Association between global DNA hypomethylation in leukocytes and risk of breast cancer

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Background: Global DNA hypomethylation may result in chromosomal instability and oncogene activation, and as a surrogate of systemic methylation activity, may be associated with breast cancer risk. Methods: Samples and data were obtained from women with incident early-stage breast cancer (I–IIIa) and women who were cancer free, frequency matched on age and race. In preliminary analyses, genome-wide methylation of leukocyte DNA was determined by measuring 5-methyldeoxycytosine (5-mdC), as well as methylation analysis of the LINE-1 repetitive DNA element. Further analyses used only 5-mdC levels. Logistic regression models were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for risk of breast cancer in relation to amounts of methylation. Results: In a subset of samples tested (n = 37), 5-mdC level was not correlated with LINE-1 methylation. 5-mdC level in leukocyte DNA was significantly lower in breast cancer cases than healthy controls (P = 0.001), but no significant case–control differences were observed with LINE-1 methylation (P = 0.176). In the entire data set, we noted significant differences in 5-mdC levels in leukocytes between cases (n = 176) and controls (n = 173); P value < 0.001. Compared with women in the highest 5-mdC tertile (T3), women in the second (T2; OR = 1.49, 95% CI = 0.84–2.65) and lowest tertile (T1; OR = 2.86, 95% CI = 1.65–4.94) had higher risk of breast cancer (P for trend ≤0.001). Among controls only and cases and controls combined, only alcohol intake was found to be inversely associated with methylation levels. Conclusion: These findings suggest that leukocyte DNA hypomethylation is independently associated with development of breast cancer.

Introduction

Altersations of DNA methylation, which influence gene expression and genome integrity, are an important component of cancer development (1). Although localized hypomethylation and hypermethylation of specific genes has been studied more extensively, global DNA hypomethylation is a hallmark of most cancer genomes (2–6), including breast cancer (7–11). Hypomethylation has been proposed to contribute to malignancy by activating oncogenes (12,13), inducing genomic instability (14) and causing chromosome instability (4,15–17).

Materials and methods

Study population

Data and samples were obtained from the Roswell Park Cancer Institute (RPCI) Databank and Biorepository (DBBR), a shared Core resource (29). In the DBBR, patients newly diagnosed with cancer consent to provide a blood sample and to complete an in-depth epidemiologic questionnaire that includes a food frequency questionnaire, information on reproductive history, family history of cancer, supplement use, comorbidities, prescription and non-prescription medication use, smoking, alcohol consumption, lifetime physical activity, height and weight from young adulthood to present and demographic factors. Permission is granted for linkage of data and samples with medical record information and for use by investigators with protocols approved by the RPCI Institutional Review Board. Visitors and family members of patients are consented as healthy controls, who can be matched by sex, age, residence and race for patients with other types of cancers. Relationships with patients are noted so that they are not included in the same population, avoiding overmatching. Blood samples are drawn prior to surgery or chemotherapy and processed and frozen within 1 h of phlebotomy. This study of hypomethylation and breast cancer risk was approved by the RPCI Institutional Review Board.

Study participants

For these analyses, we obtained DNA and data from the DBBR from patients with early stage (I, II and IIIa) histologically confirmed breast cancer, ages 35–75. Controls were free of malignant diseases, with the exception of non-melanoma skin cancer, and were frequency matched to cases on age and race. DNA was extracted from whole blood using Flexigene kits and was stored at

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Table I. Characteristics of breast cancer cases and controls, RPCI, DBBR, 2004–2007 (Controls matched on age and races)

