Genetic polymorphisms of the methylenetetrahydrofolate reductase gene, plasma folate levels and breast cancer susceptibility: a case–control study in Taiwan

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Introduction

Several epidemiological investigations suggest that adequate folate intake may be important in the prevention of breast cancer, particular among women who regularly consume alcohol (1–5). Folate in the form of methylenetetrahydrofolate provides the methyl groups required for intracellular methylation reactions. Folate also functions as a coenzyme in the synthesis of purines and thymidylate for DNA (6). Low folate status may thus alter DNA methylation and thereby influence genes stability and expression (6,7). Diminished folate status may also result in uracil misincorporation into DNA, leading to chromosome breaks and DNA repair disruption (6,8).

Therefore, folate metabolism disturbance may lead to aberrant DNA synthesis and DNA methylation and may be a factor in carcinogenesis.

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme residing at a critical metabolic branch point in folate metabolism; it catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate (5,10-methylTHF) into 5-methyltetrahydrofolate (5-methylTHF), which directs the folate pool towards homocysteine remethylation into methionine at the expense of DNA and RNA biosynthesis (9). The potential influence of MTHFR on DNA methylation and on the availability of uridylates and thymidylates for DNA synthesis and repair makes MTHFR a potential candidate cancer-predisposing gene. Polymorphisms have been identified in the gene encoding MTHFR (10,11). Two common polymorphisms have been described in the MTHFR gene, both single nucleotide substitutions resulting in amino acid changes (C677T→Ala222Val and A1298C→Glu429Ala) (10,11). These variant genotypes are associated with increased thermolability and substantial decrease in enzymatic activity in vitro (12,13). Several epidemiological studies have shown that the low-activity variant MTHFR may reduce the risk for colon cancer (14–16) and acute lymphocytic leukemia (17). Conversely, the same variants have also been associated with an increased risk for various cancers, including endometrial cancer (18), cervical intraepithelial neoplasia (19), esophageal squamous cell carcinoma (20), gastric cancer (21), bladder cancer (22) and squamous cell carcinoma of the head and neck (23).

Following the previously reported associations between the polymorphisms of the MTHFR gene and various cancers, a number of studies have examined how the MTHFR polymorphisms influence the risk for breast cancer (24–34). Most of these studies have reported on the MTHFR C677T polymorphism and risk for breast cancer with mixed results. Accumulating evidence implicates that breast carcinogenesis could be initiated through activation of proto-oncogenes by hypomethylation of their promoter regions (35), or through inactivation of tumor suppressor genes by hypermethylation (35), or through alteration of estrogen receptor gene methylation patterns (36). Alternatively, breast tumorigenesis could be triggered by strand break and DNA mutation caused by

Abbreviations: CI, confidence interval; MTHFR, methylenetetrahydrofolate reductase; OR, odds ratio.

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Materials and methods

Case and control selection

This case–control study was conducted at the Tri-Service General Hospital, Taipei, Taiwan, from January to December in 2004. Based on the hospital chart number, the cases involved 146 women consecutively selected from subjects with a first confirmed histopathologic diagnosis of breast carcinoma in the age range of 20–80 years. The histopathological profile included 117 cases of infiltrating ductal carcinoma and 29 cases of intraductal or intralobular carcinoma. Control subjects comprising individuals without a history of cancer were simultaneously recruited from the health examination clinics of the same hospital during the same study period. Based on the availability of control subjects, 1–2 individuals were individually matched to cases by age (±5 years). There were 139 case–control sets with 1 case matched to 2 controls, and 7 sets with 1 case and 1 control, resulting in a total of 146 cases and 285 controls included in this study.

Collection of questionnaire data and blood specimens

Once case patients and control subjects agree to participate, written informed consent was obtained from all subjects. The research protocol was approved by the Institutional Review Board at the Tri-Service General Hospital, Taipei. All participants underwent personal interview administered by well-trained interviewers in conformance with institutional guidelines for studies including human subjects. Data were collected on sociodemographic characteristics, menstrual and reproductive history, menopausal status, lifestyle and medical history as well as family history of breast and other cancers. More specifically, in this study, menopausal status was defined as last menstruation after 1 year free of menstrual cycle and no attempt was made to distinguish between women with artificial and those with natural menopause. Immediately after the interview, a 10 ml blood sample was drawn into coded EDTA-treated tubes and centrifuged at 3000 r.p.m. for 10 min at room temperature within 10 h of collection. Plasma, buffy coat and red blood cells were separated and stored at −70°C until subsequent analysis. Because questionnaire data and biospecimens were obtained prior to cases’ acceptance with surgery and radiotherapy or chemotherapy, any influence of treatment is unlikely.

