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Gene-Specific Promoter Methylation Status in Hormone-Receptor-Positive Breast Cancer Associates with Postmenopausal Body Size and Recreational Physical Activity

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Abstract

Introduction: Breast cancer, the leading cancer diagnosis among American women, is positively associated with postmenopausal obesity and little or no recreational physical activity (RPA). However, the underlying mechanisms of these associations remain unresolved. Aberrant changes in DNA methylation may represent an early event in carcinogenesis, but few studies have investigated associations between obesity/RPA and gene methylation, particularly in postmenopausal breast tumors where these lifestyle factors are most relevant.

Methods: We used case-case unconditional logistic regression to estimate odds ratios (ORs) and 95% Confidence Intervals (CI) for the associations between body mass index (BMI=weight [kg]/height [m^2]) in the year prior to diagnosis, or RPA (average hours/week), and methylation status (methylated vs. unmethylated) of 13 breast cancer-related genes in 532 postmenopausal breast tumor samples from the Long Island Breast Cancer Study Project. We also explored whether the association between BMI/RPA and estrogen/progesterone-receptor status (ER+PR+ vs. all others) was differential with respect to gene methylation status. Methylation-specific PCR and the Methy Light assay were used to assess gene methylation.

Results: BMI 25-29.9kg/m^2, and perhaps BMI ≥ 30kg/m^2, was associated with methylated *HIN1* in breast tumor tissue. Cases with BMI ≥ 30kg/m^2 were more likely to have ER+PR+ breast tumors in the presence of unmethylated *ESR1* (OR=2.63, 95% CI 1.32-5.25) and women with high RPA were more likely to have ER+PR+ breast tumors with methylated *GSTP1* (OR=2.33, 95% CI 0.79-6.84).

Discussion: While biologically plausible, our findings that BMI is associated with methylated *HIN1* and BMI/RPA are associated with ER+PR+ breast tumors in the presence of unmethylated *ESR1*and methylated *GSTP1*, respectively, warrant further investigation. Future studies would benefit from enrolling greater numbers of postmenopausal women and examining a larger panel of breast cancer–related genes.

Keywords

Body mass index, Physical activity, Gene methylation, Breast cancer, Epidemiology

Introduction

Breast cancer remains the leading cause of cancer-related illness in the United States (US), and may be influenced by a number of

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environmental [1], reproductive and lifestyle [2] factors. There is abundant research showing that elevated body mass and physical inactivity are associated with increased risk of postmenopausal breast cancer [3,4], but the mechanisms driving these associations are unresolved [5]. Given the large proportion of women who are inactive in the US [6] and the steadily increasing rates of obesity [7], understanding the underlying mechanism for the observed association between these lifestyle factors and breast carcinogenesis is of paramount importance.

DNA methylation is an epigenetic alteration that can modify gene expression [8] and is known to been related to breast carcinogenesis [9,10]. Specifically, hypermethylation of tumor suppressor genes has been associated with clinical/pathological factors for breast cancer, as well as mortality in our study population [11]. Some investigators have hypothesized that elevated body mass and/or physical inactivity may affect DNA methylation through increased estrogen [12,13] and chronic inflammation[14,15]; but to date, only three studies have examined associations between body mass and gene-specific methylation in breast tumors [16-18]. This previous research was limited by examining a very small (<5) subset of genes [16,17] and some studies did not stratify by menopausal status [17,18]. No previous study has considered associations between physical activity and gene methylation of breast tumors.

The goals of our study were two-fold. First, we aimed to assess the potential association between body mass index (BMI) or recreational physical activity (RPA) in relation to promoter methylation status, assessed in a panel of 13 breast cancer-related genes measured in tumor tissue (*APC, BRCA1, CCND2, CDH1, DAPK1, ESR1, GSTP1, HIN1, CDKN2A, PGR, RARβ, RASSF1A* and *TWIST1*). These genes may play an important role in breast carcinogenesis and their promoter regions have been frequently methylated in breast tumors [19]. Second, we explored whether associations between BMI/RPA and breast cancer subtypes, defined by estrogen and progesterone receptor (ER/PR) status, were modified by gene promoter methylation.

We hypothesized that: (1) breast tumors from postmenopausal women with elevated body size/physical inactivity would have a greater prevalence of methylation than tumors from postmenopausal women with lower body mass/high physical activity; and (2) elevated body size/physical inactivity would differentially associate with ER+PR+ breast cancer when we also consider the gene-promoter methylation status of the tumor (methylated vs. unmethylated).

Materials and Methods

We utilized case-only 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 [20]. Institutional Review Board approval was obtained by all participating institutions.

Study population

Case women were English-speaking female residents of Nassau and Suffolk counties, Long Island, New York (NY), newly diagnosed with a first primary breast cancer between August 1, 1996 and July 31, 1997. Participants were identified using rapid case ascertainment via daily or weekly contact with pathology departments of all 28 hospitals on Long Island, and three tertiary care hospitals in New York City. At diagnosis, participants were aged 20-98 years and 67% were postmenopausal. Approximately 94% of study participants self-reported their race as white, 4% as black, and 2% as other, which was consistent with the underlying racial/ethnic distribution in these two NY counties at the time of data collection.

