Title
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Permalink
https://escholarship.org/uc/item/1523f69k

Journal
Breast cancer research and treatment, 77(3)

ISSN
0167-6806

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Publication Date
2003-02-01

DOI
10.1023/a:1021843019755

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Peer reviewed
Report

Breast cancer risk and *methylenetetrahydrofolate reductase* polymorphism

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Key words: alcohol, breast cancer, folate, *methylenetetrahydrofolate reductase*, *MTHFR*, postmenopausal

Summary

**Objective.** Methylenetetrahydrofolate reductase (*MTHFR*), a polymorphic enzyme involved in folate metabolism, plays a role in DNA biosynthesis, methylation, and repair in actively dividing cells. Because breast-cell division occurs in women with active ovulatory cycles, polymorphisms in the *MTHFR* gene could be a risk factor for breast cancer.

**Methods.** We genotyped 352 clinic-based study subjects for *MTHFR*, 105 subjects with breast cancer and 247 with benign breast disease, histopathologically classified as high-risk or low-risk for breast cancer. Questionnaire data were collected prior to biopsy to blind subjects and interviewers to diagnoses.

**Results.** Premenopausal women with the *MTHFR* polymorphism had a threefold increased breast cancer risk (OR = 2.8; 95%CI: 1.02–7.51) compared to the clinic-based controls with benign breast disease. Results were similar using either low- or high-risk controls. However, risk for postmenopausal women was not elevated (OR = 0.8; 95%CI 0.4–1.4). No significant interaction between genotype and smoking or alcohol was found, but polymorphic *MTHFR* decreased the likelihood of drinking alcohol (OR = 0.5; 95%CI 0.3–0.9).

**Conclusion.** These data suggest that polymorphic *MTHFR* increases risk of premenopausal, but not postmenopausal, breast cancer. These findings should be explored with a larger sample size in order to analyze gene–environment interactions between *MTHFR* and folate. Once the intricate relationship between diet and breast cancer has been elucidated, new cancer control initiatives can be considered such as folate chemoprevention trials in high-risk individuals.

Introduction

Methylenetetrahydrofolate reductase (*MTHFR*) has been implicated in carcinogenesis: the biochemical product of *MTHFR* (*N*⁵-methyl THF) is required for DNA methylation, while the substrate of *MTHFR* (*N*⁵, *N*¹⁰-methylene THF) is essential for both purine nucleotide biosynthesis and methylation of deoxuryridine monophosphate (dUMP) to dTMP (Figure 1). DNA methylation is an important epigenetic determinant of gene regulation of protooncogenes and tumor suppressor genes, as well as the estrogen receptor gene. Breast cancerogenesis could be initiated through activation of protooncogenes by hypomethylation of their promoter regions [1]; or through inactivation of tumor suppressor genes by hypermethylation [2]; or through alteration of estrogen receptor gene methylation patterns [3].

A common polymorphism has been described that increases the thermolability of *MTHFR*, resulting in elevated plasma levels of homocysteine and decreased 5-methyltetrahydrofolate levels [4]. Based on these findings we hypothesized that variant *MTHFR* alleles might alter the risk of breast cancer. Considering the fact that cell kinetics of breast tissue stem cells are closely linked to ovarian hormones, disruption of these essential biochemical processes should have the greatest effect in women with active ovulatory cycles.
In order to test these hypotheses we conducted a case-control study of premenopausal and postmenopausal breast cancer. This is the first paper to present an analysis of the risk of breast cancer from polymorphisms in \textit{MTHFR}. Subjects were recruited from breast centers in Orange County, CA, where study subjects were undergoing breast biopsy. They were classified either as a breast cancer case or as breast cancer free, subsequent to epidemiologic data collection.

\textbf{Materials and methods}

\textbf{Subjects}

Women older than 39 years of age with a suspicious breast mass detected clinically and/or by diagnostic mammography were enrolled in a previously described case-control study in Orange County, CA [5]. Prior to the biopsy date, a self-administered risk factor questionnaire was distributed to subjects. Subjects were instructed to return them by mail before receiving their diagnosis, which we confirmed, otherwise the data were invalidated to assure that subjects were blinded. It was necessary to have 17 subjects complete questionnaires in the breast center while they waited for diagnostic results. Of 535 eligible patients, 352 subjects were included in this analysis, while 86 declined participation and four refused blood donation. An additional 93 subjects were excluded from the analysis because they failed to complete all questionnaires prior to receiving diagnostic results \((n = 51)\) or had incomplete questionnaire/genetic data \((n = 9)\). Because the \textit{MTHFR} allele frequency differs considerably by race/ethnicity [6] and because we did not have sufficient study subjects for a subanalysis in different categories, we only included white non-Hispanics in our analysis. This required the exclusion of 33 subjects (15 Asians, 16 Hispanics and two Blacks).

