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ORIGINAL ARTICLE

Exposures in early life: associations with DNA promoter methylation in breast tumors

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There is evidence that epigenetic changes occur early in breast carcinogenesis. We hypothesized that early-life exposures associated with breast cancer would be associated with epigenetic alterations in breast tumors. In particular, we examined DNA methylation patterns in breast tumors in association with several early-life exposures in a population-based case–control study. Promoter methylation of *E-cadherin*, *p16* and *RAR-β*$_2$ genes was assessed in archived tumor blocks from 803 cases with real-time methylation-specific PCR. Unconditional logistic regression was used for case–case comparisons of those with and without promoter methylation. We found no differences in the prevalence of DNA methylation of the individual genes by age at menarche, age at first live birth and weight at age 20. In case–case comparisons of premenopausal breast cancer, lower birth weight was associated with increased likelihood of *E-cadherin* promoter methylation (OR = 2.79, 95% CI, 1.15–6.82, for ⩽2.5 *v.* 2.6–2.9 kg); higher adult height with *RAR-β*$_2$ methylation (OR = 3.34, 95% CI, 1.19–9.39, for ⩾1.65 *v.* <1.60 m); and not having been breastfed with *p16* methylation (OR = 2.75, 95% CI, 1.14–6.62). Among postmenopausal breast cancers, birth order was associated with increased likelihood of *p16* promoter methylation. Being other than first in the birth order was inversely associated with likelihood of ⩾1 of the three genes being methylated for premenopausal breast cancers, but positively associated with methylation in postmenopausal women. These results suggest that there may be alterations in methylation associated with early-life exposures that persist into adulthood and affect breast cancer risk.

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Key words: breast cancer, early-life exposure, epidemiology, epigenetics, promoter methylation

Introduction

There is evidence that fetal and early-life exposures play a critical role in development of breast cancer in adulthood.[1] The development of the breast is a progressive process, initiated in the embryonic period, continuing throughout childhood particularly during puberty, with terminal differentiation during the first full-term pregnancy.[2,3] Age at menarche and age of first live birth are well-established risk factors for breast cancer. Other exposures related to the *in utero* environment including infant birth size (birth weight and birth length), mother's age at delivery, birth order and mother's pre-eclampsia may also affect risk.[4–6] There is some evidence suggesting an inverse association of exposure to breast milk in infancy with breast cancer risk.[7,8] Childhood and adolescent body mass index (BMI) also appear to be inversely associated with breast cancer risk.[9,10] Moreover, there is a consistently positive association between height and breast cancer.[1,11] The biological mechanisms underlying the observed associations are not clear; one possible mechanism is that there are epigenetic alterations resulting from these exposures, which play a role in breast carcinogenesis.

Commonly found in human neoplasias, epigenetic alterations in response to early-life exposures may influence the risk of adult diseases,[2,12,13] including cancer. Both global hypomethylation and gene-specific promoter hypermethylation are prominent features of breast tumors.[14] The *E-cadherin*, *p16* and *RARβ*$_2$ genes are tumor suppressor genes involved in key cellular processes including cell cycle regulation, cell-to-cell interaction, hormone- and receptor-mediated cell signaling, apoptosis and angiogenesis.[15] Promoter methylation of these genes occurs frequently in breast cancer cell lines and breast tumors.[16–18]

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A recent animal study reported that *in utero* and lactational exposure to environmental contaminants decreased the expression of *p16* gene and slightly increased methylation of CpG sites in the promoter region of *p16* in prepubertal female offspring rats.[19] In addition, there is both *in vivo* and *in vitro* evidence that prenatal and early postnatal nutrition can influence patterns of DNA methylation and cause changes in gene expression in the offspring.[12,13] In a study from the Netherlands, individuals periconceptionally exposed to famine during the Dutch Hunger Winter of 1944–1945 were found to have persistent hypomethylation of *IGF2* compared with their unexposed, same-sex siblings.[20] To our knowledge, there are no studies of the associations of early-life exposures with DNA methylation in breast tumors.

To better understand the relationship of early-life exposures with gene-specific promoter hypermethylation in breast tumor, we evaluated DNA promoter methylation status of *E-cadherin*, *p16* and *RARβ*$_2$ genes in breast tumors in a population-based study.

