, and apoptosis (5, 6). Preliminary evidence suggests that tumor DNA methylation levels may yield prognostic information for patients with prostate cancer (7). Several candidate gene studies have shown

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that promoter region DNA hypermethylation is associated with features of more aggressive prostate cancer such as higher Gleason score ( $\geq 7$ ) or advanced stage, as well as with biochemical (PSA) recurrence (8–27). However, these early studies primarily examined the promoter region in only a few candidate genes, and validation of methylation results in independent patient cohorts has received little attention.

In the present study, we evaluated candidate genes reported in prior studies to have aberrant promoter region methylation profiles in subsets of patients with prostate cancer. The goal of the study was to determine whether there was confirmatory evidence for differential methylation profiles of 14 candidate genes in tumor tissue derived from a cohort of patients with prostate cancer with long-term follow-up for disease-related outcomes.

## Materials and Methods

### Study population

Data and tumor tissue samples used for the present study were available from a cohort of 407 patients who had radical prostatectomy as primary therapy for clinically localized prostate cancer and who participated in prior population-based studies (28, 29). Baseline data collection included an in-person interview, blood draw, and consent for obtaining pathology reports and tumor tissue from radical prostatectomy samples. All patients signed informed consent and procedures were Institutional Review Board (IRB) approved. Information on biopsy and pathologic Gleason score, PSA level at diagnosis, tumor stage, primary treatment, vital status, and underlying cause of death was collected from the Seattle–Puget Sound Surveillance, Epidemiology, and End Results (SEER) cancer registry and data were coded according to SEER guidelines (30).

Prostate cancer recurrence events and vital status were based on two follow-up patient surveys, completed in 2004–2005 and in 2010–2011, as well as medical record reviews and linkage with the cancer registry. Mailed follow-up surveys were completed by 85% (first follow-up survey) and 76% (second follow-up survey) of eligible patients with prostate cancer; as of the second follow-up survey, 50 (3.5%) patients were lost to follow-up. These surveys collected information on use of secondary therapies, follow-up PSA results, test results from bone scans, MRIs, CTs, and follow-up prostate bed and lymph node biopsies. Four criteria were used to classify a patient as having a recurrence event: (i) rising PSA (i.e., PSA  $\geq 0.2$  ng/mL); (ii) receipt of secondary treatment [androgen deprivation therapy (ADT) or orchiectomy, radio-, or chemotherapy]; (iii) a positive bone scan, MRI, CT, or prostate bed or lymph node biopsy showing prostate cancer; and/or (iv) a physician's diagnosis of tumor recurrence. If it was unclear whether or not a patient had experienced a recurrence, medical records were reviewed. Patients who died of prostate cancer before the follow-up surveys were coded as recurred. The patient cohort is linked to the cancer

registry to ascertain vital status. For deceased patients, underlying cause of death was obtained from the registry and copies of death certificates were also reviewed to confirm whether a patient died of prostate cancer or another cause. On the basis of these criteria, 104 (26%) men were classified as having recurred; 303 men had no evidence of disease recurrence.

Of the 104 men with recurrence events, 25 were classified on the basis of a rising PSA, 55 received secondary treatment, and 24 had a positive bone scan, biopsy, CT or MRI, or died of prostate cancer. The average follow-up period for the patient cohort was 11.7 years (range, 2.0–19.9). All patients who remained recurrence free had a minimum follow-up of 6.96 years.

### Selection of genes for analysis

Candidate genes previously shown to be associated with recurrence or features of more aggressive disease (i.e., Gleason score  $\geq 7$ , metastasis, prostate cancer death) in at least two prior publications were selected for validation in our cohort (8–27). We evaluated CpGs in the transcriptional start sites for 14 such genes: *ABHD9*, *APC*, *ASC*, *CD44*, *CDH13*, *GPR7*, *GSTP1*, *HOXD3*, *MDR1*, *PITX2*, *PTGS2*, *RAR $\beta$* , *RASSF1A*, and *RUNX3*. In addition, we examined the methylation