Data collection

Interviews were completed for 82.1% (n=1508) of eligible cases, and occurred within 3 months of diagnosis (before initiation of chemotherapy) for most case participants [20]. Tumor tissue was excised prior to the initiation of chemotherapy or radiation for all case participants. Written informed consent was obtained from all study participants prior to the study interview.

For LIBCSP cases, study investigators obtained archived pathology blocks for the first primary breast cancer from the 31 hospitals in Long Island and adjacent areas. Tumor blocks were successfully retrieved for 962 women [21] and tumor tissue from 532 postmenopausal participants were available for this study. Cases with tumor blocks available for methylation analysis (vs. those without tumor tissue available) were more likely be older (mean age 59.6 vs. 57.9 years), postmenopausal (70.7% vs. 64.6%), and have an invasive tumor (87.8% vs. 80.1%). Other demographic and clinical/pathological characteristics were similar between the two groups [19].

Body size, physical activity and covariate assessment

Body size and physical activity were assessed as part of the interviewer-administered structured questionnaire that was completed shortly after diagnosis. BMI in the year prior to diagnosis was calculated for each participant based on the following formula: weight (kg)/height (m^2). RPA was assessed using a modified instrument developed by Bernstein and colleagues [22]. RPA from menopause to reference date was used to estimate postmenopausal RPA as previously described [23] and defined as inactive, low RPA (≤9.23 hrs/wk) and high RPA (>9.23 hrs/wk) based on the control median.

During the interview participants were also asked about their demographic characteristics; reproductive, environmental, and medical histories (including family history of breast cancer); cigarette smoking and alcohol use; and use of exogenous hormones. Menopausal status was derived using information on the last menstrual period and gynecologic surgeries, combined with data on pregnancy, lactation, and use of hormone replacement therapy as previously described [24].

Gene-specific promoter DNA methylation assessment

DNA extraction from the archived tumor tissue was performed as previously described [25]. For methylation analysis, a panel of 13 genes known to be involved in breast carcinogenesis was selected. Promoter methylation of *ESR1*, *PGR* and *BRCA1* was determined by methylation-specific (MSP)-PCR as described previously [25,26]. The Methy Light assay was used for determining the methylation status of the remaining genes [27,28]. The percentage of methylation was calculated by the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_T = (C_{T,Target} - C_{T,Actin})_{sample} - (C_{T,Target} - C_{T,Actin})_{fully\ methylated\ DNA}$[29] and multiplying by 100. The MSP-PCR assay for *ESR1*, *PGR* and *BRCA1* promoter methylation generated dichotomous outcomes (i.e. methylated vs. unmethylated). Conversely, Methy Light assay yielded percentage of methylation for gene promoters that were subsequently dichotomized into methylated or unmethylated cases using a 4% cut-off as reported in previous literature [30]. The numbers of assayed samples and corresponding methylation frequencies for the selected genes are summarized in Xu et al. [19]. The main reason for missing methylation data was insufficient DNA, primarily due to small tumor size.

Hormone Receptor (HR) subtype assessment

We abstracted data recorded on the medical record to ascertain breast cancer subtype defined by HR status [20]. ER/PR status of the first primary breast cancer was available from the medical record for 65.6% of cases (N=990), of which 67.7% (N=670) were postmenopausal and included in these analyses.

Statistical Methods

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

We previously reported the relationship between gene-promoter methylation with demographic and clinical-pathological characteristics of the LIBCSP breast cancer cases by menopausal status [11,31]. The study reported here focuses on: (1) whether BMI and/or RPA are associated with gene methylation in postmenopausal breast tumors; and (2) whether the association between BMI and/or RPA and ER/PR subtype is differential with respect to gene methylation

status. To address these aims, we employed a case-case approach, and thus we relied solely upon data collected among postmenopausal case participants of the LIBCSP (n=532) [32].

To assess whether BMI or RPA was associated with gene-specific promoter methylation levels measured in case tumor tissue, we used logistic regression [32] to estimate odds ratios (ORs),and corresponding 95% confidence intervals (CIs) with case groups characterized by tumor methylation status (methylated vs. unmethylated for each marker). With this approach the ORs estimate the likelihood of a case possessing a methylated gene-promoter given their body size/physical activity status.

To determine whether the association between BMI or RPA and ER/PR receptor status was differential with respect to gene-specific promoter methylation, we used logistic regression to estimate ORs (95% CIs) with case groups characterized by both gene methylation status (methylated vs. unmethylated) and ER/PR status (ER+PR+ vs. all others: ER-PR-, ER+PR-, ER-PR+). With this approach the ORs estimate the likelihood of an ER+PR+ case given both gene methylation and body size/physical activity status. If the sample size in any strata of BMI/RPA and gene promoter methylation was less than ≤ 5, the OR (95% CI) was not estimated. In addition to comparing ER+PR+ breast cancer cases to all others, we also considered the comparison of ER+PR+ cases (primarily Luminal A and B subtypes) to ER-PR- cases (exclusively HER2 and triple negative subtypes) to better understand of potential associations with intrinsic subtypes.

