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Promoter methylation of *E- cadherin*, *p16*, and *RAR-β₂* genes in breast tumors and dietary intake of nutrients important in one-carbon metabolism

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Abstract

Aberrant DNA methylation plays a critical role in carcinogenesis, and the availability of dietary factors involved in one carbon metabolism may to contribute to aberrant DNA methylation. We investigated the association of intake of folate, vitamins B_2, B_6, B_{12}, and methionine with promoter methylation of *E- cadherin*, *p16*, and *RAR-β₂* genes in archived tumor tissues from incident, primary breast cancer cases in a population-based case-control study. Real time methylation-specific PCR was performed on 803 paraffin-embedded samples; usual dietary intake was queried from a food frequency questionnaire. Unconditional logistic regression was used to derive adjusted odds ratios (OR) and 95% confidence intervals (CI) for likelihood of promoter methylation for high compared to low intake of those one-carbon nutrients. Overall, in case-case comparisons, dietary intakes of folate, vitamins B_2, B_6, B_{12}, and methionine were not associated with likelihood of promoter methylation of *E- cadherin*, *p16*, and *RAR-β₂* for all cases combined or within strata defined by menopausal status and ER status in this study. This finding, however, does not exclude the possibility that intake of such nutrients might have the ability to modulate promoter methylation in normal, or pre-malignant (dysplastic) breast tissue.

Keywords

one-carbon nutrient; breast cancer; epidemiology; epigenetics; promoter methylation

Introduction

One carbon metabolism provides methyl groups for a variety of biological process including methylation of DNA, RNA and protein, as well as for synthesis of purines and pyrimidines for DNA synthesis (1–3). Collectively, nutrients involved in this metabolism are sometimes called “one-carbon nutrients”. Folate, the principle methyl donor, plays a central role in the conversion of methionine to *S*-adenosylmethione (SAM) (1, 2). Other nutrients including

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vitamins B_2, B_6, B_{12}, and the amino acid methionine are also involved in the one carbon metabolism pathway, potentially affecting genomic DNA methylation and synthesis and thereby causing dysregulation of gene expression (2, 4–8). In addition, alcohol intake has been reported to interfere with one carbon metabolism in a number of ways including negative effects on folate absorption, utilization and excretion, and on activity of metabolic enzymes (1, 9, 10). Findings regarding the association of folate, vitamins B_2, B_6, B_{12} and methionine with breast cancer risk has been inconsistent (11–13).

Aberrant DNA methylation, including global hypomethylation and gene-specific hypermethylation of promoter regions, is a common epigenetic alteration in breast carcinogenesis. (14–17). Little is known about the etiology of these alterations in methylation. Diets deficient in folate and other one carbon nutrients may explain the observed changes, at least in part. There is evidence from pre-clinical models that these one carbon nutrients can affect both genomic DNA methylation as well as the methylation of the promoter region of some important tumor suppressor genes (2, 18–20). Moreover, in clinical trials restriction of dietary folate intake has been shown to alter genomic methylation of circulating white blood cells (21, 22). To our knowledge, associations between dietary one-carbon nutrients and DNA methylation in breast cancer have been investigated in only two other studies, both indicating an association of folate intake with DNA hypermethylation of one or more genes (23, 24).

The *p16* gene, a tumor suppressor gene, is important in cell cycle regulation (16). There is evidence that *p16* methylation may be critical in a cascade of events leading to increased proliferation and genetic instability in human breast cells (25). Dietary folate deficiency has been shown to increase *p16* promoter methylation in an animal model (20) as well as in head and neck squamous cell carcinoma (26). In addition, other genes involved in cell adhesion (*E-cadherin*) (27) and hormone and receptor-mediated cell signaling (i.e. *RAR-*β_2 (retinoic acid-binding receptor-β_2)) (17) seem to be responsive to folate status. The expression of the *E-cadherin* gene was lower with decreasing folate in a human colonic epithelial cell line (28). Loss of expression of *E - cadherin* caused by promoter methylation occurs frequently in breast cancer (17), suggesting that folate status might affect the *Wnt* pathway through epigenetic alterations. Promoter methylation of *E - cadherin*, *p16*, and *RAR-*β_2 genes has also been shown to occur more frequently in breast tumors than benign or adjacent nonmalignant breast tissue (29, 30) and is associated with poorly differentiated breast tumors, distant metastasis of breast tumors, and estrogen receptor (ER) status of breast tumors (31–35).

