Hypermethylation of CpG Islands in Primary and Metastatic Human Prostate Cancer

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ABSTRACT

Aberrant DNA methylation patterns may be the earliest somatic genome changes in prostate cancer. Using real-time methylation-specific PCR, we assessed the extent of hypermethylation at 16 CpG islands in DNA from seven prostate cancer cell lines (LNCaP, PC-3, DU-145, LAPC-4, CWR22Rv1, VCaP, and C42B), normal prostate epithelial cells, normal prostate stromal cells, 73 primary prostate cancers, 91 metastatic prostate cancers, and 25 noncancerous prostate tissues. We found that CpG islands at GSTP1, APC, RASSF1a, PTGS2, and MDRI were hypermethylated in >85% of prostate cancers and cancer cell lines but not in normal prostate cells and tissues; CpG islands at EDNRB, ESR1, CDKN2a, and hMLH1 exhibited low to moderate rates of hypermethylation in prostate cancer tissues and cancer cell lines but were entirely unmethylated in normal tissues; and CpG islands at DAPK1, TIMP3, MGMT, CDKN2b, p14/ARF, and CDH1 were not abnormally hypermethylated in prostate cancers. Receiver operator characteristic curve analyses suggested that CpG island hypermethylation changes at GSTP1, APC, RASSF1a, PTGS2, and MDRI in various combinations can distinguish primary prostate cancer from benign prostate tissues with sensitivities of 97.3–100% and specificities of 92–100%. Hypermethylation of the CpG island at EDNRB was correlated with the grade and stage of the primary prostate cancers. PTGS2 CpG island hypermethylation portended an increased risk of recurrence. Furthermore, CpG island hypermethylation patterns in prostate cancer metastases were very similar to the primary prostate cancers and tended to show greater differences between cases than between anatomical sites of metastasis.

INTRODUCTION

Prostate cancer remains the second leading cause of cancer-related deaths among men in the United States (1). Much of the morbidity and mortality from prostate cancer, as with most other solid organ cancers, arises from metastatic disease. However, prostate cancer is often slow-growing and can be effectively treated by radical retropubic prostatectomy when the cancer is still small, well-differentiated, and organ-confined (2). Currently, the early detection of human prostate cancer features digital rectal examination and serum prostate-specific antigen (PSA) determination (3, 4). However, because of their limited sensitivity and specificity, these methods cannot reliably identify early-stage prostate cancer (5–7). Therefore, the development of a more sensitive and specific set of markers that could facilitate the early detection of prostate cancer at the earliest stages could improve the current standard of care.

Aberrant DNA methylation patterns have commonly been associated with human cancers (8). Hypermethylation of the CpG island at the promoter of GSTP1 has been described as one of the earliest and most commonly found genome alterations arising during prostate carcinogenesis, present in >90% of prostate cancer cases but not in normal prostate tissues (9, 10). Detection of GSTP1 CpG island hypermethylation changes in DNA from urine and other bodily fluids has been reported to identify prostate cancers with sensitivities approaching 75% (11–13). However, using GSTP1 CpG island hypermethylation as the only marker for molecular screening and diagnosis of prostate cancer presents some potential limitations. First, the theoretical maximum sensitivity for the screening test can only be as high as the frequency of GSTP1 CpG island hypermethylation in the primary cancer tissues. Additionally, although normal prostate tissues do not exhibit GSTP1 CpG island hypermethylation, screening tests using GSTP1 CpG island hypermethylation as the only marker might not be able to distinguish prostate cancer from other cancers, because several other cancers also exhibit GSTP1 CpG island hypermethylation (8). These limitations could potentially be overcome if multiple sensitive and specific molecular markers were identified and used simultaneously to sensitively and uniquely identify prostate cancers. Autopsy studies have shown that there is a 64% prevalence of small, asymptomatic, organ-confined prostate cancer among men between 60 and 70 years of age (14). Clearly, not all of these men progress to symptomatic or metastatic disease. Consequently, there may be a danger in over-diagnosis of prostate cancer because many men seem to die with but not from prostate cancer. This has become a rising controversy in the era of PSA screening for prostate cancer (15). Therefore, it would be useful to identify molecular markers that cannot only sensitively and specifically diagnose early-stage prostate cancer but also help identify men that would later progress to having symptomatic or metastatic disease. To identify such molecular markers and generate new hypotheses regarding the epigenetic mechanisms involved in prostate cancer progression, we used a candidate gene approach to quantitatively assess the methylation status of CpG islands located in the regulatory regions of 16 genes (GSTP1, APC, RASSF1a, PTGS2, MDRI, HIC1, EDNRB, ESR1, CDKN2a, CDKN2b, p14/ARF, MGMT, hMLH1, TIMP3, DAPK1, CDH1) in 2 normal prostate cell lines, 7 prostate cancer cell lines, 25 benign prostate tissues, 73 primary prostate cancer tissues with a wide spectrum of tumor stage and grade, and 91 metastatic prostate cancer tissues.

MATERIALS AND METHODS

Cell Culture. LNCaP, PC-3, CWR22Rv1, and DU-145 cell lines were obtained from American Type Culture Collection (Rockville, MD). Prostate epithelial cells (PrEC) were obtained from Cambrex (East Rutherford, NJ). These cells were propagated in vitro as per the respective vendors’ recommendations. LAPC-4, C4–2B, VCaP cell lines and normal prostate stromal cells (4ST) were generously provided by Dr. John T. Isaacs (Johns Hopkins University, Baltimore, MD). C4–2B and VCaP cell lines were propagated in vitro in RPMI 1640 (Life Technologies Inc., Grand Island, NY) containing 10% fetal bovine serum (Bio-Whittaker, Walkersville, MD). LAPC-4 and 4ST cell lines were propagated in vitro in Iscove’s media (Life Technologies, Inc.) containing 10% fetal bovine serum and 1 mM R1881. All cells were grown in 5% CO₂/95% air at 37°C.
sections were examined at 60-µm intervals after staining with H&E to ensure the absence of cancer cells.

