

DNA methylation as a promising landscape: A simple blood test for breast cancer prediction

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Abstract Breast cancer is the most common malignancy among women worldwide. Risk assessment is one of the main services delivered by cancer clinics. Biomarker analysis on different tissues including the peripheral blood can provide crucial information. One of the potential epigenetic biomarkers (epimarkers) is introduced as the peripheral blood DNA methylation pattern. This study was conducted to evaluate the potential value of peripheral blood epimarkers as an accessible tool to predict the risk of breast cancer development. WBC's DNA was the focus of several case-control studies at both genome wide and candidate gene levels to reveal epigenetic changes accounting for predisposition to breast cancer, leading to suggest that ATM, TITF1, SFRP1, NUP155, NEUROD1, ZNF217, DBC2, DOK7 and ESR1 genes and the LINE1, Alu and Sat2 DNA elements could be considered as the potential epimarkers. To address that by which mechanisms WBC's DNA methylation patterns could be linked to the propensity to breast cancer, several contemplations have been offered. Constitutional epimutation during embryonic life, and methylation changes secondary to either environmental exposures or tumor-mediated immune response, are the two main mechanisms. One can deduce that epimarkers based on their potential properties or regulatory impacts on cancer-related genes may be employed for risk

prediction, prognosis, and survival inferences that are highly required for breast cancer management toward personalized medicine.

Keywords Epimarker · WBC DNA methylation · Breast cancer · Risk prediction

Introduction

Breast cancer, with a lifelong risk of one in nine, is the most common cancer among women [1]. The prognosis of breast cancer, in particular its survival rate, crucially depends on its earlier detection. In terms of any interventions, these points highlight the need for regular life-time screening tests because of the disease frequency, and the feasibility and safety of such tests since they may be repeated many times.

Adopting a comprehensive screening approach including genetic molecular test could be highly recommended for this intervention. In the case of hereditary breast cancer, there are specific blood tests, BRCA1/2 mutation analysis that only covers less than 10 % of breast cancer cases in total, relative to sporadic form of the disease. For the latter form, although some new molecular tests namely particular sets of SNPs [2] have been added to the regular screening methods such as mammography, predicting the risk of breast cancer development in the absence of familial history is still a big challenge. The current screening methods are criticized by both the low sensitivity [3, 4] and overdiagnosis pitfalls [5, 6].

Since each screening molecular test only suggests a relative risk for predisposition to breast cancer, providing a more inclusive set of these tests could collectively infer more precise estimation for risk assessment. In this regard, coupling of epigenetic test to conventional molecular panel may even confer more values, as it could uncover new dimension of molecular

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architecture relative to cancer susceptibility. It is well known that epigenetic alterations, especially DNA methylation, are involved in tumorigenesis [7]. These alterations include gene-specific hypermethylation and global genomic hypomethylation that are well defined in tumor tissues [8]. However, recent studies have identified some epigenetic changes in the WBC genome among breast cancer patients which propose the predictive value of these changes as new biomarkers. Epigenetic biomarkers (epimarkers) are noteworthy, because of the stability of DNA molecules, dynamic nature of epigenetic changes, and availability of different ways for its measurement.

The purpose of this review is to introduce epimarkers as potential markers in risk prediction, diagnosis, and prognosis of breast cancer.

Peripheral blood epimarkers

In the case of identifying epimarker in the peripheral blood shown in Table 1, we have two alternatives: methylation in DNA of circulating tumor cell and WBC. Since cell-free DNA of circulating tumor cells originates from the tumor mass, it can be regarded as a source of diagnostic rather than predictive information. Presence of circulatory tumor DNA in low amounts and possibility of their contamination with normal cell DNA are serious limitations to consider them as reliable source. However, undergoing techniques may overcome these limitations to catch circulatory tumor cell DNA in good quantity and purity, giving them a momentum to be apply for diagnostic and prognostic purposes [9]. On the contrary, high amount and good quality of WBC DNA and more importantly its predictive potentials and implications concluded by several case-control studies make it a practical source to examine the epigenetic changes of WBCs before and during breast cancer development. Although there is no known mechanistic correlation between WBC epigenomic pattern and tumor development by now, such epigenetic changes may be looked as an independent risk factor quite long before breast cancer development. Since WBC DNA is accessible easily, its epigenetic along with its genetic influences on cancer propensity could be repeatedly evaluated in specified time intervals.

