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Validation study of genes with hypermethylated promoter regions associated with prostate cancer recurrence

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Abstract

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

Methods—We used the Infinium® HumanMethylation450 BeadChip to assess DNA methylation in tumor tissue from 407 patients with clinically localized PCa who underwent radical prostatectomy. Recurrence status was determined by follow-up patient surveys, medical record review, and linkage with the 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 to 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 to those without recurrence. Evidence was strongest for *HOXD3* (lowest $P = 9.46\text{x}10^{-6}$), with higher average methylation across promoter region CpGs associated with reduced recurrence-free survival ($P = 2\times10^{-4}$). DNA methylation profiles did not differ by recurrence status for the other genes.

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Conclusions—These results validate the association between promoter hypermethylation of *ADHB9* and *HOXD3* and PCa recurrence.

Impact—Tumor DNA methylation profiling may help distinguish PCa patients at higher risk for disease recurrence.

Keywords

DNA methylation; prostate cancer recurrence; hypermethylation; HOXD3

Introduction

Prostate cancer (PCa) is one of the most common malignancies and the second leading cause of cancer-related death in American men, responsible for over 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 PCa 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 PCa patients (7). Several candidate gene studies have shown that promoter region DNA hypermethylation is associated with features of more aggressive PCa such as higher Gleason score (≥ 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 PCa patients. 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 PCa patients 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 (RP) as primary therapy for clinically localized PCa 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 (RP) samples. All patients signed informed consent and procedures were 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 SEER cancer registry and data were coded according to SEER guidelines (30).

PCa 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 PCa patients; 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 ≥ 0.2 ng/mL); (ii) receipt of secondary treatment (androgen deprivation therapy (ADT) or orchiectomy, radiation, or chemotherapy)); (iii) a positive bone scan, MRI, CT, or prostate bed or lymph node biopsy showing PCa; 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 that died of PCa prior to 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 PCa or another cause. Based on 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 based on a rising PSA, 55 received secondary treatment, and 24 had a positive bone scan, biopsy, CT or MRI, or died of PCa. 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 ≥7, metastasis, PCa 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*, *RARβ*, *RASSF1A* and *RUNX3*. In addition, we examined the methylation status of CpG sites in the 5’ region and gene body for these candidate genes.

Sample preparation and tumor DNA extraction

FFPE blocks from RP specimens were used to make H&E stained slides, which were reviewed by a prostate cancer pathologist to confirm the presence and location of PCa within the blocks. Areas containing ≥ 75% tumor tissue had two 1-mm tumor tissue plugs

per patient taken for DNA extraction. For 20 patients, adjacent non-tumor (benign) prostate tissue plugs were also taken for DNA extraction.

Extraction of tumor DNA from the FFPE cores was completed using the Recover All Total Nucleic Acid Isolation Kit (Ambion/Applied Biosciences, Austin, TX). The standard manufacturer's protocol was followed, except that the elution step was performed twice to maximize DNA yield. Purified DNA was quantified (PicoGreen) and each aliquot was labeled with a unique patient ID, tracked, and stored at −80°C. The average yield of DNA was 3 μg. 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, Irvine, CA) according to the manufacturer's protocol. Controls on the array were used to track the bisulfite conversion efficiency. The Infinium® HumanMethylation450 BeadChip array (Illumina Inc., San Diego, CA) 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 2 negative controls. Outcome events (recurrence, PCa 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 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-value < 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 were excluded based on 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 PCa recurrence compared to those with no evidence for recurrence (35). The data were normalized using subset-quantile within array normalization (SWAN) (36) available in the minfi package. The β-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 β-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 β- 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 based on the average β-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 PCa recurrence. Patients were categorized into low methylation and high methylation groups by the third quartile of the β-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 3rd 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 3rd 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, pathological 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. Boxplots were generated for each significantly hypermethylated CpG site. Paired t-tests were performed on the corresponding M-values, which were normally distributed.

