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Genome-wide DNA Methylation Profiling of Cell-Free Serum DNA in Esophageal Adenocarcinoma and Barrett Esophagus[1,2]

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Abstract

Aberrant DNA methylation (DNAm) is a feature of most types of cancers. Genome-wide DNAm profiling has been performed successfully on tumor tissue DNA samples. However, the invasive procedure limits the utility of tumor tissue for epidemiological studies. While recent data indicate that cell-free circulating DNAm (cfDNAm) profiles reflect DNAm status in corresponding tumor tissues, no studies have examined the association of cfDNAm with cancer or precursors on a genome-wide scale. The objective of this pilot study was to evaluate the putative significance of genome-wide cfDNAm profiles in esophageal adenocarcinoma (EA) and Barrett esophagus (BE, EA precursor). We performed genome-wide DNAm profiling in EA tissue DNA ($n = 8$) and matched serum DNA ($n = 8$), in serum DNA of BE ($n = 10$), and in healthy controls ($n = 10$) using the Infinium HumanMethylation27 BeadChip that covers 27,578 CpG loci in 14,495 genes. We found that cfDNAm profiles were highly correlated to DNAm profiles in matched tumor tissue DNA ($r = 0.92$) in patients with EA. We selected the most differentially methylated loci to perform hierarchical clustering analysis. We found that 911 loci can discriminate perfectly between EA and control samples, 554 loci can separate EA from BE samples, and 46 loci can distinguish BE from control samples. These results suggest that genome-wide cfDNAm profiles are highly consistent with DNAm profiles detected in corresponding tumor tissues. Differential cfDNAm profiling may be a useful approach for the noninvasive screening of EA and EA premalignant lesions.

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Introduction

DNA methylation (DNAm) is a fundamental epigenetic modification in which a methyl group is added to the carbon-5 position of the cytosine ring within the CpG dinucleotide [1]. Extensive research has revealed that DNAm is widely implicated in all crucial changes in cancer cells, such as tumor-suppressor gene silencing, oncogene activation, and defective DNA repair [2,3]. Indeed, aberrant DNAm has been detected in a variety of cancers, including esophagus, colon, breast, liver, kidney, and lung [4–10]. To date, however, most studies of cancer DNAm have investigated DNAm in tissue-extracted DNA. The invasive procedure and the likely existence of tissue heterogeneity limit the utility of tissue DNA for epidemiologic studies. Therefore, it is desirable to develop less invasive and more accessible

Abbreviations: EA, esophageal adenocarcinoma; DNAm, DNA methylation; cfDNA, cell-free circulating DNA

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approaches that can substitute or complement tissue DNA for DNAm studies.

The presence of cell-free DNA (cfDNA) in the plasma/serum in healthy individuals and, in higher amounts, in cancer patients was demonstrated three decades ago [11,12]. However, it is only recently possible to use cfDNA as a marker for cancer diagnosis or progression [13,14]. In cancer patients, cfDNA carries the same mutations (K-*ras*, N-*ras*, *p53*) as those found in corresponding tumor tissues [15,16]. CfDNA also carries other features of the primary tumor, including microsatellite instabilities, loss of heterozygosity, and epigenetic [13,17,18]. Interestingly, DNAm patterns detected in cfDNA are in high concordance with patterns observed in corresponding primary tumor tissues [19,20]. Indeed, individual cfDNA methylation (cfDNAm) markers have been linked to different types of cancer, including esophageal adenocarcinoma (EA) [21–27]. However, there has been relatively little attention on the association of cfDNAm with cancer precursor; no studies have examined cfDNAm profiles in relation to cancer precursor and cancers on a genome-wide scale. The objective of this study was to compare the concordance of genome-wide DNAm profiles between tumor tissues and sera and to assess the performance of genome-wide cfDNAm profiles in differentiating EA, EA precursor (Barrett esophagus, BE), and control.

Materials and Methods

Genomic DNA Extraction from Tissue and Serum

Matched serum and tissue samples were obtained from eight randomly selected EA patients. Additional serum samples were collected from 10 BE patients and 10 healthy controls [28,29]. EA and BE were incident cases of histologically confirmed patients, and all subjects were recruited from Massachusetts General Hospital (Boston, MA). EA is defined as a tumor center located at or above the gastroesophageal junction and had at least two-thirds of the bulk tumor located in the esophagus; and BE is defined as pathologically confirmed intestinal metaplasia [30]. Controls were among healthy friends and non–blood-related family members of hospitalized patients. Controls were recruited at the same institutions in the same period as EA cases [29]. Pathologist-identified tissue regions that have more than 70% tumor cells without definite evidence of necrosis are considered as tumor tissues and used for DNA extractions. Peripheral venous blood sample was drawn for each subject, and the serum sample was separated within 2 hours. The serum was isolated by centrifugation at 2000 rpm for 10 minutes at 40°C and stored at −80°C until analysis. CfDNA was extracted from 800-μl aliquots of serum using the Maxwell 16 blood DNA kit (Promega, Madison, WI). Tissue DNA was isolated from 100 mg of EA tumor tissue by Maxwell 16 tissue DNA purification kit. The study was approved by the Human Subjects Committee of Massachusetts General Hospital and Harvard School of Public Health (Boston, MA).

