GENOMICS INFORMATICS PROTEOMICS METABOLOMICS

ATCTGATCCTTCTGAACGGAACTAATTTCAA
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Genome-wide Monitoring of CpG Island Methylation With Agilent's Tiling Microarray Technology

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Abstract

DNA methylation profiling has emerged as an important technique for understanding the role methylation plays in cellular processes and as a potential diagnostic and prognostic indicator in diseases such as cancer. Of increasing importance is the ability of the researcher to observe genome-wide methylation patterns in multiple samples. We have combined tiling CpG Island Microarrays with the methyl DNA immunoprecipitation method (mDIP; Weber et al., 2005) to compare relative methylation patterns across CpG islands in two model systems—X-chromosome inactivation and cancer profiling.

Introduction

Methylation of DNA is "epigenetic"—a heritable modification that regulates gene expression without changing the DNA sequence itself. It occurs throughout the genome and plays an important role in gene regulation and chromatin structure. Specifically, methylation in higher eukaryote genomes is known to be associated with regulatory processes including embryonic development, chromatin structure and stability, gene silencing, genomic imprinting, X-inactivation, and cancer.

Many studies have focused on the methylation state of specific genomic sites thought to play important roles in cellular processes. These sites include dense clusters of CG dinucleotides, referred to as "CpG islands," which occur throughout the genome, but are often found in gene promoter regions. In somatic cells, genes with highly methylated CpG islands are "hypermethylated"—they exhibit little or no mRNA expression and are considered silenced. In contrast, promoter CpG islands tend to be "hypomethylated" and are often not subject to repression.

Methylation plays a key role in the developmental process of X-inactivation. In female mammalian cells, one of the two X-chromosomes is silenced to compensate for the increased gene dosage compared to male cells, which contain only a single X-chromosome. DNA methylation is one step of the inactivation process, and is thought to contribute to the silencing of the chromosome.

Many types of cancer display hypermethylated regions that are characteristic markers of a transformed or aberrant phenotype. In certain tumors, putative tumor suppressor genes have been shown to be both hypermethylated and silenced (for review see Jones and Baylin, 2002). The correlation of altered or aberrant DNA methylation with various cancers highlights the great promise of determining CpG island methylation patterns in driving disease pathology and progression profiling.

Several methods for monitoring DNA methylation on a genome-wide basis have been previously described. One promising method—methyl DNA immunoprecipitation (mDIP)—uses an antibody against 5-methyl-cytosine to enrich for regions of methylated DNA (Weber et al., 2005). Relative methylation levels within a given sample are analyzed by generating mDIP-enriched DNA, which is then labeled and competitively hybridized against labeled input genomic DNA on a microarray (see **Figure 1** for workflow overview).

Validation of the Agilent CpG island microarray was performed with mDIP-enriched DNA using two human model systems—X-inactivation (normal male versus normal female) and tumor profiling (comparing normal cell lines to lung cancer cell lines). Comprehensive coverage of all CpG islands in the genome allowed us to scan entire chromosomes and focus on specific regions of interest.

Materials and Methods

Bead Preparation

For each immunoprecipitation (IP), 50 µl of pan-mouse IgG magnetic beads (Invitrogen P/N 11041) were washed twice in PBS + 0.5% bovine serum albumin (BSA) before incubation with 5 µl anti 5-methyl cytosine antibody (Eurogentec P/N BI-MECY-0100) in PBS + 0.05% BSA at 4°C on a rotator overnight. Beads were then washed three times and resuspended in 50 µl using PBS + 0.05% BSA for all steps.

DNA Preparation and Immunoprecipitation

4 µg genomic DNA was suspended in 200 µl PBS and mechanically sheared to an average length of ~500 bp. 250 ng sheared genomic DNA was retained as the input fraction. The remaining sheared DNA was denatured at 95°C for 10 minutes, cooled on ice, then incubated with the antibody/bead complex in PBS + 5 µg/ml BSA + 25 µg/ml yeast tRNA at 4°C on a rotator overnight.