Histopathological criteria were used to stratify the clinic-based control population with benign breast disease into two groups of individuals having either high- or low-risk of developing future breast cancer [7]. Women with atypical hyperplasia and women with proliferative disease without atypia were classified as high-risk, while women without proliferative disease were defined as low-risk.
The Institutional Review Board at the University of California, Irvine, approved the study proposals as well as the manner in which informed consent was obtained from subjects.

**MTHFR genotype**

Samples were genotyped for MTHFR allele 677CC [8] and classified as variant homozygous (TT corresponding to val/val), variant heterozygous (CT corresponding to val/ala), and wild-type homozygous (CC corresponding to ala/ala). The variant allele is considered ‘thermolabile’, because the activity of the encoded enzyme is reduced at 37°C or higher. Heterozygotes have up to 60% reduced enzymatic activity, while heterozygotes are in an intermediate range [9]. A specific set of primers was used to amplify a 198 bp fragment that contained the polymorphic allele. The primers MTHFR_A (5′-TGA AGG AGA AGG TGT CTG CGG GA-3′) and MTHFR_B (5′-AGG ACG GTG CGG TCA GAG TG-3′) were used at 50 pmol concentration, with 200 µmol dNTPs, PCR buffer, and 0.5 unit Taq polymerase (Gibco, Inc., Rockville, MD). Genomic DNA concentration was adjusted to 200 ng per 50 µl PCR reaction and denatured at 95°C for 2 min prior to the reaction. The PCR reaction was conducted in an MJ Research thermocycler under the following conditions: 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 30 cycles. The restriction endonuclease HinfI (New England Biolabs, Beverly, MA) was used to digest 10 µl of the amplification product in the presence of the appropriate buffer (NEB#2) to determine the presence of MTHFR 677CC, CT, or TT polymorphic sites, respectively.

**Statistical analysis**

We used unconditional logistic regression to determine the risk of breast cancer for subjects with at least one variant allele of MTHFR (CT and TT combined) was not elevated (OR = 1.1; P = 0.65; Table 2) compared to the clinic-based control subjects, adjusting for age and family history of breast cancer. However, we found premenopausal women to be at risk for breast cancer if they had a variant allele of MTHFR. In this clinic-based population there was a significant, threefold increased breast cancer risk (OR = 2.8; P < 0.05) (Table 2) for premenopausal, but not postmenopausal women (OR = 0.8; P = 0.45). This risk remained unchanged in premenopausal women that drank at least one and a half drinks per week (drink defined as one can of beer, one glass

**Table 1. Allele frequency of MTHFR for case and control subjects, Orange County, CA**

|        | Premenopausal | Postmenopausal | Total |
|--------|---------------|----------------|-------|
| Case subjects |               |                |       |
| MTHFR CCa | 8 (32%)       | 34 (43%)       | 42 (40%) |
| MTHFR CTb | 15 (60%)      | 43 (54%)       | 58 (55%) |
| MTHFR TTb | 2 (8%)        | 3 (4%)         | 5 (5%)  |
| Clinic-based control subjects |               |                |       |
| MTHFR CC | 51 (51%)      | 61 (41%)       | 112 (45%) |
| MTHFR CT | 41 (41%)      | 70 (48%)       | 111 (45%) |
| MTHFR TT | 8 (8%)        | 16 (11%)       | 24 (10%) |
| Total   | N = 125       | N = 227        | N = 352 |
| MTHFR CC | 59 (47%)      | 95 (42%)       | 154 (44%) |
| MTHFR CT | 56 (45%)      | 113 (50%)      | 169 (48%) |
| MTHFR TT | 10 (8%)       | 19 (8%)        | 29 (8%)  |

a Wild type at position 677.

