The influence of one-carbon metabolism on gene promoter methylation in a population-based breast cancer study

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Abnormal methylation in gene promoters is a hallmark of the cancer genome; however, factors that may influence promoter methylation have not been well elucidated. As the one-carbon metabolism pathway provides the universal methyl donor for methylation reactions, perturbation of this pathway might influence DNA methylation and, ultimately, affect gene functions. Utilizing approximately 800 breast cancer tumor tissues from a large population-based study, we investigated the relationships between dietary and genetic factors involved in the one-carbon metabolism pathway and promoter methylation of a panel of 13 breast cancer-related genes. We found that *CCND2*, *HIN1* and *CHD1* were the most "dietary sensitive" genes, as methylation of their promoters was associated with intakes of at least two out of the eight dietary methyl factors examined. On the other hand, some micronutrients (i.e., B_2 and B_6) were more "epigenetically active" as their intake levels correlated with promoter methylation status in 3 out of the 13 breast cancer genes evaluated. Both positive (hypermethylation) and inverse (hypomethylation) associations with high micronutrient intake were observed. Unlike what we saw for dietary factors, we did not observe any clear patterns between one-carbon genetic polymorphisms and the promoter methylation status of the genes examined. Our results provide preliminary evidence that one-carbon metabolism may have the capacity to influence the breast cancer epigenome. Given that epigenetic alterations are thought to occur early in cancer development and are potentially reversible, dietary modifications may offer promising venues for cancer intervention and prevention.

Introduction

Promoter hypermethylation of cancer-related genes, accompanied by global hypomethylation, is a common feature of tumor cells.[1] While large numbers of studies have been performed to investigate the functional consequence of aberrant DNA methylation, little is known about what might influence the DNA methylation status of the genome. Since epigenetic processes are dynamic, reversible and susceptible to exogenous factors, identifying environmental or lifestyle factors that influence the epigenome may offer a unique opportunity for chemoprevention or diet-based interventions that target epigenetic pathways.

Increasing evidence suggests that certain constituents in food and dietary supplements have the capacity to influence the epigenome and, ultimately, an individual's risk of developing cancer.[2,3] Among such compounds, micronutrients in folate-mediated one-carbon metabolism are of particular interest.[4] The universal methyl donor in the cellular reactions, *S*-adenosylmethionine (SAM), is generated in the one-carbon pathway. The key micronutrients in this pathway are folate, methionine and several B vitamins (i.e., B_2, B_6 and B_{12}) that are essential co-factors for one-carbon transfer reactions. Other methyl donors, such as choline and betaine, can also affect SAM status, primarily through choline-mediated one-carbon metabolism, and ultimately impact DNA methylation. It has been shown that dietary methyl donors are capable of modulating methylation patterns in both animal models and humans.[5-8] Furthermore, there are reports showing that functional polymorphisms in one-carbon-metabolizing genes can also influence DNA methylation status.[4,9,10] Nevertheless, human data on whether one-carbon metabolism influences DNA methylation are limited and inconsistent. This issue has been explored primarily in colon cancer[11,12] and to a much lesser extent in breast cancer.

In this study, we chose a panel of 13 genes that had been shown to play an important role in breast carcinogenesis and

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Table 1. Genes selected for methylation analysis and their putative functions

Gene	Full name	Group	Function	Assayed sample no.	Methylated samples (%)
ESR1	estrogen receptor 1	steroid hormone receptor	hormone binding, DNA binding and activation of transcription	851	44.8
PGR	progesterone receptor	steroid hormone receptor	mediates the physiological effects of progesterone	851	11.9
BRCA1	breast cancer 1, early onset	tumor suppressor	maintaining genomic stability	851	59.0
APC	adenomatous polyposis coli	tumor suppressor	an antagonist of the Wnt signaling; Defects cause familial adenomatous polyposis	800	48.4
p16/ CDKN2A	cyclin-dependent kinase inhibitor 2A	tumor suppressor	cell cycle control	777	3.6
HIN1/ SCGB3A1	secretoglobin, family 3A, member 1	tumor suppressor	growth-inhibitory cytokine, regulates epizthelial cell differentiation.	765	62.9
RASSF1A	Ras association domain family member 1	tumor suppressor	involved in cell cycle control	765	85.2
DAPK1	death-associated protein kinase 1	tumor suppressor	a positive mediator of gamma-interferon induced programmed cell death	765	14.1
GSTP1	glutathione S-transferase pi 1	detoxification	xenobiotic metabolism	765	27.8
CCND2	cyclin D2	oncogene	regulators of CDK kinases	765	19.6
TWIST1	twist homolog 1	transcription factors	cell lineage determination and differentiation	765	15.3
CDH1	E-cadherin	tumor suppressor	cell proliferation, invasion and/or metastasis	765	5.8
RARβ	retinoic acid receptor, beta	steroid hormone receptor	mediates cellular signalling in embryonic morphogenesis, cell growth and differentiation	765	27.6

