Identification of Differentially Methylated Genes in Normal Prostate Tissues from African American and Caucasian Men


Abstract

Purpose: Aberrant DNA methylation changes are common somatic alterations in prostate carcinogenesis. We examined the methylation status of six genes in prostate tissue specimens from African American (AA) and Caucasian (Cau) males.

Experimental Design: We used pyrosequencing to quantitatively measure the methylation status of GSTP1, AR, RARβ2, SPARC, TIMP3, and NKX2-5. Real-time PCR was used to determine gene expression, and gene reactivation was analyzed by 5-aza-2′-deoxycytidine and/or trichostatin A treatment.

Results: Statistical analysis showed significantly higher methylation in the prostate cancer tissue samples in comparison with matched normal samples for GSTP1 (P = 0.0001 for AA; P = 0.0008 for Cau), RARβ2 (P < 0.001 for AA and Cau), SPARC (P < 0.0001 for AA and Cau), TIMP3 (P < 0.0001 for AA and Cau), and NKX2-5 (P < 0.0001 for AA; P = 0.003 for Cau). Overall, we observed significant differences (P < 0.05) in the methylation level for all genes, except GSTP1, in the AA samples in comparison with the Cau samples. Furthermore, regression analysis revealed significantly higher methylation for NKX2-5 (P = 0.008) and TIMP3 (P = 0.039) in normal prostate tissue samples from AA in comparison with Cau, and a statistically significant association of methylation with age for NKX2-5 (P = 0.03) after adjusting for race.

Conclusion: Our findings show higher methylation of several genes in prostate tissue samples from AA in comparison with Cau and may potentially contribute to the racial differences that are observed in prostate cancer pathogenesis. Clin Cancer Res; 16(14); 3539–47. ©2010 AACR.

Prostate cancer (PCa) is a common malignancy and a leading cause of cancer death among men in the United States. PCa cells are known to carry a variety of genetic defects, including gene mutations, deletions, translocations, and amplifications, which endow the cells with new capabilities for dysregulated proliferation, inappropriate survival, tissue invasion and destruction, immune system evasion, and metastasis (1). More recently, it has become apparent that PCa cells also carry epigenetic defects, including changes in cytosine methylation patterns and chromatin structure and/or organization, which are equivalent to genetic changes effecting and maintaining neoplastic and malignant phenotypes (2). For human PCa, abundant evidence has accumulated to suggest that somatic epigenetic alterations may appear earlier during cancer development, as well as more commonly and consistently, than genetic changes (3). Furthermore, epigenetic changes tend to arise in association with age (4) and/or in response to chronic or recurrent inflammation leading to cell and tissue damage (5).

Epigenetic inactivation of genes in cancer cells is largely based on transcriptional silencing by aberrant CpG methylation of CpG-rich promoter regions (6, 7). Aberrant promoter methylation of GSTP1, encoding the π-class glutathione S-transferase (GST), an enzyme capable of detoxifying electrophilic and oxidant carcinogens, remains the most common somatic genome abnormality (>90% of cases) reported thus far for PCa, appearing earlier and more frequently than other gene defects that arise during PCa development (8). Since the recognition that the GSTP1 CpG was frequently hypermethylated in PCa, more than 40 genes have been reported to be targets of DNA hypermethylation–associated epigenetic gene silencing in PCa cells (9). Despite the increasing number of aberrantly methylated genes in PCa, only a few genes show promise as PCa biomarkers for early diagnosis and disease risk assessment.

In this study, we sought to investigate DNA methylation changes in prostate tissue samples from African American...
(AA) men in comparison with Caucasian (Cau) men to identify methylated genes that could be potentially useful as "ethnic-sensitive" biomarkers for the detection of PCa.