Benign prostates were obtained from 13 brain-dead transplant tissue donors, ages 4-52, whose families consented to prostate removal for research purposes. After removal of transplantable organs under cardiac bypass, the entire prostate was removed under sterile conditions within 30 min of circulatory arrest and was snap-frozen and stored at -80°C. Twelve consecutive, 6-µm frozen sections were obtained from each prostate. The first and twelfth section were stained with H&E and examined to ensure the absence of cancerous or dysplastic epithelia. The remaining 10 sections were used for DNA extraction.

Metastatic prostate cancer samples were obtained at autopsy from 28 men dying of prostate cancer between 1995 and 2001. Autopsies were performed between 1.5 and 21 h after death (mean 5.9 h). All 28 men had undergone chemical or surgical castration therapy before death. Metastatic prostate cancers were collected and snap frozen. Tissue samples were isolated during processing to avoid cross-contamination and immediately entered into a tracking database. Maximal tumor sample purity was obtained through serial crosscut microdissection at 300-µm intervals, with an average tumor purity >85% based on histological examination. One to six anatomically separate metastases were studied for each of the 28 subjects, for a total of 87 metastatic samples. Metastatic site anatomical categories studied include bone, lymph node, liver, adrenal, cranial subdural metastasis, and intraprostatic cancer found at autopsy.

DNA was isolated from all tissues as described previously (18).

**Bisulfite Modification of DNA Samples and Real-Time Methylation-Specific PCR (RT-MSP).** One µg of sample DNA was subjected to sodium bisulfite modification using the CGPathGene DNA modification kit (Soroligos Co., Norcross, GA). RT-MSP was carried out using a technique similar to the MethyLight assay described previously (19). Briefly, bisulfite-treated DNA was amplified using real-time PCR with oligonucleotide primers and Taqman probe complementary to a region of the MYOD1 promoter that did not contain any CpG dinucleotides but did contain non-CpG cytosines to ascertain the amount of converted input templates in each sample. Hypermethylation of the 16 CpG islands included in this study was then examined by real-time PCR amplification of bisulfite-modified DNA using oligonucleotide primers and Taqman probes specific for a fully methylated bisulfite-converted portion of each CpG island such that only CpG islands that were methylated at every CpG dinucleotide interrogated by the primers and probes would be amplified and generate fluorescent signal. Primer and probe oligonucleotide sequences for MYOD1 (20), GSTP1 (20), APC (21), RASSF1a (22), PTGS2 (21), MDR1, HIC1 (21), EDNRB, ESRI (21), Cdkn2a (21), Cdkn2b (21), p14ARF (21), MGMT (21), hMLH1 (21), TIMP3 (21), DAPK1, and CDH1 (21) are listed in Table 2. All PCR reactions were carried out on an iCycler real-time thermal cycler (Bio-Rad, Hercules, CA) at 95°C for 8.5 min followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The EDNRB reaction was carried out under the same conditions except that an annealing temperature of 64.5°C was used. Each PCR reaction was carried out in a 25-µl volume containing 1× AmpliTaq Gold PCR buffer II (Applied Biosystems, Foster City, CA).
DNA METHYLATION PATTERNS OF PROSTATE CANCER

Fig. 1. Normalized index of methylation (NIM) for prostate cancer cell lines and normal prostate epithelial and stromal cells. Degree of hypermethylation at 16 CpG islands was assessed using quantitative real-time methylation-specific PCR (RT-MSP) and was normalized to the amount of bisulfite-converted input as described in “Materials and Methods.” The NIM was color-scaled between white (representing no methylation detected) and red (indicating that >99% of bisulfite-converted input copies were methylated). SssI treated universally methylated WBC DNA (Univ. Meth.) served as the positive control and, by definition, had a NIM of 1.0. WBC DNA was taken from the WBCs of healthy volunteers and served as a negative control. PDEC, normal prostate epithelial cells; 4ST, normal prostate stromal cells. LNCaP, C4-2B, PC-3, DU-145, LAPC-4, CWR22Rv1, VCaP are prostate cancer cell lines. * indicates NIM values < 0.20 but greater than the calculated threshold for each CpG island.

Table 1: DNA methylation NIM scores for each gene of interest in the 73 prostate cancer cases with Gleason score of 5 and 6.

<table>
<thead>
<tr>
<th>Gene</th>
<th>NIM Score</th>
<th>Mean NIM</th>
<th>Median NIM</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYOD1</td>
<td>0.98</td>
<td>0.98</td>
<td>0.98</td>
<td>0.01</td>
</tr>
<tr>
<td>H11001</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.01</td>
</tr>
<tr>
<td>H11022</td>
<td>0.96</td>
<td>0.97</td>
<td>0.97</td>
<td>0.01</td>
</tr>
<tr>
<td>H11025</td>
<td>0.95</td>
<td>0.95</td>
<td>0.95</td>
<td>0.01</td>
</tr>
<tr>
<td>H11026</td>
<td>0.94</td>
<td>0.94</td>
<td>0.94</td>
<td>0.01</td>
</tr>
<tr>
<td>H11027</td>
<td>0.93</td>
<td>0.93</td>
<td>0.93</td>
<td>0.01</td>
</tr>
<tr>
<td>H11028</td>
<td>0.92</td>
<td>0.92</td>
<td>0.92</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Pathological stage was coded such that organ-confined cancers and cancers with capsular penetration but no other extra-prostatic involvement were considered low stage; cancers with involvement of the seminal vesicles and cancers that metastasized to pelvic lymph nodes were considered high stage. Degree of hypermethylation at 16 CpG islands was assessed using quantitative real-time methylation-specific PCR (RT-MSP) and was normalized to the amount of bisulfite-converted input as described in “Materials and Methods.” The NIM was color-scaled between white (representing no methylation detected) and red (indicating that >99% of bisulfite-converted input copies were methylated). SssI treated universally methylated WBC DNA (Univ. Meth.) served as the positive control and, by definition, had a NIM of 1.0. WBC DNA was taken from the WBCs of healthy volunteers and served as a negative control. PDEC, normal prostate epithelial cells; 4ST, normal prostate stromal cells. LNCaP, C4-2B, PC-3, DU-145, LAPC-4, CWR22Rv1, VCaP are prostate cancer cell lines. * indicates NIM values < 0.20 but greater than the calculated threshold for each CpG island.

