Promoter Hypermethylation in White Blood Cell DNA and Breast Cancer Risk

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Abstract
The role of gene-specific methylation in white blood cells (WBC) as a marker of breast cancer risk is currently unclear. We determined whether promoter hypermethylation in blood DNA of candidate tumor suppressor genes frequently methylated in breast tumors can be used as a surrogate biomarker for breast cancer risk. Promoter methylation of BRCA1, CDH1 and RARβ was analyzed in WBC DNA from a population-based sample of 1,021 breast cancer patients and 1,036 controls by the MethyLight assay. Gene-specific promoter methylation in the DNA of 569 tumor tissue samples was also analyzed to determine the correlation of methylation levels with blood from the same individual. Hypermethylation of BRCA1 (OR: 1.31; 95% CI: 0.98-1.75) in WBC was associated with an increased risk of breast cancer when positive methylation was defined as ≥0.1% methylated. There was lack of concordance between tumor tissue and paired WBC DNA methylation. These results provide limited support that hypermethylation of BRCA1 in WBC DNA may be useful for determination of breast cancer risk. Additional studies with larger numbers of genes are needed to fully understand the relationship between WBC methylation and breast cancer risk.

Key words: Breast cancer, Promoter methylation, BRCA1, white blood cell DNA

Introduction

Aberrant hypermethylation in promoter regions of genes is now recognized as an important and early event in carcinogenesis [1]. To date, CpG island hypermethylation has been shown to inactivate more than 70 genes in breast tumor tissues [2]. Among these genes, breast cancer-related tumor suppressor genes, BRCA1, CDH1 and RARβ are frequently hypermethylated in breast cancer [3-5]. Numerous studies have confirmed the hypothesis that aberrant methylation of specific genes contributes to the malignant phenotype of breast cancer [6-9].

It is increasingly recognized that tumor DNA can be found in the bloodstream of cancer patients and that this DNA frequently contains the same genetic and epigenetic alterations as DNA isolated from an individual’s tumor [10-12]. This suggests that detec-
tion of tumor DNA in blood may serve as an early and more accessible marker for diagnosis of breast cancer. However, the frequency of aberrant methylation in white blood cells (WBC) as a potential biomarker of risk has not been extensively investigated. We hypothesized that aberrant promoter methylation of BRCA1, CDH1 and RARβ would be detectable in WBC DNA of breast cancer patients and there would be a correlation between methylation in tumor tissue and blood DNA but with more frequent methylation in tissue DNA.

In the present study, we determined whether methylation in BRCA1, CDH1 and RARβ in WBC DNA differed between cases and controls in the Long Island Breast Cancer Study Project (LIBCSP). Since tumor methylation for these genes was available for a large subset of the cases, we also determined the correlation between methylation status in tumor and WBC DNAs from cases.

Materials and Methods

Study population and data collection

We utilized the resources from the case-control component of the LIBCSP, a population-based investigation. Details of the study participants and design have been described previously [13-15]. In brief, eligible case participants included English speaking adult female residents of Nassau and Suffolk counties on Long Island, NY. Eligible case women were of all ages and races and newly diagnosed with in situ or invasive breast cancer between August 1, 1996, and July 31, 1997. Potentially eligible controls were frequency-matched to the expected age distribution of the cases by 5-year age groups and identified through random digit dialing for women age <65 years and the Health Care Finance Administration rosters for women age ≥65 years. Controls were defined as women who resided in the same Long Island counties as the cases, but who had no personal history of breast cancer.

The interviewer-administered structured case-control questionnaire was used to assess a number of personal, demographic and breast cancer-related characteristics. The questionnaire was completed by 82.1% of eligible cases (n = 1,508) and 62.8% of eligible controls (n = 1,556) and was administered within a few months of diagnosis. Among women who completed the questionnaire, 73.1% of cases (n = 1,102) and 73.3% of controls (n = 1,141) donated a blood sample. Of these, 1,021 cases and 1,036 controls with enough DNA for MethyLight analysis were included in the present study. The study protocol was approved by the Institutional Review Boards of the collaborating institutions.

