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Survey of Differentially Methylated Promoters in Prostate Cancer Cell Lines[1]*

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Abstract

DNA methylation and copy number in the genomes of three immortalized prostate epithelial and five cancer cell lines (LNCaP, PC3, PC3M, PC3M-Pro4, and PC3M-LN4) were compared using a microarray-based technique. Genomic DNA is cut with a methylation-sensitive enzyme *Hpa*II, followed by linker ligation, polymerase chain reaction (PCR) amplification, labeling, and hybridization to an array of promoter sequences. Only those parts of the genomic DNA that have unmethylated restriction sites within a few hundred base pairs generate PCR products detectable on an array. Of 2732 promoter sequences on a test array, 504 (18.5%) showed differential hybridization between immortalized prostate epithelial and cancer cell lines. Among candidate hypermethylated genes in cancer-derived lines, there were eight (*CD44*, *CDKN1A*, *ESR1*, *PLAU*, *RARB*, *SFN*, *TNFRSF6*, and *TSPY*) previously observed in prostate cancer and 13 previously known methylation targets in other cancers (*ARHI*, *bcl-2*, *BRCA1*, *CDKN2C*, *GADD45A*, *MTAP*, *PGR*, *SLC26A4*, *SPARC*, *SYK*, *TJP2*, *UCHL1*, and *WIT-1*). The majority of genes that appear to be both differentially methylated and differentially regulated between prostate epithelial and cancer cell lines are novel methylation targets, including *PAK6*, *RAD50*, *TLX3*, *PIR51*, *MAP2K5*, *INSR*, *FBN1*, and *GG2-1*, representing a rich new source of candidate genes used to study the role of DNA methylation in prostate tumors.

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Introduction

Aberrant DNA methylation of CpG sites is among the earliest and most frequent alterations in cancer [1,2]. In many cases, DNA methylation at CpG, in or near the promoter or first exon of a gene, is associated with gene ''silencing.'' Multiple different methylases and proteins that either bind methylated DNA or unmethylated CpG are associated with: 1) transmitting the methylation status to other proteins in the chromatin; 2) maintaining the DNA methylation profile during replication; and 3) changing the methylation profile during differentiation of cells. Thus, as a result of methylation at multiple CpG sequences, chromatin structure in the promoter may be altered, preventing normal interaction with transcriptional machinery. If this occurs in genes critical to growth inhibition, the resulting silencing of transcription could promote tumor growth. Hypermethylation has been shown to be commonly associated with transcriptional inactivation for classic tumor suppressor genes, genes important for cell cycle regulation, and genes that mediate DNA mismatch repair [3].

At present, several molecular biology methods are routinely used to determine the methylation status of a CpG island. Among these, bisulfite nucleotide sequencing is a technique used for a detailed mapping of methylated cytosine residues within a gene promoter [4,5]. Restriction landmark genome scanning (RLGS) is a two-dimensional gel electrophoresis method that has been used to study genetic and epigenetic changes, including DNA methylation [6–8]. Microarrays allow many DNA sequences to be queried in parallel especially when the targets can be made into reduced complexity representations [9,10]. Using this method, the binding profile of proteins that interact specifically with methylated DNA sequences can be detected by chromatin immunoprecipitation (ChIP) [11–14]. Alternatively, DNA methylation can be detected directly by cleavage of the genome with a 5-methylcytosine–sensitive restriction enzyme. In one method, methylation at the methylation-sensitive restriction sites for *Bst*UI and *Hpa*II preserves certain methyl-insensitive *Mse*I fragments that are otherwise cleaved if the site is unmethylated. Difference is amplified polymerase chain reaction (PCR) products indicate differences in *Bst*UI and *Hpa*II methylation [15–17].

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Another method is to cleave with a methyl-sensitive restriction enzyme, size fractionate, and hybridize fractions to a microarray. As methylation will change the size of cleavage products, they will be in a different fraction [18–20]. A protocol for detecting methylation differences between two genomes using this class of methods is outlined in Figure 1. The protocol relies on the occurrence of two methyl-sensitive cleavage sites in close proximity. If the restriction sites are both unmethylated, they can be cleaved and primers can be ligated. When the distance between the ligated primers is short enough, the fragment can be amplified efficiently by PCR. If, on other hand, the DNA is methylated at one of the cleavage sites, it will not be cut at that site and a longer fragment will be produced. In most cases, this longer fragment will be sufficiently long that the PCR of the fragment does not occur efficiently. During PCR, thousands of cleavage–ligation fragments from unmethylated parts of the genome amplify with varying efficiencies, and their representation in the final pool of amplified products depends on the efficiency of their amplification. However, the efficiency of amplification of any particular fragment should remain similar between experiments. Thus, in general, differences in the starting amount of a particular fragment will be preserved in the same ratio after PCR. The reduction in complexity while preserving ratios relies on the same principles as those previously published methods for comparative genomic hybridization (CGH) [9] and expression analysis [10]. These differences are measured on an array of genomic regions; in this case, we monitored methylation changes on an array 2732 promoter–first exon regions [21,22].