|                        | Cases, N = 179 | Controls, N = 180 | P value<sup>a</sup> | OR (95% CI)<sup>b</sup> |
|------------------------|----------------|-------------------|---------------------|--------------------------|
| **Age**                |                |                   |                     |                          |
| 30–39                  | 6 (3.4)        | 8 (4.4)           |                     |                          |
| 40–49                  | 43 (24.0)      | 42 (23.3)         |                     |                          |
| 50–59                  | 55 (30.7)      | 65 (36.1)         |                     |                          |
| 60–69                  | 54 (30.2)      | 47 (26.1)         |                     |                          |
| 70+                    | 21 (11.7)      | 18 (10.0)         | P = 0.764           |                          |
| **Race**               |                |                   |                     |                          |
| Caucasian              | 168 (93.9)     | 170 (94.4)        | 1.00                |                          |
| African-American       | 11 (6.2)       | 10 (5.6)          | P = 0.812           | 0.91 (0.91–2.21)         |
| **Family history of BRCA** |            |                   |                     |                          |
| No                     | 122 (68.2)     | 142 (78.9)        | 1.00                |                          |
| Yes                    | 29 (16.2)      | 37 (20.6)         | P = 0.740           | 0.89 (0.51–1.54)         |
| Missing                | 28 (15.6)      | 1 (0.6)           |                     |                          |
| **Menopausal status**  |                |                   |                     |                          |
| Premenopausal          | 59 (33.0)      | 73 (40.6)         | 1.00                |                          |
| Postmenopausal         | 90 (50.3)      | 102 (56.7)        | P = 0.699           | 1.00 (0.51–1.98)         |
| Missing                | 30 (16.8)      | 5 (2.8)           |                     |                          |
| **Education**          |                |                   |                     |                          |
| Below or at high school| 41 (22.9)      | 36 (20.0)         | 1.00                |                          |
| Above high school      | 107 (59.8)     | 143 (79.4)        | P = 0.107           | 0.66 (0.39–1.12)         |
| Missing                | 31 (17.3)      | 1 (0.6)           |                     |                          |
| **BMI**                |                |                   |                     |                          |
| <25                    | 47 (26.3)      | 52 (28.9)         | 1.00                |                          |
| 25–29.9                | 43 (24.0)      | 63 (35.0)         | 0.74 (0.43–1.30)    |                          |
| 30+                    | 55 (30.7)      | 49 (27.2)         | P = 0.200           | 1.27 (0.73–2.24)         |
| Missing                | 34 (19.0)      | 16 (8.9)          |                     |                          |
| **Smoking habit**      |                |                   |                     |                          |
| Never smoker           | 75 (41.9)      | 85 (47.2)         | 1.00                |                          |
| Ever                   | 74 (41.3)      | 87 (58.3)         | P = 0.870           | 0.94 (0.61–1.47)         |
| Missing                | 30 (16.8)      | 8 (4.4)           |                     |                          |
| **Alcohol consumption habit** |          |                   |                     |                          |
| Never                  | 36 (20.1)      | 32 (17.8)         | 1.00                |                          |
| Ever                   | 115 (64.3)     | 147 (82.7)        | P = 0.182           | 0.67 (0.39–1.16)         |
| Missing                | 28 (15.6)      | 1 (0.6)           |                     |                          |
| **Alcohol consumption frequency** | |                   |                     |                          |
| Never                  | 36 (20.1)      | 32 (17.8)         | 1.00                |                          |
| Below median<sup>c</sup> | 18 (10.1)     | 37 (20.6)         | 0.55 (0.29–1.05)    |                          |
| At or above median     | 44 (24.6)      | 39 (21.7)         | P = 0.061           | 1.26 (0.74–2.16)         |
| Missing                | 42 (23.5)      | 19 (10.6)         |                     |                          |
| **Age at menarche**    |                |                   |                     |                          |
| <13                    | 71 (39.7)      | 89 (49.4)         | 1.00                |                          |
| 13+                    | 77 (43.0)      | 78 (43.3)         | P = 0.346           | 1.22 (0.78–1.91)         |
| Missing                | 31 (17.3)      | 13 (7.2)          |                     |                          |
| **Age at first birth** |                |                   |                     |                          |
| <25                    | 76 (42.5)      | 76 (42.2)         | 1.00                |                          |
| 25+                    | 43 (24.0)      | 60 (33.3)         | 0.69 (0.41–1.16)    |                          |
| Nulliparous            | 28 (15.6)      | 38 (21.1)         | P = 0.356           | 0.74 (0.41–1.34)         |
| Missing                | 32 (17.9)      | 6 (3.3)           |                     |                          |
| **Parity**             |                |                   |                     |                          |
| 1–2 children           | 69 (38.6)      | 74 (41.1)         | 1.00                |                          |
| 3+                     | 52 (29.0)      | 64 (35.6)         | 1.25 (0.69–2.26)    |                          |
| Nulliparous            | 28 (15.6)      | 38 (21.1)         | P = 0.707           | 1.06 (0.57–1.97)         |
| Missing                | 30 (16.8)      | 4 (2.2)           |                     |                          |
| **Age at menopause**   |                |                   |                     |                          |
| <50                    | 37 (41.1)      | 54 (52.9)         | 1.00                |                          |
| 50+                    | 45 (50.0)      | 45 (44.1)         | P = 0.207           | 1.41 (0.78–2.54)         |
| Missing                | 8 (8.9)        | 3 (2.9)           |                     |                          |
| **HRT**                |                |                   |                     |                          |
| Never                  | 44 (48.9)      | 43 (42.1)         | 1.00                |                          |
| Ever                   | 44 (48.9)      | 58 (56.7)         | P = 0.307           | 0.73 (0.41–1.30)         |
| Missing                | 2 (2.2)        | 1 (1.0)           |                     |                          |
| **Multivitamin use**   |                |                   |                     |                          |
| Never or occasionally  | 67 (37.3)      | 60 (33.3)         | 1.00                |                          |
| Regularly              | 84 (46.9)      | 118 (65.6)        | P = 0.048           | 0.63 (0.40–0.98)         |
| Missing                | 28 (15.6)      | 2 (1.1)           |                     |                          |
DNAs were also run as controls. Pyrosequencing was done on duplicate samples using sodium bisulfite DNA conversion, and methylated and unmethylated polymerase chain reaction cycling conditions and materials were described for quantitative pyrosequencing of sodium bisulfite-converted DNA (31). Primers, the methylation status of LINE-1 repetitive DNA element was analyzed using quantitative bisulfite pyrosequencing within 37 subjects was 4.9%. All analyses were done on duplicate samples. Mean coefficient of variation and DKO, which lacks both DNA methyltransferases DNMT1 and DNMT3b in methylated and unmethylated DNA from colorectal cancer cell line HCT116. Global genomic DNA methylation analysis