**Table 1.** Distributions for selected characteristics of the prostate cancer patient cohort by recurrence status

Characteristic	Recurrence	No recurrence
	(n = 104) n (%)	(n = 303) n (%)
Age at diagnosis, y		
35–54	33 (31.7)	92 (30.4)
55–59	18 (17.3)	77 (25.4)
60–64	34 (32.7)	86 (28.4)
65–69	12 (11.5)	28 (9.2)
70–74	7 (6.7)	20 (6.6)
Mean age ( $\pm$ SE)	58.5 (7.18)	58.2 (7.10)
PSA at diagnosis, ng/mL		
0–3.9	13 (12.5)	54 (17.8)
4–9.9	45 (43.3)	189 (62.4)
10–19.9	24 (23.1)	28 (9.2)
20 +	15 (14.4)	14 (4.6)
Missing	7 (6.7)	18 (5.9)
Pathologic stage		
Local	47 (45.2)	235 (77.6)
Regional	57 (54.8)	68 (22.4)
Gleason score		
2–5	8 (7.7)	37 (12.2)
6	16 (15.4)	136 (44.9)
7 (3 + 4)	45 (43.3)	101 (33.3)
7 (4 + 3)	17 (16.4)	16 (5.3)
8–10	18 (17.3)	13 (4.3)

status of CpG sites in the 5' region and gene body for these candidate genes.

### Sample preparation and tumor DNA extraction

Formalin-fixed, paraffin-embedded (FFPE) blocks from radical prostatectomy specimens were used to make hematoxylin and eosin (H&E)-stained slides, which were reviewed by a prostate cancer pathologist to confirm the presence and location of prostate cancer within the blocks. Areas containing  $\geq 75\%$  tumor tissue had two 1-mm tumor tissue plugs per patient taken for DNA extraction. For 20 patients, adjacent nontumor (benign) prostate tissue plugs were also taken for DNA extraction.

Extraction of tumor DNA from the FFPE cores was completed using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion/Applied Biosciences). The standard manufacturer's protocol was followed, except that the elution step was performed twice to maximize the DNA yield. Purified DNA was quantified (PicoGreen) and each aliquot was labeled with a unique patient ID, tracked, and stored at  $-80^{\circ}\text{C}$ . The average yield of DNA was 3  $\mu\text{g}$ . A tumor DNA aliquot (500 ng) for each patient was shipped to Illumina, Inc. for completion of assays.

### DNA methylation arrays

Samples were bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research) according to the man-

ufacturer's protocol. Controls on the array were used to track the bisulfite conversion efficiency. The Infinium HumanMethylation450 BeadChip array (Illumina, Inc.) was used to measure genome-wide CpG methylation using beads with target-specific probes designed to interrogate individual CpG sites on bisulfite-converted genomic DNA (31). Measurements were run on DNA samples aliquoted on seven 96-well plates. Across the plates, we included a duplicate sample for 18 patients and randomly assigned these duplicates to separate plates. We additionally included replicate tumor DNA samples from two patients on every plate. All plates also contained Illumina controls and two negative controls. Outcome events (recurrence and prostate cancer death) were distributed randomly across plates such that similar numbers of events were represented on each plate. Laboratory personnel were blinded to outcome events as well as to the location of duplicate and replicate samples on plates.

### Data processing and analysis

Failed samples were identified by using the detection  $P$  value metric according to the standard protocols (Illumina, Inc). A sample was excluded if less than 95% of the CpG sites for that sample on the array were detected with a detection  $P < 0.05$ , resulting in 32 exclusions. The detection  $P$  value metric was also used to filter out individual CpG sites with detection  $P > 0.01$ ; no CpG sites

**Table 2.** Number of CpG sites by location in 14 candidate genes evaluated for differential methylation profiles in prostate cancer patients with recurrence versus no recurrence

