A functional 19-base pair deletion polymorphism of dihydrofolate reductase *(DHFR)* and risk of breast cancer in multivitamin users[1–3]

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ABSTRACT

Background: Dihydrofolate reductase (DHFR) converts dihydrofolate (DHF) into tetrahydrofolate (THF) and plays an essential role in cell metabolism and cellular growth. Folic acid from multivitamins needs to be reduced by DHFR before it participates in cellular reactions.

Objectives: We examined the relation of a 19-base pair (bp) deletion polymorphism of the *DHFR* gene with the risk of breast cancer by using data from the Long Island Breast Cancer Study Project, a population-based case-control study. We also investigated the transcriptional effect of this deletion polymorphism.

Design: Dietary data and habitual use of multivitamins were assessed from a modified Block food-frequency questionnaire (FFQ). Genotypes of *DHFR* were ascertained from 1062 case subjects and 1099 control subjects by allele-specific polymerase chain reaction. Unconditional logistic regression was used to estimate odds ratios (ORs) and 95% CIs.

Result: Although the *DHFR* 19-bp deletion polymorphism was not associated with overall breast cancer risk, we observed a borderline significant additive interaction ($P = 0.06$) between the *DHFR* genotype and multivitamin use. The −19-bp allele was associated with greater breast cancer risk in multivitamin users (51.2% of the study population) with an OR of 1.26 (95% CI: 0.96, 1.66) and 1.52 (95% CI: 1.08, 2.13) for the +/− and −/− genotypes, respectively (P for trend = 0.02) than in multivatimin nonusers. A dose-dependent relation (P for trend < 0.001) between *DHFR* expression and the deletion genotype was observed. Compared with the subjects with the 19-bp +/+ genotype, subjects with the −/− genotype had 4.8-fold DHFR mRNA levels.

Conclusions: The *DHFR* 19-bp deletion polymorphism affects the transcription of *DHFR* gene in humans. Multivitamin supplements may place a subgroup of women (ie, those with the −19-bp allele) at elevated risk of developing breast cancer. *Am J Clin Nutr* 2007; 85:1098–102.

KEY WORDS Folate, dihydrofolate reductase, DHFR, one-carbon metabolism, breast cancer, multivitamins, prospective study, epidemiology, diet

INTRODUCTION

Folate and its derivatives play a central role in transporting the methyl moiety in one-carbon metabolism (**Figure 1**) that facilitates the cross-talk between DNA synthesis and methylation (1). Given that few modifiable risk factors have been identified for breast cancer, there has been intense interest in the potential anticarcinogenic role of folate in breast cancer. Several epidemiologic studies, including 3 large prospective cohorts—the Nurses' Health Study, the Canadian National Breast Screening Study, and the Iowa Women's Health Study—suggest that adequate folate intake is important in the prevention of breast cancer, particularly in women with moderate alcohol consumption (2–4). However, no effects of folate consumption on the risk of breast cancer were observed in 2 other cohorts, including the American Cancer Society's Cancer Prevention Study (5) and Melborne Collaborative Cohort (6). Adding to these conflicting results, the latest report from a large cohort, the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial, indicated an ≈20% increase of breast cancer risk associated with folic acid supplemental use of ≥400 µg/d in postmenopausal women (7).