Materials and methods

Study population

Briefly, the Western New York Exposures and Breast Cancer Study (WEB Study) was a population-based case–control study, including female residents of Erie and Niagara counties in western New York State who were diagnosed with primary, histologically confirmed, incident breast cancer between 1996 and 2001, and were 35–79 years of age. This report includes data from cases only. Among 1638 eligible cases, 1170 (72%) participated in the study. All participants provided informed consent, and the protocol was approved by the Institutional Review Boards of all the participating institutions.

Extensive in-person interviews and self-administered questionnaires were administered to participants, including queries regarding demographic factors, family history of breast cancer, medical history, alcohol drinking and smoking history, physical activity and other breast cancer risk factors. Information on menstrual and reproductive history included age at menarche, outcome and duration of each pregnancy and age at each live birth. Participants were also queried regarding their birth weight and whether they were breastfed by their mothers or not. In addition, participants were asked to recall their body weight for each decade of their lives from age 20 until 12–24 months before diagnosis for cases. Current height was measured by trained interviewers according to a standardized protocol. Participants provided information on the age of their mothers at the time of their birth and on their place in the birth order in their families. Data included information on menstruation and menopausal status. Women were considered postmenopausal if their menses had ceased permanently and naturally, or if they had undergone any of the following conditions: a bilateral oophorectomy, a hysterectomy without removal of the ovaries and were older than 50 or radiation or other medical treatment, which resulted in permanent cessation of their menses and were older than 55.

Information on tumor size, histological grade and cancer stage was abstracted from medical records by trained research nurses using a standardized protocol. Estrogen receptor (ER) status was determined by a single pathologist, by immunohistochemical analysis as described previously.[21]

Tumor block promoter methylation determination

Archived tumor blocks were successfully obtained from 920 (78.6%) of all participant breast cancer cases. Tumor samples were microdissected from fixed microscope slides. Bisulfite modification was performed on 2 μg of tumor DNA isolated from the dissected tissue in accordance with methods described elsewhere.[21,22] Promoter methylation of *E-cadherin*, *p16* and *RAR-β*$_2$ was determined by the fluorescence-based version of methylation-specific PCR using real-time PCR amplification of bisulfite converted DNA in an ABI 7900HT real-time PCR system as previously described.[21,22] Briefly, each reaction contained 5 μl of Taqman Universal Master Mix, 4.5 μl of bisulfite-treated DNA and 0.5 μl of a 60× assay by design premix containing the primers and probes that were designed for each respective gene (Applied Biosystems, Carlsbad, CA, USA); primers and probes sequences were published previously.[21] As a control to check for modified viable DNA, we used an assay for the *ACTB* gene with primers and probes specifically designed for CpG-free sites within the gene sequence, thus amplifying the modified DNA regardless of the methylation status. If the *ACTB* result was negative (i.e. no amplification signal was detected), the DNA was not used in subsequent assays, and remodification was attempted; the other three genes were assayed only if *ACTB* was positive. Each individual DNA sample was assayed in triplicate for each gene for quality control purposes. In addition, as a positive control, universally methylated DNA (CpGenome; Norcross, GA, USA) was used along with water blanks as a negative control. We had successful promoter methylation results for 803 cases.

Statistical analysis

Early-life characteristics of participating cases with and without promoter methylation of each specific gene were compared using ANOVA for continuous variables and the χ^2-test for categorical variables. Birth weight was classified into three categories: $<$2.5, 2.6–3.9 and $>$3.9 kg. Maternal age at delivery was categorized into three groups: $<$25, 25–30 and $\geqslant$30 years. Tertile distributions among cases were used to categorize data on age at menarche and adult height. Age at first birth was classified into three groups: $<$23, 23–30 and $\geqslant$30 years among parous cases. Weight at 20 years of age was divided into two groups with a cutoff based on the median in controls.

