One-Carbon Metabolism, *MTHFR* Polymorphisms, and Risk of Breast Cancer

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Abstract

Accumulating evidence from epidemiologic studies suggests that risk of breast cancer is reduced in relation to increased consumption of folate and related B vitamins. We investigated independent and joint effects of B vitamin intake as well as two polymorphisms of a key one-carbon metabolizing gene [i.e., *methylenetetrahydrofolate reductase* (*MTHFR*) *677C>T* and *1298A>C*] on breast cancer risk. The study uses the resources of a population-based case-control study, which includes 1,481 cases and 1,518 controls. Significant inverse associations between B vitamin intake and breast cancer risk were observed among non-supplement users. The greatest reduction in breast cancer risk was observed among non-supplement users in the highest quintile of dietary folate intake [odds ratio (OR), 0.61; 95% confidence interval (95% CI), 0.41-0.93] as compared with non-supplement users in the lowest quintile of dietary folate intake (high-risk individuals). The *MTHFR 677T* variant allele was associated with increased risk of breast cancer (*P*, trend = 0.03) with a multivariate-adjusted OR of 1.37 (95% CI, 1.06-1.78) for the *677TT* genotype. The *1298C* variant allele was inversely associated with breast cancer risk (*P*, trend = 0.03), and was likely due to the linkage of this allele to the low-risk allele of *677C*. The *MTHFR*-breast cancer associations were more prominent among women who did not use multivitamin supplements. Compared with *677CC* individuals with high folate intake, elevation of breast cancer risk was most pronounced among *677TT* women who consumed the lowest levels of dietary folate (OR, 1.83; 95% CI, 1.13-2.96) or total folate intake (OR, 1.71; 95% CI, 1.08-2.71). From a public heath perspective, it is important to identify risk factors, such as low B vitamin consumption, that may guide an effective prevention strategy against the disease. (Cancer Res 2005; 65(4): 1606-14)

Introduction

There is considerable interest in identifying risk factors associated with breast cancer that can be modified to reduce the risk of the disease. Accumulating evidence from epidemiologic studies suggests a protective role of folate and related B vitamins against breast cancer, especially among alcohol users. Four large prospective epidemiologic studies on these associations have been published (1–4); three found that adequate folate intake may reduce the risk of breast cancer. In the large Nurses' Health Study (1), a significant reduction in risk associated with total folate as well as folate from multivitamin supplements was observed among women with daily consumption of ≥15 g of alcohol, a known folate antagonist. Similar results have also been observed on dietary folate in the Canadian National Breast Screening Study (2) and the Iowa Women's Health Study (3). However, in a recent study on 1303 postmenopausal breast cancer cases in the American Cancer Society Cancer Prevention Study II Nutrition cohort (N = 66,561), no effect of folate on risk of breast cancer was apparent (4). In addition to these four prospective studies focusing on dietary folate intake, two other prospective studies on biological methyl levels also suggest that higher plasma B vitamin levels are associated with lower risk of breast cancer (5, 6). Most of these findings corroborate evidence from case-control studies conducted in the United States (7, 8), Italy (9, 10), and China (11).

Breast cancer is a manifestation of abnormal genetic as well as epigenetic changes. Interruption of one-carbon metabolism may be important in breast cancer etiology as it facilitates the cross-talk between genetic and epigenetic processes by playing critical roles in both DNA methylation and DNA synthesis (Fig. 1). One-carbon metabolism is a network of interrelated biological reactions that provide essential cofactors for the production of *S*-adenosylmethionine, the primary methyl donor for methylation, as well as the methyl group in methylation of dUMP to dTMP for DNA synthesis [reviewed by Choi and Mason (12)]. A low methyl supply induces DNA global hypomethylation (13) as well as deficient methylation of dUMP to dTMP leading to uracil misincorporation (14). Folate deficiency results in interruption of DNA repair capability (15), which may lead to DNA strand breaks, enhanced mutagenesis, and apoptosis.

Folate (as well as methionine and choline) is the major source of methyl groups from foods (16); dietary folate depletion alone is a sufficient perturbing force to diminish the methyl pool (17). Other B vitamins, such as vitamins B_2, B_6, and B_{12}, are also key cofactors for one-carbon metabolism that involves a constellation of genes including *methylenetetrahydrofolate reductase* (*MTHFR*). MTHFR is at a critical metabolic branch point of one-carbon metabolism; it carries out the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which directs the folate pool towards remethylation of homocysteine to methionine, at the expense of thymidylate synthesis (Fig. 1). A single nucleotide polymorphism of the *MTHFR* gene (*677C>T*) is associated with an alanine-to-valine substitution and is correlated with enzyme thermolability and reduced enzyme activity (18). Individuals with the *677TT* genotype tend to accumulate 5,10-methylenetetrahydrofolate intracellularly at the expense of 5-methyltetrahydrofolate,

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whereas individuals with the *677CC* or *677CT* genotypes have predominantly 5-methyltetrahydrofolate intracellularly (19). Additionally, the *677TT* genotype has been shown to correlate with suboptimal folate status in terms of decreased folate and increased homocysteine levels in serum or plasma (20). A second common polymorphism in the C-terminal regulatory domain of the gene, *MTHFR 1298A>C* (Gln>Ala), has also been identified (21), but its function remains controversial.

Despite ample epidemiologic evidence and strong biological plausibility, few studies have examined whether functional polymorphisms in one-carbon metabolizing genes modify the risk of breast cancer associated with dietary intake of folate and other methyl-related nutrients. The only report on folate-gene interactions comes from the Shanghai Breast Cancer Study conducted in China (22), in which the *MTHFR 677C>T* polymorphism was not an independent predictor of breast cancer risk, whereas individuals with the *677TT* genotype had elevated risk of breast cancer when dietary folate consumption was low. Because of the dietary pattern in Chinese women being different from their counterparts in western countries, it is not clear whether these findings would be reproduced in the U.S. population. We used the resources of the Long Island Breast Cancer Study Project, a U.S. population-based study, to examine the independent and joint effects of B vitamin intake and related metabolizing genes on risk of breast cancer.