Ingested folate must be fully reduced by dihydrofolate reductase (DHFR), a key enzyme in one-carbon metabolism, before being used in cell metabolism (8). The *DHFR* gene encodes the enzyme that catalyzes the conversion of dihydrofolate (DHF) into tetrahydrofolate (THF). It plays an essential role in cell metabolism and cellular growth (8) by shuttling the methyl group with the use of THF for de novo synthesis of a variety of essential metabolites. Although folate naturally found in foods are not fully absorbed through the intestinal lining, they are predominantly in the fully reduced form, 5-methyl-THF; meanwhile, the fully unreduced (eg, folic acid) and partially reduced (eg, DHF) forms are also found (9). In contrast, folate in multivitamin supplements and food fortification is purely folic acid, the fully unreduced form that requires the action of DHFR before it can
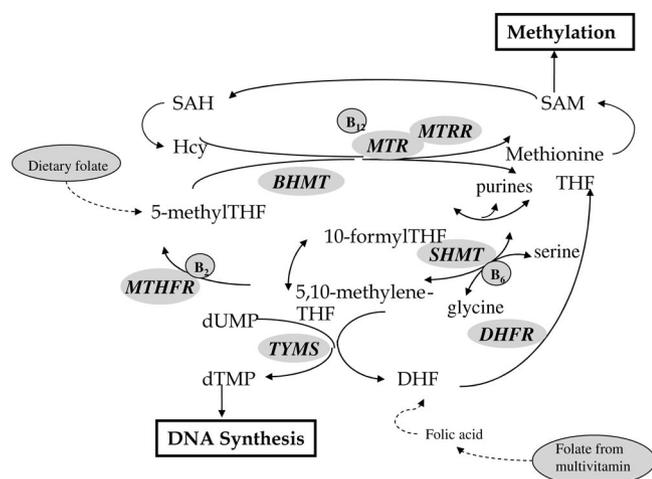
FIGURE 1. Schematic illustration of the one-carbon metabolism pathway. Key genes involved in one-carbon metabolism include methylenetetrahydrofolate reductase (*MTHFR*), thymidylate synthase (*TS*), methionine synthase (*MTR*), methionine synthase reductase (*MTRR*), serine hydroxymethyltransferase (*SHMT*), dihydrofolate reductase (*DHFR*), and betaine-homocysteine methyltransferase (*BHMT*). 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) is the substrate for synthesis of both thymidylate and 5-methylTHF. The latter reaction directs the folate pool toward remethylation of homocysteine (Hcy) to methionine. SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine.

participate in cellular reactions (8). These properties led us to hypothesize that alteration of DHFR function may affect folate utilization from supplement use.

A 19-bp deletion (hereafter, the 19-bp deletion allele is referred to as the $-$19-bp allele) polymorphism in intron 1 of the *DHFR* gene was identified (10). This polymorphism may be functional, because the $-$19-bp allele removes a putative transcription factor binding site for Sp1, which affects gene regulation (11). However, there is no direct mechanistic evidence to illustrate the functionality of this polymorphism.

To the best of our knowledge, the 19-bp deletion polymorphism of *DHFR* has not been investigated in the context of any human cancer. Herein, we examined the association of *DHFR* polymorphism and risk of breast cancer in the Long Island Breast Cancer Study Project (12), a population-based case-control study. We also explored the functionality of this deletion polymorphism by examining *DHFR* expression levels with respect to different genotypes.

SUBJECTS AND METHODS

Study population

We utilized the resource of Long Island Breast Cancer Study Project, which was designed to determine whether the risk of breast cancer is associated with polycyclic aromatic hydrocarbon–DNA adducts and organochlorine compounds. Detailed descriptions of the study have been published elsewhere (12, 13). Briefly, case subjects (hereafter termed cases) were women residents of Nassau and Suffolk counties on Long Island, NY, who were of any age or race, spoke English, and were newly diagnosed with in situ or invasive breast cancer between 1 August 1996 and 31 July 1997. Control subjects (hereafter termed controls) were frequency-matched to the expected age distribution of the cases, and identified through random-digit dialing for women