Laboratory assays

Genomic DNA was extracted from EDTA-treated blood samples using proteinase K digestion followed by phenol–chloroform extraction and ethanol precipitation. Genotyping for the MTHFR C677T and A1298C polymorphisms were performed using the PCR–RFLP methods reported by Froost et al. (12) and Weisberg et al. (13). Briefly, a fragment containing the polymorphic site was amplified by PCR and subsequently digested with restriction endonuclease. Fragments were analyzed on 2% agarose gels stained with ethidium bromide. For each set of reactions, a negative control containing H2O instead of DNA to check for contaminations was added. Based on the HinII RFLP analysis for MTHFR C677T polymorphism, a single undigested band at 198 bp represents a homozygous wild-type allele, two bands at 175 and 23 bp represents a homozygous mutant allele. With regards to the MTHFR A1298C polymorphism, the A→C substitution at nucleotide 1298 creates an MboII site. After restriction enzyme analysis, the MTHFR 1298 wild-type homozygotes produce two fragments: 25 and 215 bp; the variant homozygotes show only one fragment of 240 bp. To ensure quality control, genotyping was performed with blinding to case–control status, and a 10% masked, random sample of subjects was repeatedly tested and the results were concordant for all masked duplicated sets. Overall, genotyping data were obtained from 142 cases and 285 controls for C677T and A1298C polymorphisms. In addition, plasma levels of folate were determined using a radioassay kit (Bio-Rad, Richmond, CA) according to the manufacturer’s instructions. Plasma samples were grouped in case–control sets. Thawing was performed in ice water and under dim yellow light. Laboratory personnel were unaware of the case–control status. Each case–control set was analyzed in the same batch using the same reagents. During the assay process, we interspersed replicate plasma samples, which were labeled to preclude their identification by the laboratory personnel, to assess laboratory precision. The mean coefficient of variation for 22 pairs of replicate plasma samples was 6.8%.

Statistical analysis

Differences between cases and controls in age at menarche, age at first full-term pregnancy, age at menopause and the parity numbers were tested using the Student’s t-test. Because the average quantity of cigarettes and alcohol consumed by Chinese women is not large in Taiwan, habitual cigarette smoking was defined as smoking cigarettes at least once a week for more than 1 year. Similarly, habitual alcohol drinking was defined as consuming any alcoholic beverage at least once a week for more than 1 year. Distributions in cigarette smoking and alcohol intake status, menopausal status, as well as use of post-menopausal hormone and oral contraceptives between cases and controls were evaluated using the Chi-square test. For data on MTHFR alleles and genotypes, deviation from Hardy–Weinberg equilibrium was tested using the Chi-square test. Linkage disequilibrium between the MTHFR 677 C>T and 1298 A>C polymorphisms was calculated as D’, which ranges from 0 (no linkage disequilibrium) to 1 (complete linkage disequilibrium). The EH linkage utility program (39) was used to determine Chi-square statistics and P-values for tests of allelic association between polymorphic markers.

To measure associations between MTHFR genotypes and breast cancer risk, individuals were grouped according to the genotype. Subjects with the homozygous wild-type genotype (i.e. 677CC and 1298AA) were considered as the reference category. Conditional logistic regression, which preserves the matching of cases and controls, was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs). We also assessed the possible modifying effect by plasma folate levels on the risk associated with MTHFR genotypes using stratified analyses. All statistical tests were based on two-sided probabilities using Statistical Analysis System Software (v. 8.0; SAS Institute, NC, USA).