We formally assessed evidence for multiplicative interaction using a likelihood ratio test [33], comparing multivariable models with and without cross-product terms to represent the interaction between BMI or RPA and a gene-specific methylation marker (a priori α=0.05). A significant interaction indicates that the odds of a case possessing the ER+PR+ breast cancer subtype, given BMI (or RPA) level, are statistically different across strata of gene-specific methylation.

We identified potential confounders based on the known epidemiology of breast cancer and analysis of causal diagrams [34]. For all models, potential confounders included: race (white/black/other); family history of breast cancer (yes/no); and history of benign breast disease (yes/no). Confounders were added in the model if they their inclusion changed the exposure estimate >10% [35]. None of the covariates assessed resulted in a >10% change in estimate, therefore only 5-year age group remained in our final case-case models.

Results

Associations between postmenopausal BMI and gene promoter methylation for the 13 breast cancer-related genes are shown in Table 1. Women with BMI 25-29.9kg/m^{2}were more likely to have methylated *HIN1* breast tumors (OR=1.57, 95%CI: 1.03-2.39). Although we observed elevated likelihood of methylated *HIN1* in breast tumors among women with BMI ≥ 30kg/m^2, the estimate was less pronounced and included the null (OR=1.44, 91% CI: 0.94-2.23). The remaining methylated gene promoters did not appear to be associated with postmenopausal BMI. We observed no differences in the likelihood of gene promoter methylation breast cancer in association with postmenopausal RPA for any of the 13 genes examined (Table 2).

We hypothesized that postmenopausal BMI or RPA may differentially associate with ER+PR+ breast cancer in strata of gene-promoter methylation. We found that obesity was associated with ER+PR+ breast cancer among women with unmethylated *ESR1* (OR=2.63; 95% CI: 1.32-5.25) (Table 3); the corresponding OR among cases with methylated *ESR1* was 1.24 (95% CI: 0.62-2.48) (multiplicative p for interaction=0.004). Similarly, we found that high RPA women with methylated *GSTP1* were more likely to have ER+PR+ breast cancer (OR=2.33; 95% CI: 0.79-6.84) than high RPA women with unmethylated *GSTP1* (OR=1.05; 95% CI: 0.53-2.10) (Table 4). We observed a multiplicative interaction (p=0.03) between *GSTP1* promoter methylation, postmenopausal RPA and ER+PR+ breast cancer, but given the small proportion of women with methylated *GSTP1* our estimates were imprecise. We were unable to estimate the ORs, due to the low prevalence tumor methylation, in several markers (e.g. *CDH1, p16, PR* and *RASSF1A*). With the remaining gene promoters that we considered, we identified no differential associations between BMI or RPA and ER+PR+ breast cancer.

The associations between postmenopausal BMI and breast cancer, defined by *ESR1* methylation and estrogen-receptor status, were robust, and remained significant (p=0.019), when we compared ER+PR+breast cancer to ER-PR- breast cancer only (Supplemental Table 1). For postmenopausal RPA and *GSTP1* methylation, however, our estimates were less robust, and were of borderline statistical significance (p=0.068) when comparing ER+PR+ breast cancer to ER-PR-breast cancer (Supplemental Table 2).

Discussion

In this population-based study, we found that women with postmenopausal BMI 25-29.9kg/m^2, and perhaps BMI ≥30kg/m^2, were more likely to have methylated *HIN1* breast cancer. We also observed a two-fold increase in the likelihood of ER+PR+ breast cancer among postmenopausal obese women with unmethylated *ESR1* tumors and among postmenopausal highly active women with methylated *GSTP1* tumors. Our findings are biologically plausible, as discussed below.

Table 1: Age-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for the association between postmenopausal body mass index (BMI) and breast cancer, as defined by gene-specific promoter methylation (comparing methylated vs. unmethylated cases), Long Island Breast Cancer Study Project (1996-1997).

	BMI (<25kg/m^2)				BMI (25-29.9kg/m^2)				BMI (≥30kg/m^2)		
Genes	**Methylated/ Unmethylated**	**OR**	**95% CI**		**Methylated/ Unmethylated**	**OR**	**95% CI**		**Methylated/ Unmethylated**	**OR**	**95% CI**
APC	110/106	1.00	reference		90/89	0.97	(0.65, 1.45)		76/71	1.03	(0.68, 1.57)
BRCA1	127/102	1.00	reference		117/76	1.24	(0.84, 1.82)		86/73	0.95	(0.63, 1.42)
CDH1	16/193	1.00	reference		11/156	0.86	(0.39, 1.91)		6/143	0.50	(0.19, 1.31)
CYCLIND2	43/166	1.00	reference		40/127	1.20	(0.73, 1.97)		33/116	1.15	(0.69, 1.93)
DAPK	29/180	1.00	reference		26/141	1.13	(0.64, 2.01)		27/122	1.43	(0.81, 2.55)
ESR1	106/122	1.00	reference		81/110	0.85	(0.58, 1.25)		78/81	1.11	(0.74, 1.66)
GSTP1	55/154	1.00	reference		46/121	1.06	(0.67, 1.67)		42/107	1.12	(0.70, 1.79)
HIN	118/91	1.00	reference		112/55	1.57	(1.03, 2.39)		97/52	1.44	(0.94, 2.23)
CDKN2A	7/202	1.00	reference		9/164	1.67	(0.61, 4.61)		6/137	1.28	(0.42, 3.89)
PR	34/196	1.00	reference		21/172	0.70	(0.39, 1.25)		15/144	0.60	(0.31, 1.14)
RARB	64/145	1.00	reference		49/118	0.93	(0.60, 1.46)		37/112	0.76	(0.47, 1.23)
RASSF1A	176/33	1.00	reference		146/21	1.28	(0.71, 2.32)		122/27	0.86	(0.49, 1.52)
TWIST	36/173	1.00	reference		30/137	1.05	(0.61, 1.79)		22/127	0.84	(0.47, 1.51)