Global methylation levels measured by 5-mdC and LINE-1, which were normally distributed, were compared using general linear model in 37 samples (19 cases and 18 controls). In the full study population, after standardization between batches and exclusion of 1% outliers of 5-mdC distribution, 5-mdC levels were normally distributed in both cases (n = 176) and controls (n = 173) assessed by skew and kurtosis test. Continuous variables and amounts of global methylation were categorized into dichotomous or tertiled variables based on the distribution among controls. Chi-square test for categorical variables was used to compare characteristics between cases and controls. To identify potential predictors of methylation levels and/or factors that may modify the association between methylation levels and breast cancer risk, univariate associations between DNA methylation and each lifestyle or demographic factor were assessed among controls and among all participants after adjustment for case-control status. Age, body mass index, pack-years of tobacco use, frequency of alcohol intake and parity were assessed as both continuous and categorical variables. Relationships between methylation levels and demographic and lifestyle variables were assessed using standardized β-coefficients. For body mass index, pack-years of tobacco use, frequency of alcohol intake and parity, categorical variables were entered as continuous variables 0, 1, 2 to determine standardized β-coefficients. Logistic regression models were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association between genomic methylation levels and breast cancer risk. For potential confounding, we examined known risk factors for breast cancer, i.e., age (categorical variable, 30–39, 40–49, 50–59, 60–69 and 70+), race (Caucasian and African-American), family history of breast cancer (yes and no), menopausal status (premenopausal and postmenopausal), body mass index (<25, 25–29.9 and 30+), smoking habit (never and ever), pack-years (never smoker, below median and at or above median), alcohol consumption (never and ever), alcohol consumption frequency (never drinking, below median and at or above median), age at menarche (<13 and 13+), age at first birth (<25, 25+ and nulliparous), parity (1–2 children, 3+ and nulliparous) and multivitamin use (never or occasionally and regularly) (shown in Table I), variables reported to affect methylation in the literature (age and race) (22,33–36), variables associated with methylation in the data set (frequency of alcohol consumption) and laboratory variability (batches). While none of the variables assessed changed OR estimates by at least 10%, age, race and batch were kept in all final models. Unordered polychotomous logistic regression models were used to compare different subtype of breast cancer cases. Linear trends for categorical variables were assessed by a two-sided likelihood ratio test.

| Table I. Continued |
|---------------------|
| Histological grade  |
| I                   | 18 (10.1) |
| II                  | 51 (28.5) |
| III                 | 108 (60.3) |
| AJCC stage          |
| I                   | 115 (64.3) |
| II                  | 57 (31.8) |
| III                 | 7 (3.9) |
| Hormone status      |
| ER+/PR+             | 111 (62.0) |
| ER-/PR– or ER+/PR–  | 23 (12.9) |
| ER–/PR–             | 41 (22.9) |
| Tumor size          |
| <1 cm               | 138 (77.1) |
| 1–2 cm              | 38 (21.2) |
| ≥3 cm               | 3 (1.7) |
| Lymph node involvement |
| No                  | 135 (75.4) |
| Yes                 | 44 (24.6) |
| Metastasis          |
| No                  | 179 (100.0) |

AJCC, American Joint Committee on Cancer; BMI, body mass index; BRCA, breast cancer; HRT, hormone replacement therapy.

*P value by Chi-square test.

aOR adjusting for age and race.

bMedian pack-year = 0.7 pack-year.

cMedian total alcohol consumption frequency = 1.7 per week for alcohol consumption.

dAmong postmenopausal women.

| Table II. Comparison of global methylation levels in leukocyte DNA measured by two methods (N = 37) |
|--------------------------------------------------|
| Median   | Cases, n = 19 | Controls, n = 18 | P value* |
| 5-mdC    |              |                  |          |
| Mean     | 3.98         | 4.33              | 0.001    |
| Median   | 3.98         | 4.33              |          |
| Interquartile range | 0.45         | 0.27              |          |
| LINE-1   |              |                  | 0.176    |
| Mean     | 74.70        | 73.90             |          |
| Median   | 74.50        | 73.50             |          |
| Interquartile range | 1.00         | 1.70              |          |

*Assessed by general linear model adjusting for age and race.

–70°C until analysis. For these analyses, we requested data and samples from 179 cases, who met the above eligibility criteria, and 180 matched controls.