Unconditional logistic regression was used for case–case comparisons of those with and without promoter methylation

to evaluate odds ratios (ORs) and 95% confidence intervals (95% CIs) for the associations of early-life exposures with the likelihood of promoter methylation. All analyses were adjusted by age, education level and race. Because we had previously found an association of methylation with ER status,[21] we also adjusted for ER status. Potential confounding effect by PR status and other known risk factors of breast cancer, including family history of breast cancer, were further examined, and no appreciable confounding was observed. We also found that maternal age at delivery did not modify the effect of both birth weight and birth order, and adjusting it categorically produced similar results. Those results are not shown. We evaluated associations stratifying on menopausal status. All statistical tests were based on two-sided probability. Statistical analyses were performed using SAS, version 9.2 (SAS Institute, Cary, NC, USA).

Results

We previously reported case–control associations between early-life exposures and breast cancer.[10] Analyses here were limited to case–case comparisons in relation to DNA methylation.

Demographic characteristics of cases with and without promoter methylation of *E-cadherin*, *p16* and *RAR-β_2* genes have been shown in detail elsewhere.[22] Briefly, there were no differences in the methylation frequency by age at diagnosis, race, education, menopausal status, age at menopause and recent BMI. Table 1 shows comparisons of cases with and without promoter methylation of the three genes for early-life exposure factors. After adjusting for current age, cases with promoter methylation of *E-cadherin* gene were of younger age at first birth than those without methylation of this gene.

Table 1. *Descriptive characteristics of breast cancer cases and controls by hypermethylation status of* E-cadherin, p16 *and* RAR-β_2, *WEB Study 1996–2001*[a]

	E-cadherin		*p16*		*RAR-β_2*	
	M ($n = 161$)	UM	M ($n = 208$)	UM	M ($n = 221$)	UM
Age (years)	58.0 ± 11.8	57.4 ± 11.2	58.0 ± 11.2	57.4 ± 11.3	57.4 ± 11.3	57.6 ± 11.3
Postmenopausal	111 (68.9%)	455 (70.9%)	147 (70.7%)	419 (70.4%)	157 (71.0%)	409 (70.3%)
Early-life exposure factors[b]						
Birth weight (kg)						
≤2.5 (below average)	21 (13.9%)	75 (12.6%)	19 (9.9%)	77 (13.9%)	25 (12.6%)	71 (13.0%)
2.6–3.9 (average)	110 (72.9%)	446 (75.0%)	152 (79.6%)	404 (72.8%)	145 (72.9%)	411 (75.1%)
>3.9 (above average)	20 (13.2%)	74 (12.4%)	20 (10.5%)	74 (13.3%)	29 (14.5%)	65 (11.95)
Birth order						
First born	52 (32.5%)	222 (35.0%)	61 (29.6%)	213 (36.2%)	78 (35.8%)	196 (34.0%)
Other than first born	108 (67.5%)	412 (65.0%)	145 (70.4%)	375 (63.8%)	140 (64.2%)	380 (66.0%)
Having been breastfed						
Yes	62 (48.1%)	251 (49.2%)	81 (49.1%)	232 (48.9%)	76 (42.9%)	237 (51.3%)
No	67 (51.9%)	259 (50.8%)	84 (50.9%)	242 (51.1%)	101 (57.1%)	225 (48.7%)
Maternal age at delivery (years)						
<25	55 (36.7%)	251 (41.0%)	76 (39.0%)	230 (40.5%)	81 (39.1%)	225 (40.4%)
25–30	48 (32.0%)	184 (30.0%)	58 (29.7%)	174 (30.6%)	71 (34.3%)	161 (29.0%)
>30	47 (31.3%)	178 (29.0%)	61 (31.3%)	164 (28.9%)	55 (26.6%)	170 (30.6%)
Age at menarche (year)	12.5 ± 1.5	12.6 ± 1.6	12.6 ± 1.6	12.6 ± 1.6	12.6 ± 1.5	12.5 ± 1.6
Age at first live birth (year)[c]	23.4 ± 4.9*	24.5 ± 4.8	24.0 ± 4.7	24.4 ± 4.9	24.0 ± 4.5	24.3 ± 5.0
Nulliparous women	30 (18.6%)	111 (17.3%)	42 (20.2%)	99 (16.7%)	33 (14.9%)	108 (18.6%)
Parity[c]	2.9 ± 1.5	2.8 ± 1.4	3.0 ± 1.5	2.8 ± 1.4	2.9 ± 1.5	2.8 ± 1.4
Adult height (m)	1.62 ± 0.07	1.62 ± 0.07	1.63 ± 0.06	1.62 ± 0.07	1.63 ± 0.06	1.62 ± 0.07
Weight at 20 years (kg)	55.2 ± 8.8	55.3 ± 9.3	55.2 ± 9.0	55.3 ± 9.2	56.1 ± 10.7	54.5 ± 8.5

WEB Study, Western New York Exposures and Breast Cancer Study; M, methylated; UM, unmethylated.