Sample collection and DNA preparation

Blood samples were collected at the time of the case-control interview by trained field staff and DNA was isolated from blood specimens using the methods previously described [15]. DNA was available for 1,021 cases and 1,036 controls. Archived pathology blocks from 962 (63.8%) women were successfully retrieved from the 33 hospitals in the Long Island study area. Isolation of tumor tissues from paraffin sections and tumor DNA were as previously described [16, 17].

Methylation analysis

DNAs first underwent bisulfite modification using the CpGnome DNA Modification Kit (Chemicon International, Purchase, NY) following the manufacturer’s protocol. Sodium bisulfite-treated WBC DNA was analyzed by the MethyLight technique as described previously [18]. The primers and probes for BRCA1, CDH1, RARβ and β-actin (ACTB) were previously described [10, 19, 20]. Specificity of the reactions for methylated DNA was confirmed separately using CpGenome Universal methylated and unmethylated DNAs (Chemicon, MA, USA). Relative quantification was determined based on the threshold cycles of the gene of interest and of the internal reference gene. The percentage of methylation at a specific locus was calculated by the 2^{-ΔΔCT} method [21]. For the 2^{-ΔΔCT} method to be valid, the amplification efficiencies of the test genes and reference gene, ACTB must be approximately equal. This was examined using real-time PCR and Taqman detection of serial dilutions of DNA with a 100-fold range and gene-specific primers of each gene and ACTB. The ΔΔCT (CTTarget gene - CTReference) was calculated for each DNA dilution and a plot of the log DNA dilution vs ΔCT was made. All amplification efficiencies were similar (data not shown). All samples were assayed in duplicate and the MethyLight assay was further validated by using mixtures of fully methylated and unmethylated DNA to give 0, 0.1, 0.5, 1, 5, 10, 25, 50 or 100% methylation.

Methylation data obtained from tumor tissue DNAs have been described in prior publications [16,17]. Among women who donated blood, tumor tissue data were available for 569, 515 and 515 cases for BRCA1, CDH1 and RARβ, respectively. BRCA1 methylation in tumor tissue was determined by methylation-specific PCR (MSP) as described previously [16]. The CpG sites of BRCA1 covered in MSP were overlapped with those measured with the MethyLight assay. The MethyLight assay was used for determining the methylation status of CDH1 and RARβ in tumor tissue as described previously [17].
Statistical Analysis

Statistical analysis was performed using the SAS 9.1 (Cary, NC). The χ2 test was used to examine differences in the distributions of variables between cases and controls with methylation data. Among those with WBC methylation data, there were no significant case-control differences in the distributions of menopausal status, but cases were more likely to report a family history of breast cancer than controls (18.8% vs. 14.2%; P=0.006). Cases with tumor data on gene methylation did not differ in age or family history from those with WBC methylation data. Cases with BRCA1 methylation data were somewhat less frequently postmenopausal (62%) than those with data on CDH1 and RARβ (66%).

Odds ratios (ORs) and corresponding 95% confidence intervals (CIs) for the association between methylation status and breast cancer risk were estimated by unconditional logistic regression [22]. We explored several constructions for the methylation exposure variable including binary models, where methylation status by MethyLight was categorized as positive if values were ≥1%, ≥0.5%, and ≥0.1%, respectively; a 3-level categorical model (unmethylated, <1% methylated, and ≥1% methylated); as well as continuous modeling. Potential confounders of the methylation-breast cancer association were identified through the literature and included: first degree family history of breast cancer (yes/no); body mass index (BMI = weight in kilograms/height in meters squared) (categorical); physical activity (categorical); smoking history (ever/never); and alcohol history (ever/never). Covariates were included in the final model if their inclusion changed the exposure estimate by >10% [23]. Only family history of breast cancer, BMI and physical activity altered the estimate by >10% [23]. Final models were therefore adjusted for each of these variables as well as 5-year age group.

The kappa coefficient (and corresponding 95% confidence intervals) was used to compare the proportion of subjects defined as hypermethylated in WBC vs. tumor tissue for each of the three breast cancer-related tumor suppressor genes, BRCA1, CDH1 and RARβ [24].

