Research Paper

Global DNA Methylation, Measured by the Luminometric Methylation Assay (LUMA), Associates with Postmenopausal Breast Cancer in Non-Obese and Physically Active Women

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Abstract

Introduction: Little is known about how modifiable lifestyle factors interact with the epigenome to influence disease. Body mass index (BMI, weight kg/height m2) and physical activity are associated with postmenopausal breast cancer, but the mechanisms are not well-understood. We hypothesized that BMI or physical activity may modify the association between markers of global DNA methylation and postmenopausal breast cancer risk.

Methods: Resources from a population-based case-control study (~1300 postmenopausal women) were used to construct logistic regression models. We explored whether the association between breast cancer and global methylation, assessed using the luminometric methylation assay (LUMA) and long interspersed elements-1 (LINE-1) methylation in white blood cell DNA, was modified by BMI or recreational physical activity (RPA).

Results: The LUMA-breast cancer association was modified by BMI (multiplicative p=0.03) and RPA (p=0.004). Non-obese women in the highest quartile of LUMA experienced a greater than two-fold increased risk of postmenopausal breast cancer (BMI<25kg/m2: OR=2.16; 95% CI=1.35, 3.57 and BMI 25-29.9kg/m2: OR=2.96; 95% CI=1.69, 5.19) compared to women in the lowest LUMA quartile. Similar increases in the LUMA-breast cancer association were observed among women who were physically active (moderate RPA: OR=2.62; 95% CI=1.44, 4.75 and high RPA: OR=2.62; 95% CI=1.53, 4.49). Estimates among obese and inactive women were less pronounced and imprecise. Although we observed statistical interactions (p<0.05) between BMI and RPA with LINE-1, we were unable to discern any clear associations with breast cancer.

Conclusions: The association between LUMA and postmenopausal breast cancer risk may be modified by postmenopausal body size and physical activity.

Key words: Obesity, physical activity, global DNA methylation, breast cancer, epidemiology

Introduction

Breast cancer remains the leading cause of cancer-related illness in the United States, and is thought to be the result of both abnormal genetic and epigenetic change (1). DNA methylation is an epigenetic modification that may influence cancer development by altering gene expression and genome integrity (2). While gene-specific methylation in target tissue has been investigated widely, global hypomethylation in peripheral blood DNA has been related to the cancer genome (3) and may be a useful surrogate biomarker for studying lifestyle or environmental effects in large epidemiologic studies where tumor tissue is limited. Although global loss of DNA methylation can lead to genomic instability, alter gene transcription, and increase mutation rates (4), little is known about how modifiable lifestyle factors influence DNA methylation status of the genome (5).

Elevated body mass and physical inactivity have long been associated with increased risk of postmenopausal breast cancer (6,7). Both have been shown to be associated with markers of global methylation in some studies, likely though their downstream effects on hormones and inflammation (8-11). Lifestyle factors may therefore play an important role in the association between global methylation and breast cancer risk.

The goals of this study were two-fold. We aimed to examine potential modification of the global DNA methylation-breast cancer association by (1) body size and (2) physical activity using two independent, but complementary, methods to assess global methylation in white blood cell (WBC) DNA: long interspersed elements-1 (LINE-1) methylation and the luminometric methylation assay (LUMA). LINE-1 methylation approximates levels in repetitive elements (12) and is a commonly used surrogate marker of genome-wide DNA hypomethylation (13), while LUMA measures levels of 5-^{m}C in the C^{m}CGG motif therefore approximating methylation levels at gene promoters rather than the total genome (14). We hypothesized that body size/physical activity would modify the association between global DNA methylation and postmenopausal breast cancer risk. To our knowledge, this is the first study to systematically address the interplay between body size or physical activity, DNA methylation and postmenopausal breast cancer risk using both LUMA and LINE 1.

Materials and Methods

We utilized resources from the case-control component of the Long Island Breast Cancer Study Project (LIBCSP), a population-based study. Details of the parent study have been reported previously (15).

Institutional Review Board approval was obtained by all participating institutions.

Study population

LIBCSP participants were English-speaking female residents of Nassau and Suffolk counties, Long Island, New York. LIBCSP case women were newly diagnosed with a first primary breast cancer between August 1, 1996 and July 31, 1997, and were identified through daily or weekly contact with local hospital pathology departments. Population-based controls were randomly selected using random digit dialing for women under age 65 and Health Care Finance Administration rosters for women ages 65 and older. LIBCSP controls were frequency matched to the expected age distribution of case women by 5-year age groups. At diagnosis/date of identification, participants were aged 20-98 years, 67% were postmenopausal and 94% were white.