Table 2: Age-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for the association between postmenopausal recreational physical activity (RPA) and breast cancer, defined by tumor gene-specific promoter methylation (comparing methylated vs. unmethylated cases), in the Long Island Breast Cancer Study Project (1996-1997).

	Inactive			Low RPA(≤ 9.23 hrs/wk)			High RPA (>9.23 hrs/wk)		
Genes	**Methylated/ Unmethylated**	**OR**	**95% CI**	**Methylated/ Unmethylated**	**OR**	**95% CI**	**Methylated/ Unmethylated**	**OR**	**95% CI**
APC	69/67	1.00	reference	93/86	1.05	(0.67, 1.64)	72/70	1.00	(0.62, 1.60)
BRCA1	84/68	1.00	reference	117/71	1.34	(0.87, 2.07)	85/66	1.04	(0.66, 1.64)
CDH1	10/119	1.00	reference	14/164	1.00	(0.43, 2.33)	4/131	0.37	(0.11, 1.19)
CYCLIND2	31/98	1.00	reference	34/144	0.76	(0.44, 1.33)	28/107	0.81	(0.45, 1.46)
DAPK	21/108	1.00	reference	24/154	0.82	(0.44, 1.56)	26/109	1.21	(0.64, 2.29)
ESR1	74/77	1.00	reference	85/101	0.87	(0.57, 1.34)	62/89	0.73	(0.46, 1.15)
GSTP1	38/91	1.00	reference	41/137	0.73	(0.43, 1.22)	37/98	0.90	(0.53, 1.54)
HIN	76/53	1.00	reference	111/67	1.16	(0.73, 1.85)	91/44	1.44	(0.87, 2.38)
CDKN2A	9/124	1.00	reference	6/166	0.48	(0.17, 1.39)	5/133	0.52	(0.17, 1.59)
PR	19/133	1.00	reference	22/166	0.92	(0.48, 1.77)	18/133	0.95	(0.48, 1.89)
RARB	43/86	1.00	reference	51/127	0.81	(0.50, 1.33)	32/103	0.62	(0.36, 1.06)
RASSF1A	110/19	1.00	reference	154/24	1.13	(0.59, 2.18)	111/24	0.79	(0.41, 1.53)
TWIST	22/107	1.00	reference	29/149	0.96	(0.52, 1.76)	22/113	0.94	(0.49, 1.80)

Table 3: Age-adjusted odds ratios (ORs) and 95% CIs (CIs) for the association between postmenopausal body mass index (BMI) and ER+PR+ breast cancer (vs. all others cases: ER-, PR-, ER+PR-, ER-PR+) considering gene-specific methylation status of the tumor (methylated vs. unmethylated), the Long Island Breast Cancer Study Project (1996-1997).