Results

Men who recurred (mean age = 58.5 years) were slightly older than non-recurrent (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 to 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 1). The significant *P*-values ranged between $2.57x10^{-3}$ and $2.95x10^{-4}$ for *ABHD9* (Table 3). Confirmatory evidence was strongest for *HOXD3*, with 9 out of 11 promoter region CpG sites having *P*-values between $1.24x10^{-3}$ and $9.46x10^{-6}$.

For the 7 CpG sites in *ABHD9* that were significantly hypermethylated, higher methylation in 4 CpG sites was associated with shorter times to recurrence: sites cg26010734 and cg15826897 were strongest with *P*-values $9.26x10^{-7}$ and $2x10^{-4}$ respectively, while sites cg18366919 and cg08457898 were marginally significant with *P*-values of 0.033 and 0.037. For *HOXD3*8 of 9 significantly hypermethylated promoter region CpG sites were related to time to PCa recurrence. Among these CpGs, cg13316854 and cg24704177 were the strongest with *P*-values of $4.73x10^{-5}$ and $2x10^{-4}$, 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*-value = 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 (Figure 1). Average methylation level across promoter region CpGs of *HOXD3* was strongly associated with time to recurrence (log-rank test *P*-value = $2x10^{-4}$). The median time for recurrence-free survival was 19.4 years and 17.0 years, respectively, in the low and high methylation groups. Based on Cox models adjusting for age, Gleason score, PSA at diagnosis and pathological stage, the hazard ratio comparing patients in the high methylation group to those in the low methylation group was 1.16 (95% 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 to low average methylation had Gleason scores of ≥ 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 to 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 to patients in the low methylation group where only 47.5% had Gleason scores ≥ 7 (P = 0.007). In terms of stage, for *HOXD3* more patients in the high compared to low average methylation group had regional as opposed to localized disease, 42.2% versus 26.9%, respectively (*P* = 0.006).

All of the hypermethylated CpG sites in *ABHD9* and *HOXD3* that were significantly associated with PCa recurrence also showed higher levels of methylation in prostate tumor versus histologically benign adjacent prostate tissue from the same patients (Figures 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 $4.4x10^{-5}$ for *ABHD9* and $1.3x10^{-3}$ for *HOXD3*.

Discussion

The results of this validation study confirm the association between PCa 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 $P = 9.46x10^{-6}$), 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 PCa when compared to patients in the low average methylation group.

