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DNA hypermethylation and clinicopathological features in breast cancer: the Western New York Exposures and Breast Cancer (WEB) Study

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Abstract

Aberrant DNA hypermethylation of gene promoter regions has been increasingly recognized as a common molecular alteration in carcinogenesis. We evaluated the association between major clinicopathological features and hypermethylation of genes in tumors among 803 incidence breast cancer cases from a large population-based case–control study conducted in Western New York State. DNA samples were isolated from archive paraffin embedded tumor tissue and were analyzed for hypermethylation status of the *E-cadherin*, *p16*, and *RAR-β_2* genes using real time methylation-specific polymerase chain reaction. The frequencies of hypermethylation were 20.0% for *E-cadherin*, 25.9% for *p16*, and 27.5% for *RAR-β_2* genes. For postmenopausal women, hypermethylation of *E-cadherin* tended to be more likely in progesterone receptor (PR) negative than in PR-positive tumors (odds ratio (OR), 1.41; 95% confidence interval (CI), 0.91–2.18). Hypermethylation of *p16* tended to be more frequent among estrogen receptor (ER) negative cases than ER-positive cases (OR, 1.51; 95% CI, 1.01–2.32). Hypermethylation of *RAR-β_2* gene was inversely associated with histological and nuclear grade of breast cancer.

Keywords

Hypermethylation; Estrogen receptor; Progesterone receptor; Breast cancer; Epidemiology

Introduction

Aberrant DNA hypermethylation has been increasingly recognized as a frequent molecular alteration in cancer [1, 2]. This epigenetic modification occurs at the cytosines of CpG dinucleotide-rich regions, which are mostly unmethylated in normal tissues. Hypermethylation of CpG islands in gene promoter regions of many tumor suppressor and DNA repair genes is associated with chromatin condensation, delaying replication, inhibiting initiation of transcription and silencing of genes [3]. For breast tumors, there is evidence of hypermethylation of functionally important genes including those involved in DNA repair (*BRCA1*) [4], cell cycle regulation (*p16*) [3], cell adhesion (*E-cadherin)* [5], hormone and receptor-mediated cell signaling (*ER* (estrogen receptor) and RAR-β_2 (retinoic acid-binding receptor-β_2)) [6], regulation of cell transcription (*HOXA5* (homeo box A5)) [7], and other functions [6]. There are suggestions that aberrant hypermethylation may be useful as a biomarker, with implications for breast cancer etiology, diagnosis and management.

Recent studies have focused on identifying the gene-specific hypermethylation profile of different tumors [3, 8–12]. Some studies have evaluated the association between gene hypermethylation and biological or clinical properties of breast tumors [13–19]. An association between CpG island hypermethylation of *p16* and *RAR-β_2* genes and poorly differentiated breast tumors was found in two previous studies [14, 19]. Promoter hypermethylation of *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) was associated with larger tumor size and higher histologic grade [17]. Methylation of *GSTP1* (glutathione *S*-transferase π 1) and/or *RAR-β_2* was found to be associated with breast cancer cases with sentinel lymph node metastasis [15]; and *HIN-1* (high in normal-1) and *RAR-β_2* had greatly higher methylation frequencies in bone, brain, and lung metastases than the primary breast tumors [18]. Some studies reported no apparent association between methylation distribution phenotypes and tumor size, grade, stage, or lymph node status [13, 16]. Because of generally small sample sizes for existing studies, as well as differences in analytic methods and selection of genes, there still remains uncertainty regarding the relation between gene hypermethylation and breast tumor clinical characteristics.

The aim of the present study was to evaluate the association between the major clinicopathological features of breast cancer and methylation of three genes: *p16*, *E-cadherin*, and *RAR-β_2* among primary breast cancer cases from a large scale population-based case–control study. These genes are known to be important in breast cancer development and progression, and are frequently hypermethylated in breast tumors, leading to down-regulation of expression of their gene products.

Materials and methods

A population-based case–control study of breast cancer, the Western New York Exposures and Breast Cancer (WEB) Study was conducted in 1996–2001. Eligible cases were women diagnosed with primary, histologically confirmed, incident breast cancer, age 35–79, current residents of Erie or Niagara Counties in New York State, and with no previous cancer history other than nonmelanoma skin cancer. Among 1,627 eligible cases, 1,170 (72%) participated. Cases were interviewed within 1 year of diagnosis; most were interviewed within 3–6 months following diagnosis. All participants provided informed consent, and the study protocol was approved by the Institutional Review Boards of the University at Buffalo and of all the participating hospitals.