**Materials and Methods**

**Cell lines and tissue samples**

The human PCa cell lines PC3, DU145, and LNCaP were obtained from the American Type Culture Collection. The immortalized normal prostate epithelial cell line pNT1A was obtained from the European Collection of Cell Culture. All cell lines were cultured in the recommended medium in the presence of 10% fetal bovine serum (Invitrogen) unless otherwise stated. We collected matched pairs of normal and PCa tissue samples from both AA and Cau male patients with PCa (Table 1A; age range, 52-75 years) at the time of radical prostatectomy. The prostate tissue samples were obtained with informed consent and maintained by the Baylor Specialized Program of Research Excellence in the PCa tissue bank. The pathologic status was confirmed before processing, and we chose tumor samples that had >70% cancer, and in some cases we needed to microdissect to get that level of enrichment. Matched normal samples were confirmed free of tumor by histologic examination. In addition, normal prostate tissue samples were obtained from the George Washington University Hospital pathology tissue bank. The samples were collected by transrectal ultrasound needle biopsy from men with an elevated serum PSA level of ≥4 ng/mL and an abnormality detected by digital rectal examination. Needle biopsies from AA and Cau males (age range, 47-85 years) were determined to be noncancerous. The mean age was 62.7 ± 7.2 and 62.4 ± 8.8 years (P = 0.89) for AA and Cau, respectively. Fifteen of 18 and 12 of 13 biopsies (P = 0.53) were from the left mid-zone of the prostate of AA and Cau, respectively. The remaining samples were either from the left apex or the right mid-zone (Table 1B).

**Bisulfite modification, PCR, and pyrosequencing analysis**

High molecular weight genomic DNA extracted from prostate tissues and WBC was modified using sodium bisulfite treatment (10). Briefly, genomic DNA (2 μg) was denatured in 0.3 mol/L NaOH at 37°C for 15 minutes; sodium bisulfite and hydroquinone were added to final concentrations of 3.1 mol/L and 0.5 mmol/L, respectively. The reaction was incubated at 50°C for 16 hours and de-salted using Wizard DNA purification resin (Promega) according to the instruction of the manufacturer. Bisulfite modification was completed by DNA desulfonation in 0.3 mol/L NaOH at 37°C for 15 minutes. Modified DNA was precipitated with ethanol, washed in 70% ethanol, dried, and dissolved in 50 μL of TE buffer. The PCR primers were designed to assay the methylation status of CpGs within 0.5 kb from the transcription start site (primer sequences are shown in Table 2). The CpG islands interrogated are shown in Supplementary Data. Either one-step or two-step PCR reactions were carried out using 2 μL of bisulfite-converted genomic DNA and either one or two sets of different bisulfite PCR primers in a standard PCR reaction mix. One of the primers (reverse primer) in the final PCR reaction was biotinylated to create a ssDNA template for the pyrosequencing reaction. Where indicated, we used a previously described amplification protocol (11) based on the universal primer approach. Briefly, the biotinylated reverse primer was substituted with a 5’ tailed unlabeled reverse primer and a biotinylated universal primer at a ratio of 1:9 in the PCR reaction. The integrity of the PCR product was verified on 1.5% agarose gels with ethidium bromide staining. The PCR product was immobilized on streptavidin-Sepharose beads (Amersham), washed, and denatured, and the biotinylated strands were released into annealing buffer containing the sequencing primer. Pyrosequencing was done using the PSQ HS96 Gold SNP Reagents on a PSQ 96HS machine (Qiagen). PCR primer sequences and sequencing primer sequences are listed in Table 2. Bisulfite-converted DNA from blood of normal volunteers and blank reactions, with water substituted for DNA, served as negative control and bisulfite-converted SstI methylase–treated blood DNA served as a positive control. Each bisulfite PCR and pyrosequencing reaction was done at least twice.