					Gene-specific methylation status						
		All breast cancer cases			Methylated breast tumor			Unmethylated breast tumor			
Genes	**Body mass index**	**ER+PR+/ all others**	**OR**	**(95% CI)**	**ER+PR+/ all others**	**OR**	**(95% CI)**	**ER+PR+/ all others**	**OR**	**(95% CI)**	**p for interaction**
APC											
	BMI (<25kg/m^2)	79/80	1.00	reference	41/39	1.00	reference	38/41	1.00	reference	0.266
	BMI (25-29.9kg/m^2)	77/50	1.55	(0.96, 2.49)	35/27	1.24	(0.64, 2.43)	42/23	1.89	(0.95, 3.75)	
	BMI (≥30kg/m^2)	73/39	1.89	(1.15, 3.12)	35/22	1.51	(0.76, 3.02)	38/17	2.39	(1.16, 4.92)	
BRCA1											
	BMI (<25kg/m^2)	87/82	1.00	reference	51/45	1.00	reference	36/37	1.00	reference	0.300
	BMI (25-29.9kg/m^2)	82/56	1.36	(0.86, 2.10)	46/36	1.12	(0.62, 2.03)	36/20	1.84	(0.89, 3.80)	
	BMI (≥30kg/m^2)	78/41	1.79	(1.10, 2.91)	44/23	1.70	(0.89, 3.24)	34/18	1.93	(0.93, 4.04)	
CDH1											
	BMI (<25kg/m^2)	78/80	1.00	reference	6/7	1.00	reference	72/73	1.00	reference	--
	BMI (25-29.9kg/m^2)	70/48	1.48	(0.91, 2.41)	3/5	not estimated		67/43	1.57	(0.95, 2.60)	
	BMI (≥30kg/m^2)	76/38	2.05	(1.25, 3.38)	4/2	not estimated		72/36	2.03	(1.21, 3.40)	
CYCLIND2											
	BMI (<25kg/m^2)	78/80	1.00	reference	15/17	1.00	reference	63/63	1.00	reference	0.763
	BMI (25-29.9kg/m^2)	70/48	1.48	(0.91, 2.41)	18/14	1.46	(0.54, 3.93)	52/34	1.51	(0.86, 2.64)	
	BMI (≥30kg/m^2)	76/38	2.05	(1.25, 3.38)	18/9	2.26	(0.78, 6.56)	58/29	2.00	(1.13, 3.52)	
DAPK											
	BMI (<25kg/m^2)	78/80	1.00	reference	11/10	1.00	reference	67/70	1.00	reference	0.290
	BMI (25-29.9kg/m^2)	70/48	1.48	(0.91, 2.41)	10/9	1.02	(0.29, 3.62)	60/39	1.59	(0.94, 2.69)	
	BMI (≥30kg/m^2)	76/38	2.05	(1.25, 3.38)	17/6	2.59	(0.73, 9.18)	59/32	1.93	(1.12, 3.34)	
ESR1											
	BMI (<25kg/m^2)	86/82	1.00	reference	40/36	1.00	reference	46/46	1.00	reference	0.004
	BMI (25-29.9kg/m^2)	82/55	1.41	(0.89, 2.22)	39/15	2.33	(1.11, 4.93)	43/40	1.05	(0.58, 1.92)	
	BMI (≥30kg/m^2)	78/41	1.81	(1.12, 2.94)	33/24	1.24	(0.62, 2.48)	45/17	2.63	(1.32, 5.25)	
GSTP1											
	BMI (<25kg/m^2)	78/80	1.00	reference	18/16	1.00	reference	60/64	1.00	reference	0.224
	BMI (25-29.9kg/m^2)	70/48	1.48	(0.91, 2.41)	19/16	1.04	(0.40, 2.68)	51/32	1.69	(0.96, 2.98)	
	BMI (≥30kg/m^2)	76/38	2.05	(1.25, 3.38)	20/14	1.28	(0.49, 3.33)	56/24	2.49	(1.38, 4.51)	
HIN											
	BMI (<25kg/m^2)	78/80	1.00	reference	46/33	1.00	reference	32/47	1.00	reference	0.130
	BMI (25-29.9kg/m^2)	70/48	1.48	(0.91, 2.41)	48/32	1.07	(0.56, 2.02)	22/16	2.00	(0.91, 4.40)	
	BMI (≥30kg/m^2)	76/38	2.05	(1.25, 3.38)	54/19	2.03	(1.02, 4.05)	22/19	1.72	(0.80, 3.70)	
p16											
	BMI (<25kg/m^2)	75/80	1.00	reference	1/3	not estimated		74/77	1.00	reference	--
	BMI (25-29.9kg/m^2)	75/50	1.59	(0.98, 2.57)	2/3	not estimated		73/47	1.61	(0.98, 2.63)	
	BMI (≥30kg/m^2)	71/39	1.94	(1.17, 3.20)	3/2	not estimated		68/37	1.91	(1.15, 3.19)	
PR											
	BMI (<25kg/m^2)	87/82	1.00	reference	9/18	1.00	reference	78/64	1.00	reference	--
	BMI (25-29.9kg/m^2)	82/56	1.36	(0.86, 2.16)	6/7	1.71	(0.44, 6.61)	76/49	1.25	(0.76, 2.04)	
	BMI (≥30kg/m^2)	78/41	1.79	(1.10, 2.91)	8/1	not estimated		70/40	1.43	(0.86, 2.38)	
RARB											
	BMI (<25kg/m^2)	78/80	1.00	reference	18/27	1.00	reference	60/53	1.00	reference	0.598
	BMI (25-29.9kg/m^2)	70/48	1.48	(0.91, 2.41)	17/19	1.30	(0.53, 3.18)	53/29	1.60	(0.89, 2.88)	
	BMI (≥30kg/m^2)	76/38	2.05	(1.25, 3.38)	18/12	2.25	(0.87, 5.78)	58/26	1.97	(1.09, 3.57)	

RASSF1A												
	BMI (<25kg/m²)	78/80	1.00	reference	64/67	1.00	reference	14/13	1.00	reference	--
	BMI (25-29.9kg/m²)	70/48	1.48	(0.91, 2.41)	61/43	1.50	(0.89, 2.52)	9/5	not estimated		
	BMI (≥30kg/m²)	76/38	2.05	(1.25, 3.38)	67/26	2.69	(1.53, 4.75)	9/12	0.67	(0.21, 2.15)	
TWIST											
	BMI (<25kg/m²)	78/80	1.00	reference	9/17	1.00	reference	69/63	1.00	reference	0.317
	BMI (25-29.9kg/m²)	70/48	1.48	(0.91, 2.41)	12/10	2.44	(0.73, 8.13)	58/38	1.38	(0.81, 2.35)	
	BMI (≥30kg/m²)	76/38	2.05	(1.25, 3.38)	13/7	3.51	(1.03, 11.96)	63/31	1.86	(1.07, 3.22)	

Table 4: Age-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for the association between postmenopausal recreational physical activity (RPA) and ER+PR+ breast cancer (vs. all others: ER-PR-, ER+PR-, ER-PR+) considering gene-specific methylation status of the tumor (methylated vs. unmethylated), the Long Island Breast Cancer Study Project (1996-1997).