Extensive in-person interviews and self-administered questionnaires were used to ascertain information on potential confounding factors, breast cancer risk factors and anthropometric measures. 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.

The pathological diagnosis of breast cancer was reconfirmed by a senior pathologist from Georgetown University. Information on cancer diagnosis, tumor size, histologic grade, and cancer stage (as measured by tumor-node-metastasis (TNM) stage) was abstracted from

medical charts using a standard protocol. ER/PR status was determined by immunohistochemical analysis according to previous methods [20]. The 5 μm tissue were stained in DAKO Autostainer (DAKO, Carpentaria, CA) using the Dako Cytomation EnVision + system-HRP (DAB) kit. The Allred score was used to evaluate staining for ER and PR [20]. The staining signal was scored by estimating the proportion and average intensity of positive tumor cells. A proportion score (PS) ranging from 1 to 5 was assigned that represents the estimated proportion of positive tumor cells on the entire slide. An intensity score (IS) ranging from 1 to 3 was assigned that estimated the average staining intensity of positive tumor cells. The PS and IS were added to obtain a total score (TS) ranging from 2 to 8. A score of 3 or more was considered as positive, while a score of 2 or less was considered as negative. *P53* mutation were identified using Affymetrix p53 Gene Chip System (Affymetrix, Santa Clara, CA) as previously described [21]. The presence of mutation was subsequently confirmed by bidirectional sequencing using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the Mega BACE 1000 DNA Analysis System (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) according to the manufacturers' instructions.

Of the women with breast cancer in the WEB study, we was able to obtain archived tumor blocks for 922 of them. Of those we obtained, only tissue blocks with tumors were analyzed. Genomic tumor DNA isolated from tissue block was modified by bisulfite modification according to previous methods [22]. Briefly, genomic tumor DNA (2 μg in 20 μl DNA) was treated with of 3 M NaOH (2 μl) and incubated at 50°C for 20 min. Subsequently, 500 μl of the freshly prepared hydroquinone-bisulfite solution (2.5 M sodium metabisulfite, 2 M NaOH and 125 mM hydroquinone [pH 5.0]) was added to each DNA sample and placed on a 70°C heat block for 2 h in the dark. After the incubation, 1 ml of purification resin (Promega, Madison WI) was added to the DNA and subsequently processed through a minicolumn purification protocol (Promega, Madison WI) using a vacuum unit according to the manufacturer's instructions.

Hypermethylation of *E-cadherin*, *p16*, and *RAR-β_2* was determined by real time methylation-specific polymerase chain reaction (MSP). As a control to check for modified viable DNA, we used a β-actin assay. If the β-actin result was negative, the DNA could not be used in subsequent assays, and re-modification was attempted. If β-actin was positive, then the other three genes were assayed immediately. We had hypermethylation results for 803 cases. The experiments were performed using the ABI 7900HT real time PCR system as previously described [23]. For each gene, fluorogenic PCR was carried out in a 10 μl reaction volume in a 384 well optical tray (AbGene, Surrey U.K.). The sequences of the primers and probe used to amplify and detect methylation were: 5′-CGATCGTATTCGGCGTTTGTTT-3′ (forward primer), 5′-CCGAAAAACTACGACTCCAAAAACC-3′ (reverse primer), and 5′-FAM-TCGTTCGGCGTTTTC-MGB-3′ (proble) for *E-cadherin*, 5′-GAGTTTTCGGTTGATTGGTTGGTT-3′ (forward primer), 5′-GCCGCACCTCCTCTAC-3′ (reverse primer), and 5′-FAM-CCCGAACCGCGACCGT-MGB-3′ (proble) for *p16*, and 5′-GAGTTGTTTGAGGATTGGGATGTC-3′ (forward primer), 5′-ACGATACCCAAACAAACCCTACTC-3′ (reverse primer), and 5′-FAM-

ATCGCTCGCGTTCTC-MGB-3′ (proble) for *RAR-β_2*. Each reaction contained 5 μl of Taqman Universal Master Mix (2×), 4.5 μl of bisulfite treated DNA and 0.5 μl of a 60× assay by design premix that was designed for each respective gene (Applied Biosystems, Carlsbad CA). Thermal cycling started with an initial 10 min denaturation at 95°C followed by cycling of 95°C for 15 s and 60°C for 1 min. This cycling was repeated 45 times and followed with a 5 min extension at 72°C whereupon the data was analyzed [24]. Each individual DNA sample was assayed in triplicate for each gene for quality control purposes. Additionally, as a positive control, universally methylated DNA (CpGenome; Norcross, GA) was used along with water blanks as a negative control.

