Lack of association between methylenetetrahydrofolate reductase genetic polymorphisms and postmenopausal breast cancer risk

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Abstract. Published data on the association between methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms and breast cancer risk are inconclusive. We investigated the independent and the combined effects of two commonly occurring polymorphisms, *MTHFR* 677C>T (rs1801133) and *MTHFR* 1298A>C (rs1801131), as well as their interaction with the use of hormone replacement therapy (HRT), to determine their potential contribution to breast cancer risk. We studied 530 breast cancer cases and 270 controls of the same age and ethnicity participating in a case-control study of postmenopausal women. The duration of HRT use was ascertained through a postal questionnaire. Genotyping was conducted by TaqMan® allelic discrimination. Adjusted odds ratios and 95% confidence intervals were calculated using logistic regression. No significant association was observed between either the individual or the combined *MTHFR* genotypes and the risk of postmenopausal breast cancer. Additionally, no effects resulting from the interaction between *MTHFR* genotypes and HRT use were detected. Therefore, our data do not support the hypothesis that genetic variation in the *MTHFR* gene is implicated in the aetiology of postmenopausal breast cancer.

Introduction

Folates, a group of water-soluble B-vitamins, are important nutritional factors and play an integral role in maintaining DNA stability by regulating DNA biosynthesis, DNA repair and DNA methylation. In the form of 5,10-methylenetetrahydrofolate, folates are required for *de novo* synthesis of both purines and the pyrimidine nucleoside thymidine. In the form of 5-methyltetrahydrofolate, they are involved in the remethylation of homocysteine to methionine, the precursor of S-adenosylmethionine, which is the principal methyl donor in most cellular reactions (1).

Folate deficiency induces and accelerates carcinogenesis by perturbing each of these processes. Low dietary folate intake or impaired folate absorption or metabolism lead to purine and thymidine depletion and incorporation of uracil into DNA during DNA synthesis. The consequences are DNA strand breakage and chromosomal aberrations. Furthermore, cytosine methylation is altered, which may induce both gene-specific DNA hypermethylation and global DNA hypomethylation, potentially decreasing essential tumor-suppressor activation and increasing inappropriate proto-oncogene activation (2).

Polymorphisms in the genes involved in the transport of folate or its metabolism – thymidilate synthetase (*TS*), methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*) and methionine synthase reductase (*MTRR*) – may result in allozymes with altered activity and are thus believed to cause interindividual differences in cancer risk susceptibility. It has been suggested that under conditions of limiting folate, one-carbon units are directed preferentially through the methionine cycle to facilitate methylation reactions at the expense of DNA synthesis and repair (3). MTHFR catalyzes the irreversible reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydorfolate (Fig. 1) and is thus a critical enzyme redirecting the pool of folate from DNA synthesis and repair to DNA methylation (4).

Epidemiologic studies of breast cancer in association with MTHFR status have focused on two common gene variants. The most common variant of the *MTHFR* gene is 677C>T, which encodes a thermolabile variant of the enzyme with an alanine-to-valine substitution at position 222. The substitution results in a reduction of enzyme activity. Homozygotes (677TT) have approximately 30% and heterozygotes (677CT) have approximately 65% of the activity of homozygous wild-types (677CC), respectively (5). Another polymorphism of the *MTHFR* gene is 1298A>C, which encodes a variant of the enzyme with a glutamate-to-alanine substitution at position 429 and has also been related to decreased enzyme activity (6). Homozygotes (1298CC) had approximately 60% of the activity of the homozygous wild-types (1298AA) (7).

The aim of the present study was to investigate the independent and the combined effects of two commonly occurring polymorphisms, *MTHFR* 677C>T (rs1801133) and *MTHFR* 1298A>C (rs1801131), as well as their interaction

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with the use of hormone replacement therapy (HRT), to determine their potential contribution to breast cancer risk in postmenopausal women.