Results

The baseline characteristics of cases and controls are summarized in Table I. The mean age of the cases was 50.5 years (median 49 years, range 26–76 years), and this was 49.2 years for controls (median 49 years, range 26–76 years). There were no significant differences between cases and controls in terms of age at menarche, age at menopause and parity number. No significant differences were found between cases and controls in terms of alcohol intake status, menopausal status, and the use of post-menopausal hormone

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases (n = 146)</th>
<th>Controls (n = 285)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at enrollment (years)</td>
<td>50.5 (9.5)</td>
<td>49.2 (8.6)</td>
<td>0.154</td>
</tr>
<tr>
<td>Age at menarche (years)</td>
<td>13.8 (1.6)</td>
<td>13.7 (1.6)</td>
<td>0.774</td>
</tr>
<tr>
<td>Age at first birth (years)</td>
<td>26.0 (4.0)</td>
<td>26.5 (3.7)</td>
<td>0.328</td>
</tr>
<tr>
<td>Age at menopause (years)</td>
<td>48.9 (4.9)</td>
<td>48.5 (5.4)</td>
<td>0.590</td>
</tr>
<tr>
<td>Number of parity</td>
<td>2.5 (1.1)</td>
<td>2.3 (0.9)</td>
<td>0.090</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Percentage</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarette smoking</td>
<td>10.6</td>
<td>4.9</td>
<td>0.027</td>
</tr>
<tr>
<td>Alcohol intake</td>
<td>11.0</td>
<td>13.0</td>
<td>0.543</td>
</tr>
<tr>
<td>Post-menopausal women</td>
<td>45.4</td>
<td>46.5</td>
<td>0.832</td>
</tr>
<tr>
<td>Post-menopausal hormone use</td>
<td>24.1</td>
<td>31.2</td>
<td>0.127</td>
</tr>
<tr>
<td>Oral contraceptive use</td>
<td>18.4</td>
<td>16.2</td>
<td>0.561</td>
</tr>
</tbody>
</table>

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and oral contraceptives. However, smokers were significantly more common in cases than in controls (10.6% versus 4.9%, \( P = 0.027 \)).

Figure 1 represents the risk of breast cancer in relation to plasma folate levels. Higher plasma levels of folate tended to be associated with a lower risk of breast cancer in analysis controlling for matching factors; the OR comparing the highest with the lowest tertile was 0.52 (95% CI: 0.26–1.05).

The frequencies of \( \text{MTHFR} \) alleles and genotypes by case-control status and the association between \( \text{MTHFR} \) genotypes and breast cancer risk are reported in Table II. The frequencies of the \( \text{MTHFR} \) 677T allele was 0.31 for the cases and 0.33 for the controls. The observed frequencies of the three \( \text{MTHFR} \) 677 genotypes among controls (CC: 46.3%; CT: 42.1%; TT: 11.6%) did not differ from those expected under the Hardy–Weinberg equilibrium (\( P = 0.474 \)). When the \( \text{MTHFR} \) 677CC genotype was used as the reference group, the 677CT heterozygotes appeared to have a slightly lower risk for breast cancer (matched OR: 0.77; 95% CI: 0.50–1.18), whereas no significant deviation was observed to the 677TT homozygotes (matched OR: 1.00; 95% CI: 0.51–1.94). Overall, the combined variant genotypes (677CT + TT) appeared to be associated with a slightly lower risk for breast cancer (matched OR: 0.81; 95% CI: 0.54–1.21). For the A1298C polymorphism, the \( \text{MTHFR} \) 1298C allele frequency was 0.16 for the cases and 0.23 for controls. The observed frequencies of the three \( \text{MTHFR} \) 1298 genotypes among controls (AA: 60.3%; AC: 33.3%; CC: 6.4%) were also in accordance with the Hardy–Weinberg equilibrium (\( P = 0.324 \)). When the \( \text{MTHFR} \) 1298AA genotype was used as the reference group, the 1298AC heterozygotes had a significantly decreased risk for breast cancer (matched OR: 0.52; 95% CI: 0.32–0.85). The risk also appeared to reduce for 1298CC homozygotes (matched OR: 0.80; 95% CI: 0.34–1.90), although this reduction did not reach statistical significance. Overall, the combined variant genotypes (1298AC + CC) were associated with a 43% reduction in risk of breast cancer (matched OR: 0.57; 95% CI: 0.36–0.89).

