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Genome-Wide Methylation Analysis of Prostate Tissues Reveals Global Methylation Patterns of Prostate Cancer

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Altered genome methylation is a hallmark of human malignancies. In this study, high-throughput analyses of concordant gene methylation and expression events were performed for 91 human prostate specimens, including prostate tumor (T), matched normal adjacent to tumor (AT), and organ donor (OD). Methylated DNA in genomic DNA was immunoprecipitated with anti-methylcytidine antibodies and detected by Affymetrix human whole genome SNP 6.0 chips. Among the methylated CpG islands, 11,481 islands were found located in the promoter and exon 1 regions of 9295 genes. Genes (7641) were methylated frequently across OD, AT, and T samples, whereas 239 genes were differentially methylated in only T and 785 genes in both AT and T but not OD. Genes with promoter methylation and concordantly suppressed expression were identified. Pathway analysis suggested that many of the methylated genes in T and AT are involved in cell growth and mitogenesis. Classification analysis of the differentially methylated genes in T or OD produced a specificity of 89.4% and a sensitivity of 85.7%. The T and AT groups, however, were only slightly separated by the prediction analysis, indicating a strong field effect. A gene methylation prediction model was shown to predict prostate cancer relapse with sensitivity of 80.0% and specificity of 85.0%. These results suggest methylation patterns useful in predicting clinical outcomes of prostate cancer. *(Am J Pathol 2013, 182: 2028–2036; http://dx.doi.org/10.1016/j.ajpath.2013.02.040)*

Prostate cancer is one of the most prevalent malignancies among American men, with approximately 280,000 new cases diagnosed annually. Each year up to 28,050 patients with prostate cancer die in the United States alone, and mortality from prostate cancer is only second to lung carcinoma in the United States.[1] Although most prostate cancers are indolent and responsive to the available hormone therapies, a significant number of cases become hormone refractory and metastatic. The precise cause of prostate cancer progression has remained elusive, despite extensive research efforts and recent advances in our understanding of this disease. Comprehensive gene expression and genome analyses have suggested that a global pattern of gene expression and copy number alterations exist for prostate cancer.[2–4] The related gene products include critical molecules in signaling pathways, DNA replication, cell growth, cell cycle checkpoints, and apoptosis.[5–8]

Hypermethylation of the gene promoter is a well-known epigenetic event that silences gene expression and is a critical regulatory component in normal physiology, mediating gene imprinting for inactivation of the X chromosome[9] and tissue-specific gene expression,[10] and in pathological processes, mediating inactivation of tumor suppressor genes and promoting tumorigenesis.[11,12] Addition of a methyl group to the cytosine residue of CpG dinucleotides by methyltransferase creates a binding motif for methyl-cytosine binding proteins, methyl-CpG-binding domain (MBD) or methyl-CpG-binding protein 2 (MeCP2), which in turn produces steric hindrance at the CpG clusters located in the promoter regions to transcriptional activators or repressors.[13,14] Silencing of genes involved in cell cycle control, cell survival, DNA damage repair, and signal transduction is the characteristics of cancer cells.[15–23] There is a lack of global

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correlation of CpG island methylation and gene expression in prostate cancer. To map the epigenetic regulation leading to altered expression of hundreds of genes in prostate cancer, we performed a genome-wide concordance analysis of gene methylation and expression in matched prostate tumor (T), benign prostate tissues adjacent to cancer (AT), and organ donor prostate without history of urological disease or any malignancy (OD). We found unique methylation profiles that distinguished the T, AT, and OD samples.

Materials and Methods

Genomic DNA Preparation

Ninety-one specimens of prostate cancer and adjacent benign prostate tissues and organ donor prostates were obtained from University of Pittsburgh Tissue Bank in compliance with institutional regulatory guidelines (Supplemental Table S1). To ensure high purity ($\geq$80%) of tumor cells, needle-microdissection was performed by pathologists to isolate the tumor cells from adjacent normal tissues ($\geq$3-mm distance from the tumor). For AT and OD samples, similar needle-microdissections were performed to achieve 80% epithelial purity. Genomic DNA of these T and AT tissues was extracted with a commercially available tissue and blood DNA extraction kit (Qiagen, Hilden, Germany). The protocols of tissue procurement and procedure were approved by Institution Board of Review of the University of Pittsburgh.