Materials and Methods

Subjects. The Long Island Breast Cancer Study Project was designed to analyze whether the risk of breast cancer is associated with PAH-DNA adducts and organochlorine compounds. A detailed description of the study has been published elsewhere (23). In brief, cases were English-speaking women (>97% of all residents) newly diagnosed with a primary *in situ* or invasive breast cancer between August 1, 1996, and July 31, 1997, and who were residents of Long Island (Nassau and Suffolk counties) in New York at the time of their diagnosis. All cases were confirmed by the physician and medical record. Among a total of 2,030 eligible cases, we were able to obtain the permission of physician for 1,837 cases (90.5%). Physician refusal was commonly due to illness of the patient. Control women were a sample of current residents of Nassau and Suffolk counties who spoke English and who were frequency matched to the expected age distribution of case women by 5-year age groups. Potentially eligible control women were identified by Waksberg's method of random digit dialing (24) for those under 65 years of age, and by Health Care Finance Administration (HCFA) rosters for those 65 years of age and older. The response rate to the random digit dialing telephone screener was 77.9%, which is applicable only to the control respondents who are under age 65 years (and comprise 57.9% of the control group). The response rate to the HCFA rosters was 41.8%. Overall, 1,508 cases (82.1%) and 1,556 controls (62.7%) completed the in-home interview.

Dietary Assessment. A modification of the Block food frequency questionnaire (FFQ; refs. 25, 26), which has been previously validated (25, 27), was used to assess dietary intake in the year before the interview. This instrument was self-given and completed by 1,481 (98.2%) of cases and 1,518 (97.6%) of control participants in an average of 36 minutes. Response for this component (23) did not seem to vary with age of respondent. Dietary intake values for one-carbon related micronutrients, folate (the bioactive ingredient is vitamin B_9—folic acid), vitamins B_1 (thiamin), B2 (riboflavin), B_3 (niacin), and B_6 (pyridoxine), were calculated from the FFQ based on food items, serving sizes, and consumption frequencies. We also examined total consumption for each B vitamin by summing dietary intake and supplemental sources of these micronutrients. Use of vitamin supplements was queried on the FFQ. Conversion of FFQ data to daily intakes of B vitamins was carried out using the National Cancer Institute's DietSys, version 3.

Genotyping Methods. We obtained a 40 mL blood specimen from 1,102 (73.1%) cases and 1,141 (73.3%) control subjects. DNA was isolated utilizing methods previously described (28). Genotypes of the *MTHFR 677C>T* and *1298A→C* polymorphisms were ascertained by previously published methods (29). About an additional 10% of the study population were included as quality control samples; the rate of concordance was 98% and 99% for the *MTHFR 677C>T* and *1298A>C* polymorphisms, respectively. All laboratory personnel were blinded to the case-control as well as quality control status of the specimens.

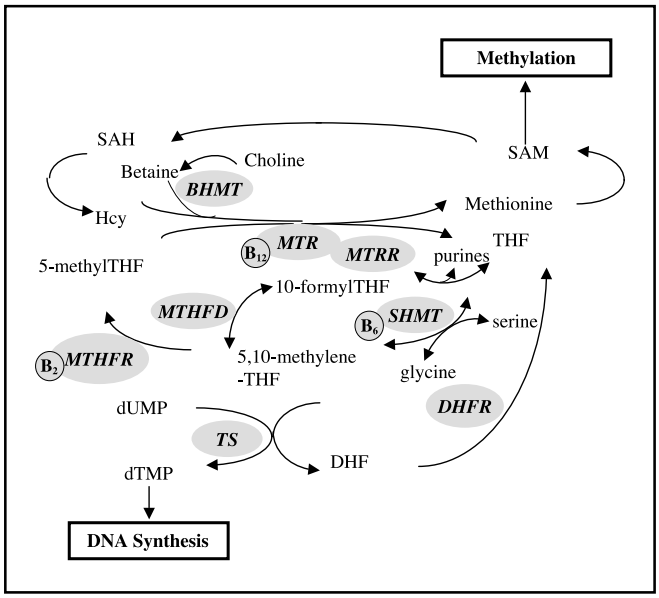

Figure 1. Schematic illustration of one-carbon metabolism noting the cross-talk between genetic (DNA synthesis) and epigenetic (methylation) processes. Key genes involved in one-carbon metabolism include *methylenetetrahydrofolate reductase* (*MTHFR*), *thymidylate synthase* (*TS*), *methionine synthase* (*MTR*), *methionine synthase reductase* (*MTRR*), *serine hydroxymethyltransferase* (*SHMT*), *dihydrofolate reductase* (*DHFR*), and betaine-homocysteine methyltransferase (*BHMT*). Vitamins B_2, B_6, and B_{12} are cofactors in the pathway. *MTHFR* is at a critical metabolic branch point of the metabolic pathway, carrying out the irreversible conversion of 5,10-methylenetetrahydrofolate (*5,10-methyleneTHF* to *5-methylTHF*), which directs the folate pool toward remethylation of homocysteine (*Hcy*) to methionine at the expense of thymidylate synthesis. *SAM, S*-adenosylmethionine; *SAH, S*-adenosylhomocysteine.

Other Study Variables. Information on other key covariates considered as potential confounders and/or effect modifiers was obtained during the structured, interviewer-given, in-person, 2-hour main questionnaire. The distribution of risk factors for breast cancer from the main study population (1,508 cases and 1,556 controls who completed the main questionnaire) has been published in detail elsewhere (23). Similar distributions were observed among the subset of the 1,481 of cases and 1,518 control participants who also completed the FFQ (30). Distribution of risk factors for breast cancer as well as B vitamin intake from the subpopulation from, of which we were able to ascertain the *MTHFR* genotype (data not shown), was comparable with those identified and reported for the full study population (23).

