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NEWSLETTER

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Application Note

Genome-Wide Detection of DNA Methylation Changes in Lymphomas Using the Infinium® HumanMethylation27 BeadChip

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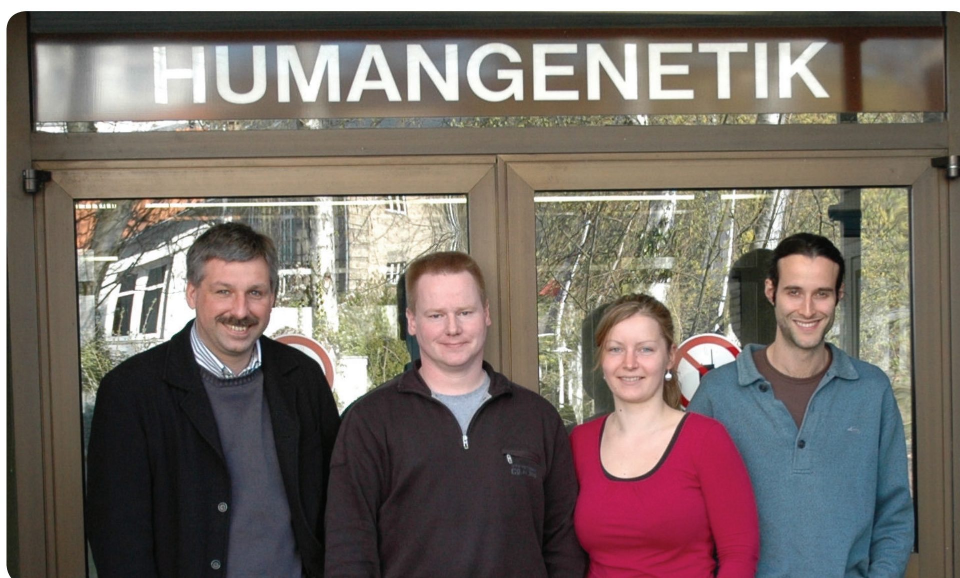
INTRODUCTION

It is well known that DNA methylation plays an important role in the regulation of gene expression, genome stability, imprinting, and DNA repair[1]. Aberrations in the "normal" DNA methylation patterns observed in promoter regions and repetitive sequences have been shown to be involved in a wide range of diseases, including cancer[2-4]. In fact, global hypomethylation of DNA repeats in cancer cells is known to contribute to chromosomal instability[5,6] while regions of hypermethylation in tumor suppressor genes result in gene silencing[2-4].

Studying these regions of differential methylation has previously been performed using PCR approaches, such as methylation-specific PCR (MSP) or bisulfite sequencing (BS). These methods are extremely targeted and do not provide a comprehensive look at the entire genome. In order to obtain a global picture of aberrant DNA methylation



From left to right, Reiner Siebert, Ole Ammerpohl, Julia Richter, and Jose I. Martin-Subero.

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events in cancer, recent efforts have focused on the development of methylation-specific microarrays[7]. These array techniques are commonly based either on bisulfite treatment of the DNA, or enrichment of methylated DNA sequences by immunoprecipitation or digestion with methylation-specific endonucleases[8]. Immunoprecipitation and enzymatic digestion do not allow quantitative measuring of the methylation status of specific CpGs. Rather, they provide a rough picture of the whole methylome. Methods linking bisulfite treatment to methylation-specific oligonucleotide microarrays allow quantification of individual CpGs, but are limited by the usually low number of regions that can be interrogated simultaneously[9,10].

The recently launched Infinium HumanMethylation27 BeadChip enables the simultaneous quantification of absolute DNA methylation levels of selected CpG sites in over 14,000 human genes. This provides a specific, yet genome-wide, view of methylation patterns in the genome in minimal time. Here we demonstrate the use of the HumanMethylation27 BeadChip for genome-wide methylation analysis of lymphoma cell lines.

MATERIALS AND METHODS

Samples Analyzed

DNA from seven lymphoma cell lines and six non-malignant samples, for use as controls, of hematopoietic origin were analyzed. Of the seven lymphoma samples, six cell lines were run in duplicate and one in sextuplet. Four of the controls were hybridized once, one in duplicate, and the remaining control in quadruplet. Additionally, unmethylated, methylated, and hemimethylated DNAs were hybridized in quadruplet.

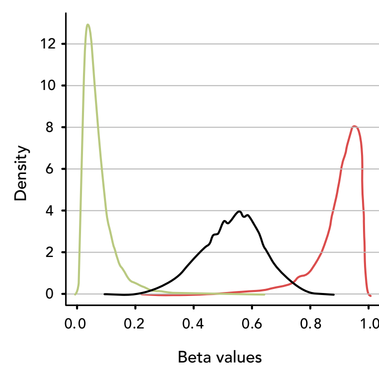
Illumina's Infinium Methylation Assay

The HumanMethylation27 BeadChip consists of 12 arrays, each of which enables analysis of more than 14,000 well-annotated genes. Taking into account that roughly 28,000 CpG dinucleotides* are mapped to the BeadChip, each of the 14,000 genes is statistically represented by two CpG dinucleotides.