tumor progression. For example, steroid hormone genes (e.g., *ESR1* and *PGR*) and tumor suppressors (e.g., *BRCA1, APC* and *p16[INK4a]*) are well-established breast cancer-related genes. More importantly, promoters of these genes are frequently methylated in breast tumor tissues and this aberrant methylation has been associated with malignant phenotypes and survival rates of breast cancer cases.[13-22] Thus, promoter methylation was proposed to be the underlying mechanism for the loss of gene function.[23,24]

We previously reported that one-carbon metabolism influenced breast cancer risk and survival in the population-based Long Island Breast Cancer Study Project (LIBCSP).[25-27] Herein, we investigate the influence of one-carbon metabolism (i.e., dietary intake of co-factors and genetic polymorphisms of genes) on promoter methylation status of a panel of breast cancer-related genes.

Results

Promoter methylation of 13 breast cancer-related genes was assessed in up to 851 breast tumor samples from the LIBCSP, including 104 in situ and 747 invasive tumors. **Table 1** lists the genes examined in this study, with their established or proposed functions. Also shown in **Table 1** are number of tumor samples examined for each gene's methylation status. The main reason for missing methylation data was an insufficient amount of DNA yielded from the tumor blocks.

We have previously reported the relationship of promoter methylation of the same panel of genes with demographic and clinical-pathological characteristics in the population.[28,29] This study focuses on the influence of diet on promoter methylation of the same panel of genes. Dietary methyl constituents examined in this study include folate, methionine, choline, betaine, B vitamins (B_2, B_6 and B_{12}), and alcohol (a folate antagonist). Interrelationships between dietary intake of these methyl constituents and 13 breast cancer genes are summarized in **Table S1**.

There are multiple gene-nutrient associations; however, the effects were moderate in size, 2-fold in general, when the high nutrient intake categories were compared to low ones (in tertiles). Although folate, methione and choline are considered key methyl donors in one-carbon metabolism, there is little evidence that these three micronutrients were associated with promoter methylation of any of the 13 breast cancer genes; the only exception is the inverse relationship between folate and *CCND2* methylation (**Table S1**). Interesting patterns emerge from the gene-nutrient matrix (**Table 2**). Two co-factors for one-carbon metabolizing enzymes, B_2 (for *MTHFR*) and B_6 (for *cSHMT*), were associated with 3 of the 13 breast cancer genes (**Fig. 1**) and two of these genes, *HIN1* and *CDH1*, were both associated with B_2 and B_6. Furthermore, both positive and inverse associations between dietary intake and promoter methylation were observed (**Fig. 1**). Another interesting observation from the gene-nutrient matrix is

that promoter methylation of several genes (*CCND2*, *HIN1* and *CHD1*) appeared to be associated with multiple (at least two) dietary micronutrients (**Fig. 2**). While high intake of methyl constituents positively associated with *HIN1* promoter methylation (with B_2 and B_6), inverse associations were observed for *CCND2* (with folate and B_6) and *CDH1* (with B_2, B_6 and B_{12}).

We then explored associations between 13 functional polymorphisms in one-carbon metabolizing genes and gene promoter methylation status (**Table S2**). Several suggestive dose-dependent relations were observed (in other words, if there was a corresponding increase in the magnitude of the odd ratios (ORs) with an increase in the number of variant alleles for a particular polymorphism, then that association was considered a positive dose-dependent relationship and, conversely, if the magnitude of the ORs decreased with the number of alleles, then that association was considered to be an inverse dose-dependent relationship): the *TYMS* 2R allele, *ESR1* hypermethylation; the *DHFR* 19 bp deletion allele, *CDH1* hypomethylation; *CHDH* rs12676 T allele, *GSTP1* hypermethylation; and *CHDH* rs9001 C allele, *GSTP1* hypomethylation. However, unlike the gene-nutrient matrix above, we did not observe any clear patterns between one-carbon polymorphism and promoter methylation.

