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Evaluation of Androgen Receptor Function in Prostate Cancer Prognosis and Therapeutic Stratification

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Evaluation of Androgen Receptor Function in Prostate Cancer
Prognosis and Therapeutic Stratification

While prostate cancers initially respond to androgen ablation therapy, tumors often become treatment resistant as tumor cells develop mechanisms to evade the treatment. Dysfunction of the androgen receptor frequently observed in castration resistant stages of prostate cancer. We reasoned that early knowledge of androgen receptor dysfunction can predict the course of prostate cancer progression. We proposed an approach for monitoring potential dysfunctions of the androgen receptor by measuring the expression of a panel of genes directly regulated by androgen receptor. We examined human prostate cancer tissues (surgery specimens) at early stages of the disease and matched with longitudinal follow up data. We have completed the evaluation of 140 patients by monitoring mRNA expression levels of ERG, PSA, PMEPA1, ODC1, AMT1 and GAPDH levels. Also, we have completed the evaluation of 80 whole-mounted sections of radical prostatectomy specimens by immunohistochemistry assessing AR, ERG, NKX3.1 and PSA proteins. This study addresses the association of AR function defects (decreased or attenuated expression of AR regulated genes) with unfavorable clinical features, as well as, if expression levels of the androgen regulated gene panel is indicative of biochemical recurrence-free survival. Major findings emerging from our PCRP/CDMRP award revealed that monitoring the levels of AR regulated proteins may support disease progression-associated clinical-pathologic features. In contrast, assessment of AR regulated genes at mRNA levels show promising performance in supporting the prediction of biochemical recurrence–free survival in low grade tumors. In summary, the study results indicate the utility of monitoring the defects of AR function in prostate cancer.

Prostate cancer, Androgen receptor function, Androgen regulated genes, Therapeutic stratification
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1. INTRODUCTION:

This PCRP/CDMRP/DOD Idea Development Award supported project was initiated in 2011. The original award was for three years and was granted with a one year no-cost extension. This final report encompasses the entire project period from 2011 to 2015 and delineates the potential clinical application of findings resulted from the accomplishments of the award supported research.

The proposal’s idea originated from the clinical observation that prostate cancer is initially driven by the male hormone androgen acting through the androgen receptor. However, in some patients androgen receptor becomes dysfunctional at late stages of tumorigenesis. These patients are less likely to show sustained response to androgen ablation therapy and more likely to die from incurable metastatic prostate cancer. Thus, early knowledge of the androgen receptor dysfunctions, that would make prostate tumors refractory to routine androgen ablation therapy, should help in patient stratification for new emerging therapeutic strategies. Indeed, while prostate cancers initially respond to androgen ablation therapy, tumors often become treatment resistant as tumor cells develop mechanisms to evade the treatment. In this castration resistant (CRPC) stage of prostate cancer the dysfunction of the androgen receptor (AR) has been recognized (Schmidt and Tindall Curr Drug Targets 2013; Dobi, Sreenath et al., Book chapter 2013; Yuan et al., Oncogene 2014; Crawford et al., J Urol 2015).

The scope of this proposal is to predict the course of prostate cancer progression by monitoring a panel of AR regulated genes. To monitor the functional status of androgen receptor in prostate tumors a panel of six androgen inducible genes were selected (Xu et al., J Urol 2000; Xu et al., Genomics 2000; Segawa et al., Oncogene 2002). This panel includes tissue (KLK3 (PSA)), AR stability regulator (PMEPA1), transcription factor (NKX3.1), polyamine biosynthesis (ODC1, AMD1) and oncogene (ERG) protein coding genes. Selected genes are either direct targets of AR or are tightly regulated by AR and have demonstrated prostate associated expression encompassing major biological functions regulated by AR in the human prostate. Expression levels of the selected genes were determined in radical prostatectomy specimens. CPDR Biospecimen Bank linked to comprehensive clinico-pathologic patient data base and longitudinal follow-up were used for evaluation of quantitative gene expression values in RP specimens for 1) positive or negative correlation with disease progression as defined by Gleason grade, pathological stage and biochemical recurrence; 2) feasibility of gene expression measurement in clinical assays (specificity, sensitivity and reproducibility).

We reasoned that AR function in prostate tumor cells can be precisely defined by measuring the expression of AR regulated gene panel (ARP), including (KLK3 (PSA), PMEPA1, TMPRSS2-ERG (ERG), NKX3.1, AMD1 and ODC1) towards defining AR function by the Androgen Receptor Function Index (ARFI) (Figure 1).

In our approach we leveraged the mechanistic understanding of direct AR regulated genes. Thus, our approach is distinct from the empirically design OncotypeDX Prostate mRNA-based AR-regulated gene set that has been recently introduced to clinical practice by Genomic Health Inc.

![Figure 1. Androgen Receptor Function Index (ARFI) as Potential Indicator of CaP Stratification.](image-url)
A second distinctive feature and major strength of promising data emerging from our PCRP/CDMRP/DOD award is that monitoring AR function at the protein expression levels of panel of androgen regulated genes may provide an IHC-based more easily adaptable and cost effective approach in routine pathology settings. In our approach we have addressed the association of AR function defects with unfavorable clinical features, if the ARFI is indicative of BCR and metastasis-free survival. Leveraging the high representation of African American men in the CPDR data and tissue bank we evaluated the performance of monitoring AR-regulated genes in both Caucasian American and African American men in response to the emerging need for biomarkers that performs equally well in ethnic groups within the United States. We have completed a collaborative evaluation of castration resistant prostate cancers (CRPC). In support of the central hypothesis of this proposal, the result revealed a thus far unrecognized type of CRPCs with dispensed AR function. In summary, the observed readouts of AR dysfunction may provide a promising tool for improved prognostic accuracy and patient stratification at early stages of prostate cancer.

The objective of this proposal is to predict the course of prostate cancer progression by monitoring a panel of AR regulated genes in stratifying patients for treatment modalities. We have reported the association of decreased expression of androgen regulated genes with features of prostate cancer progression (Sterbis, Gao et al. 2008; Dobi, Furusato et al. 2010; Sharad Epigenetics 2014; Gsponer et al, 2014). Other reports have also noted a signature of attenuated AR function in late stage, especially in metastatic prostate cancer in human specimens (Varambally, Yu et al. 2005), (Hermans, van Marion et al. 2006), (Tomlins, Mehra et al. 2007) (Mendiratta, Mostaghel et al. 2009). Assessment of AR function by measuring the expression of androgen regulated genes as part of a gene panel has been recently shown to improve the prediction of the presence or absence of adverse pathology at the time of diagnosis (Klein et al., Eur Urol 2014; Cullen et al., Eur Urol 2014).

To develop readouts for AR function in CaP cells, this PCRP/CDMRP Idea Development grant award focused on the quantitative measurements of AR regulated genes in carefully isolated benign and tumor cells and in prostate tumor tissues. The findings reported here summarize the accomplishments of the proposal towards the evaluation of androgen receptor function towards the following specific aims:

Aim #1: To establish the Androgen Receptor Function Index (ARFI) by assessing the AR regulated gene panel as a quantitative measure of in vivo AR functional status in prostate cancer at the time of radical prostatectomy.

Aim #2: To define the utility of AR-regulated proteins by the AR Function Index, ARFI.

2. KEY WORDS:
Prostate cancer, androgen receptor, androgen receptor regulated genes, prostate cancer progression PSA, NKX3.1, PMEPA1, ERG, ODC1, AMD1

3. ACCOMPLISHMENTS:
a) Specific objectives and major activities
Towards Aim#1, Task 1: to establish the Androgen Receptor Function Index (ARFI) by assessing the AR regulated gene panel (ARP) as a quantitative measure of in vivo AR functional status in prostate cancer at the time of radical prostatectomy.

Step 1 plan (Months 1-6):
Institutional Review Board regulatory review and approval processes will occur at two separate institutions: the Walter Reed Army Medical Center (WRAMC) and the Uniformed Services University of the Health Sciences (USUHS). While IRB approval is still ongoing, probes and primers will be designed following the same principles we have been using for TaqMan and will be tested for specificity and sensitivity by using copy number defined dilutions of cDNA clones of ARP.

Completed IRB approval in Month 10 and the Task completed by Month 12 (100%).