Based on this apparent relationship of folate and other one carbon nutrients with DNA methylation, we hypothesized that tumors from women with breast cancer with lower dietary intake of one-carbon nutrients would have higher prevalence of methylation than those from women with higher intake. We evaluated associations of reported dietary intakes of folate, methionine, and vitamins B_2, B_6, B_{12} with promoter methylation of *E - cadherin*, *p16*, and *RAR-*β_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 of New York State, who were diagnosed with primary, histologically confirmed, incident breast cancer between 1996 and 2001 and were between 35 and 79 years of age. This report includes data from cases only. Among 1638 eligible cases, 1,170 (72%) participated. All

participants provided informed consent. The protocol was approved by the Institutional Review Boards of all of the participating institutions.

Data on demographics, and other breast cancer risk factors were collected during detailed in-person interview conducted by trained interviewers. The Health Habits and History food frequency questionnaire (FFQ) was used to query habitual dietary consumption 12–24 months before diagnosis (36, 37) with some small adaptations specific to dietary practices in Western New York. Nutrient intake from the FFQ was calculated using the DIETSYS (version 3.7) nutrient analysis software developed specifically for the food frequency questionnaire. Additionally, information was collected on vitamin and mineral supplement use. Total nutrient intake, intake from supplements and diet, was calculated by adding dietary intake with supplementary intake from either multiple supplement use or singular supplement use. The bioavailability of naturally occurring folate in food varies greatly as these forms of folate are labile and easily oxidized; and synthetic folic acid obtained from supplements is much more bioavailable (38). To account for this difference in the absorption of folate from foods and folic acid from supplements, dietary folate equivalents were examined. Folic acid intake from supplements was converted to dietary folate equivalents by multiplying the supplemental amount by 1.7 and this amount was added to food folate intake to determine total folate from diet and supplements (39).

The diagnosis of breast cancer from the medical record was reconfirmed by a single pathologist from Georgetown University based on review of archived tumor blocks. Information on tumor size, histological grade, and cancer stage (as measured by tumor-node-metastasis (TNM) stage) was abstracted from medical records by trained research nurses using a standardized protocol. ER status was determined by the study pathologist, by immunohistochemical analysis as described in detail previously (35).

Tumor block promoter methylation determination

Among the 1170 breast cancer cases in the WEB study, we were able to obtain archived tumor blocks for 920 (78.6%). Tumor samples were microdissected from fixed microscope slides. Bisulfite modification was performed on tumor DNA isolated from tissue blocks in accordance with methods described previously (35, 40). Promoter methylation of *E-cadherin*, *p16*, and *RAR-β_2* was determined by the fluorescence based version of methylation-specific PCR (MSP) using real time PCR amplification of bisulfate converted DNA in an ABI 7900HT real time PCR system as previously described (35, 41). Briefly, each reaction contained 5 μl of Taqman Universal Master Mix (29), 4.5 μl of bisulfite treated DNA and 0.5 μl of a 60X assay by design premix containing the primers and probes that were designed for each respective gene (Applied Biosystems, Carlsbad CA); the primers and probes used in this study have been described previously (35). As a control to check for modified viable DNA, we used an assay for the *ACTB* gene with primers and probes specially 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 could not be used in subsequent assays, and re-extraction and modification was attempted; the other 3 genes being assayed only if *ACTB* was positive. 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. We had successful promoter methylation results for 803 cases.