Table 1 Comparison of peripheral blood epimarkers

Source of epimarkers	Characteristic				
	Amount	Purity	Diagnostic value	Predictive value	Noninvasive accessibility
WBC DNA methylation	High	Originated only from WBCs	No	Yes	Yes
Circulatory tumor DNA methylation	Low	Heterogeneous mixture of normal cells and tumor DNA	Yes	No	Yes

History of WBC DNA methylation as epimarker in cancers

The quest for WBC-based epimarkers was launched in 2001, when Karen et al. [10] showed a relationship between hypomethylation of P53 gene in WBC and risk of lung cancer development. Consequently, methylation alterations in different kind of cancers have been studied, concluding that WBC DNA methylation can be considered as a surrogate biomarker for the risk of cancer development, including breast cancer (Table 2) [27–37].

How methylation of WBC genome was explored in breast cancer?

According to previous studies mentioned in Table 2, there are two ways for discovering epimarkers in WBC: searching for methylation pattern alteration at target gene (candidate gene approach) and at the epigenomic level (whole genome approach). In the candidate gene approach, researchers evaluate genes involved in breast cancer carcinogenesis or genes that are mostly methylated in breast tumors. Although this approach is logical for DNA methylation studies of circulatory tumor cell, in the case of WBCs, because of their unknown role in breast cancer, may not embrace methylation pattern as of the tumor. For this reason, whole genome approach has been more popular, recently. In this regard, a WBCs gene which its methylation alteration pattern is associated with the breast cancer risk will be disclosed, which does not necessarily mean its direct role in carcinogenesis.

Evidences based on candidate gene approach

BRCA1, because of the crucial role of its mutations in hereditary breast cancer, has been considered as a natural choice to conduct a candidate gene analysis. In terms of epigenetic changes, BRCA1 promoter hypermethylation has received good attention as it offers another alternative inactivation mechanism for this critical gene. Some studies have shown an association between WBCs *BRCA1* methylation changes and the risk of breast cancer, which was more significant among sporadic breast cancer cases than the hereditary ones

Table 2 DNA methylation epimarker survey in WBC genome for breast cancer risk prediction