The data were further analyzed to examine the combined \( \text{MTHFR} \) genotype distribution among study subjects. Among 276 1298AA subjects, 103 (37.3%) carried a 677CC genotype and 50 (18.1%) carried a 677TT genotype. The 677T and 1298C alleles only occurred in trans; no subject presented a 1298CC/677TT genotype configuration. A strong linkage disequilibrium was observed between the 677T>C and 1298A>C polymorphisms among both the cases and controls (\( D^* = 0.902 \) for the cases and 1.000 for the controls). \( \text{MTHFR} \) haplotype frequency estimations for combinations of 677TT and A1298C alleles demonstrated the following statistically significant case-control differences (\( P = 0.016 \)): 0.53, 677C/1298A; 0.30, 677C/1298C; 0.16, 677T/1298A and 0.01, 677T/1298C among cases and 0.44, 677C/1298A; 0.33, 677C/1298C and 0.23, 677T/1298A among controls. The combined effects of the \( \text{MTHFR} \) 677 and 1298 genotypes on risk for breast cancer were also analyzed (see Table II). When \( \text{MTHFR} \) 677CC and 1298AA genotypes were considered as the reference group, the wild-type genotype at 677 locus combined with the variant genotypes at 1298 locus (677CC and 1298AC/CC) were associated with a 23% decrease in risk for breast cancer (matched OR: 0.77; 95% CI: 0.46–1.27). The variant genotypes at 677T locus combined with the wild-type genotype at 1298 locus (677CT/TT and 1298AA) were associated with a 41% decrease in the risk (matched OR: 0.59; 95% CI: 0.33–1.04). The variant genotypes at both 677 and 1298 loci (677CT/TT and 1298AC/CC) were associated with a 74% reduction in the risk (matched OR: 0.26; 95% CI: 0.10–0.63).

Because the \( \text{MTHFR} \) gene is important in folate metabolism, it is plausible that plasma folate levels may modify the effect of germ-line variants in the \( \text{MTHFR} \) gene, thereby impacting the risk for breast cancer. Thus, we examined the \( \text{MTHFR} \)–breast cancer association according to plasma folate levels. As shown in Table III, a significantly inverse association of the combined \( \text{MTHFR} \) 677C>T and 1298A>C polymorphisms with the breast cancer risk were observed for women with lower levels of plasma folate, whereas a less distinct pattern was observed among women with higher levels of plasma folate.
Discussion

In this study, we concomitantly evaluated the associations of the two common polymorphisms in MTHFR (C677T and A1298C), a gene that plays a central role in folate metabolism, to the risk for breast cancer. The effects of these genotypes were also evaluated in conjunction with plasma folate levels. This study showed that plasma folate level was inversely associated with the risk for developing breast cancer. Furthermore, we observed a significantly reduced risk (74% reduction) associated with the compound MTHFR variant genotypes at both 677 and 1298 loci. More interestingly, the reduction in breast cancer risk associated with the MTHFR polymorphisms was more strengthened among women with low plasma folate levels. Our findings demonstrate the relevance of folate metabolism in the susceptibility to breast cancer among the Taiwanese female population.

The MTHFR enzyme is part of a complex metabolic entity involving both generation of the universal methyl-group donor S-adenosylmethionine (SAM), the methyl donor for most biological transmethylation reactions in the body, and DNA synthesis via the creation of nucleotides (11). Thus, functional polymorphisms in MTHFR and their associations with cancer risk are of great interest. Indeed, numerous epidemiological studies have examined the relationship between MTHFR polymorphisms and cancer risk but have generated conflicting results. Several epidemiological studies have shown that the low-activity variant of MTHFR C677T was associated with a decreased risk for colon cancer (14–16) and acute lymphocytic leukemia (17). In contrast, the same variants have also been associated with an increased risk for various cancers, including endometrial cancer (18), cervical intraepithelial neoplasia (19), esophageal squamous cell carcinoma (22), gastric cancer (21), and bladder cancer (22). Moreover, the results from previous association studies on the MTHFR polymorphisms and breast cancer risk are also inconsistent. Several clinical studies have reported that the MTHFR C677T variants are associated with an increased risk of early-onset breast cancer before the age of 40 (24), breast carcinoma in pre-menopausal women (25,26), or in those with bilateral breast cancer or combined breast and ovarian cancer (27). In addition, population-based epidemiological studies have indicated a gene–nutrient interaction between MTHFR polymorphisms and dietary folate intake in breast carcinogenesis, in which the MTHFR 677 C>T polymorphism was associated with elevated risk for breast cancer when dietary folate consumption was low (28,29).