Statistical Analysis. Using the Cpg island methylation data for the 73 primary prostate cancers and the 25 normal prostate tissues, the optimal sensitivity and specificity with each DNA methylation marker for diagnosis of primary prostate cancer was determined by receiver operator characteristic (ROC) curve analysis using the MedCalc software (MedCalc Software, Mariakerke, Belgium). The area under the ROC curve and the methylation threshold yielding the optimal sensitivity and specificity were calculated for each DNA methylation marker. In addition, using the same thresholds, the sensitivity, specificity, positive predictive value, and negative predictive value for each of the methylation markers individually and in various combinations were calculated.

Pathological stage was coded such that organ-confined cancers and cancers with capsular penetration but no other extra-prostatic involvement were considered low stage; cancers with involvement of the seminal vesicles and cancers that metastasized to pelvic lymph nodes were considered high stage. Using the NIM at each gene as a dichotomous independent variable with NIM > the median for each gene designated as high methylation and NIM < the median designated as low methylation and early or late stage as the dependent variable, a univariate logistic regression analysis was performed for each gene to determine associations between the methylation data and pathological stage. Gleason score was coded such that Gleason scores of 3 + 2, 3 + 3, and 4 + 3 were defined as moderate grade and Gleason scores 4 + 4, 4 + 5, and 5 + 4 were defined as high grade. A univariate logistic regression analysis, with the NIM at each gene as a dichotomous independent variable (using the same parameters as above) and moderate or high grade as the dependent variable, was performed for each gene to determine associations between the quantitative methylation data and Gleason score. These univariate logistic regression analyses were performed using the SAS statistical software package (SAS Institute, Inc., Cary, NC).

Biochemical and clinical prostate cancer recurrence data were available for 36 of the 73 patients with primary prostate cancer that underwent radical prostatectomy. For each gene, a NIM less than the median for that gene among the 36 subjects was defined as low methylation, whereas a NIM greater than the median was defined as high methylation. A Kaplan-Meier analysis was carried out, and recurrence curves were plotted for high versus low methylation at each gene locus. Kaplan-Meier recurrence curves were also generated for samples with Gleason score 5 and 6 versus Gleason score 7 versus Gleason score 8 and 9, samples with high versus low pathological stage as defined previously, samples with age greater than the median versus age less than the median, and samples with preoperative serum PSA greater than the median versus PSA less than the median. The statistical significance of differences in the rate of recurrence for each curve was analyzed by the log-rank test. These analyses were carried out using the GraphPad Prism 4 software package (GraphPad Software Inc., San Diego, CA). Recurrence data were also analyzed by fitting the data to a Cox proportional hazards model. Each of the variables listed above were first individually fit to a univariate Cox proportional hazards models. The only exception to this was that age and serum PSA were treated as continuous variables in this analysis. All variables with P > 0.1 were eliminated from a multivariate Cox proportional hazards model. Several rounds of elimination from the multivariate Cox proportional hazards model were carried out until all remaining covariates attained P < 0.1. This analysis was performed using the SAS statistical software package.

To analyze the relationship between the anatomical site category (bone, lymph node, liver, and so forth) of metastasis and the methylation pattern of the metastatic sample, we compared the overall variability in the NIM among all genes and specimens between site categories (σ_y^2) to the variability among all genes and specimens within site categories (σ_x^2) by implementing an analysis of molecular variance approach that extended traditional ANOVA concepts to facilitate variance-based hypotheses. The NIM, z, was defined as the ratio of the number of methylated copies of each gene of interest to the number of bisulfite-converted, MYOD1 copies (y). For each specimen and site...
category, \( y \) would remain the same for all \( G = 16 \) genes examined. As a preprocessing step, values of \( z \geq 1 \) were truncated to values of 1. To accommodate subjects with multiple metastatic deposits from the same site category, a composite NIM for each gene was constructed by the use of singular value decomposition (23) applied to a matrix of multiple NIM data vectors among genes formed for each subject and site category. As opposed to defining the average or median NIM among multiple metastatic deposits from the same site category by subject for each gene, a singular value decomposition approach retains the dependence among genes. Because no analytic distributions are imposed on the NIM data, the method is nonparametric in its approach to inference. We formally compared the two variances, \( \sigma_b^2 \) and \( \sigma_w^2 \) by examining the null hypothesis, \( H_0 : \sigma_b^2 = \sigma_w^2 \) versus the alternative, \( H_a : \sigma_b^2 \neq \sigma_w^2 \). We considered the overall ratio, among all specimens and genes, of between- to within-site variability, pooled across all site categories, denoted by the statistic, \( \delta = (SSB)/(SSW) \), where SSW summarizes within-site variability through the sum of squared deviations between each NIM, \( z \), and the mean NIM within each site category, and SSB summarizes between-site variability through the sum of squared deviations between site-specific NIM means. As an overall measure of methylation index variability among all specimens and genes, \( \delta > 1 \) indicates that the between-site variability is greater than the variability between subjects and genes within site categories. That is, the average NIM variability between site categories is larger than the average NIM variability within site categories. On the other hand, \( \delta < 1 \) indicates that the within-site variability, overall, between subjects and genes, is greater than the overall between-site variability. That is, the variability within site categories is on average greater than the variability when all specimens among all subjects and sites are pooled together. To test the null hypothesis of equal variances, \( H_0 \), we used the method of bootstrap and sample with replacement of the \( n = 36 \) specimens, \( M = 500 \) times, calculating the ratio statistic, \( \delta \), for each \( m \)-th bootstrap sample, denoted by \( \delta_m \). On the basis of these statistics, we estimated the significance of \( \delta \) by \( p = M^{-1} \sum I(\delta_m \geq \delta) \), where \( I(\geq a) \) is a binary indicator function with the value 1 if \( x \geq a \) and 0 otherwise. In addition

