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DNA Methylation Profiling Distinguishes Three Clusters of Breast Cancer Cell Lines

Siyuan Zheng^{a)} and Zhongming Zhao^{a),b),c)}

Zhongming Zhao: zhongming.zhao@vanderbilt.edu

^{a)}Department of Biomedical Informatics, Vanderbilt University School of Medicine, 2525 West End Avenue, Suite 600, Nashville, TN, 37203, USA (phone: +1-615-343-9158; fax: +1-615-936-8545

^{b)}Department of Psychiatry, Vanderbilt University School of Medicine, Nashville, TN, 37212, USA

^{c)}Department of Cancer Biology, Vanderbilt-Ingram Cancer Center, Vanderbilt, University Medical Center, Nashville, TN, 37232, USA

Abstract

Methylation change plays an important role in many cellular systems, including cancer development. During recent years, genome-wide or large scale methylation data has become available thanks to rapid advances in high-throughput biotechnologies. So far, researchers have always used gene expression profiling to study disease subtypes and related therapies. In this study, we investigated methylation profiles in 30 breast cancer cell lines using methylation data generated by microarray technologies. Strong variation of the number of methylation peaks was found among these 30 cell lines; however, more peaks were found in the upstream regions than downstream regions of genes. We further grouped the methylation profiles of these cell lines into three consensus clusters. Finally, we performed an integrative analysis of breast cancer cell lines using both methylation and gene expression profiling data. There was no significant correlation between methylation profiling subtypes and gene expression profiling subtypes, suggesting the complex nature of methylation in the regulation of gene expression. However, we found basal B cell lines appeared exclusively in two methylation clusters. Although these results are preliminary, this study suggests that methylation profiling might be promising in disease subtype classification and the development of therapeutic strategies.

Introduction

Breast cancer is among the top threats to public health worldwide. Approximately 1 in 8 women is expected to be diagnosed with breast cancer over the course of her lifetime [1]. Recently, tremendous efforts have been made to understand the pathogenesis of this cancer, and striking improvements have been achieved in its diagnosis and treatment [2–5]. Technological innovations over the past two decades have empowered these advances, expanding our knowledge of breast cancer to an unprecedented level [6–7]. The breast cancer genome and transcriptome have been intensively interrogated to identify the causative gene deregulations, aiming to unveil the driving forces behind this disease. Subtypes have also been recognized, mainly identified through large scale gene expression studies [8–9]. The most recent findings included the identification of six triple-negative breast cancer (TNBC) subtypes using clustering analyses of gene expression (GE) profiles from 21 breast cancer data sets [10]. The robust gene signatures have been used for

prognosis, diagnosis, and drug treatment of this cancer, as well as the prediction of treatment responses [3, 10–11].

Cell lines are cultured tumor cells that are first resected from patients and then grown under controlled laboratory conditions. Because of their availability and homogeneity, they are widely used as models for cancer research. So far, there are numerous breast cancer cell lines available (e.g., those listed at <http://icbp.lbl.gov/cc/celllines.php>), representing the genetic background of different breast cancers. Despite their extensive usage, a long lasting concern is that cell lines have acquired many additional genetic characteristics within the original tumors due to selective pressures in the *in vitro* culture environment. Recently, several studies have been conducted to examine the genomic properties of these cell lines, aiming to provide principles and guidelines regarding the use of them as breast cancer models [12–14].

Copy number alterations and gene expressions of more than 51 cell lines were investigated, suggesting that they are important models for studying breast cancer, including identifying molecular features for predicting responses to target therapies or other physiological perturbation [12]. However, compared to gene expression or other genetic alteration analyses, epigenetic properties have not yet been well-examined. In this study, we profiled the methylation patterns of 30 breast cancer cell lines from the previous compilation of 51 cell lines [12, 15]. We found that, similar to gene expression profiling, methylation profiling could separate the cell lines into clusters. Although this is a pilot study, these results shed light on our understanding of the epigenetic mechanisms in breast cancer, and they are likely useful for future breast cancer diagnosis and targeted therapies.