Interviews were completed for 82.1% (n=1508) of eligible cases and 62.8% (n=1556) of controls, and occurred within 3 months of diagnosis (before completion of the first course of treatment) for most case participants (15). At the time of the case-control interview, trained phlebotomists obtained a non-fasting 40 mL blood sample from 73.1% of case and 73.3% of control participants. DNA was isolated from blood specimens using the methods previously described (16). Written informed consent was obtained from all participants prior to the study interview.

Global Methylation

LUMA assessment in the LIBCSP has been described in detail (14) and followed the modified protocol described by Bjornsson *et al*. (17). LUMA levels were expressed as a percentage based on the following equation: methylation (%) = [1-(*Hpa*II ΣG/ΣT)/(*Msp*I ΣG/ΣT)]*100 (17,17). LINE-1 methylation profiling for the LIBCSP has also been described in detail (14). A pre-validated pyrosequencing-based methylation assay (18,19) was used to assess 4 CpG sites in the promoter region of LINE-1. Methylation status at each of the 4 loci were analyzed individually as a T/C single nucleotide polymorphism using QCpG software (Qiagen) and subsequently averaged to provide an overall percentage 5^{m}C status. For quality control randomly selected samples were replicated to examine batch effects (corresponding CV% <1%). Additionally, cases and controls were assayed at the same time with laboratory personnel blinded to both case-control and quality control status.

Body Size, Physical Activity and Covariate Assessment

Body size and physical activity were assessed as part of the interviewer-administered structured ques-

tionnaire. Body mass index (BMI) at reference date was calculated for each participant based on the following formula: weight (kg)/height (m²). Recreational physical activity (RPA) was assessed using a modified instrument developed by Bernstein and colleagues (20). RPA from menopause to reference date was used to estimate postmenopausal physical activity as previously described (21).

During the baseline interview participants were also asked about their demographic characteristics; reproductive, menstrual, environmental, and medical histories (including family history of breast cancer); cigarette smoking and alcohol use; and use of exogenous hormones.

Statistical Methods

All statistical analyses were performed using SAS statistical software version 9.1 (SAS Institute, Cary, NC).

The relationship between LUMA and LINE-1 global methylation in WBC DNA and breast cancer incidence has been previously reported among all women in the LIBCSP study population, regardless of menopausal status (14). Because this investigation focuses on postmenopausal breast cancer only, where associations with BMI and RPA are most relevant, we first estimated associations between LUMA, LINE-1 and breast cancer incidence among postmenopausal women only.

We subsequently assessed whether BMI or RPA was associated with global methylation levels measured in the blood of controls only. We conducted unconditional logistic regression to estimate odds ratios (ORs) and 95% confidence intervals (CI) (22), with dichotomous global methylation markers as the outcome. We additionally used linear regression to estimate the association between BMI or RPA and global methylation levels.

Finally, we used a case-control approach to determine whether the association between global methylation (measured by LUMA and LINE-1) and postmenopausal breast cancer risk varied within strata of BMI or RPA. We conducted unconditional logistic regression (22) to estimate ORs and corresponding 95% CIs for the joint effects of: (1) BMI and LUMA; (2) RPA and LUMA; (3) BMI and LINE-1; as well as (4) RPA and LINE-1, respectively. Multiplicative interaction was assessed by comparing multivariable models with and without cross-product terms to represent the interaction between BMI or RPA and the individual methylation marker (LUMA or LINE-1) using an a priori p<0.05 (23). For case-control analyses methylation levels were categorized into quartiles based on the distribution among controls. Given LUMA, a global measurement of promoter methyla-

tion, was positively associated with overall breast cancer risk in our study population (14) we used the lowest quartile as the referent group. In contrast, LINE-1 hypomethylation is hypothesized to represent decreased genomic integrity. Therefore the highest quartile of LINE-1 was selected as the referent category. We categorized BMI using the standard World Health Organization classifications (<25.0 kg/m²; 25.0–29.9 kg/m²; and ≥30 kg/m²) and RPA based on the following definitions: inactive (no RPA), moderate RPA (≤9.23 hrs/wk), and high RPA (>9.23 hrs/wk).