Discussion

Changes in DNA methylation have been shown to play important roles in breast cancer development; these alterations are potentially reversible.[30] The panel of genes chosen in this study was based on their important role in breast carcinogenesis and tumor progression. These genes are frequently methylated in breast cancer and promoter methylation was proposed to be the underlying mechanism for the loss of function.[23,24]

To date, few studies have identified factors, especially modifiable lifestyle factors, that are capable of influencing the epigenome.[3] In this study, we focus on the one-carbon metabolism, in which potentially modifiable factors are involved. The goal of this study was to examine the potential influence of nutrient intake and genetic polymorphisms in the key enzymes involved in the one-carbon metabolic pathway on promoter methylation of breast cancer-related genes. Our study, based on a large population-based case-control study, is the first to systematically examine lifestyle factors that may influence the breast cancer epigenome, and may lead to the development of better preventive strategies for the disease.

Several interesting findings emerged from our study. First, from a panel of 13 breast cancer related genes, promoter methylation of *CCND2*, *HIN1* and *CHD1* appeared to be more "sensitive" to dietary modulation, as they were correlated with at least 2 out of 8 micronutrients examined in the study. In the meantime, two one-carbon micronutrients, vitamin B_2 and B_6, appeared to be more "epigenetically active" as they were associated with promoter methylation of 3 out of 13 genes examined. While vitamin B_2 is co-factor for the rate-limiting enzyme in folate-mediated one-carbon metabolism, MTHFR, vitamin B_6 is the co-factor of another critical enzyme, cSHMT, involved in regenerating the methyl-carrying folate in the pathway. These results suggest that

Table 2. Summary of significant trends between one-carbon micronutrient intake and gene promoter methylation status

	ESR1	PGR	BRCA1	APC	p16	CDH1	RARβ	TWIST1	CCND2	GSTP1	RASSF1A	HIN1	DAPK1
Folate									↓				
Methionine													
Choline													
Betaine				↑									
B_2						↓		↑				↑	
B_6						↓			↓			↑	
B_{12}						↓							
Alcohol		↓											

↑ indicates positive association, i.e., higher intake is associated with higher likelihood for a case to possess a methylated promoter for the gene of interest; ↓ indicates inverse association, i.e., higher intake is associated with lower likelihood for a case to possess a methylated promoter for the gene of interest. Cells without an arrow are those with insignificant trend test results.

intake of certain B vitamins may affect the supply of key co-factors required for maintaining proper functions of one-carbon metabolism in the cell, thus influencing the metabolic output of methyl donors for methylation reactions.

Second, associations between high intake of one-carbon micronutrients and promoter methylation are not unidirectional; both positive (hypermethylation) and inverse (hypomethylation) relationships were observed. For example, high B_2 intake was associated with lower likelihood of having a methylated promoter for *CDH1* but with higher likelihood of having a methylated promoter for *TWIST1* and *HIN1*. These results may reflect the complex regulatory mechanisms in the cell. One-carbon metabolism provides the methyl-donor (i.e., SAM) for the methylation reaction; interruption of this pathway by suboptimal nutrients intake or by suboptimal enzyme activity caused by genetic polymorphism may influence methyl supply. However, the regulation of the methyltransferases (e.g., DNMT3) is not known, especially for gene specific methylation. Genomic structure, sequence dependence and genetic mutation could all play a role.