Results

Features of Breast Cancer Cell Lines

A total of 30 breast cancer cell lines were collected for methylation profiling analysis. A summary of these cell lines was provided in Table 1. Molecular experiments have been conducted previously to determine the biomarker statuses in those cell lines, including *ER* (encoding estrogen receptor), *PR* (encoding progesterone receptor), *HER2* (encoding human epidermal growth factor receptor 2) and *TP53* (encoding tumor suppressor P53) (see <http://www.atcc.org> and <http://icbp.lbl.gov/cc/celllines.php> and references [12, 14]). Expression subtypes were also established in two independent studies; these subtypes were named luminal, basal A and basal B [12, 14]. While most cell lines have the same classification, cell line SUM190PT has a discordant assignment. There is a clear correlation pattern between the expression subtypes and the marker status. For example, 100% of the *ER* positive and *PR* positive cell lines have luminal subtype, but no basal A or basal B subtypes have either of these two markers expressed. Additionally, 70% (7 out of 10) of the *HER2* positive cell lines have the luminal subtype while the remaining 30% (3 out of 10) have the basal A subtype. No positive *HER2* is found in the basal B subtype.

Methylation Characteristics Varied Among Breast Cancer Cell Lines

We next examined the methylation status of these 30 breast cancer cell lines using the data generated by the Agilent 244K array, which covered 237,202 probes. The analysis methods are shown in the “Experimental Part” section. We first measured the methylation peaks in the whole genic regions. As shown in Table 2, the number of methylation peaks varied greatly among these cell lines, ranging from 17 peaks in SUM159PT to 3256 peaks in MDAMB468. For all the cell lines, the median number of peaks was 470. There was no significant correlation between the number of methylation peaks of the breast cancer cell lines and their expression subtypes (Spearman correlation test, $r = -0.18$, $p = 0.35$).

To further explore methylation status in genes, we classified the methylation peaks that mapped into gene regions into three groups: gene body region, upstream and downstream gene region. The upstream and downstream regions were defined as 1000 bp long upstream and downstream of the gene body regions. Remarkably, for all cell lines, there were more peaks mapped to gene body regions than upstream or downstream regions. This feature is most likely due to the larger region of gene body compared to up/down stream regions. Of note, we found more peaks localized to upstream regions, reflecting that the promoter regions of genes are the primary locations for methylation regulation [16–18].

Gene Methylation Profiling Revealed Three Robust Subtypes

We next examined whether methylation profiling could be utilized to separate breast cancer cell lines into distinct subtypes. This analysis is unique and important because, so far, subtype analysis has prevalently been done for breast cancer by gene expression profiling. Because of the intrinsic noise within the methylation data, we first selected the most representative probes for the corresponding genes. The underlying rationale is that probes constituting peak regions represent the real signals of cell line methylation status. Therefore, we identified all the probes in methylation peak regions. For robustness and reliability, only those probes that appeared in more than 5 cell lines were selected for the following clustering analysis.

We used a consensus clustering method [19]. To determine how many clusters would provide the best performance in subtype classification, we compared the results by setting a varying number of clusters. As shown in Fig. 1, the consensus score reached its maximum when k was set at 3, suggesting that we should separate these cell lines into three subclasses. When k was set at 4, it generated five classes. For these five classes, we noticed that the three clusters at the right bottom in Fig. 1 likely reflected finer classification of the largest subclass generated by setting $k=3$. We then used the SAM (Significance Analysis of Microarrays) method [20] to find the most correlated probes within each subclass.

The methylation pattern of each subclass is shown in Fig. 2. There is a small set of probes showing high methylation level in cluster 1 (red probes in Fig. 2). A similar size of the probe set was observed to be highly methylated in cluster 2. Conversely, we found a large probe set whose methylation expression was high in cluster 3, suggesting that cluster 3 shares strong methylation regulation.

To explore additional biological insights, we annotated the expression subtypes (luminal, basal A and basal B) to these three clusters (see top panel in Fig. 2). We found no significant correlation (Spearman correlation test, $r = -0.19$, $p = 0.32$) between these two levels of biological information, suggesting that methylation might be too complex and dynamic to reflect gene expression change. However, we observed basal B cell lines occurred exclusively in clusters 2 and 3. Note that cluster 3 is highly methylated compared to other two clusters. Future investigation of the methylation regulation in this specific basal B subtype is warranted.