Candidate gene	Total No. CpG sites evaluated <sup>a</sup>	No. CpG sites evaluated in promoter region <sup>b</sup>	No. CpG sites evaluated in 5' region <sup>b</sup>	No. CpG sites evaluated in gene body region <sup>b</sup>
<i>ABHD9</i>	20	12 (7)	11 (8)	6 (1)
<i>APC</i>	39	26 (0)	22 (0)	2 (0)
<i>ASC</i>	19	12 (2)	3 (0)	4 (0)
<i>CD44</i>	32	5 (0)	6 (0)	21 (0)
<i>CDH13</i>	61	10 (0)	4 (1)	47 (0)
<i>GPR7</i>	13	10 (4)	3 (1)	0 (0)
<i>GSTP1</i>	19	11 (0)	2 (0)	6 (0)
<i>HOXD3</i>	28	11 (9)	10 (9)	7 (1)
<i>MDR1</i>	31	7 (0)	25 (3)	11 (0)
<i>PITX2</i>	69	17 (1)	13 (0)	51 (0)
<i>PTGS2</i>	17	9 (3)	2 (0)	6 (0)
<i>RAR<math>\beta</math></i>	29	11 (0)	11 (0)	12 (0)
<i>RASSF1A</i>	56	40 (0)	30 (0)	46 (0)
<i>RUNX3</i>	91	40 (0)	5 (0)	74 (0)

<sup>a</sup>The total number of CpG sites evaluated is greater than the sum of the CpG sites in each region for some genes because certain CpGs have multiple annotations due to alternative transcription start sites. The total number of CpG sites evaluated in each gene was used to determine statistical significance based on  $t$  tests of differential methylation between patients with recurrence versus no recurrence, with Bonferroni correction for multiple testing within each gene.

<sup>b</sup>Shown in parentheses is the number of CpG sites with significantly higher methylation (i.e., hypermethylated) in patients with prostate cancer recurrence compared with those with no evidence of recurrence.

were excluded on the basis of this criterion. In addition, only men of European descent were included because evidence suggests that methylation patterns vary by ancestry (32, 33).

The minfi package (34) implemented in R statistical computing software was used to calculate methylation levels in tumor tissue from patients who experienced prostate cancer recurrence compared with those with no evidence for recurrence (35). The data were normalized using subset-quantile within array normalization (SWAN; ref. 36) available in the minfi package. The  $\beta$  value was calculated as a measure of methylation level at each CpG locus [intensity of the methylated allele / (intensity of the unmethylated allele + intensity of the methylated allele + 100)], with  $\beta$  values ranging from 0 (unmethylated) to 1 (100% methylated) as an estimate of the percentage of DNA methylation (34). An  $M$  value, defined as a logit transformation of the  $\beta$  value that is approximately normally distributed, was also estimated for each CpG site. The number of CpG sites examined per gene ranged from 13 to 91, and a CpG site was considered to be differentially methylated if it was significant based on a  $t$  test on the  $M$  values after Bonferroni correction for multiple tests within each gene. A gene was considered to be validated as a prognostic marker if at least 50% of the promoter region CpG sites were significantly more highly methylated on the basis of the average  $\beta$  value (i.e., hypermethylated) in recurrent versus nonrecurrent patients. As a secondary aim, we also compared the methylation profile

of CpGs in the 5' region and the gene body of the 14 candidate genes by recurrence status.

In other secondary analyses, we investigated the genes with confirmatory evidence in relation to time to prostate cancer recurrence. Patients were categorized into low-methylation and high-methylation groups by the third quartile of the  $\beta$  values for each significantly hypermethylated CpG site. Log-rank tests were performed to determine whether the low and high average methylation groups had differential times to recurrence. We further calculated the average methylation level of the CpG sites within the promoter region for each confirmed gene; the third quartile for the average was 0.592 for *ABHD9* and was 0.572 for *HOXD3*. We defined low and high average methylation as below or above the third quartile, respectively. Kaplan–Meier plots were generated for patients with low and high average methylation levels, and a log-rank test was performed to test whether time to recurrence differed between the two groups. Cox models were fitted to estimate the effect of DNA methylation on time to recurrence, adjusting for age, PSA at diagnosis, pathologic stage, and Gleason score. Chi-square tests were performed to test if Gleason score or stage of disease varied between the low- and high-methylation groups. In addition, differential methylation for paired tumor-adjacent benign tissue ( $n = 20$ ) was evaluated. Box plots were generated for each significantly hypermethylated CpG site. Paired  $t$  tests were performed on the corresponding  $M$  values, which were normally distributed.

