iCommunity NEWSLETTER

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Application Note

Barrett's Esophagus and Esophageal Adenocarcinoma Epigenetic Biomarker Discovery Using Infinium® Methylation

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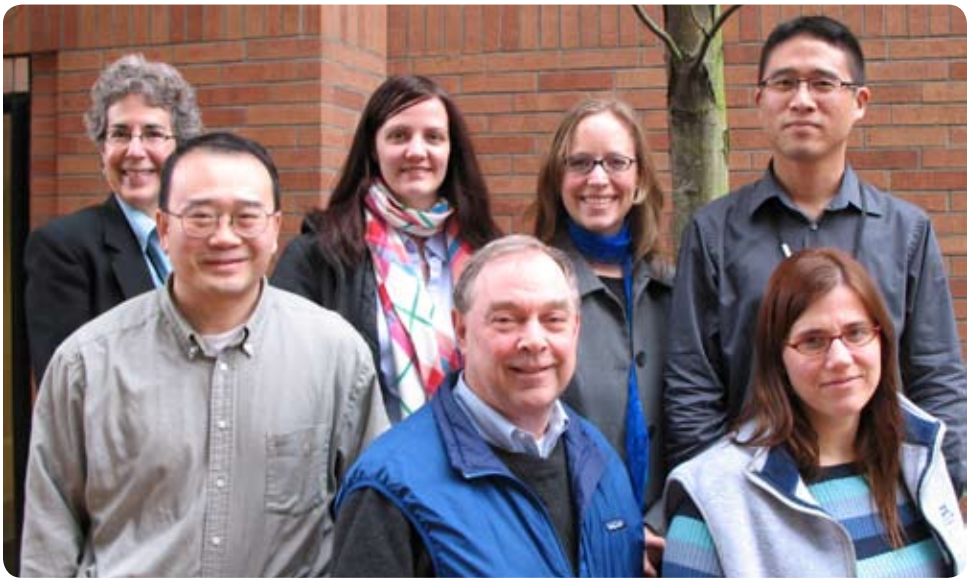
INTRODUCTION

There is a growing body of evidence that epigenetic alterations are not only common, but may precede major genetic changes that lead to cancer[1–3]. Selected gene-specific hypo- and hyper-methylation of CpG islands have been shown to cause overexpression of oncogenes and transcriptional silencing of tumor suppressor genes, respectively, possibly leading to clonal evolution[4,5]. Therefore, we pursued an epigenetic evaluation of DNA samples from individuals diagnosed with Barrett's esophagus (BE).

BE is a premalignant condition in which the normal squamous epithelium is replaced by a specialized intestinal metaplasia in response to acid reflux. It is the only known precursor to esophageal adenocarcinoma (EA). Methylation changes specific to BE/EA samples have been previously identified[6,7]. These observations lead us to hypothesize that BE tissue has a unique, tissue-specific methylation



From top left to bottom right, Carissa Sanchez, Heather Kissel, Patricia Galipeau, Dennis Chao (Bottom) Xiaohong Li, Brian Reid, Jessica Arnaudo.

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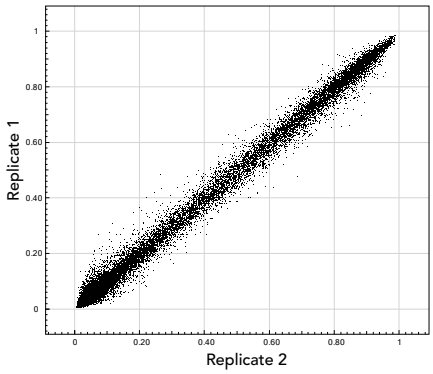
FIGURE 1. COMPARISON OF METHYLATION LEVELS BETWEEN REPLICATES



The beta methylation values at 28,245 CpG sites in two replicate biological samples were compared. This BeadStudio plot shows excellent agreement between the same DNA sample that was bisulfite-treated and run in replicate.

signature that distinguishes it from adjacent squamous and gastric tissue, regardless of the stage of neoplastic progression. In addition, methylation events are selected during progression to EA.

Previous evaluations of CpG methylation in BE/EA studies have been limited by the inability to characterize the methylation status of large numbers of genes at once, allowing only 1–20 genes per sample to be evaluated in a single study. The Infinium HumanMethylation27 BeadChip from Illumina® represents a significant technological advance in epigenetic studies. This platform characterizes the methylation status of over 27,500 CpG sites across more than 14,000 genes. It provides semi-quantitative methylation data without the use of a standard curve or reference locus and is technically compatible with small, endoscopic biopsy and surgical resection specimens.