The χ^2 test was used to compare the distributions of the methylation status of individual genes and various features of breast cancer. Unconditional logistic regression was employed to estimate odds ratios (ORs) and 95% confidence intervals (CIs). We did a case–case comparison of those with to those without hypermethylation of a particular gene in their tumor, examining the likelihood of hypermethylation according to a tumor charateristic. All models were adjusted for age at diagnosis, education level, and race. Potential confounding effects from other demographic factors and known breast cancer risk factors, including age at menarche, age at menopause, parity, family history of breast cancer, and body mass index (BMI), were also examined and no appreciable confounding was observed. All statistical tests were based on two-sided probability. Statistical analyses were conducted using SAS Version 9.1 (SAS Institute, Cary, NC).

Results

Among women with available hypermethylation results, 736 (92.2%) cases were Caucasians and 67 (7.6%) were other racial groups including African-Americans (55), American Indian (3), Hispanic (3), Asian (2), and others (4). Approximately 70.5% of the cases were postmenopausal at diagnosis with mean age at diagnosis 57.0 years, and mean tumor size 1.8 cm. Additional primary clinical and pathologic characteristics of the women with breast cancer are shown in Table 1. The frequency of hypermethylation was 20.0% for *E-cadherin*, 25.9% for *p16*, and 27.5% for *RAR-β_2* gene (Table 2). Promoter region CpG hypermethylation for any one of the three genes was identified in 485 (60.4%) of 803 primary breast tumors. Fifty-one (6.4%) showed hypermethylation for two genes and only 1 (0.1%) tumor for all three genes. The frequencies of hypermethylation for *E-cadherin*, *p16*, and *RAR-β_2* genes were similar for pre- and postmenopausal cases.

The relationship between individual gene hypermethylation status and clinical and pathologic features of breast cancer were evaluated stratifying by menopausal status in Table 3. Associations varied somewhat by menopausal status, but tests for multiplicative interaction were not significant ($P > 0.05$). For premenopausal women, while there was no association of hypermethylation with tumors stratified on ER status or PR status, in examination of tumors with either ER− or PR-positive status there was a higher likelihood of hypermethylation of *p16* (OR, 2.21; 95% CI, 1.05–4.61), in a model adjusting for age at diagnosis, race, and education level. Among postmenopausal women, hypermethylation of *E-cadherin* tended to be more frequent among PR-negative cases (OR, 1.41, 95% CI, 0.91–2.18), although confidence intervals included the null. Patients with ER-negative tumors

were more likely to have hypermethylation of the *p16* gene (OR, 1.51, 95% CI, 1.01–2.32). No associations were observed for tumors that were both ER-negative and PR-negative and the hypermethylation of any gene. Histological grade was inversely associated with hypermethylation of *RAR-β_2* gene (OR, 0.59 and 0.69; 95% CI, 0.37–0.94 and 0.44–1.07, for moderate and poorly differentiated tumors, respectively); further tumors with poorer nuclear grade were inversely associated with hypermethylation of *RAR-β_2* gene. We observed no associations between hypermethylation of *E-cadherin*, *p16*, and *RAR-β_2* with tumor size, stage, metastases, or *p53* mutation among both pre- and postmenopausal women.

We also evaluated hypermethylation in the three genes together (Table 4). There was a tendency for tumors with hypermethylation in at least one gene to be more likely to be PR-negative among postmenopausal women (OR, 1.39; 95% CI, 0.95–2.04), although the confidence interval include the null. Among premenopausal women, nodal invasion or metastatsis was associated with hypermethylation in at least one gene. No associations were observed between other clinicopathologic features of breast cancer and hypermethylation in at least one gene.

We analyzed the data among Caucasians alone; and the estimates and the confidence intervals were similar to results in the overall population (data not shown).

