Methylation of the PMEPA1 gene, a negative regulator of the androgen receptor in prostate cancer

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Abbreviations: PMEPA1, prostate transmembrane protein androgen induced 1; AR, androgen receptor; CaP, cancer of the prostate; CA, Caucasian American; AA, African American; LCM, laser capture microdissection; SMAD, Mothers against decapentaplegic homolog; TGF-β, transforming growth factor-beta; PI3K, phosphatidylinositol-3-kinase

The prostate transmembrane protein androgen induced 1 (PMEPA1) gene is highly expressed in prostate epithelial cells and is a direct transcriptional target for the androgen receptor (AR). AR protein levels are controlled by the AR-PMEPA1 negative feedback loop through NEDD4-E3 ligase. Reduced expression of PMEPA1 observed in prostate tumors, suggests that loss of PMEPA1 may play critical roles in prostate tumorigenesis. This study focuses on epigenetic mechanisms of reduced PMEPA1 expression in the cancer of the prostate (CaP). Benign (n = 77) and matched malignant (n = 77) prostate epithelial cells were laser capture micro-dissected from optimum cutting temperature embedded frozen prostate sections from 42 Caucasian American (CA) and 35 African American (AA) cases. Purified DNA specimens were analyzed for CpG methylation of the PMEPA1 gene. PMEPA1 mRNA expression levels were evaluated by qRT-PCR. Analysis of PMEPA1 methylation and mRNA expression in the same tumor cell populations indicated a significant inverse correlation between mRNA expression and methylation in CaP (P = 0.0115). We noted higher frequency of CpG methylation within the evaluated first intronic region of the PMEPA1 gene in prostate tumors of CA men as compared with AA. In CaP cell lines, PMEPA1 expression was induced and AR protein levels were diminished in response to treatment with the DNA methyltransferase inhibitor, 5-aza-2’-deoxycytidine (decitabine). Cell culture-based studies demonstrated that decitabine restores PMEPA1 expression in AR-positive CaP cell lines. This report reveals the potential role of PMEPA1 gene methylation in the regulation of AR stability. Thus, downregulation of PMEPA1 may result in increased AR protein levels and function in CaP cells, contributing to prostate tumorigenesis.

Introduction

Androgens and the androgen receptor (AR) play central roles in the normal growth, differentiation and physiological function of the prostate gland.1 It has also been established that AR dysregulation leads to the progression of cancer of the prostate (CaP).2,3,4 To suppress androgen-dependent CaP growth, currently used therapeutic agents inhibit the binding of androgens to AR or the biosynthesis of androgens.5,6 Although most CaP initially respond to androgen ablation, its therapeutic effect is short lived and patients eventually develop castration resistant CaP.5,6

The AR binds to AR-responsive elements (AREs) and regulates the transcription of androgen responsive genes controlling differentiation and growth.1,4,7,8 Dysfunction of the androgen axis contributes to CaP through numerous mechanisms, including increased AR expression, intra-tumoral androgen synthesis, AR splice variants, mutations of the AR and androgen metabolizing enzymes.2,5,9 In a subset of advanced CaP, AR signaling is bypassed in favor of AR independent pathways.2,3,10,11

The PMEPA1 gene was identified in our laboratory as a highly androgen-induced gene in a screen for androgen regulated genes in CaP cells.12,13 PMEPA1 is predominantly expressed in the prostate gland and is directly regulated by AR.14 PMEPA1 spans 55–60 kb on chromosome 20 (20q13.31-q13.33) and the PMEPA1 protein is highly conserved among vertebrates, suggesting a critical role in the homeostasis of prostate. Functional
analysis of PMEPA1 has revealed that it is a NEDD4 E3-ligase binding protein and plays a role in downregulation of AR through a negative feedback loop between AR and PMEPA1. Inhibition of PMEPA1 leads to increased AR levels in CaP cells. Thus, decrease or loss of PMEPA1 mRNA expression that is frequently observed in CaP may result in gain of AR function. Studies also suggest that PMEPA1 is involved in other cancers through regulation of the TGF-β, PI3K and WNT pathways. These findings highlight the cellular context-dependent role of PMEPA1 in normal and malignant conditions.