aged $<$65 y and through the Center for Medicare and Medicaid Services rosters for women aged $\geq$65 y. Eligible controls were women who spoke English and resided in the same Long Island counties as the cases, but with no personal history of breast cancer. In-person interviews were completed for 82.1% of cases ($n = 1508$) and 62.8% of controls ($n = 1556$). Of those who completed an interview, 73.1% of cases ($n = 1102$) and 73.3% of controls ($n = 1141$) donated a blood sample (12). As previously reported (12), an increase in breast cancer in women from Long Island was found to be associated with lower parity, late age at first birth, little or no breastfeeding, a family history of breast cancer, and increasing income and education. Results were similar when the analyses were restricted to respondents who donated blood or for those with DNA available for these analyses (14). Dietary intake in the year before the interview was assessed with a modified Block Food Frequency Questionnaire (FFQ) (15, 16). The frequency and portion sizes data were translated to daily intakes of nutrients from both dietary and supplement sources by using the National Cancer Institute's DietSys version 3. Habitual use of multivitamin supplements was also obtained from the FFQ. The questions included multivitamin use over the past 10–15 y before the interview, type of multivitamin, and dosage. These multivitamins were assigned a 400 μg folic acid dose. The study protocol was approved by the Institutional Review Boards at the collaborating institutions.

Genotyping

DNA was isolated from the blood specimens by using the methods previously described (13). Genotypes were determined by modified PCR amplification by using allele-specific primers described elsewhere (10). The products were separated on 3% agarose gels and visualized with ethidium bromide. About 10% of the study population were randomly duplicated and included as quality control samples; the concordance rate was 99%. The call rate for our genotyping assay was 96%. The main reason for those genotypes not ascertained was insufficient amount of DNA left for genotyping.

DHFR expression

We obtained blood samples from 36 healthy blood donors from the New York Blood Center. The lymphocytes were homogenized by using the QIAshredder Mini Spin Column (QIAGEN, Valencia, CA); total RNA was then isolated by using RNeasy Mini-Kit (QIAGEN) with DNase treatment (RNase-Free DNase Kit; QIAGEN). The purity of the isolated total RNA was analyzed by absorption at 260 nm and 280 nm, and the concentration was measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

β-actin was used as an internal control to normalize the *DHFR* mRNA levels. The primers used in the real-time RT-PCR were as follows: *DHFR* forward: 5′-TTCCGGCGAGACATGGCAGGGCAA-3′; *DHFR* reverse: 5′-CGCCCGCAGACGCCTGGGAA-3′; β-*actin* forward: 5′-ACTGGAACGGTGAAGGTGAC-3′; and β-*actin* reverse: 5′-GTGGACTTGGGAGAGGACTG-3′.

Real-time qPCR was performed by using an ABI 7900HT instrument (Applied Biosystems, Foster City, CA), and the signal was detected with SYBR Green I (Invitrogen, Eugene, OR). Total RNA was reverse-transcribed and subsequently amplified by AccuRT, a thermostable magnesium activated reverse

transcriptase–DNA polymerase that was provided generously by Roche Molecular Systems (Alameda, CA). The PCR specificity was checked by melting curve analysis. Ct values (the cycle number at the point at which the fluorescence from the template crosses the threshold) for each reaction were determined with the instrument's software, SDS 2.2, choosing the threshold at 0.76. Measurements were performed in triplicate from which the median value was chosen for statistical analyses. Levels of *DHFR* mRNA were normalized to the housekeeping gene, β-*actin*. The relative expression level was calculated as follows: $1/(2e\Delta Ct)$, where $\Delta Ct = Ct_{(DHFR)} - Ct_{(\beta\text{-}actin)}$. As one can see, the higher the ΔCt values, the lower the *DHFR* expression relative to β-*actin*.

Statistical analysis

The Hardy–Weinberg equilibrium was tested with the Pearson goodness-of-fit statistic to compare the observed and expected genotype frequencies in cases and controls, respectively (17). Univariate analyses were done to compare distributions of covariates in cases and controls. Unconditional logistic regression was used to estimate odds ratios (ORs) and corresponding 95% CIs for the association between the *DHFR* 19-bp deletion polymorphism and breast cancer, with adjustment for the frequency-matching variable age (as a continuous variable) in addition to other potential confounding factors (18, 19).

