DNA Methylation Screening of Primary Prostate Tumors Identifies *SRD5A2* and *CYP11A1* as Candidate Markers for Assessing Risk of Biochemical Recurrence

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Introduction

Prostate Cancer (PCa) is the second-leading cause of cancer-related deaths among American men. Currently, the best way to determine the probability of a prostate tumor becoming aggressive after the "Intermediate" stage involves an invasive prostate biopsy procedure to observe the pathology (ie. the organization of cells) of the tumor. This procedure may produce unintended harmful consequences to the patient and may over- or under- diagnose a patients disease as well. A typical treatment for supposed "serious cases" of the disease is to remove the prostate in a procedure called radical prostatectomy (RP). Post-RP, after the prostate is removed, doctors continue to observe the patient to ensure that there is no evidence of disease (NED). However, occasionally doctors detect levels of the prostate-specific antigen (PSA) which indicate the patient has experienced biochemical recurrence (BCR) and that there may be residual prostate tissue/tumor in the body. It is critical that we discover novel prognostic biomarkers to better predict a patient's disease outcome in a less invasive manner.

PCa is dependent on the androgen biosynthesis pathway (ABP). During normal prostate development and development within the tumor (tumorigenesis), the androgen receptor (a transcription factor which turns on/off genes) is activated by dihydrotestosterone (DHT). DHT is a product of the ABP. The ABP however has been shown to be dysregulated in different stages of tumor development in PCa [1,2]. Monitoring the changing dysregulation of the gene expression of the ABP may help determine which tumors will develop BCR or NED.

Tumors within patients can shed their DNA into the blood stream [3-5]. This is cell-free DNA (cfDNA). Scientists can observe the tumor-specific cfDNA in the blood because occasionally tumor-DNA has specific characteristics which are different from the DNA found everywhere else in the body. One of the characteristics which can be observed in tumor-specific DNA is "methylation" status. DNA methylation is an additional molecular mark on DNA which can alter gene expression and accumulates or decreases in specific areas of DNA, known as promoter regions, as cancer progresses. Regions which have more DNA methylation are called "CpG islands". We may be able to detect different levels of DNA methylation at these CpG Islands within promoter regions in DNA found in the blood. These differences could be used to predict diseases and how a patients cancer is progressing as well.

Abstract

BACKGROUND: Altered DNA methylation in CpG islands of gene promoters has been implicated in prostate cancer (PCa) progression and can be used to predict disease outcome. In this study, we determine whether methylation changes of androgen biosynthesis pathway (ABP)-related genes in patients' plasma cell-free DNA (cfDNA) can serve as prognostic markers for biochemical recurrence (BCR).
METHODS. Methyl-binding domain capture sequencing (MBDCap-seq) was used to identify differentially methylated regions (DMRs) in primary tumors of patients who subsequently developed BCR or not, respectively. Methylation pyrosequencing of candidate loci was validated in cfDNA samples of 86 PCa patients taken at and/or post-radical prostatectomy (RP) using univariate and multivariate prediction analyses.
RESULTS. Putative DMRs in 13 of 30 ABP-related genes were found between tumors of BCR (n=12) *versus* no evidence of disease (NED)(n=15). *In silico* analysis of The Cancer Genome Atlas data confirmed increased DNA methylation of two loci - *SRD5A2* and *CYP11A1*, which also correlated with their decreased expression, in tumors with subsequent BCR development. Their aberrant cfDNA methylation was also associated with detectable levels of PSA taken after patients' post-RP. Multivariate analysis of the change in cfDNA methylation at all of CpG sites measured along with patient's treatment history predicted if a patient will develop BCR with 77.5% overall accuracy.
CONCLUSIONS. Overall, increased DNA methylation of *SRD5A2* and *CYP11A1* related to androgen biosynthesis functions may play a role in BCR after patients' RP. The correlation between aberrant cfDNA methylation and detectable PSA in post-RP further suggests their utility as predictive markers for PCa recurrence.

Materials and Methods

DNA Methylation Quantification of Prostate Normal and Tumor Tissue and Cell-free Plasma: The first DNA methylation quantification method we used was Methyl-binding domain capture sequencing (MBDCap-seq)(Fig. 1A). MBDCap-seq identified "large" differentially methylated regions (DMRs) in primary tumors of patients who later experienced recurrent PCa (biochemical recurrence, BCR) or did not (no evidence of disease, NED), respectively. The second method we used for quantifying DNA methylation was pyrosequencing. Pyrosequencing is more specific for measuring the percentage of methylation at potential sites of DNA methylation (CpG sites). We measured DNA methylation of cfDNA samples from 86 PCa patients taken at and/or post-radical prostatectomy (RP) using univariate and multivariate prediction analyses.

