

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

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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/organization, which are equivalent to genetic changes effecting and maintaining neoplas-

tic 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

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Translational Relevance

Prostate cancer (PCa) cells carry a myriad of genomic defects including genetic and epigenetic alterations. Epigenetic changes such as DNA methylation appear early and are more common and consistent than genetic defects. This makes DNA methylation changes very attractive as biomarkers for PCa detection. Furthermore, because epigenetic DNA methylation changes are reversible, epigenetic drugs are being explored for reversing somatic epigenetic defects. Identification of novel DNA methylation genes that serve as biomarkers would improve future clinical practice in PCa disease detection. In addition, the prevalence of DNA methylation changes in different ethnic groups could potentially lead to designing "ethnic-sensitive" biomarkers and epigenetic drugs for PCa. Our study describes the methylation status of regulatory genes in prostate tissues from African Americans and Caucasians and discusses how the prevalence of methylation changes can help us to understand PCa disparity and lead to the design of ethnic-sensitive biomarkers for PCa detection.

(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 noncaerous. 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 μ g) 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 desalted 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 μ L 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 μ L 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 SssI 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

Table 1. Patient clinical and demographic characteristics by race

A								
	AA				Cau			
T2	17 (44)				28 (43)			
T3a	18 (46)				31 (58)			
T3b	4 (10)				6 (9)			
N1	0 (0)				2 (3)			
Gleason	6.4				6.8			

B								
No.	AA				Cau			
	Core	PCa GS	PSA (ng/mL)	Age (y)	Core	PCa GS	PSA (ng/mL)	Age (y)
1	RM	NEG (GS = 0)	0.5	54	LM	NEG (GS = 0)	1.5	65
2	LM	NEG (GS = 0)	0.2	63	LA	NEG (GS = 0)		58
3	LA	NEG (GS = 0)	6.3	60	LM	NEG (GS = 0)	4.1	65
4	LM	NEG (GS = 0)	4.9	65	LM	NEG (GS = 0)	6.2	59
5	LA	NEG (GS = 0)	3.6	68	LM	NEG (GS = 0)	3.8	60
6	LM	NEG (GS = 0)	1.5	71	LM	NEG (GS = 0)	4.1	59
7	LM	NEG (GS = 0)	5.7	61	LM	NEG (GS = 0)	5.1	76
8	LM	NEG (GS = 0)	4.8	47	LM	NEG (GS = 0)	4.9	64
9	LM	NEG (GS = 0)	0.5	67	LM	NEG (GS = 0)	8.7	85
10	LM	NEG (GS = 0)	6.2	65	LM	NEG (GS = 0)	9.0	56
11	LM	NEG (GS = 0)	1.4	65	LM	NEG (GS = 0)	4.9	72
12	LM	NEG (GS = 0)	4.9	70	LM	NEG (GS = 0)	2.1	59
13	LM	NEG (GS = 0)	4.7	59	LM	NEG (GS = 0)	1.7	55
14	LM	NEG (GS = 0)	7.7	54				
15	LM	NEG (GS = 0)	9.3	61				
16	LM	NEG (GS = 0)	14.1	55				
17	LM	NEG (GS = 0)	5.0	76				
18	LM	NEG (GS = 0)	5.0	68				

NOTE: A, matched pairs of normal and PCa tissue samples obtained from AA and Cau male patients with PCa at the time of radical prostatectomy. The staging (T) and Gleason score are shown. Data are number of cases with percentage in parentheses. N1 is positive pelvic lymph nodes (age range, 52-75 y). B, needle biopsies of normal prostate tissue samples from AA and Cau male patients (age range, 47-85 y) with elevated serum PSA level and an abnormality detected by digital rectal examination. The mean age was 62.7 ± 7.2 and 62.4 ± 8.8 y ($P = 0.89$) for the AA and Cau groups, 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 samples, respectively. The remaining samples were either from the left apex or the right mid-zone. Gleason score of 0 indicates noncancerous.

Abbreviations: GS, Gleason score; LA, left apex; LM, left mid-zone; RM, right mid-zone; NEG, Negative.