Fig. 2. The normalized index of methylation (NIM) for normal prostate tissues, tumor-adjacent benign prostate tissues, and primary prostate cancers at 16 CpG islands. A, NIM at 16 CpG islands for normal prostate tissues taken from 13 transplant tissue donors. B, NIM at 16 CpG islands for tumor-adjacent benign prostate cancer tissues microdissected from 12 of the 73 patients undergoing radical prostatectomy for organ-confined prostate cancer. C, NIM at 16 CpG islands for primary prostate cancer tissues obtained from 73 patients at the time of radical prostatectomy. * indicates NIM values < 0.20 but greater than the calculated threshold for each CpG island. Univ. Meth., universally methylated; PIN, prostatic intraepithelial neoplasia.
to formally comparing variances, we characterized the observed differential variability within- and between-site categories, in terms of individual genes, by estimating their proportionate contribution to the statistic, $\delta$. To this end, we calculated the ratio statistic, $\delta$, within each gene, and defined a proportionate gene contribution by $\theta_i = \delta_i / \sum \delta_i$. By ranking genes according to the measure $\theta_i$, we calculated the relative importance of individual genes in characterizing observed differences between the within-site variability and the between-site variability.

RESULTS

Methylation of CpG Islands in Prostate Cancer Cell Lines and Normal Prostate Epithelial and Fibroblast Cells. The NIM at each of the 16 CpG islands for 7 prostate cancer cell lines (LNCaP, PC-3, DU-145, LAPC-4, CWR22Rv1, VCaP, and C42B) and the normal PrEC and 4ST cells are shown in Fig. 1. CpG islands at GSTP1 (all cell lines but CWR22Rv1), APC (all cell lines except DU-145), RASSF1a (all cell lines), PTGS2 (all cell lines), and MDR1 (all cell lines except LNCaP) were frequently methylated in the prostate cancer cell lines but were not methylated in the PrEC or 4ST cells. There was often a high prevalence of fully methylated copies of these CpG islands in these cell lines, indicated by NIM values $>0.5$. As a convention, when we refer to the prevalence of methylation for a sample at a particular locus, we mean the fraction of copies of that locus that are methylated at every interrogated CpG dinucleotide, as estimated by the NIM. The CpG island at HIC1 was methylated in all of the prostate cancer cell lines and also methylated in the PrEC cells but not in the 4ST prostate stromal cells. CpG islands at EDNRB (all cell lines except LAPC-4), ESR1 (PC-3), CDKN2a (PC-3, CWR22Rv1), TIMP3 (PC-3, DU-145), and DAPK1 (PC-3) were variably methylated in the prostate cancer cell lines, but completely unmethylated in the PrEC and 4ST cells. CDKN2b, p14/ARF, hMLH1, MGMT, and CDH1 were not methylated in any of the prostate cancer cell lines or normal cells. None of the CpG islands were methylated in DNA from WBCs.

Methylation of CpG Islands in Benign Prostate Tissues and Primary Prostate Cancers. A summary of the NIM for the benign prostate and primary prostate cancer tissues at each of the 16 CpG islands examined is shown in Fig. 2. As with the prostate cancer cell lines, the CpG islands at GSTP1 (94%), APC (90%), RASSF1a (96%), PTGS2 (88%), and MDR1 (88%) were frequently hypermethylated in a large percentage of the 73 primary prostate cancers and had, on average, a high prevalence of fully methylated alleles (mean and median NIM $>0.25$) but were almost never methylated in the benign tissues (Figs. 2 and 6). Indeed, even the tumor-adjacent benign tissues, taken from 12 of the 73 patients with prostate cancer undergoing radical prostatectomy, did not show any significant levels of methylation at these CpG islands (Fig. 2B). Only benign prostate tissues containing large regions of high-grade prostatic intraepithelial neoplasia (PIN) were methylated to a significant level above a threshold NIM level at any of the examined CpG islands (Fig. 2B). For the CpG islands at GSTP1, APC, RASSF1a, PTGS2, and MDR1, this threshold was defined as the NIM threshold that results in the optimal sensitivity and specificity for distinguishing the primary prostate cancers from the benign tissues. For the other CpG islands, this background threshold was determined by taking the mean NIM plus two SDs for 10 identical replicates of the WBC reactions for each CpG island. The CpG island at the HIC1 gene was methylated in 100% of the prostate cancer specimens but was also methylated in 100% of the benign prostate specimens. CpG islands at EDNRB, ESR1, and CDKN2a, had low to moderate frequencies (49%, 19%, 6%, respectively) of methylation in the primary prostate cancers but were entirely unmethylated in benign tissues (Fig. 2). The CpG island at MGMT was methylated in a single case of the primary prostate cancers (1%; Fig. 2C). CpG islands at hMLH1, DAPK1, TIMP3, CDKN2b, p14/ARF, CDH1, showed lack of methylation in all of the primary prostate cancers as well as the benign tissues (Fig. 2).