Global genomic DNA methylation analysis

5-mdC levels were determined by liquid chromatography–electrospray ionization tandem mass spectrometry after hydrolysis of DNA (1 μg) as described previously (30). Briefly, global DNA methylation was expressed as the ratio of 5-mdC to guanine and was determined directly using guanine as the internal standard based on the assumption that (guanine) = (5-mdC) + (cytosine). Methylated and unmethylated DNA from colorectal cancer cell line HCT116 and DKO, which lacks both DNA methyltransferases DNMT1 and DNMT3b in HCT116, was run in every batch as quality control to standardize between batches by adjusting the difference of methylation level of quality control. All analyses were done on duplicate samples. Mean coefficient of variation within 37 subjects was 4.9%.

Quantitative bisulfite pyrosequencing

The methylation status of LINE-1-repetitive DNA element was analyzed using quantitative pyrosequencing of sodium bisulfite-converted DNA (31). Primers, polymerase chain reaction cycling conditions and materials were described previously (32). Non-CpG cytosines served as internal controls to verify efficient sodium bisulfite DNA conversion, and methylated and unmethylated DNAs were also run as controls. Pyrosequencing was done on duplicate samples and pyrosequencing assays were performed a minimum of two times.
Potential interactions were evaluated using cross-product terms and likelihood ratio test statistics comparing models with and without the cross-product term.

To assess the dose-response relationship of genomic methylation with breast cancer risk, a smoothing spline of the predicted ORs as methylation level increase continuously in the final logistic regression model was drawn using STATA graph. Additionally, global methylation levels were categorized into four groups using cut off values based on the curve; after stratifying by % genomic methylation 4.286 that met OR = 1.0 in the smoothing spline, we cut off midpoint of methylation range in each strata. Midpoint was 3.809 in the lower range of 3.332–4.266 below 4.286 of methylation level and 4.843 in the upper range of 4.286–5.399 above 4.286 of methylation level, respectively. Thus, the range of each category was 3.332–3.809, 3.809–4.286, 4.286–4.843 and 4.843–5.399, respectively. All statistical analyses were performed using STATA version 9.0 (Stata corporation, College Station, TX).

Discussion

In the pilot study, two measures of global methylation in peripheral blood samples 5-mdC and LINE-1 were not correlated. In the entire study, mean levels of global methylation, measured as the percentage of 5-mdC in leukocyte DNA, were significantly lower in breast cancer cases than in controls and were independently associated with increased breast cancer risk in a dose-dependent manner. Except for frequency of alcohol intake, lifestyle and demographic factors did not predict methylation levels. However, hypomethylation was suggested as a stronger risk factor for breast cancer among women with a family history of breast cancer and among never smokers. Methylation levels were not associated with clinicopathological characteristics of the breast tumors.

As reviewed by Laird (20), there is no consensus, to date, on the best technique for assessing methylation profiles. Repetitive DNA sequences (e.g. LINE-1, Alu, SAT2 and SAT1) are all comparatively rich in CpG dinucleotides and contain a large portion of total DNA methylation levels in the genome (4,26). Thus, genome-wide changes in DNA methylation are postulated to affect methylation levels in repetitive DNA sequences. A previous study reported that methylation levels in repetitive sequences assessed by methylation-specific polymerase chain reaction techniques using MethylLight were correlated with 5-mdC levels (37); however, there were no associations between methylation levels in LINE-1 measured by pyrosequencing and 5-mdC level in our study. A possible reason for this could be differential sensitivity to detect subtle changes of methylation patterns between the distinct assays used to measure 5-mdC and LINE-1. In addition, LINE-1 methylation status is probably not always predictive of total 5-mdC levels, as the latter measurement reflects the overall contribution of a diverse set of DNA sequence elements including both CpG island and non-CpG island regions.

Consistent with findings from this study, genomic DNA hypomethylation status in leukocyte DNA has recently been associated with risk of colorectal, head and neck and bladder cancer (21–24). Lim et al. (21) reported a dose-dependent inverse association between global 5-mdC levels and risk of asymptomatic colorectal adenoma (OR = 5.8, 95% CI = 2.0–16.6 for lowest tertile versus highest tertile, P for trend = 0.002). In the Spanish Bladder Cancer Study, global methylation levels measured by 5-mdC were also lower among bladder cancer patients than among controls (OR = 2.67, 95% CI = 1.77–4.03). When further stratified by smoking status, current smokers in the lowest methylation quartile had the highest risk of bladder cancer compared with never smokers in the highest methylation quartile (OR = 25.51, 95% CI = 9.61–67.76, P for interaction = 0.06) (22). In another study, hypomethylation of LINE-1 was associated with increased risk of squamous cell cancer of the head and neck (OR = 1.6, 95% CI = 1.1–2.4) (24). In contrast, Wischwendter et al. (38) reported that methylation of Alu-repetitive elements in blood DNA, another surrogate marker correlated with 5-mdC (37), did not differ between breast cancer cases and controls. We also did not observe a difference in methylation levels between breast cancer cases and controls when methylation levels were assessed by LINE-1-repetitive DNA sequences, although associations with 5-mdC levels were evident.