Step 2 plan (Months 6-22):
RNA samples for QRT-PCR will be obtained from radical prostatectomy specimens of 110 CaP patients following our established strategy. Total RNA from laser-captured microdissected (LCM) normal and cancer cells from either formalin fixed paraffin embedded (FFPE) or OCT embedded and H&E stained frozen prostate sections of radical prostatectomy specimens (5-10,000 epithelial cells per sample) will be acquired from the CPDR Biospecimen Bank. Total RNA will be quantified by using RiboGreen fluorometric method. The total RNA isolated from the paired tumor and normal LCM epithelium specimens will be converted to cDNA. The expression levels of ARP genes (PSA/KLK3, PMEPA1, NKX3.1, ODC1, AMD1 and ERG/TMPRSS2-ERG) will be determined in the matched tumor and normal prostate tissue-derived cDNA samples by real time QRT-PCR (TaqMan).

Completed the mRNA expression analyses of 140 tumor samples (77 cases by QRT-PCR, 63 cases by NanoString) by Month 40 (100%): Total RNA from laser-captured microdissected (LCM) normal and cancer cells from both formalin fixed paraffin embedded (FFPE) and OCT embedded and H&E stained frozen prostate sections of radical prostatectomy specimens were acquired from the CPDR Biospecimen Bank. Total RNA were quantified by using RiboGreen fluorometric method. The total RNA isolated from the paired tumor and normal LCM epithelium specimens were converted to cDNA. The expression levels of AR-regulated genes (PSA/KLK3, PMEPA1, NKX3.1, ODC1, AMD1 and ERG/TMPRSS2-ERG) were determined in the matched tumor and normal prostate tissue-derived cDNA samples from frozen tissues by real time QRT-PCR (TaqMan). In a new approach the study design was focused on low grade disease (Gleason 3+3 or 3+4; no positive margins, no extracapsular extension, and no seminal vesicle invasion) with (N= 21) or without (N=42) recurrence (BCR or Mets). NanoString analysis was performed on 63 macrodissected tumor cDNA samples. The advantage of the NanoString platform is the reliable quantitative data by mRNA copy number on FFPE-derived mRNA in multi-plex analyses. The expression levels of AR-regulated genes (PSA/KLK3, PMEPA1, ODC1, AMD1 and ERG) were determined in 63 macrodissected FFPE-derived tumor samples.

Step 3 plan (Months 23-40):
AR panel expression data will be analyzed by informatic and statistical methods for positive or negative correlation with aggressiveness of prostate cancer, as defined by Gleason grade, pathological stage, biochemical recurrence and for feasibility of gene expression measurement in a clinical assay (specificity, sensitivity and reproducibility). Cumulative index will be used for quantitative definition of AR function (ARF index) towards determine the stratification power of AR gene panel at the time of radical prostatectomy. This index will be incorporated into nomograms modeling time-to-event data, including prediction of disease progression, combined with established clinical and pathological characteristics that predict this study endpoint.
Completed the QRT-PCR evaluation of ARP mRNA expression data showing correlation between the expression of AR regulated genes. However, the analysis did not show correlation of cumulative mRNA levels as defined by QRT-PCR with disease progression. Taking a new, more quantitative approach using NanoString platform the analyses of ARP mRNA in FFPE-derived tumor samples (N=63) revealed 74% specificity and 76% sensitivity for predicting BCR-free survival among patients with low-grade index tumors: Gene AR panel expression data were analyzed by informatic and statistical methods for positive or negative correlation with aggressiveness of prostate cancer, as defined by Gleason grade, pathological stage, biochemical recurrence and for feasibility of gene expression measurement in a clinical assay including specificity and sensitivity. Cumulative index were used for quantitative definition of AR function towards determine the stratification power of AR gene panel at the time of radical prostatectomy. Due to the low number of events incorporation of ARFI into nomograms (modeling time-to-event) was not feasible. However we were able to complete the assessment of AR specificity and sensitivity for predicting disease progression in low grade disease.

Towards Aim#2, Task 2: to define the utility of AR-regulated proteins by the AR Function Index, ARFI.

Step 1 plan (Months 12-30):
IHC will be set up and optimized with antibodies against ARP gene products. Whole-mounted sections of RP specimens with prostate cancer will be assayed in a cohort of over 110 patients by immunohistochemistry. The staining intensities will be determined according to percent of cells positive. The intensity will be scored and a combination of measurements will be calculated by multiplying the percent of positive cells with the degree of intensity, which will result in an IHC intensity score. The sum of staining intensity scores will be expressed as the cumulative IHC staining index of AR regulated gene panel.

Completed the evaluation of index tumors of 80 whole-mounted radical prostatectomy specimen indicating 74% specificity and 54% sensitivity in predicting biochemical recurrence and/or metastasis among Caucasian American men. The observed 74% specificity in detecting AR dysfunction may support the early identification of patients with higher risk for progression among CA patients. Attenuated expression of PSA and NKX3.1 was consistently observed in index tumors of both AA and CA men with Gleason score 8-10 carcinoma. Although the overall pattern of AR regulated genes were similar except ERG, that was absent in majority of index tumors of AA men the panel showed only 52% specificity and 47% sensitivity for predicting BCR/Metastasis in AA men: IHC was optimized with antibodies against AR regulated gene protein products (PSA, NKX3.1, ERG and AR). Whole-mounted sections of RP specimens with prostate cancer were assayed in a cohort of 80 patients by immunohistochemistry. The staining intensities were determined according to percent of cells positive. The intensity was scored and combination of measurements was calculated by multiplying the percent of positive cells with the degree of intensity, which resulted in an IHC intensity score. The sum of staining intensity scores were expressed in a four color scale as the cumulative IHC staining index of AR regulated gene panel.

Step 2 plan (Months 12-40):
To establish concordance between the expression of ARP mRNA and proteins branched-chain DNA (b-DNA) signal amplification method will be used. Adjacent 4 μm-thick section will be selected from each of the FFPE whole-mount prostate samples.
The b-DNA approach in this step was replaced with microarray analyses comparing ARFI mRNA to immunostaining of ARP proteins in adjacent sections of whole mounted prostates.

Step 3 plan (Months 31-44):

Incorporate the AR gene panel cumulative indexes into predictive nomograms for prostate cancer progression defining predictive power and utility for patient stratification. Protein ARP protein panel will be assessed in formalin fixed paraffin embedded (FFPE) tissues by evaluating 110 whole-mounted radical prostatectomy specimens. From the IHC staining of products of the ARP will be summarized in a cumulative index for patient stratification. IHC scores and a cumulative pathology scores will be established for the tumor foci in the sections. Cumulative IHC score will be evaluated alone and by combining with nomograms modeling time-to-event data incorporating the biochemical recurrence within eight years of follow up. Post-operative predictive value of existing, validated nomograms will be assessed by using the patient cohort. The IHC-derived ARP values, as a single cumulative index, will be incorporated into validated nomograms (Kattan) modeling time-to-event data, including prediction of CaP progression, combined with established clinical and pathological characteristics that predict this study endpoint. The concordance index, will be used to assess the improvement in model fit upon inclusion of AR function index.

Completed the development of a numeric IHC scoring system from the assessment of 80 whole-mounted radical prostatectomy specimens and the evaluation of sensitivity and specificity of ARP IHC in predicting BCR/distant metastasis. From the IHC staining of products of the ARP were expressed in a four color scale representing the cumulative index. IHC scores and disease progression (BCR/metastasis) were correlated to ARP. Due to the low number of events nomograms, modeling time-to-event, was not feasible. However, the performance characteristics of ARP IHC was calculated resulting in 74% specificity and 54% sensitivity in predicting BCR/distant metastasis in support of unfavorable clinical-pathologic features. This performance was observed only among CA patients.

b. Significant results and key outcomes:

By the end of the first reporting period Institutional Review Board approval was obtained from the Walter Reed National Military Medical Center (WRNMMC) and from the Uniformed Services University of the Health Sciences (USUHS). Primers and probes were prepared and QC-ed for qRT-PCR assays for assessing the expression of PSA/\textit{KLK3}, \textit{PMEPA1}, \textit{NKX3.1}, \textit{ODC1}, \textit{AMD1}, \textit{AR} and \textit{ERG} (\textit{TMPRSS2-ERG}) genes. The quality control of primers and probes for the detection of endogenously expressed AR panel genes has been completed, target sequences were confirmed by DNA sequencing of PCR products and sensitivity and specificity was confirmed.