Third, we found suggestive evidence that functional polymorphisms in one-carbon metabolizing genes could influence promoter methylation. However, we did not pursue the analyses of gene-diet interactions due to limited power. There are few other reports studying gene promoter methylation status in relation to one-carbon metabolism. One study examined seven genes (*RARβ2*, *CDH1*, *ESR1*, *BRCA1*, *CCND2*, *p16* and *TWIST*) among ~200 tumors.[31] They found that patients who were homozygous for the *TYMS* double tandem repeat (2R/2R) showed a trend for more frequent promoter methylation (by methylated gene counts) in their breast cancers, and patients who were homozygous for the *MTHFD1* G1958A polymorphism showed a significantly higher frequency of methylation. However, none of the individual methylated CpG sites showed a significant association with any
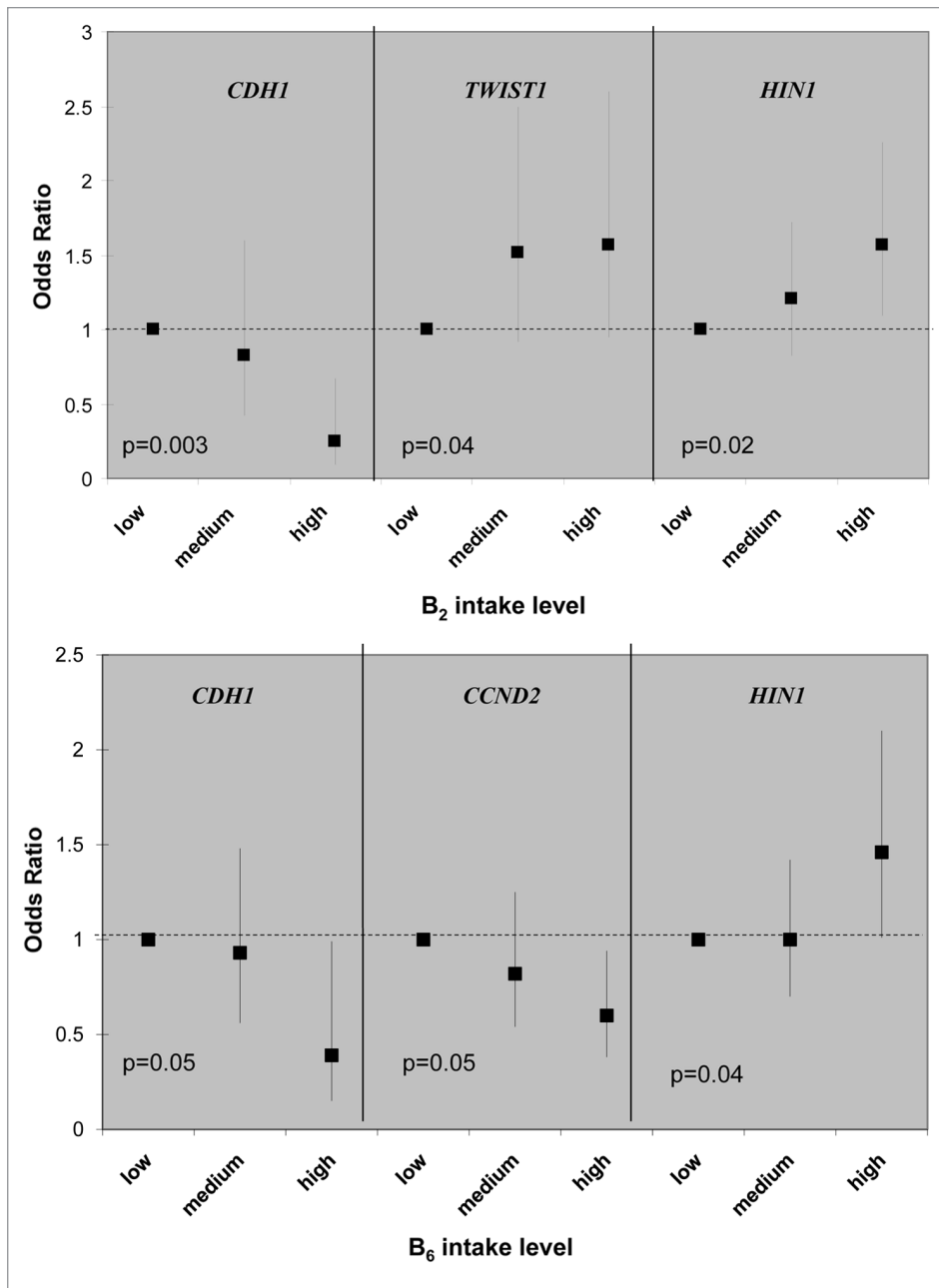
Figure 1. Example of epigenetically active micro-nutrients for association between gene promoter methylation status and dietary intake of one-carbon related nutrients. Odd ratios (ORs) and 95% confidence intervals are shown on the y-axis, comparing medium and high intake to the low intake group. p values are of the test for liner trend of ORs.

The majority of these published papers are on colorectal cancer, focusing on one or few of the nutrients (e.g., folate), SNPs (*MTHFR* C677T) and/or gene methylation status (*p16*, *hMLH*).[9,33-35] These results are far from conclusive. Variations in sample sizes, and the use of different assays to detect methylation status, could have contributed to the inconsistency.