					Gene-specific methylation status						
		All breast cancer cases			Methylated breast tumor			Unmethylated breast tumor			
Genes	**Recreational physical activity[a]**	**ER+PR+/ All others**	**OR**	**(95% CI)**	**ER+PR+/ All others**	**OR**	**(95% CI)**	**ER+PR+/ All others**	**OR**	**(95% CI)**	**p for interaction**
APC											
	Inactive	55/41	1.00	reference	25/21	1.00	reference	30/20	1.00	reference	0.164
	Low RPA	69/61	0.84	(0.50, 1.44)	32/31	0.87	(0.40, 1.87)	37/30	0.82	(0.39, 1.73)	
	High RPA	73/39	1.40	(0.80, 2.45)	40/21	1.60	(0.73, 3.52)	33/18	1.22	(0.55, 2.74)	
BRCA1											
	Inactive	63/45	1.00	reference	35/27	1.00	reference	28/18	1.00	reference	0.259
	Low RPA	74/61	0.87	(0.52, 1.45)	44/39	0.88	(0.45, 1.70)	30/22	0.87	(0.39, 1.96)	
	High RPA	76/44	1.23	(0.72, 2.10)	46/23	1.55	(0.76, 3.16)	30/21	0.92	(0.41, 2.07)	
CDH1											
	Inactive	55/39	1.00	reference	5/4	not estimated		50/35	1.00	reference	--
	Low RPA	67/61	0.78	(0.45, 1.33)	4/7	not estimated		63/54	0.82	(0.47, 1.44)	
	High RPA	69/37	1.32	(0.75, 2.34)	2/1	not estimated		67/36	1.31	(0.72, 2.36)	
CYCLIND2											
	Inactive	55/39	1.00	reference	12/13	1.00	reference	43/26	1.00	reference	0.115
	Low RPA	67/61	0.78	(0.45, 1.33)	10/13	0.83	(0.26, 2.59)	57/48	0.72	(0.39, 1.34)	
	High RPA	69/37	1.32	(0.75, 2.34)	18/7	2.82	(0.87, 9.16)	51/30	1.03	(0.53, 2.00)	
DAPK											
	Inactive	55/39	1.00	reference	11/6	1.00	reference	44/33	1.00	reference	0.145
	Low RPA	67/61	0.78	(0.45, 1.33)	7/11	0.34	(0.09, 1.35)	60/50	0.90	(0.50, 1.62)	
	High RPA	69/37	1.32	(0.75, 2.34)	14/6	1.36	(0.33, 5.53)	55/31	1.33	(0.71, 2.51)	
ESR1											
	Inactive	63/45	1.00	reference	30/21	1.00	reference	33/24	1.00	reference	0.194
	Low RPA	73/60	0.87	(0.52, 1.45)	32/32	0.70	(0.33, 1.47)	41/28	1.06	(0.52, 2.18)	
	High RPA	76/44	1.24	(0.72, 2.11)	31/15	1.44	(0.63, 3.32)	45/29	1.13	(0.56, 2.29)	
GSTP1											
	Inactive	55/39	1.00	reference	12/16	1.00	reference	43/23	1.00	reference	0.030
	Low RPA	67/61	0.78	(0.45, 1.33)	16/10	2.33	(0.76, 7.17)	51/51	0.53	(0.28, 1.01)	
	High RPA	69/37	1.32	(0.75, 2.34)	18/11	2.33	(0.79, 6.84)	51/26	1.05	(0.53, 2.10)	
HIN											
	Inactive	55/39	1.00	reference	33/18	1.00	reference	22/21	1.00	reference	0.207
	Low RPA	67/61	0.78	(0.45, 1.33)	43/33	0.69	(0.33, 1.45)	24/28	0.82	(0.37, 1.84)	
	High RPA	69/37	1.32	(0.75, 2.34)	51/18	1.55	(0.70, 3.40)	18/19	0.92	(0.38, 2.21)	
p16											
	Inactive	54/41	1.00	reference	2/2	not estimated		52/39	1.00	reference	--
	Low RPA	66/61	0.82	(0.48, 1.40)	1/4	not estimated		65/57	0.85	(0.49, 1.47)	
	High RPA	70/39	1.36	(0.77, 2.39)	3/1	not estimated		67/38	1.32	(0.74, 2.35)	
PR											
	Inactive	63/45	1.00	reference	5/8	not estimated		58/37	1.00	reference	--
	Low RPA	74/61	0.87	(0.52, 1.45)	4/10	not estimated		70/51	0.88	(0.51, 1.52)	
	High RPA	76/44	1.23	(0.72, 2.10)	10/5	not estimated		66/39	1.08	(0.61, 1.91)	
RARB											
	Inactive	55/39	1.00	reference	13/19	1.00	reference	42/20	1.00	reference	0.183
	Low RPA	67/61	0.78	(0.45, 1.33)	16/19	1.23	(0.46, 3.24)	51/42	0.57	(0.29, 1.10)	
	High RPA	69/37	1.32	(0.75, 2.34)	16/12	1.96	(0.70, 5.49)	53/25	1.01	(0.49, 2.06)	
RASSF1A											
	Inactive	55/39	1.00	reference	49/29	1.00	reference	6/10	1.00	reference	--
	Low RPA	67/61	0.78	(0.45, 1.33)	57/51	0.66	(0.36, 1.19)	10/10	1.72	(0.44, 6.64)	
	High RPA	69/37	1.32	(0.75, 2.34)	57/32	1.06	(0.56, 1.98)	12/5	not estimated		
TWIST											
	Inactive	55/39	1.00	reference	8/10	1.00	reference	47/29	1.00	reference	0.360
	Low RPA	67/61	0.78	(0.45, 1.33)	9/12	0.86	(0.24, 3.15)	58/49	0.73	(0.40, 1.34)	
	High RPA	69/37	1.32	(0.75, 2.34)	11/6	2.39	(0.61, 9.46)	58/31	1.16	(0.61, 2.19)	