**RNA extraction, cDNA preparation, and reverse transcription-PCR**

Total RNA extracted from cells and prostate tissues using TRIzol reagent (Invitrogen) was used in cDNA synthesis using the Invitrogen SuperScript first-strand synthesis
system for reverse transcription-PCR (RT-PCR) and according to the manufacturer’s protocol (primers used are listed in Table 2). Either SYBR Green or TaqMan assay (designed for NKX2-5) was used to quantitatively measure mRNA expression. Real-time PCR was carried out in a Bio-Rad iCycler real-time thermal cycler as described previously (12) and incorporating optimized PCR reaction conditions for each gene. First, a DNA standard was generated for each gene using PCR amplification of the coding sequence. The PCR product concentration was determined to calculate the copy number. A dilution series of each gene (10^8-10^2 copies) was used as a cDNA standard for the real-time PCR. The threshold cycle (Ct) in the PCR cycle at which fluorescence exceeds background was then converted to copy number based on a cDNA standard curve generated. Each experiment was carried out in duplicate.

Treatment with 5-aza-2′-deoxycytidine and/or trichostatin A

pNT1A, DU145, PC3, and LNCaP cells were seeded at 5×10^5/100-mm tissue culture dish. After 24 hours of incubation, the culture medium was changed to medium containing 5-aza-2′-deoxycytidine (5′-aza-dC; 5 μmol/L) for 96 hours and/or trichostatin A (TSA; 250 nmol/L)
for an additional 24 hours. Total RNA extracted from cells and tissues using TRIzol reagent (Invitrogen) was first used in first-strand DNA (cDNA) synthesis using the Invitrogen SuperScript first-strand synthesis system and then used in real-time quantitative PCR as previously described (13). Mock-treated cells were cultured similarly.

Data analysis
The methylation index at each gene promoter and for each sample was calculated as the average value of mC/(mC + C) for all examined CpG sites in the gene and expressed as the percentage of methylation. Statistical significance was judged by the appropriate Mann-Whitney \( t \) test, Student \( t \) test, Pearson correlation, and simple and multiple regression methods used to compare the categorical variables of methylation changes in cancer by race and cancer \( \times \) race interactions, or methylation changes in normal prostate tissues by race, age, and race \( \times \) age interaction (summary in Supplementary Table). We used a cutoff corresponding to average methylation in normal

<table>
<thead>
<tr>
<th>Table 2. Primer sequences used in the pyrosequencing analysis</th>
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<tbody>
<tr>
<td><strong>Pyrosequencing</strong></td>
</tr>
<tr>
<td>GSTP1 (1st step)</td>
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<td>GSTP1 (2nd step)</td>
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<tr>
<td>AR (1st step)</td>
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<tr>
<td>AR (2nd step)</td>
</tr>
<tr>
<td>RAR( \beta ) 2 (1st step)</td>
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<tr>
<td>RAR( \beta ) 2 (2nd step)</td>
</tr>
<tr>
<td>SPARC (1st step)</td>
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<tr>
<td>SPARC (2nd step)</td>
</tr>
<tr>
<td>TIMP3 (1st step)</td>
</tr>
<tr>
<td>TIMP3 (2nd step)</td>
</tr>
<tr>
<td>NKX2-5 (1st step)</td>
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<tr>
<td>NKX2-5 (2nd step)</td>
</tr>
</tbody>
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RT-PCR

