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Exploration of Euchromatin Cell-Free DNA Methylation in Breast Cancer

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ABSTRACT

Circulating cell-Free DNA (cfDNA) is emerging as a non-invasive liquid biopsy biomarker for personalized and precision cancer management. While extensive tissue-based DNA methylation profiling at global and gene levels have been documented, studies regarding methylation status of cfDNA at the sub-genome scale as well as correlation with that of tissue-derived genomic DNA have yet to be explored. The ability to specifically interrogate DNA methylation status of the transcriptionally active regions within chromosomes, i.e., euchromatin, not only fulfills the knowledge gap but also provides a much needed longitudinal and real-time insight for early cancer detection and intervention. We have developed a proprietary technology for selective enrichment of euchromatin cfDNA and analyzed the 5-methylcytosine (5-mC) content in these circulating nucleocomplexes. Paired tissue and plasma DNA (n=28) were obtained from breast cancer patients at various stages with ages and genders matched to the control arm (n=21). Quantitative measurement of DNA methylation was determined by ELISA-based assays. Our results revealed significant lower methylation levels from breast cancer cohort as compared to the cancer-free group ($P<0.01$). Most importantly, the methylation quantification dataset from euchromatin cfDNA strongly correlated with that of tissue genomic DNA ($R^2=0.72$). This is the first report on euchromatin cfDNA methylation and provides promising outcomes for its future clinical application.

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Introduction

Cancer is a multi-stage process fueled by a combination of deregulated epigenetic changes and genetic alterations in DNA sequence [1]. Comprehensive detection of DNA mutation is relatively difficult and time-consuming because it could occur randomly in any nucleotide of a particular gene. In contrast, aberrant DNA methylation usually takes place in defined CpG islands within the regulatory region of the genes and it's easier to measure methylation level in a quantitative manner [1, 2]. Given the greater consistency of DNA methylation changes in cancer compared to mutations, methylation is thus a promising target for biomarker development.

Deregulated DNA methylation has been detected in a variety of cancers, including esophagus, colon, breast, liver, kidney, and lung [3–7]. DNA used for epigenetic analysis is usually extracted from tumor tissues harvested after surgical operation or biopsy, the invasive procedure and the existence of tumor heterogeneity thus limit its clinical utility as a monitoring biomarker. Recently, both genetic and epigenetic alterations found in genomic DNA extracted from the primary tumor could be detected in peripheral blood as cell-free DNA (cfDNA), opening the door for a non-invasive, real-time and longitudinal epigenetic surrogate endpoint [8-11]. Most importantly, the methylation patterns detected in cfDNA are in high concordance with patterns observed in corresponding primary tumor tissues [12, 13].

Metabolic nucleic acids in the form of nucleoprotein complexes when released into bloodstream are protected from circulating DNase/RNase digestion, while naked forms of nucleic acids are rapidly degraded. Within the chromosome secondary and tertiary structures, euchromatin regions which comprise the most active portion of genome, such as enhancers, transcribed exons, or active promoters, has been shown to display much higher protein/DNA ratios than those of non-coding inactive heterochromatin regions [14]. Because of the loosen structure of euchromatin, DNA strands are widely exposed and readily accessible to transcriptional machinery than those tightly packed heterochromatins. This unique feature highlights an opportunity for selective separation and enrichment of high-quality, biologically active and functional euchromatin DNA complexes from circulation. In principle, our proprietary approach requires no prior information about circulating DNA/protein complex composition and is very sensitive since little starting material is sufficient. Most importantly, taking into account the fact that the vast majority of human genome doesn't code for proteins (over 98%), euchromatin-based selective enrichment technology thus makes perfect sense for cfDNA clinical application than other randomly blind-extraction methodologies.

Genome-wide and individual cfDNA methylation marker or panel that differentiate control and breast cancer plasma have been reported [15–18]. However, epigenetic study on sub-chromosomal level (between global and specific gene/panel) is still lacking. Given the fact that euchromatin region is rich in gene concentration, and is often under active transcription, and that more than 90% of the human genome is euchromatic, it will be of great interest to selectively enrich well-protected, higher-molecular-weight, functional euchromatin cfDNA for the accurate quantification of methylation levels and profiles, consequently, the improvement of sensitivity and specificity of

current screening such as mammography and ultrasound [19]. Moreover, tumors that display global epigenetic alterations should benefit from targeted therapies that restore these global patterns, representing the first examples of personalized therapies developed from epigenetic knowledge. However, treatment with currently approved epigenetic drugs, e.g., DNA methyltransferase inhibitors 5-Aza-CR and 5-Aza-CdR, is rather broad, and yet to be defined epigenetic cancer subtypes that might respond differently [20-22]. In this regard, our euchromatin cfDNA methylation profiling could further improve the treatment efficiency by selecting the most responsive patients.

The objective of this study is to apply the euchromatin cfDNA approach to compare the DNA methylation levels in breast cancer and healthy cohorts, and to further correlate DNA methylation profiles between matched tumor tissues and plasma samples.