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 (C_t) 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 μ mol/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/

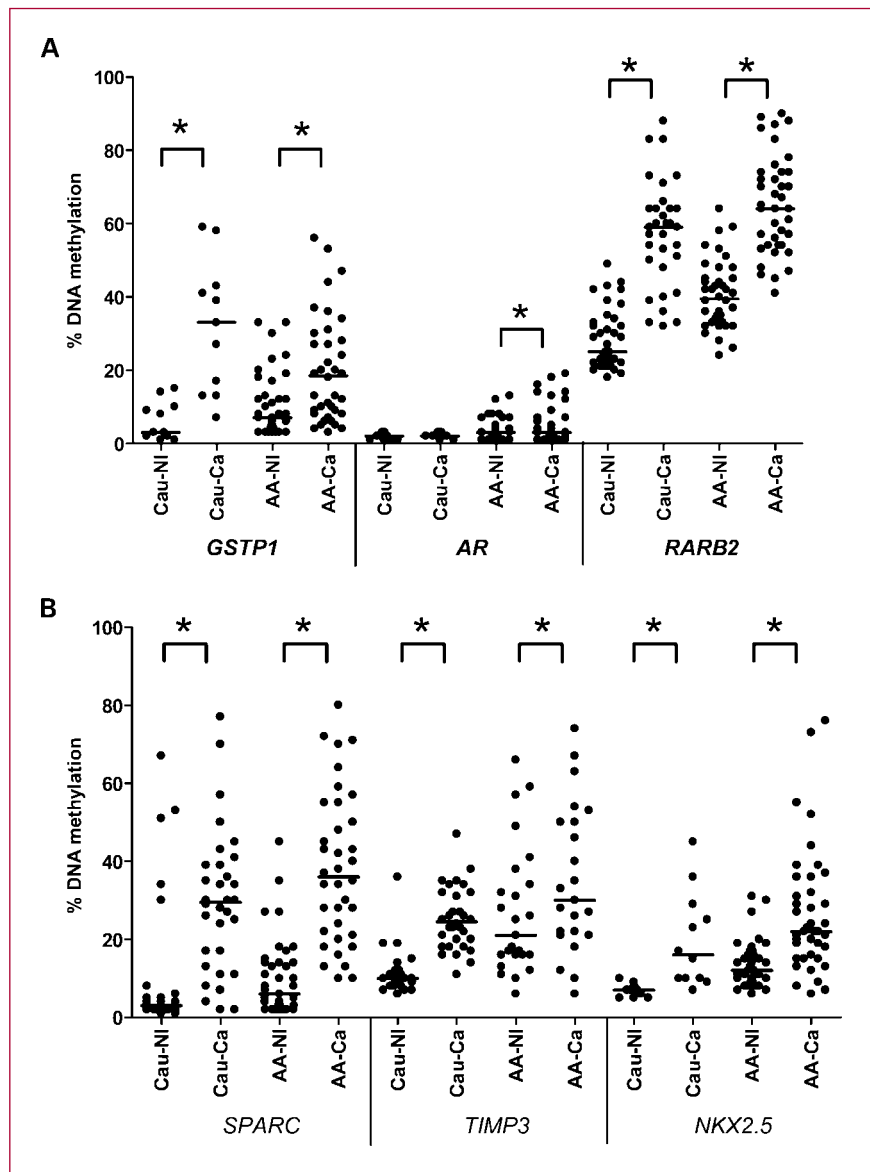
(mC + C) for all examined CpG sites in the gene and expressed as the percentage of methylation. Statistical significance was judged by the appropriate Mann-Whitney *t* test, Student *t* test, Pearson correlation, and simple and multiple regression methods used to compare the categorical variables of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