<table>
<thead>
<tr>
<th><strong>Forward</strong></th>
<th><strong>Reverse</strong></th>
<th><strong>PCR size (bp)</strong></th>
</tr>
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<tbody>
<tr>
<td>SPARC (STD)</td>
<td>TGTGCGAGCTGGATGAGAAC</td>
<td>GATGTAATTAGGTTGTA TGGTACCTGGTGAGCAGCT</td>
</tr>
<tr>
<td>SPARC</td>
<td>TGTGCGAGCTGGATGAGAAC</td>
<td>GTGGCAGGAGATCTGAGAGCAGCAG</td>
</tr>
<tr>
<td>TIMP3 (STD)</td>
<td>CCGGAACTGGGGGGAAGGGAGG</td>
<td>AGCGGAGGAAAGGGAGGAGG</td>
</tr>
<tr>
<td>TIMP3</td>
<td>GGGGAAAGAGCTGGTCAGGGAAGG</td>
<td>AGCGGAGGAGAGCAGGACGAG</td>
</tr>
<tr>
<td>NKX2-5 (STD)</td>
<td>GCCGCAGGCTTCAGCAAGAG</td>
<td>GCCGCAGGCTTCAGCAAGAG</td>
</tr>
<tr>
<td>NKX2-5</td>
<td>CAGTCTACCTGAGGTCAAGG</td>
<td>GGGCAGGCTTCAGCAAGAG</td>
</tr>
</tbody>
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NOTE: Primers used in the RT-PCR analysis are also shown, where STD represents the primers used to generate cDNA standards. U represents universal primer sequence—GGGACACCGCTGATCTTTA.
tissues + 2 SD to call a cancer as methylation positive (above the cutoff). The sensitivity, specificity, and accuracy of each individual or two combined candidate biomarkers were calculated. Sensitivity was defined as the number of true-positive (methylated in cancer) cases divided by the number of true-positive plus false-negative (not methylated in cancer) cases, and specificity was defined as the number of true-negative (not methylated in normal tissue) cases divided by the number of true-negative plus false-positive (methylated in normal tissue) cases. The accuracy of the test is measured by the area under the receiver operating characteristic (ROC) curve. Data analysis was done using either Prism 4 software (GraphPad Software, Inc.) or SPSS for Windows (version 13.0, SPSS). Significance was set at $P < 0.05$.

**Results**

We investigated the DNA methylation status for a panel of genes in matched normal and PCa tissue samples from radical prostatectomy specimens obtained from AA and Cau cancer patients who had undergone radical prostatectomy. Y axis, percentage of methylated cytosines in the samples as obtained from pyrosequencing. X axis, normal and PCa tissues obtained from AA and Cau. *, $P < 0.05$ (Mann-Whitney U test). A, quantitative methylation analysis for GSTP1, AR, and RARB2. B, quantitative methylation analysis for SPARC, TIMP3, and NKX2-5.
these genes in 40 DNA samples from matched normal and PCA tissue samples obtained from AA males and between 12 and 40 DNA samples from matched normal and PCA tissue samples obtained from Cau males who underwent radical prostatectomy for PCa (age range, 52–75 years). Table 1 shows a summary of patient clinical data for the two groups, with Cau samples showing a nonsignificant slightly higher Gleason score. For each gene studied, the percentage of methylation at a specific promoter was compared between the matched normal and PCA tissue samples (Fig. 1). There was considerable variation in the percentage of CpG island methylation in the individual patient samples studied, presumably reflecting both random variability in tissue composition and variable methylation level per cell. The variable range of methylation could also reflect differences in genetic susceptibility to methylation, lifestyle, or other environmental exposures (including diet) and the random nature of the methylation event. The results showed significant hypermethylation for GSTP1 ($P < 0.0001$ for AA; $P = 0.0008$ for Cau), RARβ2 ($P < 0.001$ for AA and Cau), TIMP3 ($P < 0.0001$ for AA and Cau), and NKX2-5 ($P < 0.0001$ for AA; $P = 0.003$ for Cau) in the PCA tissue samples when compared with the matched normal tissue samples. The AR gene showed low prevalence of methylation in the prostate tissue samples from both AA and Cau males. Regression analysis to examine whether methylation frequency in the normal and PCA tissue samples differed by race showed significantly higher methylation prevalence for AR ($P = 0.006$), RARβ2 ($P < 0.0001$), SPARC ($P = 0.02$), TIMP3 ($P < 0.0001$), and NKX2-5 ($P = 0.002$) for AA in comparison with Cau samples, whereas PCA × race interaction showed significance for GSTP1 ($P = 0.0069$; Supplementary Table). In addition, ROC analysis (Fig. 2) was done to compare the adjacent normal prostate and matched cancer tissues in AA, Cau, and both (Cau + AA) samples. The areas under the ROC curves were as follows: for GSTP1, $0.969$ (Cau) and $0.811$ (AA); for AR, $0.59$ (Cau) and $0.531$ (AA); for RARβ2, $0.969$ (Cau) and $0.922$ (AA); for SPARC, $0.75$ (Cau) and $0.921$ (AA); for TIMP3, $0.875$ (Cau) and $0.668$ (AA); and for NKX2-5, $0.984$ (Cau) and $0.857$ (AA). For the analysis in the Cau + AA samples, the areas under the ROC curves were $0.844$ for GSTP1, $0.536$ for AR, $0.925$ for RARβ2, $0.878$ for SPARC, $0.705$ for TIMP3, and $0.884$ for NKX2-5. The strength of the ROC curves indicates differences in the sensitivity of DNA methylated genes in the AA and Cau samples. Overall, the DNA methylation of GSTP1, RARβ2, SPARC, and NKX2-5 genes indicates their potential as predictive genes for PCa detection in both AA and Cau male samples.