Statistical analysis

Characteristics including reported dietary intake of participating cases with and without promoter methylation of each gene were compared with the Student's *t*-test for continuous

variables and the χ^2 test for categorical variables. In addition, the likelihood of promoter methylation according to dietary methyl donor intake was examined with a case-case comparison of those with promoter methylation of a gene compared to those without. For these latter analyses, unconditional logistic regression models were used to estimate multivariate adjusted odds ratios (ORs) and 95% confidence intervals (95% CIs) for quartiles of folate, vitamins B_2, B_6, B_{12}, and methionine intake, using the highest intake category as the referent. Tests for dose-response relationship over the categories of intake were estimated by fitting the models with exposure variables included as continuous variables. All models were adjusted for age at diagnosis, education level, race, and total energy intake. Furthermore, potential confounding effects of other demographic factors, known breast cancer risk factors, and dietary intakes of other nutrients were examined, and the covariates included in the model were those that influenced the OR by more than 10%. Because alcohol consumption can affect one-carbon metabolism through its negative impact on folate absorption and concentration of vitamin B_{12} (9), we further examined potential interaction between dietary one-carbon nutrients intake and alcohol consumption for each of the specific genes by evaluation of a multiplicative term in the regression model. Intakes of one-carbon nutrients and alcohol in these logistic regression analyses with the interactions were stratified into low or high intake using the median intakes as cut-off points. All statistical tests were based on two-sided probability. Analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC).

Results

Demographic characteristics of cases with and without promoter methylation of *E - cadherin*, *p16*, and *RAR-β_2* gene are shown in Table 1. The frequency of promoter methylation was 20% (161) for *E - cadherin*, 25.9% (208) for *p16*, and 27.5% (221) for *RAR-β_2*. Mean levels of dietary folate, vitamins B_2, B_6 and B_{12} intakes were similar among cases with methylated of each of the three genes compared to those without methylation; intakes of dietary methionine among cases with methylated *p16* gene were lower than among those with unmethylated *p16*.

Likelihood of promoter methylation in breast tumors by intake of dietary folate, vitamins B_2, B_6, B_{12}, and methionine is shown in Table 2. In these case-case comparisons, there was no association of dietary intake of folate, vitamins B_2, B_6, or B_{12} with promoter methylation of *E – cadherin*, *p16*, or *RAR-β_2* gene. For cases with lower intake of dietary methionine, compared to those with the highest, there was decreased likelihood of methylated *E - cadherin* gene (OR = 0.60; 95% CI, 0.31–1.16; *p* trend 0.05). There were no differences in these associations by menopausal or ER status (data not shown). We also evaluated the associations of methylation with intake of folate, vitamins B_2, B_6 and B_{12} from supplements; there were no associations with promoter methylation (data not shown). Additionally, results for supplemental and total intake (diet plus supplements) of folate, vitamins B_2, B_6, B_{12}, and methionine were similar to those for dietary intake (data not shown). When we analyzed associations between intake of dietary one-carbon nutrients and likelihood of tumors with promoter methylation in at least one gene, no association was observed. We further conducted analysis stratified on menopausal status and ER status.

We evaluated joint effects of alcohol consumption and intake of one-carbon nutrients on promoter methylation in breast tumors (Table 3). *E – cadherin* promoter methylation was approximately two times more prevalent among cases with low intake of dietary folate and high alcohol than for those with high folate and low alcohol (OR, 2.05, 95% CI, 1.16–3.62). Similarly, high alcohol and low dietary vitamin B_{12} was associated with increased prevalence of *E – cadherin* methylation (OR, 1.85, 95% CI, 1.04–3.27). The difference in likelihood of methylation was associated with alcohol intake, not of the other nutrients. No

multiplicative interaction for any of the combinations of alcohol and dietary one-carbon nutrients was observed.

Discussion

In this study, we investigated the potential association of intake of folate, methionine, and vitamins B_2, B_6, B_{12} with promoter methylation of *E-cadherin*, *p16* and *RAR-β_2* genes in breast cancer. We did not observe any difference in the likelihood of promoter methylation based on diet, supplemental or total intake of the selected nutrients for any of these genes. We did see increased likelihood of *E – cadherin* promoter methylation associated with high alcohol and low dietary folate intakes but that the interaction was not greater than multiplicative and the increase in risk was primarily associated with alcohol consumption.

Frequencies of promoter methylation for *p16* and *RAR-β_2* genes in our sample were similar to previous reports (31); *E-cadherin* promoter methylation frequency was somewhat lower than has been reported previously (29, 31, 34). This difference may be related to the particular characteristics of our sample, sample size in this or other studies or it may depend on the sensitivity of the MSP assay and differences in MSP assay design. 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.