b Variant (677C → T).
Table 2. Risk of breast cancer from polymorphisms in the MTHFR gene: multivariate models using different control groups\textsuperscript{a}

|               | Premenopausal |                | Postmenopausal |                | Overall |                |
|---------------|--------------|----------------|----------------|----------------|---------|----------------|
|               | Cases Controls OR\textsuperscript{b}(95% CI) | Cases Controls OR\textsuperscript{b}(95% CI) | Cases Controls OR\textsuperscript{b}(95% CI) |
| Clinic-based benign breast disease controls | | | | | |
| MTHFR 677CC\textsuperscript{c} | 7 (29%) 51 (52%) 1.00 (referent) | 34 (43%) 58 (41%) 1.00 (referent) | 41 (39%) 109 (45%) 1.00 (referent) |
| MTHFR 677CT/TT\textsuperscript{d} | 17 (71%) 48 (48%) 2.77 (1.02–7.51) | 46 (57%) 85 (59%) 0.80 (0.44–1.44) | 63 (61%) 133 (55%) 1.13 (0.69–1.85) |
| Clinic-based benign breast disease controls: low-risk | | | | | |
| MTHFR 677CC | 7 (29%) 23 (50%) 1.00 (referent) | 34 (43%) 27 (33%) 1.00 (referent) | 41 (39%) 50 (39%) 1.00 (referent) |
| MTHFR 677CT/TT\textsuperscript{d} | 17 (71%) 23 (50%) 2.27 (0.76–6.81) | 46 (57%) 54 (67%) 0.54 (0.26–1.09) | 63 (61%) 77 (60%) 0.90 (0.51–1.60) |
| Clinic-based benign breast disease controls: high-risk | | | | | |
| MTHFR 677CC | 7 (29%) 22 (49%) 1.00 (referent) | 34 (43%) 27 (52%) 1.00 (referent) | 41 (39%) 49 (51%) 1.00 (referent) |
| MTHFR 677CT/TT\textsuperscript{d} | 17 (71%) 23 (51%) 3.08 (1.00–9.50) | 46 (57%) 25 (48%) 1.37 (0.65–2.87) | 63 (61%) 48 (49%) 1.60 (0.87–2.92) |

\textsuperscript{a} One case and five control subjects are excluded due to missing questionnaire data for covariates in the multivariate model. For 17 controls undergoing only fine needle aspirations or core biopsies there was insufficient tissue surrounding the fibroadenoma to classify the proliferative state of the lesion into low- or high-risk. Therefore, they were used only in the analyses including all controls.

\textsuperscript{b} Adjusted odds ratio for age and family history of breast cancer.

\textsuperscript{c} Wild type MTHFR 677CC (ala/ala) is referent.

\textsuperscript{d} Defined as at least one variant allele of MTHFR: 677CT (ala/val) or 677TT (val/val).
of wine, or one shot of liquor; \( n = 283 \) (OR = 2.9; 95%CI 0.9–8.9). In order to further explore the impact of the MTHFR variant on breast cancer risk, we stratified the analysis by control group. The odds ratio for premenopausal women using high-risk controls was 3.1 (\( P = 0.05 \)) compared to 2.3 (\( P = 0.14 \)) using low-risk controls, although these findings were not statistically significant (Table 2).

### Discussion

Our data show that MTHFR variants are a risk factor for premenopausal breast cancer in the analysis of both the low- and high-risk control groups. The literature consistently shows little or no risk of developing future breast cancer for women with non-proliferative benign breast disease but an increased risk for women with proliferative disease or atypical hyperplasia [7]. However, our results show little difference between breast cancer models using either low- or high-risk benign breast disease controls.

The finding that only premenopausal women, and not postmenopausal women, show an association between breast cancer and MTHFR variants may be related to an early phase of breast cancer pathogenesis: exposure of breast tissue to ovarian hormones drives cell kinetics of breast tissue stem cells, resulting in carcinogenesis [10]. Dividing cells are particularly susceptible to alterations in DNA synthesis, DNA repair, and DNA methylation. Since the MTHFR polymorphism affects these biochemical processes it is possible that premenopausal women with cyclic ovarian activity and thus with active breast cell division would be at increased risk for breast cancer.

In order to verify our findings from the clinic-based study, population-based control subjects with no history of cancer were randomly selected from Orange County residents [11] using random digit dialing methodology (98% of households have a phone in Orange County, CA). Of 145 women who agreed to participate in the study and to donate either a blood sample or a cheek cell sample, we analyzed and genotyped the 61 women who were >39 years of age and had no history of breast biopsy. We only included white non-Hispanics in our analysis and the median age for the population-based control population was 59 years.