Activation of PMEPA1 transcription by decitabine in LNCaP and LAPC4 cells with demethylation of CpG residues within the PMEPA1 promoter downstream sequences suggested a role for DNA methylation in regulating PMEPA1 in CaP. The current study focuses on the evaluation of the methylation and expression of the PMEPA1 gene in primary prostate tumors and in CaP cell culture models. The results underscore the role of DNA methylation in silencing the PMEPA1 gene with potential implications in the AR degradation pathway.

**Results**

PMEPA1 is frequently methylated in prostate cancer

We evaluated the methylation of the PMEPA1 gene in human prostate tumors. As a positive control, we analyzed the promoter methylation of GSTPI, a gene known to be methylated in the majority of CaPs. Laser capture microdissection (LCM) was used for the precise isolation of tumor and matched non-adjacent normal cells from 77 CaP cases. Genomic DNA and total RNA were isolated from each specimen.

We optimized the methylated-DNA precipitation and methylation-sensitive restriction enzymes (COMPARE-MS) assay for 2 ng of purified genomic DNA obtained from LCM-dissected tumor cells. Methylation analysis was performed for CpG-rich sequences of PMEPA1 gene (20q13.31-q13.33) and GSTPI gene (11q13) that is highly methylated in CaP and a LINE1 repetitive DNA element that has been shown to harbor methylation in the human genome.

The cohort (42 CA and 35 AA patients) was designed to address the frequency of PMEPA1 methylation. PMEPA1 gene methylation was observed in 28 of 77 cases (36%), whereas GSTPI gene was detected in 50 of 77 (65%) cases. As expected GSTPI methylation was highly prevalent. In this study, the cancer cells were isolated by LCM yielding low amounts of DNA suitable only for COMPARE-MS, an assay that has been shown to robustly enrich for methylated DNA with high sensitivity and specificity. However, limitations in the sensitivity to detect methylated alleles are expected with low amount of DNA input DNA from LCM microdissected primary tumor cells. This likely explains the slightly lower rate of GSTPI methylation observed in this study compared with previously published studies.

The cohort (42 CA and 35 AA patients) was designed to address the frequency of PMEPA1 methylation. PMEPA1 gene methylation was observed in 28 of 77 cases (36%), whereas GSTPI methylation was detected in 50 of 77 (65%) cases (Fig. 1B). As expected GSTPI methylation was highly prevalent. In this study, the cancer cells were isolated by LCM yielding low amounts of DNA suitable only for COMPARE-MS, an assay that has been shown to robustly enrich for methylated DNA with high sensitivity and specificity. However, limitations in the sensitivity to detect methylated alleles are expected with low amount of DNA input DNA from LCM microdissected primary tumor cells. This likely explains the slightly lower rate of GSTPI methylation observed in this study compared with previously published studies.

The observed methylation of PMEPA1 prompted us to investigate whether methylation affected the expression level of PMEPA1 in CaP. Thus, we evaluated the level of PMEPA1 gene expression from the same tumor samples that were evaluated for DNA methylation. Patient matched non-adjacent normal epithelial cells
were also analyzed for quantitative PMEPA1 expression (n = 77). Matched tumor over normal relative PMEPA1 expressions are summarized in Figure 2A. Consistent with our previous report,15 PMEPA1 expression was reduced in two-thirds of CaP cases. Both Caucasian American (CA) (65%) and African American (AA) (62.9%) groups showed similar frequencies of decreased PMEPA1 mRNA expression in CaP cells in comparison to matched normal epithelial cells.

Overall, methylation of the PMEPA1 gene significantly correlated with reduced mRNA expression (P = 0.0115) (Fig. 2B). The analysis revealed that 82% of patients, who have methylated PMEPA1 gene, have lower PMEPA1 expression in tumors compared with matched normal epithelium (Fig. 2B). Taken together these findings suggest that DNA methylation plays major roles in silencing the PMEPA1 in CaP. In this study, assessment of clinico-pathological data (Table 1) did not reveal correlation of biochemical recurrence with PMEPA1 methylation status or expression that is likely due to the limited sample size.

Differential PMEPA1 methylation between CA and AA patients

An unexpected findings of this study relates to differential methylation of PMEPA1 between the CA and AA CaP (P = 0.0064) despite similar patterns of PMEPA1 mRNA expression. These data suggested for additional mechanisms involved in downregulation of PMEPA1 in AA CaP.