Statistical Method. Unconditional logistic regression analysis was conducted to estimate odds ratios (OR) and 95% confidence intervals (95% CI) for associations of individual B vitamins and *MTHFR* genotype with breast cancer risk. Age at reference date (defined as date of diagnosis for cases and date of identification for controls, and categorized as: <44, 45-54, 55-64, 65-74, 75+ years) was included in all models. Univariate analyses were done to compare distributions of covariates and/or confounding variables among cases and controls. Variables that were independently related to disease risk were included as adjustment terms for multivariate analyses. These included family history of breast cancer in a first-degree relative (yes/no), history of benign breast disease (yes/no), education (<high school, high school graduate, some college, college graduate, post-college), and body mass index at age 20 (≤18, >18-19, >19-21, >21-22, >22 kg/m^2). We also included several established risk factors in the

multivariate analyses although these were not significantly associated with breast cancer risk in our study population. These included menopausal status (pre- or postmenopausal), age at menarche (≤11, >11-12, >12-13, >13-14, >14 years), age at menopause (≤45, >45-48, >48-50, >50-53, >53 years), and energy intake (≤902, >902-1147, >1147-1399, >1399-1745, >1745 kcal/d). These covariates were included in models as indicator variables. Although age-adjusted and multivariate-adjusted analyses yielded similar results, only those from multivariate analyses were presented.

We calculated the risk of breast cancer for intake of B vitamins from dietary sources alone as well as for combined intake from diet and supplements. The B vitamins that were explored included folate, vitamins B_1, B_2, B_3, and B_6. Intakes of these nutrients were categorized into quantiles based on the distribution among the controls; those in the lowest quantile were considered as the referent category. For *MTHFR*, subjects were grouped according to the genotype; individuals with the homozygous wild-type genotype (i.e., *677CC* and *1298AA*) were considered as the referent group. Stratified analyses were done by multivitamin use (any or none), menopausal status (pre or post), and breast cancer type (invasive or *in situ*). Tests for trend were done by treating each categorized variable as a continuous term and entering the variable into a logistic regression model. To test the degree of correlation between B vitamin subtypes, Spearman correlation coefficients were analyzed based on deciles of intake for each individual B vitamin.

Log likelihood tests were done to evaluate effect modification on a multiplicative scale. The likelihood ratio statistic was calculated by comparing the difference of the log likelihood value for a model with a cross-product term for two main effect variables with the log likelihood value for a model without the cross-product term. For example, to assess the folate-*MTHFR* interaction, folate intake (low, medium, and high) and the *MTHFR 677C>T* genotype (*677CC*, *677CT*, and *677TT*) were categorized into tertiles; cross-product terms were created using these categories and were included in the model as indicator variables. ORs were then calculated to compare variable combinations with the lowest-risk referent category in unconditional logistic regression analysis.

Linkage disequilibrium between the *MTHFR 677C>T* and *1298A>C* polymorphisms was calculated as D', which ranges from 0 (no linkage disequilibrium) to 1 or −1 (complete linkage disequilibrium; ref. 31). The EH linkage utility program (32) was used to determine χ^2 statistics and P values for tests of allelic association between polymorphic markers. All statistical analyses were done using SAS Version 8.0.

Results

A detailed distribution of established and suspected risk factors for breast cancer for the study population has been published previously (23). Overall, 94% of the cases and 93% of the controls were Caucasians. The mean age of the cases was 58.8 years compared with 57.0 years in the controls ($P = 0.001$). This is mainly a result of decreased response rate among control women over age 75 years. Many established risk factors were confirmed in this study population including parity, breast-feeding, age at first birth, and family history of breast cancer (23). The prevalence of use of hormone replacement and oral contraceptives was 26.3% and 31.6% of study subjects, respectively.

B Vitamins and Breast Cancer Risk. Table 1 reports the risk of breast cancer in relation to intake of B vitamins from food sources

Table 1. Multivariate-adjusted ORs and 95% CI for associations of daily intake of B vitamins with risk of breast cancer in the Long Island Breast Cancer Study Project, 1996-1997

Nutrient type	Quintiles of dietary intake					
	Q1 (lowest intake)	Q2	Q3	Q4	Q5	*P*, trend*
Dietary folate						
Range (μg/d)	≤159	>159-216	>216-279	>279-356	>356	
Cases/controls	314/296	276/297	308/296	265/297	263/296	
OR (95% CI)†	1.00 (referent)	0.89 (0.70-1.13)	1.00 (0.78-1.29)	0.85 (0.65-1.11)	0.85 (0.64-1.14)	0.29
Total folate (diet + supplements)						
Range (μg/d)	≤208	>208-330	>330-561	>561-722	>722	
Cases/controls	309/300	305/294	256/295	280/299	276/294	
OR (95% CI)†	1.00 (referent)	1.03 (0.81-1.31)	0.85 (0.67-1.08)	0.93 (0.73-1.18)	0.95 (0.74-1.22)	0.43
Vitamin B_1 (thiamin)						
Range (mg/d)	≤0.72	>0.72-0.95	>0.95-1.16	>1.16-1.48	>1.48	
Cases/Controls	344/307	300/290	259/292	273/303	250/290	
OR (95% CI)†	1.00 (referent)	0.87 (0.67-1.13)	0.74 (0.56-0.99)	0.72 (0.53-0.98)	0.69 (0.49-0.96)	0.02
Vitamin B_2 (riboflavin)						
Range (mg/d)	≤0.95	>0.95-1.30	>1.30-1.62	>1.62-2.12	>2.12	
Cases/Controls	331/293	296/302	265/295	271/296	263/296	
OR (95% CI)†	1.00 (referent)	0.85 (0.66-1.09)	0.78 (0.59-1.02)	0.80 (0.59-1.07)	0.76 (0.55-1.04)	0.13
Vitamin B_3 (niacin)						
Range (mg/d)	≤9.8	>9.8-12.9	>12.9-15.7	>15.7-19.9	>19.9	
Cases/Controls	337/296	292/299	252/293	287/299	258/295	
OR (95% CI)†	1.00 (referent)	0.85 (0.65-1.10)	0.75 (0.56-1.00)	0.85 (0.63-1.16)	0.78 (0.56-1.09)	0.24
Vitamin B_6 (pyridoxine)						
Range (mg/d)	≤0.84	>0.84-1.15	>1.15-1.42	>1.42-1.84	>1.84	
Cases/controls	309/300	321/297	269/295	275/300	252/290	
OR (95% CI)†	1.0 (referent)	1.09 (0.85-1.40)	0.92 (0.70-1.20)	0.91 (0.68-1.21)	0.87 (0.64-1.18)	0.17

*P value for trend for categorical variables.