We next analyzed the methylation pattern in tissue punches of normal prostate collected from AA (18 samples; mean age, $62.7 ± 7.2$ years) and Cau males (13 samples; mean age, $62.4 ± 8.8$ years; $P = 0.89$; Table 1B). For each gene studied, the percentage DNA methylation at a specific promoter region was expressed as a function of age for both the AA and Cau male samples (Fig. 3). We observed methylation of the GSTP1, RARβ2, and TIMP3 genes but this did not significantly correlate with age in both AA and Cau. The SPARC and AR genes were entirely unmethylated in the Cau male samples, although we observed some methylation in the AA samples. Regression analysis showed significantly higher methylation for TIMP3 gene ($P = 0.039$) and NKX2-5 gene ($P = 0.008$) in the AA samples when compared with the Cau samples.
In addition, we observed a significant association between age and methylation level for NKX2-5 gene ($P = 0.03$) in the AA samples when compared with the Cau samples. Furthermore, we observed a modest association of NKX2-5 methylation ($P = 0.09$; Supplementary Table) for a race by age interaction. These results support the observation we made above of higher methylation of several genes in prostate tissue samples from AA in comparison with Cau. In addition, our data suggest that, for some genes, higher methylation differences maybe associated with aging.

To investigate if methylation is associated with the silencing of SPARC, TIMP3, and NKX2-5 genes in PCa tissues, we performed expression analysis by quantitative RT-PCR. The results presented in Fig. 4 showed a significantly higher level of gene expression for SPARC ($P = 0.0213$) and TIMP3 ($P < 0.0001$), but not NKX2-5 ($P = 0.12$), in the matched normal prostate tissues when compared with the PCA tissues. Our data suggest an inverse association between DNA methylation and gene expression for SPARC, TIMP3, and NKX2-5. We observed that in cancer samples that showed higher methylation,
this was associated with low levels of gene expression, whereas the normal prostate samples had lower methylation and higher gene expression level to indicate that methylation leads to some loss of gene expression.

To verify that the expression of the hypermethylated genes can be restored, prostate cell lines were treated with the demethylating agent 5′-aza-dC, the histone deacetylase inhibitor TSA, or the combination of the two drugs, and gene expression was analyzed by quantitative RT-PCR (Fig. 5). The cell lines chosen for the analysis had high methylation levels for the genes tested and all had very low levels of expression at baseline. Results indicate at least a 2-fold increase in expression in response to 5′-aza-dC treatment for all genes analyzed in at least one cancer cell line, except for TIMP3 gene, for which we did not detect any significant effect on expression level. The data support the observation we made above that silencing is related to DNA methylation. Treatment with the histone deacetylase inhibitor TSA alone did not restore gene expression for SPARC. On the other hand, TIMP3 and NKX2-5 showed increased expression after treatment with either TSA alone or the combination of 5′-aza-dC and TSA in at least one PCA cell line. The data show that both methylation and histone deacetylation seem to play a role in silencing the expression of TIMP3 and NKX2-5, although methylation seems to play a dominant role in NKX2-5 gene silencing.