To monitor the functional status of androgen receptor in prostate tumors a panel of six androgen inducible genes were selected. This panel includes tissue (\textit{KLK3}(PSA)), AR stability regulator (\textit{PMEPA1}), transcription factor (\textit{NKX3.1}), polyamine biosynthesis (\textit{ODC1}, \textit{AMD1}) and oncogene (\textit{TMPRSS2-ERG}) protein coding genes. Selected genes are either direct targets of AR or are tightly regulated by AR and have

Figure 2. Timetable of the androgen dose and time kinetic evaluation of target genes in VCaP cell line.
demonstrated prostate associated expression encompassing major biological functions regulated by AR in the human prostate. The primers and probes have been obtained, and tested by using cDNA from VCaP prostate cancer cell line endogenously expressing all target genes, *PMEPA1* (Locus ID: GXL_128240), *TMPRSS2-ERG* fusion A (Locus ID: GXL_39091), *KLK3* (PSA) (Locus ID: GXL_32002), *NKKX3.1* (Locus ID: GXL_29827), *AMD1* (Locus ID: GLX_261249), and *ODC1* (Locus ID: GXL_75806). PCR amplicons were isolated and target regions were confirmed by DNA sequencing. To assess the performance of primers and probes assessing androgen dose and time kinetic response of target genes, VCaP cells were grown in androgen depleted conditions for four days. To induce androgen regulated genes, synthetic androgen (R1881) was added to the cell cultures at 0, 0.1, 1.0 and 10nM concentrations and cells were harvested at 0, 12, 24 and 48h time points (Figure 2.). To monitor changes in cell morphology in response to R1881 induction, cells were assessed at 0, 12, 24 and 48h time points. The observed cell morphology was consistent with the expected time and dose kinetic response of VCaP cells to R1881 treatment (Figure 3.).

Evaluating protein products of three of the target genes (*PSA*, *NKX3.1* and *ERG*) that was previously shown robust response to androgen induction in hormone responsive prostate cancer cell culture models (Xu et al., 2001; Segawa et al., 2002; Tomlins et al., 2005), VCaP cells were harvested and cell lysates were prepared at 0, 12, 24 and 48h time points in the 0, 0.1, 1.0 and 10 nM R1881 treatment groups. Expression of PSA, *NKX3.1* and ERG proteins were assessed by immunoblot assays. As expected, the experiment revealed androgen hormone dose dependent expression of the assayed proteins (Figure 4.).

For the evaluation of ARP target gene expressions RNA was isolated from VCaP cells at 0, 12, 24 and 48h in the 0, 0.1, 1.0 and 10 nM R1881 treatment groups. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in triplicates and one control reaction without RT were performed for each RNA sample by using primer and probe sets for *KLK3* (PSA), *TMPRSS2-ERG* (fusion junction “A” (Tomlins et al., 2005)), *NKKX3.1*, *PMEPA1*, *ODC1* and *AMD1* genes and expression values were expressed relative to *GAPDH*. Expression results were calculated from the average CT (threshold cycle) values of triplicates (Figure 5.). Specific primers and probes were designed for the qRT-PCR analyses of *PSA/KLK3, PMEPA1, NKKX3.1, ODC1, AMD1* and *TMPRSS2-ERG* genes. Quality control of primer and probe sets has been completed. Kinetic and androgen dose response of endogenously expressed levels of ARFI genes in VCaP cells indicate the sensitivity of detection. Sequence analyses of target gene amplicons confirmed the specificity of detecting ARFI genes.
In Year#2 of the award RNA samples for qRT-PCR were obtained from radical prostatectomy specimens from 77 patients enrolled in the Center for Prostate Disease Research (CPDR) program between 1996 and 2010. Clinicopathologic data were obtained from the CPDR database. Optimum cutting temperature (OCT) embedded RP tissues specimens from 42 Caucasian American (CA) and 35 African American (AA) men due to reported differences in incidence and mortality. None of the patients had received androgen deprivation therapy. Biochemical recurrence (BCR) was defined as two consecutive post-operative PSA values greater than 0.2 ng/mL measured at >= 8 weeks post-operatively. Total RNA from laser-captured microdissected (LCM) normal and matching cancer cells from OCT embedded and H&E stained frozen prostate sections of radical prostatectomy specimens were prepared. Total RNA were isolated from paired tumor and normal LCM epithelium specimens. Expression levels of AR panel genes (PSA/KLK3, PMEPA1, ERG(TMPRSS2-ERG) and GAPDH) were determined by real time QRT-PCR (TaqMan). Clinico-pathologic data were obtained from the CPDR database. The analysis showed positive correlation between the expressions of ARP genes (ERG vs. PMEPA1 and ERG vs. PSA) in tumors of AA patients that has not observed within the CA group. We have further extended the focused evaluation of PMEPA1 gene due to its emerging role in regulating AR activity (Sharad et al., Epigenetics 2014).

To assess the concordance between the expression of ARP mRNA and proteins we have completed the direct comparison of ARP IHC results from index tumors of 40 prostate cancer cases to mRNA expression data obtained by Affymetrix microarrays. The result showed 90% concordance between detecting ERG protein or ERG mRNA validating the identical origin of tumor samples assessed by IHC and by gene expression experiments. However, the overall concordance between the cumulative indexes of AR panel proteins and the expression of AR panel genes were only 50% likely due to notable differences between the protein and mRNA levels of the NKX3.1 and PSA (KLK3) genes and to challenges in matching frozen ex-vivo biopsies used in mRNA expression to the protein assessments by IHC of index tumors in whole mounted RP specimens. (Figure 6 and 7).

Figure 5. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of KLK3(PSA), TMPRSS2-ERG(ERG), NKX3.1, PMEPA1, ODC1 and AMD1 genes in VCaP cells demonstrate the activation of ARFI genes in response to increasing doses of R1881 at 48h time point. Experiments were performed in triplicates and expression levels relative to GAPDH are shown as fold changes.
Staining intensities have been determined and intensity was scored and a combination of measurements was calculated. Assessment of ARP genes by IHC showed remarkable accuracy in identifying biochemical recurrence (BCR) and metastasis in tumors with poorly differentiated (PD) morphology (14 out of 15 BCR/metastasis). In contrast, detection of RNA levels of ARP genes performs better in predicting favorable outcome in tumors with well differentiated (WD) morphology, precisely identifying 13 cases with BCR-free survival (no-BCR) and no metastasis out of 14 cases classified as intact AR (Figure 7).

These findings suggested different utility of detecting ARP genes by IHC when comparing to measuring mRNA levels. Detection of ARP proteins may complement the clinical-pathologic features of higher grade tumors supporting the prediction of BCR. In contrast, detecting attenuated expression of ARP mRNA levels in index tumors with well-to-modarately differentiated morphology may support the prediction of favorable outcome.
Encouraged by the promising data observed by the analysis of ARP proteins (Figure 7.), in Year #3-4 we have extended the ARP protein IHC analyses examining 80 whole-mounted FFPE radical prostatectomy specimens. We have focused this experiment to address ARP IHC performance in both CA and AA men leveraging the high representation of AA men enrolled to the CPDR’s Biospecimen Bank and National Database. In this study we carefully matched patients by clinicopathologic features including time range of RP. We have developed a numeric ARP IHC score (0-3) from the percentage and staining and intensity of index tumors. The numeric scores were further validated and refined to a three color IHC scale to represent ARP readout (Figure 8). With the exception of ERG mRNA detection in index tumors, frequency of attenuated mRNA expression of AR-regulated genes showed similar overall frequencies in AA or CA patients. The specificity of detecting attenuated ARP mRNA was 74% with a sensitivity of 53% in CA. ERG frequencies were shown to be significantly lower (28%) in AA men (Rosen et al., 2012) and only 17% in high grade tumors (Farrell et al., 2014). Therefore, in AA patients detection of ARP mRNA was less informative due to the high frequency of ERG negative index tumors.