To begin the experiment, 1 µg genomic DNA from each sample was bisulfite converted, and 200 ng of the converted DNA was used for amplification. Treating DNA with bisulfite converts unmethylated cytosines to uracils, creating readily detectable, artificially induced single nucleotide variations.

Amplified DNA was hybridized to the HumanMethylation27 BeadChip and the arrays were imaged using a BeadArray™ Reader. Image processing and intensity data extraction were performed according to Illumina's instructions. Each methylation data point is represented by fluorescent signals from the M (methylated) and U (unmethylated) alleles. These signals were used to compute a Beta value, which is a quantitative measure of DNA methylation ranging from 0, for completely unmethylated cytosines, to 1, for completely methylated cytosines.

FIGURE 1. THE HUMANMETHYLATION27 BEADCHIP PROPERLY QUANTIFIES DNA METHYLATION LEVELS



Density plot of methylation profiles generated with the Infinium BeadChip from unmethylated (green line), hemimethylated (black line), and methylated (red line) control DNAs.

Data analysis, including unsupervised hierarchical clustering and differential methylation analysis, was performed with the BeadStudio Methylation Module.

Methylation-Specific PCR, Bisulfite Sequencing, and Pyrosequencing

To validate the DNA methylation data generated by BeadArray technology, conventional PCR-based methylation analyses

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* Please note that the BeadChip used for these experiments was a pre-release version. The current Infinium HumanMethylation27 BeadChip contains just over 27,000 sites.

(MSP and BS) were performed as previously described[11]. For BS, a total of ten clones were sequenced. Pyrosequencing (PS) was performed according to standard protocols with brief modifications[12] and evaluated with the Pyro Q-CpG 1.0.9 software (Biotage, Uppsala, Sweden).

RESULTS

Unmethylated, Hemimethyated, and Methylated Control Samples

In vitro methylated and unmethylated DNAs, as well as a 1:1 mixture of them (to create a hemimethylated mixture), showed mean methylation levels of 0.070, 0.894, and 0.530, respectively. This indicates that the HumanMethlyation27 BeadChip is able to properly quantify DNA methylation levels (*Figures 1, 4A, and 5*).

Reproducibility

Coefficients of determination (r^2) were calculated to measure the reproducibility of samples hybridized in replicate. A mean r^2 of 0.977 (min: 0.955, max: 0.988, SD: 0.011) was obtained, confirming the high reproducibility of the HumanMethylation27 BeadChip (*Figure 2A*). The interarray reproducibility (one sample hybridized onto two different slides) was also high (*Figure 2B*). We found no significant differences between results obtained from samples that were bisulfite converted by Illumina or in our lab, arguing for a stable assay producing reproducible results (*Figure 2C*).

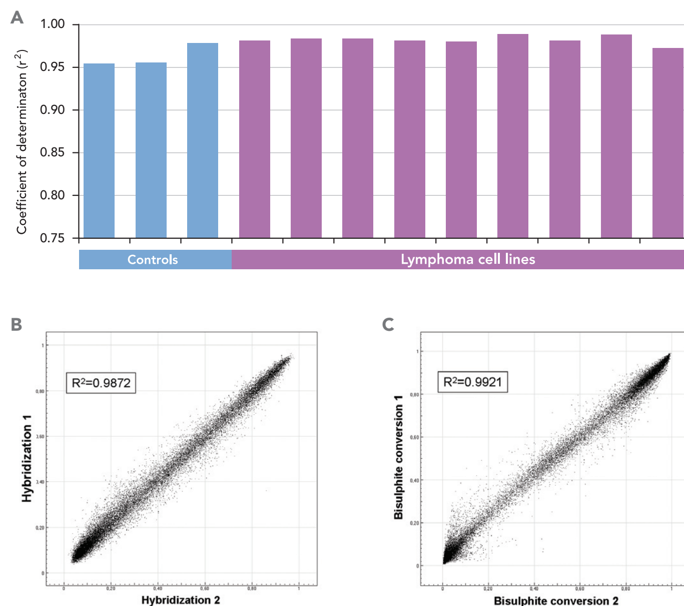
Validation of Results

To assess the reliability of results obtained using the Infinium methylation array, data were validated by MSP, PS, and BS of selected genes and cell lines. For data validation by MSP, five lymphoma cell lines were studied with a total of 12 published primer sets, for methylated and unmethylated alleles, targeting genes known to be hypermethylated in cancer.