Discussion

During the past decade, technological advances have allowed researchers to generate numerous genetic and genomic datasets for biological and biomedical studies, especially in cancer studies [21–22]. In particular, genomic scale technologies empower investigations in a largely unbiased manner. Complex diseases, including cancer as the most focused research area, have been under extensive investigation, gleaning many important findings related to both molecular mechanisms and clinical therapies. Microarray technology is among the most widely used platforms, and it has been applied to interrogate gene expression, DNA

copy number alteration, genome-wide association studies, genome-wide microRNA assay, and DNA methylation assay. Because of its genome scale capability, high-dimensional microarray data is often used to stratify biological samples, such as tumor samples, to provide insights into disease pathogenesis with the goal to develop new tools for diagnosis and prognosis [23–24]. Researchers have shown that microarray data outperforms histological data to classify disease subtypes [25].

So far, most class discovery studies have been based on gene expression microarrays. Methylation arrays are rarely used for this purpose because this technology is newly developed and methylation change is dynamic. It has been thought that methylation signals captured by arrays are highly transient, and there is substantial stochastic noise in the data. However, numerous studies during the past a few years have suggested methylation plays an important role in gene silencing, genomic imprinting, X-chromosome inactivation, and tumorigenesis [26]. Thus, it is necessary to explore whether methylation profiling can be used for disease status studies and biomarker discovery, including classification of subtypes of diseases like cancer.

In this study, we used methylation microarray data to examine subclasses in 30 breast cancer cell lines. We showed that the featured gene methylation profiles could robustly classify these cell lines into three subclasses. Consistent with previous studies that showed clinically related phenotypes defined by methylation patterns [27], our results provided additional evidence that methylation could be used to stratify cancer phenotypes. Our results also revealed that methylation regulation predominantly occurs in the upstream gene region, consistent with many single gene methylation assays. With the increase of matured microarray technology designed for methylation, and the fact that single-base resolution of methylomes can now be generated by next generation sequencing technologies, disease subtype classification and biomarker discovery using methylation data is expected to accelerate in the near future.

Conclusions

In this study, we investigated the methylation profiles of 30 breast cancer cell lines using the methylation data generated by microarray technologies. We developed novel strategies to measure methylation levels using methylation peaks in genic regions. We found strong variation of the number of methylation peaks among these 30 cell lines. However, more peaks were found in the upstream regions than downstream regions of genes. We further grouped the methylation profiles of these cell lines into three consensus clusters, from which some interesting features of methylation could be detected. Finally, we performed an integrative analysis of breast cancer cell lines using both methylation and gene expression profiling data. We did not find a correlation between methylation profiling subtypes and gene expression profiling subtypes, suggesting the complex nature of methylation related to the regulation of gene expression. However, we found basal B cell lines appeared exclusively in methylation clusters 2 and 3. Overall, this study suggests that methylation profiling might be promising in disease subtype classification and development of therapeutic strategies.

Experimental Part

A total of 30 breast cancer cell lines were assayed for DNA methylation using microarray technology following the DMH protocol [5]. The DMH protocol enables one to capture the GC-rich region when digesting the genome. The GC-rich fragments are then subjected to fluorescent labeling and microarray hybridization. Breast cancer cell lines and pooled normal controls were hybridized simultaneously to the Cy5 and Cy3 channels of an Agilent

244K CGI array, which covers 237,220 probes. Details of the DMH protocol and experiment procedure are described in references [5, 15].

In our analysis, the resulting images of the above arrays were scanned and preprocessed using the Bioconductor *limma* package [28]. “Median” method and “scale” method were selected for within and between array normalizations, respectively. The methylation probe signals were smoothed using a running median method, with the window size set to 600 base pairs (bp). Peak values were identified using the *Ringo* package [29]. The minimum number of probes for a peak region was set to 3 and the maximum length of the peak was set to 600 bp. Featured probes were selected to present at least 5 times in the peaks among all breast cancer cell lines. The methylation profile matrix represented by these probes was further used for consensus clustering analysis. The probes were mapped to corresponding genes including gene body and 1000 bp upstream and downstream of the gene body, using the similar protocol in the previous work [30].