In this application note, we describe the use of the Infinium HumanMethylation27 BeadChip to measure CpG methylation in samples obtained from individuals with Barrett's esophagus. We validate these data by Pyrosequencing and demonstrate that achieved results are similar to those for an earlier experiment performed using Illumina's GoldenGate® Methylation Assay.

SAMPLES AND DATA ACQUISITION

We obtained 60 fresh, frozen biopsies from 24 patients diagnosed with BE. The patients represented a cross-section of all stages of BE, from early BE (no genomic instability and no progression to cancer over mean 11.59 years) to advanced EA. Samples from three different tissue types, including Barrett's epithelium and adjacent normal proximal squamous and distal gastric tissue, were collected from 18 of these patients. EA samples from visible tumors were collected from surgical resections of six patients.

The methylation fraction of >27,500 individual CpG sites was measured in all the samples using Illumina's Infinium Methylation Assay. As input to the assay, 500 ng of DNA was bisulfite-converted using the EZ DNA Methylation Gold kit (Zymo Research). After bisulfite conversion, the methylation level at each CpG site was determined by measuring the methylation fraction (beta), defined as the fraction of methylated signal over the total signal (unmethylated + methylated). The Infinium Assay includes redundant, built-in, bisulfite conversion quality controls that measure the conversion rate of non-CpG cytosines and background signal. Using controls evaluated with Illumina BeadStudio software, we determined that non-methylated cytosines in all 72 biological samples were efficiently bisulfite converted, providing a within-assay assessment of background signal and bisulfite conversion rate.

REPRODUCIBILITY AND VALIDATION OF THE METHYLATION RESULTS

Reproducibility

The Infinium Methylation Assay was highly reproducible, showing excellent agreement of CpG site methylation fractions between replicates (average r^2=0.98 for 12 pairs of replicates, with a range of 0.97 to 0.99). Figure 1 shows a typical high level of concordance between two replicates of a single DNA sample processed in independent bisulfite treatments and amplified in two different Infinium Assay reactions.

"The Infinium Methylation Assay was highly reproducible, showing excellent agreement of CpG site methylation fractions between replicates"

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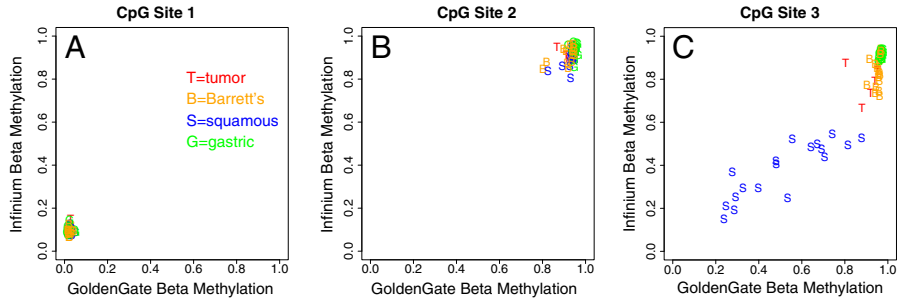
FIGURE 2. CONSISTENT GOLDENGATE AND INFINIUM METHYLATION RESULTS



Methylation fractions of three CpG sites measured by Infinium and GoldenGate Assays. The plots show that the results from the Infinium Assay were generally consistent with those from the GoldenGate Assay.

Comparison of Infinium HumanMethylation27 BeadChip with the GoldenGate Methylation Cancer Panel I

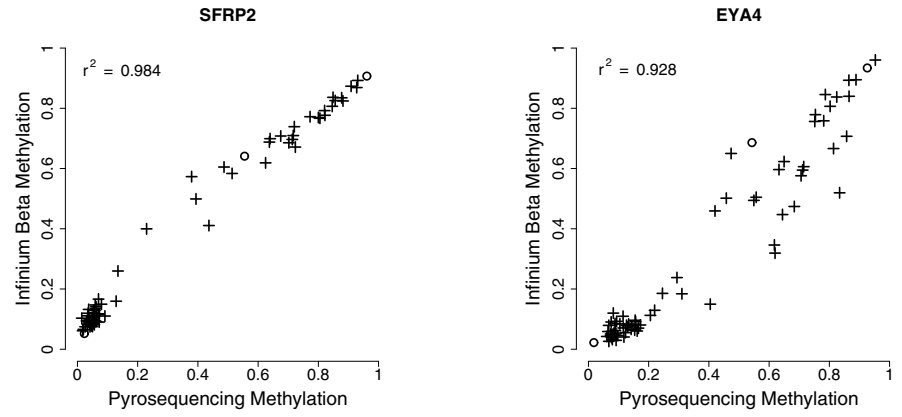
We found that the measurements of methylation levels from the new Infinium Methylation Assay agreed with our earlier study conducted on the GoldenGate platform using Illumina's Methylation Cancer Panel I, which included 1,536 CpG sites across 808 genes. The two experiments used independent biopsies from the same patients. We compared the beta methylation values between the GoldenGate and Infinium Assays at the 117 CpG sites that are targeted on both platforms. Although the assays were performed in different biopsy samples, used different probes, and were based on different technologies, we found general agreement of beta methylation values between the two assays (Figure 2). We found 60 of 117 sites to have significantly different methylation fractions between the normal squamous and the BE samples on the GoldenGate platform (Wilcoxon test $p<0.05$). Fifty-three of the 60 sites, or 88%, also had significantly different fractions on the Infinium platform. Thus, we find that the Infinium platform produced results consistent with our GoldenGate study.