Patients and methods

Patients and controls. In this study, 530 postmenopausal women diagnosed with invasive primary breast cancer between January 1, 2006 and December 31, 2008 at the Institute of Oncology Ljubljana were enrolled. All were of Caucasian ethnic origin and aged between 50-69 years at the time of diagnosis. The control group consisted of 270 postmenopausal women randomly selected from the outpatient clinic records of the Department of Obstetrics and Gynaecology, University Medical Centre Ljubljana, that were of the same age and ethnicity, and had no history of breast cancer.

Women were invited to participate through a postal questionnaire. Overall response rates were 82.5% for cases and 73.2% for controls. Complete data for all variables considered in the multivariate model were available for 78.4% of cases and 70.9% of controls. Of these controls, 38.1% donated a blood sample. The number of cases included in genotype analyses was therefore proportionally decreased by random selection to gain the 2:1 ratio appropriate for case-control comparisons. The final analysis thus included 800 postmenopausal women: 530 cases and 270 controls.

In addition to general information (socioeconomic status, weight and height), data on reproductive factors (age at menarche, number of pregnancies, age at first delivery, number of deliveries, breastfeeding and age at menopause), exogenous hormone use [oral contraceptives (OC) and HRT], family history of breast or ovarian cancer (first-degree relatives), smoking and alcohol consumption were collected. OC and HRT use for <1 year was considered no use. Women were assumed to be postmenopausal if they had no periods for at least 12 months before the reference date or had undergone a bilateral oophorectomy.

Informed written consent was obtained from all women enrolled in the study. The study protocol was approved by the National Medical Ethics Committee of the Republic of Slovenia (No. 61/06/07).

Genotyping. In case patients, DNA was extracted from formalin-fixed paraffin-embedded normal breast tissues using the HP PCR Template Preparation kit (Roche Diagnostics GmbH, Mannheim, Germany). In controls, genomic DNA was extracted from whole blood using the FlexiGene DNA kit 250 (Qiagen GmbH, Hilden, Germany). Genotyping for the polymorphisms *MTHFR* 677C>T and *MTHFR* 1298A>C was conducted by the TaqMan® allelic discrimination method. For *MTHFR* 677C>T, 1.1% of the samples failed. For *MTHFR* 1298A>C, 0.9% of the samples failed. Samples that failed to be genotyped were scored as missing. Reliability was assessed by random selection of 5% of samples in which all genotypes were confirmed by sequencing using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Werterstadt, Germany). Concordance was 100% for all genotypes.

Statistical analyses. T-tests (for means) and Chi-square tests (for frequencies) were carried out to detect differences in baseline characteristics between cases and controls. Observed genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium with the Chi-square goodness-of-fit test. Odds ratios (ORs) for breast cancer risk and the corresponding 95% confidence intervals (CI) were calculated using logistic regression analysis. The homozygous wild-type genotype, as determined by the more common of the homozygous genotypes, served as a reference category, with the heterozygous genotype and homozygous variant genotypes being collapsed into one category. Effect modification by the different genetic variants was investigated for the association between HRT use (never or <1, 1-5 and ≥5 years) and breast cancer risk. All analyses were adjusted by body mass index (BMI; <25, 25-30 and ≥30 kg/m^2), years of OC use (never or <1, 1-5, 5-10 and ≥10 years), years of HRT use (never or <1, 1-5 and ≥5 years) and smoking at the time of diagnosis (non-smokers, 1-10 cigarettes per day and ≥10 cigarettes per day). Potential confounding effects of other known breast cancer risk factors were also examined, but none of those had a substantial effect on the ORs. A P-value of <0.05 was considered statistically significant. All statistical analyses were performed using SPSS 18.0 software package.