To our knowledge, there are only two previous studies of dietary intake of methyl donors in relation to breast cancer. In a case control study of 304 African-American breast cancer cases, there was increased likelihood of methylated *ER* α gene associated with low dietary folate intake (≤443.9 μg/day) compared to higher intake (23). In a recent study of 162 breast tumors, Christensen *et al* (24) measured 1,413 autosomal CpG loci associated with 773 cancer-related genes, and applied unsupervised clustering, a recursively partitioned mixture modeling (RPMM), of all CpG loci to reveal eight distinct methylation classes. They reported that alcohol intake and total dietary folate were significantly associated with methylation class membership; however, they also did not find associations between dietary folate and methylation of CpG loci in *E-cadherin*, *p16* and *RAR-β_2* genes in breast tumors (24). These data suggested that the effect of folate status on DNA methylation may be gene specific (2). It is also possible that putative protective effects of intake of one-carbon nutrients on breast cancer may also be from mechanisms other than the DNA methylation pathway, such as effects on histone methylation (42). Further human studies are needed to evaluate the effects of one-carbon nutrients on other epigenetic gene regulatory mechanisms as well as promoter methylation.

Although our observations suggest that dietary intake of one-carbon vitamins have limited impact on promoter methylation of these critical tumor suppressor genes in existing breast cancers, one should not conclude from these data that availability of one-carbon nutrients cannot impact gene methylation in the human breast. Intervention trials in which human subjects have undergone dietary folate deprivation have clearly shown effects on genomic methylation in circulating white blood cells (21, 22). Moreover, data from a limited number of observational studies have implied that diminished dietary intake of one-carbon nutrients is associated with hypermethylation of tumor suppressor genes in colonic and head & neck cancers (26, 43, 44), although this has not been an entirely consistent finding (45). The endogenous factors that drive gene hypermethylation in established cancers are poorly understood, but nevertheless appear to be quite potent (46). It is entirely feasible, therefore, that alterations in a methylation phenotype produced by environmental factors of modest potency, such as the diet, affect only gene methylation in pre-neoplastic tissue, which appears to be considerably more plastic in this regard. Such a concept is also consistent with the observations from animals studies, where the aberrant gene methylation induced by

methyl-deficient has been most readily demonstrated in normal, rather than neoplastic, tissue (2, 19). Such an impact could still affect the carcinogenic process.

There are several strengths and weakness which should be taken into account in assessment of these results regarding diet and methylation in tumors. Strengths of our study include the population-based study design, large sample size with archived tissues obtained from breast cancers, and detailed information on a wide range of potential confounders. Additionally, the FFQ used in the study has been studied and is consistent with other instruments of this kind; it includes most foods usually consumed by the population in Western New York State. However, several limitations, common to other observational studies on diet and cancer, need to be considered. There are well known limitations of the FFQ for assessment of usual intake and this non-differential error would result in bias toward the null in estimation of associations. Another concern of this study is recall bias; cases may differentially recall exposures of dietary because of their cancer diagnosis. However, this bias would not affect these case-case comparisons of tumor characteristics, particularly in that the participants would not be aware of their methylation status. Although the mean level of dietary folate intake for cases in our study was lower than the previous study examining this question (23), it was similar to that reported in the National Health and Nutrition Examination Surveys (NHANES) study during the same period (47). The lower intakes in our population may partly account for the lack of an association with DNA methylation in breast cancer in our study or it may be that there is truly no association. In spite of the relatively large size of the study, the statistical power in subgroups of our study remained limited due to the low frequencies of the promoter methylation, reducing our ability to identify weak associations. Furthermore, we were unable to obtain archived tumor tissue for all breast cancer cases. Compared to those for whom we were unable to obtain tissue, cases with breast tumor tissue were slightly younger at diagnosis and tended to have tumors of more advanced stage; they were similar in terms of tumor size, histological grade, nuclear grade, ER and PR status. It is not likely that there would be a difference in selection of breast cancer cases by promoter methylation for specific gene. Both age and TNM stage are unrelated to methylation of these genes in this population (35); therefore selection bias is not likely, particularly for the case-case comparisons. Another concern with interpretation of these findings is that our study was limited to an examination of methylation in three genes. While these genes were chosen because they are frequently methylated in breast tumors and because there was some evidence that they would be responsive to folate status in breast carcinogenesis, we cannot rule out that methyl donor intake may affect promoter methylation of genes other than those included in our study. Examination of intake of one-carbon nutrients in relation to other genes remains an important area of inquiry. In addition, the methodology used in this study was limited to 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, but it is possible that in some women, aberrant methylation of these genes in sequences that we did not study also affects their expression.