Sensitivity and Specificity of DNA Methylation Markers for Diagnosis of Primary Prostate Cancer. Because a collection of benign prostate and primary prostate cancer samples were studied, sensitivity, specificity, positive predictive value, and negative predictive value were determined to assess the usefulness of hypermethylation at each of the CpG islands studied in distinguishing primary prostate cancer from benign prostate tissue. This analysis was performed using ROC curves, and optimal thresholds of methylation yielding the maximal sensitivity and specificity for each methylation marker were determined. This analysis revealed that hypermethylation at the GSTP1, APC, MDR1, PTGS2, and RASSF1a CpG islands were the most powerful in sensitively and specifically distinguishing primary prostate cancer from benign prostate tissues. Each of these markers had areas under the ROC curve of $>0.9$ and sensitivities and specificities between 85–100% (Fig. 3). Additionally, using the markers in combination provided even more diagnostic power than using a single marker alone. For instance, hypermethylation at the GSTP1 CpG island in combination with hypermethylation at the MDR1 CpG island yields a sensitivity of 97.3% while maintaining a specificity of
Table 3 Summary of diagnostic information using CpG island methylation at various loci individually and in combination

<table>
<thead>
<tr>
<th>Loci</th>
<th>Sensitivity (95% Confidence Interval)</th>
<th>Specificity (95% Confidence Interval)</th>
<th>PPVa (95% Confidence Interval)</th>
<th>NPV (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTP1</td>
<td>93.2 (84.7–97.7)</td>
<td>100.0</td>
<td>100.0</td>
<td>83.3 (65.2–94.4)</td>
</tr>
<tr>
<td>APC</td>
<td>90.4 (81.2–96.0)</td>
<td>96.0</td>
<td>98.5 (92.0–100.0)</td>
<td>77.4 (58.9–90.4)</td>
</tr>
<tr>
<td>MDR1</td>
<td>87.7 (77.9–94.2)</td>
<td>100.0</td>
<td>100.0</td>
<td>73.5 (55.6–87.1)</td>
</tr>
<tr>
<td>PTGS2</td>
<td>87.7 (77.9–94.2)</td>
<td>92.0</td>
<td>97.0 (94.3–100.0)</td>
<td>71.9 (53.3–86.3)</td>
</tr>
<tr>
<td>RASSF1a</td>
<td>95.9 (88.4–99.1)</td>
<td>100.0</td>
<td>100.0</td>
<td>89.3 (71.8–97.7)</td>
</tr>
<tr>
<td>GSTP1 or MDR1</td>
<td>97.3 (90.5–99.7)</td>
<td>100.0</td>
<td>100.0</td>
<td>92.6 (75.7–99.1)</td>
</tr>
<tr>
<td>GSTP1 or MDR1 or APC</td>
<td>98.6 (92.6–100.0)</td>
<td>96.0 (79.6–99.3)</td>
<td>98.6 (92.6–100.0)</td>
<td>96.0 (79.6–99.9)</td>
</tr>
<tr>
<td>GSTP1 or APC or MDR1 or PTGS2</td>
<td>100.0 (95.0–100.0)</td>
<td>92.0 (73.9–98.8)</td>
<td>97.3 (90.7–99.7)</td>
<td>100.0 (85.0–100.0)</td>
</tr>
<tr>
<td>GSTP1 or APC or MDR1 or PTGS2 or RASSF1a</td>
<td>100.0 (95.0–100.0)</td>
<td>92.0 (73.9–98.8)</td>
<td>97.3 (90.7–99.7)</td>
<td>100.0 (85.0–100.0)</td>
</tr>
</tbody>
</table>

a PPV, positive predictive value; NPV, negative predictive value.

100% (Table 3). However, for the tissues examined in this study, a point of diminishing returns is probably crossed when using more than three markers at once because the marginal increase in sensitivity is accompanied by a decrease in specificity (Table 3).

Correlation of Methylation Data with Pathological Stage, Gleason Score, and Recurrence. The utility of the NIM at each CpG island in predicting pathological stage and Gleason score was determined by logistic regression analysis. Of all of the methylation mark-

Fig. 4. Kaplan-Meier curve assessment of risk of biochemical recurrence as a function of Gleason score, pathological stage, and PTGS2 methylation. A, Gleason score. B, pathological stage. C, normalized index of methylation (NIM) at PTGS2. This analysis was performed for 36 of the 73 patients treated with radical prostatectomy for whom clinical recurrence data were available. In each panel, the differences in the rates of recurrence for each group were statistically significant by log-rank test.
ers, only EDNRB CpG island hypermethylation correlated with pathological stage and Gleason score to a statistically significant extent. The odds ratio for high pathological stage (involvement of the seminal vesicles or metastatic disease) using the NIM at the EDNRB CpG island was 3.193 (\(P = 0.040\)). The odds ratio for high Gleason score (4/7, 4/8, 4/9) as predicted by the NIM at the EDNRB CpG island was 4.615 (\(P = 0.0050\)).

Additionally, the potential for predicting recurrence after radical retropubic prostatectomy using the NIM at each CpG island as a marker was investigated. A Kaplan-Meier analysis using the median NIM as the cut-point for each CpG island to distinguish high levels from low levels of methylation showed that of all of the CpG islands, patients with hypermethylation at the PTGS2 CpG island greater than the median had a higher rate of recurrence than patients with PTGS2 CpG island hypermethylation below the median (\(P = 0.0017\); Fig. 4C). As a validation of the patient population used, known predictors of biochemical recurrence, such as Gleason score and pathological stage (24) were also tested by Kaplan-Meier analysis to see if they predicted recurrence in this patient population. High Gleason score and high pathological stage did predict a statistically significantly greater rate of recurrence than low or moderate Gleason score or low pathological stage (Fig. 4, A and B). Also, age at the time of surgery did not correlate with risk of recurrence with statistical significance (\(P = 0.24\)).

The univariate Cox proportional hazards models revealed that of all of the variables tested, only high as opposed to low PTGS2 CpG island hypermethylation, increasing preoperative serum PSA, high as opposed to low pathological stage, and Gleason scores of 7, 8, and 9 compared with Gleason scores of 5 and 6, increased the risk of recurrence (Table 4A). However, when entered into a multivariate Cox proportional hazards model, only high PTGS2 CpG island hypermethylation predicted for increased risk of recurrence independently of Gleason score and pathological stage. High pathological stage was only marginally predictive of recurrence independently of Gleason score. Therefore, of all of the input variables tested simultaneously in the multivariate Cox proportional hazards model, only PTGS2 methylation, Gleason score, and pathological stage independently correlated with recurrence in the final model (Table 4B).