†Adjusted for age, family history of breast cancer in first-degree relative, history of benign breast disease, educational attainment, body mass index at age 20, and kilocalories per day.

only as well as total folate from food and supplements. The focus of the analyses was on folate because of its central role in transporting the methyl moiety in one-carbon metabolism. We found no association of dietary folate or total folate with risk of breast cancer. Vitamins B_2 and B_6 are directly involved in one-carbon metabolism as cofactors. Vitamins B_1 and B_3, on the other hand, participate in energy production and are not directly involved in the one-carbon pathway. Information on another key one-carbon related vitamin, B_{12}, was not available for this population. Overall, slight reductions of breast cancer risk (OR, < 1) were observed among people with increased consumption of these B vitamins, with the strongest effect seen for vitamin B_1, for which significantly reduced breast cancer risk was observed in the highest three quintiles of consumption (*P*, trend = 0.02). Given that B vitamins from food sources overlapped, we examined the degree of correlation between B vitamin subtypes. Spearman coefficients ranged from a low of 0.41 between total folate and vitamin B_3 to a high of 0.90 between dietary folate and vitamin B_1.

In our study population, about 50% of the participants were multivitamin supplement users and 97% of women in the highest quintile of total folate intake were supplement users. Use of multivitamin supplements was not associated with breast cancer risk in the Long Island Breast Cancer Study Project (30). Associations between dietary B vitamins and breast cancer risk presented in Table 1 did not change after including multivitamin use (yes, no) in the multivariate models. We did stratified analyses with respect to multivitamin use (Table 2). For every B vitamin we examined, stronger inverse B vitamin-breast cancer associations were observed among non-supplement users compared with the users; the *P* for trend across the quintiles of intakes was 0.06 for dietary folate,

Table 2. Multivariate-adjusted ORs and 95% CI stratified by supplement use for associations of daily intake of B vitamins with risk of breast cancer in the Long Island Breast Cancer Study Project, 1996-1997

Nutrient type	Quintiles of dietary intake					
	Q1 (low)	Q2	Q3	Q4	Q5	*P*, trend*
Folate						
Supplement use = no						
Cases/Controls	189/164	142/154	164/148	125/134	93/133	
OR (95% CI)†	1.0 (referent)	0.82 (0.59-1.16)	0.98 (0.69-1.38)	0.83 (0.57-1.23)	0.61 (0.41-0.93)	0.06
Supplement use = yes						
Cases/controls	125/132	134/143	144/148	140/163	170/163	
OR (95% CI)†	1.0 (referent)	0.99 (0.69-1.42)	1.04 (0.71-1.51)	0.91 (0.62-1.35)	1.11 (0.73-1.67)	0.75
Vitamin B_1						
Supplement use = no						
Cases/controls	193/157	163/150	125/137	133/148	99/141	
OR (95% CI)†	1.0 (referent)	0.81 (0.56-1.16)	0.66 (0.44-1.00)	0.62 (0.40-0.97)	0.45 (0.28-0.74)	0.002
Supplement use = yes						
Cases/controls	151/150	137/140	134/155	140/155	151/149	
OR (95% CI)†	1.0 (referent)	0.95 (0.66-1.37)	0.83 (0.55-1.24)	0.85 (0.55-1.31)	0.96 (0.61-1.53)	0.81
Vitamin B_2						
Supplement use = no						
Cases/controls	186/157	161/154	135/139	118/143	113/140	
OR (95% CI)†	1.00 (referent)	0.81 (0.57-1.15)	0.77 (0.52-1.14)	0.69 (0.45-1.04)	0.62 (0.39-0.99)	0.05
Supplement use = yes						
Cases/controls	145/136	135/148	130/156	153/153	150/156	
OR (95% CI)†	1.0 (referent)	0.82 (0.57-1.19)	0.74 (0.49-1.10)	0.88 (0.57-1.34)	0.81 (0.51-1.28)	0.60
Vitamin B_3						
Supplement use = no						
Cases/controls	187/147	150/152	121/138	144/153	111/143	
OR (95% CI)†	1.0 (referent)	0.73 (0.50-1.06)	0.65 (0.43-0.99)	0.69 (0.44-1.08)	0.57 (0.35-0.94)	0.05
Supplement use = yes						
Cases/controls	150/149	142/147	131/155	143/146	147/152	
OR (95% CI)†	1.0 (referent)	0.97 (0.68-1.40)	0.84 (0.57-1.25)	0.99 (0.65-1.51)	0.98 (0.61-1.57)	1.00
Vitamin B_6						
Supplement use = no						
Cases/controls	172/153	184/159	131/142	119/146	107/133	
OR (95% CI)†	1.0 (referent)	1.08 (0.76-1.52)	0.81 (0.55-1.20)	0.73 (0.48-1.10)	0.70 (0.45-1.09)	0.03
Supplement use = yes						
Cases/controls	137/147	137/138	138/153	156/154	145/157	
OR (95% CI)†	1.0 (referent)	1.11 (0.78-1.60)	0.98 (0.67-1.44)	1.11 (0.75-1.65)	0.99 (0.65-1.52)	0.96

*P value for trend for categorical variables.

†Adjusted for age, family history of breast cancer in first-degree relative, history of benign breast disease, educational attainment, body mass index at age 20, and kilocalories per day.