Genome-wide DNA Methylation Analysis

We used the Illumina Infinium HumanMethylation27 BeadChip (Illumina, San Diego, CA) to analyze DNAm profiles. The BeadChip contains 27,578 highly informative CpG loci covering more than 14,495 genes [31]. DNA samples were bisulfite converted, then whole-genome amplified (WGA), enzymatically fragmented, and hybridized to the array. The assay was performed according to the manufacturer's instructions and was done at the BioMedical Genomics Center, University of Minnesota.

Data Analysis

After scanning of the BeadChip, data files were managed using the Illumina BeadStudio software Methylation module. Each CpG site on the BeadChip is represented by two bead types representing the methylated (M) and unmethylated (U) state at that site. The methylation value for each CpG locus is expressed as a β value, representing a continuous measurement from 0 (unmethylated) to 1 (completely methylated) according to the following calculation: β value = (signal intensity of M probe) / [(signal intensity of M + U probes) + 100]; the average β values is based on the average intensity of all U and M CpG probes for a given locus.

Average β values were analyzed without normalization as recommended by Illumina. The differences of DNAm levels (β value) between groups were analyzed using Student's t test. Correlations of mean DNAm levels of 27,578 CpG loci between tissue and serum DNA samples were analyzed using the Pearson test. We selected the most differentially methylated loci (a β value difference of >0.23 [32] and a $P \leq .0000006$ between groups) to run hierarchical clustering analysis. The choice of 0.23 as a criterion for a difference in β was based largely on replicate experiments by Illumina that showed that the HumanMethylation27 BeadChip could reliably detect a difference in $\beta < 0.20$ with a less than 1% false-positive rate [32]. Unsupervised hierarchical clustering analysis was carried out using the R software program.

Results

Average DNA yields (range) were 446.6 ng (350.0-627.7 ng), 426.5 ng (268.1-784.8 ng), 154.8 ng (145.1-172.2 ng), and 156.4 ng (128.2-182.1 ng) for EA sera, matched EA tissue, BE sera, and control sera samples, respectively. The OD 260/280 ratio, a measure of DNA purity with respect to protein contamination, ranged from 1.32 to 1.80. Comparison of the number of CpG sites that could be successfully interrogated (as determined by BeadStudio software) revealed that the call rates in both tissue DNA samples and sera DNA samples were

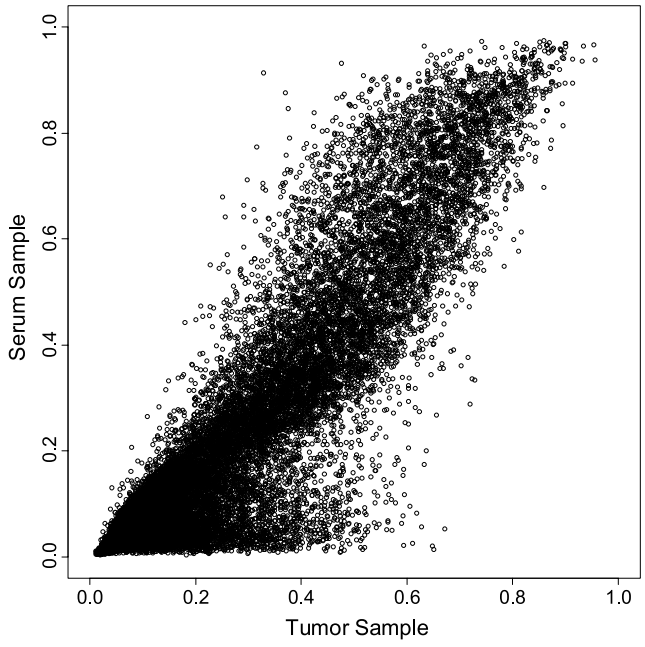

Figure 1. Correlation of DNAm levels (β values) between serum DNA samples and matched tissue DNA samples ($r = 0.92$).