**Table 3.** Genes with over 50% of the promoter region CpG sites having higher methylation in tumor tissue from prostate cancer patients with recurrence versus no recurrence

Candidate gene	Illumina CpG site	Avg. $\beta$ value: recurrence	Avg. $\beta$ value: no recurrence	$P^a$	CpG location	Third IQR
<i>ABHD9</i>	cg18366919	0.581264	0.522453	0.000295	Island	0.649
<i>ABHD9</i>	cg26010734	0.507842	0.442909	0.000385	N_Shore	0.565
<i>ABHD9</i>	cg17476026	0.429670	0.357897	0.000540	N_Shore	0.525
<i>ABHD9</i>	cg17399362	0.512639	0.432249	0.000838	N_Shore	0.614
<i>ABHD9</i>	cg08457898	0.569149	0.518281	0.001475	N_Shore	0.643
<i>ABHD9</i>	cg16184495	0.589635	0.530117	0.001796	N_Shore	0.672
<i>ABHD9</i>	cg15826897	0.483290	0.434591	0.002572	N_Shore	0.535
<i>HOXD3</i>	cg13316854	0.438143	0.364137	9.46E-06	Island	0.476
<i>HOXD3</i>	cg24704177	0.453488	0.377573	2.93E-05	Island	0.498
<i>HOXD3</i>	cg06145336	0.671013	0.594325	3.33E-05	Island	0.742
<i>HOXD3</i>	cg01014615	0.646853	0.575173	9.87E-05	Island	0.719
<i>HOXD3</i>	cg12634591	0.488850	0.419412	0.000159	N_Shore	0.538
<i>HOXD3</i>	cg09387749	0.646414	0.581765	0.000419	N_Shore	0.715
<i>HOXD3</i>	cg25938806	0.456353	0.391304	0.000618	N_Shore	0.502
<i>HOXD3</i>	cg02773086	0.623405	0.569983	0.001001	N_Shore	0.691
<i>HOXD3</i>	cg18702197	0.426674	0.362498	0.001237	Island	0.472

Abbreviation: IQR, interquartile range.

<sup>a</sup> $P$  values with Bonferroni correction for multiple testing within each gene.

## Results

Men who recurred (mean age, 58.5 years) were slightly older than nonrecurrent (mean age, 58.2 years) patients, but the difference was not significant ( $P = 0.65$ ). They were also more likely to have higher Gleason scores and higher diagnostic PSA values, and were more likely to have pathologically defined regional as opposed to localized-stage disease (Table 1).

Results showed excellent concordance across the 450K CpG sites, with a median Pearson  $r^2$  of 0.98 for blind duplicates, and correlations  $>0.99$  for replicates across plates. Quality control results from the GenomeStudio control panel fell within parameters outlined in the Illumina GenomeStudio Methylation Module users guide (37).

The number of CpG sites evaluated per gene and the number within each region (i.e., promoter, 5', gene body) that were more highly methylated in patients who experienced recurrence compared with those without evidence of recurrence is shown in Table 2. The number of hypermethylated promoter region CpG sites with significant  $P$  values based on a  $t$  test and Bonferroni correction for multiple testing within each gene ranged from 0 to 9. For *ABHD9* and *HOXD3*, over 50% of the promoter region CpG sites were significantly hypermethylated (Table 3). These two genes also had  $>50\%$  of the 5' region CpG sites hypermethylated in patients with recurrence versus no recurrence (Supplementary Table S1). The significant  $P$  values ranged between  $2.57 \times 10^{-3}$  and  $2.95 \times 10^{-4}$  for *ABHD9* (Table 3). Confirmatory evidence was strongest for *HOXD3*, with 9 of 11 promoter region CpG sites having  $P$  values between  $1.24 \times 10^{-3}$  and  $9.46 \times 10^{-6}$ .