Discussion

To better understand the role of promoter hypermethylation status in the natural history of breast carcinogenesis and as a molecular predictor of disease progression, we evaluated the association between gene hypermethylation and clinicopathological characteristics of primary breast tumors. Promoter methylation for at least one gene was found in 66.9% of the breast tumors. Hypermethylation frequencies of individual genes reported in previous studies on breast cancer have varied widely. Frequencies for *p16* and *RAR-β_2* genes in our sample were similar to those reported previously [14]. However, the frequency for *E-cadherin* hypermethylation (20.0%) was lower than other reports; in those the frequency of *E-cadherin* hypermethylation ranged between 39 and 80% [14–16]. This variation may depend on the sensitivity of the MSP assay, differences in MSP assay design, and by sample size or other differences in the populations under study. In our study, we used the same assay conditions for each tumor DNA sample and positive and negative internal controls; our MSP analysis was reliable.

Encoded by the *E-cadherin* gene, the transmembrane glycoprotein *E-cadherin* is involved in maintaining homotypic cell-to-cell adhesion of differentiated epithelial tissues. Loss of *E-cadherin* expression has been related to loss of differentiation, increased invasiveness, and decreased patient survival [25, 26]. Although mutations and deletions in *E-cadherin* gene have been reported in cancers including lobular breast carcinoma [27–29], in most breast carcinomas, *E-cadherin* mutations have been found to be rare or absent. Promoter hypermethylation of the gene might play a role in alteration of *E-cadherin* expression. Hypermethylation of the *E-cadherin* promoter has been shown to be associated with loss of *E-cadherin* expression in breast cancer cell lines and primary ductal and lobular breast cancers [25, 30–32]. Previous studies have found a correlation between reduced *E-cadherin*

expression and loss of ER and PR [33, 34]. Consistent with those investigations, a positive association between hypermethylation of *E-cadherin* gene and negative PR status was observed in our population. We did not see any association of *E-cadherin* hypermethylation with stage or metastasis.

The *p16* gene, one of the most commonly inactivated tumor suppressor genes in human cancer [35], is a cyclin-dependent kinase inhibitor that regulates progression through the G_1 phase of the cell-cycle [35]. Down-regulation of *p16* expression caused by promoter hypermethylation occurs frequently in breast cancer. There is evidence that *p16* hypermethylation is an early and likely critical step in breast cancer development [36, 37]. We found some tendency for hypermethylation in *p16* to occur more often in ER-negative cancer patients than ER-positive among postmenopausal women. We did not observe associations of *p16* hypermethylation with other clinic or pathological features of breast cancer.

The protein coded by *RAR-β_2* functions in inhibition of proliferation, apoptosis, and senescence. The gene is methylated frequently in breast cancer [14, 15, 18] and even normal breast tissue [38], which may result in loss of expression and a loss of control of cellular proliferation. Unlike previous studies, in which there was found a positive association between *RAR-β_2* hypermethylation and metastasis in breast tumor [15, 18], we found no associations between hypermethylation of *RAR-β_2* gene and metastasis and lymphovascular invasion. However, we found inverse associations between histological and nuclear grade and *RAR-β_2* hypermethylation among postmenopausal women.

The strengths of this study include the population-based study design and a relatively large sample size, leading to relatively stable risk estimates. Nevertheless, the statistical power in subgroups of our study remained limited due to the low frequencies of the hypermethylation, which limited our ability to identify weak associations. We cannot rule out the possibility that hypermethylation of genes other than those included in our study may be related to clinicopathological features of breast cancer. Further our inability to obtain the paraffin embedded breast tumor tissue for 24.7% of cases may have led to selection bias. Comparing with cases without breast tumor tissue, cases with breast tumor tissue had slightly younger age at diagnosis and higher TNM stage of breast tumor. However, they had similar tumor size, frequencies of histological grade, nuclear grade, ER and PR status.

In summary, our data suggest that *E-cadherin* hypermethylation is associated with higher histological grade and PR-negative tumors, *p16* hypermethylation is associated with ER-negative tumors, and *RAR-β_2* hypermethylation is inversely related to histological and nuclear grade. Although there appears to be little support from results for a distinctive promoter hypermethylation phenotype in breast cancer, further research is needed to assess the association of these characteristics of tumors with other breast cancer risk factors to better understand their etiology.