Technical validation of Infinium HumanMethylation27 BeadChip with Pyrosequencing

The Infinium Methylation Array was validated using Pyrosequencing. Assays were designed and optimized to sequence the same CpG sites for nine different genes

"...highly quantitative Pyrosequencing data shows strong correlation with data obtained using the high-density Infinium Assay"

FIGURE 3. HIGH CORRELATION BETWEEN INFINIUM HUMANMETHYLATION27 BEADCHIP AND QUANTITATIVE PYROSEQUENCING DATA



Comparison of methylation fractions in EYA4 and SFRP2 assessed using Pyrosequencing (x-axis) and the Infinium methylation platform (y-axis beta methylation value) in the same bisulfite-treated biological samples (+) and methylation controls (0).

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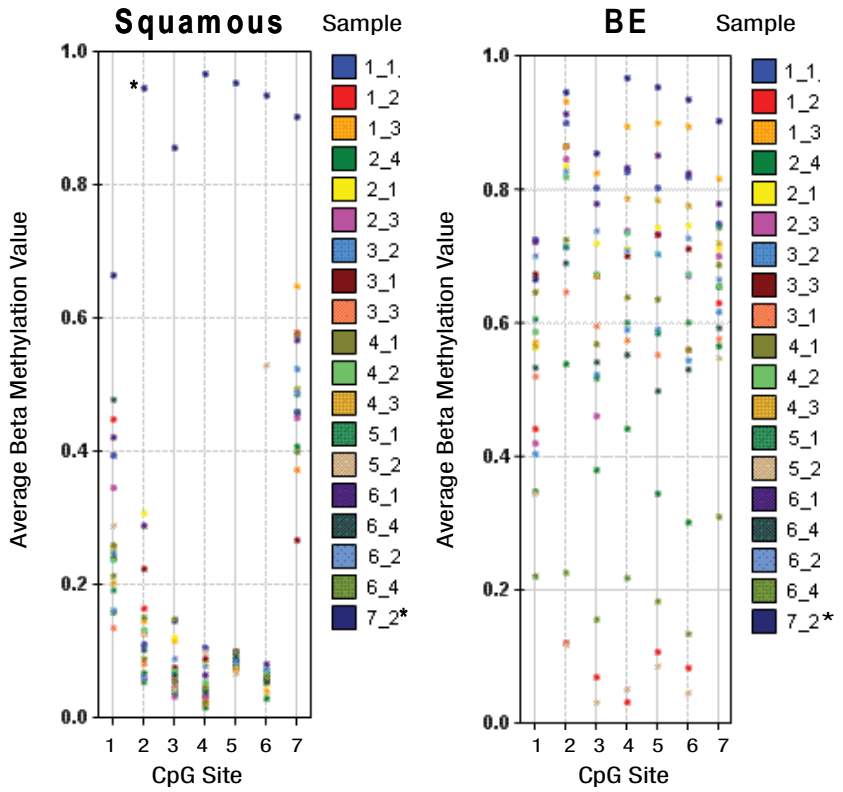
FIGURE 4. APC METHYLATION LEVELS AT MULTIPLE CPG SITES



Average beta methylation values (y-axis) for seven CpG sites associated with APC in squamous tissue (left panel) and BE tissue (right panel). Each sample is represented as a different color, as customized within the BeadStudio software.
*Methylated control

assayed on the Infinium platform (two of the nine are shown in Figure 3). Seventy-two tissue samples and three technical controls were evaluated. The highly quantitative Pyrosequencing data shows strong correlation with data obtained using the high-density Infinium Assay across the spectrum of beta methylation values (average r^2=0.91, range=0.82–0.98).