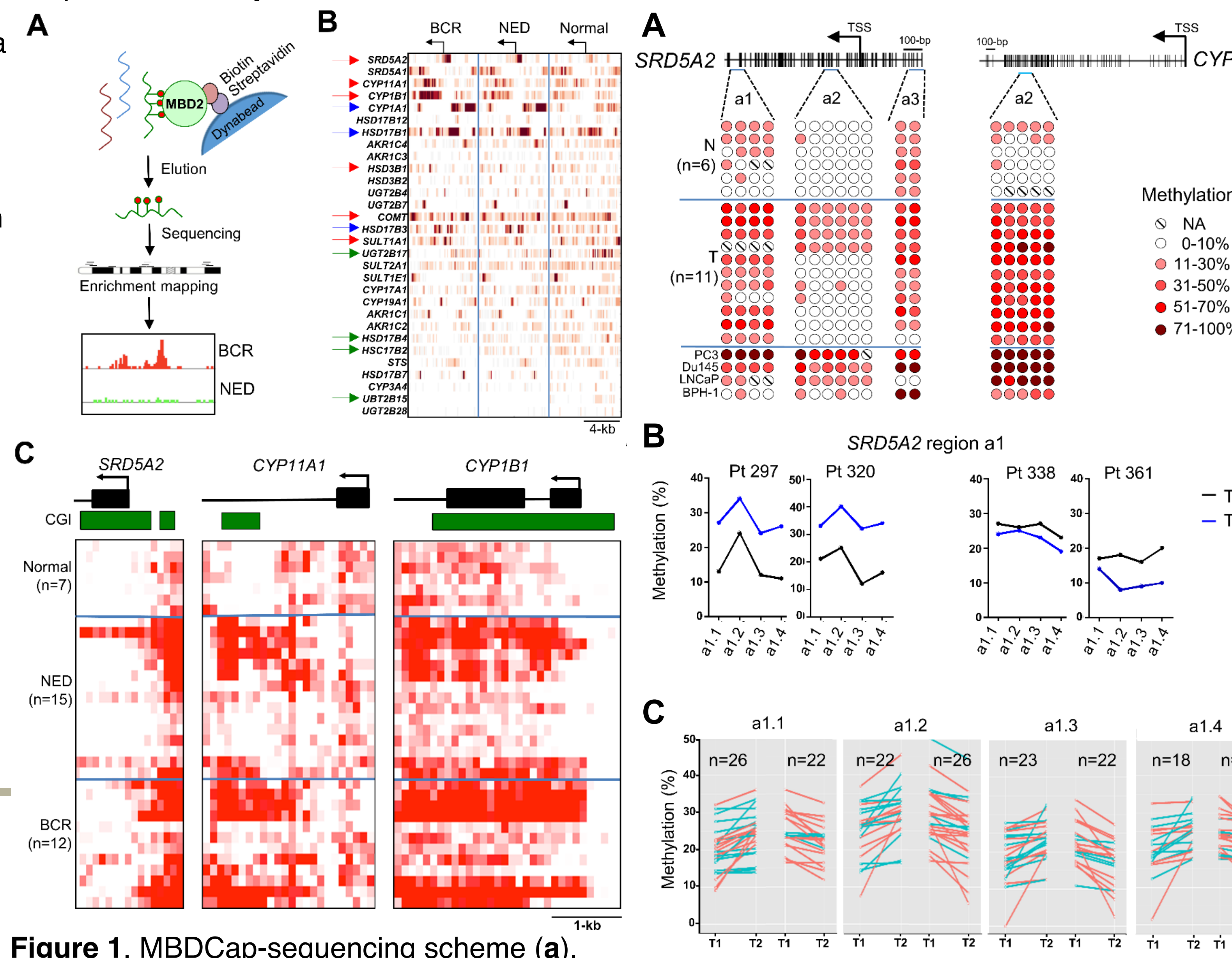

Figure 1. MBDCap-sequencing scheme (**a**). Tornado plot of differentially methylated genes in the ABP (**b**). Certain genes of the ABP indicating significant differences in DNA methylation (**c**).