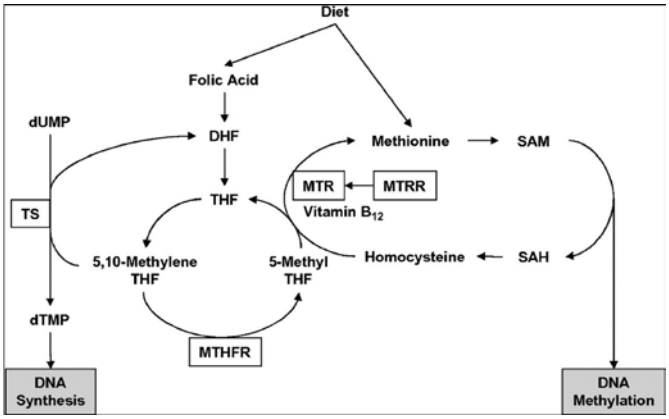

Figure 1. Folate metabolism [modified by Suzuki *et al* (4)]. THF, tetrahydrofolate; DHF, dihydrofolate; dUMP, deoxyuridine monophoshate; dTMP, deoxythymidine monophoshate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; TS, thymidilate synthetase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase.

Results

Distribution of selected characteristics for cases and controls were consistent with most established risk factors and are presented in Table I. Rather unexpectedly, significantly more women in the control group were using HRT (65.8% of controls vs. 29.6% of cases). Therefore, HRT use was highly significantly associated with a decrease in breast cancer risk (1-5 years of HRT use: OR=0.22, 95% CI 0.14-0.32; ≥5 years of HRT use: OR=0.23, 95% CI 0.16-0.34). When adjusted for confounding effects, none of the two genetic variants (*MTHFR* 677C>T and *MTHFR* 1298A>C) was associated with breast cancer risk (Table II).

Next, we examined the possible combined effects of the *MTHFR* 677C>T and *MTHFR* 1298A>C genotypes on breast

Table I. Characteristics of the study population.

Variable	Cases (n=530)	Controls (n=270)	P-value
Education/highest degree obtained (%)			<0.001
Primary school	30.7	6.3	
Secondary school	59.2	70.3	
University, PhD	10.1	23.4	
BMI (%)[a]			<0.001
<25 kg/m^2	33.6	52.2	
25-30 kg/m^2	40.7	35.1	
≥30 kg/m^2	25.7	12.7	
Mean ± SD age at menarche (years)	13.7±1.8	13.5±2.1	0.021
Mean ± SD age at spontaneous menopause (years)	50.5±3.7	50.4±3.9	0.711
Nulliparity (%)	5.3	3.4	0.089
Mean ± SD number of full-term pregnancies[b]	1.8±0.9	1.7±0.9	0.127
Mean ± SD age at first delivery (years)[b]	24.0±4.6	24.9±4.8	0.012
Women that breastfed (%)	86.4	90.3	0.344
Mean ± SD duration of breastfeeding (months)[c]	8.1±8.7	7.7±7.4	0.269
OC use (%)	42.1	54.7	0.001
Duration of OC use (%)			0.002
0<1 year	57.9	45.3	
1-5 years	14.4	22.7	
5-10 years	12.7	17.1	
≥10 years	15.0	14.9	
HRT use (%)	29.6	65.8	<0.001
Duration of HRT use (%)			<0.001
0<1 year	70.4	34.2	
1-5 years	14.3	33.1	
≥5 years	15.3	32.7	
Regimen of HRT (%)[d]			0.487
Combined, estrogen plus progestin	71.2	67.8	
Estrogen only	28.8	32.2	
First degree family history of breast or ovarian cancer (%)	18.1	15.5	0.138
Smoking (%)	20.2	15.9	0.041

[a]Calculated as weight in kilograms divided by height in meters squared at the age of the diagnosis. [b]Among women who had a full term pregnancy. [c]Among those who ever breastfed. [d]Among those who ever used HRT.

cancer risk. The reference group consisted of individuals with the putatively most advantageous combinations of the genotypes: low-risk genotypes, i.e., the presence of the homozygous CC genotype for *MTHFR* 677C>T and the AA genotype for *MTHFR* 1298A>C. An association between the combined *MTHFR* genotypes and breast cancer risk was also not detected (Table III).