Immunoprecipitated DNA Elution and Purification

Beads were washed three times with PBS + 0.05% Triton X-100. IP-enriched DNA was then eluted from the beads by incubation at 65°C for five minutes in 150 µl TE + 1% SDS. The elution was repeated with an additional 150 µl TE + 1% SDS. Elutions were combined and treated with 2.5 µl 20 mg/ml proteinase K (Invitrogen P/N 25530049) at 50°C for two hours. Samples were then extracted with one volume of phenol:chloroform:isoamyl alcohol (24:24:1), ethanol precipitated with 1/25 volume 5M NaCl + 1.2 µl glycogen (20 µg/ml) and two volumes of 100% ethanol at -80°C for thirty minutes, and pelleted in a microfuge. Precipitated DNA was washed with 70% ethanol and dried in a speedvac for five minutes.

Cyanine 3 and Cyanine 5 Dye Labeling

The immunoprecipitated DNA pellet (80% of total IP as described above) and 200 ng input DNA each were suspended in 30 µl water for labeling. Samples were labeled with Cyanine

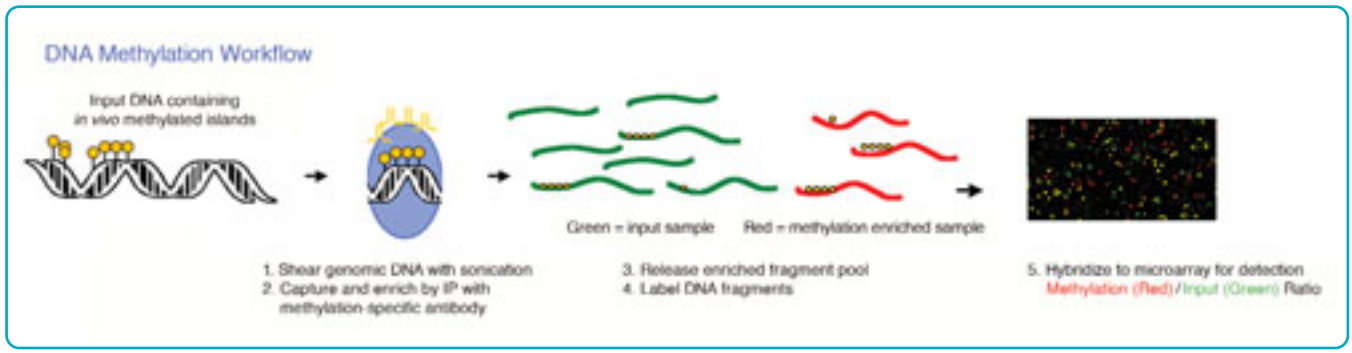

Figure 1. Affinity-based isolation of methylated DNA. The methyl DNA immunoprecipitation (mDIP) method uses a monoclonal antibody against 5-methylcytosine to isolate methylated DNA from the genome. mDIP is one example of enriching methylated DNA. The resulting fraction of methylated DNA is purified, Cyanine 5 (Cy5)-labeled, and competitively hybridized against similarly Cyanine 3 (Cy3)-labeled "input" genomic DNA onto a single microarray. The microarray is washed, scanned, and analyzed with Agilent Feature Extraction Software. Relative DNA methylation levels for each probe/CpG island are reflected in changes in Cyanine 5/Cyanine 3 ratios.

3- and Cyanine 5-dUTP nucleotides, respectively, using the Agilent Genomic DNA Labeling Kit Plus (P/N 5188-5309). Samples were purified on Microcon™ YM-30 columns (Millipore P/N 42410) and eluted in 30 µl TE.

Microarray Hybridization and Analysis

Hybridization of labeled input and IP DNA was carried out essentially as described in the Agilent aCGH protocol except hybridizations were performed at 67°C for forty hours. The Agilent Human CpG Island Microarray (P/N G4492A) contains ~237,000 oligonucleotide probes covering 21 MB of CpG islands (average probe spacing ~100 bp). Microarrays were scanned on an Agilent microarray scanner (P/N G2565BA) and images analyzed with Agilent Feature Extraction Version 9.1 software using aCGH protocols and settings. Cy3 and Cy5 signals were normalized to obtain a median log2 ratio of zero and data was visualized with Agilent CGH Analytics Version 3.4.