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Table 1

Hypermethylation status of *E-cadherin*, *p16*, and *RAR-β_2* for pre- and postmenopausal women, WEB Study, 1996–2001[a]

Clinicopathologic factors	Premenopausal wemen						Postmenopausal women					
	E-cadherin		*p16*		*RAR-β_2*		*E-cadherin*		*p16*		*RAR-β_2*	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Age at diagnosis (yr)												
< 50	45	164	49	160	58	151	8	30	4	34	14	24
50–59	5	23	12	16	6	22	31	150	48	133	50	131
60–69	–	–	–	–	–	–	38	169	60	147	56	151
≥70	–	–	–	–	–	–	34	106	35	105	37	103
P-value		0.65		0.03		0.48		0.41		0.12		0.63
Education (yr)												
< 12	3	5	2	6	2	6	10	36	13	33	13	33
12	18	49	13	54	21	46	46	187	61	172	68	165
≥12	29	133	46	116	41	121	55	232	73	214	76	211
P-value		0.16		0.37		0.64		0.92		0.92		0.79
Tumor size (cm)[b]												
< 1.0 (0.9)	5	28	11	22	9	24	29	84	25	88	39	74
1.0–1.7 (0.9–1.4)	15	56	14	57	18	53	22	120	40	102	38	104
1.8–2.6 (1.5–1.9)	19	36	11	44	16	39	22	69	27	64	22	69
≥2.7 (2.0)	7	48	15	40	14	41	28	138	42	124	46	120
P-value		0.03		0.38		0.96		0.11		0.59		0.37
Metastatic disease at presentation												
No	28	120	37	111	39	109	87	318	109	296	107	298
Yes	19	62	21	60	23	58	19	107	27	99	39	87
P-value		0.42		0.88		0.74		0.11		0.22		0.32
Vascular/lymph invasion												
No	30	117	35	112	39	108	78	309	105	282	102	285
Yes	18	64	22	60	23	58	21	110	31	100	39	92
P-value		0.78		0.61		0.66		0.30		0.43		0.45
Histological grade												

Clinicopathologic factors	Premenopausal wemen						Postmenopausal women					
	E-cadherin		*p16*		*RAR-β2*		*E-cadherin*		*p16*		*RAR-β2*	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Well	3	16	0	19	8	11	13	50	19	44	20	43
Moderate	15	51	19	47	20	46	36	138	46	128	40	134
Poor	26	84	26	84	24	86	44	159	55	148	53	150
P-value		0.75		0.03		0.13		0.97		0.85		0.39
Nuclear grade												
I	5	25	9	21	9	21	24	91	29	86	33	82
II	19	56	18	57	22	53	41	149	49	141	47	143
III	25	89	27	87	27	87	40	155	52	143	50	145
P-value		0.62		0.76		0.62		0.97		0.96		0.74
TNM stage												
0	7	22	7	22	11	18	10	52	14	48	17	45
I	15	65	21	59	20	60	53	202	72	183	67	188
II_a/II_b	21	67	17	71	20	68	26	128	40	114	48	106
III/IV	2	21	9	14	10	13	4	20	2	22	8	16
P-value		0.40		0.25		0.12		0.71		0.18		0.69
ER status												
+	28	112	36	104	43	97	81	310	92	299	111	280
−	16	64	22	58	16	64	29	124	48	105	39	114
P-value		1.0		0.77		0.08		0.64		0.06		0.50
PR status												
+	28	120	40	108	39	109	59	266	78	247	94	231
−	16	51	15	52	19	48	46	156	56	146	53	149
P-value		0.40		0.47		0.76		0.20		0.34		0.50
ER/PR												
Both +	23	98	29	92	36	85	56	240	70	226	86	210
Either +	10	32	17	25	8	34	22	85	27	80	29	78
Both −	11	39	9	41	13	37	26	94	37	83	30	90
P-value		0.77		0.04		0.40		0.80		0.31		0.70
p53 mutation												

Clinicopathologic factors	Premenopausal wemen						Postmenopausal women					
	E-cadherin		*p16*		*RAR-β2*		*E-cadherin*		*p16*		*RAR-β2*	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Wild	30	119	38	111	40	109	66	276	96	246	92	250
Mutant	15	49	16	48	17	47	25	104	34	95	38	91
P-value		0.59		0.94		0.97		0.98		0.71		0.58