In a methylation oligonucleotide microarray study of 304 frozen prostatectomy samples, Cottrell et al. previously reported that promoter region methylation of *ABHD9* was significantly increased in patients who experienced early PSA recurrence versus non-recurrent men (Bonferroni-adjusted $P < 0.05$) (10). The significance of the result with respect to biochemical recurrence was then confirmed in a cohort of 605 RP patients (12). Little is known about the function of *ABHD9* (A*bhydrolase 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 et al. 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 Methy Light technology in a cohort of 232 RP patients (14). They observed that promoter hypermethylation of *HOXD3* was associated with higher Gleason scores (≥ 7 vs. ≤ 6, $P < 0.001$) and more advanced stage (14). In a subsequent report from the same group of investigators that utilized the same cohort of patients, the association of *HOXD3* with biochemical recurrence was examined. Liu et al. found that increased levels of promoter methylation for any two of a panel of three genes, *APCHOXD3* and *TGFβ2*, was predictive of biochemical recurrence ($P = 0.017$) (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 PCa (41–45). A gene expression profile predicting PCa 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 PCa 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 vs. 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 promotor 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 et al. (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 PCa during follow-up. Also similar to earlier studies, our work was based on use of tumor tissue obtained at surgery, and for clinical utility ideally prognostic biomarker tests for PCa 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 PCa recurrence highlights the potential of differential DNA methylation as a prognostic biomarker for stratifying patients with more aggressive tumors. *HOXD3* in particular appears 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 the 11 *HOXD3* CpGs examined were more heavily methylated in tumor compared to 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. Based on these promising results, future evaluation of the *HOXD3* DNA methylation profile in tumor tissue as a biomarker for PCa outcomes is needed to assess its clinical utility as a prognostic tool.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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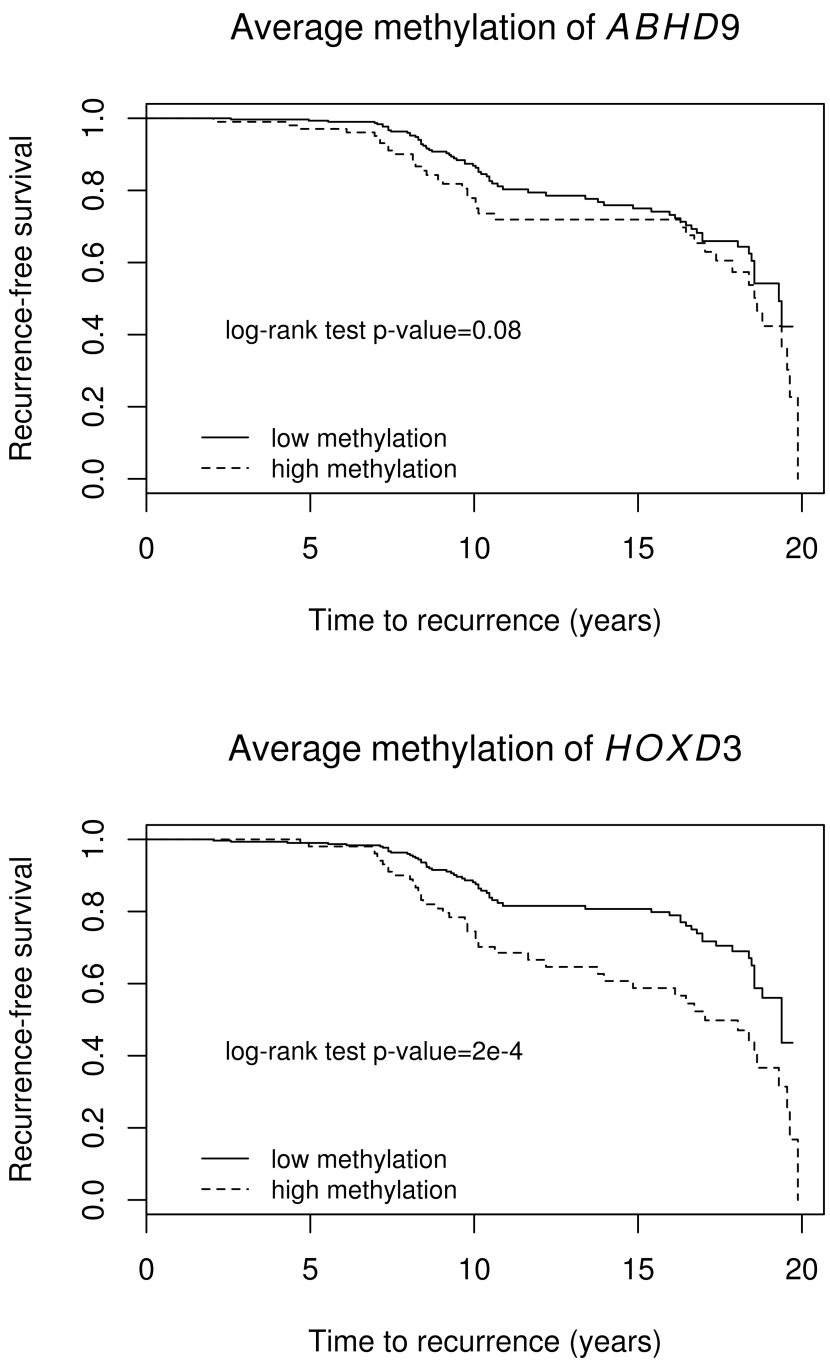

Figure 1.
Kaplan-Meier plots for time to prostate cancer recurrence according to the average promoter region methylation in *ABHD9 and HOXD3*.