Figure 3 shows strong correlation between HumanMethylation27 BeadChip and MSP data. For several genes, we found a nearly perfect match of the results. The slight differences observed with both methods can be easily explained by the fact that the CpGs studied by the MSP primers and the BeadChip were not always identical. These data indicate that the CpGs studied with the

"...the Human-Methylation27 BeadChip is able to properly quantify DNA methylation levels"

FIGURE 2. HIGH REPRODUCIBILITY OF HUMANMETHYLATION27 BEADCHIP ANALYSES



(A) Reproducibility of the HumanMethylation27 BeadChip analyses as measured by coefficients of determination (r^2) in controls and lymphoma cell lines. (B–C) Scatter plot of two samples hybridized to two independent arrays (r^2= 0.9872; panel B) and a DNA sample bisulfite-converted in two different laboratories (Illumina and our lab) (r^2= 0.9921; panel C).

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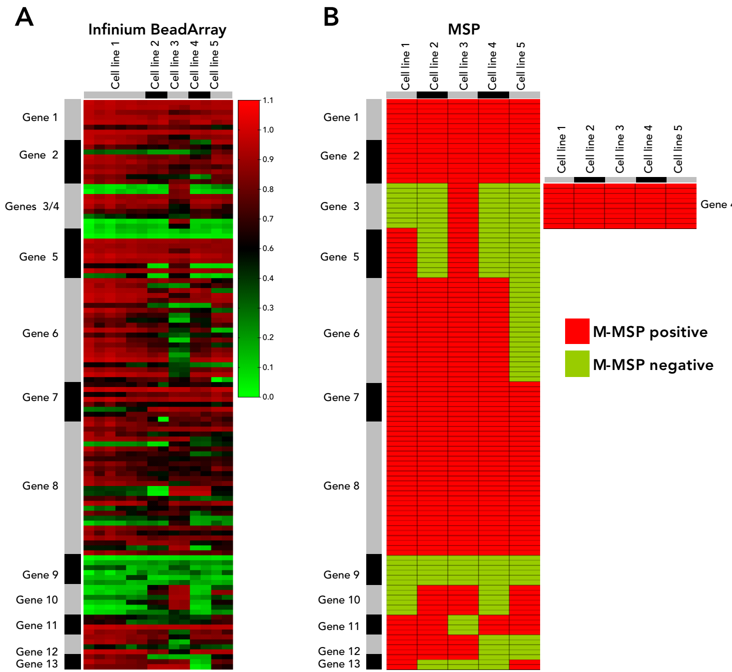
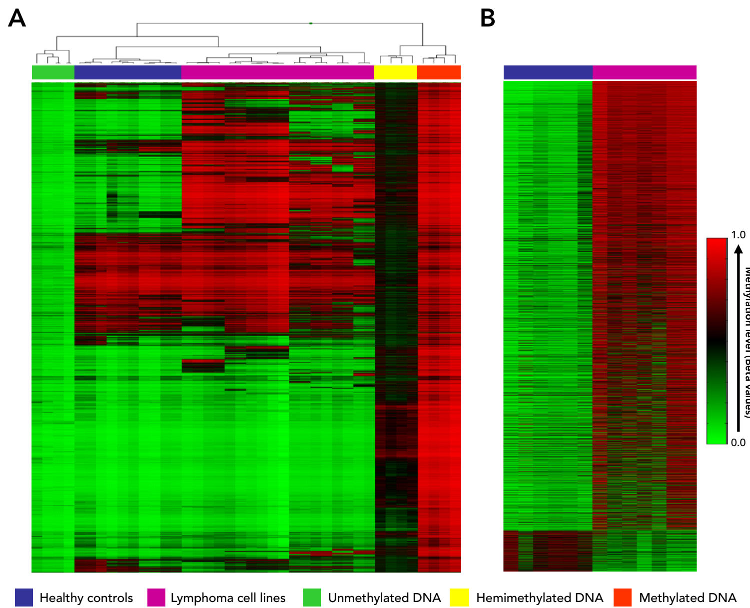
FIGURE 3. GOOD CORRELATION BETWEEN INFINIUM HUMAN METHYLATION27 AND MSP DATA



Comparison of results obtained from five lymphoma cell lines by the Infinium HumanMethylation27 BeadChip (A) or MSP (B)

"Figure 3 shows strong correlation between Human-Methylation27 BeadChip and MSP data. For several genes, we found a nearly perfect match of the results."

FIGURE 4. METHYLATION DIFFERENCES IN LYMPHOMA VS. NORMAL CELLS



(A) 2D-Cluster analysis of DNA methylation levels from 26,492 CpGs in lymphoma cell lines (18 hybridizations, 7 samples), negative controls (10 hybridizations, 6 samples), as well as artificially unmethylated, hemimethylated, and methylated DNA. X-chromosomal CpGs were excluded from this analysis. (B) Differential methylation analysis between controls and lymphoma cell lines (CpGs with a $p < 0.01$ are displayed).