We noted a higher frequency of PMEPA1 methylation (50%) in CA CaP in comparison to AA (20%) (Fig. 3A and B). The difference in PMEPA1 methylation status between these two groups remained striking (CA: 57%; AA: 17%, P = 0.0014) even when only tumor cells with well-differentiated morphology were compared (Fig. 3C). In contrast, the methylation status of the GSTP1 gene showed no significant difference (69% in CA and 60% in AA) (Fig. 3A and B). In the current study design the patient cohort represented nearly equal number of AA and CA cases. While both clinical (2.5-fold higher mortality in AA CaP patients) and cancer genome differences (low frequency of TMPRSS2-ERG in AA patients) have been consistently noted in literature between CA and AA patients,26-28 additional evaluations of differential PMEPA1 methylation between CA and AA are warranted.

Association of PMEPA1 methylation with AR in prostate cancer cell lines

Association of PMEPA1 methylation with reduced expression of PMEPA1 in CaP specimens provided a rational for testing the methylation status of PMEPA1 in CaP cell lines. We used AR-positive (VCaP, LNCaP, and LAPC4) and AR-negative (DU145 and PC3) cell culture models to analyze the methylation of the PMEPA1 gene. GSTP1 methylation was monitored as an established methylated gene since its methylation status has been established for these CaP cell lines.23,28 The results indicated that PMEPA1 is methylated only in AR positive cell lines and not in AR negatives (Fig. 4A). Consistent with previous reports,29,30 this study also showed that GSTP1 is methylated in both AR positive and negative CaP cell lines. These observations suggest a cellular context dependent relationship between PMEPA1 methylation and AR and therefore gain of AR function may be favored by decreased PMEPA1 expression involving epigenetic mechanisms such as DNA methylation.

PMEPA1 expression is induced by the DNA methyltransferase inhibitor decitabine in AR positive CaP cell lines

PMEPA1 methylation may lead to the silencing of PMEPA1 resulting in elevated AR protein levels and AR signaling. To further test this hypothesis, we treated the VCaP, LNCaP, and LAPC4 cell lines with low doses of decitabine. Western blot analysis revealed dose-dependent induction of PMEPA1 protein in response to decitabine treatment (Fig. 4B). Consistent with the protein data qRT-PCR analysis also showed decitabine dose-dependent increases of PMEPA1 mRNA expression (Fig. 4B). Taken together, these observations suggested that methylation contributes to the silencing of PMEPA1 gene in AR positive CaP cell lines. We also monitored the response to decitabine treatment on AR protein levels. Consistent with the PMEPA1-AR negative feedback model decrease in the expression of AR protein was observed in response to decitabine treatment (Fig. 4C).

Silencing of PMEPA1 may result in enhanced AR functions

PMEPA1 protein recruits AR to the NEDD4-1 ubiquitin ligase for degradation.16 Thus PMEPA1 modulates levels of AR. Cancer-associated silencing of PMEPA1 may result in elevated levels of AR and increased AR signaling (Fig. 5A). We have
evaluated the relationship of PMEPA1 levels with AR activity. For monitoring the AR activity, we assessed the expression of the known AR regulated gene, KLK3 (PSA) gene by PMEPA1 knockdown in LNCaP cells. Reduced expression of PMEPA1 protein was observed in PMEPA1 siRNA treated LNCaP cells (Fig. 5B). As expected, both AR and PSA protein levels were increased in response to PMEPA1 knockdown (Fig. 5B). Thus, the silencing of the PMEPA1 gene leads to enhanced AR activity in CaP by eliminating a negative regulatory control of AR protein levels.

### Discussion

Emerging data continues to underscore the critical roles of PMEPA1 as an androgen regulated NEDD4 E3 ligase binding protein in maintaining AR protein levels in prostate epithelial cells. Therefore, decrease or loss of PMEPA1 expression and function may have major impact on gain of AR function and CaP progression. The goal of this study was to elucidate the molecular basis for the reduced or lost expression of PMEPA1 in CaP. The results presented here demonstrate that promoter methylation is a major mechanism involved in silencing the PMEPA1 gene in CaP. The potential contribution of genomic methylation in the modulation of PMEPA1 expression was further supported using CaP cell lines. AR positive cells (LNCaP, LAPC4, and VCaP) showed methylation of the PMEPA1 gene, suggesting that the methylation may indeed contribute to the repression of PMEPA1. Increased expression of PMEPA1 in response to decitabine treatment of CaP cell lines was reflected both at mRNA and protein levels. The high frequency of methylation silencing of PMEPA1 in CaP samples provides further support for the hypothesis that PMEPA1 may negatively control prostate tumorigenesis through the AR axis (Fig. 5A and B). Similar to other cancers, methylation has been shown to play a major role in the CaP genome. GSTP1 methylation represents one of the earliest genomic alterations in CaP onset. Other genes of functional relevance have also been shown to be methylated in CaP.