Additionally, we investigated whether the *MTHFR* 677C>T and *MTHFR* 1298A>C genotypes in interaction with HRT use (never or <1, 1-5 and ≥5 years) affect the risk of postmenopausal breast cancer. No interaction effects between *MTHFR* genotypes and HRT use were found (Table IV). Additionally, no interaction was observed between the genetic variants and breast cancer risk with respect to HRT regimen (estrogen monotherapy vs. combined, estrogen plus progestin therapy) (data not shown).

Discussion

In this case-control study of postmenopausal women we found no association between either the independent or the combined effects of two commonly occurring polymorphisms, *MTHFR* 677C>T and *MTHFR* 1298A>C, and breast cancer.

Previous attempts to explore the relationship between these two polymorphisms and breast cancer have yielded conflicting results (4,8,9,10), although two recent meta-analyses, involving 15.260 cases and 20.411 controls, and 16.480 cases and 22.388 controls, concluded that the functional *MTHFR* 677C>T polymorphism may play a low penetrance role in the development of breast cancer (11,12). By contrast, a clear inverse association has been consistently observed between the *MTHFR* 677TT genotype and colorectal cancer, especially with high levels of folate intake and low levels of alcohol intake (13). It has

Table II. Genetic variation in *MTHFR* gene and risk of postmenopausal breast cancer.

Genotype	Cases, n (%)	Controls, n (%)	OR (95% CI)[a]	p-value
MTHFR 677C>T				
CC	222 (42.5)	108 (40.1)	1.0	
CT	238 (45.6)	124 (46.1)	0.95 (0.67-1.35)	0.792
TT	62 (11.9)	37 (13.8)	0.88 (0.52-1.50)	0.642
CT/TT	300 (57.5)	161 (59.9)	0.94 (0.68-1.31)	0.707
MTHFR 1298A>C				
AA	258 (49.2)	131 (48.7)	1.0	
AC	219 (41.8)	117 (43.5)	0.89 (0.63-1.26)	0.510
CC	47 (9.0)	21 (7.8)	1.04 (0.57-1.92)	0.897
AC/CC	266 (50.8)	138 (51.3)	0.91 (0.66-1.27)	0.592

[a]Adjusted for BMI (<25, 25-30 and ≥30 kg/m^2); years of OC use (never or <1, 1-5, 5-10 and ≥10); years of HRT use (never or <1, 1-5 and ≥5); smoking (non-smokers, 1-10 cigarettes per day and ≥10 cigarettes per day).

Table III. Combined effects of *MTHFR* 677C>T and *MTHFR* 1298A>C genotypes and risk of postmenopausal breast cancer.

Genotype		Cases/Controls	OR (95% CI)[a]	p-value
MTHFR 677C>T	*MTHFR* 1298A>C			
CC	AA	66/26	1.0	
CC	AC/CC	156/82	0.66 (0.37-1.18)	0.164
CT/TT	AA	191/105	0.68 (0.39-1.20)	0.186
CT/TT	AC/CC	109/56	0.72 (0.39-1.32)	0.290

[a]Adjusted for BMI (<25, 25-30 and ≥30 kg/m^2); years of OC use (never or <1, 1-5, 5-10 and ≥10); years of HRT use (never or <1, 1-5 and ≥5); smoking (non-smokers, 1-10 cigarettes per day and ≥10 cigarettes per day).

Table IV. HRT use, genetic variation in *MTHFR* gene and risk of postmenopausal breast cancer.