Data are shown as Means ± S.D. (continuous variables) or *n* (%) (categorical variables).

All continuous variables were analyzed with ANOVA tests, and all categorical variables were analyzed with χ^2-tests.

[a] Subjects with missing values were excluded from the analysis.

[b] Comparisons of early-life exposure factors between participating cases with and without promoter methylation of specific gene were adjusted for age.

[c] Among parous women.

*Comparison of methylated with unmethylated cases, $P < 0.05$.

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There were no differences in other early-life exposure factors comparing cases with or without methylation of *E-cadherin*, *p16* or *RAR-β₂* genes.

Likelihood of promoter methylation in premenopausal breast tumors by early-life exposures are shown in Table 2. There was increased likelihood of tumors with *E-cadherin* methylation among those with lower birth weight (⩽2.5 kg) compared with average birth weight (2.6–3.9 kg; OR = 2.79, 95% CI, 1.15–6.82). Compared with those who had been breastfed, there was increased likelihood of methylation for *p16* gene in premenopausal tumors of cases who had not been breastfed (OR = 2.75, 95% CI, 1.14–6.62). There was a reduction of likelihood of *p16* methylation among participants reporting maternal age at delivery between 25 and 30 years compared with those with earlier maternal age at delivery (OR = 0.41, 95% CI, 0.18–0.90). In addition, adult height was associated with increased likelihood of *RAR-β₂* methylation, the adjusted OR and 95% CI for the comparison of the highest compared with the lowest tertile was 3.34 (95% CI, 1.19–9.39). There was no association of birth order of the participant in her family, her own age at menarche, age of first live birth or weight at age 20 with the likelihood of promoter methylation of *E-cadherin*, *p16* or *RAR-β₂* gene in premenopausal breast tumors.

Table 3 shows results among postmenopausal women. There was greater likelihood of *p16* promoter methylation associated with being other than first born in the birth order (OR = 1.78, 95% CI, 1.17–2.70). Age at first live birth between 23 and 29 years was associated with reduced likelihood of *E-cadherin* methylation, whereas age at first live birth ⩾30 years was not associated with likelihood *E-cadherin* methylation. There was no association of the likelihood of

Table 2. *Association between early-life exposures and promoter methylation of* E-cadherin, p16 *and* RAR-β₂ *genes in breast tumors among premenopausal women: case–case comparisons*