Figure 8. Performance of ARP protein detection by IHC in poorly differentiated and well to moderately differentiated index tumors in Caucasian American (CA) and African American (AA) Men. Follow up range: 10-17 years; B= BCR; M= Metastasis. ARFI scores: IHC color intensities and percentage of staining of index tumors were calculated in a numeric scale of 0-3 and were converted to color codes as follows: red: normal, positive >2.4 (80-100%); yellow: decreased 1.2-2.4 (40-80%); green: absent, negative <1.2 (0-40%). Specificity and sensitivity for adverse clinical features, such as BCR and/or metastasis within a 10-17 years of follow up were assessed. Decreased expression of PSA and NKX3.1 was consistently observed in index tumors of both AA and CA men with poorly differentiated index tumors. The overall pattern of AR regulated genes were similar except ERG, that was absent in majority of index tumors of AA men. In CA patients the ARP IHC score showed 74% specificity and 54% sensitivity for predicting BCR and or metastasis. In AA men 52% the specificity and 47% sensitivity was observed likely due to the high frequency of ERG negative tumors.
To evaluate the performance of ARP mRNA detection, in Year # 4 we have employed the quantitative technology platform, NanoString. The NanoString platform is suitable for the analyses of RNA isolated from FFPE tissues, probes can be multiplexed yielding absolute quantitation by counting the copy number of detected mRNAs. The expression levels of AR-regulated genes (ERG, PSA/KLK3, PMEPA1, ODC1, and AMD1) were determined in 63 macrodissected FFPE-derived tumor samples by NanoString platform. The study design was focused on low grade disease (Gleason 3+3 or 3+4; no positive margins, no extracapsular extension, and no seminal vesicle invasion) with (N= 21) or without (N=42) recurrence (BCR or distant Metastasis). NanoString analysis was performed on all 63 macrodissected tumor cDNA samples (Figure 9.).

Leveraging the observation that measuring mRNA levels of the ARP suggested better performance in predicting favorable outcome in low grade disease (Figure 7) we evaluated the prognostic potential of androgen regulated genes by the NanoString platform. **Analyses revealed that the ARP gene panel differentiates the BCR group from the BCR-free group with 74% specificity and 76% sensitivity.**

The remarkable separation power of androgen regulated genes (ERG, AMD1, PMEPA1, ODC1 and PSA) is shown by significantly lower frequency of attenuated ARP gene expression within the patient group with BCR-free survival (No-BCR) and by the frequent detection of decreased ARFI gene expression within the BCR group. Green in the heat map represents index tumors for each patient with gene expression below the cutoff value.

**Figure 9. Assessment of ARFI gene expression in tumor mRNA samples of low grade disease indicates high specificity for confirming BCR-free survival.** BCR group is marked by BCR or No-BCR in the heading. **Copy number cutoff** for BCR of ERG, AMD1, PMEPA1, ODC1 and KLK3/PSA mRNA is indicated on the right. **Red color marks normal expression levels, green indicates decreased expression of ARFI genes below the copy number cutoff values in tumors.**

**c) Other achievements:**

We have completed a collaborative study with Dr. Lukas Bubendorf, University of Basel directly addressing the correlation of ERG protein expression (as the result of androgenic activations) and ERG gene rearrangements on the progression to castration resistant prostate cancer (CRPC) (Figure 10.). In this study design we examined tissue microarray from 114 hormone naïve and 117 CRPCs. We analyzed the expression of ERG oncoprotein by IHC and ERG rearrangement status by

Figure 10. The presence of ERG fusions with absent ERG protein indicates a thus far unrecognized subset of CRPCs with dispensed AR pathway.
fluorescence in-situ hybridization. Also, we monitored the protein expression levels of AR and the proliferation marker Ki67. Consistent with previous reports the *TMPRSS2-ERG* gene fusion status showed correlation with the presence or absence of ERG protein both in hormone naïve and in CRPC specimens \(p<0.0001\). The major fining of the study is the complete absence of ERG protein in 26% of CRPC cases harboring ERG genomic rearrangement. Thus, presence of ERG fusions with absent ERG protein revealed a thus far unrecognized subset of CRPCs with dispensed AR pathway who may not benefit from conventional therapy directed against the AR pathway (Gsponer et al., PCPD 2014). This finding is consistent with the central hypothesis of the proposal.

d) Opportunities for training and professional development

This project provided training opportunity to a 4th year Urology Resident, WRNMMC under the half year rotation to the CPDR Resident’s Translation Research Training Program. The goal of the resident’s research was to establish the numeric scoring system for the IHC staining intensities of ARP genes and draft the manuscript and meeting abstracts towards the publication of findings.

4. IMPACT:

With the support of the PCRP/CDMRP/DoD Idea Development Award we investigated the expression levels of an AR regulated gene panel, ARP to improve patient stratification for outcome prediction and for specific therapeutic approaches in prostate cancer treatment. In our approach we selected genes leveraging the mechanistic understanding of direct AR regulated genes. Thus, our approach is distinct from the empirically designed OncotypeDX Prostate mRNA-based AR-regulated gene set that has been recently introduced to clinical practice by Genomic Health Inc.

The major finding emerging from our PCRP/CDMRP award is that there is clear difference between the utility of ARP protein detection and the detection of ARP mRNA levels in prostate cancers.

1) Monitoring AR function at protein expression levels of androgen regulated genes may provide more easily adaptable and cost effective IHC-based approach in routine pathology settings. The association of AR function defects, decreased or attenuated expression of ARP proteins, showed 74% specificity in predicting BCR/distant metastasis in support of unfavorable clinical-pathologic features. This performance was demonstrated among CA patients.

2) Assessment of AR function at mRNA expression levels showed promising performance in evaluating low grade index tumors. Attenuated ARP mRNA expression was frequently observed in patients who later experienced BCR. In contrast ARP mRNA defect was rarely found in index tumors of patients with BCR-free survival yielding an overall 74% specificity and 76% sensitivity for BCR.

3) Examination of castration resistant prostate cancers our collaborative study revealed the absence of ERG protein in 26% of CRPC cases harboring *ERG* genomic rearrangement. Thus, presence of *ERG* fusions with absent ERG protein indicates a thus far unrecognized subset of CRPCs with dispensed AR pathway who may not benefit from conventional therapy directed against the AR pathway.

In summary, consistent with the central hypothesis of this proposal the study results indicate the utility of monitoring the defects of AR function in prostate cancer. The observed readouts of AR dysfunction may
provide practical tool for improved prognostic accuracy and patient stratification to predict treatment response at early stages of prostate cancer (Figure 11).

5. CHANGES/PROBLEMS:

There were no changes to the original central hypothesis and specific aims. This award has been granted for a twelve month no cost extension by the grant agency Grant Officer with an approved SOW to solidify promising findings generated under this award focused on the evaluation of the AR gene panel towards defining predictive power and utility in patient stratification. The task of the comparative assessment of mRNA and protein ARP by branched-chain DNA (b-DNA) signal amplification method has been replaced by a comparison of microarray (Affymetrix GeneChip) derived tumor gene expression data set with IHC staining from matched tumor specimens. This change was due to limitations in the accuracy of branched-chain DNA (b-DNA) signal amplification method. The change had no impact on the overall progress of the proposal. Evaluating the mRNA expression levels of ARP genes we have completed the analysis of 77 LCM sample. To extend this task we have employed a new and more quantitative technology platform, NanoString. Initial data of ARFI mRNA performance in low grade prostate cancers suggests remarkable specificity for the prediction of BCR-free survival. Building on the encouraging results we are aiming to further develop the ARP panel to pre-clinical validation phase. Although, significant correlations were noted between attenuated expression of ARP and disease progression, converting the ARP readouts to nomogram was challenging due to the overall low number of examined cases and events. However, further effort to achieve this goal is warranted.
6. PRODUCTS:

a) Peer-reviewed articles


  Acknowledgement of PCRP/CDMRP/ DOD support: yes


  Acknowledgement of PCRP/CDMRP/ DOD support: yes


b) Podium presentation

Albert Dobi, Denise Young, Lakshmi Ravindranth, Wei Huang, Aaron Brothers, Shashwat Sharad, Hua Li, Gyorgy Petrovics, David G. McLeod, Isabell A. Sesterhenn and Shiv Srivastava: Evaluation of Androgen Receptor Dysfunction to Enhance Stratification for Prostate Cancer Treatment. American Urological Association (AUA) Annual Meeting, Hungarian-American Section, May 16-21, 2014, Orlando, FL

  Acknowledgement of PCRP/CDMRP/ DOD support: yes


  Acknowledgement of PCRP/CDMRP/ DOD support: yes

c) Moderated poster presentation

Albert Dobi, Denise Young, Lakshmi Ravindranth, Wei Huang, Aaron Brothers, Shashwat Sharad, Hua Li, Yongmei Chen, Gyorgy Petrovics, David G. McLeod, Isabell A. Sesterhenn, Shiv Srivastava: Evaluation of androgen receptor function in prostate cancer. *American Urological Association (AUA) Annual Meeting*, May 16-21, 2014, Orlando, FL

  Acknowledgement of PCRP/CDMRP/ DOD support: yes

Acknowledgement of PCRP/CDMRP/ DOD support: yes

d) Poster presentations


Albert Dobi, Denise Young, Wei Huang, Lakshmi Ravindranath, Shashwat Sharad, Hua Li, Gyorgy Petrovics, David G. McLeod, Isabell A. Sesterhenn and Shiv Srivastava.: **Assessment of the Androgen Receptor Function Index (ARFI) in Prostate Cancer.** American Association for Cancer Research (AACR) Annual Meeting, April 6-10, 2013, Washington, DC


7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATION

There was no change in the active other support of the PD/PI(s) or senior/key personnel. This award supported the employment and post-doctoral training of Shashwat Sharad, PhD. He has contributed to the qRT-PCR analysis of *PMEPA1*, *PSA* and *GAPDH* genes. The Research Assistant position of this award, supported the salary of Ms. Wei Huang, MS., a full-time employee of CPDR. She has experience in key techniques pertinent to this proposal. Ms. Huang supported the completion of tasks in Aim #1 and Aim #2.