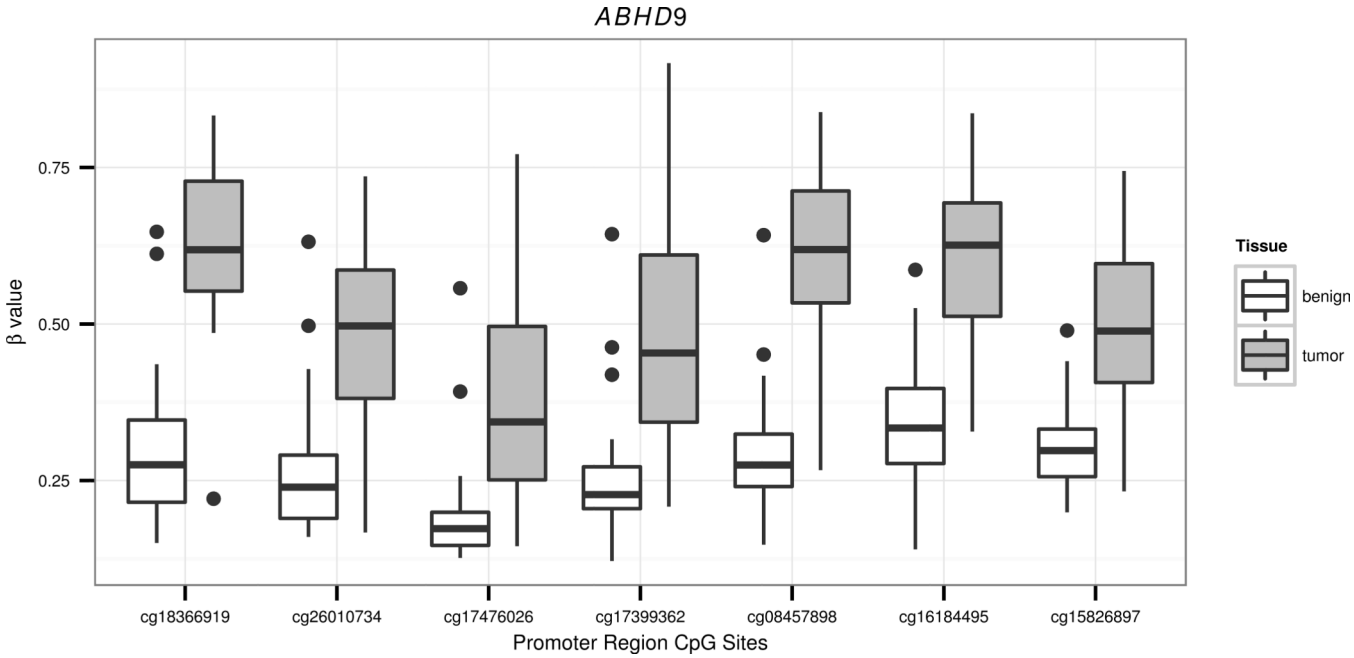

Figure 2.

Boxplots of each significantly hypermethylated CpG site in the *ABHD9* promoter region in prostate tumor tissue vs. adjacent benign prostate tissue.