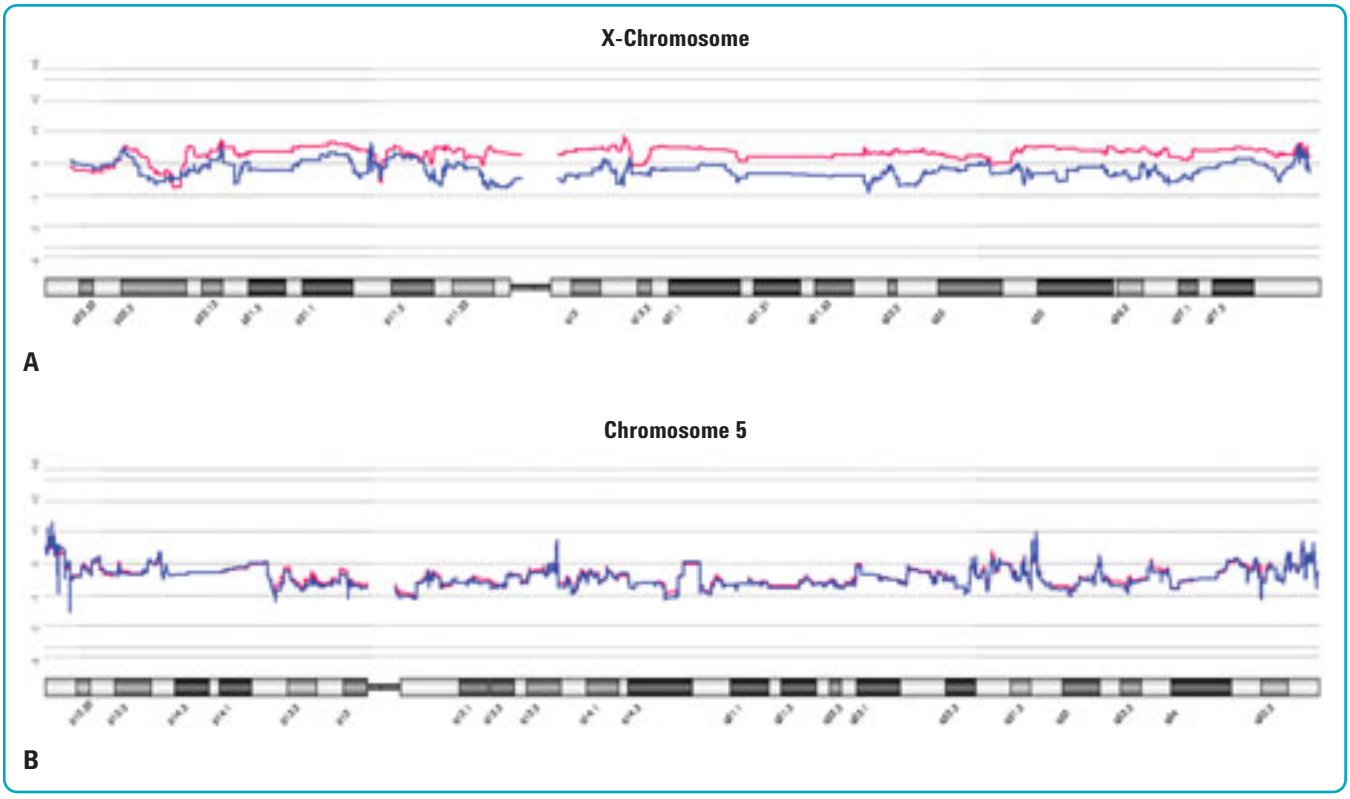

Figure 2. Comparison of methylated DNA (mDIP-enriched) between male HEL299 and female IMR90 embryonic lung fibroblast cell lines. For each sample, Cy5-labeled mDIP-enriched DNA and Cy3-labeled input genomic DNA were competitively hybridized on an Agilent Human CpG Island Microarray (P/N G4492A). Microarray data was normalized to a median $\log_2$ ratio of zero. Shown is an 80-point moving average for male (blue) and female (red) cell lines. CpG island ratios are shown across the entire X-Chromosome (**Figure 2A**) and Chromosome 5 (**Figure 2B**). Comparison of both these profiles shows higher levels of relative CpG island methylation in the female than male sample only on the X-chromosome and not the autosomal Chromosome 5.