Biostatistician Yongmei Chen, MD, MPH. (5%) effort performed the analysis of clinic-pathology data correlations. Correlation analyses include the qRT-PCR and IHC data towards developing the ARP cumulative index.

4th year Urology Resident (no salary from this award), CPT Aaron Brothers, MD, WRNMMC was under the half year rotation to the CPDR Resident’s Translation Research Training Program. The goal of the resident’s research was to establish the numeric scoring system for the IHC staining intensities of ARP genes and draft the manuscript.

Collaborators: Lukas Bubendorf, MD, and Christian Ruiz PhD, from Institute for Pathology, University Hospital Basel, University of Basel, Switzerland collaborators performed the assessment of Castration Resistant Prostate Cancers. The PI and Co-PIs of the PCRP/CDMRP award provided study design, reagents, data analysis and interpretation.

8. SPECIAL REPORTING REQUIREMENTS

N/A
9. REFERENCES


Hermans, KG, van Marion R, et al. TMPRSS2:ERG fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer. Cancer Res. 2006, 66(22): 10658-10663.


10. APPENDICES

(Attached)
ORIGINAL ARTICLE

ERG rearrangement and protein expression in the progression to castration-resistant prostate cancer


BACKGROUND: Approximately half of the prostate carcinomas are characterized by a chromosomal rearrangement fusing the androgen-regulated gene TMPRSS2 to the oncogenic ETS transcription factor ERG. Aim of this study was to comprehensively analyze the role and impact of the ERG rearrangement and protein expression on the progression to castration-resistant (CR) disease.

METHODS: We used a tissue microarray (TMA) constructed from 114 hormone naive (HN) and 117 CR PCs. We analyzed the ERG rearrangement status by fluorescence in situ hybridization and the expression profiles of ERG, androgen receptor (AR) and the proliferation marker Ki67 by immunohistochemistry.

RESULTS: Nearly half of the PC tissue specimens (HN: 38%, CR: 46%) harbored a TMPRSS2-ERG gene fusion. HN PCs with positive translocation status showed increased tumor cell proliferation (P < 0.05). As expected, TMPRSS2-ERG gene fusion was strongly associated with increased ERG protein expression in HN and CR PCs (both P < 0.0001). Remarkably, the study revealed a subgroup (26%) of CR PCs with ERG rearrangement but without any detectable ERG protein expression. This subgroup showed significantly lower levels of AR protein expression and androgen-regulated serum PSA (both P < 0.05).

CONCLUSIONS: In this study, we identified a subgroup of ERG-rearranged CR PCs without detectable ERG protein expression. Our results suggest that this subgroup could represent CR PCs with a dispensed AR pathway. These tumors might represent a thus far unrecognized subset of patients with AR-independent CR PC who may not benefit from conventional therapy directed against the AR pathway.

Prostate Cancer and Prostatic Disease advance online publication, 28 January 2014; doi:10.1038/pcan.2013.62

Keywords: castration resistance; TMPRSS2-ERG; ERG

INTRODUCTION

Prostate cancer (PC) is the most frequently diagnosed cancer among males in western countries and the second leading cause of cancer-related death. Although the mortality of PC has decreased mainly due to earlier detection, this disease still accounts for 9% of the total cancer deaths. Most PCs are nowadays diagnosed at an early stage. They initially depend on androgens for their growth and are thus referred to as hormone naive (HN) PC. Based on this dependence, the standard treatment for patients harboring these tumors is androgen-deprivation therapy (ADT). Although this therapy is initially effective, most of the treated tumors recur after a few months or years as castration-resistant (CR) PC. Mechanisms responsible for this progression are not fully understood.

PC research was revolutionized by the discovery of the TMPRSS2-ERG gene fusion in 2005. Later on, it was realized that this rearrangement was part of a whole family of gene fusions that connect the promoter region of androgen-regulated genes, most frequently the TMPRSS2 (transmembrane protease inhibitor 2) with transcription factors of the ETS (erythroblastosis virus E26 transforming sequence) family of transcription factors. Of these fusions, the rearrangement involving the genes TMPRSS2 and ERG is by far the most common (> 90%) and is present in approximately 50% of prostate tumors. The two involved genes are < 3 Mb apart on chromosome 21, and their fusion can occur through various rearrangements mechanisms, most frequently deletion of the intervening region on chromosome 21 (reviewed in Tomlins et al. and Perner et al.). This rearrangement results in an androgen regulation of the ERG gene, leading to the overexpression of this gene in prostatic adenocarcinoma (reviewed in Sreenath et al.). Despite the extensive studies about the role of the ERG rearrangement and expression, its clinical significance remains controversial. Recently, Minner et al. did not observe any prognostic impact in a larger cohort of radically operated PCs.

In CR PC, ERG rearrangement has been shown to prevail in 34–45% of the tumors. Very recently, we observed a higher frequency of ERG rearrangements (45%) in recurrent CR PC specimens and a lower frequency of 25% in metastatic CR PCs. In contrast to the rearrangement, which is present on a genomic level, ERG protein expression is more dynamic, as it depends on the presence and activation of the androgen receptor (AR). In the CR disease state, the tumor may adapt to very low levels of androgens. Thus, it is not evident if these levels are sufficient for...
the activation of ERG transcription. Data from these investigations have provided controversial results: whereas in some CR PC xenograft experiments ERG mRNA expression was not detectable, others have shown ERG protein expression in rearranged CR PC samples and xenografts. In the present study, we used a tissue microarray (TMA) consisting of 231 locally advanced PCs that were collected either before (HN) or after recurrence to ADT (CR). We used this TMA to comprehensively interrogate and characterize the ERG protein expression and rearrangement comparing HN and CR PCs. We included standard markers into our analyses known to be relevant in PC, such as AR protein expression and Ki67 labeling. Here, we show that a considerable fraction of ERG-rearranged CR PCs loses ERG protein expression. We hypothesize that this might be due to a dispersed AR pathway.

MATERIALS AND METHODS

TMA and patients

The use of clinical specimens for the construction of the castration resistance TMA (crTMA) was approved by the ethical committee of the University and the University Hospital of Basel, Basel, Switzerland. The crTMA was manufactured as previously described. Briefly, tissue cylinders with a diameter of 0.6 mm were punched from the ‘donor’ tissue blocks containing the TURP specimens using a home-made, semi-automatic robotic precision instrument. Three cores from each specimen were arrayed. The composition of the crTMA has been previously described and is summarized in Supplementary Table S3. Briefly, it is composed of 697 spots from 231 TURPs from a total of 202 patients treated with advanced, locally obstructive PC. In addition, it contains 12 specimens from BPH. Castration resistance was defined as locally obstructive recurrence and/or PSA-recurrence during ADT.

Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH)

IHC was performed according to the standard indirect immuno-peroxidase procedures. The primary antibody was omitted for negative controls. All slides were read manually by an experienced pathologist (LB). Data from AR and Ki67 were available from a previous study on the same TMA block. Briefly, the antibodies M3562 and M7240 (both DAKO, Carpinteria, CA, USA) were used for AR and Ki67 staining, respectively. The anti-ERG mouse monoclonal antibody 9F7 was from Bicore Medical (Concord, CA, USA). FISH analysis for detection of ERG rearrangement was performed as previously reported. Images were obtained by usage of the AXIO Imager.A1 microscope equipped with an AxioCam and the AxioVision 4.6 software (all from Zeiss, Jena, Germany).