We found the distribution of CC, CT, and TT genotypes in the population-based control subjects to be 56, 41, and 3%, respectively, and the allele frequency of the variant allele to be 24%. A frequency of 3% for homozygosity of MTHFR 677TT is slightly lower than previously reported in the literature (range: 4.8–30.2%; for review see [6]), which could indicate a maldistribution in these population-based controls. Nevertheless, we repeated the logistic regression analysis above with cases versus the population-based controls and found the overall OR for MTHFR variants to be only marginally elevated (OR = 2.1; 95%CI 1.1–4.1), controlling for age and family history of breast cancer. However, similar to the findings above, a stratified analysis of premenopausal women revealed a multivariate-adjusted OR of 5.3 (95%CI 1.2–22.2), while the risk for postmenopausal women was only minimally elevated (OR = 1.6; 95%CI 0.7–3.4). While consistent with the findings from the clinic-based controls, the main limitation to this ancillary analysis is that the clinic-based population, although internally comparable, may not be representative of the general Orange County population from which the population-based controls were selected. Despite the fact that the number of population-based controls is smaller than the number of breast cancer case subjects, the validity of the ancillary findings should not be affected by the number of controls as long as the ratio of exposed to unexposed controls is a representative ratio of the source population. However, sample size will affect the variance of our point estimate.

Polymorphic MTHFR variants have been found to be associated with decreased risk of colorectal cancer [12–14]—for a review see [15]—and of acute lymphatic leukemia [16]. In contrast, MTHFR variants have been shown to be associated with an increased risk of endometrial and ovarian tumors [17, 18]. This discrepancy can be explained by differences of MTHFR expression in different tissues. Our data indicate that individuals with at least one polymorphic variant of MTHFR (heterozygous and homozygous combined) are at increased risk for premenopausal breast cancer. Recently, a case series of Israeli women with breast and ovarian cancer found that the polymorphic allele is significantly more common in cases of bilateral breast cancer as compared with unilateral cancer or with combined breast and ovarian cancer [19].

Because dietary folate deficiency has been suggested as a risk factor for breast cancer, it is possible that genetically controlled folate availability also modifies the risk for breast cancer, because reduced MTHFR enzyme capacity results in diminished 5-methyltetrahydrofolate availability for homocysteine
to be converted to methionine by the methionine synthetase (Figure 1). Methionine is converted to S-adenosylmethionine, which in turn is a substrate for the methyltransferase involved in DNA methylation. Our finding that individuals with a polymorphic variant of MTHFR were at increased risk for premenopausal breast cancer may be attributable to perturbed DNA methylation. Alterations in methylation patterns can result in protooncogene activation, tumor suppressor gene inactivation, or estrogen receptor gene misregulation.

Alcohol consumption has been shown to increase breast cancer risk [20] possibly by reducing folate utilization. It is therefore interesting that we also found that the variant allele of MTHFR decreases the likelihood of drinking alcohol by half in the combined case-control population (OR = 0.5; 95% CI 0.3–0.9). We speculate that the additional reduction of folate utilization by alcohol in individuals with already reduced capacity due to MTHFR variant results in some physiological effect that reduces tolerance to alcohol. Although we found no significant interactions, it is conceivable that women with both high intakes of alcohol and MTHFR variant could be at particularly high risk for breast cancer. Inadequate intake of dietary folate could additionally amplify risk. If such an effect is indeed present, detection will require larger sample sizes than the present study.

Our results could explain inconsistent findings in the epidemiologic literature on dietary folate and breast cancer, and we suggest the need for an analysis of the effect of dietary folate stratified by MTHFR genotype. Clearly, the present results need to be duplicated and expanded in other epidemiologic studies to include gene–environment interactions with factors that influence DNA methylation such as folate and alcohol, methionine, and vitamin B12 intake. Our data indicate that MTHFR may increase the risk of premenopausal breast cancer. Additional evidence consistent with our findings could support chemoprevention trials to test these modifiable risk factors in high-risk subjects, polymorphic at the MTHFR locus.

Acknowledgments

We wish to thank John West, MD, Breast Care Center of Orange, Orange, CA, Edward White, MD, Saddleback Breast Center, Laguna Hills, CA, and John Butler, MD, Department of Surgery, University of California, Irvine, CA, for their assistance in recruiting our participants. We thank Shu Yuan Liao, MD, Department of Pathology, University of California, Irvine, CA, for her work on the histopathology. We also thank our research assistants for their diligent efforts at data and sample collection and processing: Irene Masunaka, Randy Williams, Presley Hayes, and Tara McKittrick, Epidemiology Division, Department of Medicine, University of California, Irvine. This research was supported by funds from the University of California Breast Cancer Research Program, grant 1RB-0295; National Institutes of Health, National Cancer Institute grant 5 U01 CA58860-09, and a University of California at Irvine Cancer Center Seed Grant.

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