Whereas, several studies have reported that the MTHFR 1298CC genotype (29,34) and MTHFR compound heterozygosity (677CT and 1298AC) (34) were associated with a reduced risk of developing breast cancer. Nevertheless, lack of an association of MTHFR polymorphisms with breast cancer was also observed in a number of epidemiological studies (30–33). In this study, a significantly decreased risk for breast cancer was observed in subjects who carried the compound MTHFR variant genotypes at both 677 and 1298 loci. Moreover, this MTHFR–associated reduction in the risk was more evident among women with lower plasma folate levels. Intriguingly, our current study findings are broadly inconsistent with what has been observed for colorectal neoplasia that decreased risk with homozygous MTHFR 677 variant genotype was strengthened in those with high dietary folate (14–16). Currently, no clear explanation for these conflicting results can be given. It is likely that variations of study size and design, particularly ethnicity, may result in inconsistent findings. It is also likely that these inconsistent study findings may suggest that the effects of folate metabolism on carcinogenesis are highly complex; appear to depend on cell type, target organ and stage of transformation; and are gene- and site-specific (40). Indeed, the MTHFR is situated at the branch point of one-carbon metabolism balancing DNA methylation and synthesis. It is hypothesized that an imbalance between biological methylation and nucleotide synthesis that is responsible for MTHFR-related carcinogenesis. Based on this hypothesis, a possible explanation of how lower MTHFR activity could decrease the risk of breast cancer in those with lower plasma folate levels is that although circulating folate levels (5-methylTHF) tend to be low, the pool of intracellular 5,10-methyleneTHF is increased ‘backed up’ because of the reduced MTHFR activity conferred by the 677 C>T and 1298 A>C polymorphisms, which is crucial in the conversion of uracil to thymidine for DNA synthesis and repair. Expanded intracellular pools of 5,10-methyleneTHF could increase the availability of thymine and thereby enhance DNA stability (41). Our findings suggest that the MTHFR polymorphisms may protect against potential defects in DNA synthesis, which might be more crucial toward a later stage in breast tumorigenesis. We speculate that as the cells progress into carcinoma, breast epithelial cells divide faster and are more likely to be prone to nucleotide pool imbalances. In particular, dUMP may

Table III. Relationship of MTHFR polymorphisms to breast cancer risk stratified by plasma folate levels

<table>
<thead>
<tr>
<th>Plasma folate (ng/ml)a</th>
<th>MTHFRb genotype</th>
<th>No. of cases</th>
<th>No. of controls</th>
<th>ORb,c</th>
<th>95% CIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤Median</td>
<td>C677T A1298C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>AA</td>
<td>32</td>
<td>24</td>
<td>1.00</td>
<td>Reference</td>
</tr>
<tr>
<td>CC</td>
<td>AC/CC</td>
<td>28</td>
<td>67</td>
<td>0.30</td>
<td>0.15–0.60</td>
</tr>
<tr>
<td>CT/TT</td>
<td>AA</td>
<td>13</td>
<td>31</td>
<td>0.32</td>
<td>0.14–0.74</td>
</tr>
<tr>
<td>CT/TT</td>
<td>AC/CC</td>
<td>3</td>
<td>21</td>
<td>0.11</td>
<td>0.03–0.43</td>
</tr>
<tr>
<td>&gt;Median</td>
<td>CC</td>
<td>11</td>
<td>36</td>
<td>1.00</td>
<td>Reference</td>
</tr>
<tr>
<td>CC</td>
<td>AA</td>
<td>17</td>
<td>41</td>
<td>1.36</td>
<td>0.56–3.27</td>
</tr>
<tr>
<td>CC</td>
<td>AC/CC</td>
<td>33</td>
<td>45</td>
<td>2.35</td>
<td>0.96–5.73</td>
</tr>
<tr>
<td>CT/TT</td>
<td>AA</td>
<td>5</td>
<td>20</td>
<td>0.80</td>
<td>0.24–2.65</td>
</tr>
</tbody>
</table>

aPlasma folate was categorized based on the median value among control subjects.
bMTHFR, methylenetetrahydrofolate reductase; OR, odds ratio; CI, confidence interval.
cAdjusted for age at enrollment.

Acknowledgements

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