### Table 1. Clinicopathologic characteristics

|                         | Caucasian American | African American |
|-------------------------|--------------------|------------------|
| **Number of patients**  | 42                 | 35               |
| **Gleason Sum**         |                    |                  |
| 6                      | 17 (41.5)          | 14 (41.2)        |
| 7                      | 15 (36.6)          | 16 (47.1)        |
| 8 to 10                | 9 (21.9)           | 4 (11.8)         |
| **Pathological T stage**|                    |                  |
| pT2                    | 17 (42.5)          | 14 (41.2)        |
| pT3–4                  | 23 (57.5)          | 20 (58.8)        |
| **Surgical margin status** |                |                  |
| Negative               | 39 (92.9)          | 28 (82.4)        |
| Positive               | 3 (7.1)            | 6 (17.6)         |
| **PSA at diagnosis**    |                    |                  |
| Median (range)         | 5.2 (1.1,23.4)     | 6.6 (3.2,98.7)   |
| **Seminal vesicle invasion** |                |                  |
| Negative               | 39 (92.9)          | 28 (82.4)        |
| Positive               | 3 (7.1)            | 6 (17.6)         |
| **Age at RP**          |                    |                  |
| Mean (SD)              | 61.1 (8.4)         | 59.4 (8.3)       |
| Median (range)         | 62.1 (40.2,73.6)   | 59.8 (45.7,91.9) |
| **BMI**                |                    |                  |
| Mean (SD)              | 26.2 (3.5)         | 27.3 (4.9)       |
| Median (range)         | 26 (20,34)         | 28 (19,42)       |

Studies from our and other groups have shown that PMEPA1 is a multifunctional protein. However, PMEPA1 functions is likely cellular context dependent and may be different in the presence or absence of AR. In normal prostate epithelial cells a negative feedback loop between AR and PMEPA1/NEDD4 regulates AR. In CaP, loss of PMEPA1 appears to be critical in gain of AR function and CaP progression. Out of the AR context, a negative feedback loop between TGF-β and PMEPA1 has been noted through direct interaction of PMEPA1 with SMAD3/4.
supporting cancer progression. Thus, gain or loss of \textit{PMEPA1} expression and function may indeed cancer cell type dependent.

The differential methylation of \textit{PMEPA1} gene between CA (50\%) and AA patients (20\%) was an unexpected finding. Emerging data on CaP genomes is beginning to clarify genomic differences between ethnic groups. CaP-associated somatoge- netic and epigenetic differences between various ethnic groups are increasingly recognized.\textsuperscript{26,27,33-37} However, the distinct biology is not well understood and warrants further investigations, including the observed \textit{PMEPA1} methylation difference between CA and AA patients.

A significant effort has been devoted toward developing inhibitors targeting epigenetic modification of the cancer genome including FDA approved DNMT inhibitors (azacitidine and decitabine) for Myelodysplastic Syndrome and HDAC inhibitors (vorinostat and romidepsin) for Cutaneous T Cell Lymphoma.\textsuperscript{38,39} With the increasing knowledge of epigenetic alterations linked to CaP, such as in \textit{GSTPI} or AR axis, more targeted therapeutic strategies may be possible. \textit{PMEPA1} may represent a new promising target for epigenetic drugs or new class of drugs complementing AR axis inhibitors.

In conclusion, the \textit{PMEPA1} gene is methylated in CaP primary prostate tumors suggesting that methylation may contribute to the silencing of \textit{PMEPA1}. \textit{PMEPA1} gene expression levels correlated with its DNA methylation status. \textit{PMEPA1} methyla- tion status displayed an intriguing difference between CA and AA patients. These data, along with our earlier observation, indicate that reduced or lost \textit{PMEPA1} expression in CaP cells may lead to elevated AR levels. Our study provides insights into the role of methylation in the CaP-associated silencing of \textit{PMEPA1} with potential impact on the AR axis in malignant prostate. Targeting AR for degradation by \textit{PMEPA1} may synergize current therapeutic approaches.