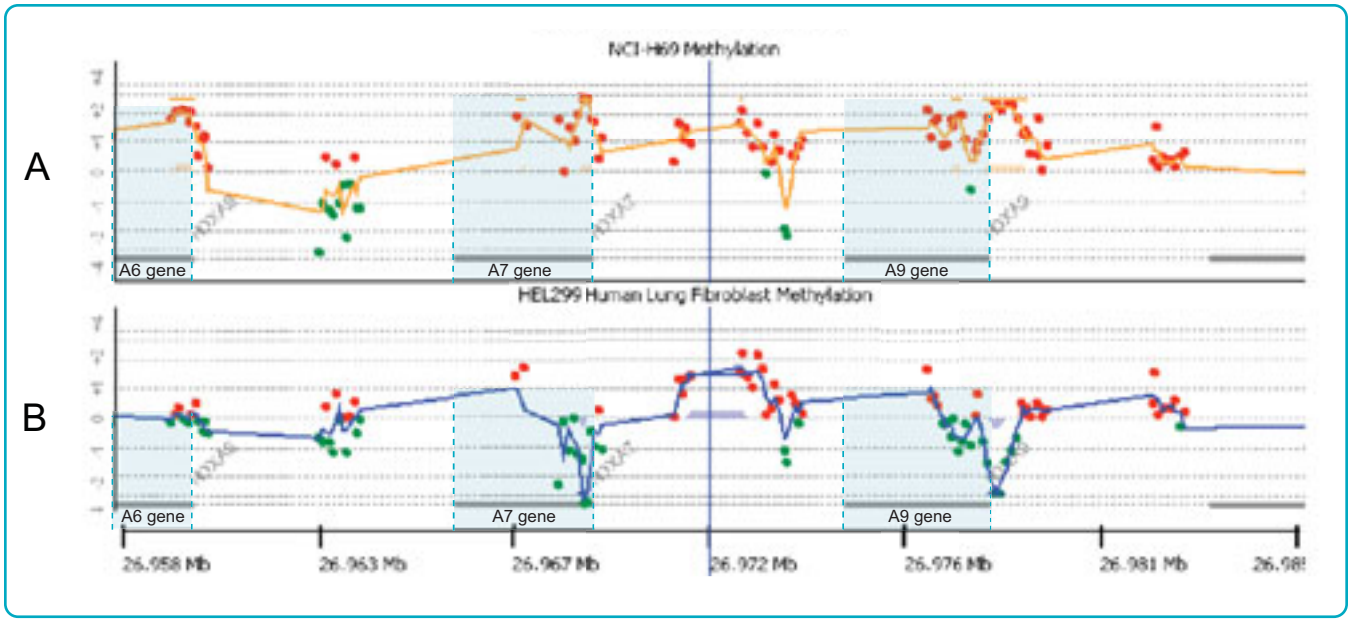

Figure 3. Methylation comparison of the HoxA gene cluster between tumor and normal lung cell lines. Methylated DNA was immunoprecipitated and labeled from genomic DNA (as previously described) from the small cell lung carcinoma NCI-H69 cell line (**Figure 3A**) and the normal embryonic lung fibroblast HEL299 cell line (**Figure 3B**). For each line, samples of both Cy5-labeled mDIP-enriched DNA and Cy3-labeled input genomic DNA were competitively hybridized to an Agilent Human CpG Island Microarray (P/N G4492A). Microarrays were processed, scanned on an Agilent Scanner (P/N G2565BA), and data normalized to a median $\log_2$ ratio of zero. The methylation profile for a portion of the Hox locus on Chromosome 7 is shown. Every point represents an individual CpG island microarray probe. Each colored line (orange for tumor, blue for normal) represents a three-point moving average of the $\log_2$ ratio. Regions of more methylation (red) and less methylation (green) are indicated. Highly methylated regions have $\log_2$ ratios significantly above zero while less methylated regions have log ratios significantly below zero. Profiles clearly show significant differences between the normal and cancer samples at HoxA6, HoxA7, and HoxA9 promoter regions.