For the seven CpG sites in *ABHD9* that were significantly hypermethylated, higher methylation in four CpG sites was associated with shorter times to recurrence: sites cg26010734 and cg15826897 were strongest with  $P$  values  $9.26 \times 10^{-7}$  and  $2 \times 10^{-4}$ , respectively, while sites cg18366919 and cg08457898 were marginally significant with  $P$  values of 0.033 and 0.037. For *HOXD3*, eight of nine significantly hypermethylated promoter region CpG sites were related to time to prostate cancer recurrence. Among these CpGs, cg13316854 and cg24704177 were the strongest with  $P$  values of  $4.73 \times 10^{-5}$  and  $2 \times 10^{-4}$ , respectively.

Patients in the low versus high average methylation groups for *ABHD9* did not differ significantly in terms of recurrence-free survival (log-rank test  $P = 0.08$ ). The median time to recurrence was 19.3 years in the low methylation group and 18.6 years in the high methylation group (Fig. 1). Average methylation level across promoter region CpGs of *HOXD3* was strongly associated with time to recurrence (log-rank test  $P = 2 \times 10^{-4}$ ). The median time for recurrence-free survival was 19.4 and 17.0 years, respectively, in the low- and high-methylation groups. On the basis of Cox models adjusting for age, Gleason score, PSA at diagnosis, and

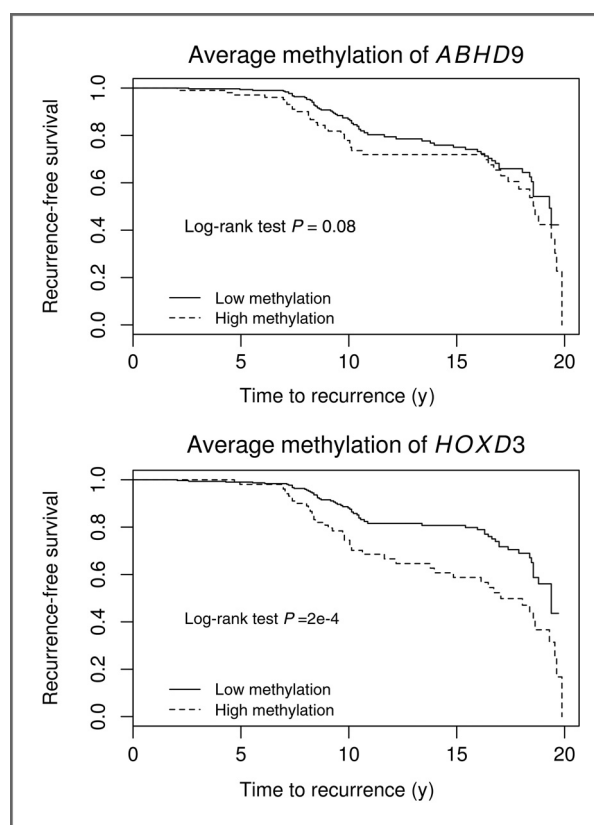


Figure 1. Kaplan-Meier plots for time to prostate cancer recurrence according to the average promoter region methylation in *ABHD9* and *HOXD3*.

pathologic stage, the HR comparing patients in the high-methylation group to those in the low-methylation group was 1.16 [95% confidence interval (CI), 0.77–1.75] for *ABHD9* and 1.70 (95% CI, 1.14–2.54) for *HOXD3*.

Patients in the high-methylation group for both *ABHD9* and *HOXD3* had higher Gleason scores and more advanced stage. For *ABHD9*, a greater proportion of patients with high compared with low average methylation had Gleason scores of  $\geq 7$ , 66.7% versus 46.6%, respectively ( $P = 0.001$ ); more patients also had regional as opposed to localized-stage disease (high methylation = 40.2% compared with low methylation = 27.5%;  $P = 0.02$ ). Similarly, for *HOXD3*, a higher proportion of patients in the high-methylation group had tumors with Gleason scores of 7 or greater (63.7%) compared with patients in the low-methylation group where only 47.5% had Gleason scores  $\geq 7$  ( $P = 0.007$ ). In terms of stage, for *HOXD3*, more patients in the high compared with low average methylation group had regional as opposed to localized disease, 42.2% versus 26.9%, respectively ( $P = 0.006$ ).