Methylation of CpG Islands in Metastatic Prostate Cancers. The methylation profile of 91 metastatic deposits (83 anatomically distinct metastases from autopsy specimens from 28 subjects and 8

**Fig. 5.** The normalized index of methylation (NIM) for metastatic prostate cancers from various sites. The degree of hypermethylation at 16 CpG islands was assessed for 83 metastatic prostate cancers obtained from anatomically distinct sites at the time of autopsy from 28 men that died from metastatic prostate cancer. Heavy horizontal lines separate specimens by patient. *, indicates NIM values < 0.20 but greater than the calculated threshold for each CpG island. **, indicates pelvic lymph node (LN) metastases obtained from eight patients at the time of intended radical prostatectomy for presumed organ-confined prostate cancer. U. Meth, universally methylated.

### Table 4 Variables that predict increased risk of recurrence with statistical significance in Cox proportional hazards models

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio</th>
<th>95% Confidence interval</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Univariate Cox proportional hazards model results</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Preoperative serum PSA</td>
<td>1.04</td>
<td>1.00–1.07</td>
<td>0.05</td>
</tr>
<tr>
<td>Organ confined/capsular penetration</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seminal vesicle involvement/metastases</td>
<td>3.11</td>
<td>1.26–7.70</td>
<td>0.01</td>
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<tr>
<td>Gleason score</td>
<td></td>
<td></td>
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<tr>
<td>5 or 6</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>3.37</td>
<td>0.92–12.39</td>
<td>0.07</td>
</tr>
<tr>
<td>8 or 9</td>
<td>7.00</td>
<td>1.69–29.00</td>
<td>0.01</td>
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<tr>
<td>PTGS2 NIM</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Less than median</td>
<td>1.00</td>
<td></td>
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<tr>
<td>Greater than median</td>
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<td>1.07–7.44</td>
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<td><strong>B. Multivariate Cox proportional hazards model results</strong></td>
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<td>Pathological stage</td>
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<td>Organ confined/capsular penetration</td>
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<tr>
<td>Seminal vesicle involvement/metastases</td>
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<tr>
<td>5 or 6</td>
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<td>7</td>
<td>4.95</td>
<td>1.11–22.19</td>
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<tr>
<td>8 or 9</td>
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<td>1.84–44.49</td>
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<tr>
<td>PTGS2 NIM</td>
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<tr>
<td>Less than median</td>
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<td></td>
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<tr>
<td>Greater than median</td>
<td>4.26</td>
<td>1.36–13.36</td>
<td>0.01</td>
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metastases from pelvic lymph nodes taken at the time of intended radical prostatectomy) was examined (Fig. 5). Four of the 87 metastases samples from the autopsy subjects could not be amplified by RT-MSP. The methylation pattern in the metastases was remarkably similar to that of the primary prostate cancers, such that the same CpG islands were hypermethylated in both the metastatic and primary prostate cancer specimens (Figs. 2C and 5) at similar frequencies (Fig. 6A). The only notable difference in the methylation patterns between the primary and metastatic prostate cancer specimens is that the metastatic prostate cancers had greater mean and median NIM values than the primary prostate cancers (Fig. 6, B and C). Also, the sum of the NIM across all genes, excluding HIC1, called the aggregate-normalized index of methylation was greater in the metastases than in the primary prostate cancers ($P < 0.0001$; Fig. 7).

An interesting qualitative feature of the methylation profile of the metastatic cancers is that the methylation pattern is strikingly homogeneous across all metastatic sites from any given patient. For example, subject 22 (Fig. 5) has some degree of hypermethylation at the hMLH1 CpG island in his intraprostatic cancer, and this hypermethylation is maintained at every one of his three metastatic deposits. This is especially noteworthy because hMLH1 hypermethylation is very infrequent in prostate cancer. As another example, in subject 32 (Fig. 5), the CpG island at MDR1 is not hypermethylated in the intraprostatic cancer nor in any of the metastatic deposits. Again, this is especially telling because MDR1 is hypermethylated in $>85\%$ of prostate cancers but is not hypermethylated in any of the five prostate cancer lesions in this patient. This trend appears to hold true for all CpG islands and patients with only a few exceptions. At least in a qualitative sense, it appears as though the pattern of CpG island hypermethylation is maintained in a clonal manner even through the process of metastatic progression. Furthermore, it appears as though the methylation pattern of metastatic cancers in each category (lymph node, bone, liver, and so forth) of anatomical site involvement is heterogeneous across different subjects. For instance, the methylation pattern of specimens from all lymph node metastases or all bone metastases appears to be heterogeneous. Therefore, it seems that the methylation pattern is not influenced by the site of metastasis to a great extent.

To test this hypothesis in a quantitative fashion, we used an analysis of molecular variance approach to assess whether the variability of the methylation profile within site categories was greater or less than the variability of the methylation profile across all sites and subjects. In the experiment, $SSB = 22.339$, $SSW = 117.642$, and thus, $\delta = 0.189$. For, $M = 500$ bootstrap samples, $P < 0.0001$, and thus, we reject $H_0$. Therefore, the data indicate evidence of differential variability when comparing between- to within-sites, overall, among all subjects and genes. Because $\delta < 1$, there is a significantly larger variability in the NIM profile within site categories than there is across all sites and patients. This result suggests that the NIM among subjects and genes
is unlikely to be site-specific. The \textit{GSTP1} CpG island contributed the most to the observed differences in NIM variability.