	E-cadherin			*p16*			*RAR-β₂*		
	M	UM	OR (95% CI)[a]	M	UM	OR (95% CI)[a]	M	UM	OR (95% CI)[a]
Birth weight (kg)									
⩽2.5	10	18	2.79 (1.15–6.82)	6	22	0.70 (0.27–1.85)	7	21	1.00 (0.39–2.57)
2.6–3.9	28	135	1.0	45	118	1.0	42	121	1.0
>3.9	9	26	1.69 (0.70–4.05)	8	27	0.79 (0.33–1.88)	12	23	1.61 (0.72–3.60)
Birth order									
First born	14	49	1.0	20	43	1.0	23	40	1.0
Other than first born	35	134	0.83 (0.40–1.69)	40	129	0.68 (0.35–1.30)	40	129	0.52 (0.27–1.02)
Maternal age at delivery (years)									
<25	15	70	1.0	27	58	1.0	22	63	1.0
25–30	18	53	1.85 (0.81–4.20)	12	59	0.41 (0.18–0.90)	18	53	0.76 (0.35–1.63)
>30	13	54	1.25 (0.54–2.89)	19	48	0.82 (0.41–1.67)	20	47	1.21 (0.58–2.49)
Having been breastfed									
Yes	9	43	1.0	9	43	1.0	12	40	1.0
No	34	119	1.21 (0.50–2.93)	45	108	2.75 (1.14–6.62)	47	106	1.18 (0.53–2.62)
Age at menarche (years)									
<12	11	48	0.81 (0.37–1.81)	18	41	1.66 (0.81–3.38)	15	44	0.83 (0.40–1.75)
12–13	27	95	1.0	27	95	1.0	32	90	1.0
>13	12	44	0.97 (0.44–2.14)	16	40	1.42 (0.68–2.96)	17	39	1.28 (0.62–2.64)
Age at first live birth (years)									
<23	18	51	1.0	16	53	1.0	20	49	1.0
23–29	20	70	0.92 (0.42–2.00)	23	67	0.81 (0.41–1.61)	23	67	0.63 (0.30–1.33)
⩾30	5	34	0.48 (0.15–1.50)	11	28	1.28 (0.61–2.66)	11	28	0.80 (0.31–2.04)
Adult height (m)									
<1.60	10	40	1.0[b]	11	39	1.0[b]	7	43	1.0[b]
1.60–1.64	16	62	0.94 (0.37–2.37)	19	59	1.11 (0.46–2.68)	23	55	3.00 (1.13–7.97)
⩾1.65	24	85	1.04 (0.40–2.74)	31	78	1.39 (0.56–3.46)	34	75	3.34 (1.19–9.39)
Weight at 20 years (kg)									
<54.4	22	79	1.0	23	78	1.0	27	74	1.0
⩾54.4	28	108	0.97 (0.51–1.85)	38	98	1.29 (0.71–2.37)	37	99	1.08 (0.59–1.96)

M, methylated; UM, unmethylated; OR, odds ratio; CI, confidence interval; ER, estrogen receptor.

[a] ORs and 95% CIs adjusted for age, race, education and ER status.

[b] ORs and 95% CIs adjusted for age, race, education, ER status, weight at 20 years, weight 2 years ago and maternal height.

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Table 3. *Association between early-life exposures and promoter methylation of* E-cadherin, p16 *and* RAR-β_2 *genes in breast tumors among postmenopausal women: case–case comparisons*

	E-cadherin			*p16*			*RAR-β_2*		
	M	UM	OR (95% CI)[a]	M	UM	OR (95% CI)[a]	M	UM	OR (95% CI)[a]
Birth weight (kg)									
⩽2.5	11	57	0.77 (0.38–1.54)	13	55	0.66 (0.34–1.26)	18	50	1.03 (0.57–1.85)
2.6–3.9	82	311	1.0	107	286	1.0	103	290	1.0
>3.9	11	48	0.86 (0.42–1.73)	12	47	0.68 (0.35–1.34)	17	42	1.20 (0.65–2.22)
Birth order									
First born	38	173	1.0	41	170	1.0	55	156	1.0
Other than first born	73	278	1.18 (0.76–1.83)	105	246	1.78 (1.17–2.70)	100	251	1.14 (0.77–1.69)
Maternal age at delivery (years)									
<25	40	181	1.0	49	172	1.0	59	162	1.0
25–30	30	131	1.02 (0.60–1.73)	46	115	1.37 (0.86–2.20)	53	108	1.35 (0.86–2.11)
>30	34	124	1.20 (0.72–2.02)	42	116	1.26 (0.78–2.04)	35	123	0.80 (0.49–1.29)
Having been breastfed									
Yes	53	208	1.0	72	189	1.0	64	197	1.0
No	33	140	1.06 (0.64–1.77)	39	134	0.79 (0.49–1.26)	54	119	1.30 (0.83–2.04)
Age at menarche (years)									
<12	25	103	0.94 (0.56–1.58)	32	96	0.93 (0.58–1.49)	33	95	0.88 (0.55–1.40)
12–13	64	249	1.0	85	228	1.0	89	224	1.0
>13	22	103	0.77 (0.45–1.33)	30	95	0.83 (0.51–1.35)	35	90	1.00 (0.63–1.60)
Age at first live birth (years)									
<23	49	163	1.0	59	153	1.0	62	150	1.0
23–29	31	169	0.56 (0.33–0.95)	49	151	0.89 (0.56–1.42)	58	142	1.00 (0.64–1.56)
⩾30	8	44	0.56 (0.24–1.28)	8	44	0.51 (0.22–1.16)	14	38	0.90 (0.45–1.81)
Adult height (m)									
<1.60	40	156	1.0[b]	49	147	1.0[b]	60	136	1.0[b]
1.60–1.64	32	152	0.94 (0.55–1.61)	50	134	1.18 (0.73–1.91)	48	136	0.78 (0.49–1.26)
⩾1.65	39	147	1.24 (0.71–2.18)	48	138	1.17 (0.70–1.98)	49	137	0.71 (0.43–1.17)
Weight at 20 years (kg)									
<54.4	53	219	1.0	70	202	1.0	76	196	1.0
⩾54.4	58	236	1.04 (0.68–1.58)	77	217	1.08 (0.74–1.58)	81	213	0.97 (0.67–1.41)