All the hypermethylated CpG sites in *ABHD9* and *HOXD3* that were significantly associated with prostate cancer recurrence also showed higher levels of

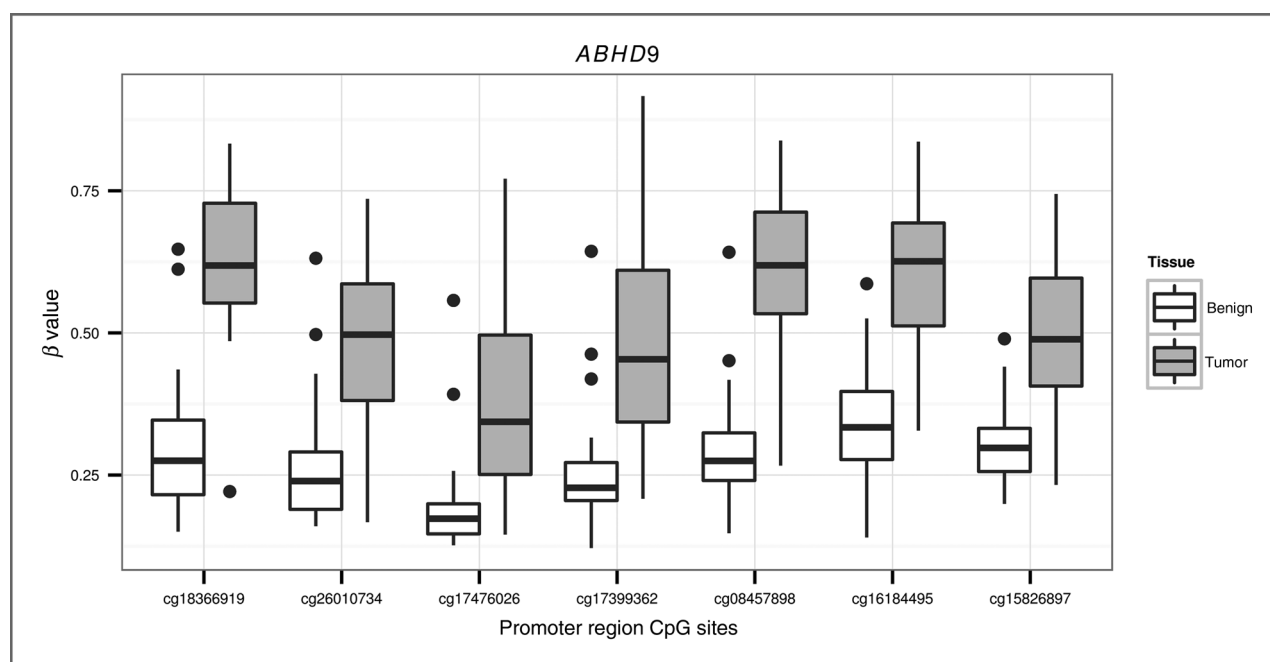


Figure 2. Box plots of each significantly hypermethylated CpG site in the *ABHD9* promoter region in prostate tumor tissue versus adjacent benign prostate tissue.

methylation in prostate tumor versus histologically benign adjacent prostate tissue from the same patients (Figs. 2 and 3). Tumor tissue had much higher methylation levels: the mean difference ranged from 0.19 to 0.32 for *ABHD9* and 0.16 to 0.23 for *HOXD3*. All paired *t* tests were significant with *P* values less than  $4.4 \times 10^{-5}$  for *ABHD9* and  $1.3 \times 10^{-3}$  for *HOXD3*.

## Discussion

The results of this validation study confirm the association between prostate cancer recurrence and promoter hypermethylation for two of the 14 candidate genes evaluated: *ABHD9* (located at 19p13.12) and *HOXD3* (at 2q31.1). Confirmatory evidence was most compelling for *HOXD3* (lowest *P* =  $9.46 \times 10^{-6}$ ), which was a strong predictor of time to recurrence. In addition, patients with high average methylation across promoter region CpG sites in *HOXD3* had shorter disease-free survival and were more likely to have Gleason scores of 7 or greater and to have regional as opposed to localized-stage prostate cancer when compared with patients in the low average methylation group.