Table 2. Primer sequences used in the pyrosequencing analysis

	Pyrosequencing		
	Sense	Antisense	Sequencing primer
<i>GSTP1</i> (1st step)	AAGGAGGTTAGGG GTAAGAGTTATA	CCAAAACCTCCCCAATAC	GGGGGAGGATGTTAAG
<i>GSTP1</i> (2nd step)	GAGTTAGGGGGAGGATGT	5'-Biotin-CCAAAACCTCCCCAATAC	
<i>AR</i> (1st step)	TAGGAAGTAGGGG TTTTTLAGGGTTAG	ACCCAACCCACCTCCTTACCT and U-ACCTCCCCTTTCTTTCTCC	TTGTTTTTTTAAA GTTATTAGGTA
<i>AR</i> (2nd step)	GTAGGGGTTTTTTA GGGTTAGAGTTAG	5'-Biotin-U	
<i>RARβ2</i> (1st step)	AGTTGGGTATT TGAAGGTTA	TACCCAAACAACCCCTACTC and U-CCCAAACAACCCCTACTC	GGGACACCGC TGATCGTTA
<i>RARβ2</i> (2nd step)	AAGTAGTAGGAAGT GAGTTGTTAGA	5'-Biotin-U	
<i>SPARC</i> (1st step)	GGTGTAAATTATAGAAG GGAAAGGTTGGG	U-CCTATTACCTATCTCTAAAC CCCTCCACATT	
<i>SPARC</i> (2nd step)	AAGGTTGGGAGGGGGTTA TATATATTTAG	5'-Biotin-U	TTAGGGTAGTT TGAAGGAT
<i>TIMP3</i> (1st step)	GGTGGGTGGGTG TTAGTTGG	U-CAAACCCTCTACCCCTTCTC	
<i>TIMP3</i> (2nd step)	GGTTTTGGTTGGGT TAGAGATA	5'-Biotin-U	GGTTAGAGATATT TAGTGGTTTA
<i>NKX2-5</i> (1st step)	GAGAGTAGGGTTG GGGAATATG	AACCCCTAACCAATAACAAACT and U-CAATAACAACTAAATCC CCCTCCTCTA	GGTAGTTTTTT TGATGGTG
<i>NKX2-5</i> (2nd step)	TAAGGTTTTGGTAGTT TTTTTGTATGG	5'-Biotin-U	
RT-PCR			
	Forward	Reverse	PCR size (bp)
<i>SPARC</i> (STD)	TGTGCGAGCTGGATGAGAAC	GATGTA CTGTG CATTGTCCAGGTC	599
<i>SPARC</i>	TGTGCGAGCTGGATGAGAAC	GTGGCAGGAAGAGTCGAAGG	128
<i>TIMP3</i> (STD)	CCAAGGTGGTGGGAAGAAG	AGCGGGAAGGGAGGGAAG	547
<i>TIMP3</i>	GGGGAAGAAGCTGGTAAAGGAG	AGCAGGTA CTGGTA CTGTTGAC	171
<i>NKX2-5</i> (STD)	GCCGCCTTCAAGCCAGAG	GCCCCACCAGCTCCAGAG	474
<i>NKX2-5</i>	CACGTCCACGCAGGTCAAG (TaqMan probe: CTGGTCCTG CCGCTGCCGCTTG)	GCCCCACCAGCTCCAGAG	88

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—GGGACACCGCTGATCGTTA.

Fig. 1. Quantitative DNA methylation analysis in human prostate tissues. The percent DNA methylation levels of promoter CpG islands were analyzed in bisulfite-modified genomic DNA extracted from matched pairs of normal (NI) and PCa (Ca) tissue samples 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 t test). A, quantitative methylation analysis for *GSTP1*, *AR*, and *RARB2*. B, quantitative methylation analysis for *SPARC*, *TIMP3*, and *NKX2-5*.



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 males with PCa to determine the prevalence of methylation changes in the two groups. We analyzed a total of six genes, including *GSTP1*, *AR*, and *RARB2*, which we and others have previously shown to be hypermethylated in PCa tissue samples from Cau males (4, 14, 15); in addition, we also analyzed *SPARC*, *TIMP3*, and *NKX2-5*, which we have identified as being hypermethylated in the gene promoter using methylated CpG island amplification coupled with representational difference analysis in PCa cell lines (16). We analyzed the methylation status of

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), *RAR β 2* ($P < 0.001$ 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) 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* ($P = 0.006$), *RAR β 2* ($P < 0.0001$), *SPARC* ($P = 0.02$), *TIMP3* ($P < 0.0001$), and *NKX2-5* ($P = 0.002$) for AA in comparison with Cau samples, whereas PCa \times 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 *RAR β 2*, 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 *RAR β 2*, 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*, *RAR β 2*, *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*, *RAR β 2*, 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.