Besides age-adjusted models, we also evaluated for potential confounding. A priori, we did not expect genotype status to be associated with many of these factors, but because of their potential association with breast cancer, we considered the following: family history of breast cancer in a first-degree relative, history of benign breast disease, education, body mass index (BMI) at age 20 y, BMI at diagnosis, alcohol drinking, parity, ever lactation, contraceptive use, hormone replacement therapy (HRT) use, age at menarche, months of lactation, age at first birth, number of miscarriages, history of fertility problems, race, religion, and marital status. If addition of the selected covariate to the logistic regression model changed the effect estimate by 10% or more, then the covariate was considered a confounder (18). None of the variables met such criteria, so only the age-adjusted model results are presented.

We evaluated interactions on additive and multiplicative scales between *DHFR* genotypes and multivitamin supplement use by using indicator terms for those with the genotypes only, exposures only, and those with both the genotypes and exposures of interest. Multiplicative interactions were examined by a comparison of the log-likelihood statistic for models that included a multiplicative interaction term in the logistic regression model to those without (19). The magnitude of an additive interaction effect was determined by estimating the interaction contrast ratio (ICR) and 95% CI departures from additive effects with the following formula (20) using PROC LOGISTIC program in SAS (21):

$$ICR = OR_{eg} - OR_e - OR_g + 1 \qquad (1)$$

where OR_{eg} is the OR for exposure with variant genotype, OR_e is the OR for exposure with a wild-type genotype, and OR_g is the OR for variant genotype in nonexposed. The 95% CI of the ICR was obtained from ICR ± 1.96 SE (ICR). An ICR > 0 implies a greater than additive interaction (positive interaction), whereas an ICR = 0 implies no interaction and an ICR < 0 implies a less than an additive effect (negative interaction).

TABLE 1
Effects of *DHFR* genotypes and breast cancer risk

Genotype	Case subjects	Control subjects	OR (95% CI)[1]
		n (%)	
Overall			
19 bp +/+	335 (31.5)	375 (34.1)	1.00 (reference)
19 bp +/−	518 (48.8)	518 (47.1)	1.14 (0.94, 1.38)
19 bp −/−	209 (19.7)	206 (18.7)	1.18 (0.93, 1.51)[2]
Multivitamin user			
19 bp +/+	157 (29.3)	202 (35.3)	1.00 (reference)
19 bp +/−	258 (48.1)	268 (46.8)	1.26 (0.96, 1.66)
19 bp −/−	121 (22.6)	103 (18.0)	1.52 (1.08, 2.13)[3]
Multivitamin nonuser			
19 bp +/+	170 (33.3)	170 (33.3)	1.00 (reference)
19 bp +/−	252 (49.4)	243 (47.5)	1.03 (0.78, 1.36)
19 bp −/−	88 (17.3)	98 (19.2)	0.95 (0.66, 1.36)

[1] Adjusted for age (continuous).

[2] *P* = 0.06 for additive interaction between *DHFR* deletion polymorphism and multivitamin use.

[3] *P* for trend = 0.02.

ANOVA was carried out to examine mean *DHFR* expression levels with respect to genotypes. All statistical analyses were performed by using SAS version 9.1 (SAS Institute, Cary, NC).

RESULTS

The *DHFR* −19-bp genotypes were ascertained in 1062 cases and 1099 controls. The *DHFR* −19-bp allele frequencies were 0.40 and 0.42 in the cases and controls, respectively. The genotype distribution was in agreement with Hardy-Weinberg Equilibrium in both cases (*P* = 0.73) and controls (*P* = 0.25). Overall, the *DHFR* 19-bp deletion polymorphism was not significantly associated with breast cancer risk. Compared with individuals with the 19-bp +/+ genotype, those with the −/−-genotype had an OR of 1.18 (95% CI: 0.93, 1.51) (**Table 1**). The association between *DHFR* and breast cancer did not differ significantly with respect to menopausal status (pre- or postmenopausal) and cancer type (in situ versus invasive; data not shown).