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Fig. 1. Consensus clustering matrix of 30 breast cancer cell lines

K defines the number of clusters in the data set. Setting $k=3$ had the best consensus score. Row and column represent samples.

Fig. 2. Consensus clustering of the methylation profiles using the featured probes

The 30 breast cancer cell lines were stratified into three clusters based on their methylation profiles. The methylation based clusters were further correlated with gene expression based clusters. Each row denotes a representative probe, and each column denotes a cell line. Colors represent the normalized methylation levels, as illustrated in the figure key.

Table 1

Summary of 30 Breast Cancer Cell Lines

Cell line	Neve et al. ^(a)	Kao et al. ^(b)	ER ^(d)	PR ^(c)	HER2 ^(c)	TP53 ^(c)
HCC1599	NA	A	-	-	-	-
600MPE	L	NA	+	-	NA	-
AU565	L	NA	-	-	+	+
BT549	B	B	-	-	-	+
CAMA1	L	NA	+	-	NA	+
HCC1007	L	L	+	-	+	+
HCC1143	A	A	-	-	-	+
HCC1428	L	L	+	+	-	-
HCC1954	A	A	-	-	+	NA
HCC202	L	L	-	-	+	-
HCC2157	A	A	-	+	+	+
HCC2185	L	L	-	-	-	+
HCC3153	A	A	-	-	-	-
HCC38	B	NA	-	-	-	+
HS578T	B	B	-	-	-	+
LY2	L	NA	+	-	NA	NA
MCF10A	B	B	-	-	-	NA
MCF7	L	L	+	+	-	+
MDAMB175	L	L	+	-	-	NA
MDAMB361	L	L	+	-	+	-
MDAMB435	B	NA	-	-	NA	+
MDAMB436	B	B	-	-	-	-
MDAMB468	A	A	-	-	-	+
SKBR3	L	L	-	-	+	+
SUM149PT	B	B	-	-	-	+
SUM159PT	B	NA	-	-	NA	-
SUM190PT	A	L	-	-	+	NA
T47D	L	L	+	+	-	+

Cell line	Neve et al. ^{a)}	Kao et al. ^{b)}	ER ^{a)}	PR ^{c)}	HER2 ^{c)}	TP53 ^{c)}
UACC812	L	L	+	-	+	-
ZR7530	L	L	+	-	+	-

^{a)}Reference: Neve et al. [12].

^{b)}Reference: Kao et al. [14].

^{c)}Status determined from ATCC (<http://www.atcc.org>) and <http://icbp.lbl.gov/ccc/cellines.php>.

NA: not available.

Table 2

Summary of Methylation Peaks Identified in Each Breast Cancer Cell Line

Cell line	Body	Upstream ^{a)}	Downstream ^{a)}	Total
HCC1599	196	31	9	299
600MPE	235	80	10	350
AU565	45	9	1	62
BT549	969	512	39	1535
CAMA1	248	98	20	442
HCC1007	84	17	3	116
HCC1143	1369	748	55	2074
HCC1428	320	125	19	468
HCC1954	784	353	35	1237
HCC202	97	43	5	166
HCC2157	194	69	16	317
HCC2185	31	10	1	51
HCC3153	51	12	0	89
HCC38	399	222	17	616
HS578T	1314	727	62	2000
LY2	322	88	15	540
MCF10A	324	90	18	472
MCF7	1571	841	63	2414
MDAMB175	28	10	0	48
MDAMB361	820	422	44	1310
MDAMB435	681	366	26	1067
MDAMB436	14	3	0	32
MDAMB468	2069	1168	85	3256
SKBR3	547	161	13	740
SUM149PT	235	113	9	347
SUM159PT	7	0	1	17
SUM190PT	368	217	13	597
T47D	586	292	37	923
UACC812	1328	722	58	2080
ZR7530	143	50	4	228

^{a)} Gene up/down stream region was defined to have 1000 bp length.