Our findings and others (21–24) indicate that global genomic hypomethylation of leukocyte DNA may be an independent risk factor for multiple cancer types, possibly due to resulting genomic instability. Lower DNA repair capacity in lymphocytes has been associated with increased risk of breast cancer (39,40), and genomic instability measured by micronuclei frequency, levels of DNA single-strand breaks and alkali-labile lesions, sister chromatid exchange and/or spontaneous chromosomal aberrations in peripheral blood cells has...
been shown to be higher among breast cancer cases compared with healthy controls and women with benign breast disease (25,41–43). Global DNA hypomethylation levels in both normal and cancer cells, however, show the same pattern of hypomethylation with low folate status (44), and methylation patterns appear to vary between different cell types (8). Methylation levels are also probably to differ between cancer DNA and leukocyte DNA; therefore, the use of peripheral leukocyte DNA methylation as a surrogate marker of genomic instability in target tumor tissue should be evaluated in further studies.

There are reports that environmental exposure to nutritional, chemical and physical factors can alter methylation status and produce different phenotypes (45–47). Except for an inverse association between methylation levels and frequency of alcohol consumption, we did not find any demographic or lifestyle predictors of methylation.

### Table III. Predictors of global methylation assessed by univariate general linear model (cases = 176 and controls = 173)

| Variables                                | Among controls | Among all participants (cases + controls) |
|------------------------------------------|----------------|------------------------------------------|
|                                          | Mean ± SE of 5-mdC | β   | P value  | Mean ± SE of 5-mdC | β   | P value  |
| Total                                    | 4.38 ± 0.03      |      |          | 4.28 ± 0.02        |      |          |
| Age (continuous)                         | 4.41 ± 0.04      | 0.001 | 0.730    | 4.30 ± 0.03        |      | <0.001   |
| < Median (55)                            | 4.34 ± 0.04      | −0.068 | 0.208    | 4.26 ± 0.03        | −0.043 | 0.263    |
| Race                                     | 4.38 ± 0.03      |      |          | 4.27 ± 0.02        |      |          |
| Caucasian                                | 4.41 ± 0.08      | −0.032 | 0.781    | 4.31 ± 0.06        | −0.036 | 0.646    |
| African-American                         | 4.39 ± 0.06      | 0.012 | 0.730    | 4.28 ± 0.04        | 0.004 | 0.248    |
| Batch (category on date)                 | 4.36 ± 0.03      |      |          | 4.27 ± 0.02        |      |          |
| Yes                                      | 4.36 ± 0.03      | 0.069 | 0.300    | 4.29 ± 0.05        | 0.030 | 0.532    |
| No                                       | 4.43 ± 0.06      |      |          | 4.29 ± 0.05        |      |          |
| Education                                | 4.35 ± 0.07      |      |          | 4.25 ± 0.04        |      |          |
| ≥ high school                            | 4.38 ± 0.03      | 0.033 | 0.624    | 4.28 ± 0.02        |      | 0.036    |
| BMI (continuous)                         | 4.37 ± 0.04      |      |          | 4.26 ± 0.03        |      | 0.002    |
| <25                                      | 4.34 ± 0.04      |      |          | 4.26 ± 0.03        |      |          |
| 25–29.9                                  | 4.39 ± 0.06      | 0.012 | 0.730    | 4.28 ± 0.04        | 0.009 | 0.717    |
| Smoking habit                            | 4.38 ± 0.04      |      |          | 4.25 ± 0.03        |      |          |
| Never                                    | 4.37 ± 0.04      | −0.007 | 0.904    | 4.30 ± 0.03        | 0.043 | 0.272    |
| Ever                                     | 4.37 ± 0.04      |      |          | 4.25 ± 0.03        |      | <0.001   |