Cutoffs, data analysis and statistics

For protein expression analysis of AR, Ki67 and ERG, the percentage of positive tumor cells was noted by an experienced pathologist (LB) and used as score. For dichotomous stratification of ERG, samples with any specific positivity were considered as ERG positive (that is, cutoff > 0) and were considered negative in reference to endothelial ERG-positive staining. Cutoffs for definition of low or high for Ki67 labeling index were used as previously described. For correlation studies between different markers, every evaluable spot was considered for the analysis, that is, the analyses were performed on a ‘spot-by-spot’ basis. All other analyses (that is, descriptive tables, association with clinical data, such as treatment status, cm, ct and survival data) were performed on a ‘one-value-per-biopsy’ basis, thereby considering only one value per biopsy/specimen. If more than one spot/value per biopsy/specimen was available, the spot with the maximal score was included in the analysis. Statistical analysis was performed with the R Framework Version 3.0.1 including the ‘coin’ package. Differences between two groups were analyzed with the Wilcoxon’s rank-sum test; differences between more than two groups were analyzed using the Kruskal–Wallis rank-sum test for metric variables, for example, expression score. Fisher’s exact test were used to analyze contingency tables. Survival curves were plotted by usage of the Kaplan–Meier method, and differences were assessed using the log-rank test. P-values < 0.05 were considered as statistically significant.

RESULTS

ERG expression and TMPRSS2-ERG rearrangement in HN and CR PC and association with clinicopathological features

To interrogate ERG protein expression and rearrangement by IHC and FISH, respectively, in the context of progression to castration resistance, we used the recently described crTMA that was constructed for this purpose. In addition, we included IHC data for AR and Ki67 expression from a previous study. For ERG expression analysis, 78 (68%) and 88 (77%) out of 114 HN and 117 CR TURPs, respectively, were evaluable (Figure 1). Of note, only cases with unequivocal nuclear staining for ERG in endothelial cells were considered as evaluable. ERG FISH analysis was successful in 94 (83%) and 94 (81%) of the 114 and 117 HN and CR PCs, respectively. ERG protein positivity, as well as the presence of ERG rearrangement, showed similar distributions between HN and CR PC (Table 1a). The success rate of ERG protein positivity in 47% (37/78) and 40% (35/88) of the HN and CR PC samples. Similarly, 38% (36/94) and 47% (44/94) of the same samples showed ERG rearrangement. High-grade prostate intraepithelial neoplasias were not present in this TMA and thus not analyzed in this study. We did not observe ERG positivity in the 10 evaluable BPH samples present on this TMA. In addition, the crTMA comprises a unique set of 36 matched PC samples from the same patients before (HN) and after hormonal ablation therapy (CR). The analysis of this subset revealed a change of ERG status in individual patients to be rare (1/21 and 2/30 for IHC and FISH, respectively; Supplementary Table S1).

We next investigated a potential association between ERG status and clinicopathological features, such as cm and ct stages, and Gleason pattern. ERG status was not differentially distributed across different cm and ct stages (data not shown). Interestingly, only ERG protein expression but not ERG rearrangement revealed a significant decrease of positivity toward higher Gleason pattern. This was true in HN (P = 0.004) as well as in CR PCs (P = 0.019) (Table 1b). As PCs of higher Gleason pattern are characterized by higher tumor cell proliferation, we investigated a potential correlation between ERG status and Ki67 labeling index. We did not observe a correlation between ERG protein expression and increased tumor cell proliferation. This was also true for ERG rearrangement. However, stratification into HN and CR revealed that the proliferation index in ERG-rearranged HN was significantly higher than in those HN where ERG was not rearranged (55% vs 38%, P < 0.05, Supplementary Table S2).

No significant association of ERG status with overall survival of HN or CR PC patients

We analyzed the potential impact of ERG protein expression and rearrangement on overall survival. In both cohorts, HN as well as CR, neither ERG status nor ERG rearrangement were related to patient prognosis in Kaplan–Meier survival analysis (Supplementary Figure S1).

Decreasing correlation of TMPRSS2-ERG translocation with protein expression of ERG in CR PC

It is well established that ERG protein expression is dependent on the presence of an ERG rearrangement in prostatic adenocarcinoma. Here we investigated the power of this correlation in the cohort of the crTMA, which is composed of highly advanced PCs before (HN) and after ADT (CR). As expected, a high correlation between ERG rearrangement and ERG protein expression was observed (P < 0.0001). This was also true if PC samples were stratified according to their hormonal treatment status HN and CR (P < 0.0001, Table 2). Intriguingly, whereas in HN PCs, the number of FISH-IHC discordant results were minimal (7% FISH positive, but ERG negative and 9% FISH negative, but ERG positive), in CR PCs, 26% (13 spots) of the ERG-rearranged samples did not show detectable ERG protein expression (Table 2). This surprisingly large
group of \( {\text{ERG}} \)-rearrangement positive, but \( {\text{ERG}} \)-protein-negative PC samples in the CR, but not in the HN group, can hardly be explained by a technical phenomenon. These findings rather suggest that losing the high concordance between \( {\text{ERG}} \) FISH and \( {\text{ERG}} \) IHC toward more advanced PC samples may be due to the existence of a specific subset of CR PC patients whose tumors have lost the ability of expressing the \( {\text{ERG}} \) protein despite the presence of an \( {\text{ERG}} \) rearrangement. Of note, these 13 spots were from 11 different TURPs from 10 distinct patients.

We next interrogated the association between the AR protein expression and the \( {\text{ERG}} \) status. As previously described,\(^{18}\) AR protein expression was present in almost all analyzed PC samples and maximal (score = 100) in >90% of the specimens. Overall, we were not able to see a significant association between \( {\text{ERG}} \) rearrangement or positivity and AR expression (\( P > 0.05 \) both, data not shown). To analyze whether the \( {\text{ERG}} \) FISH vs IHC discrepancy in CR PC with \( {\text{ERG}} \) rearrangement but absent \( {\text{ERG}} \) protein is due to a loss of AR, we stratified the PCs into the different \( {\text{ERG}} \) subgroups according to the two treatment status. Although AR expression was present at high levels (score 90–100) in almost all PC samples, independent of the \( {\text{ERG}} \) status, only \( {\text{ERG}} \)-rearrangement-positive CR PCs with absent \( {\text{ERG}} \) protein were characterized by lower levels of AR protein (\( P = 0.002 \), Figure 2a). Further, we interrogated a correlation of \( {\text{ERG}} \) protein expression with serum protein levels of the AR target gene PSA in the subgroup of \( {\text{ERG}} \)-rearranged CR PCs. As expected, the group of \( {\text{ERG}} \)-rearrangement positive and \( {\text{ERG}} \)-protein-negative CR PC samples had lower PSA levels than \( {\text{ERG}} \)-rearranged- and \( {\text{ERG}} \)-protein-positive samples (\( P < 0.05 \), Figure 2b). However, it must be considered that PSA serum information was only available for four \( {\text{ERG}} \)-rearrangement-positive but \( {\text{ERG}} \)-protein-negative CR PC samples.

DISCUSSION

The rearrangement of the \( {\text{ERG}} \) gene\(^2\) and its associated expression in PC\(^{23}\) has been the subject of numerous studies. Depending on the cohort used, the prevalence of the rearrangement and protein expression varies extensively (15–80%).\(^{17}\) Most of the studies have focused on the analysis of material from surgically resected prostates. In this study, we interrogated the \( {\text{ERG}} \) status on gene and protein levels in TURP specimens originating from HN and CR prostate tumors. For this purpose, we used a TMA specifically constructed for the analysis of disease progression.\(^{18}\)
We observed an overall ERG positivity rate of 43% of both ERG rearrangement and IHC positivity across all PC samples. This is similar to recent reports that found ERG protein positivity in 47% and 52% of the PC samples\(^\text{11,24,25}\) and ERG rearrangement in 47–55%.\(^\text{11,26,27}\) Stratification into HN and CR PC revealed a broader range (38–47%), but no significant differential positivity between these two groups could be detected. Concordantly, in the matched patient cohort, virtually all of the patients retained their ERG status after recurring under ADT. Although earlier reports that had focused on ERG RNA expression analysis or were based on tissues from xenografts had reported controversial prevalence rates in CR PC,\(^\text{28}\) our findings are in line with a very recent study by Teng \textit{et al.}\(^\text{29}\) in which the authors observed the ERG expression in 37% of human CR PCs. These data strongly suggest that even lower levels of circulating androgens, as is the case under ADT therapy in patients with CR disease, are sufficient to sustain ERG expression in ERG-rearranged PC.