M, methylated; UM, unmethylated; OR, odds ratio; CI, confidence interval; ER, estrogen receptor.
[a] ORs and 95% CIs adjusted for age, race, education and ER status.
[b] ORs and 95% CIs adjusted for age, race, education, ER status, weight at 20 years, weight 2 years ago and maternal height.

RAR-β_2 methylation with any of the early-life exposures; case–case comparisons were generally close to the null.

In addition, we investigated the associations of those early-life exposure factors with the likelihood of promoter methylation in at least one of the three genes in tumors stratified on menopausal status (Table 4). Among premenopausal cases, adult height was associated with increased likelihood of tumors with promoter methylation of at least one gene (OR = 2.88, 95% CI, 1.25–6.61, for the highest *v.* lowest tertile). Among premenopausal cases, being other than first born was associated with reduced likelihood of promoter methylation of at least one gene (OR = 0.38, 95% CI, 0.19–0.77); although a positive association between birth order and likelihood of promoter methylation in at least one gene (OR = 1.77, 95% CI, 1.23–2.55, for being other than first born compared with first born) was observed among postmenopausal breast cases. In addition, among postmenopausal breast cases, there was an inverse association between age at first live birth and likelihood of promoter methylation in at least one gene (OR = 0.51, 95% CI, 0.27–0.97, for age at first live birth ⩾30 *v.* <23 years). Other early-life exposures were not associated with the likelihood of promoter methylation in at least one of the three genes in tumors in both premenopausal and postmenopausal women.

Discussion

To our knowledge, this study is the first to assess associations of early-life exposures with promoter methylation in breast tumors in a large population-based study. We found positive associations between lower birth weight and *E-cadherin* promoter methylation, between not having been breastfed

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Table 4. *Case–case comparisons of early-life exposure and promoter methylation of at least one gene in breast tumors across menopausal status*

	Any *v.* none			Any *v.* none		
	Any one	None	OR (95% CI)[a]	Any one	None	OR (95% CI)[a]
	Premenopausal			Postmenopausal		
Birth weight (kg)						
⩽2.5 (below average)	20	8	1.47 (0.60–3.57)	39	59	0.87 (0.40–1.15)
2.6–3.9 (average)	104	89	1.0	267	126	1.0
>3.9 (above average)	28	7	2.31 (0.94–5.66)	37	22	0.82 (0.46–1.47)
Birth order						
First born	51	12	1.0	124	87	1.0
Other than first born	106	63	0.38 (0.19–0.77)	250	101	1.77 (1.23–2.55)
Maternal age at delivery (years)						
<25	60	25	1.0	135	86	1.0
25–30	45	26	0.67 (0.33–1.36)	116	45	1.59 (1.02–2.47)
>30	45	22	0.84 (0.42–1.69)	104	54	1.21 (0.79–1.87)
Having been breastfed						
Yes	30	22	1.0	172	89	1.0
No	112	41	1.87 (0.91–3.83)	115	58	1.02 (0.67–1.57)
Age at menarche (years)						
<12	40	19	1.15 (0.59–2.25)	85	43	0.95 (0.61–1.47)
12–13	78	44	1.0	213	100	1.0
>13	42	14	1.78 (0.86–3.67)	79	16	0.78 (0.50–1.21)
Age at first live birth (years)						
<23	47	22	1.0	152	60	1.0
23–29	60	30	0.93 (0.46–1.88)	126	74	0.68 (0.44–1.05)
⩾30	26	13	0.97 (0.40–2.35)	29	23	0.51 (0.27–0.97)
Adult height (m)						
<1.60	27	23	1.0[b]	136	60	1.0[b]
1.60–1.64	51	27	1.69 (0.79–3.63)	119	65	0.84 (0.53–1.32)
⩾1.65	82	27	2.88 (1.25–6.61)	122	64	0.83 (0.51–1.34)
Weight at 20 years (kg)						
<54.4	65	34	1.0	179	93	1.0
⩾54.4	95	43	1.17 (0.67–2.05)	198	96	1.08 (0.76–1.54)