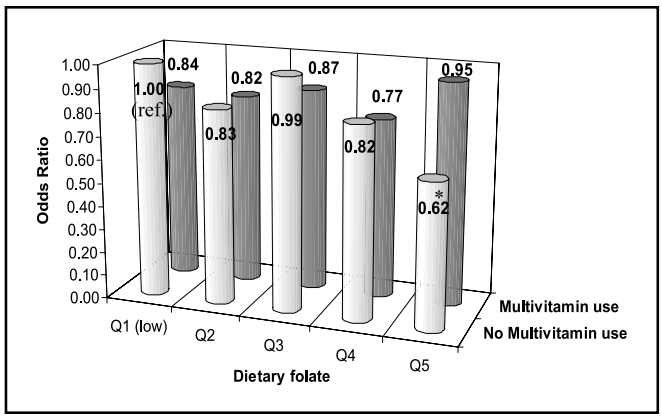

Figure 2. Relationship between dietary folate intake and risk of breast cancer with rspect to multivitamin use in the Long Island Breast Cancer Study Project, 1996-1997. The ORs were adjusted for age, family history of breast cancer in first-degree relatives, history of benign breast disease, education, body mass index at age 20, and daily caloric intake. *P*, interaction = 0.04. *, $P < 0.05$.

0.002 for vitamin B_1, 0.05 for vitamins B_2 and B_3, and 0.03 for vitamin B_6 among non-supplement users. Compared with non-supplement users in the lowest quintile of B vitamin intakes, non-supplement users in the highest quintile of folate, B_1, B_2, and B_3 had significantly lower risks of breast cancer with ORs of 0.61 (95% CI, 0.41-0.93), 0.45 (95% CI, 0.28-0.74), 0.62 (95% CI, 0.39-0.99), and 0.57 (95% CI, 0.35-0.94), respectively. A reduced but nonsignificant reduction (OR, 0.70; 95% CI, 0.45-1.09) was also observed for B_6. The risk reduction was not apparent among supplemental users. Fig. 2 illustrates joint effects of dietary folate and supplement use on breast cancer risk. Compared with high-risk individuals (lowest quintile of dietary folate intake and no supplemental use), the greatest reduction of breast cancer risk (OR, 0.61; 95% CI, 0.41-0.93) was observed among individuals with the highest intake of dietary folate but who do not use multivitamin supplements (*P*, interaction = 0.04). Controlling for uses of hormone replacement and oral contraceptives did not significantly change the results reported in Table 2 and Fig. 2. Stratified analyses by these variables also yielded comparable results. The associations of B vitamin intake and breast cancer risk did not substantially vary with menopausal status (pre- versus postmenopausal) or disease type (invasive versus *in situ*).

***MTHFR* Polymorphisms and Breast Cancer Risk.** The *MTHFR 677C>T* genotypes were ascertained from 1,063 cases and 1,104 controls. The genotype distribution was in agreement with Hardy-Weinberg equilibrium in both cases ($P = 0.30$) and controls ($P = 0.96$). The *677T* allele frequency of 40% of the cases was higher than that of controls (37%). The *677T* variant allele was associated with increased breast cancer risk in a dose-dependent fashion (Table 3). Compared with individuals with the *677CC* wild-type genotype, those with the *677TT* genotype had an age-adjusted OR of 1.34 (95% CI, 1.04-1.73; *P*, trend = 0.04). After adjusting for additional risk factors including family history of breast cancer in a first-degree relative, history of benign breast disease, education, and body mass index at age 20, the dose-dependent relationship remained elevated with a multivariate-adjusted OR of 1.37 (95% CI, 1.06-1.78) for *677TT* and a *P* value for trend of 0.03.

The *MTHFR 1298A>C* polymorphism was ascertained from 1,062 cases and 1,103 controls (Table 3). The genotype distributions were in agreement with the Hardy-Weinberg equilibrium ($P = 0.89$ for cases; $P = 0.84$ for controls). We observed an inverse association of the *1298C* allele and risk of breast cancer in a dose-dependent fashion (*P*, trend = 0.03); the *1298CC* genotype conferred a significantly lower risk of breast cancer compared with the *1298AA* genotype (OR, 0.73; 95% CI 0.53-1.00). This relationship was not modified by menopausal status or stage of breast cancer.

A high degree of linkage disequilibrium was observed between the *677C>T* and *1298A>C* polymorphisms ($D' = -0.54$, $P < 0.001$). The negative sign of the D' indicates that the *677C-1298C* (or *677T-1298A*) alleles were linked. When combined genotypes were examined, individuals who are homozygous with risk alleles at both loci (*677TT-1298AA*) had significant significantly elevated risk of breast cancer (OR, 1.82; 95% CI 1.17-2.85) compared with those who are homozygous with low-risk alleles (*677CC-1298CC*). Combined heterozygosity did not modify the disease risk; individuals who were heterozygous at both loci (*677CT-1298A*C) had similar risk as those with the *677CC-1298CC* genotype (OR, 1.09; 95% CI, 0.72-1.66; Table 3).

We also examined the *MTHFR*-breast cancer association according to menopausal status (pre- versus postmenopausal). Comparable results were observed in both groups for the *677C>T* polymorphism (data not shown). The inverse association of the *1298A>C* polymorphism with breast cancer risk was only present in postmenopausal women with a multivariate-adjusted OR of 0.65 (95% CI, 0.44-0.96; *P*, trend = 0.02). The *MTHFR*-breast cancer associations did not differ significantly with respect to *in situ* and invasive cases.

Gene-Environment Interactions in Breast Cancer. When the *MTHFR*-breast cancer relationship was examined according to supplement use, dose-dependent relations were only apparent among non-supplement users (Table 4), with *P* values for trend of 0.005 and 0.02 for the *677C>T* and *1298A>C* polymorphisms, respectively. In this subgroup, the *677TT* genotype was associated with a 70% increase in breast cancer risk (95% CI, 1.14-2.52) whereas the *1298CC* genotype was associated with a 38% reduction in risk (95% CI, 0.38-1.01).