[a] Low RPA ≤ 9.23 hours/week, High RPA > 9.23 hours/week

Supplemental Table 1: Age-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for the association between postmenopausal body mass index (BMI) and ER+PR+ breast cancer (vs. ER-PR- breast cancer) considering *ESR1* methylation status of the tumor (methylated vs. unmethylated), the Long Island Breast Cancer Study Project (1996-1997).

				ESR1 methylation status						
	All breast cancer cases			Methylated breast tumor			Unmethylated breast tumor			
Body mass index (BMI)	ER+PR+/ ER-PR-	OR[c]	95% CI[d]	ER+PR+/ ER-PR-	OR	95% CI	ER+PR+/ ER-PR-	OR	95% CI	p for interaction
BMI[d] (<25kg/m^2)	86/37	1.00	reference	40/15	1.00	reference	46/22	1.00	reference	0.019
BMI (25-29.9kg/m^2)	82/22	1.50	(0.81, 2.78)	39/8	1.79	(0.68, 4.70)	43/14	1.42	(0.64, 3.13)	
BMI (≥30kg/m^2)	78/19	1.74	(0.92, 3.28)	33/13	0.99	(0.41, 2.38)	45/6	3.76	(1.39, 10.15)	

Supplemental Table 2: Age-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for the associations between postmenopausal recreational physical activity (RPA) and ER+PR+ breast cancer (vs. ER-PR- breast cancer) considering the *GSTP1* methylation status of the tumor (methylated vs. unmethylated), the Long Island Breast Cancer Study Project (1996-1997).

				GSTP1 methylation status						
	All breast cancer cases			Methylated breast tumor			Unmethylated breast tumor			
Recreational physical activity (RPA)[a]	ER+PR+/ ER-PR-	OR[d]	95% CI[e]	ER+PR+/ ER-PR-	OR	95% CI	ER+PR+/ ER-PR-	OR	95% CI	p for interaction
Inactive	55/18	1.00	reference	12/6	1.00	reference	43/12	1.00	reference	0.068
Low RPA[d] (≤ 9.23 hrs/wk)	67/27	0.84	(0.42, 1.68)	16/3	not estimated		51/24	0.60	(0.27, 1.34)	
High RPA (>9.23 hrs/wk)	69/18	1.28	(0.61, 2.69)	18/5	1.85	(0.46, 7.48)	51/13	1.11	(0.46, 2.68)	

[a] Low RPA ≤ 9.23 hours/week, High RPA > 9.23 hours/week

Inactivation of tumor suppressor genes by promoter hypermethylation is a common epigenetic alteration in breast carcinogenesis [36,37]. These alterations are known to occur more frequently in breast tumor tissue than adjacent nonmalignant tissue [36,37] and have been associated with the clinicopathologic parameters of breast cancer [10]. Gene-promoter hypermethylation may therefore be an important event in breast carcinogenesis.

Increased BMI and physical inactivity are risk factors for postmenopausal breast cancer [4,38], and their influence on endogenous estrogens are well-documented [39,40]. *In vivo* and *in vitro* data suggest estrogen may induce aberrant DNA methylation, altering several genes implicated in breast carcinogenesis [41,42]. Specifically, estrogens were reported to induce promoter hypermethylation of *CDH1* and *CDKN2A* in non-malignant breast cells of humans [43]. In addition to increased levels of estrogen, central adiposity has been associated with chronic low-grade inflammation [44]. Several studies have shown greater frequency of promoter methylation in *CDKN2A*, *CDH1*, *BRCA1*, and *MLH1* among patients with chronic inflammatory disease compared with patients without [14,15]. Moreover, clinical data indicate that weight loss (≥ 5% initial body weight) was associated with significantly lower promoter methylation of *TNF-α* in peripheral blood [45]. Physical activity has similarly been found to reduce levels of pro-inflammatory markers [46].