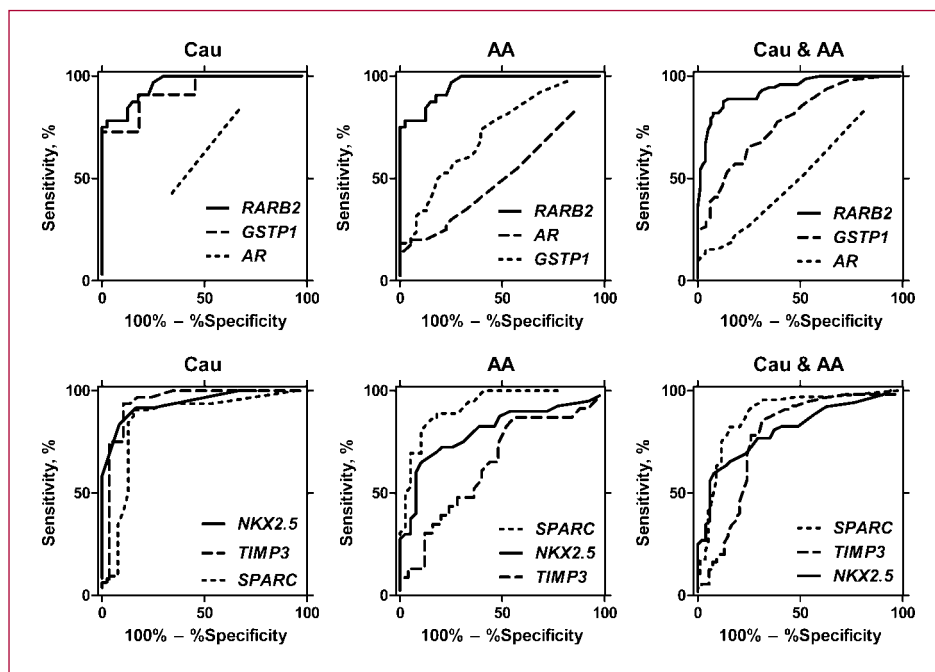


Fig. 2. ROC curves for methylation analysis in PCa tissue samples from AA, Cau, and both (AA + Cau). The areas under the ROC curves in Cau samples are 0.969 for *GSTP1*, 0.59 for *AR*, 0.969 for *RAR β 2*, 0.75 for *SPARC*, 0.875 for *TIMP3*, and 0.984 for *NKX2-5*. The areas under the ROC curves in AA samples are 0.811 for *GSTP1*, 0.531 for *AR*, 0.922 for *RAR β 2*, 0.921 for *SPARC*, 0.668 for *TIMP3*, and 0.857 for *NKX2-5*. The areas under the ROC curves in both Cau + AA samples are 0.844 for *GSTP1*, 0.536 for *AR*, 0.925 for *RAR β 2*, 0.878 for *SPARC*, 0.705 for *TIMP3*, and 0.884 for *NKX2-5*.

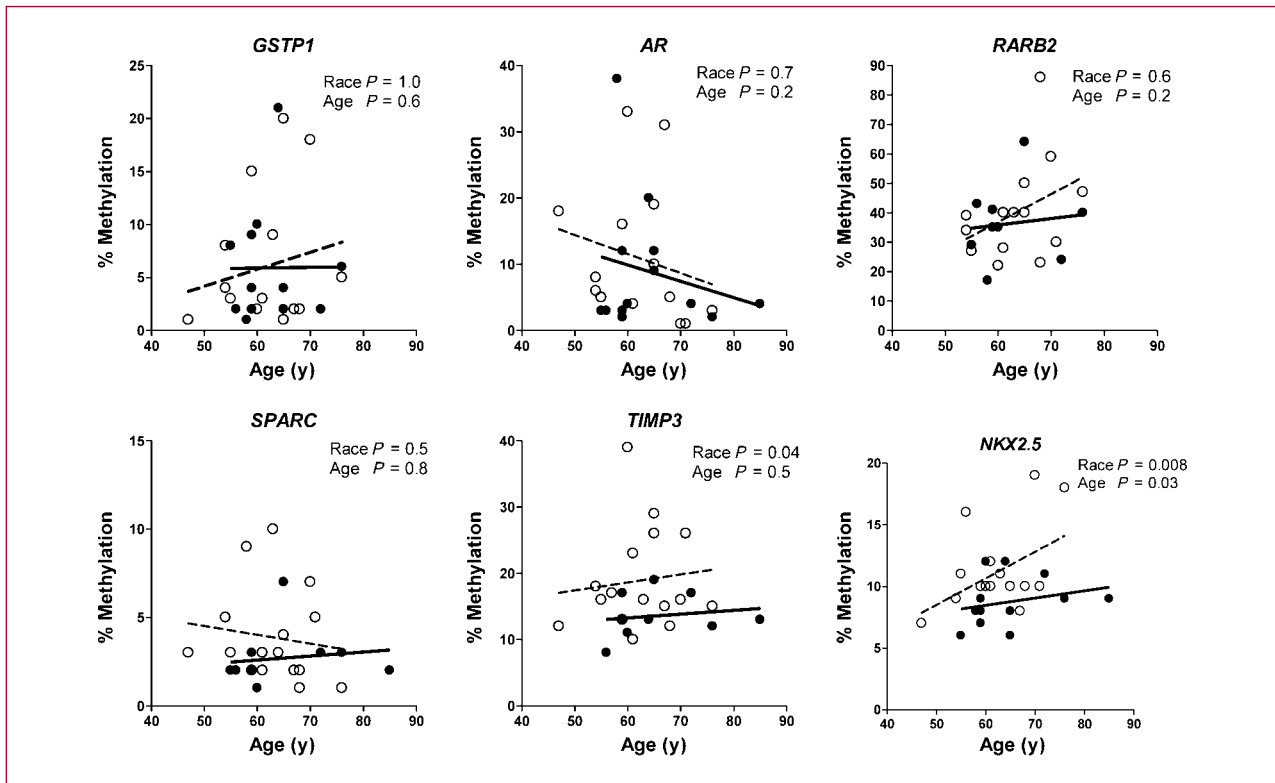
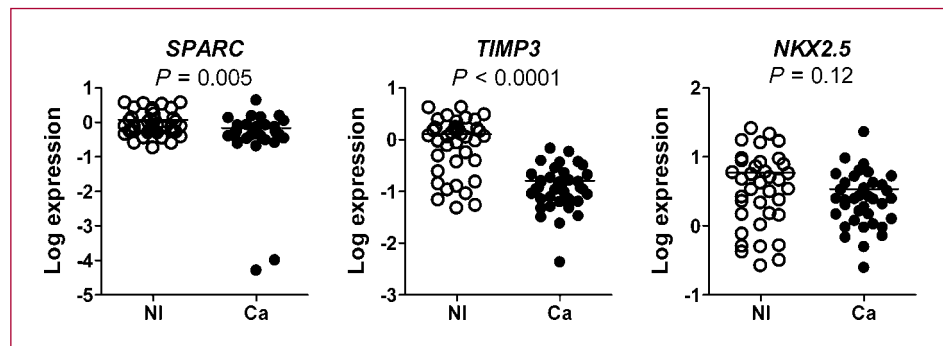