Results and Discussion

The study cohort consisted of twenty-eight breast cancer patients who at diagnosis ranged from 32 to 88 years old (median age 55 years old), with 11% stage I, 36% stage II, 11% stage III and 42% stage IV cases. Twenty-one age- and gender-matched healthy subjects were also included. Patient clinical characteristics revealed 50% invasive ductal carcinoma, 39% ductal carcinoma in situ (DCIS) and 11% undefined tumor (Table 1).

Table 1: Patient cohort demographics and clinical characteristics

Parameter	Number (n=28)	Patient Percentage
Gender	Female	100%
Median Age	55	(32-88)
Race		
White	17	61%
Black	3	11%
Undefined	8	28%
Breast Cancer		
Invasive/ Infiltrating Ductal	14	50%
DCIS	11	39%
Undefined	3	11%
Overall Clinical Stage		
Stage I	3	11%
Stage II	10	36%
Stage III	3	11%
Stage IV	12	42%

We first considered whether histologically normal tissue was epigenetically distinct from tumor mass. To do this, we compared tissue DNA methylation values for breast cancer (n = 28) and cancer-free (n = 21) groups. At the global methylation level, significant lower 5-mC concentration was detected in breast cancer cohort than in cancer-free group (mean ± SD; 54.93 ± 7.38 % vs. 87.94 ± 8.08 %, $p < 0.01$), consistent with the observation of global hypomethylation in breast tumor (Figure 1). Next, we investigated euchromatin cfDNA methylation levels in plasma samples from both breast cancer (n = 28) and control (n = 21) arms. Quantification of cfDNA methylation averaged of 5.73% (± 0.85%) in breast cancer and 14.45% (± 1.98%) in cancer-free control (Figure 2). In agreement with tissue biopsy data, plasma samples showed significant lower euchromatin cfDNA methylation in breast cancer compared to cancer-free cohort with p value less than 0.001.

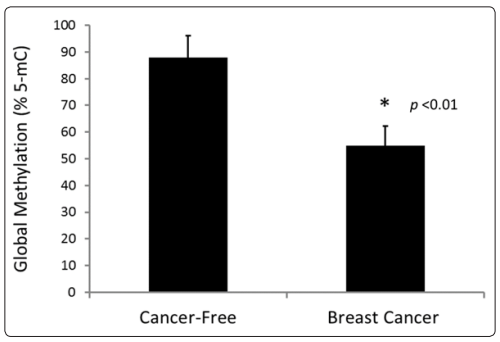

Figure 1: Global methylation of tissue genomic DNA from breast cancer patients and matched healthy cohort.

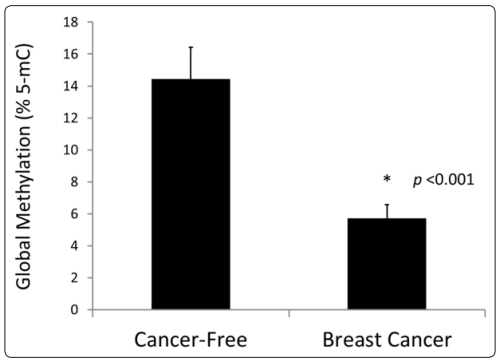

Figure 2: Global methylation of euchromatin cfDNA from breast cancer patients and matched healthy cohort

Finally, we evaluated the sensitivity of liquid biopsy to detect tumor-specific differential methylation. To assess the performance of liquid biopsies at capturing the euchromatin cfDNA methylation patterns reminiscent of the tumor, we next examined the DNA methylation profiles of each biopsy type and how similar they were to each other. To do this, methylation levels of cfDNA and corresponding tumor genomic DNA were quantified using 5-methylcytosine (5-mC) specific ELISA kits, and data were normalized to total DNA input. The most striking observation was the strong correlation between liquid and tissue biopsies (R^2=0.72; Figure 3). Comparing DNA methylation dataset of the plasma samples with the tumor tissues, it was notable that such high degree of association occurred in each individual patient. Patients with higher percentage of 5-mC in euchromatin cfDNA consistently showed higher levels of 5-mC in their matched tumor genomic DNA, the same patterns were also observed in the cases with lower concentrations of 5-mC. Collectively, our findings demonstrated that the majority of euchromatin cfDNA methylation detectable in liquid biopsy originated from tumor DNA, despite the fact that each patient had different stage of breast cancer and thus shed different cfDNA amount into the bloodstream.

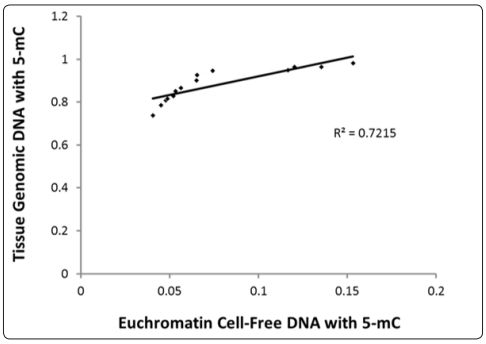

Figure 3: Strong correlation of global methylation between matched plasma euchromatin cfDNA and tissue genomic DNA

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