Understanding the etiology of aberrant DNA methylation will likely be informative in understanding breast carcinogenesis and in targeting prevention efforts. There are indications that dietary one-carbon nutrients intake may contribute to aberrant DNA methylation. In our study, we found no association between one-carbon nutrients intake and likelihood of gene promoter methylation for *E- cadherin*, *p16* or *RAR-β_2* in breast tumors or any indication of more than additive interaction for folate and alcohol for *E- cadherin* promoter methylation. The fact that we examined this question only in tumor tissue, and the fact that several potent forces appear to drive aberrant DNA methylation in neoplasms that may override the effects of altered nutrient availability, may be a responsible for the lack of associations..

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Table 1

One-carbon nutrients intake and other characteristics of breast cancer cases, cases with (M) and without (UM) promoter methylation, WEB Study 1996–2001

	E-cadherin		*p16*		*RAR-β2*	
	M (n = 161)[§]	**UM (n = 742)[§]**	**M (n = 208)[§]**	**UM (n = 595)[§]**	**M (n = 221)[§]**	**UM (n = 582)[§]**
Age, y [†]	58.0 ± 11.8	57.4 ± 11.2	58.0 ± 11.2	57.4 ± 11.3	57.4 ± 11.3	57.6 ± 11.3
Race/ ethnicity						
White	148 (91.9%)	594 (92.5%)	192 (92.3%)	550 (92.4%)	210 (95.0%)	532 (91.4%)
Non-white	13 (8.1%)	48 (7.5%)	16 (7.7%)	45 (7.6%)	11 (5.0%)	50 (8.6%)
ER status						
+	114 (71.3%)	446 (70.0%)	136 (65.7%)	424 (71.9%)	163 (74.8%)	397 (68.6%)
–	46 (28.7%)	191 (30%)	71 (34.3%)	166 (28.1%)	55 (25.2%)	182 (31.4%)
Alcohol consumption						
Never	20 (12.6%)	110 (17.3%)	46 (22.3%)	84 (14.2%)	41 (18.7%)	89 (15.4%)
Ever	139 (87.4%)	527 (82.7%)	160 (77.7%)	506 (85.8%) [a]	178 (81.3%)	488 (84.6%)
Dietary intake						
Folate intake (μg/day) [†]	281.7 ± 178.4	280.7 ± 178.4	275.2 ± 143.2	282.9 ± 158.9	285.5 ± 175.0	279.2 ± 146.6
Vitamin B_2 (mg/day) [†]	1.7 ± 1.2	1.6 ± 1.2	1.5 ± 1.2	1.7 ± 1.2	1.7 ± 1.2	1.6 ± 1.2
Vitamin B_6 (mg/day) [†]	1.4 ± 1.0	1.4 ± 1.1	1.4 ± 1.0	1.5 ± 1.1	1.4 ± 1.0	1.4 ± 1.1
Vitamin B_{12} (μg/day) [†]	3.3 ± 2.2	3.3 ± 2.1	3.1 ± 2.1	3.3 ± 2.2	3.3 ± 2.1	3.3 ± 2.2
Methionine (g/day) [†]	1.2 ± 0.9	1.1 ± 0.8	1.1 ± 0.4 [a]	1.2 ± 0.6	1.2 ± 0.7	1.2 ± 0.5

[§] M – methylated; UM – unmethylated;

[†] Mean ± SD;

[a] Comparison of methylated to unmethylated cases $p < 0.05$.