Authors	Year	Patients	Methods	Results
Widschwendter M. et al. [7]	2008	353 post menopause breast cancer patients and 730 post menopause controls	Methyl light assay for four genes categories (<i>ERT, DMHR, PCGT, MBC</i>)	Hypermethylation of 6 genes (<i>NUP155, ZNF217, PTGS2, TITFI, NEUROD1, and SFRP1</i>) in cases compared to controls.
Flanagan JM. et al. [11]	2009	14 patient with bilateral breast cancer and 14 controls (confirmed by 190 cases and controls)	Enzyme based enrichment and microarray	Hypermethylation of <i>ATM</i> (intragenic repetitive element) in patients
Choi J. et al. [12]	2009	18 patients 19 controls (confirmed by 179 cases and 180 controls)	5- <i>mdC</i> (5- methyl deoxy cytosine) was measured by chromatography and LINE1 methylation by quantitative pyrosequencing	Hypomethylation of 5- <i>mdC</i> in patients
Cho YH. et al. [8]	2010	40 patients 40 controls	Methyl light for 8 tumor suppressor gene and repetitive elements (<i>sat2, LINE1, Alu</i>)	Hypomethylation of <i>Sat2</i> in WBC of patients
Wong EM. et al. [13]	2011	255 patients 169 controls	Methylight& MS-HRM for <i>BRCA1</i>	Hypermethylation of <i>BRCA1</i> in patients.
Iwamoto T. et al. [14]	2011	200 patients 200 controls 162 tumor tissues	MSP (<i>BRCA1</i> promoter)	Hypermethylation of <i>BRCA1</i> promoter in patients.
Wojdacz TK. et al. [15]	2011	75 breast tumors 75PB from the same patients	MS-HRM (<i>RASSF1, APC, BRCA1</i>)	No association between methylation in WBC genome and breast tumors.
Wu HC. et al. [16]	2011	31 females with a family history of breast cancer 20 females without a family history	<i>LINE1, sat2, Alu</i> with MethylLight <i>LINE1</i> with pyrosequencing	Hypomethylation of <i>Alu</i> and <i>LINE1</i> in females with a family history of breast cancer.
Bosviel R. et al. [17]	2012	900 patients 900 controls	qMSP (<i>BRCA1</i>)	No significant difference between the cases and controls.
Pang D. et al. [18]	2012	72 patients with hereditary breast cancer 30 patients with sporadic breast cancer	Bisulfite sequencing (<i>BRCA1</i> promoter)	<i>BRCA1</i> was more methylated in sporadic than hereditary breast cancer. The methylation pattern was different in these two forms of breast cancer.
Brennan K. et al. [19]	2012	667 patients 769 controls from three large cohorts	Methyl light assay for two intragenic repetitive elements(<i>mvp2a</i> and <i>mvp2b</i>) and <i>LINE1</i>	Hypermethylation of <i>ATM</i> [intragenic repetitive element MVP2a] in patients.
Wu HC. et al. [20]	2012	282 breast cancer patients 347 unaffected sisters	<i>LINE1, sat2, Alu</i> with MethylLight <i>LINE1</i> with pyrosequencing	Association between <i>sat2</i> methylation and the risk of breast cancer.
Kitkumthorn N. et al. [21]	2012	216 patients with six different cancers(nasopharynx, lung, liver, bile duct, breast and colon) 144 controls	<i>LINE 1</i> by COBRA	<i>LINE1</i> was hypomethylated in colon and nasopharynx cancer patients.
Hansmann T. et al. [22]	2012	641 patients with familial breast and ovarian cancer without <i>BRCA1/2</i> mutation	Bisulfate pyrosequencing for(<i>ATM, BRCA1, BRCA2, RAD51C, PTEN</i> and <i>TP53</i>)	Hypermethylation of <i>RAD51C</i> and <i>BRCA1</i> in normal cells of familial breast and ovarian cancer patients.
Xu X. et al. [23]	2013	298 breast cancer patients 612 controls	Whole genome methylation analysis with array	250 differentially methylated regions in cases compared with control
Heyn H. et al. [24]	2013	15 monozygotic twins discordant for breast cancer	Whole genome methylation analysis with array	Hypermethylation of <i>DOK7</i> in patients.
Anjum SH. et al. [25]	2014	72 <i>BRCA1</i> mutation carrier 72 without <i>BRCA1</i> mutation	Whole genome methylation analysis with array	Specific methylation signature in WBC of <i>BRCA1</i> mutation carriers that is useful for predicting of sporadic breast cancer and survival
Kuchiba A. et al. [26]	2014	384breast cancer patients 384 controls	Global methylation measured by LUMA	Global genomic hypomethylation in breast cancer patients

MSP methylation-specific PCR, qMSP quantitative methylation-specific PCR, MS-HRM methylation-sensitive high-resolution melting, PB peripheral blood, COBR4 combined bisulfite restriction analysis

[14, 22, 38]. Such association was also stronger among cases with early age at the onset [age ≤ 40] and high-grade tumors. Therefore, some researchers suggested that in sporadic breast cancer, the first hit may influence somehow *BRCAl* methylation according to the Knudson's two hit theory, while such pivotal role has been given to germ line mutation in hereditary breast cancer [18, 22, 39–42]. However, while that potential value for *BRCAl* methylation as a candidate biomarker has not been approved by other studies [15, 17, 43], a very recent investigation on sporadic breast cancer concludes that WBC methylation signature may have such value conditioned to prior presence of *BRCA1* mutations [25]. In this study, WBC DNA methylation signature in *BRCA1* mutation carriers could predict breast cancer risk (area under curve 0.65). Also, the mentioned signature could predict mortality (area under curve 0.67) in sporadic breast cancer patients.

In addition to *BRCAl*, other genes which are involved in breast carcinogenesis such as estrogen receptor target genes (ERT), polycomb group target genes (PcGT) and *ATM* also have been studied [7, 11], which suggest *ATM*, *NUP155*, *ZNF217*, *PTGS2*, *TITF1*, *NEUROD1* and *SFRP1* as potential biomarkers for breast cancer. It should be noted that methylation of *ATM* was in a repetitive element in the gene body and because of notable overlap between methylation in cases and controls; it seems that this marker alone is not sufficient as a diagnostic test (which area under curve of 0.59 shows insufficient specificity as a diagnostic biomarker).