| Pack-year (continuous)                   | 4.38 ± 0.04      | 0.001 | 0.534    | 4.25 ± 0.03        |      | 0.766    |
| Never smoker                             | 4.37 ± 0.06      |      |          | 4.30 ± 0.05        |      |          |
| Below median                             | 4.37 ± 0.06      |      |          | 4.27 ± 0.04        | 0.009 | 0.716    |
| At or above median                       | 4.37 ± 0.06      |      |          | 4.27 ± 0.04        | 0.009 | 0.716    |
| Alcohol consumption habit                | 4.45 ± 0.07      |      |          | 4.33 ± 0.05        |      |          |
| Never                                    | 4.36 ± 0.03      |      |          | 4.26 ± 0.02        |      | −0.076   |
| Ever                                     | 4.37 ± 0.04      |      |          | 4.30 ± 0.03        |      | 0.109    |
| Alcohol drinking frequency (continuous)  | 4.45 ± 0.07      |      |          | 4.33 ± 0.05        |      |          |
| Never                                    | 4.42 ± 0.04      |      |          | 4.33 ± 0.05        |      |          |
| < median                                 | 4.30 ± 0.04      |      |          | 4.23 ± 0.03        |      | 0.036    |
| + median                                 | 4.39 ± 0.06      |      |          | 4.30 ± 0.03        |      |          |
| Age at menarche                          | 4.41 ± 0.04      |      |          | 4.30 ± 0.03        |      |          |
| < 13                                     | 4.35 ± 0.04      |      |          | 4.26 ± 0.03        |      | −0.036   |
| + 13                                     | 4.41 ± 0.04      |      |          | 4.30 ± 0.03        |      | 0.375    |
| Age at first birth                       | 4.34 ± 0.04      |      |          | 4.28 ± 0.03        |      |          |
| < 25                                     | 4.36 ± 0.05      |      |          | 4.25 ± 0.03        |      |          |
| + 25                                     | 4.37 ± 0.06      | 0.062 | 0.080    | 4.31 ± 0.04        | 0.013 | 0.596    |
| Nulliparous                              | 4.47 ± 0.06      |      |          | 4.27 ± 0.04        |      | 0.002    |
| Parity (continuous)                      | 4.36 ± 0.04      |      |          | 4.26 ± 0.03        |      |          |
| Nulliparous                              | 4.34 ± 0.04      |      |          | 4.26 ± 0.03        |      |          |
| 1–2                                      | 4.33 ± 0.03      |      |          | 4.31 ± 0.03        |      | 0.021    |
| ≥ 3                                      | 4.38 ± 0.04      |      |          | 4.28 ± 0.03        |      |          |
| Menopausal status                        | 4.38 ± 0.04      |      |          | 4.28 ± 0.03        |      |          |
| Premenopausal                            | 4.37 ± 0.04      |      |          | 4.27 ± 0.03        |      | −0.013   |
| Postmenopausal                           | 4.38 ± 0.04      |      |          | 4.28 ± 0.03        |      | 0.736    |
| Age at menopause                         | 4.38 ± 0.04      |      |          | 4.28 ± 0.03        |      |          |
| ≤ 50                                     | 4.46 ± 0.05      |      |          | 4.31 ± 0.04        |      |          |
| ≥ 50                                     | 4.27 ± 0.06      | −0.042 | 0.220    | 4.22 ± 0.04        | −0.026 | 0.271    |
| HRT                                      | 4.37 ± 0.03      |      |          | 4.28 ± 0.02        |      |          |
| Never                                    | 4.38 ± 0.05      |      |          | 4.27 ± 0.04        |      | −0.012   |
| Ever                                     | 4.37 ± 0.03      |      |          | 4.25 ± 0.03        |      | 0.303    |
| Multivitamin use                         | 4.39 ± 0.05      |      |          | 4.31 ± 0.03        |      |          |
| Never or occasionally                    | 4.37 ± 0.03      |      |          | 4.25 ± 0.03        |      | −0.023   |
| Regularly                                 | 4.37 ± 0.03      |      |          | 4.31 ± 0.03        |      |          |