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Table 2

Dietary intake of one-carbon nutrients and *E-cadherin*, *p16*, and *RAR-β_2* promoter methylation in breast tumors: case-case comparison

	E-cadherin			*p16*,			*RAR-β_2*		
Quartile of nutrients	**M**	**U**	**OR (95% CI) [1]**	**M**	**U**	**OR (95% CI) [1]**	**M**	**U**	**OR (95% CI) [1]**
Folate [a]									
Q4 (highest)	36	141	1.0	40	137	1.0	52	125	1.0
Q3	35	163	0.95 (0.54–1.58)	57	141	1.18 (0.73–1.91)	53	145	0.96 (0.60–1.52)
Q2	42	158	1.11 (0.64–1.92)	53	147	0.93 (0.56–1.54)	51	149	0.98 (0.59–1.60)
Q1 (lowest)	48	180	1.17 (0.66–2.08)	58	170	0.76 (0.44–1.31)	65	163	1.15 (0.69–1.92)
p trend			0.48			0.19			0.61
Vitamin B_2 [b]									
Q4 (highest)	44	143	1.0	42	145	1.0	59	128	1.0
Q3	38	159	0.74 (0.44–1.24)	42	155	0.83 (0.50–1.39)	57	140	0.92 (0.58–1.46)
Q2	37	161	0.69 (0.39–1.21)	70	128	1.43 (0.86–2.38)	45	153	0.68 (0.41–1.14)
Q1 (lowest)	42	179	0.69 (0.37–1.28)	54	167	0.70 (0.39–1.27)	60	161	0.87 (0.50–1.51)
p trend			0.25			0.56			0.36
Vitamin B_6 [c]									
Q4 (highest)	38	136	1.0	38	136	1.0	55	119	1.0
Q3	41	166	0.93 (0.55–1.56)	53	154	1.06 (0.64–1.73)	51	156	0.78 (0.49–1.25)
Q2	38	164	0.82 (0.47–1.44)	56	146	1.04 (0.62–1.77)	50	152	0.85 (0.51–1.41)
Q1 (lowest)	44	176	0.91 (0.49–1.68)	61	159	0.88 (0.50–1.58)	65	155	1.10 (0.64–1.89)
p trend			0.70			0.65			0.62
Vitamin B_{12} [d]									
Q4 (highest)	39	145	1.0	45	139	1.0	56	128	1.0
Q3	41	173	0.95 (0.57–1.59)	54	160	0.90 (0.56–1.45)	52	162	0.76 (0.48–1.20)
Q2	43	160	1.05 (0.62–1.78)	55	148	0.96 (0.59–1.56)	56	147	0.91 (0.56–1.46)
Q1 (lowest)	38	164	0.94 (0.53–1.68)	54	148	0.82 (0.48–1.39)	57	145	1.00 (0.60–1.66)
p trend			0.94			0.54			0.78
Methionine [e]									
Q4 (highest)	41	151	1.0	38	154	1.0	57	135	1.0

	E-cadherin			*p16,*			*RAR-β₂*		
Quartile of nutrients	**M**	**U**	**OR (95% CI)** [1]	**M**	**U**	**OR (95% CI)** [1]	**M**	**U**	**OR (95% CI)** [1]
Q3	50	158	1.11 (0.67–1.86)	61	147	1.51 (0.91–2.50)	58	150	0.97 (0.60–1.55)
Q2	36	158	0.74 (0.41–1.33)	42	152	0.91 (0.52–1.62)	45	149	0.81 (0.48–1.38)
Q1 (lowest)	34	175	0.60 (0.31–1.16)	67	142	1.41 (0.76–2.60)	61	148	1.45 (0.65–2.04)
p trend			0.05			0.68			0.74

[1] Odds ratios and 95% confidence intervals adjusted for age, race, education, energy, lifetime alcohol intake.

[a] Quartile cutoff point for dietary folate among cases (μg/day): 178.49, 252.32, 347.65.

[b] Quartile cutoff point for dietary vitamin B_2 among cases (mg/day): 1.16, 1.63, 2.18.

[c] Quartile cutoff point for dietary vitamin B_6 among cases (mg/day): 1.06, 1.46, 1.91.

[d] Quartile cutoff point for dietary vitamin B_{12} among cases (μg/day): 2.15, 3.32, 5.10.

[e] Quartile cutoff point for dietary methionine among cases (g/day): 0.86, 1.12, 1.45.