Evidences based on whole genome approach

Since there are uncertainties behind candidate gene(s) selection, and the logic for representative value of peripheral blood for epigenetic changes in diseased breast tissue, whole genome approach could be considered as preferred alternative. Both hypermethylation and hypomethylation changes of WBC at genomic level could accompany with cancer developments.

According to the results of several genomic studies, hypermethylation changes of some genes in WBC genome have been evident in different cancers, including bladder, head and neck, pancreas, and breast cancers [23, 31, 33, 44, 45]. As a result, a number of genes have been nominated as potential epimarkers for epigenetic predisposition to breast cancer with prediction accuracy of 65.8 % [23].

Analysis of global genomic hypomethylation as a well-known epigenetic manifestation in cancer has been referred by several whole genome studies. Recent studies have reported that such genomic hypomethylation continuum can be evident even at WBC DNA level and may detect high-risk women before developing breast cancer suggesting its value as predictive biomarker [26, 30, 32, 36]. In this regard, some researchers have suggested that methylation analysis of

repetitive elements such as *LINE1*, *sat2* and *Alu* in WBC may be a surrogate for genomic hypomethylation. Based on this approach, some studies have shown that these repetitive elements in the leukocytes of healthy women with a family history of breast cancer are hypomethylated [16, 46] as well as breast cancer patients compared to controls [8, 21, 47].

Another way for evaluating global hypomethylation is measurement of 5-methyl cytosine (5mc) amounts in the genome. Xu and Ambrosone in two case-control studies showed that 5mc (not methylation of repetitive element) decreases in the WBC genome in breast cancer patients [12, 48]. The correlation between global 5mc and methylation of repetitive elements was not reported in another study [49], which makes it an unresolved controversy.

Limitation in previous studies

Some recent evidence has indicated that different epigenetic markers in the WBC genome could be considered as potential biomarkers for risk estimation of breast cancer. However, it is necessary to reassess these findings through studies with larger sample sizes. Most of the previous investigations were case-control studies, and the past history of their participants was not provided. It is important to note that many factors such as demographic characteristics, environmental exposures, and even genetic susceptibility can change the leukocyte methylation signatures [50]. Therefore, these factors may confound the interpretation of results and should be taken into account in future studies, which have been properly respected by Brennan et al. study [19], which summoned women from three large cohorts and followed them for 2–4 years and examined their past history along with their blood samples that were taken before cancer development. Another limitation of these studies is choosing the right candidate genes in the WBC genome that may reflect methylation deviation in breast cancer patients. Therefore, it is highly recommended to adhere with prospective and whole genome approaches, meanwhile considering other risk factors which affect the methylation signature in WBCs.

Biological relevance between WBC genome methylation and cancers

In spite of the documented association between WBC genome methylation and breast cancer, there is still a major debate in this issue: “how such association could be explained at mechanistic level?” Here, five explanations for the association between WBC DNA methylation status and breast cancer are overviewed:

First, there is a constitutional epimutation which means that some epigenetic changes can be observed in normal tissues

(blood) as well as in tumor tissues [13, 51–57]. In this case, constitutional epimutation can be considered as a consequence of germ line alteration that is not fully understood. However, it is clear that in germ line development, there are two phases of demethylation and remethylation which any remained errors in each phase can be passed through in the germ line cells [11]. In addition, constitutional epimutation can occur in early developmental stages at intrauterine phase (Fig. 1—rational 2). Second, some evidence have indicated that genetic variation could lead to specific epigenetic signatures [33, 58]; so, genetic susceptibility may lead to epigenetic alteration (Fig. 1—rational 1).

Third, lifetime exposures to environmental influences could change epigenomic map of somatic cell/stem cell, which in turn is a result of direct or indirect effect by mutation of driver gene that control DNA methylation [44] (Fig. 1—rational 3). Such exposures may lead to the methylation changes in whole body somatic cells or only in specific tissues like hematopoietic stem cells. Considering the fact that DNA methylation is a lengthy process and blood cells have a short life span, their methylation condition may have been acquired from certain subgroups of hematopoietic cells.