Hormonal and inflammatory mediators have the capacity to induce and maintain promoter methylation facilitating the growth and survival of tumors, but to our knowledge, few studies have examined associations between body size and methylation status of breast tumors [16-18]. Consistent with our findings, Tao and colleagues [16] observed no association between body size and methylation of *CDH1*, *CDKN2A*, and *RAR-β2* among postmenopausal case women; associations by ER/PR status were not reported. Naushad and colleagues [17] examined the association between BMI and methylation of *Ec*-SOD, *RASSF1*, *BRCA1,* and *BNIP3*. BMI was significantly positively associated with *Ec*-SOD, *RASSF1* and *BRCA1* methylation but inversely associated with *BNIP3*. Most recently, Hair and colleagues [18] reported significant associations between BMI and methylation of 2 loci among all breast tumors and 21 loci specific to ER+ tumors, but did not examine menopause-specific associations. The association between body size and breast cancer risk is known to vary by menopausal subgroups [47]. It is therefore likely that obesity-associated methylation sites also differ by menopausal status. While we employed a biologically driven candidate gene approach, our study improves on the prior research by including a larger number of candidate genes, exploring associations by ER/PR status, and focusing on postmenopausal women. Further, it is the first study to consider the association between physical activity and gene methylation in postmenopausal breast tumors.

In our findings reported here, elevated postmenopausal BMI more strongly associated with ER+PR+ breast cancer among women with unmethylated *ESR1*. The ER protein is coded for by *ESR1* and gene silencing of *ESR1* by DNA methylation is often observed in breast tissues that do not express ER (e.g. ER-) [48]. Estrogens have long been hypothesized to underlie the positive association between obesity and postmenopausal breast cancer risk [39]. Our observation of stronger and more precise associations between postmenopausal obesity and ER+PR+ breast cancer among women where *ESR1* is active (unmethylated) is biologically reasonable and suggests that methylation-mediated silencing of the *ESR1* gene may alleviate the role of obesity-related estrogen in postmenopausal breast carcinogenesis.

We similarly found that the odds of being an ER+PR+ breast cancer case was enhanced among women engaging in high postmenopausal RPA in the presence of *GSTP1* methylation. *GSTP1* is involved in a wide range of detoxification reactions which protect cells from carcinogens [49]. The 5' region of *GSTP1* is rich in CpG islands and its methylation has been associated with loss of GSTP1 expression [50], breast carcinogenesis [51] and ER+PR+ case status [52]. The immediate systemic response to physical activity is an increase in reactive oxygen species production; it is therefore biologically plausible that reduced GSTP1 expression via DNA methylation may enhance risk of breast cancer, specifically ER+PR+ breast cancer.

Strengths of our epidemiologic study include: (1) our novel examination of the potential role of physical activity, as well as obesity, in the association between tumor methylation and breast cancer; (2) restricting eligibility to postmenopausal breast cancer, where the associations with obesity and physical activity are most pronounced; (3) our population-based design, which enhances generalizability and facilitates quantification of any study bias due to subject selection; (4) relatively large sample size, which facilitates examining subgroup associations as we did here; (5) detailed exposure assessment of our anthropometic measures, which reduces the likelihood of random measurement error; (6) our case-case approach, which substantially reduces the likelihood of recall bias given that both the "case" group and our "comparison" group had breast cancer (and it is highly unlikely that misreporting of anthropometric-related information is differential with respect to methylation or HR status[53]); and (7) we only considered associations for which we had *a priori* strong biologic rationale, mitigating concerns regarding multiple comparisons.

There are also several limitations to consider when examining methylation in tumors in an epidemiologic study. First, we

were unable to obtain archived tumor tissue for all LIBCSP case participants, which may result in selection bias; however, we were able to identify and consider potential sources of this error. Second, we were underpowered to explore potential variation by intrinsic subtype (Luminal A, Luminal B, HER2 and triple negative) given our study population primarily consisted of postmenopausal white women with low proportion of HER2- tumors. Third, gene-promoter methylation analyses were constrained by sample size for several of the genes we considered, and thus future studies should consider enlarging study enrollment. Fourth, we had a limited panel of 13 biologically relevant genes for analyses. Although this is four times that of the one previous investigation focused on obesity, gene methylation and postmenopausal breast cancer [16], we were unable to explore all the mechanistic pathways that may be involved in this association. Finally, classification of methylation status is not universally defined and our cutoff of 4% may not be biologically relevant for all the genes assessed.

In summary, using data from a large population-based sample, we found that BMI may associate with *HIN1* methylation status of postmenopausal breast tumor tissue. Notably, we also observed that both postmenopausal body size and physical activity may increase the likelihood of ER+PR+ breast cancer (which is the most common subtype diagnosed among American women [54]) in the absence and presence of *ESR1* and *GSTP1* methylation, respectively. While our results require confirmation in larger studies of postmenopausal women with greater number of genes, they suggest that DNA methylation may play an important role in understanding mechanisms underlying the associations between body size, physical activity 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.

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Ethical Statement

Institutional Review Board approval was obtained by all participating institutions.

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