We examined interactions between *MTHFR* polymorphisms and folate intake in relation to breast cancer risk. With respect to the *677C>T* polymorphism, compared with low-risk individuals (*677CC* genotype with high folate intake), elevation of breast cancer risk was the most pronounced among *677TT* women who consumed the lowest levels of dietary folate (OR, 1.83; 95% CI, 1.13-2.96) or had the lowest total folate intake (OR, 1.71; 95% CI, 1.08-2.71), although the interactions for both models were not significant on a multiplicative scale (Fig. 3). Similar associations were observed for every other B vitamin we examined; the *677TT* individuals had the highest ORs when their dietary consumption was in the lowest category of vitamins B_1, B_2, B_3, and B_6 at 2.06 (95% CI, 1.25-3.40), 1.88 (95% CI, 1.17-3.01), 2.05 (95% CI, 1.25-3.38), and 2.36 (95% CI, 1.47-3.88), respectively. No indication of effect modification by the *1298A>C* polymorphism was apparent in the study; the *P* for interaction was 0.84 for dietary folate and 0.94 for total folate.

Discussion

Low consumption of folate and related B vitamins has been implicated as one of the few modifiable risk factors associated with breast cancer (1–3). Findings from our study lend support to the concept that folate as well as other B vitamins may have anti-carcinogenic properties against breast cancer, especially among individuals who do not use multivitamin supplements. The study population was recruited between 1996 and 1997, just before the

Table 3. ORs and 95% CI for *MTHFR* polymorphisms with risk of breast cancer in the Long Island Breast Cancer Study Project, 1996-1997

Genotype	No. cases (%)	No. controls (%)	OR (95% CI)*	OR (95% CI)†
677C>T				
677CC	398 (37.4)	440 (39.9)	1.0 (referent)	1.0 (referent)
677CT	476 (44.8)	509 (46.1)	1.04 (0.87-1.26)	1.05 (0.87-1.27)
677TT	189 (17.8)	155 (14.0)	1.34 (1.04-1.73)	1.37 (1.06-1.78)
P, trend‡			0.04	0.03
1298A>C				
1298AA	558 (52.5)	536 (48.6)	1.0 (referent)	1.0 (referent)
1298AC	417 (39.3)	457 (41.4)	0.88 (0.74-1.06)	0.87 (0.72-1.05)
1298CC	87 (8.2)	110 (10.0)	0.77 (0.56-1.04)	0.73 (0.53-1.00)
P, trend			0.05	0.03
Combined genotypes				
677CC-1298CC	63 (6.0)	69 (6.3)	1.0 (referent)	1.0 (referent)
677CC-1298AC	188 (17.8)	198 (18.0)	1.04 (0.70-1.55)	1.09 (0.72-1.65)
677CC-1298AA	146 (13.8)	172 (15.6)	0.93 (0.62-1.40)	0.97 (0.63-1.49)
677CT-1298CC	17 (1.6)	32 (2.9)	0.58 (0.30-1.15)	0.60 (0.29-1.23)
677CT-1298AC	207 (19.6)	218 (19.8)	1.04 (0.70-1.54)	1.09 (0.72-1.66)
677CT-1298AA	251 (23.7)	259 (23.6)	1.06 (0.72-1.56)	1.12 (0.75-1.68)
677TT-1298CC	6 (0.6)	9 (0.8)	0.73 (0.25-2.17)	0.82 (0.27-2.49)
677TT-1298AC	22 (2.1)	41 (3.7)	0.59 (0.32-1.09)	0.63 (0.33-1.20)
677TT-1298AA	158 (14.9)	102 (9.3)	1.70 (1.11-2.59)	1.82 (1.17-2.85)

*Adjusted for age.
†Adjusted for age, family history of breast cancer in first-degree relative, history of benign breast disease, educational attainment, body mass index at age 20, and kilocalories per day.
‡*P* value for trend for categorical variables.

Food and Drug Administration–mandated folate fortification in the U.S. food supply in January of 1998. Nevertheless, our population-based study consists of women with rather healthy dietary habits with respect to B vitamin intake. For example, the median intakes of total and dietary folate were 433 and 242 μg/d, respectively, both of which are higher than the Recommended Dietary Allowances of 180 μg/d for non-pregnant and non-nursing women aged 15 years and older. Only 18% of the women in our study population fell below the Recommended Dietary Allowances. The relatively sufficient intake of folate and one-carbon related B vitamins may explain the lack of overall associations between these micronutrients and breast cancer risk. It is interesting to see that compared with high-risk individuals (non-supplement users with lowest quintile of dietary folate intake), women in the highest quintile of dietary folate intake who do not use supplements had even lower risk of breast cancer (OR, 0.61; 95% CI, 0.41-0.93) than those with comparable dietary folate intake but who also use supplements at the same time (OR, 0.95; 95% CI, 0.67-1.33), although the confidence intervals were overlapping. It is very unlikely that supplement use can abolish the reduction in risk associated with dietary folate; however, it is possible that those non-supplement users who are in the highest category of dietary folate intake may belong to a unique subgroup of women who have other healthy lifestyles that have not been identified or controlled for in this analysis. Although supplement users in our study overall have higher mean dietary folate intake [consistent with the notion that supplement users are more health conscious and have a healthier lifestyle (33, 34)], the mean dietary folate consumption was actually lower in supplement users in the highest quintile of dietary folate intake. This finding seems to suggest that folate from food sources may have stronger anti-carcinogenic effects than the synthetic folate found in supplements. Alternatively, it has been reported that supplement users may be less healthy in terms of increased use of prescription drugs as well as increased number of health visits in the previous year among the elderly population (35). Nevertheless, interpretation of this surprising finding is speculative and warrants caution.