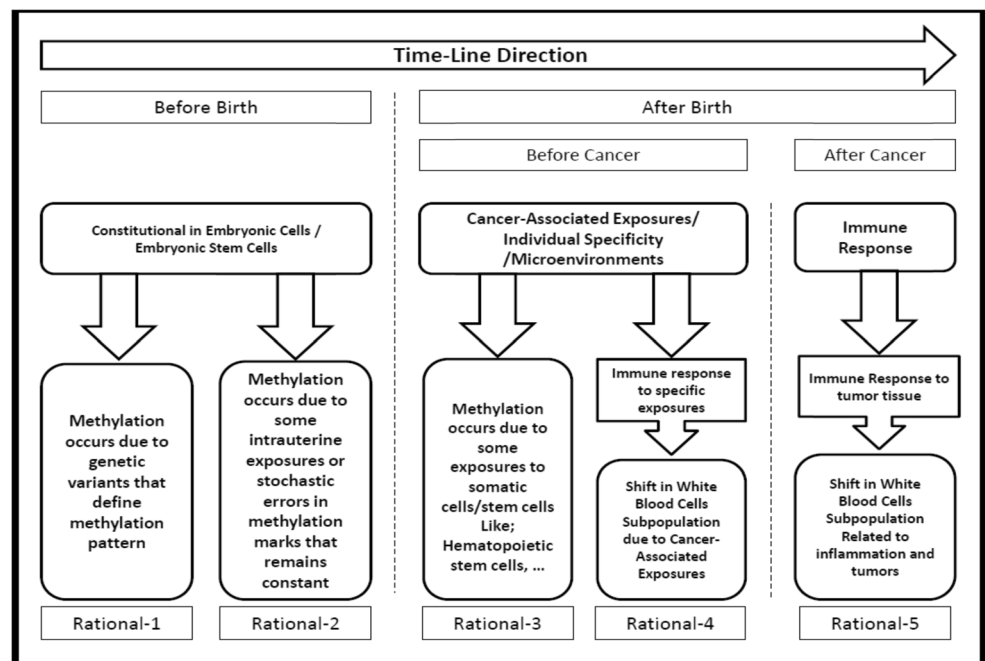
The other concerns are connected to the nature of immune system response that is defined by clonal-specific expansion of leukocytes. There are evidences that different cancers evoke immune responses in specific manner mirrored by proliferation of particular subset of these immune cells. So, if one considers that this activation is somehow followed by epigenetic changes at activated clones, inspection of these changes could show new information. Therefore, the point is how we

could find the main cellular types/clones to explore their methylation signature patterns according to the existing type of the cancer. However, this assumption could still be sound if we consider that the differential shift or polarization of the immune cells can be also due to cancer-associated environmental exposures in addition to cancer inflammation itself [20, 59] (Fig. 1—rational 4 and 5).

Future applications of peripheral blood epimarkers

Since epimarkers can help to identify high-risk women for breast cancer in population, and as epigenetic changes are reversible [60], it seems that reversing the epigenetic alterations and turning cells back to their normal epigenetic state can be considered as a future modality in prevention of breast cancer. On the other hand, because of potentiality of epimarkers, they can be used for various purposes. Epigenetic alterations, similar to genetic variations, can change the prognosis and survival parameters in cancer. Some studies on the ovaries, stomach, and pancreas cancer patients suggested that hypermethylation of some genes in the WBC genome affects the survival [61–63]. Another study by Zhuang et al. suggested that DNA methylation can be used as a prognostic biomarker [64]. Therefore, in near future, different applications may be defined for epimarkers: therapeutic (e.g., for epidrugs efficacy assessment), prognostic (for patient survival assessment), predictive (to adjust the best treatment options), and recurrence (for estimation of cancer relapse). In this view, new comprehensive studies holding more statistical power

Fig. 1 Potential mechanisms for association between WBC DNA methylation and cancers



and less confounding cracks could pave the way for the era of epigenetic biomarkers in medicine.

Conclusion

Recent investigations have identified some epigenetic alterations in the WBC genome of breast cancer patients that could predict the risk of breast cancer. Although the current findings are quite promising, it seems that application of WBC methylation pattern as a sensible predicting test for breast cancer requires more search and research, for example by designing a prospective and whole genome studies. It is well known that many factors such as the lifestyle and environmental exposures could affect the WBC methylation signature and should be considered in future studies.

Conflicts of interest None

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