For all models, potential confounders included first degree family history of breast cancer (yes/no), history of benign breast disease (yes/no), race (white/black/other), use of oral contraceptives (ever/never), hormone replacement therapy (ever/never), age at menarche (continuous), parity (continuous), lactation history (ever/never), age at first birth (continuous), age at menopause (continuous), total daily calories (continuous), alcohol history (ever/never), and smoking history (never, current, former). Confounders were included in the final model if their inclusion changed the exposure estimate >10% (24). None of the above covariates met our criterion and final models were adjusted only for 5-year age group.

Results

Table 1 shows the frequency (%) of the key covariates used in our analyses (age, BMI, RPA, LUMA and LINE-1) by case control status among LIBCSP postmenopausal participants. LUMA levels were associated with increased risk of postmenopausal breast cancer (Quartile 4 [Q4] vs. Quartile 1 [Q1]: OR=2.20; 95% CI=1.62, 3.00), whereas LINE-1 methylation levels were not (Q4 vs. Q1: OR=0.86; 95% CI=0.64, 1.16).

In **Table 2**, we present the association between LUMA and postmenopausal breast cancer risk within strata of postmenopausal BMI and RPA, respectively. The association between LUMA and postmenopausal breast cancer risk was stronger among women with BMI<25 (OR=2.16; 95% CI=1.35, 3.47) or BMI 25-29 (OR=2.96; 95% CI=1.69, 5.19), compared to women with BMI≥30 (OR=1.46; 95% CI=0.78, 2.73) (multiplicative interaction *p*=0.03). Similarly, the association between LUMA and postmenopausal breast cancer risk was greater than 2-fold among women with low (OR=2.62; 95% CI=1.53, 4.49) and high (OR=2.62; 95% CI=1.44, 4.75) RPA, whereas the association among inactive women was less in magnitude and included the null (OR=1.63; 95% CI=0.86, 3.11) (multiplicative interaction *p*=0.004).

We observed multiplicative interactions (*p*<0.05) between LINE-1 methylation and both BMI and RPA, but our estimates were imprecise and we were unable

to discern any clear patterns of association **(Table 3)**. Furthermore, among control women neither postmenopausal BMI nor RPA were associated with LUMA in logistic or linear regression models (data not shown).

Discussion

In this population-based case-control study, we observed a greater than 2-fold increased risk of postmenopausal breast cancer when women in the highest quartile of LUMA were compared to women in the lowest. This association was modified by both BMI and RPA. LUMA was associated with the greatest risk of postmenopausal breast cancer among women with BMI<30 and among women who were physically active. Although we observed a significant multiplicative interaction (p<0.05) between LINE-1 and both BMI and RPA, we were unable to identify any clear relationship. We therefore conclude that, in our data, the association between LUMA and postmenopausal breast cancer risk is modified by both BMI and RPA.

To our knowledge, this is the first epidemiologic study to examine the association between genome-wide DNA methylation, assessed using LUMA, BMI or RPA and human cancer of any site. Lower global methylation can lead to genomic instability and changes in gene transcription which may impact normal cell growth and increase the likelihood of tumorigenesis (25). Given the cost and tissue specificity (26) of whole-genome methylation profiling, global methylation levels, assessed by LUMA, in readily available specimens (e.g. blood) may be feasible when studying lifestyle or environmental effects in large populations (27).

TABLE 1. Distributions of key covariates among postmenopausal cases and controls, Long Island Breast Cancer Study Project (1996-1997).

	Cases		Controls	
	No	%	No	%
Age at reference (years)[a]				
<40	12	1.2	15	1.5
40-44	28	2.8	33	3.3
45-49	99	9.8	111	11.2
50-54	179	17.8	201	20.3
55-59	170	16.9	192	19.4
60-64	180	17.9	185	18.7
65-69	184	18.3	123	12.4
70-74	99	9.8	74	7.5
75-79	35	3.5	38	3.8
≥80	80	2.0	18	1.8
Body mass index (BMI)[a]				
BMI (<25kg/m²)	386	39.0	439	45.3
BMI (25-29.9kg/m²)	340	34.3	309	31.9
BMI (≥30kg/m²)	264	26.7	222	22.9
Recreational physical activity (RPA)[b]				
Inactive	254	30.1	223	27.8
Low RPA (≤ 9.23 hrs/wk)	329	38.9	290	36.1
High RPA (>9.23 hrs/wk)	262	31.0	290	36.1
Luminometric methylation assay[c]				
Quartile 1 (<0.43)	119	17.3	164	24.1
Quartile 2 (0.43<0.56)	126	18.3	177	26.0
Quartile 3 (0.56<0.66)	186	27.0	175	25.7
Quartile 4 (≥0.66)	259	37.5	165	24.2
Long interspersed elements-1[c]				
Q4 (≥80.4)	186	26.7	156	22.8
Q3 (78.7<80.4)	175	25.1	180	26.4
Q2 (77.0<78.7)	155	22.2	171	25.0
Q1 (<77.0)	182	26.1	176	25.8