The goal of the study was to examine whether the folate-breast cancer association is modified by polymorphisms of the folate-metabolizing gene, *MTHFR*, in the hope of clarifying how folate may be protective against breast cancer. We observed an increased susceptibility of breast cancer among women with the *MTHFR 677TT* genotype. This result corroborated the findings from the Nurses' Health Study (6) that low plasma folate levels conferred higher risk of breast cancer. We also observed an elevated risk of breast cancer in *677TT* individuals that was even stronger if the consumption of dietary or total folate was low. One possible mechanism is that low folate intake as well as slow metabolism associated with the *MTHFR* polymorphism results in a suboptimal methyl supply inside the body and, in turn, increased breast cancer risk through an epigenetic process such as aberrant DNA methylation. As in many neoplasia, the hallmark feature of global hypomethylation and region-specific hypermethylation is present in breast cancer. In a study by Soares et al. (36) on 136 breast cancer cases, DNA methylation of breast tumors was significantly less than that of adjacent as well as normal parenchyma. A statistically significant correlation was found between global hypomethylation and the disease stage, the tumor size, and histologic grade of tumor. Subjects with the *MTHFR 677TT* genotype have been shown to possess a lower degree of genomic DNA methylation in peripheral

lymphocytes compared with the wild-type *677CC* subjects; an inverse correlation between RBC folate and DNA methylation status was also apparent (37). A follow-up analysis using a new quantitative method also showed that genomic DNA methylation in peripheral blood mononuclear cells directly correlated with folate status and inversely correlated with plasma homocysteine levels; and when analyzed according to folate status, only the *677TT* subjects with low levels of folate accounted for the diminished DNA methylation (38). In another recent study (39) on 233 cancer patients (with colorectal, breast, and lung tumors), carriers of the *MTHFR 677T* allele showed a lower level of methylation in the genome (P = 0.002) and tumors (P = 0.047). Additionally, tumors from patients with a variant genotype of another one-carbon metabolizing gene, methionine synthase, showed promoter hypermethylation in a large panel of tumor suppressor genes including $p16^{INK4A}$ and *BRCA1*, both of which are important in mammary tumorigenesis.

There are several reports on the association of the *MTHFR 677C>T* polymorphism with breast cancer risk (22, 40–44); most were clinic-based studies that had limited sample sizes and were restricted to specific ethnic [e.g., Jewish (40)] or clinical characteristics [e.g., <40 years of age with bilateral breast cancer (41)]; results from these studies were variable. The only population-based results come from the Shanghai Breast Cancer Study which consisted of women 25 to 64 years of age in which multivitamin use was low (22). In this Chinese population, *MTHFR* polymorphism was not an independent predictor of breast cancer risk. However, the *MTHFR 677C>T* polymorphism significantly modified the risk of breast cancer associated with dietary folate consumption (22), a finding that is consistent with our current study. These findings give support to the notion that dietary folate may be protective against breast cancer.

It is worth pointing out that the main effect of the *MTHFR 677C>T* polymorphism on breast cancer risk is different from its effect on colorectal cancer. Although high folate status reduced the risk of both cancers, the *MTHFR 677TT* genotype was associated with a decreased risk of colorectal cancer (45, 46) and an increased risk of breast cancer. In the meantime, interactions between folate and *MTHFR* were similar in both diseases; the highest risk was observed among *677TT* individuals with low folate intake (45, 46). Because the MTHFR is situated at the critical junction of one-carbon metabolism balancing DNA methylation and synthesis (Fig. 1), reduced MTHFR activity conferred by the *677C→T* polymorphism may tilt the balance in favor of the DNA synthesis pathway at the expense of methyl supply (i.e., *S*-adenosylmethionine) for methylation reactions. The opposite effects of this polymorphism seem to suggest that colon and breast cancer may have different underlying etiologic pathways. This hypothesis needs to be investigated in mechanistic studies using cell lines or animal models.

Functionality of the *MTHFR 1298A>C* polymorphism has not been well established. Individuals with combined heterozygosity for *677CT-1298AC* showed reduced enzyme activities, elevated plasma homocysteine, and decreased plasma folate, similar to those with the *677TT* genotype (21); however, these findings were not entirely reproducible in other studies (29, 47). Our results confirmed that the two *MTHFR* polymorphisms were in strong linkage disequilibrium. The apparent reduced breast cancer risk associated with *1298CC*

Table 4. ORs and 95% CI for *MTHFR* polymorphisms with risk of breast cancer stratified by supplement use in the Long Island Breast Cancer Study Project, 1996-1997

Genotype	No. cases (%)	No. controls (%)	OR (95% CI)*	OR (95% CI)†
677C>T				
Supplement use = yes				
677CC	216 (40.2)	221 (38.3)	1.0 (referent)	1.0 (referent)
677CT	222 (41.3)	271 (47.0)	0.84 (0.65-1.09)	1.18 (0.82-1.68)
677TT	99 (18.4)	85 (14.7)	1.20 (0.85-1.70)	0.83 (0.64-1.09)
P, trend ‡			0.68	0.74
Supplement use = no				
677CC	174 (34.1)	213 (41.7)	1.0 (referent)	1.0 (referent)
677CT	247 (48.4)	230 (45.0)	1.33 (1.02-1.75)	1.36 (1.03-1.81)
677TT	89 (17.5)	68 (13.3)	1.55 (1.07-2.27)	1.70 (1.14-2.52)
P, trend			0.007	0.005
1298A>C				
Supplement use = yes				
1298AA	275 (51.3)	282 (49.1)	1.00 (referent)	1.00 (referent)
1298AC	214 (39.9)	235 (40.9)	0.94 (0.73-1.20)	0.93 (0.72-1.20)
1298CC	47 (8.8)	57 (9.9)	0.81 (0.53-1.24)	0.83 (0.54-1.29)
P, trend			0.34	0.38
Supplement use = no				
1298AA	274 (53.7)	249 (48.4)	1.00 (referent)	1.00 (referent)
1298AC	198 (38.8)	214 (41.6)	0.85 (0.66-1.11)	0.80 (0.61-1.05)
1298CC	38 (7.5)	51 (9.9)	0.71 (0.45-1.12)	0.62 (0.38-1.01)
P, trend			0.09	0.02

*Adjusted for age.
†Adjusted for age, family history of breast cancer in first-degree relative, history of benign breast disease, educational attainment, body mass index at age 20, and kilocalories per day.
‡*P* value for trend for categorical variables.