Although no correlation of ERG status with clinico-pathological features, such as cM or cT stage, was found, we observed that at least for the protein expression, positive ERG status was associated with lower Gleason pattern (Table 1b). Of note, this TMA was not tailored for the analysis of the Gleason pattern, as most (97%) of the arrayed PCs show a high Gleason pattern (4 or 5) (Supplementary Table S3). In previous studies, TMPRSS2-ERG-negative PCs have already been associated to the highest Gleason pattern.

<table>
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<tr>
<th>Table 1. Overview of the ERG status on the castration resistance tissue microarray (crTMA)</th>
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<tr>
<td>(a)</td>
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<td><strong>ERG status</strong></td>
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<td><strong>Immunohistochemistry</strong></td>
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<td>4</td>
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<tr>
<td>5</td>
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<td><strong>CR</strong></td>
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<td>3</td>
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<tr>
<td>4</td>
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<tr>
<td>5</td>
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Abbreviations: CR, castration resistant; FISH, fluorescence in situ hybridization; HN, hormone naive; NS, not significant; PC, prostate cancer.

(a) ERG status was significantly different between BPH and all PCs, but not between HN and CR. Fisher’s exact tests were used for comparisons.

(b) HN and CR prostate cancer samples without ERG protein expression are characterized by higher Gleason pattern. This association was not true for ERG rearrangement. The \(\chi^2\) test was used for comparison between the groups: Not rearranged vs rearranged and ERG \(+\) vs ERG \(-\) in HN samples. Fisher’s exact test was used for CR samples.

We observed an overall ERG positivity rate of 43% of both ERG rearrangement and IHC positivity across all PC samples. This is similar to recent reports that found ERG protein positivity in 47% and 52% of the PC samples\(^\text{11,24,25}\) and ERG rearrangement in 47–55%.\(^\text{11,26,27}\) Stratification into HN and CR PC revealed a broader range (38–47%), but no significant differential positivity between these two groups could be detected. Concordantly, in the matched patient cohort, virtually all of the patients retained their ERG status after recurring under ADT. Although earlier reports that had focused on ERG RNA expression analysis or were based on tissues from xenografts had reported controversial prevalence rates in CR PC,\(^\text{28}\) our findings are in line with a very recent study by Teng \textit{et al.}\(^\text{29}\) in which the authors observed the ERG expression in 37% of human CR PCs. These data strongly suggest that even lower levels of circulating androgens, as is the case under ADT therapy in patients with CR disease, are sufficient to sustain ERG expression in ERG-rearranged PC.

Although no correlation of ERG status with clinico-pathological features, such as cM or cT stage, was found, we observed that at least for the protein expression, positive ERG status was associated with lower Gleason pattern (Table 1b). Of note, this TMA was not tailored for the analysis of the Gleason pattern, as most (97%) of the arrayed PCs show a high Gleason pattern (4 or 5) (Supplementary Table S3). In previous studies, TMPRSS2-ERG-negative PCs have already been associated to the highest
Gleason category studied. Similarly, we observed that a high fraction of tumors with Gleason Pattern 5 is ERG negative: 67% and 70% for HN and CR PCs by IHC, respectively, as well as 70% for HN PC by FISH (Table 1b). Interestingly, the lower number of ERG FISH-positive CR PCs with Gleason Pattern 5 (58%) might be explained by the higher number of ERG-discrepant CR PC samples in this study (see below).

As expected, we found a strong correlation between genomic rearrangement and protein expression in HN as well as in CR PCs, confirming that ERG expression depends on the presence of the ERG rearrangement, also in more advanced CR PCs. Stratification into the different four FISH (negative/positive) and disease (HN/CR) subgroups revealed that in the subgroup of ERG-rearranged CR PC the rate of discordant samples was surprisingly high (26%), suggesting that every fourth ERG-rearranged CR PC will no longer express the ERG protein. As the discordance rates in the other three groups were between 7% and 9%, the high discordance rate of 26% might be attributed to the defects of androgen signaling. Very recently, Teng et al. had also reported a decrease in the serum PSA. As expected, we found a strong correlation between genomic rearrangement and protein expression in HN as well as in CR PCs, confirming that ERG expression depends on the presence of the ERG rearrangement, also in more advanced CR PCs. Stratification into the different four FISH (negative/positive) and disease (HN/CR) subgroups revealed that in the subgroup of ERG-rearranged CR PC the rate of discordant samples was surprisingly high (26%), suggesting that every fourth ERG-rearranged CR PC will no longer express the ERG protein. As the discordance rates in the other three groups were between 7% and 9%, the high discordance rate of 26% might be attributed to the defects of androgen signaling. Very recently, Teng et al. had also reported a decrease in the serum PSA.

ERG FISH positive but IHC negative), only four out of the 13 stained positive for neuroendocrine markers, thus suggesting that neuroendocrine trans-differentiation alone cannot explain the characteristics of this subgroup. The four poorly differentiated neuroendocrine CR PCs included two small cell prostate carcinomas and two large cell neuroendocrine carcinomas. Further studies are needed to investigate the specific characteristics of this ERG FISH-positive but ERH IHC-negative subset of PCs on a molecular level and to define the role of ERG rearrangement and expression.

A limitation of our study is that our cohort comprises locally advanced and obstructive tumors from palliative TURPs. Materials from TURPs for TMA construction must be rigorously examined before construction to exclude areas with technical artifacts originating from the resection procedure (for example, heat/mechanical damage). However, PC specimens from these TURPs represent very valuable tissue samples, especially in the context of hormonal ablation. In this study, the stratification into different disease states (HN/CR) and FISH positivity groups limited the sample number in the different subgroups. Thus, studies with larger cohorts of HN and CR PC samples from TURPs are needed to further assess these findings and to evaluate the AR-downstream signaling pathways in the distinct HN/CR ERG subgroups. Despite these limitations, in this study we were able to show the prevalence of ERG positivity in HN and CR PC and that this positivity is not differentially distributed between these two disease groups. Importantly, we provide evidence for the existence of an ERG-rearranged PC subset of cases that has apparently lost the ability to express androgen-regulated genes.

CONFLICT OF INTEREST

The Henry M. Jackson Foundation for the Advancement of Military Medicine has filed a patent application on the mouse monoclonal anti-ERG antibody, 9FY, on which ST, AD and SS are co-inventors and has been licensed to the Biocare Medical. The study was conducted independent of any involvement from Biocare Medical. The Brigham and Women’s Hospital and the University of Michigan have filed a patent on ETS gene rearangements in prostate cancer, on which SP is a co-inventor and the diagnostic field of use has been licensed to GenProbe. GenProbe has not played a role in the design and conduct of the study, in the collection, analysis or interpretation of the data and had no involvement in the preparation, review or approval of the manuscript. All the other authors declare no conflict of interest.

Figure 2. Differential androgen receptor (AR) and PSA protein expression in ERG-rearranged castration-resistant (CR) prostate cancer (PC). (a) AR protein expression in hormone naive (HN) and CR PC. Only ERG-rearranged CR PCs without AR expression (discordant samples) showed significantly lower levels of AR protein expression. (b) Serum PSA levels in ERG-rearranged CR PC. The discordant samples (see Figure 2a) showed reduced levels of serum PSA. Statistical test used: Wilcoxon rank sum. n.s., not significant.
ACKNOWLEDGEMENTS

This work was supported by the Krebsforschung Schweiz (KFS-02780-02-2011) to CR, by the German Research Foundation (Deutsche Forschungsgemeinschaft (DFG), Emmy-Noether-Program, PE1179-2/1), the Rudolf-Becker-Foundation and the Wilhelm-Sander-Foundation (No. 2011.077.1) to SP, by the RO1 DK065977 to SS and by the DoD, CDMRP, PC073614 to SS and AD. We thank Thuy Nguyen and Petra Hirschmann for excellent technical support.