OR, odds ratio; CI, confidence interval; ER, estrogen receptor.
[a] ORs and 95% CIs adjusted for age, race, education and ER status.
[b] ORs and 95% CIs adjusted for age, race, education, ER status, weight at 20 years, weight 2 years ago and maternal height.

and *p16* promoter methylation and between adult height and *RAR-β_2* methylation in premenopausal breast tumors. Among postmenopausal cases, being other than first born was positively associated with the likelihood of *p16* promoter methylation. The likelihood of promoter methylation of the individual genes, *E-cadherin*, *p16* or *RAR-β_2* did not differ by maternal age at delivery, age at menarche, age of first live birth and weight at 20 years of age in either strata of menopause. Although none of the exposures we studied was consistently associated with alterations of all three of the genes studied, our findings suggested changes in methylation for individual genes in association with particular exposures.

Unlike most organs where most development occurs during embryogenesis and the early postnatal period, development of the breast occurs from the *in utero* period, to infancy through puberty;[2] breast tissue is not fully differentiated until after the first full-term pregnancy.[2,3] Therefore, prenatal and early-life exposures might influence susceptibility to breast cancer in adulthood. Most previous studies found a positive association between higher than average birth weight and risk of pre-menopausal breast cancer.[4–6,9] However, results from several studies also suggested increased breast cancer risk associated with lower than average birth weight (<2.5 kg or 5.5 pounds), particularly in young women.[4,23,24] Birth weight, a proxy for intrauterine environment, has been linked to maternal hormones and other maternal exposures, including nutrition, alcohol and tobacco smoking.

There is evidence that early-life exposures may have an impact on methylation in tissues. Recent studies reported a significant correlation between global *LINE-1* methylation in cord blood and low birth weight.[25] Maternal smoking during pregnancy is strongly associated with increased risk of low

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birth weight[26] and has been found to be inversely associated with genomic DNA demethylation in adulthood.[27] Results from the Children's Health Study showed that children exposed to prenatal tobacco smoke had significantly increased promoter methylation of *AXL* and *PTPRO* genes.[28] Poor maternal nutrition or reduced intake of micronutrients during gestation could also contribute to low birth weight.[20,29] Animal studies have shown histone modification and aberrant DNA methylation of different genes in rats exposed to intrauterine under nutrition.[30] In the current study, we observed an association between lower birth weight and increased likelihood of *E-cadherin* promoter methylation in premenopausal breast tumors. These data support the hypothesis that prenatal environment/development may lead to aberrant DNA methylation, and further influence adult disease risk.

We found that not having been breastfed was significantly associated with increased likelihood of *p16* promoter methylation in premenopausal breast tumors. Some studies have found an inverse association between being breastfed in infancy and premenopausal breast cancer risk,[7,10,31] while others have not.[32–34] A protective effect associated with breastfeeding in infancy may be partly related to exposure to growth factors, enzymes and hormones in breast milk.[35] Early introduction of cow milk proteins (complementary feeding) has been proposed to induce intestinal mucosa inflammation and increase gut permeability.[36] Inflammation has been found to alter the DNA promoter methylation pattern and critical gene regulation in several studies.[37,38] The mechanism by which early-life exposure to breast milk and/or complementary feeding may influence breast cancer risk in adulthood is still unclear. Further studies, including animal studies, are needed to examine the association of not being breastfed in infancy with aberrant DNA methylation.