Table 3

Interactions between dietary intake of one-carbon nutrients and alcohol consumption and likelihood of promoter methylation of *E-cadherin, p16* and *RAR-*β_2 in breast tumors: case-case comparison

	E-cadherin			***p16,***			***RAR-*β_2**		
	M	**U**	**OR (95% CI)** [1]	**M**	**U**	**OR (95% CI)** [1]	**M**	**U**	**OR (95% CI)** [1]
Folate/ alcohol intake [a]									
High/ Low	28	149	1.0	48	129	1.0	51	126	1.0
High/ High	43	155	1.74 (1.00–3.01)	49	149	0.91 (0.56–1.47)	54	144	0.89 (0.56–1.43)
Low/ Low	39	174	1.31 (0.73–2.34)	59	154	0.77 (0.47–1.25)	61	152	1.05 (0.65–1.70)
Low/ High	51	164	2.05 (1.16–3.62)	52	163	0.69 (0.42–1.14)	55	160	0.90 (0.55–1.47)
p interaction			0.77			0.97			0.89
Vitamin B_2/ alcohol intake [b]									
High/ Low	38	147	1.0	42	138	1.0	57	123	1.0
High/ High	49	155	1.60 (0.96–2.69)	42	162	0.87 (0.53–1.43)	59	145	0.86 (0.55–1.35)
Low/ Low	34	176	0.86 (0.48–1.56)	65	145	1.21 (0.73–2.00)	55	155	0.77 (0.47–1.27)
Low/ High	45	164	1.43 (0.81–2.54)	59	150	1.12 (0.67–1.89)	50	159	0.68 (0.41–1.13)
p interaction			0.93			0.84			0.95
Vitamin B_6/ alcohol intake [c]									
High/ Low	30	145	1.0	45	130	1.0	52	123	1.0
High/ High	49	157	1.74 (1.03–2.95)	46	160	0.84 (0.51–1.36)	54	152	0.83 (0.52–1.61)
Low/ Low	37	178	1.01 (0.56–1.82)	62	153	0.88 (0.53–1.45)	60	155	1.00 (0.61–1.63)
Low/ High	45	162	1.56 (0.87–2.78)	55	152	0.85 (0.51–1.41)	55	152	0.92 (0.56–1.52)
p interaction			0.73			0.67			0.74
Vitamin B_{12}/ alcohol intake [d]									
High/ Low	29	155	1.0	49	135	1.0	53	131	1.0
High/ High	51	163	2.00 (1.18–3.42)	50	164	0.89 (0.55–1.43)	55	159	0.82 (0.52–1.31)
Low/ Low	38	168	1.35 (0.77–2.37)	58	148	0.92 (0.58–1.47)	59	147	1.03 (0.65–1.64)
Low/ High	43	156	1.85 (1.04–3.27)	51	148	0.84 (0.51–1.38)	54	145	0.96 (0.59–1.56)
p interaction			0.29			0.93			0.71
Methionine/ alcohol intake [e]									

	E-cadherin			*p16,*			*RAR-β_2*		
	M	**U**	**OR (95% CI) [1]**	**M**	**U**	**OR (95% CI) [1]**	**M**	**U**	**OR (95% CI) [1]**
High/ Low	35	146	1.0	52	129	1.0	55	126	1.0
High/ High	57	167	1.65 (1.00–2.71)	49	175	0.71 (0.45–1.14)	61	163	0.84 (0.54–1.31)
Low/ Low	32	177	0.69 (0.39–1.24)	55	154	0.63 (0.38–1.03)	57	152	0.92 (0.57–1.50)
Low/ High	37	152	1.07 (0.60–1.92)	52	137	0.70 (0.42–1.18)	48	141	0.84 (0.50–1.40)
p interaction			0.87			0.17			0.81

[1]Odds ratios and 95% confidence intervals adjusted for age, race, education, energy, menopausal status, age at menopause, and smoking status.

Cutoff point for lifetime total alcohol (oz): 697.18.

[a]Cutoff point for diet folate (mcg/day):252.32.

[b]Cutoff point for diet vitamin B_2 (mg/day):1.63.

[c]Cutoff point for diet vitamin B_6 (mg/day):1.46.

[d]Cutoff point for diet vitamin B_{12} (mcg/day):3.32.

[e]Cutoff point for diet methionine (g/day):1.12.