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

[b] Cut-off point of tumor size among postmenopausal women

Table 2

Frequencies (%) of hypermethylated genes, WEB Study, 1996–2001

Genes	Overall Methylated cases (%)	Premenopausal Methylated cases (%)	Postmenopausal Methylated cases (%)
E-cadherin	161 (20.0)	50 (21.1)	111 (19.6)
p16	208 (25.9)	61 (25.7)	147 (26.0)
RAR-ß2	221 (27.5)	64 (27.0)	157 (27.7)
None	266 (33.1)	77 (32.5)	189 (33.4)
Any one	485 (60.4)	146 (61.6)	339 (59.9)
Any two	51 (6.4)	13 (5.5)	38 (6.7)
Three	1 (0.1)	1 (0.4)	0 (0.0)

Table 3

Association of *E-cadherin*, *p16*, and *RAR-β2* hypermethylation with selected clinicopathological factors for breast cancer, WEB Study, 1996–2001[a]

	E-cadherin			*p16*			*RAR-β2*		
	Yes	**No**	**OR[b] (95% CI)**	**Yes**	**No**	**OR[b] (95% CI)**	**Yes**	**No**	**OR[b] (95% CI)**
Pre-menopausal									
Tumor size (cm)									
1.0	5	28	1.0	11	22	1.0	9	24	1.0
1.0–1.7	15	56	1.43 (0.57–3.62)	14	57	0.37 (0.16–0.84)	18	53	0.97 (0.43–2.15)
1.8–2.6	19	36	2.65 (1.06–6.62)	11	44	0.41 (0.17–0.97)	16	39	1.06 (0.46–2.45)
≥2.7	7	48	0.76 (0.26–2.23)	15	40	0.59 (0.26–1.34)	14	41	0.94 (0.40–2.19)
Metastatic disease at presentation									
No	28	120	1.0	37	111	1.0	39	109	1.0
Yes	19	62	1.35 (0.69–2.63)	21	60	1.02 (0.55–1.91)	23	58	1.20 (0.65–2.23)
Vascular/lymph invasion									
No	30	117	1.0	35	112	1.0	39	108	1.0
Yes	18	64	1.12 (0.57–2.18)	22	60	1.17 (0.63–2.18)	23	58	1.19 (0.64–2.18)
Histological grade									
Well	3	16	1.0	0	19		8	11	1.0
Moderate	15	51	1.61 (0.64–4.04)	19	47		20	46	0.89 (0.42–1.91)
Poor	26	84	1.74 (0.75–4.06)	26	84		24	86	0.61 (0.30–1.25)
Nuclear grade									
I	5	25	1.0	9	21	1.0	9	21	1.0
II	19	56	2.28 (0.83–6.24)	18	57	0.64 (0.29–1.44)	22	53	0.88 (0.40–1.95)
III	25	89	1.78 (0.67–4.73)	27	87	0.65 (0.31–1.38)	27	87	0.67 (0.31–1.45)
TNM stage									
0	7	22	1.0	7	22	1.0	11	18	1.0
I	15	65	0.67 (0.28–1.62)	21	59	0.77 (0.34–1.74)	20	60	0.81 (0.36–1.84)
II_a/II_b	21	67	0.94 (0.41–2.17)	17	71	0.52 (0.23–1.19)	20	68	0.70 (0.31–1.58)
III/IV	2	21	0.25 (0.05–1.26)	9	14	1.52 (0.52–4.39)	10	13	2.03 (0.70–5.91)
ER status									
+	28	112	1.0	36	104	1.0	43	97	1.0