The age-adjusted ICR suggested deviations from the additive scale for the *DHFR* deletion allele combined multivitamin supplement use (ICR: 0.43; 95% CI: −0.02, 0.87; *P* = 0.06), which indicated an additive interaction with borderline significance between the *DHFR*-deletion polymorphism and multivitamin use. Furthermore, examination of the association between *DHFR* and breast cancer by multivitamin use showed a stronger effect of the *DHFR* genotype in multivitamin users (Table 1). About one-half (51.2%) of the study population used multivitamins on a regular basis (≥1 time/wk) over the 10–15 y before enrollment. As previously reported (16), in our study population, multivitamin use was not significantly associated with the risk of breast cancer (OR: 0.95; 95% CI: 0.83, 1.10). However, as shown in Table 1, in these 536 multivitamin users, the 19-bp −/− genotype was associated with a 52% increase in breast cancer risk (95% CI: 1.08, 2.13) compared with individuals with the +/+ genotype. The *DHFR* −19-bp allele has an additive effect on the risk of breast cancer in this group (*P* for trend = 0.02). No apparent association between the *DHFR* 19-bp deletion polymorphism and the risk of breast cancer was observed in nonusers. Furthermore, there were no significant interactions between the

TABLE 2
Quantification of *DHFR* mRNA in 3 different genotypes[1]

	DHFR genotype			
	+/+ (n = 9)	+/− (n = 22)	−/− (n = 5)	P[2]
ΔCt[3]	4.5 ± 1.1[4]	3.4 ± 1.4	2.0 ± 0.8	0.003
DHFR/β-*actin* mRNA levels[5]	0.06 ± 0.07	0.15 ± 0.14	0.29 ± 0.18	0.013
Increase in expression (-fold)	1 (ref)	2.4	4.8	

[1] *DHFR* mRNA was standardized to β-*actin* mRNA.

[2] ANOVA was used to test for significant differences between means of expression of different genotypes by comparing their variances.

[3] $\Delta Ct = Ct_{(DHFR)} - Ct_{(\beta\text{-}actin)}$.

[4] $\bar{x} \pm SD$ (all such values).

[5] Relative mRNA level $= 1/(2e \, \Delta Ct)$.

DHFR 19-bp deletion polymorphism and dietary or total folate intake (data not shown).

As an attempt to investigate the function of the *DHFR* polymorphism, we quantified *DHFR* mRNA levels from freshly collected lymphocytes of 36 healthy blood donors from the New York Blood Center. The mean ΔCt of these 36 samples was 3.4, indicating that *DHFR* expression level was ≈10% of β-*actin* expression. Nine individuals had the 19-bp +/+ genotype, 22 had the +/− genotype, and 5 had the −/− genotype. The mean ΔCt values were 4.5, 3.4, and 2.0 for the 19-bp +/+, +/−, and −/− genotypes, respectively, translating to 6%, 15%, and 29% expression levels relative to β-*actin* (**Table 2**). These numbers represented a significant dose-dependent relation (*P* for trend < 0.001) between *DHFR* expression and the deletion genotype. Compared with the 19-bp +/+ genotype, the +/− and −/− genotypes had a 2.4-fold and 4.8-fold increase in mRNA levels, respectively (Table 2).

DISCUSSION

Despite intense interest in potential preventive effects of folate against breast cancer, the role of folate in breast carcinogenesis is far from elucidated. Although several large cohort studies suggested that higher folate intake was associated with lower breast cancer risk, particularly in moderate alcohol drinkers (2–4), the latest results from a large screening trial indicated that overconsumption of folate, particularly from vitamin supplements, was detrimental in terms of breast cancer risk (7). Folic acid from vitamin supplements and food fortification needs to be reduced before participating in cellular reactions (8). Folic acid fortification of the US diet was implemented by the Food and Drug Administration in January 1998. Subjects of the Long Island Brest Cancer Study Project were recruited between 1996 and 1997, before this mandate. Thus, we anticipate little contribution of folic acid to dietary folate from food fortification; however, more than one-half of these women habitually took multivitamin supplements.