**DISCUSSION**

We comprehensively examined the methylation profile of CpG islands at 16 cancer-related genes in the entire spectrum of prostate cancer disease progression, from benign prostate, to primary prostate cancer, to metastatic prostate cancer, using a collection of prostate cell lines and tissues. These 16 CpG islands were chosen because they were each found to be hypermethylated in other human cancers and are known to have many functions in carcinogenesis, including tumor suppression, modulation of inflammation, detoxification, and drug resistance (8, 22, 25–38). The relatively large sample sizes and number of genes examined, the unique collection of cancer and benign tissues, the tissue processing and purification techniques used in the study design, and the quantitative nature of the assays used in this study are well-suited to accomplishing its goals. In particular, by careful microdissecting all of the primary and metastatic prostate cancer tissues to >70% purity of cancer epithelia, and by normalizing all methylation data to input bisulfite-converted DNA, we were able to estimate the fraction of cancer DNA that was methylated at a particular locus as represented by the NIM at that locus for all of the cancer specimens.

Strikingly, the methylation pattern of the prostate cancer cell lines, the primary prostate cancer tissues, and the metastatic prostate cancer tissues were very similar in that the frequency of hypermethylation at each of the CpG islands was very consistent across all of these specimens (Fig. 6A). These similarities suggest that, at least from a DNA methylation point of view, the prostate cancer cell lines, in general, possess the same “hypermethylation fingerprint” as the primary and metastatic prostate cancers. Furthermore, this fingerprint was consistently maintained across many of the prostate cancer disease severities sampled in this study: from organ-confined, well differentiated cancers, to metastatic, poorly differentiated prostate cancers. In fact, some of these methylation changes may occur even in high-grade PIN lesions, considered to be precursors to primary prostate cancer (39), because two of the benign tissues containing large areas of high-grade PIN were significantly methylated at the \textit{APC} and \textit{PTGS2} CpG islands (Fig. 2B). This data adds to previous reports showing aberrant \textit{GSTP1} CpG island methylation in approximately 70% of high-grade PIN lesions (40, 41). These observations support a hypothesis that many of these DNA methylation derangements take place very early in the pathogenesis of prostate cancer and are consistently maintained during disease progression.

Additionally, we demonstrated that this prostate cancer hypermethylation fingerprint has great potential for the sensitive and specific diagnosis of prostate cancer. CpG island hypermethylation at \textit{GSTP1}, \textit{APC}, \textit{PTGS2}, \textit{MDR1}, and \textit{RASSF1a} was found at an extremely high frequency in the prostate cancer tissues but was not found in the normal tissues. An ROC curve analysis revealed that these markers could each yield sensitivities >88% and specificities >92%. When used in various combinations, sensitivities approached 100% and maintained specificities >92%. A few previous studies have reported the efficacy of \textit{GSTP1} CpG island hypermethylation in the diagnosis of prostate cancer in various bodily fluids with sensitivities approaching 75% (11–13). It may be possible to significantly increase the sensitivity and specificity of diagnosis of prostate cancer in bodily fluids and biopsy specimens if several of the markers identified in the current study are used in combination. Additionally, this methylation fingerprint is unique to prostate cancers and can distinguish it from cancers arising in other organs (Fig. 8).

We also examined whether the markers found in this study would correlate with known markers of disease severity. By logistic regression analysis, the NIM at the \textit{EDNRB} CpG island greater than the median directly correlated with high pathological stage (odds ratio = 3.2, \textit{P} = 0.04) and with high Gleason score (odds ratio = 4.615, \textit{P} = 0.005). We also directly investigated the correlation between hypermethylation at the various CpG islands and the risk of biochemical recurrence. To our knowledge, this is the first study to directly examine this relationship in prostate cancer. In this study, by using a Kaplan-Meier analysis, we found that of all of the CpG islands tested, only \textit{PTGS2} CpG island hypermethylation was correlated with recurrence (\textit{P} = 0.0017; Fig. 4). Additionally, a multivariate Cox proportional hazards model indicated that this correlation between high \textit{PTGS2} CpG island hypermethylation and increased risk of recurrence was independent of Gleason score and pathological stage (Table 4, A and B). Therefore, \textit{PTGS2} hypermethylation may be useful as a clinical marker along with Gleason score and pathological stage in assessing patient prognosis after treatment by radical prostatectomy. However, this association must be further tested in a much larger series of consecutive patients.

Of these CpG islands, only those at \textit{GSTP1} (9) encoding a phase II detoxification enzyme, \textit{RASSF1a} (42, 43), a putative tumor suppressor gene, and \textit{EDNRB} (44–46) have been reported previously to be frequently methylated in prostate adenocarcinoma. The \textit{EDNRB} gene encodes the endothelin receptor type B, a protein with a role in potentiating vasoconstriction that is well established (47). The 5’ regulatory region of the \textit{EDNRB} gene contains a complex CpG island (48) and methylation at certain CpG dinucleotides in this island can lead to the silencing of this gene in these tissues (45). We have shown that \textit{EDNRB} CpG island hypermethylation is a highly specific marker for prostate cancer that could become a useful clinical tool in assessing disease severity.