	E-cadherin			*p16*			*RAR-β₂*		
	Yes	**No**	**OR[b] (95% CI)**	**Yes**	**No**	**OR[b] (95% CI)**	**Yes**	**No**	**OR[b] (95% CI)**
−	16	64	1.06 (0.52–2.13)	22	58	1.07 (0.57–2.02)	16	64	0.62 (0.32–1.21)
PR status									
+	28	120	1.0	40	108	1.0	39	109	1.0
−	16	51	1.42 (0.69–2.90)	15	52	0.74 (0.37–1.50)	19	48	1.26 (0.65–2.44)
ER/PR									
Both +	23	98	1.0	29	92	1.0	36	85	1.0
Either +	10	32	1.34 (0.58–3.09)	17	25	2.21 (1.05–4.61)	8	34	0.54 (0.23–1.28)
Both −	11	39	1.08 (0.48–2.44)	9	41	0.64 (0.28–1.49)	13	37	1.00 (0.47–2.12)
p53 mutation									
Wild	30	119	1.0	38	111	1.0	40	109	1.0
Mutant	15	49	1.15 (0.56–2.34)	16	48	1.0 (0.50–1.98)	17	47	1.02 (0.52–2.00)
Postmenopausal									
Tumor size (cm)									
< 0.9	29	84	1.0	25	88	1.0	39	74	1.0
0.9–1.4	22	120	0.59 (0.33–1.06)	40	102	1.32 (0.79–2.21)	38	104	0.83 (0.51–1.37)
1.5–1.9	22	69	1.04 (0.57–1.90)	27	64	1.43 (0.81–2.56)	22	69	0.73 (0.41–1.30)
≥2.0	28	138	0.67 (0.39–1.16)	42	124	1.17 (0.70–1.94)	46	120	0.88 (0.55–1.41)
Metastatic disease at presentation									
No	87	318	1.0	109	296	1.0	107	298	1.0
Yes	19	107	0.67 (0.39–1.15)	27	99	0.75 (0.46–1.21)	39	87	1.24 (0.80–1.93)
Vascular/lymph invasion									
No	78	309	1.0	105	282	1.0	102	285	1.0
Yes	21	110	0.77 (0.45–1.31)	31	100	0.84 (0.53–1.33)	39	92	1.18 (0.76–1.83)
Histological grade									
Well	13	50	1.0	19	44	1.0	20	43	1.0
Moderate	36	138	1.32 (0.77–2.25)	46	128	1.12 (0.70–1.80)	40	134	0.59 (0.37–0.94)
Poor	44	159	1.43 (0.86–2.40)	55	148	1.18 (0.74–1.86)	53	150	0.69 (0.44–1.07)
Nuclear grade									
I	24	91	1.0	29	86	1.0	33	82	1.0
II	41	149	1.38 (0.82–2.34)	49	141	1.03 (0.64–1.64)	47	143	0.67 (0.42–1.05)

	E-cadherin			*p16*			*RAR-β₂*		
	Yes	**No**	**OR[b] (95% CI)**	**Yes**	**No**	**OR[b] (95% CI)**	**Yes**	**No**	**OR[b] (95% CI)**
III	40	155	1.38 (0.81–2.35)	52	143	1.11 (0.69–1.77)	50	145	0.68 (0.43–1.07)
TNM stage									
0	10	52	1.0	14	48	1.0	17	45	1.0
I	53	202	0.99 (0.59–1.66)	72	183	1.19 (0.73–1.92)	67	188	1.02 (0.63–1.65)
II_a/II_b	26	128	0.80 (0.44–1.45)	40	114	1.09 (0.63–1.86)	48	106	1.30 (0.77–2.19)
III/IV	4	20	0.79 (0.25–2.50)	2	22	0.28 (0.06–1.25)	8	16	1.42 (0.56–3.62)
ER status									
+	81	310	1.0	92	299	1.0	111	280	1.0
−	29	124	0.92 (0.57–1.48)	48	105	1.51 (1.01–2.32)	39	114	0.86 (0.56–1.32)
PR status									
+	59	266	1.0	78	247	1.0	94	231	1.0
−	46	156	1.41 (0.91–2.18)	56	146	1.25 (0.83–1.87)	53	149	0.86 (0.58–1.28)
ER/PR									
Both +	56	240	1.0	70	226	1.0	86	210	1.0
Either +	22	85	1.18 (0.68–2.04)	27	80	1.06 (0.64–1.76)	29	78	0.91 (0.56–1.48)
Both −	26	94	1.27 (0.76–2.14)	37	83	1.42 (0.89–2.25)	30	90	0.81 (0.50–1.30)
p53 mutation									
Wild	66	276	1.0	96	246	1.0	92	250	1.0
Mutant	25	104	1.01 (0.60–1.69)	34	95	0.91 (0.58–1.45)	38	91	1.13 (0.72–1.76)

[a] Numbers for some analyses are less than total for entire group because of missing variables