BMI, body mass index; BRCA, breast cancer; HRT, hormone replacement therapy.

*Least square means adjusted for group (case and control) status.

*Adjusting for group (case and control) status.

*Adjusting for menopausal status.
Table IV. Association between global DNA methylation level classified by the tertiles of the distribution of controls and breast cancer (cases = 176 and controls = 173)

| 5-mdC | Cases, n (%) | Controls, n (%) | OR (95% CI)* |
|-------|--------------|----------------|--------------|
| All^b | Mean ± SD    | 4.18 ± 0.34    | 4.38 ± 0.36  | <0.001       |
|       | T3           | 33 (18.8)      | 58 (33.5)    | 1.0 (reference) |
|       | T2           | 49 (27.8)      | 58 (33.5)    | 1.49 (0.84–2.65) |
|       | T1           | 94 (53.4)      | 57 (33.0)    | 2.86 (1.65–4.94) |
|       | P for trend  | <0.001         |              |              |
| Menopausal status | |               |              |              |
| Premenopausal | T3 | 11 (19.0) | 21 (30.0) | 1.0 (reference) |
|               | T2 | 18 (31.0) | 26 (36.6) | 1.27 (0.48–3.35) |
|               | T1 | 29 (50.0) | 24 (33.8) | 2.21 (0.88–5.57) |
|               | P for trend | 0.074         |              |              |
| Postmenopausal | T3 | 14 (15.9) | 34 (35.1) | 1.0 (reference) |
|               | T2 | 24 (27.3) | 31 (32.0) | 1.88 (0.82–4.31) |
|               | T1 | 50 (56.8) | 32 (33.0) | 3.76 (1.73–8.19) |
|               | P for trend | 0.001         |              |              |
| P for interaction | Age at diagnosis | | | 0.410 |
| <55 | T3 | 16 (22.2) | 31 (33.7) | 1.0 (reference) |
|     | T2 | 18 (25.0) | 33 (35.9) | 0.96 (0.40–2.26) |
|     | T1 | 38 (52.8) | 28 (30.4) | 2.50 (1.13–5.50) |
|     | P for trend | 0.013         |              |              |
| 55+ | T3 | 17 (16.4) | 27 (33.3) | 1.0 (reference) |
|     | T2 | 31 (29.8) | 25 (30.9) | 1.89 (0.84–4.26) |
|     | T1 | 56 (53.9) | 29 (35.8) | 2.81 (1.29–6.11) |
|     | P for trend | 0.009         |              |              |
| P for interaction | Family history of breast cancer | | | 0.947 |
| No | T3 | 21 (17.7) | 42 (30.9) | 1.0 (reference) |
|    | T2 | 35 (29.4) | 47 (34.6) | 1.50 (0.75–2.99) |
|    | T1 | 63 (52.9) | 47 (34.6) | 2.60 (1.35–4.98) |
|    | P for trend | 0.003         |              |              |
| Yes | T3 | 5 (17.2) | 15 (41.7) | 1.0 (reference) |
|     | T2 | 7 (24.1) | 11 (30.6) | 2.08 (0.50–8.66) |
|     | T1 | 17 (58.6) | 10 (27.8) | 5.73 (1.49–21.97) |
|     | P for trend | 0.000         |              |              |
| P for interaction | BMI (by median) | | | 0.345 |
| Below 26.5 | T3 | 9 (15.3) | 18 (25.4) | 1.0 (reference) |
|            | T2 | 14 (23.7) | 26 (36.6) | 1.08 (0.36–3.29) |
|            | T1 | 36 (61.0) | 27 (38.0) | 2.36 (0.88–6.28) |
|            | P for trend | 0.044         |              |              |
| At or higher 26.5 | T3 | 15 (17.9) | 32 (37.2) | 1.0 (reference) |
|            | T2 | 25 (29.8) | 28 (32.6) | 2.05 (0.88–4.77) |
|            | T1 | 44 (52.4) | 26 (30.2) | 3.98 (1.77–8.95) |
|            | P for trend | 0.001         |              |              |
| P for interaction | Smoking habit | | | 0.820 |
| Never smoker | T3 | 7 (9.5) | 27 (32.9) | 1.0 (reference) |
|               | T2 | 22 (29.7) | 27 (32.9) | 3.02 (1.09–8.37) |
|               | T1 | 45 (60.8) | 28 (34.2) | 6.02 (2.29–15.78) |
|               | P for trend | <0.001        |              |              |
| Ever smoker | T3 | 18 (25.0) | 29 (34.9) | 1.0 (reference) |
|               | T2 | 20 (27.8) | 27 (32.5) | 1.17 (0.50–2.72) |
|               | T1 | 34 (47.2) | 27 (32.5) | 1.97 (0.89–4.39) |
|               | P for trend | 0.086         |              |              |
| P for interaction | Alcohol consumption habit | | | 0.084 |
| Never drinking alcohol | T3 | 9 (25.0) | 13 (40.6) | 1.0 (reference) |
|               | T2 | 10 (27.8) | 12 (37.5) | 1.35 (0.39–4.63) |
|               | T1 | 17 (47.2) | 7 (21.9) | 3.98 (1.11–14.23) |
|               | P for trend | 0.033         |              |              |
| Ever drinking alcohol | T3 | 17 (15.2) | 44 (31.4) | 1.0 (reference) |
|               | T2 | 32 (28.6) | 46 (32.9) | 1.80 (0.87–3.74) |
|               | T1 | 63 (56.3) | 50 (35.7) | 3.06 (1.55–6.05) |
|               | P for trend | 0.001         |              |              |
| P for interaction | Multivitamin use | | | 0.826 |
| Never or occasional use | T3 | 12 (17.9) | 26 (43.3) | 1.0 (reference) |
|                  | T2 | 26 (38.8) | 14 (23.3) | 4.68 (1.75–12.58) |
|                  | T1 | 29 (43.3) | 20 (33.3) | 4.04 (1.54–10.57) |
|                  | P for trend | 0.007         |              |              |
Association between methylation levels and breast cancer risk, however, did not vary by alcohol consumption in our data set, which is consistent with associations observed in bladder cancer and colorectal adenoma (21,22). Some studies have shown genomic content of 5-mdC to decline with age (45,48,49), although others show global methylation levels in human leukocytes or tumor DNA to be independent of age (22,33,34), consistent with our findings. Because the vast majority of participants in this study were Caucasian, we were unable to analyze associations between DNA methylation and race. Cigarette smoking was not associated with global methylation of leukocyte DNA in agreement with previous studies (22,35,50); however, hypomethylation was suggested as a stronger risk factor for breast cancer risk among non-smokers, which is consistent with findings from Moore et al.’s study (22). Our finding that associations between methylation levels and breast cancer risk were more profound among women with a family history of breast cancer are also consistent with studies that have noted that genetic factors can affect patterns of methylation. A recent study investigating longitudinal changes in global methylation in healthy individuals found familial clustering of methylation changes (51), suggesting genetic control of maintenance methylation. However, further large studies are needed to confirm the subgroup analysis.