Results

BRCA1, CDH1 and RARβ were rarely methylated in WBC DNA from either cases or controls with only 6, 2 and 1 subjects for BRCA1, CDH1 and RARβ, respectively, when values of >10% were considered as positive. Using ≥1% as the cutoff for positive methylation, methylation frequencies of BRCA1, CDH1 and RARβ were 1.8%, 2.0% and 1.5%, in cases and 1.3%, 1.1% and 1.5% in controls, respectively. In contrast, when we used the ≥0.1% cutoff to define positive methylation status we observed more frequent hypermethylation in WBC DNA for both cases and controls (Table 1).

We found little evidence of association between any of the genes and breast cancer risk modeling methylation as a continuous variable (Table 1). Given the low proportion of women classified as positive using ≥1% or ≥0.5% methylated definitions, our binary exposure models revealed non-significant associations between hypermethylation of BRCA1, CDH1 and RARβ and breast cancer risk. We did, however, find evidence of association upon classifying women with ≥0.1% methylation as positive. Hypermethylation of BRCA1 (OR: 1.31; 95% CI: 0.98-1.75) was associated with increased risk of breast cancer, while hypermethylation of CDH1 (OR: 0.65; 95% CI: 0.54-0.79) and RARβ (OR: 0.67; 95% CI: 0.55-0.81) was associated with reduced risk (Table 1). Our three-level categorical model revealed similar modest inverse associations with breast cancer risk for RARβ (Table 1).

Table 1. Hypermethylation in white blood cell DNA in breast cancer cases and controls, Long Island Breast Cancer Study Project, 1996-1997.

|                  | BRCA1 | CDH1 | RARβ |
|------------------|-------|------|------|
|                  | OR±   | 95% CI | OR± | 95% CI | OR± | 95% CI |
| **Continuous Model** |       |       |      |       |       |       |
| **Binary Models** |       |       |      |       |       |       |
| Un-Methylated    | 1.03  | (0.98, 1.09) | 1.04 | (0.86, 1.26) | 0.89 | (0.68, 1.17) |
| ≥1% Methylated   | 1.22  | (0.57, 2.60) | 1.73 | (0.76, 3.94) | 0.62 | (0.29, 1.34) |
| ≥0.5% Methylated | 1.07  | (0.64, 1.78) | 0.73 | (0.49, 1.07) | 0.81 | (0.51, 1.30) |
| ≥0.1% Methylated | 1.31  | (0.98, 1.75) | 0.65 | (0.54, 0.79) | 0.67 | (0.55, 0.81) |
| **Categorical Model** |       |       |      |       |       |       |
| Un-Methylated    | 1.25  | (0.58, 2.67) | 1.29 | (0.56, 2.97) | 0.53 | (0.24, 1.14) |
| 0.1−<1% Methylated | 1.32  | (0.97, 1.80) | 0.65 | (0.53, 0.78) | 0.64 | (0.55, 0.82) |
| ≥1% Methylated   | 2.60  | (0.97, 1.80) | 1.29 | (0.56, 2.97) | 0.53 | (0.24, 1.14) |

*Odds ratio: adjusted for age, family history, body mass index and physical activity
We previously analyzed promoter hypermethylation in tumor tissues for BRCA1, CDH1 and RARβ [16, 17]. Tumor tissues were more frequently hypermethylated (defined as >4% methylation) than blood DNA for all genes tested (59.3% for BRCA1 [16], 23.3% for CDH1 [17] and 44.5% for RARβ [17]). Methylation of all three genes was discordant between tumor tissue and paired WBC DNA using ≥0.1% methylated as the cutoff for positive methylation status in WBC (Table 2).

### Discussion

In a large population-based, case-control study of women with breast cancer, we found that hypermethylation of BRCA1 may be associated with breast cancer risk when using very low levels of methylation (i.e. ≥0.1%) as the cutoff for comparison. Although two prior studies reported a significantly higher frequency of WBC BRCA1 methylation in breast cancer cases [25, 26], two others found no difference [27, 28]. In contrast, WBC BRCA1 methylation at levels as high as 17% has been consistently observed in 5-43% of women with BRCA1 mutation-associated pathology but without germline BRCA1 mutations, suggesting rare constitutional methylation [25, 29]. Two studies have also suggested that methylation of specific regions in ATM may be associated with increased breast cancer risk [30, 31]. An analysis of 25 genes reported that methylation of five genes (ZNF217, NEUROD1, SFRP1, TITF1 and NUP155) in WBC was associated with breast cancer risk [32]. The original 25 genes were selected because they are estrogen receptor-α targets, known to be differentially methylated depending on hormone receptor status, known to contain stem cell polycomb group targets or known to be methylated in breast cancer.