We observed that the *DHFR* 19-bp −/− genotype, which was associated with increased gene expression in our functional study, increased the risk of breast cancer in multivitamin users in a dose-dependent fashion. This finding seems to support the latest findings from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (7)—that too much folic acid from supplements may be procarcinogenic. Higher expression of *DHFR*, presumably leading to higher enzyme activity of DHFR, may lead to depletion of the 5,10-methylene-THF pool, the critical

substrate for both DNA synthesis and homocysteine remethylation that provides the methyl donor for methylation reactions (Figure 1). High DHFR activity may tilt the balance of one-carbon metabolism in favor of DNA synthesis at the expense of methyl supply (ie, *S*-adenosylmethionine) for methylation reactions; a suboptimal methyl supply can lead to aberrant DNA methylation, which has been associated with breast carcinogenesis (22).

Our functional study showed that the *DHFR* −19-bp allele was associated with increased gene expression in a dose-dependent fashion. One possible mechanism is that this 19-bp sequence resides within an inhibitory element that regulates transcription. On removal, gene expression is up-regulated and more mRNA is made. The 19-bp deletion is located in intron 1 of the *DFHR* gene. Intron 1 is well known to be a site of regulatory sequences for some genes, eg, as either a transcription factor binding site or a site that affects splicing (23, 24). An intron 1 regulatory sequence has been documented for mouse *DHFR* (11). Although there is a report that used an in silico approach and predicted the removal of a potential Sp1 binding site by the 19-bp deletion polymorphism of human *DHFR* (25), there has been no direct laboratory evidence showing the intron 1 region of *DHFR* has any transcriptional effect before our study.

A limitation of our expression assay is that *DHFR* expression may be influenced by diet or supplement use, so that mRNA levels would be heterogeneous in samples tested due to lack of lifestyle information on these individuals. However, we were unable to find reports suggesting that environmental factors affect *DHFR* expression level. Elevated DHFR activity in response to methotrexate treatment has been reported (26), but the increase was at the protein (ie, translational) level because it was unaffected by the transcriptional inhibitor actinomycin D but was blocked by the translational inhibitor cycloheximide (27). More importantly, should there be an environmental influence on *DHFR* expression, the environmental factor would unlikely be differentially related to genotype.

The major strength of the present study lies in its study design in which subjects were drawn from a population-based sample and detailed assessment of diet and risk factors of breast cancer were obtained. The relatively large sample size allowed stratified analyses by various lifestyle factors such as multivitamin use. In our case, the sample size (536 cases and 573 controls who were multivitamin users) provided 80% power to detect an OR of 1.4 (α at 0.05 level). We also used a functional genomic approach to help us gain insight of the relation between *DHFR* and breast

cancer. The major potential limitation is the lack of measurements of folate concentrations in blood. These concentrations were not measured for the subjects in our study population because of the case-control study design; biological samples collected after disease diagnosis may have been influenced by the onset, development, or even treatment of the disease. Another limitation lies in the fact that the individuals selected for the functional study of *DHFR* were different from the main study population. Our study used the existing resource of the Long Island Breast Cancer Study Project. Like most of the existing epidemiologic studies, only whole blood was collected, and no mRNA was available for functional analyses. Nevertheless, functional results from healthy blood donors from the New York Blood Center should be generalizable to the general population including the Long Island Breast Cancer Study Project, because potential confounders such as environmental influence are unlikely to be differentially related to genotypes.

In conclusion, we reported that a common 19-bp deletion polymorphism of *DHFR* affected gene transcription. This polymorphism modifies the risk of breast cancer in multivitamin supplement users. The use of multivitamin supplements may exert adverse risks in a subgroup of women (ie, those with the −19-bp allele). If these findings are replicated by other study, it may warrant careful reconsideration of multivitamin supplement use for disease prevention.

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