Given the number or micronutrients and genetic polymorphisms examined in this study, one important issue that warrants careful consideration is that of "multiple comparison," which results in increased likelihood of false positive findings. We examined eight micronutrients and 13 genetic polymorphisms in relation to the promoter methylation status of 13 genes. At the p < 0.05 level, there could be five significant results for micronutrients and eight for polymorphisms by chance alone. There is continuing debate on whether/when/how multiple comparisons should be taken into account.[36] When evaluating results of molecular epidemiology studies, in addition to the magnitude of the p value, statistical power and the priority of the tested hypothesis, are also need to take into account.[37] In this study, we opt to report the crude p values without adjusting for multiple comparisons for the following reasons. First, instead of randomly considering multiple genes without regarding of the biological relevance but only relying on statistical significance to interpret the study findings, we chose to focus only on genes and dietary/genetic factors that have functional relevance to breast cancer. Second, using the nutrient-gene matrix, our study focuses on the patterns of micronutrients-methylation associations, rather than of the magnitude of

of the polymorphisms. We observed an *ESR1-TYMS* association with the same trend in our study (**Table S2**). Another recent study examined promoter methylation of three genes (*E-cadherin*, *p16* and *RARβ2*) among ~800 breast tumors and found no evidence that *MTHFR* C677T, A1298C and *MTR* A2756G polymorphisms were associated methylation status.[32] We did not observe significant association for these three polymorphisms in our study, either.

There are other studies on the influence of one-carbon metabolism on gene promoter methylation with limited sample size.

specific associations. Lastly, multiple comparison adjustment, such as Bonferroni method, may safeguard false positive findings, but it may increase Type II error (false negative) and reduce sensitivity,[38] thus being too conservative for exploratory purpose. Nevertheless, we need to be cautious and results from our study warrant replication in other population studies.

Several limitations of our study are discussed below. (1) In our study, tumor DNA was not available for all cases of the LIBCSP participants. Although there were some differences between those with and without tumor DNA available for our analyses

Figure 2. Example of dietary sensitive genes for association between gene promoter methylation status and dietary intake one-carbon related nutrients. Odds ratios and 95% confidence intervals were shown on the y-axis, comparing medium and high intake to the low intake group. p values are of the test for liner trend of ORs.

(as described in the Methods section), the benefit of utilizing our population-based sample was that we were able to quantify the differences between the two groups. This valuable contrast aided in our interpretation of the generalizability of our study results to the general population. It is this type of information that is often unavailable from other study populations, such as those derived from a hospital-based case series. (2) The association between one-carbon metabolism and gene promoter methylation was examined only in tumor tissues; whether the same association exists in tissues that precede malignancy (i.e., normal adjacent tissues) is not known. This limits our ability to draw definitive causal-inference. Nevertheless, our results add knowledge in the breast cancer research field and provide a rationale for future mechanistic research. (3) The dietary factors may affect methylation of multiple CpG sites in the gene promoter region. The assays used in this study examined a limited number of CpG sites in the region, which may limit the sensitivity of detecting a diet-methylation relationship. (4) In our study, dietary intake values for one-carbon related micronutrients and compounds were assessed using a modified Block food frequency questionnaire (FFQ). There is continuing debate over the accuracy of this method to assess usual adult diet. Despite these potential limitations, the ranking of intakes (which was the approach used in our analyses) was unlikely to be invalid, given that the Block FFQ has been shown to be a valid and reliable dietary assessment tool for estimating usual food intake and ranking individuals into categories of intake of micronutrients.[39,40]