Genotype	HRT use (years)					
	0 to <1		1-5		≥5	
	Cases/Controls	OR[a] (95% CI)	Cases/Controls	OR[a] (95% CI)	Cases/Controls	OR[a] (95% CI)
MTHFR 677C>T						
CC	156/38	1.0	38/34	0.29 (0.16-0.53)	28/36	0.18 (0.09-0.33)
CT/TT	210/54	0.95 (0.59-1.53)	38/55	0.16 (0.09-0.29)	52/52	0.28 (0.16-0.48)
$P_{interaction}$	0.084					
MTHFR 1298A>C						
AA	177/44	1.0	35/45	0.19 (0.11-0.35)	46/42	0.29 (0.16-0.50)
AC/CC	190/48	0.95 (0.59-1.52)	41/44	0.23 (0.13-0.40)	35/46	0.19 (0.11-0.34)
$P_{interaction}$	0.447					

[a]Adjusted for BMI (<25, 25-30 and ≥30 kg/m^2); years of OC use (never or <1, 1-5, 5-10 and ≥10); years of HRT use (never or <1, 1-5 and ≥5); smoking (non-smokers, 1-10 cigarettes per day and ≥10 cigarettes per day).

been suggested that this is in agreement with the markedly lower rates of cell division and thus lower need for nucleic acid synthesis in the postmenopausal breast compared to large bowel (14).

A study by Marchand *et al* found that the *MTHFR* 677TT genotype may confer a 40% decreased breast cancer risk in postmenopausal women using HRT (14). This is consistent with the need for increased nucleic acid resulting from the hyperproliferative effect of HRT on mammary epithelial cells and reduced *MTHFR* activity, which increases the flow towards DNA synthesis. Similarly, the weak inverse association between the *MTHFR* 677CT/TT and *MTHFR* 1298AC/CC genotypes and breast cancer in our study was more pronounced among women on HRT. However, the test for interaction of the *MTHFR* 677C>T and *MTHFR* 1298A>C polymorphisms and HRT use (never or <1, 1-5 and ≥5 years) on the risk of breast cancer did not reach statistical significance ($P_{interaction}$ 0.084 and 0.447, respectively). Whether there is any relationship between estrogens and folates in breast cancer remains to be determined. HRT is known to reduce plasma homocysteine concentrations, but independently of the *MTHFR* 677C>T polymorphism (15).

Several limitations need to be considered when interpreting the results. The analysis revealed no indication of an increased risk of breast cancer with HRT use, which is inconsistent with previous studies suggesting that HRT use is associated with a small, but significant increase in the risk of breast cancer (16). Since a low percentage of control group women agreed to provide a blood sample, the extremely opposite trend in the present study might be due to preferential participation in the study by controls with this breast cancer risk factor (HRT use) present over those without this factor. Another explanation might involve the use of retrospectively collected exposure data since, as in most observational studies, we relied on self-reports of HRT use. For this reason, a colour chart displaying all preparations ever marketed in Slovenia was included in the questionnaire to aid recall. Furthermore, women of higher socioeconomic status are more than three times more likely to undergo HRT (17). In our study population, 10.1% of cases and 23.4% of controls reported having a university degree or PhD, whereas 30.7% of cases and 6.3% of controls gave their highest level of education obtained as being primary school. Thus, given the higher education level and higher prevalence of HRT use among controls, we believe that the comprehensive medical care received by HRT users in whom no pre-existing breast abnormalities have been found by mammography examinations may explain the observed decrease in breast cancer risk with HRT use of our study.

Our study population was of medium size, and it is possible that some interactions were also not significant due to insufficient power. However, the study provides adequate power to detect clinically relevant interactions, with an OR=2.0, assuming α=0.05.

The strengths of the study include the availability of information on potential confounders and the investigation of functionally relevant genetic variants. Furthermore, for both polymorphisms assessed, the distribution of each genotype followed Hardy-Weinberg equilibrium, which indicates that no selection bias occurred among genotypes. Additionally, the study population was largely homogenous. Analyses were restricted to postmenopausal Slovenian Caucasian women; mean ages for cases and for controls did not differ significantly between the groups (p=0.432).

In conclusion, we report that breast cancer risk is unlikely to be influenced by functionally relevant variants in the *MTHFR* gene.

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