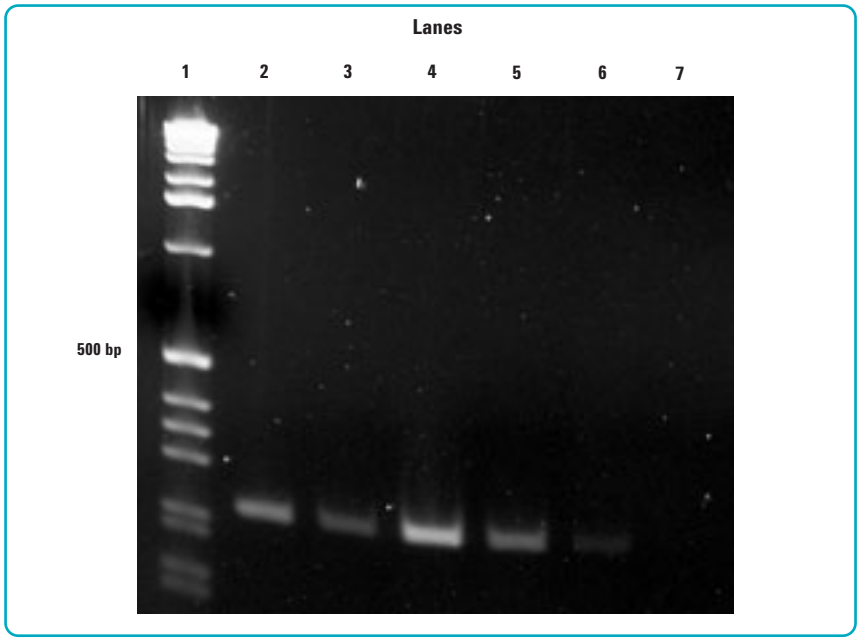

Figure 4. PCR confirmation of mDIP enrichment at the HoxA7 locus in lung cancer and normal cell lines. Using primers to a region within the HoxA7 promoter CpG island, PCR amplification was performed on both input DNA and mDIP-enriched DNA. PCR reaction products were analyzed by gel electrophoresis. Lane 1, marker; Lanes 2-3, 3% and 1% of input DNA fraction for the NCI-H69 tumor cell line; Lane 4, 3% of total mDIP-enriched NCI-H69 fraction; Lanes 5-6, 3% and 1% of input DNA fraction for the HEL299 control cell line; Lane 7, 3% of total mDIP-enriched HEL299 fraction.

Results and Discussion

Using Agilent's Human CpG Island microarray paired with the mDIP method, we analyzed the DNA methylation status of several human cell lines—male versus female, and normal versus tumor. The CpG island microarray used was designed by carefully selecting 237,220 probes from the Agilent human genome tiling probe database. Probes are spaced ~100 bp apart (on average) across 27,800 CpG islands. Probe sequences are 45-60 bp in length and have advantageous predicted hybridization and uniqueness properties.

The methylation profiles of genomic DNA from two human model systems were analyzed to demonstrate the effectiveness of combining this method with an Agilent CpG Island Microarray. In females, one of the two X-chromosomes is inactivated, and this inactive counterpart is known to have a higher degree of DNA methylation. When we compared the relative methylation states within CpG islands of male (HEL299) and female (IMR90) embryonic lung fibroblast cell lines, the female DNA had a significantly higher degree of mDIP-enrichment for the X-chromosome (**Figure 2A**). This hypermethylated state was not evident in profiles of all autosomes, as exemplified by Chromosome 5 (**Figure 2B**).

Furthermore, when we profiled the methylation pattern of DNA from the human small cell lung carcinoma NCI-H69 cell line, the promoter regions of the HoxA6, HoxA7, and HoxA9 genes within the HOX gene cluster (chromosome 7) displayed high levels of relative methylation (**Figure 3A**). The degree of methylation was greater than that found in the lung fibroblast HEL299 embryonic cell line (**Figure 3B**). mDIP enrichment was confirmed by PCR **(Figure 4)**.

Similar hypermethylation of CpG islands near HOX gene clusters has been described in stage 1 lung carcinoma and breast cancer samples (Rauch et al., 2007; Novak et al., 2006). The Novak group employed an alternative method for enriching methylated DNA and hybridized their samples to the same Agilent CpG Island microarrays. Aberrant methylation in developmentally regulated clusters such as Hox hold great promise as important diagnostic and prognostic indicators for cancer.

The two types of model systems profiled here exemplify the power of genome-wide epigenetic microarray analysis as applied to development and diseases such as cancer. Future methylation studies, in conjunction with genome-wide gene expression and chromatin profiling, will help drive better understanding of these complex processes.

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