In summary, we examined the influence of one-carbon metabolism on gene promoter methylation in 13
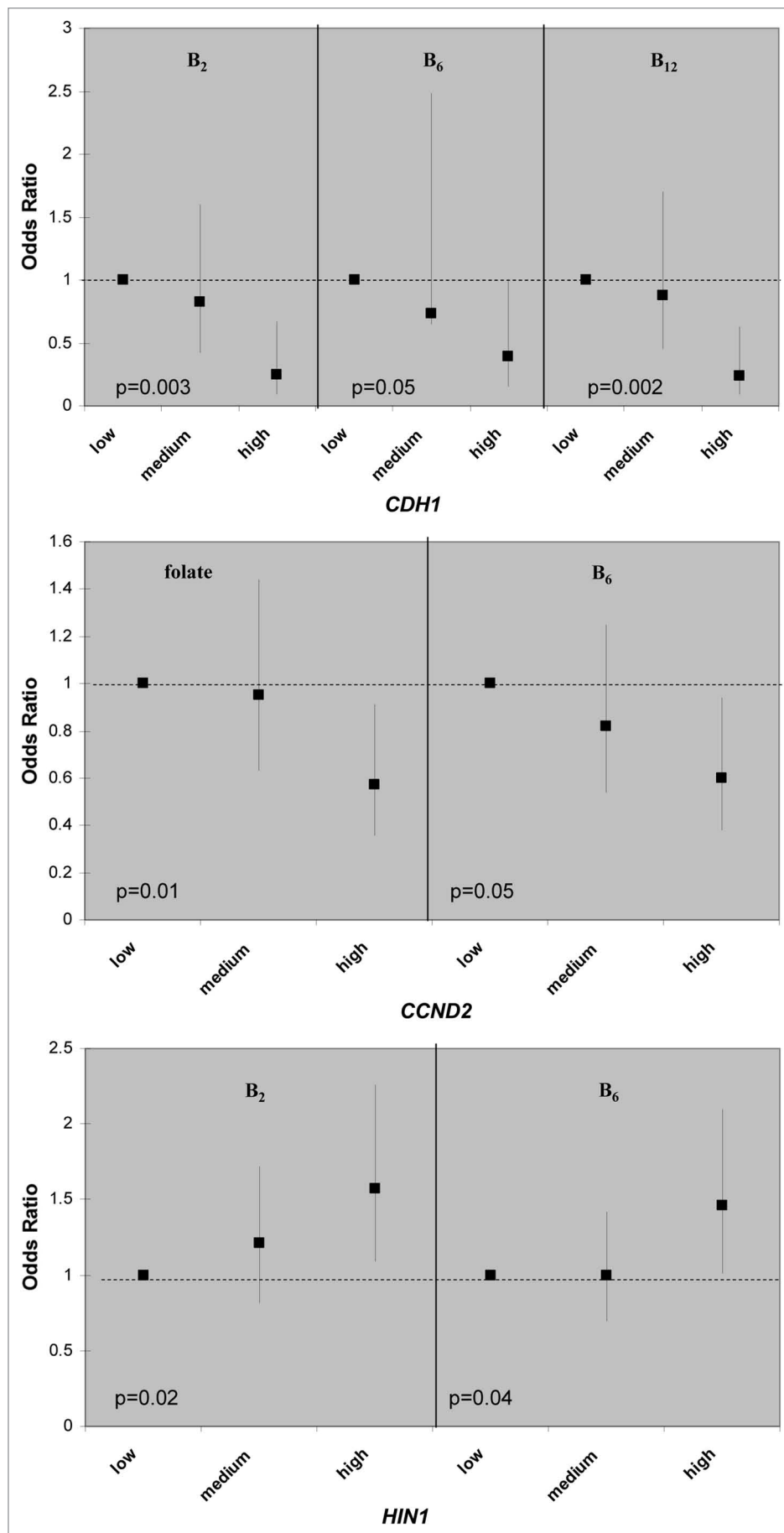
breast-cancer related genes in a relatively large epidemiologic study. Our results provide preliminary evidence that dietary nutrients might influence the breast cancer epigenome. Given that epigenetic alterations are reversible and occur early in cancer development, dietary intervention may offer promising venues for cancer prevention.

Materials and Methods

Study population. We utilized the resources of the Long Island Breast Cancer Study Project, a population-based study. The study participants included 1,504 women newly diagnosed with a first primary breast cancer in 1996–1997 and who participated in the original, parent case-control study. Details of the study design have been described in detail previously in references 41 and 42. Participant information used in this ancillary study was obtained as part of in-person interviews and medical record abstraction that were collected as part of the case-control parent study.[41] Questionnaires were administrated to assess the demographic characteristics and breast cancer-related factors. Tumor characteristics such as ER/PR status were extracted from the medical records. Among cases with this information available, 583 (58.9%) were ER+/PR+; 143 (14.4%) were ER+/PR-; 52 (5.3%) were ER-/PR+; and 212 (21.4%) were ER-/PR-.