In a methylation oligonucleotide microarray study of 304 frozen prostatectomy samples, Cottrell and colleagues previously reported that promoter region methylation of *ABHD9* was significantly increased in patients who experienced early PSA recurrence versus nonrecurrent men (Bonferroni-adjusted *P* < 0.05; ref. 10). The significance of the result with respect to biochemical recurrence was then confirmed in a cohort of 605 patients with radical pros-

tatectomy (12). Little is known about the function of *ABHD9* (*Abhydrolase domain containing 9*), which also has the alias *epoxide hydrolase 3* (*EPHX3*). Interestingly, methylation of *ABHD9* has also been reported in gastric cancer cell lines (38).

Kron and colleagues reported on the prognostic potential of *HOXD3* promoter region methylation in a genome-wide analysis of DNA methylation using Agilent human CpG island arrays (27) and quantitative MethyLight technology in a cohort of 232 patients with radical prostatectomy (14). They observed that promoter hypermethylation of *HOXD3* was associated with higher Gleason scores ( $\geq 7$  vs.  $\leq 6$ , *P* < 0.001) and more advanced stage (14). In a subsequent report from the same group of investigators that used the same cohort of patients, the association of *HOXD3* with biochemical recurrence was examined. Liu and colleagues found that increased levels of promoter methylation for any two of a panel of three genes, *APC*, *HOXD3*, and *TGF $\beta$ 2*, were predictive of biochemical recurrence (*P* = 0.017; ref. 18).

*HOXD3* is a member of the family of homeobox genes. The HOX genes are organized into four chromosomal clusters: *HOXA* at 7p15.3, *HOXB* at 17q21.3, *HOXC* at 12q13.3, and *HOXD* at 2q31 (39). HOX genes are transcription factors primarily involved in embryonic development, and control cell differentiation and proliferation and crucial cellular process (40). A rare mutation in the homeobox gene, *HOXB13*, has been associated with risk of developing familial and sporadic prostate cancer (41–45). A gene expression profile predicting prostate