**Discussion**

Ethnic differences in PCa incidence and mortality are well documented. The incidence and mortality for PCa is about 2-fold higher in AA than in Cau, with AA men experiencing among the highest rates worldwide (17). This disparity in PCa is believed to be a complex combination of environment and socioeconomic factors and genetics (for a recent review, see ref. 18). Several genetic variations in the human genome, particularly allelic variation in genes involved in pathways relevant to PCA biology, have been proposed as a genetic cause or contributor for the increased PCa risk in AA (19).

We have previously shown aberrant hypermethylation as a function of age in normal prostate tissues, which may precede and predispose to full-blown malignancy (4). Therefore, it may be possible to establish a quantitative cutoff point for the amount of methylation of some genes that would indicate the presence of cancer. In addition, the methylation prevalence may differ between ethnic groups such that methylated genes could also serve as ethnic-sensitive biomarkers for PCa detection. In the present study, we were interested in comparing the DNA methylation patterns in prostate tissue samples from AA and Cau. Previous work carried out by Enokida et al. (20) showed that GSTP1 hypermethylation was significantly higher in PCA samples from AA in comparison with Cau and Asians. Other studies did not find significant difference in GSTP1 methylation frequencies in PCA among AA and Cau (21, 22) but found higher frequency of CD44 hypermethylation in AA than in Cau (21). These studies had strictly focused on PCA tissues and benign prostatic hyperplasia (BPH) samples. However, our recent observations show that DNA methylation starts in normal prostate tissues as a function of age (4), and therefore, it is also important to analyze gene methylation in normal prostate samples from both AA and Cau. We therefore evaluated differences in DNA methylation of six genes, namely, GSTP1, AR, RAR, SPARC, TIMP3, and NKX2-5, which may play important regulatory roles in prostate disease etiology and/or progression. To our knowledge, our study is the first to analyze the methylation status of these genes in normal as well as matched pairs of normal and PCA tissue samples from AA and Cau males. Overall, we observed significant methylation prevalence in the PCA tissue samples from AA in comparison with Cau. The Cau cancer samples used in this study showed slightly higher Gleason score and similar pathologic staging when compared with the AA samples. Thus, the higher prevalence of methylation seen in the AA cancer samples is not simply reflective of differences in disease aggressiveness or stage between the two groups. In addition, regression analysis revealed significantly higher methylation for NKX2-5.
and TIMP3 genes in the normal prostate tissue samples from AA in comparison with Cau. Of the six genes that we analyzed in the normal prostate tissue samples, methylation of NKX2-5 also showed a significant association with age in AA in comparison with Cau. Our data suggest that not only is the methylation of NKX2-5 suitable as a marker for PCa detection, but it may also have increased sensitivity for detecting PCa in AA. We have analyzed the relationship between DNA methylation changes and PCa risk but did not detect any significant association between DNA methylation changes and PCa risk by race. Although the sample size used in our studies is not large, the ROC analysis shows the differential predictive potential of DNA methylation of GSTP1, RARβ2, SPARC, TIMP3, and NKX2-5 genes for PCa detection in AA and Cau samples. Thus, a larger PCa population size can confirm our observations and determine if the methylation status of these genes can provide a reliable and/or perhaps ethnic-sensitive index for the detection of PCa.

In summary, our panel of genes show significant differences in the prevalence of methylation in prostate tissue samples from AA and Cau. These genes play key regulatory roles in the pathways of prostate. Therefore, ethnic differences in the methylation pattern of these genes may contribute to the disparity associated with PCa. The genetic mechanism(s) underlying the differing prevalence of methylation in these two groups is an area of active investigation in our laboratory.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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