TABLE I. Confusion Matrix for Consensus of Predictions Made by all 3 Modeling Algorithms

	Consensus Prediction Negative PSA Recurrence	Consensus Prediction Positive PSA Recurrence	
Negative PSA Recurrence (# of predictions)	14	6	Positive Predictive Value 70%
Positive PSA Recurrence (# of predictions)	3	17	Negative Predictive Value 85%
	Sensitivity 82.35%	Specificity 73.91%	Accuracy (Kappa,SE,95% CI) 77.5% (0.55,0.131,0.294-0.806)

Sensitivity, proportion of true positive cases which are correctly categorized; Specificity, proportion of true negative cases which are correctly categorized; Positive Predictive Value (PPV), the proportion of positive cases which were categorized correctly; Negative Predictive Value (NPV), the proportion of negative cases which were categorized correctly; Accuracy, the proportion of the total predictions which were correct.

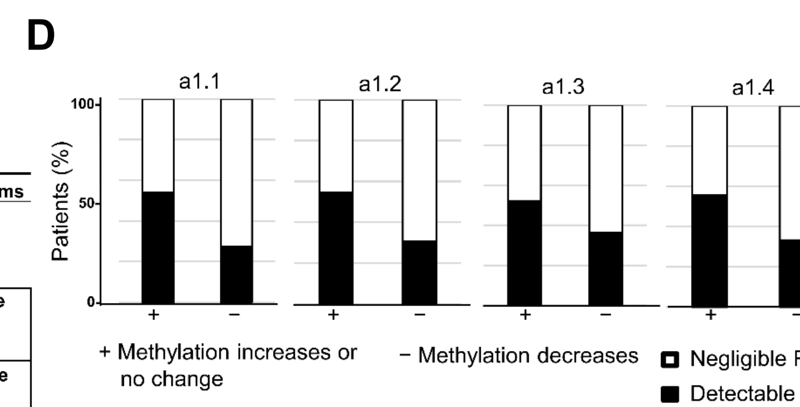

Figure 2. Pyrosequencing of tissue, cells (**a**) and plasma samples (**b**). Per CpG site, patients are grouped by whether they showed increased/stable methylation (ΔMeth%>=0,left) or decreased methylation (ΔMeth%<0,right) (**c**). The patient groups were further divided by whether or not they experienced detectable PSA recurrence (**d**).

Results

1. MBDCap-seq (Fig. 1A) results indicate that DNA methylation is significantly different between BCR and NED at the promoter regions of some of the ABP-related genes (Fig. 1B). Specifically, SRD5A2 and CYP11A1 promoter DNA methylation (Fig. 1C) were validated using an extra online database, The Cancer Genome Atlas (TCGA).
2. Pyrosequencing results indicate that tumor prostate tissues have more DNA methylation of *SRD5A2* and *CYP11A1* promoter regions (Fig. 2A) than normal counterparts.
3. Patient samples collected over time allowed us to observe the change in plasma cfDNA methylation of *SRD5A2* and *CYP11A1* (T2-T1=ΔMeth%)(Fig. 2B).
4. We found that patients which showed increases in cfDNA methylation, generally were positive for PSA above .04 ng/ml (Fig 2C and 2D).
5. Though univariate analysis of the individual CpG sites was not sufficient for predicting PSA detection post-RP (data not shown), 2 CpG sites' ΔMeth% values and a clinical factor (whether or not the patient received drug treatment) associated significantly with PSA detection. Multivariate analysis of these three factors accurately determined whether or not a patient's PSA would become detectable (Table 1).

Conclusion

By using two separate methods, we have shown that the DNA methylation of the promoter regions for some of the ABP-related genes are modified significantly between recurrent and non-recurrent PCa. Additionally, there exists a significant correlation between the hypermethylation at the promoter regions of *SRD5A2* and *CYP11A1* and their gene expression (data not shown), implicating an epigenetic role in the regulation of the ABP. Furthermore, the detectable changes in DNA methylation at *SRD5A2* and *CYP11A1* may serve as non-invasive plasma cfDNA prognostic biomarkers for BCR PCa. A broader investigation of the DNA methylation of the DNA regions beyond the promoter regions of the ABP-related genes could uncover clinically significant DMRs at other important expression regulatory regions as well.

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