We did not find any associations between global methylation levels and tumor characteristics, consistent with findings in colorectal and bladder cancer patients (21,22). Using breast tissue DNA from breast cancer cases, however, a number of studies (7,8,10,52) have found significant correlations between global hypomethylation and disease stage, tumor size and histological grade. The inconsistencies between the results in normal and tumor tissue probably arise from use of different sources of DNA and/or from differential distributions of clinical characteristics. In our study, most participants had early-stage breast cancer (3.9% of American Joint Committee on Cancer stage III, 1.7% of tumor size ≥2 cm, 24.6% of node involvement and no metastasis as shown in Table I), therefore it may lack the power to assess associations between global methylation levels and clinical features, given the small number of advanced breast cancer cases.

Limitations of our study include unexpected distributions of known breast cancer risk factors among cases and controls, which include higher family history of breast cancer, higher education and more frequent use of hormone replacement therapy among controls. One possible explanation for these observations is differential missing information among cases, which can affect the association with breast cancer as a confounder though we did not find any significant predictor of global methylation level. Another explanation is that DBBR participants were all recruited from within a comprehensive cancer center. Thus, controls within this series included community volunteers (50%), employees of RPCI (33%) and family members or friends of patients (28%). Compared with cases, employee controls were younger and more highly educated but were not significantly different for family history of breast cancer or for reproductive factors. Individuals with a family history of cancer might also be more probably to participate in a ‘cancer study’, which might explain why controls within the DBBR population were more probably to report a family history of breast cancer. The association between methylation levels with breast cancer risk, however, did not vary according to the source of the control (data not shown), and methylation levels did not vary between the different groups of controls (data not shown). Although we assessed potential effect modification, this study had a relatively small sample size for the subgroup analysis and had lack of power to find significant interactions on the association between global methylation and breast cancer risk. We did not assess dietary or genetic factors involved in 1-carbon metabolism as effect modifiers in this study, although inconsistent associations have been reported in human studies (21,22,53,54).

This study showed genomic hypomethylation of leukocyte DNA, as a surrogate of systemic methylation activity, to be associated with increased breast cancer risk, with associations being particularly strong among non-smokers and women with a family history of breast cancer. These findings provide greater understanding of factors that may modify breast cancer risk and could lead to development of a simple non-invasive blood measure of DNA hypomethylation to identify women at high risk for breast cancer.

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Table V. Association between global DNA methylation level, classified by tertiles of the distribution of controls and breast cancer stratified by clinicopathologic features

| Groupa | T1       | T2       | T3       |
|--------|----------|----------|----------|
|        | n | OR (95% CI) | n | OR (95% CI) | n | OR (95% CI) |
| Controls | 58 | 1.0 (reference) | 18 | 1.20 (0.55–2.64) | 35 | 2.31 (1.13–4.75) |
| Histological grade | | | | | | |
| I/II | 15 | 1.0 (reference) | 30 | 1.67 (0.83–3.34) | 58 | 3.25 (1.69–6.24) |
| III | 18 | 1.0 (reference) | 28 | 1.43 (0.72–2.85) | 64 | 3.27 (1.73–6.18) |
| AJCC stage | | | | | | |
| I | 20 | 1.0 (reference) | 21 | 1.58 (0.72–3.49) | 30 | 2.29 (1.08–4.88) |
| II/III | 13 | 1.0 (reference) | 29 | 1.40 (0.71–2.75) | 59 | 2.84 (1.52–5.32) |
| Hormone status | | | | | | |
| ER+ and PR+ | 21 | 1.0 (reference) | 20 | 1.79 (0.78–4.08) | 32 | 2.86 (1.31–6.27) |
| ER− or PR− | 11 | 1.0 (reference) | 34 | 1.38 (0.73–2.62) | 76 | 3.11 (1.72–5.61) |
| Tumor size | | | | | | |
| <1 cm | 25 | 1.0 (reference) | 15 | 1.81 (0.71–4.65) | 18 | 2.19 (0.87–5.49) |
| I cm+ | 8 | 1.0 (reference) | 18 | 1.52 (0.80–2.88) | 22 | 3.01 (1.65–4.50) |
| Lymph node involvement | | | | | | |
| No | 24 | 1.0 (reference) | 36 | 1.42 (0.56–3.62) | 22 | 2.49 (1.04–5.95) |
| Yes | 9 | 1.0 (reference) |

AJCC, American Joint Committee on Cancer; T1, first tertile; T2, second tertile; T3, third tertile. ORs by polychotomous logistic regression model adjusting for age (continuous variable), race (Caucasian, African-American) and batches.

*T1 methylation level < 4.25; T2 4.25 ≤ methylation level < 4.50; T3 ≥ 4.50.

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