REFERENCES


Supplementary Information accompanies the paper on the Prostate Cancer and Prostatic Diseases website (http://www.nature.com/pcan)
Supplemental information

Supplemental Figure 1. Overall survival of HN and CR PC patients. No significant association of ERG status with overall survival for HN (upper panels) or CR (lower panels) PC patients were observed.
Supplemental Table 1. ERG status in matched HN/CR patient cohort. Nearly all (IHC: 20/21, FISH: 28/30) of the PC samples retained their ERG status after recurrence to castration resistance. IHC: immunohistochemistry, FISH: fluorescence in-situ hybridization.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Status in HN</th>
<th>Status in CR</th>
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<tr>
<td>IHC</td>
<td>Negative</td>
<td>Negative</td>
<td>11 (52%)</td>
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<tr>
<td></td>
<td>Positive</td>
<td>Positive</td>
<td>9 (43%)</td>
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<tr>
<td></td>
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<td>Positive</td>
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<td></td>
<td>Positive</td>
<td>Negative</td>
<td>1 (5%)</td>
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<tr>
<td>FISH</td>
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<td>Negative</td>
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<td>13 (43%)</td>
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<td>Negative</td>
<td>1 (3%)</td>
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</table>

Supplemental Table 2. Correlation of ERG status with tumor cell proliferation. Significant association between ERG status and Ki67 LI (tumor cell proliferation) was only observed for FISH in HN PC. Fisher’s exact tests were used for comparisons. HN: hormone naïve, CR: castration resistant.

<table>
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<td>Not Rearranged</td>
<td>Rearranged</td>
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<th>CR: Low</th>
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<td>64</td>
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Supplemental Table 3. Overview of the cohort analyzed on this TMA. Summary of the HN and CR PC specimens on the crTMA used.

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<td>Median = 0.9 (± 0.7 - 1.2) years</td>
<td>Records = 109 Events = 68</td>
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Methylation of the PMEPA1 gene, a negative regulator of the androgen receptor in prostate cancer

Shashwat Sharad1, Lakshmi Ravindranath1, Michael C Haffner2, Hua Li1, Wusheng Yan1, Isabell A Sesterhenn3, Yongmei Chen1, Amina Ali1,4, Alagarsamy Srinivasan5, David G McLeod1,6, Srinivasan Yegnasubramanian7, Shiv Srivastava1, Albert Dobi1,8, and Gyorgy Petrovics1,*

1 Center for Prostate Disease Research; Department of Surgery; Uniformed Services University of the Health Sciences; Bethesda, MD USA; 2 Sidney Kimmel Comprehensive Cancer Center; Johns Hopkins University; Baltimore, MD USA; 3 The Joint Pathology Center; Silver Spring, MD USA; 4 Urology Service; Walter Reed National Military Medical Center; Bethesda, MD USA

Keywords: PMEPA1, methylation, androgen receptor, tumor suppressor, prostate cancer, laser capture microdissection

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).2–4 To suppress androgen-dependent CaP growth, currently used therapeutic agents inhibit the binding of androgens to AR or the biosynthesis of androgens.5–9 Although most CaP initially respond to androgen ablation, its therapeutic effect is short lived and patients eventually develop castration resistant CaP.5–9

The AR binds to AR-responsive elements (AREs) and regulates the transcription of androgen responsive genes controlling differentiation and growth.14 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.7,9 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 GSTP1, 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 GSTP1 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 GSTP1 methylation was detected in 50 of 77 (65%) cases (Fig. 1B). As expected GSTP1 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 GSTP1 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,55 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, assess-
ment of clinico-pathological data (Table 1) did not reveal correlation of biochemical
reappearance 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 GSTPI 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. GSTPI methylation was monitored as an
established methylated gene since its methylation status has been
established for these CaP cell lines.23,29 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 GSTPI is methylated in both AR positive
and negative CaP cell lines. These observations suggest a cellular
corelated 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 nega
tive 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. In fact, methylation and loss of expression of the *GSTP1*, a gene involved in the cellular redox maintenance, is present in a majority of CaPs. Moreover, *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. 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.

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supporting cancer progression. Thus, gain or loss of PMEPA1 expression and function may indeed cancer cell type dependent.

The differential methylation of 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.26,27,33-37 However, the distinct biology is not well understood and warrants further investigations, including the observed 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.38,39 With the increasing knowledge of epigenetic alterations linked to CaP, such as in GSTP1 or AR axis, more targeted therapeutic strategies may be possible. PMEPA1 may represent a new promising target for epigenetic drugs or new class of drugs complementing AR axis inhibitors.

In conclusion, the PMEPA1 gene is methylated in CaP primary prostate tumors suggesting that methylation may contribute to the silencing of PMEPA1. PMEPA1 gene expression levels correlated with its DNA methylation status. PMEPA1 methylation status displayed an intriguing difference between CA and AA patients. These data, along with our earlier observation, indicate that reduced or lost 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 PMEPA1 with potential impact on the AR axis in malignant prostate. Targeting AR for degradation by 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

Figure 4. (A) Methylation (+ or –) and expression (+, – or +/- intermediate) status of PMEPA1 and GSTP1 genes in AR positive and AR negative prostate cancer cell lines. VCaP, LNCaP, and LAPC4 cells harbor low PMEPA1 expression whereas no GSTP1 expression was detected in VCaP and LNCaP cells. (B) PMEPA1 expression is induced by the DNA methyl transferase inhibitor decitabine in VCaP, LNCaP, and LAPC4 cells after 14 d treatment. PMEPA1, GSTP1, and Beta-Actin protein levels were analyzed by immunoblot assays (upper panels). Gene expression was monitored by qRT-PCR (lower panels) and is shown as fold changes normalized to GAPDH control. (C) Reduced AR protein levels in LNCaP cells in response to decitabine treatment.

Figure 5. (A) Silencing of the PMEPA1 gene disrupts a negative control over AR leading to enhanced AR activity resulting in elevated levels of PSA. (B) AR and PSA protein levels are increased in response to PMEPA1 knockdown in LNCaP cells.
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 2 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′-CGTCTGCTCT GCTTAAAAACT; reverse primer, 5′-TTTGGGGAGAT GGGTTTTCAC) and **GSTP1** (forward primer, 5′-GGGACC CCTCC AGAAAGGC; reverse primer, 5′-ACTCAGGT GGCGAAGACT) 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′-CCGTCACCCC TTCTTTTGAC-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...
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.43

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.40-43 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.

qRT-PCR analysis of PMEPA1 and GAPDH expression was performed on Stratagene Mx3000P (Agilent Technologies, Inc.). The PCR primers for PMEPA1 were 5′-CATGATCCCC GAGCTGCT-3′ (forward) and 5′-TGATCTGAA AACCTCCAGC TCC-3′, (reverse) and the FAM-labeled probe was 5′-AGGCGGAGCAG TCTCCTGCGA AA-3′. GAPDH primers and probe mix were obtained from Life Technologies. All qRT-PCR reactions were performed in triplicate and data were analyzed by using MxPro v.3.2 software (Agilent Technologies Inc.). Amplification plots were evaluated and threshold cycle (CT) was set for each experiment. Multiplex measurements for target gene and GAPDH were averaged across triplicates and used to calculate standard deviation for each set. Subtraction of averaged GAPDH CT from averaged target gene CT yielded the ΔCT. Expression fold-change differences between normal and tumor was calculated by comparing ΔCT values among matched sample sets.

Cell culture

Human prostate tumor cell lines, VCaP, LNCaP were purchased from American Type Culture Collection (ATCC) and LAPC4 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 (5%) incubator at 37 °C. Cell culture and cell line treatment studies have been performed in two independent sets of experiments.

Decitabine (5-aza-2′-deoxycytidine) treatment

One million cells were seeded in T75 flasks in triplicate in the cell growth medium containing 0 μM, 2.5 μM and 5 μM of the DNA methyl transferase inhibitor, decitabine (5-aza-2′-deoxycytidine; Sigma-Aldrich) for 14 d. The media with decitabine were changed every 48 h. After 14 d cells were harvested for isolation of genomic DNA, total RNA and protein.

Transfection and siRNA knock-down of PMEPA1

The LNCP cells were seeded into 10 cm culture dishes (FALCON, Becton Dickinson) at the density of 1.5 × 10⁶ cells/ dish. The cells were incubated at 37 °C, 5% CO2 for 48 h. After incubation the cells were transfected with 50 nM of control Non-targeting (NT) siRNA and PMEPA1si RNA (5′-GTTATCACCAGGGTATATA-3′) (Dharmacon) by using Lipofectamine 2000 (Life Technologies). The cells were incubated with transfection complex 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).45

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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