Fig. 3. Age-related DNA methylated gene profiles for AA (---) or Cau (—) men. CpG islands for *GSTP1*, *AR*, *RARβ2*, *SPARC*, *TIMP3*, and *NKX2-5* were analyzed in bisulfite-modified genomic DNA extracted from fresh-frozen tissue punches of normal prostate obtained from both AA and Cau. Eighteen samples from AA and 14 samples from Cau (age range, 47–85 y) were used. Regression analysis of the association of DNA methylation changes by race and age. Y axis, percentage of methylated cytosines in the samples as obtained from pyrosequencing. Each CpG island has a different scale range. X axis, age in years.

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,

Fig. 4. Gene expression in prostate tissue samples. The relative mRNA transcript expression levels of *SPARC*, *TIMP3*, and *NKX2-5* were analyzed in 32 matched pairs of normal and PCa tissue samples by RT-PCR and expressed relative to β -actin to correct for variation in the amount of reverse-transcribed RNA. Relative expression (shown as log expression on the Y axis) was determined using gene copy number calculated from a standard curve.



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 β 2*, *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*

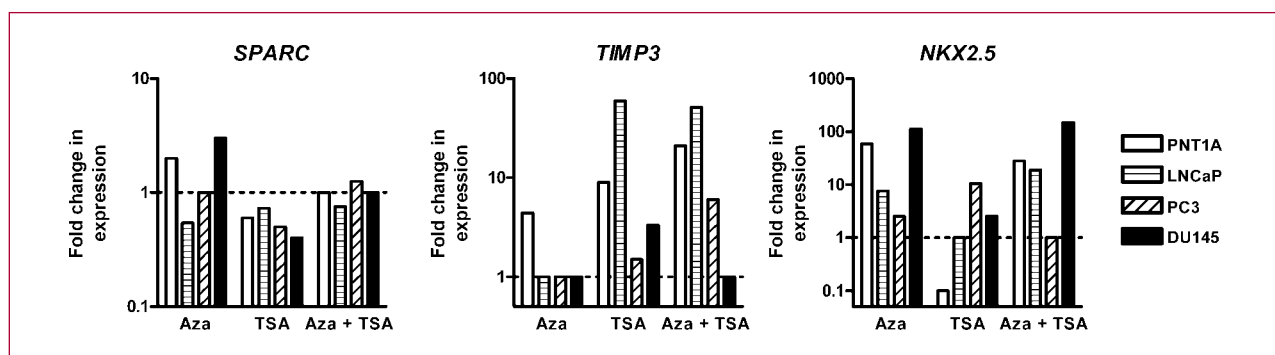


Fig. 5. Demethylation and gene expression. PCa cell lines and primary immortalized prostate epithelial cells, pNT1a, were treated with 5'-aza-dC (5 μ mol/L), TSA (250 nmol/L), or a combination of the two drugs. The cell lines chosen for the analysis had high methylation levels for the genes tested and all had very low levels of expression at baseline. The fold change in gene expression relative to mock-treated cells was determined by quantitative RT-PCR and expressed relative to β -actin to correct for variation in the amounts of reverse-transcribed RNA. Points, average values of duplicate experiments.