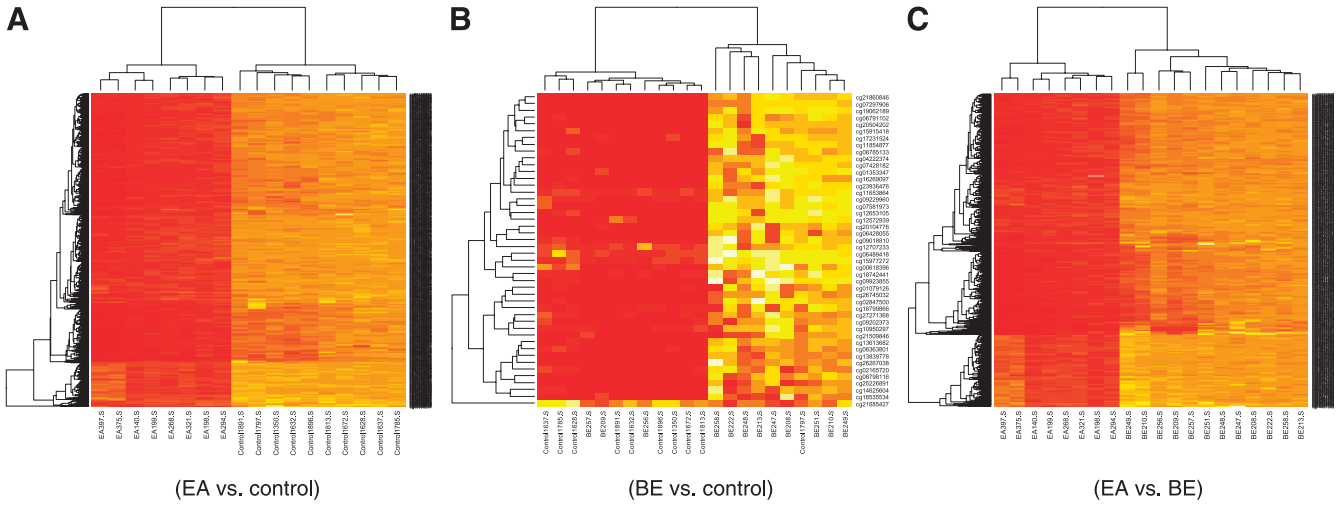

Figure 2. Hierarchical clustering of methylation values (β) from samples indicates that samples can be separated into distinct groups by cfDNAm profiles. Columns represent samples. Rows represent CpG loci. Color represents methylation level (β) from 0 to 1 as per color bar (red indicates low methylation level; yellow, high methylation level).

all greater than 99.9%. Comparison of β value distribution between EA sera DNA and matched tissue DNA showed similar patterns with high peaks of hypomethylated loci and low peaks of the hypermethylated loci (Figure W1). To investigate whether the methylation levels of sera DNA were consistent with tissue DNA samples in EA, we carried out a direct comparison of DNAm levels (β values) of 27,578 loci between tissue DNA and matched serum DNA. Figure 1 shows that highly correlated (r = 0.92) results can be achieved for matched tissue and serum DNA, suggesting that genome-wide DNAm profiles in cfDNA reflect DNAm profiles in tumor tissue DNA.

Clustering analyses showed that 911 loci perfectly discriminated between EA and control samples, 544 loci separated EA from BE samples, and 46 loci distinguished BE from control samples (Figure 2). The large number of differentiated DNAm markers is consistent with gene expression studies [33,34].

Concluding Remarks

Genome-wide DNAm profiling has been performed successfully on DNA recovered from several different types of tumor tissues including ovarian [35], breast [31,36], prostate [37], and parathyroid [38] cancers on the HumanMethylation27 BeadChip. Despite the robust methylation profiling results from tumor tissues, little information exists regarding the methylation analysis of cfDNA samples using high-density methylation arrays. To our knowledge, this study is the first to evaluate cfDNAm profiling on a genome-wide scale using the Humanmethylation27 platform. Our data, although exploratory, suggest that cfDNA obtained from serum can produce excellent DNAm profiling on a genome-wide scale and may serve as a useful tool to develop DNAm-based biomarkers for clinical application.