Adapted from Gammon et al. 2002[a]; McCullough et al.2012[b]; and Xu et al.[c]

TABLE 2. Age-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for the associations between the luminometric methylation assay (LUMA) methylation levels and postmenopausal breast cancer stratified by postmenopausal body mass index (BMI) and recreational physical activity (RPA, average hours per week) among cases (Ca) and controls (Co), Long Island Breast Cancer Study Project (1996-1997).

LUMA (Quartiles)	Ca[a]/Co[b]	OR[c]	95% CI[d]	Ca/Co	OR	95% CI	Ca/Co	OR	95% CI
	Body Mass Index								
	BMI[e] (<25kg/m²)			BMI (25-29.9kg/m²)			BMI (≥30kg/m²)		
Q1 (<0.43)	48/77	1.00	reference	32/50	1.00	reference	38/34	1.00	reference
Q2 (0.43<0.56)	48/80	0.94	(0.56, 1.56)	41/55	1.18	(0.65, 2.16)	35/40	0.83	(0.43, 1.60)
Q3 (0.56<0.66)	66/84	1.24	(0.76, 2.02)	62/54	1.82	(1.02, 3.25)	57/35	1.51	(0.80, 2.85)
Q4 (≥0.66)	98/74	2.16	(1.35, 3.47)	97/51	2.96	(1.69, 5.19)	59/38	1.46	(0.78, 2.73)
	multiplicative p = 0.033								
	Recreational Physical Activity								
	High RPA[f] (>9.23 hrs/wk)			Moderate RPA (≤ 9.23 hrs/wk)			Inactive		
Q1 (<0.43)	27/51	1.00	reference	43/54	1.00	reference	31/33	1.00	reference
Q2 (0.43<0.56)	32/65	0.89	(0.47, 1.69)	44/47	1.16	(0.65, 2.06)	38/38	1.11	(0.57, 2.18)
Q3 (0.56<0.66)	54/43	2.45	(1.31, 4.59)	51/50	1.27	(0.72, 2.23)	48/43	1.21	(0.63, 2.33)
Q4 (≥0.66)	72/53	2.62	(1.44, 4.75)	92/46	2.62	(1.53, 4.49)	59/39	1.63	(0.86, 3.11)
	multiplicative p = 0.004								

[a] Cases; [b] Controls; [c] Odds ratio; [d] Confidence interval; [e] Body mass index; [f] Recreational physical activity

TABLE 3. Age-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for the associations between long interspersed elements-1 (LINE-1) methylation profiling and postmenopausal breast cancer stratified by postmenopausal body mass index (BMI) and recreational physical activity (RPA, average hours per week) among cases (Ca) and controls (Co), Long Island Breast Cancer Study Project (1996-1997).

LINE-1 (Quartiles)	Ca[a]/Co[b]	OR[c]	95% CI[d]	Ca/Co	OR	95% CI	Ca/Co	OR	95% CI
	Body Mass Index								
	BMI[e] (<25kg/m²)			BMI (25-29.9kg/m²)			BMI (≥30kg/m²)		
Q4 (≥80.4)	69/79	1.00	reference	62/50	1.00	reference	51/44	1.00	reference
Q3 (78.7<80.4)	58/81	1.14	(0.72, 1.79)	47/61	0.64	(0.37, 1.09)	46/26	1.46	(0.77, 2.75)
Q2 (77.0<78.7)	63/78	0.93	(0.58, 1.47)	63/55	0.95	(0.56, 1.61)	44/45	0.85	(0.47, 1.52)
Q1 (<77.0)	75/76	1.14	(0.72, 1.79)	60/46	1.10	(0.64, 1.89)	51/33	1.30	(0.71, 2.38)
	multiplicative p = 0.014								
	Recreational Physical Activity								
	High RPA[f] (>9.23 hrs/wk)			Moderate RPA (≤ 9.23 hrs/wk)			Inactive		
Q4 (≥80.4)	51/50	1.00	reference	67/48	1.00	reference	46/33	1.00	reference
Q3 (78.7<80.4)	47/61	0.86	(0.48, 1.54)	60/48	1.07	(0.62, 1.85)	44/42	0.54	(0.29, 1.02)
Q2 (77.0<78.7)	43/55	0.76	(0.43, 1.34)	55/51	1.27	(0.74, 2.19)	30/39	0.74	(0.41, 1.34)
Q1 (<77.0)	45/46	1.05	(0.59, 1.86)	50/51	1.42	(0.83, 2.43)	58/41	0.97	(0.53, 1.77)
	multiplicative p = 0.049								