**Materials and Methods**

**Prostate cancer specimens and clinico-pathological data**

Radical prostatectomy (RP) specimens and clinico-pathological data were obtained from patients enrolled in the Center for Prostate Disease Research (CPDR) from 1996 to 2010 under an institutional review board-approved protocol at the Walter Reed National Military Medical Center (WRNMMC) and Uniformed Services University of the Health Sciences (USUHS) (Table 1). Optimum cutting temperature (OCT) embedded RP tissue specimens from 77 patients, including 42 CA and 35 AA, were analyzed in this study. None of these patients had received prior androgen deprivation therapy. The biochemical recurrence was defined as two consecutive post-operative PSA values (≥0.2 ng/mL) measured at ≥8 wk post-operatively.

LCM derived normal and malignant prostate epithelial cells were obtained from OCT embedded frozen sections as described
previously. Briefly, specimens obtained immediately after surgical resection of prostate, were OCT embedded and frozen on glass slides placed on dry ice. Benign and malignant cells were isolated by LCM using 6 micron frozen tissue sections archived in CPDR frozen tissue section slide library stored at -80 °C. The strategy for the evaluation of methylation and expression is described in Figure 6A and B.

Isolation of genomic DNA and total RNA from LCM derived prostate epithelial cells

Total RNA from the LCM derived specimens was isolated, purified and quantified. For genomic DNA, LCM-derived cells were lysed (buffer containing 10 mM Tris, 1 mM EDTA, 1% Tween 20) and digested with proteinase K (1 mg/ml; Roche Applied Science) at 37 °C overnight. DNA was precipitated by adding 1/10th volume of 3M sodium acetate pH 5.2 (Sigma-Aldrich) and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) (Sigma-Aldrich). For optimal recovery the DNA was precipitated by adding 2 μg of glycogen (Roche Applied Science) and equal volume of chilled isopropanol (Sigma-Aldrich). After centrifugation the DNA pellets were washed with 70% ethanol (Sigma-Aldrich) and resuspended in 25 μl of TE buffer pH 8.0 (Sigma-Aldrich). DNA quantitation of all samples was performed by using Picogreen dye (Quant-iT™ PicoGreen® dsDNA Reagent and Kits, Life Technology) and
confirmed with 260/280 ratio by NanoDrop 2000c spectrophotometer (Thermo Scientific).

**DNA Methylation assay for genomic DNA isolated from LCM derived prostate cancer cells and prostate cancer cell lines**

Based on publicly available genome wide methylation collected by the ENCODE consortium (http://genome.ucsc.edu) as well as by whole genome methylation analyses (Haffner MC and Yegansubramnian S., personal communication) the first intronic region of *PMEPA1* was selected for the analyses by the consistent differential methylation between LNCaP and primary prostate epithelium-derived PrEC cells and by the linear detection range in assaying low quantity of input DNA (Fig. S1A and B).

A combination of COMPARE-MS method, followed by Quantitative real time-Polymerase Chain Reaction (Q-PCR) was used for the evaluation of the DNA methylation. Two ng of purified genomic DNA from LCM derived prostate tumor cells was digested with 10U each of AluI and HhaI (New England Biolabs) at 37 °C for 3 h followed by heat inactivation of enzymes at 80 °C for 20 min. Methylated genomic DNA fragments were captured by recombinant MBD2 protein derived methyl-binding domain polypeptides immobilized on magnetic beads. In parallel, 2 ng of genomic DNA from normal male white blood cells (WBC), treated with 4U of CpG Methylase (M.SssI) enzyme (Zymo Research) and untreated DNA were also analyzed, serving as positive and negative controls, respectively.

Primers designed for the methylated CpG region of the *PMEPA1* gene (forward primer, 5′-CGTCTGCGCT GCTTAAAACT; reverse primer, 5′-TTTGGAGAG AT GGGTTTTCAC) and *GSTP1* (forward primer, 5′-GGGACCACTCC AGAAAGGC; reverse primer, 5′-ACTCAGCTGT GGGGAAGACT) were used for SYBR Green-based Q-PCR. *LINE1* repetitive elements, which are highly methylated in human genomic DNA, were used as positive control to ensure the recovery of methylated DNA during the COMPARE-MS assay. *LINE1* promoter (GenBank accession X58075) was amplified by the following primer set: forward primer 5′-CGCAGAAGAC GGGTGATTTC-3′ and reverse primer 5′-CCGTCACC CC TTTCTTGTAC-3′.