Several previous studies found inverse associations of birth order and breast cancer risk, either overall or among premenopausal women.[4,39] Hormonal levels varying according to parity may explain the potential associations; estrogen levels appear to be lower during successive pregnancies compared with first pregnancies.[4] There is growing evidence that higher estrogen or xenoestrogen exposure during early life could induce aberrant DNA methylation.[13,40] In the current study, we found reduced likelihood of promoter methylation of at least one gene with being other than first born among premenopausal women, possibly related to this difference in estrogen exposure. However, the possible explanation for different finding for postmenopausal women is not known.

Adult height is partly due to childhood nutrition and health.[41] Childhood malnutrition can influence DNA methylation, impacting nutrients involved in one-carbon metabolism.[2] Hughes *et al.*[42] found that individuals exposed to famine during early life (adolescence and early adulthood) had decreased risk of developing a CpG island methylator phenotype (CIMP) colorectal tumor later in life, and the degree of hypermethylation was inversely associated with early-life exposure to energy restriction. Height is also influenced by genetic factors.[43] We found that greater adult height was associated with increased likelihood of *RAR-β_2* promoter methylation in premenopausal tumors; findings did not appreciably change with adjustment for maternal height (data not shown).

As for any study of this kind, our results need to be considered in the context of the strengths and weaknesses of the data. The strengths of our study include the population-based study design, a relatively large sample of archived tumor tissues, measurement of anthropometric factors by trained interviewers and detailed information on possible confounders and disease characteristics. However, several limitations should be considered. Although the number of tumor blocks available for our study was large, the statistical power in some subgroups of our study remained limited, reducing our ability to identify weak associations. Another concern was that our inability to obtain paraffin-embedded tumor tissues for all breast cancer cases may have led to a selection bias; however, cases with and without breast tumor tissue had similar distributions of early-life exposure factors. In addition, there were similar distributions of early-life exposure factors between cases with tumor tissue but no methylation results and cases with tumor tissue and methylation results. There may have been errors in self-reported early-life exposures. There is evidence that there is not much bias in recall of reproductive history in case–control studies of breast cancer.[44] In our study, participant's birth certificates were also collected for 80 women as a validation of self-reported birth weight; reports by the study participants were well correlated with birth certificate data ($r = 0.62$).[10] Furthermore, our previous results on associations of breast cancer risk with perinatal exposures have been consistent with other studies.[1,10] It is unlikely that recall of early-life exposures would be related to gene promoter methylation. These case–case comparisons may be affected by misclassification but are not likely affected by biased recall. There may be confounding of other unmeasured factors. In particular, we did not have information on maternal or paternal education. Further studies in other populations are needed to confirm our findings and to elucidate the underlying biological mechanisms.

There are some concerns with limitations of the measurement of methylation. We examined methylation for three genes that are known to be frequently methylated in breast tumors and are known to be significant in three important pathways in breast carcinogenesis. However, we clearly are limited by the study of a small number of genes. Expansion of our findings to a large number of genes or imprinted genes will be important. Furthermore, the methodology used in our study was limited to the examination of a single CpG island in the promoter regions. It is assumed that these single regions are sentinels for gene silencing and methylation of other CpG islands, especially in tumors; however, it is possible that in some women, these genes are hypermethylated in CpG sequences that we did not study. Finally, we used real-time methylation-specific PCR to assess promoter methylation that can increase the specificity of the PCR by

interrogating more than one CpG. More specifically, we used a fluorescence-based version of the methylation-specific PCR technique because of its increased throughput by eliminating the need for gel electrophoresis.[45] This method has been found to be 10 times more sensitive than the classic methylation-specific PCR method and able to detect methylated sequences from an excess of 10,000-fold unmethylated alleles.[45] Although we are aware of the limitation of this technique given its qualitative nature compared with other quantitative methods, such as pyrosequencing, and the fact that it interrogates a limited number of CpG sites, we have followed stringent quality control criteria to ensure confidence in results. Moreover, recent findings showed that results from methylation-specific PCR are highly correlated with other quantitative methods. By using the highly specific real-time methylation-specific PCR, it is likely that our results would be reproduced by other methods.[46]

Results from our study support the hypothesis that perinatal and early-life exposures may affect DNA methylation, thereby influencing development of breast cancer in later life. Replication in other populations and the exploration of methylation of a larger number of sites are necessary to better understand this finding of potential significance in understanding the mechanism for exposures in early life and breast cancer risk.

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