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*, *RAR β 2*, *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 differ-

ences 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

- Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
- Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;349:2042–54.
- Nelson WG, Yegnasubramanian S, Agoston AT, et al. Abnormal DNA methylation, epigenetics, and prostate cancer. *Front Biosci* 2007;12:4254–66.
- Kwabi-Addo B, Chung W, Shen L, et al. Age-related DNA methylation changes in normal human prostate tissues. *Clin Cancer Res* 2007;13:3796–802.
- Hmadcha A, Bedoya FJ, Sobrino F, Pintado E. Methylation-dependent gene silencing induced by interleukin 1 β via nitric oxide production. *J Exp Med* 1999;190:1595–604.
- Feinberg AP, Tycko B. The history of cancer epigenetics. *Nat Rev Cancer* 2004;4:143–53.
- Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002;3:415–28.
- Nelson WG, De Marzo AM, Isaacs WB. Prostate cancer. *N Engl J Med* 2003;349:366–81.
- Bastian PJ, Yegnasubramanian S, Palapattu GS, et al. Molecular biomarker in prostate cancer: the role of CpG island hypermethylation. *Eur Urol* 2004;46:698–708.
- Shu J, Jelinek J, Chang H, et al. Silencing of bidirectional promoters by DNA methylation in tumorigenesis. *Cancer Res* 2006;66:5077–84.
- Colella S, Shen L, Baggerly KA, Issa JP, Krahe R. Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. *Biotechniques* 2003;35:146–50.
- Polnaszek N, Kwabi-Addo B, Peterson LE, et al. Fibroblast growth factor 2 promotes tumor progression in an autochthonous mouse model of prostate cancer. *Cancer Res* 2003;63:5754–60.
- Kwabi-Addo B, Wang J, Erdem H, et al. The expression of Sprouty1, an inhibitor of fibroblast growth factor signal transduction, is decreased in human prostate cancer. *Cancer Res* 2004;64:4728–35.
- Ellinger J, Bastian PJ, Jurgan T, et al. CpG island hypermethylation at multiple gene sites in diagnosis and prognosis of prostate cancer. *Urology* 2008;71:161–7.
- Tokumaru Y, Harden SV, Sun DL, Yamashita K, Epstein JI, Sidransky D. Optimal use of a panel of methylation markers with *GSTP1* hypermethylation in the diagnosis of prostate adenocarcinoma. *Clin Cancer Res* 2004;10:5518–22.
- Chung W, Kwabi-Addo B, Ittmann M, et al. Identification of novel tumor markers in prostate, colon and breast cancer by unbiased methylation profiling. *PLoS One* 2008;3:e2079.
- Hsing AW, Tsao L, Devesa SS. International trends and patterns of prostate cancer incidence and mortality. *Int J Cancer* 2000;85:60–7.
- Freedland SJ, Isaacs WB. Explaining racial differences in prostate cancer in the United States: sociology or biology? *Prostate* 2005;62:243–52.
- Robbins CM, Hernandez W, Ahaghotu C, et al. Association of HPC2/ELAC2 and RNASEL non-synonymous variants with prostate cancer risk in African American familial and sporadic cases. *Prostate* 2008;68:1790–7.
- Enokida H, Shiina H, Urakami S, et al. Ethnic group-related differences in CpG hypermethylation of the *GSTP1* gene promoter among African-American, Caucasian and Asian patients with prostate cancer. *Int J Cancer* 2005;116:174–81.
- Woodson K, Hayes R, Wideroff L, Villaruz L, Tangrea J. Hypermethylation of *GSTP1*, *CD44*, and *E-cadherin* genes in prostate cancer among U.S. Blacks and Whites. *Prostate* 2003;55:199–205.
- Woodson K, Hanson J, Tangrea J. A survey of gene-specific methylation in human prostate cancer among black and white men. *Cancer Lett* 2004;205:181–8.

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

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