Q-PCR was performed in duplicate with 25 μL reactions containing 1X IQ Sybr-Green Supermix (Bio Rad) and 0.4 μM forward and reverse primers under the following cycling conditions: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s on Stratagene Mx3000P (Agilent Technologies Inc.). The COMPARE-MS assay was used to analyze *PMEPA1*, *GSTP1*, and *LINE1* methylation in LCM-derived prostate tumor cell and AR positive and AR negative CaP cell

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**Figure 6.** (A) Flowchart of DNA methylation and quantitative gene expression analysis in prostate tumor specimens. (B) LCM, determination of methylation by combination of methylated DNA precipitation and methylation-sensitive restriction cleavage (COMPARE-MS), and quantitative gene expression analyses.
lines, VCaP, LNCaP, LAPC4, and DU145, PC3, respectively. The methylation status was categorized binary as present or absent. The methylation levels were normalized to the signal generated by an equal input amount of the positive control. The expression of GAPDH as the RNA input control and the target gene expression were simultaneously analyzed for each sample (in duplicates). A negative control without reverse-transcriptase enzyme was included for each specimen to rule out background signal from potential genomic DNA contamination.

*Quantitative real-time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) analysis*

TaqMan qRT-PCR using RNA from LCM derived tumor and normal epithelial cells was performed as described previously. The expression of GAPDH as the RNA input control and the target gene expression were simultaneously analyzed for each sample (in duplicates). A negative control without reverse-transcriptase enzyme was included for each specimen to rule out background signal from potential genomic DNA contamination.

*Cell culture*

Human prostate tumor cell lines, VCaP, LNCaP and LAPC4 were purchased from American Type Culture Collection (ATCC) and VCaP kindly provided by Dr Charles L. Sawyers (UCLA, CA). VCaP and LNCaP cells were maintained in DMEM (ATCC), and LAPC4 cells in RPMI-1640 (ATCC) supplemented with 10% of fetal bovine serum (Invitrogen) in a humidified CO2 incubator at 37 °C, 5% CO2 for 12 h. After incubation the transfection complex was removed and the cells were replenished with fresh complete medium. The cells were harvested 48 h post-transfection for western blot assay.

*Western blot analysis*

Cells from the cell culture experiments were lysed in Mammalian Protein Extraction Reagent (M-PER) (Pierce from Thermo Fisher Scientific Inc.) in the presence of protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Cell lysates equivalent to 25 μg of total protein were separated on 4–12% Bis-Tris gel (Life Technologies) and transferred to PVDF membrane. Membranes were incubated with primary antibodies: anti-PMEPA1 monoclonal antibody (Novus Biologicals), anti-GSTP1 polyclonal antibody (US Biologicals), anti-β-Actin monoclonal antibody (Cell Signaling), anti-PSA polyclonal antibody (Dako) and anti-AR polyclonal antibody (Santa Cruz Biotech) at 4 °C overnight and were washed before being treated with respective secondary antibodies (GE Healthcare Biosciences). Western blots were visualized by the Amersham ECL western blot detection reagent (GE Healthcare Biosciences).

*Statistical analysis*

Seventy-seven CaP patients (42 CA and 35 AA) who underwent RP for primary treatment were analyzed for PMEPA1 and GSTP1 methylation status. The cohort was designed based on our initial observations indicating higher frequency of PMEPA1 methylation in CA patients (42 CA and 8 AA). This minimum sample size (n = 77) was determined by statistical power calculation (90% power with 0.05 α, two sided chi square test). RNA and DNA were analyzed from the same specimens. PMEPA1 mRNA expression was normalized to GAPDH and reported as fold change between matched normal and tumor pairs. PMEPA1 and GSTP1 genomic DNA methylation data were reported as present or absent. Distributions of other clinico-pathological variables were examined by using Chi-square or Fisher exact tests.

Chi-square test was used to evaluate the association of PMEPA1 and GSTP1 methylation and PMEPA1 expression with race/ethnicity. Chi-square test was also used to examine the association of PMEPA1 methylation with PMEPA1 expression. Association of the combination of PMEPA1 methylation and expression groups (methylation-/high expression, methylation+/ low expression) with race was tested by using Fisher’s exact test.

*Disclosure of Potential Conflicts of Interest*

No potential conflicts of interest were disclosed.

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