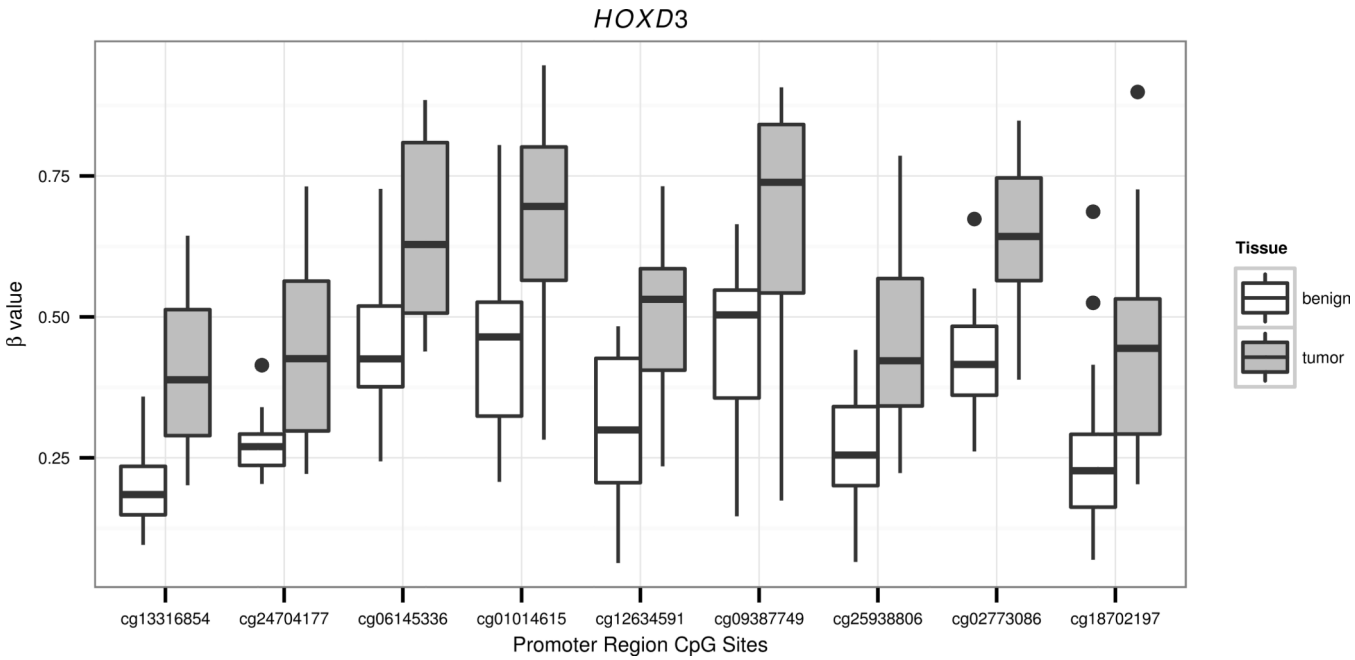

Figure 3.

Boxplots of each significantly hypermethylated CpG site in the *HOXD3* promoter region in prostate tumor tissue vs. adjacent benign prostate tissue.

Table 1

Distributions for selected characteristics of the prostate cancer patient cohort by recurrence status

	Recurrence (*n* = 104)		No recurrence (*n* = 303)	
Characteristic	**n**	**%**	**n**	**%**
Age at diagnosis (years)				
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 (± 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)
Pathological 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)

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

[a] 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 base on t-tests of differential methylation between patients with recurrence versus no recurrence, with Bonferroni correction for multiple testing within each gene.

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

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 β-value: Recurrence	Avg β-value: No recurrence	*P*-value[a]	CpG location	3rd IQR[b]
ABHD9	cg18366919	0.581264	0.522453	0.000295	Island	0.649
ABHD9	cg26010734	0.507842	0.442909	0.000385	N_Shore	0.565
ABHD9	cg17476026	0.429670	0.357897	0.000540	N_Shore	0.525
ABHD9	cg17399362	0.512639	0.432249	0.000838	N_Shore	0.614
ABHD9	cg08457898	0.569149	0.518281	0.001475	N_Shore	0.643
ABHD9	cg16184495	0.589635	0.530117	0.001796	N_Shore	0.672
ABHD9	cg15826897	0.483290	0.434591	0.002572	N_Shore	0.535
HOXD3	cg13316854	0.438143	0.364137	9.46E-06	Island	0.476
HOXD3	cg24704177	0.453488	0.377573	2.93E-05	Island	0.498
HOXD3	cg06145336	0.671013	0.594325	3.33E-05	Island	0.742
HOXD3	cg01014615	0.646853	0.575173	9.87E-05	Island	0.719
HOXD3	cg12634591	0.488850	0.419412	0.000159	N_Shore	0.538
HOXD3	cg09387749	0.646414	0.581765	0.000419	N_Shore	0.715
HOXD3	cg25938806	0.456353	0.391304	0.000618	N_Shore	0.502
HOXD3	cg02773086	0.623405	0.569983	0.001001	N_Shore	0.691
HOXD3	cg18702197	0.426674	0.362498	0.001237	Island	0.472

[a] *P*-values with Bonferroni correction for multiple testing within each gene.

[b] IQR = Interquartile range.