Evaluation of previously identified candidate epigenetic biomarkers of BE and EA

The Infinium methylation panel includes 36 genes previously evaluated for promoter methylation in the BE/EA literature. In our study, most of these genes showed evidence of CpG methylation in BE/EA samples. BE/EA samples had significantly higher methylation fractions than squamous samples from the same individuals in CpG sites associated with 30 of the 36 genes (Wilcoxon rank sum test $p<0.05$) (Figure 4 showing APC), and sites in 27 of 36 genes had CpG sites with significantly higher methylation in BE/EA samples compared to the gastric samples ($p<0.05$). Thus, we find general agreement with previous methylation studies of BE and EA.

Identifying new candidate tissue-specific and progression-associated epigenetic biomarkers of BE and EA

Methylation fractions of certain CpG sites showed non-overlapping ranges for different tissue types, enabling them to be used to identify different tissues. We found that 1,098 sites (3.9% of the 28,245 sites included on the Infinium platform) can perfectly discriminate between the BE/EA and gastric samples, 1,145 (4.1%) can discriminate between BE/EA and squamous samples, 1,022 (3.6%) can discriminate between

"Results from this pilot study agree with those from a similar study conducted using Illumina's GoldenGate platform. The Infinium Assay has the advantage of covering nearly 20 times more genes, with greatly increased CpG site coverage"

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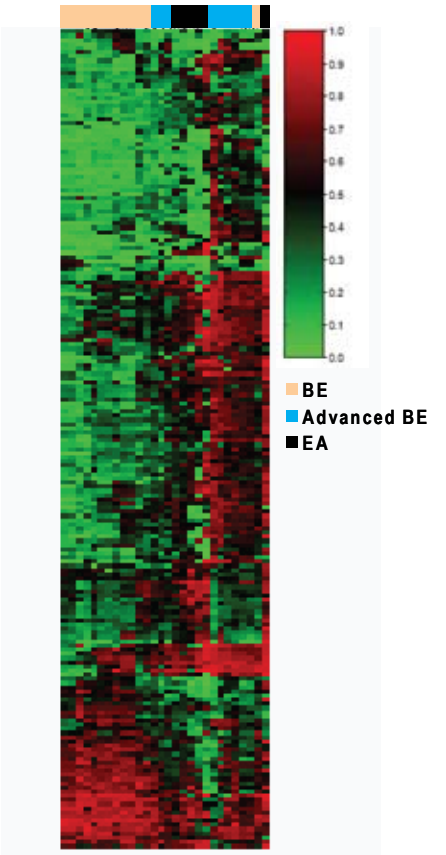
FIGURE 5. CLUSTERING WITH PROGRESS-ASSOCIATED CPG SITES



Hierarchical clustering of biopsy samples from individuals at different stages of neoplastic progression using beta methylation values for 192 progression-associated CpG sites using CpG methylation levels. Heatmap and clustering were generated using BeadStudio software.

gastric and squamous samples, and 674 sites (2.4%) can discriminate between BE and all normal samples (gastric and squamous). The large number of epigenetic differences between BE and the surrounding tissues is consistent with earlier gene expression studies comparing the three tissue types [8,9].

To identify candidate biomarkers of neoplastic progression, we compared methylation fractions of individual sites of participants at different stages of BE. One hundred ninety-two sites had a ≥0.2 difference in means between different stages of progression. Using these sites, we ran a Manhattan Hierarchical clustering algorithm (Figure 5). The extensive filter capabilities and easy column-import features of BeadStudio software allow for flexibility in generating heat maps and other graphics, which aid in visualizing these complex datasets.

SUMMARY AND CONCLUSIONS

The Infinium Methylation Assay generates highly reproducible estimates of methylation fractions of CpG sites. Results from this pilot study agree with those from a similar study conducted using Illumina's GoldenGate platform. The Infinium Assay has the advantage of covering nearly 20 times more genes, with greatly increased CpG site coverage of some of the most important genes implicated in neoplastic progression. This platform requires a very small amount of DNA (500 ng), allowing researchers to investigate methylation of a large number of genes in small tissue samples. This also minimizes measurement variability and provides the opportunity to study widespread methylation changes in combination with assays to evaluate chromosomal instability.

Additionally, we have demonstrated the power of assessing CpG island methylation using a large-scale array. With this method, we identified approximately 1,000 CpG sites that are differentially methylated between BE and adjacent normal tissue and hundreds of candidate CpG sites associated with progression to cancer. This represents a major advance in our understanding of epigenetic events during neoplastic progression in BE and EA.

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ADDITIONAL INFORMATION

To learn more about Illumina's DNA methylation solutions, visit our website at www.illumina.com.

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