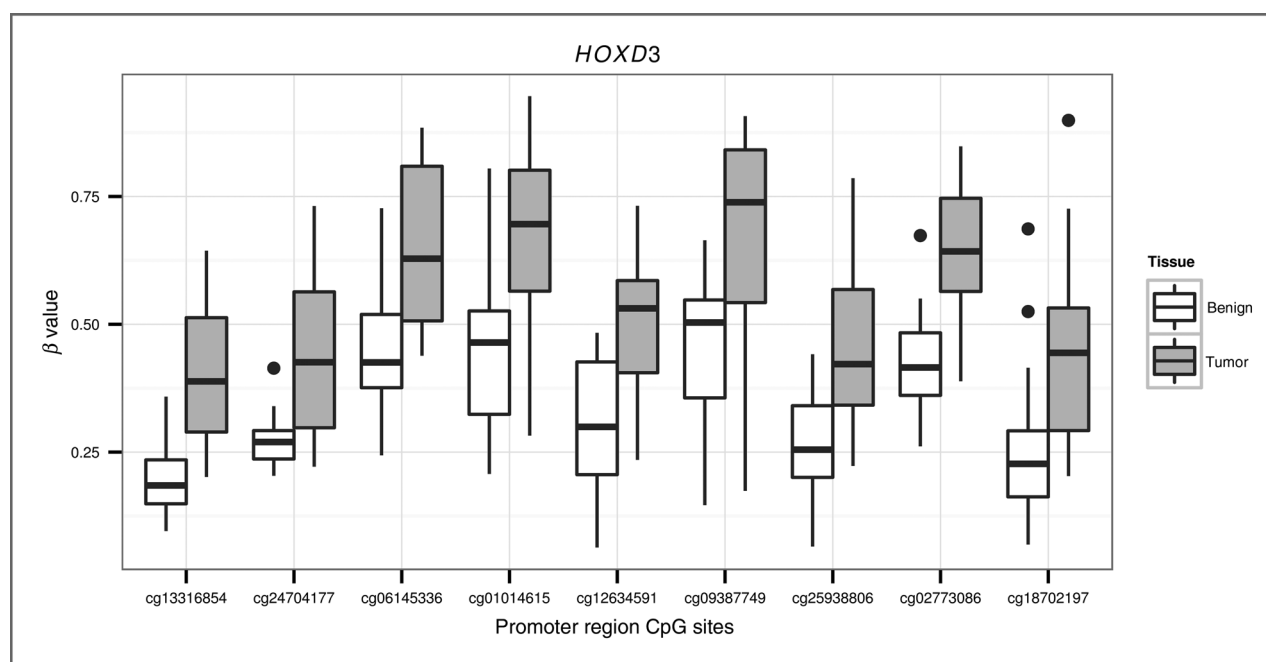


Figure 3. Box plots of each significantly hypermethylated CpG site in the *HOXD3* promoter region in prostate tumor tissue versus adjacent benign prostate tissue.

cancer recurrence contains the *HOXC6* gene (46). Methylation of homeobox genes has also been observed in multiple other cancers, including lung (47, 48) and breast (49). It is suggested that the HOX genes, including *HOXD3*, are essential for the maintenance of a differentiated tissue phenotype and that promoter region methylation of *HOXD3* may result in progressive de-differentiation of prostate cancer foci (14). Alternatively, hypermethylation of *HOXD3* may be secondary to such processes (14).

It is interesting that most of the promoter region CpG sites in *ABHD9* and several of those in *HOXD3* that were hypermethylated in the tumors of patients who recurred versus who did not recur are in CpG island shores, which may be up to 2 kb distant to promoters (50). Previously it was assumed that DNA methylation in CpG islands in the promoter region of genes was a main functional epigenetic change in cancer, but more recently shifts in the methylation boundaries from CpG islands to CpG island shores have been recognized to play a role in cancer-related epigenetic dysregulation (51). In a study of colon cancer, Irizarry and colleagues (50) showed that tissue-specific DNA methylation mainly occurs at CpG island shores rather than CpG islands, and that such methylation can alter gene expression.

As is the case with most studies of this nature, we were somewhat limited in the present study by the inclusion of biochemical recurrence as an endpoint, which may be a relatively poor predictor of adverse patient outcomes (52). However, a strength of our study

is that it also includes a number of patients also treated with secondary therapies and some who developed metastases or died of prostate cancer during follow-up. Also similar to earlier studies, our work was based on the use of tumor tissue obtained at surgery, and for clinical utility ideally prognostic biomarker tests for prostate cancer could be performed on biopsy tumor tissue to guide therapy based on the ability to distinguish tumors likely to behave aggressively. It should be noted that the genes examined in our study were selected on the basis of prior studies that only considered biochemical recurrence (8, 10–13, 16–22). Although our cohort may differ from patients included in earlier studies that only evaluated clinical features or PSA recurrence, it is a fairly large and well-characterized population-based cohort with long-term follow-up, providing a robust data resource for evaluating prognostic biomarkers.

In summary, validation of the association between promoter region hypermethylation of two candidate genes (*ABHD9* and *HOXD3*) and prostate cancer recurrence highlights the potential of differential DNA methylation as a prognostic biomarker for stratifying patients with more aggressive tumors. *HOXD3* in particular seems to be a strong candidate gene for which promoter region CpG methylation in our independent patient cohort was predictive of patient outcomes. Furthermore, 9 of 11 *HOXD3* CpGs examined were more heavily methylated in tumor compared with benign prostate tissue from the same patients; and high average methylation of *HOXD3* sites was associated with

shorter disease-free survival, higher Gleason score tumors, and more advanced disease stage. On the basis of these promising results, future evaluation of the *HOXD3* DNA methylation profile in tumor tissue as a biomarker for prostate cancer outcomes is needed to assess its clinical utility as a prognostic tool.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

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