The CpG islands at \textit{APC}, \textit{PTGS2}, and \textit{MDR1} have never previously
been reported to be frequently methylated (>85% of specimens) in human prostate cancer. The APC gene is a well characterized tumor suppressor gene and has been found to be inactivated by genetic and epigenetic mechanisms in many other human neoplasms (8, 49). One previous study has reported a moderate frequency of methylation (<30%) in the CpG island at the APC1a gene promoter in primary prostate cancers (42) whereas the current report found a frequency of 90% at this locus. This difference could be accounted for by the fact that a different set of CpG dinucleotides was interrogated in this study as compared with the previous report. PTGS2 encodes cyclooxygenase 2, the inducible isoform of the cyclooxygenase enzymes, which are the rate-limiting enzymes that convert arachidonic acid to various pro-inflammatory prostaglandins and are the primary targets for non-steroidal anti-inflammatory drugs (50). PTGS2 has been reported to be unexpressed in the normal prostate, highly expressed in a subset of proliferative inflammatory atrophy lesions of the prostate, but generally not expressed in high-grade PIN and prostate adenocarcinoma lesions at both the protein and transcript levels (51). However, the mechanism by which PTGS2 is silenced was not explored. Our finding that PTGS2 methylation occurs in 88% of prostate cancers suggests that DNA methylation may be one mechanism by which PTGS2 is silenced in prostate cancers. The MDR1 gene encodes the P-glycoprotein, which acts as an ATP-dependent efflux pump implicated in resistance to the cytotoxic actions of several antineoplastic drugs (52). An inverse correlation between methylation at CpG dinucleotides at the promoter of this gene and its expression levels has been found in many human neoplasms and thus, demethylation of CpG dinucleotides at the promoter region of the MDR1 gene is thought to underlie one mechanism of acquired drug resistance (53–55). However, the methylation status of the CpG island in the MDR1 promoter and the expression level of this gene in prostate cancers has never been reported previously. In this study, the MDR1 gene was found to be methylated in 88% of the primary prostate cancers, 89% of the metastatic prostate cancers, but in none of the benign tissues. Furthermore, a gene expression oligonucleotide microarray analysis revealed that MDR1 mRNA was not present in any of the prostate cancer cell lines or PrEC and 4ST normal cells used in this study. However, the precise role of MDR1 in prostate carcinogenesis and drug resistance has not yet been determined.

The degree of similarity between the primary and metastatic prostate cancers was somewhat surprising. The CpG islands that were frequently methylated in the primary cancers were also frequently methylated in the metastatic specimens. Also, there were really no additional CpG islands that were methylated in the metastatic prostate cancers that were not methylated in the primary prostate cancers. The only significant difference between the primary and metastatic prostate cancers was that, on average, the mean and median NIM at each of the CpG islands, and the sum of the NIM across all CpG islands (aggregate-normalized index of methylation), was greater in the metastatic prostate cancers than in the primary prostate cancers (Figs. 6, B and C, and 7). However, even this difference between the primary and metastatic prostate cancers may be because, in general, the metastatic samples had a higher percentage of cancer cells (>85% cancer cells on average by microscopic examination) in a given section than the primary prostate cancer tissue sections even after purifying for cancer cells in the primary specimens. Additionally, we found that the methylation patterns seemed to be consistently maintained for any given patient across all metastatic sites. By means of a novel statistical method called analysis of molecular variance we demonstrated that all metastases from the same category of anatomical site involvement displayed, on average, a relatively high degree of...
variability in the NIM across all genes. However, this variability was reduced by >5-fold (P < 0.0001) when specimens were pooled by patients and sites. Therefore, the methylation pattern did not correlate with the site category. Rather, the methylation pattern was largely homogeneous in any given subject between all sites. Furthermore, the intraprostatic cancers from a given patient had a methylation pattern that was very similar to the methylation pattern in the metastatic deposits obtained from the same patient. Taken together, these observations suggest that epigenetic aberrations appear to be clonally maintained during metastasis. The mechanism of this maintenance of methylation during the proliferation and metastasis of prostate cancer is still unclear. However, there is some evidence to suggest that the action of DNA methyltransferase enzymes, DNMT1 in particular, is responsible for the maintenance of methylation during cell proliferation (56–58). The mechanisms underlying the establishment of distant metastasis have been the topic of much recent study. Whether metastases arise from a rare variant in the primary cancer or from a highly prevalent population of cells prone to metastasis is unclear (59). Two studies have reported the clonal maintenance of copy number of chromosomal amplifications and other chromosomal alterations in the metastasis of human melanomas (60, 61). More recent studies, using gene expression microarray techniques, have examined the systemic gene expression changes that accompany metastatic invasion. One such report showed that the gene expression signatures of two tumors from the same patient were much more similar to each other than they were to samples from any other patient (62). Other reports have shown that a specific gene expression profile in the primary cancers, which must have occurred in a large subset of cells to be detected by gene expression microarrays, accurately predicted the propensity of these lesions to metastasize (63–65). These studies, as well as the results from the current report, examining epigenetic DNA methylation processes, support a model in which a cell that has accumulated the necessary derangements clonally proliferates in the primary cancer to form a prevalent subset of cells and, in some cases, invades and metastasizes to other sites.

Overall, the data presented in the current study suggest a model in which there is an early epigenetic catastrophe, in which several CpG islands become hypermethylated very early in the progression of prostate cancer, probably between the high-grade PIN lesion and the organ-confined, well-differentiated primary prostate cancer. The epigenetic DNA methylation changes that occurred during this catastrophe are maintained throughout the disease progression with few exceptions. Among these exceptions are an accumulation of cells that are hypermethylated at the EDNRB and the PTGS2 CpG islands, because a high degree of hypermethylation at these loci correlate directly with increasing disease severity and increased risk of recurrence, respectively. We postulate that when a cell with the requisite genetic and epigenetic derangements has proliferated clonally in the primary prostate cancer to form a significant subset of cells in the primary prostate cancer lesion, some of these proliferating cells can invade and metastasize to distant sites. The frequency to which they metastasize to a particular organ system is likely to be contingent on a probability density function that is determined by the epigenetic and genetic derangements that occurred fairly early in the disease progression. This would explain the lack of site-specificity in the methylation pattern among the metastatic prostate cancers. One important practical consequence of this early epigenetic DNA methylation catastrophe is that we could identify several markers that can be used for the sensitive and specific diagnosis of prostate cancer. Therefore, the data in this study allow us to generate several hypotheses regarding the epigenetic events that occur during prostate cancer disease progression and also provide clinically useful markers to diagnose early prostate cancer lesions and assess disease severity and prognosis.

ACKNOWLEDGMENTS

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REFERENCES

DNA METHYLATION PATTERNS OF PROSTATE CANCER

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Srinivasan Yegnasubramanian, Jeanne Kowalski, Mark L. Gonzalgo, et al.


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