Discovery and Validation of 3 Novel DNA Methylation Markers of Prostate Cancer Prognosis

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Purpose: About 15% of men experience prostate specific antigen recurrence after radical prostatectomy. A DNA methylation based molecular test could provide important information to predict which patients are most likely to experience recurrence.
Materials and Methods: We performed a genome-wide scan to find aberrantly methylated loci in prostate cancer from patients with early recurrence, high Gleason score or advanced stage. We discovered 441 candidate methylation markers and further analyzed 62 candidates in a methylation microarray study of 304 frozen prostatectomy samples.
Results: Methylation of 25 markers was significantly changed in high Gleason score (8–10) vs low Gleason score (2–6) cancers. Methylation levels of the 3 marker candidates GPR7, ABHD9 and an expressed sequence tag on chromosome 3 (Chr3-EST) were significantly increased in patients who did vs did not experience early PSA recurrence (Bonferroni correction $p < 0.05$). Furthermore, these markers were also informative when the sample set was restricted to 68 mid range Gleason score (6 or 7) samples only. We developed real-time polymerase chain reaction assays for ABHD9 and Chr3-EST, and measured methylation in paraffin embedded, formalin fixed prostatectomy samples from an independent set of 223 patients. Methylation of the 2 markers was significantly higher in patients with early PSA recurrence compared to that in patients who did not experience PSA recurrence.
Conclusions: We report that methylation of the 3 novel markers GPR7, ABHD9 and Chr3-EST is significantly associated with prostate cancer prognosis. Incorporation of these methylation markers into clinical practice will result in more accurate prediction of which patients are likely to experience PSA recurrence.

Key Words: prostate, prostatic neoplasms, microarray analysis, DNA methylation, prostate-specific antigen

Radical prostatectomy is commonly used as potentially curative treatment in patients with clinically localized prostate cancer. However, PSA recurrence and local or systemic recurrence after prostate cancer treatment with radical prostatectomy is a major problem, affecting 15% to 20% of men.[1,2] While the evidence in favor of early adjuvant therapy is not overwhelming, some studies suggest that there might be some advantage worth investigating.[3–5] The lack of a clear demonstration of a benefit to adjuvant therapy may be due to the long natural history of prostate cancer or to a lack of appropriate patient stratification. Many clinical trials that are currently under way focus on the application of adjuvant therapy in high risk populations.

To distinguish patients with high and low risk prostate cancer clinicians rely on Gleason grade, preoperative PSA and staging information. Several nomograms were developed to identify patients at high risk for recurrence.[6] A molecular based test could add to the information currently provided by clinical and histological analyses to stratify the patient population more accurately. The combined information could define a group of patients most likely to experience PSA recurrence and, therefore, receive the most benefit from effective adjuvant therapy.

A type of molecular change that could form the basis for a molecular test is DNA hypermethylation. Methylation of CpG dinucleotides in the promoter and exon regions of genes is normally associated with inactivation of the expression of the gene. As cancers are initiated and grow, the methylation of genes involved in cell division can give the cancer a growth advantage. For example, the detoxification enzyme glutathione S-transferase π is methylated in most prostate cancers and also in high grade PIN lesions, and it may be important in the transition to invasive carcinoma.[7,8]

We hypothesized that a genome-wide scan for regions that are methylated only in prostate cancers with aggressive growth properties would yield informative marker candidates that could be validated as prognostic markers in independent patient cohorts to identify the 15% of patients who have recurrence soon after surgery. In this research we discovered and validated 3 DNA methylation markers and analyzed the association of these markers with histological grade and disease recurrence.

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TABLE 1. *Patient information*

	No. Microarray (%)	No. Real-Time PCR (%)
Overall	304	223
Gleason score:		
5	5 (7.6)	8 (3.6)
6	45 (14.8)	20 (9.0)
7	55 (18.1)	114 (51.1)
8	35 (11.5)	15 (6.7)
9	20 (6.6)	12 (5.4)
Not available	126 (41.4)	54 (24.2)
Pathological stage:		
T2	89 (29.3)	93 (41.7)
T3	179 (58.9)	120 (53.8)
T4	2 (0.7)	5 (1.8)
Not available	34 (11.2)	5 (1.8)
Surgical margin:		
Pos	83 (27.3)	81 (36.3)
Neg	134 (44.1)	79 (35.4)
Not available	87 (28.6)	64 (28.7)