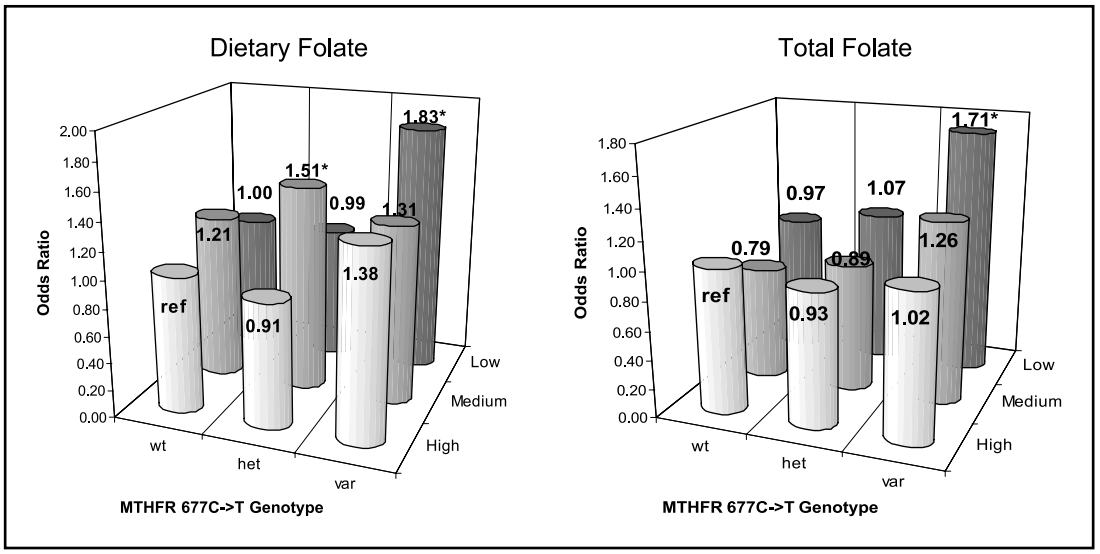

Figure 3. Interactions of the *MTHFR 677C>T* polymorphism with dietary folate (*A*) nd total folate (*B*) intake in the Long Island Breast Cancer Study Project, 1996-1997. The ORs were adjusted for age, family history of breast cancer in first-degree relatives, history of benign breast disease, education, body mass index at age 20, and daily caloric intake. *P* for interaction were 0.42 for dietary folate and 0.16 for total folate. *, $P < 0.05$.

individuals may be attributed to the fact that the *1298C* allele was highly linked with the *677C*, the low-risk allele (48). The absence of elevated risk in individuals with compound heterozygous genotype (i.e., *677CT-1298A*C) indicated that the *1298A>C* polymorphism might have limited functionality. In our study population, the frequency of *677T* and *1298C* in *cis* position (i.e., *677T-1298C* on the same chromosome) was higher than what was reported in a meta-analysis of genetically diverse populations (48). For example, the prevalence of *677TT-1298CC* genotype in our study population was 0.8% compared with 0.03% in the mixed population (48). Such discrepancy was unlikely a result of genotyping error because of the high reproducibility of paired quality control samples (i.e., 98% for *677C>T* and 99% for *1298A>C*). Alternatively, the discrepancy may reflect differences in genetic background of these populations. As shown in the meta-analysis (48), there was an increased frequency of the rare *677T-1298C* allele in Britain and Canada, possibly due to founder effect. The fact that >93% of our study subjects were Caucasians corroborates these findings.

Overall, the response rate in the Long Island Breast Cancer Study Project was lower among controls than in cases, especially among women over age 75 years (23). The study had no upper age limit, and co-morbidity among the elderly controls and the protective efforts of the subjects' families limited study participation among these older women. However, it is reassuring that in general we observed many of the established risk factors for breast cancer, including family history of breast cancer and reproductive history (23). About 73.1% and 73.3% of cases and controls who had completed the main interview donated blood. As we reported previously (23), the distribution of some breast cancer risk factors differed among blood donors and non-donors. Factors found to be associated with a decreased probability of blood donation were past smoking and increased age. Factors that were associated with an increased probability of blood donation included white or other race, use of alcohol, use of hormone replacement, practice of breast-feeding, use of hormone replacement therapy, use of oral contraceptives, and mammogram tests undergone. Other risk factors of breast cancer in our multivariate analyses, including family history, history of benign breast disease, education, body mass index at age 20, menopausal status, age at menarche, and age at menopause, did not differ with respect to donation status. Thus, there is possible bias that may influence our results. Nevertheless, it is unlikely that the choice of donating blood would differ by genotype, and the proportion of eligible subjects who donated blood is comparable with other population-based studies with a phlebotomy component (49). Thus, our results are likely to be as representative of the general population as those from other major population-based studies of breast cancer. Another limitation is the lack of measurement of biological folate status (folate in plasma or RBC). We did not measure biological folate levels for the Long Island Breast Cancer Study Project because of the case-control design of the study; biological samples were collected after disease diagnosis so the biological folate levels may have been influenced by the onset, development, or even treatment of the disease.

The major strength of this study lies in its population-based study design in which cases encompassed a broad age range and were drawn from a population-based sample. Thus, results of this study may be more generalizable than a series of cases from a narrow age range or from a single institution. In addition, the relatively large sample size allows multiple risk factors to be taken into consideration in studying associations, with the ability to conduct stratified analyses and adjustment in multivariate models.

In summary, this population-based study adds to the increasing evidence that risk of breast cancer is reduced in relation to intake of dietary folate and related B vitamins, especially among non-supplement users. Further, it seems that suboptimal folate metabolism increases the susceptibility to breast cancer, especially among those with insufficient folate intake; however, such enhanced risk may be reduced by increasing folate consumption. Although several risk factors such as family and reproductive history have been associated with breast cancer, few modifiable factors have been identified to reduce the disease risk. From a public heath perspective, it is important to identify such risk factors, such as B vitamin consumption, that may guide an effective prevention strategy against the disease.

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