This study revealed several interesting observations. First, we observed a high degree of concordance in DNAm profiles between cfDNA and EA tumor tissue DNA on a genome-wide scale. This proof-of-principle study suggests that a comprehensive analysis of cfDNAm profiles has the potential to reflect genome-wide DNAm alterations in primary tumor tissues. Second, we found that differential cfDNAm profiles can distinguish EA and BE from controls, as well as EA from BE. The present results suggest that cfDNAm profiles may be a valuable biomarker for early detection of EA. Third, we also observed a trend of increasing numbers of aberrant cfDNAm markers with controls, BE, and EA (Figure 2). Our results are in agreement with previous reports in which it was found that aberrant tissues' DNAm levels accumulated gradually with the histologic changes from normal to BE, then from BE to esophageal carcinoma [39–42]. These data suggest that cfDNAm profiles may reflect the increasing involvement of aberrant DNAm in the process of normal-BE-carcinoma sequence. However, because this is not a longitudinal study, we cannot distinguish whether the abnormal cfDNAm markers seen in the preneoplastic sera develop concurrently with adenocarcinoma, or whether the aberrant cfDNAm markers in BE represent a predisposing event that give rise to adenocarcinoma. Nevertheless, cfDNAm profiles that might identify patients with cancer or at elevated risk for developing cancer would have the potential to provide an opportunity for early intervention. Furthermore, a benefit of using cfDNAm marker is that serum can be easily obtained when biopsies are not available or when the exact position of the primary lesion is not clear. A prospective longitudinal study should help reveal whether these aberrant cfDNAm profiles in normal or BE subjects are predictive of EA.

Our results should be viewed with caution. First, for the HumanMethylation27 BeadChip, Illumina recommends a starting DNA material of 500 ng or higher to achieve better performance. In this study, the average DNA quantity was less than 500 ng and the overall methylation marker completion rate was less than 100%, suggesting that the starting DNA quantity of more than 500 ng is thus necessary to obtain more robust methylation profiling results in future studies. Moreover, the exploratory nature of this study is such that we are unable to conduct sensitivity analysis and identify DNAm markers specific to clinical features. In addition, this study is limited by the small number of tissue and serum samples from patients with EA. More works in larger sample sizes are required to assess the roles of genome-wide cfDNAm profiles in cancer diagnosis. Furthermore, cfDNAm profiling results need to be validated using quantitative real-time methods, such as MethyLight and MethDet platforms [43,44].

In conclusion, our data suggest that whole-genome amplified DNA derived from stored serum have high potential to be used for genome-wide methylation profiling. Genome-wide cfDNAm profiles may be a useful noninvasive biomarker of EA and EA precursor lesions. Further

studies on cfDNAm are needed to compare the reproducibility of the DNAm results obtained with different DNAm platforms and larger numbers of samples. In addition, comparison of DNAm performance between cfDNA obtained from fresh serum and DNA obtained from archived serum samples stored in different environmental conditions is necessary.

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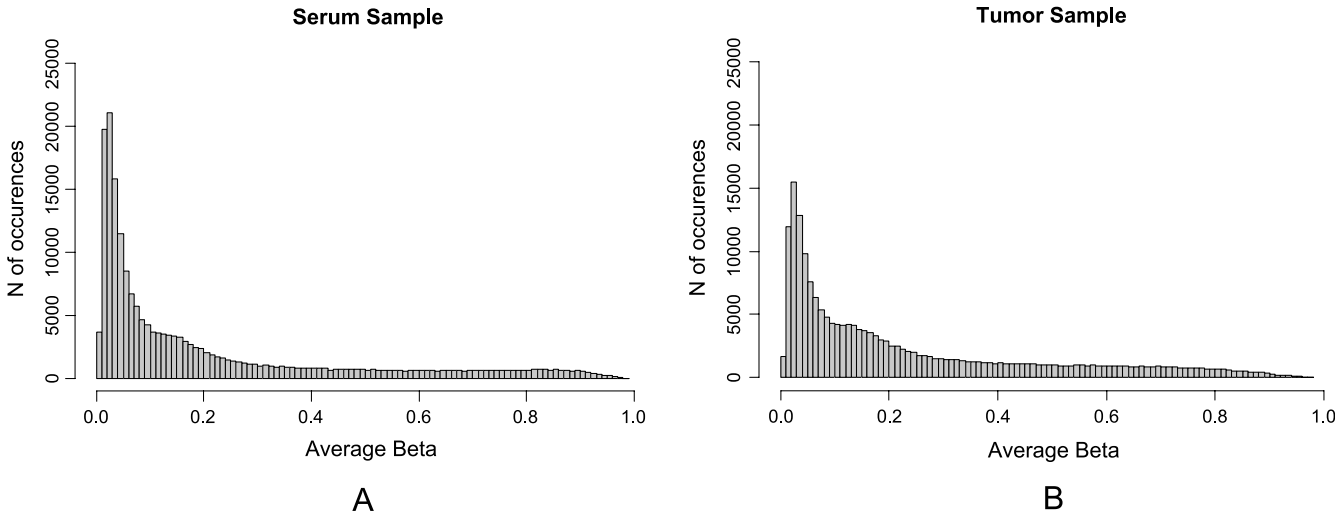

Figure W1. The β methylation value distributions in matched EA serum DNA (A) and tissue DNA (B).