MATERIALS AND METHODS

Patient Samples

Patient samples for the methylation array study came from Baylor College of Medicine (144), Charité Hospital Berlin (70), University of Muenster (63), Virginia Mason Medical Center (7) and Ardais Corporation (20). Median patient age at surgery was 60 years. Formalin fixed samples came from Stanford University (57), University of Muenster (112) and University of Regensburg (54). Median patient age at surgery was 66 years. Cancer samples were taken from archived prostatectomies. Surgery dates ranged from 1990 to 2002. Institutional review boards at all participating sites approved this study. None of the patients received any neoadjuvant or adjuvant therapy before PSA recurrence. However, information on followup and clinical parameters was available only on subsets of patients (table 1). All slides of each prostatectomy were reviewed by a pathologist at the respective sites and a representative block with at least 70% cancer cells was selected. PSA recurrence was defined based on the clinical criteria used at each institution.

DNA Extraction

DNA was extracted from all frozen prostatectomy samples using a QIAgen™ Genomic-tip 500/G kit or a similar approach. Samples from paraffin embedded prostatectomy samples were cut into 10 μ sections. Three sections per cancer were deparaffinized using 1 ml limonene for 1 hour at room temperature and lysed at 50C for 48 hours with 3 mg/ml proteinase K in a single microcentrifuge tube. DNA was extracted from the lysates using a QIAgen DNeasy® kit.

MeST Discovery

MeSTs were discovered using 2 established techniques, that is methylation sensitive arbitrarily primed PCR and methylated CpG island amplification.[9,10] Genomic DNA samples from frozen prostatectomy tissues were pooled based on clinical characteristics. The cloned and sequenced fragments were then mapped to the human genome. Overlapping sequences were combined and sequences that were primarily repetitive elements were discarded. The remaining MeSTs were scored based on proximity to a CpG island, the number of independent clones in the same region and proximity to a promoter region or gene. This scoring system along with information from published results was used to prioritize the MeSTs for further validation.

Methylation Microarray

Top marker candidates were further analyzed using a methylation oligonucleotide microarray. DNA was extracted from 304 frozen prostatectomy samples and bisulfite treated according to published protocols.[11] A total of 62 PCR fragments, representing 51 MeST candidate sequences and 11 candidates retrieved from the literature search, were amplified without methylation bias using Cy5 labeled primers specific for bisulfite converted DNA. PCR products were pooled and hybridized to a glass array containing 256 oligo pairs, each spanning 2 or 3 CpGs. For each pair 1 oligo matched the methylated (CG) version of the sequence and 1 matched the unmethylated (TG) version, as previously described.[12] The array had an average of 4 probe pairs per fragment, each oligo was present in quadruplicate and samples were hybridized to the array 4 times.

Hybridization fluorescence was measured using a GenePix™ 4000 microarray scanner. Median spot intensity of the oligos was used to calculate the log methylation ratio [log (CG/TG)] for each oligo pair on an array. For each sample and oligo pair these log methylation ratios were median averaged over hybridization replicates. Finally, for each fragment represented on the array the information from all oligo pairs corresponding to a specific fragment was combined by reporting the mean over oligo pairs of the median log methylation ratios. Statistical process control based on robust principle component analysis was applied to identify outlier hybridizations.[13] Samples with outlier hybridization were rehybridized.

Real-Time PCR

Quantitative MethyLight assays were developed for 2 top array candidates. These assays use primers outside of CpGs to amplify the bisulfite converted target sequence regardless of methylation state and a methylation specific TaqMan®-style real-time PCR probe pair is used to monitor amplification.[14] One probe labeled with FAM recognizes the methylated version of the target, while the second probe labeled with Yakima Yellow® recognizes the unmethylated version. Table 2 lists primer and probe sequences.

TABLE 2. *Quantitative MethyLight primer and probe sequences*

	Chr3-EST	ABHD9
Primer:		
Forward	TTGTAGGGTTTTTTTTGGGTT	GGTGTTAGGGTTTAGGGGTT
Reverse	CTCAAAACCCTTAAAAACATAAA	CCAAATATTTACCTAACACTCAAATA
Probe:		
Methylated DNA	ATAACCACACTACGCGCCTCC	AACTATTTTCTATCGAAACCGCCCG
Unmethylated DNA	ATAACCACACTACACACCTCCCACA	AACTATTTTCTATCAAAACCACCCACCTCT

Underlining indicates dinucleotides derived from the original CpGs.