Immunoprecipitation and Amplification of Methylated DNA

Each DNA sample was divided into two aliquots of 250 ng and was digested with either StyI or Nsp1 (New England BioLabs, Ipswich, MA) at 37°C for 2 hours, followed by ligation to the corresponding StyI or Nsp1 adapters (Affymetrix, Santa Clara, CA) at 16°C for 16 hours. The two adapter-ligated DNAs were pooled and purified with an Amicon Ultra-centrifugation filter (Millipore, Billerica, MA). Fifty nanograms of the purified DNA in 450 μL of Tris-EDTA buffer were denatured by boiling in water for 10 minutes. Methylated DNA was then immunoprecipitated with 5 μg of anti−5-methylcytosine antibody (Zymo Research, Irvine, CA) in immunoprecipitation buffer (10 mmol/L $NaPO_4$, 140 mmol/L NaCl, and 0.05% Triton X-100, pH 7) by rocking at 4°C for 2 hours. The immunocomplexes were captured with magnetic bead-protein A/G (Millipore) by shaking at room temperature for 30 minutes. Three washes were performed with 1 mL of immunoprecipitation buffer, and additional washes with 300 μL of elution buffer heated to 50°C for 10 minutes were performed until the DNA in flow-through reached zero. Methylated DNA was then eluted from the beads with 100 μL of elution buffer heated to 75°C for 5 minutes. The eluted DNA was PCR amplified with titanium DNA polymerase and primer 002 from Affymetrix SNP 6.0 reagent kit, using 30 thermal cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 65°C for 60 seconds. Amplification efficiency was assessed by resolving amplicons with 1% agarose gel electrophoresis. Samples were then purified and fragmented by incubating with DNase1 at 37°C for 35 minutes. The fragmented DNA samples (range, 100−200 bp) were biotin labeled with terminal transferase at 37°C for 4 hours and hybridized to Affymetrix human whole genome SNP 6.0 chips at 50°C for 19 hours. After washes with 6× SSPE (saline, sodium phosphate, EDTA) buffer and staining with phycoerythrin streptoavidin in an automated Affymetrix fluid station, the chips were scanned by Affymetrix GeneChip scanner 3000 7G.

Sample Baseline Genome Copy Number Analysis

To determine the baseline copy number of each sample, genome DNA of each sample was analyzed with the Affymetrix SNP 6.0 chip. Briefly, the adaptor ligated DNA was prepared from StyI and Nsp1 digestion as described in Immunoprecipitation and Amplification of Methylated DNA. The efficiency of amplification was verified by resolving amplicons with 1% agarose gel electrophoresis. The total amount of purified amplicons was in the range of 200 to 250 μg.[3,24] As described in the section above, the amplicons were DNAse1 fragmented, biotin labeled, hybridized to the Affymetrix SNP 6.0 chips, and processed for genome copy number analysis.

DNA Methylation Analysis

The hybridization signals of methylation-enriched DNA from 91 prostate tissues were analyzed by Partek Genomics Suite 6.6 (Partek, Inc., St. Louis, MO). Pair-wise copy number analyses of methylation-enriched DNA were performed with genome copy numbers of the unenriched DNA samples as baselines with criteria of marker numbers >10 and segment length >2000 bp. The segments that were detected as amplified or unchanged in comparison with the baseline copy number of the duplicate samples were considered to be enriched by the methylation-specific antibodies. These methylated fragments were then screened for CpG islands. Criteria of 50% C and G and expected CpG of 0.65 in a region of >200 bp was used to define a CpG island.[25−27] The CpG islands located in the region of 1000 bp upstream and 500 bp downstream of mRNA start site of a gene were designated as gene-associated CpG islands and were annotated through the UCSC (University of California Santa Cruz) genome build hg18. Microarray data location of Raw *.cel files of data will be available at Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo*; accession number GSE45000).

Functional Analysis of Differentially Methylated Genes

The differential profiles of methylated genes were determined by comparing the CpG islands of T/OD, AT/OD, and T/AT, respectively. For each given gene-associated CpG island, the fraction of samples that were methylated was