[a] Cases; [b] Controls; [c] Odds ratio; [d] Confidence interval; [e] Body mass index; [f] Recreational physical activity

A number of investigations have evaluated associations between biomarkers of global methylation and other cancer types, but less is known about the effect of global methylation in breast cancer, particularly with LUMA (reviewed in (28)). Delgado-Cruzata (29) reported no difference in methylation levels by LUMA between affected and unaffected sisters, while a recent hospital-based case control study conducted in a Japanese population showed breast cancer risk reductions with increasing tertiles of LUMA methylation (30). These results are in contrast to what was observed in our study population, namely that women in the highest quintile of LUMA had increased risk of breast cancer (OR=2.14; 95% CI=1.83, 3.16; *p* trend < 0.0001) compared to women in the lowest quintile (14). The same report (14) found no association with LINE-1, which is consistent with the results of a recent meta-analysis that considered all cancer outcomes (28).

Further, there are inconsistencies in associations between both BMI and physical activity with LINE-1. Elevated BMI has been shown to be associated with lower LINE-1 methylation some studies (8,9) but was not correlated with LINE-1 in others (31,32). Regarding physical activity, some studies show associations between exercise and LINE-1 (10,11), while others do not (33). Given the unclear associations between BMI or physical activity and LINE-1, as well as the uncertainty of LINE-1 methylation as a useful biomarker in epidemiologic studies of cancer (28), our findings for LINE-1 are not unexpected. While our observations for LUMA support our a priori hypothesis of modification by both BMI and physical activity, we anticipated that the positive association between LUMA and postmenopausal breast cancer risk would be strongest among women with elevated BMI and/or

inactive lifestyle. On the contrary, our data showed that high LUMA levels were more strongly associated with enhanced postmenopausal breast cancer risk among active women and women with BMI<30kg/m². Obesity/inactivity is associated with a myriad of health consequences – many of which may not be mediated by changes in DNA methylation. We anticipate that our seemingly paradoxical results may be because obese women in the lowest quartile of LUMA (Q1) are already at enhanced risk of postmenopausal breast cancer. Any additional risk caused by aberrant DNA methylation may therefore be minimal. In contrast, women with BMI <25kg/m² or high levels of physical activity have a baseline risk of breast cancer that is relatively low (as compared with women who are overweight/obese or those with lower levels of physical activity). Thus, any additional risk caused by aberrant methylation, may be substantial enough to enhance overall susceptibility to postmenopausal breast cancer among those women who are at lower risk of breast cancer.

Strengths of our study include is its population-based design; relatively large sample size; as well as detailed exposure assessment. While anthropometric data were collected systematically by trained interviewers (15) errors in reporting or differential reporting by cases and controls have the potential to bias the study results. However, is unlikely that misreporting is differential with respect to methylation status (34). While there is interest in identifying epigenetic markers in surrogate tissue that could be used to identify high risk populations, it is unknown whether these markers truly reflect the epigenome or gene regulation in the target tissue. Some studies indicate that blood-derived DNA methylation measurements (e.g. LINE-1) do not correlate with methyl-

ation in tumor tissue (31,35,36), but additional investigations are needed to understand the correlation between target and surrogate tissues using other assays, particularly LUMA.

Using data from a large population-based sample, we observed that high methylation levels, assessed via the LUMA, were inversely associated with postmenopausal breast cancer among active and non-obese women. While our results require confirmation, they suggest that body size or physical activity may play an important role in modifying the association between DNA methylation and postmenopausal breast cancer. Given the plasticity of epigenetic marks in response to cancer-related exposures, additional research is needed to clarify these mechanisms and identify specific changes likely to be involved in the pathogenesis of breast cancer.

Abbreviations

BMI: Body mass index; CI: confidence interval; LIBCSP: Long Island Breast Cancer Study Project; LINE-1: long interspersed elements-1; LUMA: luminometric methylation assay; OR: odds ratio; RPA: recreational physical activity; WBC: white blood cell.

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Conflicts of Interest

None to declare.

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