The method is applied to eight prostate epithelial cell lines—three immortalized epithelial lines and five lines derived from cancers. Differences in copy number can also be detected in this and all the other high-throughput methods referenced here. Here, copy number changes, which are also of interest, are distinguished from methylation changes by a variety methods, such as methylation-specific PCR (MSP), and by 5-aza-2′-deoxycytidine (DAC) treatment. Some of the methylation differences revealed have frequently been observed in prostate cancer, including *CD44*, *CDKN1A*, *ESR1*, *PLAU*, *RARB*, *SFN*, *TNFRSF6*, and *TSPY*, and others are previously known methylation targets in other cancers, including *ARHI*, *bcl-2*, *BRCA1*, *CDKN2C*, *MTAP*, *PGR*, *SLC26A4*, *SPARC*, *SYK*, *TJP2*, *UCHL1*, and *WIT-1*. However, most of the methylation candidates are potentially new targets that will need to be confirmed in tumors.

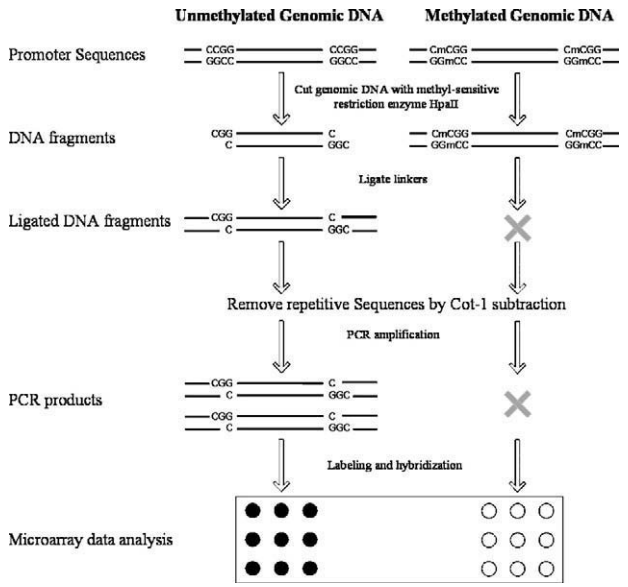

Figure 1. *Schematic of the protocol for detecting differences in HpaII fragment amplification between samples.*

Materials and Methods

Cell Culture

Human prostate epithelial cell lines 267B1, RWPE-1, and Ml-csv40 were kindly provided by Dr. J. Rhim (U.S. Navy Hospital, Bethesda, MD). LNCaP was obtained from ATCC (Manassas, VA), and PC3, PC3M, PC3M-Pro4, and PC3M-LN4 were kindly provided by Dr. Isaiah J. Fidler (M. D. Anderson Hospital Cancer Center, Houston, TX). Cells were cultured in RPMI medium containing 10% fetal bovine serum and 4 mM L-glutamine. LNCaP was also cultured in the presence of mock (PBS) or DAC (1 μm, medium changed every 24 hours; Sigma-Aldrich Co., St. Louis, MO) and the cells were harvested at 24 hours after the third dose. Genomic DNA and total RNA were extracted from cell lines using DNeasy Tissue Kit and RNeasy Mini Kit (Qiagen, Inc., Valencia, CA), respectively.

Preparation of Promoter Microarray

A detailed description of the promoter array used here has been published [21,22]. Briefly, human promoter sequences (1000 bp upstream and 500 bp downstream from transcription initiation site) were retrieved batchwise from http://genome.ucsc.edu/. PCR primers were selected using an in-house version of Primer3 (http://www.broad.mit.edu/genome_software/other/primer3.html). Promoter fragments with an average length of 1.2 kb were amplified, purified, and spotted onto UltraGAPS-coated slides (Corning, Inc., Corning, NY) in the presence of 50% DMSO. The promoter microarray contains triplicate spots of 3083 promoter sequences (2732 when duplicates are considered), 787 non-promoter controls, and 192 nonhuman controls. Many of the promoters on the array are from genes of particular relevance to cancer, and the array includes promoters from most of the genes that are known to be regulated by methylation in cancer. The array is freely available to collaborators.

Methylation Microarray Analysis

*Hpa*II (New England Biolabs, Beverly, MA) digestion was performed in 20 μl containing 0.5 μg of genomic DNA and 5 U of *Hpa*II for 2 hours at 37°C. Ten-fold overdigestion, plus monitoring of the digestion of lambda DNA mixed with human genomic DNA in a parallel reaction, were used to minimize the possibility of partial digestion. The digested fragments