[b] Odds ratios (ORs) and 95% confidence intervals (CI) were estimated with unconditional logistic model adjusted for age at diagnosis for breast cancer, race, and education

Table 4

Association between clinicopathological factors and hypermethylation of at least one gene for breast cancer, WEB Study, 1996–2001[a]

	All subjects			Premenopausal			Postmenopausal		
	Yes	No	OR[b] (95% CI)	Yes	No	OR[b] (95% CI)	Yes	No	OR[b] (95% CI)
Tumor size (cm)									
< 1.0	115	50	1.0	22	11	1.0	93	39	1.0
1.0–1.4	110	58	0.87 (0.57–1.33)	28	17	0.63 (0.27–1.47)	82	41	0.97 (0.60–1.58)
1.5–2.1	134	64	0.98 (0.65–1.47)	38	19	0.75 (0.33–1.70)	96	45	1.06 (0.66–1.69)
≥2.2	127	68	0.87 (0.58–1.31)	53	26	0.76 (0.36–1.63)	74	42	0.88 (0.54–1.45)
Metastatic disease at presentation									
No	367	186	1.0	93	55	1.0	274	131	1.0
Yes	139	68	1.05 (0.74–1.48)	61	20	1.89 (1.02–3.50)	78	48	0.78 (0.51–1.19)
Vascular/lymph invasion									
No	349	185	1.0	92	55	1.0	257	130	1.0
Yes	146	67	1.16 (0.82–1.63)	62	20	1.89 (1.03–3.47)	84	47	0.90 (0.60–1.37)
Histological grade									
Well	57	25	1.0	11	8	1.0	46	17	1.0
Moderate	157	83	0.94 (0.64–1.36)	47	19	1.11 (0.52–2.39)	110	64	0.87 (0.57–1.35)
Poor	212	101	1.05 (0.73–1.51)	71	39	0.84 (0.42–1.65)	141	62	1.17 (0.76–1.80)
Nuclear grade									
I	99	46	1.0	21	9	1.0	78	37	1.0
II	173	92	0.84 (0.57–1.23)	51	24	0.85 (0.39–1.89)	122	68	0.82 (0.53–1.27)
III	205	104	0.88 (0.61–1.29)	75	39	0.77 (0.37–1.62)	130	65	0.93 (0.60–1.45)
TNM stage									
0	57	34	1.0	22	7	1.0	35	27	1.0
I	223	112	0.94 (0.64–1.39)	49	31	0.51 (0.22–1.15)	174	81	1.15 (0.74–1.79)
II_a/II_b	160	82	0.95 (0.63–1.44)	57	31	0.59 (0.26–1.33)	103	51	1.13 (0.69–1.85)
III/IV	33	14	1.13 (0.56–2.30)	19	4	1.55 (0.43–5.59)	14	10	0.77 (0.31–1.86)
ER status									
+	352	179	1.0	95	45	1.0	257	134	1.0
−	158	75	1.11 (0.80–1.56)	52	28	0.95 (0.52–1.73)	106	47	1.22 (0.81–1.83)

	All subjects			Premenopausal			Postmenopausal		
	Yes	No	OR[b] (95% CI)	Yes	No	OR[b] (95% CI)	Yes	No	OR[b] (95% CI)
PR status									
+	307	166	1.0	98	50	1.0	209	116	1.0
−	189	80	1.31 (0.95–1.82)	46	21	1.20 (0.63–2.28)	143	59	1.39 (0.95–2.04)
ER/PR									
Both −	270	147	1.0	80	41	1.0	190	106	1.0
Either +	106	43	1.35 (0.90–2.01)	31	11	1.42 (0.65–3.10)	75	32	1.34 (0.84–2.16)
Both +	116	54	1.18 (0.81–1.72)	32	18	0.91 (0.46–1.83)	84	36	1.34 (0.85–2.11)
p53 mutation									
Wild	165	326	1.0	53	96	1.0	112	230	1.0
Mutant	61	132	1.10 (0.77–1.57)	18	46	1.42 (0.74–2.71)	43	86	0.97 (0.63–1.49)

[a] Numbers for some analyses are less than total for entire group because of missing variables

[b] Odds ratios (ORs) and 95% confidence intervals (CI) were estimated with unconditional logistic model adjusted for age at diagnosis for breast cancer, race, and education