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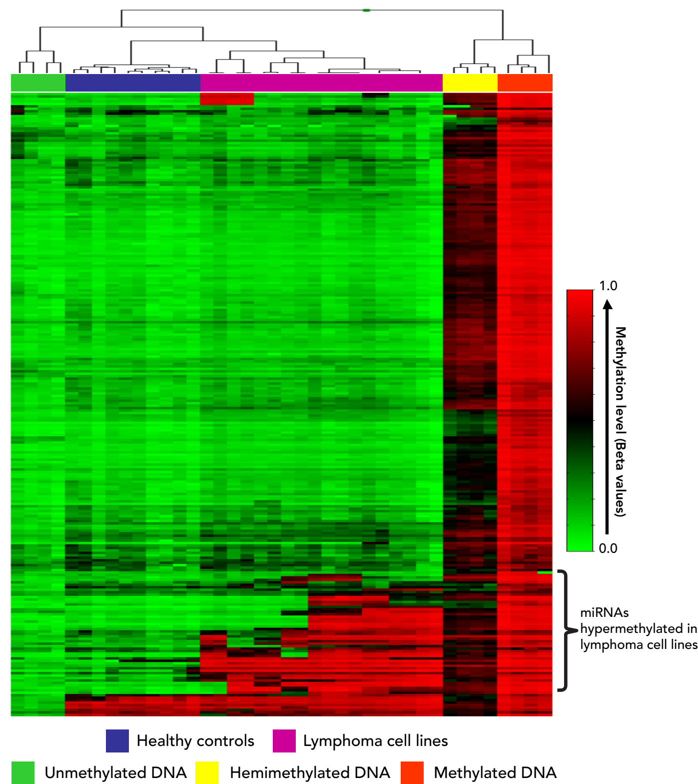
FIGURE 5. DIFFERENTIAL MIRNA METHYLATION IN LYMPHOMA VS. HEALTHY CELLS



2D-Cluster analysis of DNA methylation levels from 254 microRNA-specific CpGs in lymphoma cell lines (18 hybridizations, 7 samples), negative controls (10 hybridizations, 6 samples) as well as artificially unmethylated, hemimethylated, and methylated DNA.

"This new array will undoubtedly be a valuable research tool in the emerging epigenomics field."

BeadChip are representative for the CpG islands in the 12 validated genes.

DNA Methylation Changes in Lymphoma Cell Lines

A hierarchical cluster analysis of lymphoma cell lines and controls allowed clear differentiation according to their DNA methylation profile. As shown in *Figure 4A*, lymphoma cell lines are characterized by a large number of hypermethylated CpGs. In order to identify CpGs differentially methylated in lymphoma cell lines, we performed a differential methylation analysis using the BeadStudio software. From the 26,492 CpGs that entered this statistical analysis (CpGs on chromosome X were removed to avoid bias induced by gender), a total of 5,362 (20.2%) were significantly hypermethylated ($p < 0.01$). Using a more stringent significance level ($p < 0.001$), we still detected 3,916 (14.8%) hypermethylated CpGs. We also detected 485 ($p < 0.01$) and 127 ($p < 0.001$) CpGs hypomethylated in lymphoma cell lines as compared with normal controls (*Figure 4B*).

As the novel HumanMethylation27 BeadChip also includes 254 CpGs located in microRNAs, we investigated whether these noncoding regions were also targeted by aberrant DNA methylation in lymphoma cell lines. As shown in *Figure 5*, lymphoma cell lines show hypermethylation of microRNA-specific CpGs. Differential methylation analysis performed with BeadStudio software detected 35 ($p < 0.01$) and 22 ($p < 0.001$) significantly hypermethylated microRNA-specific CpGs. However, we did not detect any microRNA-specific CpGs showing significant hypomethylation in lymphomas as compared with normal controls.

CONCLUSIONS

Using the Infinium HumanMethylation27 BeadChip, we were able to identify over 5,000 differentially hypermethylated CpGs (representing ~2,500 genes) in lymphoma cells. Furthermore, in line with recent reports on aberrant DNA methylation of microRNAs in cancer[13], we identified a number of hypermethylated microRNA-specific CpGs in lymphoma cell lines as compared with the control samples. We also analyzed methylation profiles of primary lymphomas with the BeadChip and obtained similar results (data not shown).

From the technical point of view, the Infinium HumanMethylation27 BeadChip is highly reproducible and allows accurate quantification of methylation levels from more than 27,000 CpGs. This new array will undoubtedly be a valuable research tool in the emerging epigenomics field.

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ADDITIONAL INFORMATION

To learn more about the Infinium HumanMethylation27 BeadChip, visit www.illumina.com.

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