Archived pathology blocks for the first primary breast cancer of the LIBCSP cases were requested from the 33 hospitals in the Long Island study area and successfully retrieved for 962 women.[43] After review and processing in the laboratory by a trained breast cancer pathologist (H. Hibshoosh), tumor tissues from 859 subjects (89.3%) were available for our study analyses. We compared the demographic and clinico/pathological features between cases with or without tumor blocks available for methylation analysis in our study. Although most characteristics are similar between these two groups, some factors were different. Case women who had tumor samples available for methylation analysis tended to be older (mean age 59.6 vs. 57.9 years; p = 0.005), to have an invasive tumor (87.8% vs. 80.1%; p < 0.001), and to be post-menopausal (70.7% vs. 64.6%; p = 0.01). The study protocol was approved by the Institutional Review Boards of the collaborating institutions.

Determining gene promoter methylation in tumor DNA. Tumor block retrieval and DNA extraction were performed as previously described in reference 43 and 44. Tumor DNA first underwent bisulfite modification using the CpGnome DNA Modification Kit (Chemicon International, cat. no. S7820) following the protocol from the manufacturer. We selected a panel of 13 genes that have been implicated in breast carcinogenesis (**Table 1**) for methylation analysis. Promoter methylation of *ER*, *PR* and *BRCA1* was determined by methylation-specific PCR (MSP) as described previously in reference 44 and 45. The MethyLight assay was used for determining the methylation status of the rest of genes as described in reference 46 and 47. The percentage of methylation was calculated by the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_T = (C_{T,Target} - C_{T,Actin})_{sample} - (C_{T,Target} - C_{T,Actin})_{fully\ methylated\ DNA}$[48] and multiplying by 100. Samples containing ≥4% fully methylated molecules were designated as methylated, whereas

samples containing <4% were designated as unmethylated. The main reason for some missing methylation data was that there was not enough DNA as template for PCR reaction regardless of methods for assessing methylation status. The limiting amount of DNA is primarily due to the small size of the original tumor.

Genotyping and dietary assessment. Genotyping was conducted on 13 functional polymorphisms in the one-carbon metabolism pathway using methods described in reference 25, 26 and 49. Dietary intake values for one-carbon related micronutrients and compounds were calculated based on data collected as part of the in-person case-control interview assessed using a modified Block food frequency questionnaire (FFQ), which was self-completed by the study participants.[50,51]

Statistical analysis. The MSP-PCR assay for *ER*, *PR* and *BRCA1* promoter methylation generated dichotomized outcomes, i.e., methylated and unmethylated status. MethyLight assay, on the other hand, yielded percentage of methylation for gene promoters that were then dichotomized into methylated or unmethylated cases using a cut-off of 4%, according to previous reports in reference 47, 52 and 53. Relationships of gene promoter methylation status with demographic and clinical-pathological characteristics were examined using chi-square test. Associations between methylation status and diet or genetic factors were analyzed in a case-case setting using unconditional logistic regression.[54] With this approach, the odds ratios (ORs) and 95% confidence interval (CI) represent the likelihood of a case possessing a methylated promoter for the gene of interest given certain nutrient intake levels or genotypes. Nutrient intakes in the year prior to the case interviews were divided into tertiles based on the distributions in cases with methylation data. Tests for linear trend of ORs were calculated by regressing on the median values of each tertile.[54] For genetic polymorphisms, an ordered categorical variable by assigning a score to each genotype [i.e., 0 (no variant allele), 1 (carrying one variant allele) and 2 (carrying two variant alleles)] was used as the variable to test for trend.

Potential confounders included age at diagnosis (entered as 5-year age groups), ER/PR IHC status [positive group: ER and PR both positive (ER+/PR+) and negative group: all others (ER+/PR-, ER-/PR+, ER-/PR-)], cancer type (in situ vs. invasive), menopausal status (pre- vs. post-), race and family history of breast cancer and benign breast. If eliminating a covariate from the full regression model changed the effect estimate by 10% or more, the covariate was considered a confounder and kept in the model.[54] None of the covariates tested met this criterion and, thus, only results from the age-adjusted model are presented. Because of the exploratory nature of this study, we choose to report the crude p values without adjusting for multiple comparison; the implications of this approach are discussed in the 'Discussion' section. All statistical analyses were performed using SAS statistical software version 9.1(SAS Institute, Cary, NC).

Note

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