Here we used the MethylLight assay to determine WBC methylation for three candidate genes frequently methylated in breast cancer. This method allows rapid analysis of large numbers of samples and provides continuous data on % methylation calculated versus a “fully methylated” standard sample, which in this study was purchased from a commercial source. Previously, we have analyzed methylation for this type of material and found methylation levels ranging from 85-95%. This suggests that levels of methylation may not be accurate although relative methylation levels should be valid. In addition, there is no generally accepted cutoff for use in determining hypermethylation in WBC DNA. In our prior study of tumor tissue, we used 4% methylation as the cutoff since this value had been used previously [33]. However, one recent study used 0.1% methylation as the cutoff with cancer samples [34]. Thus, for WBC methylation we tested different cutoffs for the definition of hypermethylation including ≥1%, 0.5% or 0.1%. While the number of subjects with hypermethylation increased as the cutoff was lowered, only for BRCA1 was there a suggestion of a positive association between hypermethylation and breast cancer risk. The inverse relationships between methylation of CDH1 and RARβ and breast cancer risk are difficult to explain. Many more controls are considered positive for methylation when using the criteria ≥ 0.1% in CDH1 (n=684) and RARβ (n=404) than for BRCA1 (n=104) and how this impacts results is unclear. The OR for CDH1 became inverted as the cutoff was lowered suggesting the data are unstable. In addition, the biological relevance of these very low levels of methylation is also unclear suggesting the results should be interpreted with caution.

Our results showed a discordance of hypermethylation of the genes tested between WBC and tumor tissue, similar to results observed previously for BRCA1 [35]. This finding suggests that a direct link between WBC BRCA1 methylation and development of breast cancer is still questionable.

One of the strengths of our study is the large sample size from a population-based case-control study (1,021 cases and 1,036 controls). In addition, the MethylLight assay we used is known to be sufficiently sensitive to detect methylated DNA present at low levels of methylation.

### Table 2. Promoter hypermethylation in breast tumors, among cases, and paired white blood cell DNA using >0.1% methylated as classification for +/− in blood. Long Island Breast Cancer Study Project, 1996-1997.

| Methylation Status | BRCA1 (%) | CDH1 (%) | RARβ (%) |
|--------------------|-----------|----------|----------|
| Tumor, UM / WBC, UM | 202 (35.5) | 166 (32.2) | 184 (35.7) |
| Tumor, UM / WBC, M | 29 (5.1) | 230 (44.7) | 102 (19.9) |
| Tumor, M / WBC, UM | 288 (50.6) | 41 (8.0) | 148 (28.8) |
| Tumor, M / WBC M | 50 (8.8) | 78 (15.1) | 81 (15.7) |

Kappa Statistic (95% CI): 0.019 (−0.030, 0.068) 0.048 (−0.016, 0.112) −0.003 (−0.088, 0.082)

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* Promoter methylation in tumor tissue was classified as +/- for BRCA1 and ≥4% for CDH1 and RARβ

* Unmethylated

* White Blood Cell

* Methylated

* Confidence Interval

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levels. However, despite these strengths, analyses were based on small numbers of women with hypermethylation because WBC DNA was rarely hypermethylated. Furthermore, the relatively modest number of tumor suppressor genes investigated precluded further analyses of the possible relationship between methylation levels and breast cancer risk. Thus, further studies with additional genes are needed to establish the usefulness of WBC DNA methylation as a marker of risk.

In conclusion, we examined methylation levels in tumor and WBC DNA from in situ or invasive ductal breast cancer patients and in WBC DNA from population-based controls to identify epigenetic markers of breast cancer risk. Obtained results suggest that hypermethylation of these three genes in WBC DNA may be associated with breast cancer risk, but additional studies on the biological significance of low-level methylation is needed to fully understand the observed results.

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Conflicts of Interest
No potential conflicts of interests were disclosed.

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