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Methylenetetrahydrofolate Reductase and Thymidylate Synthase Polymorphisms are not Associated with Breast Cancer Risk or Phenotype

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Abstract. *Background: Aberrations in folate metabolism contribute to the risk of cancer via effects on the synthesis, methylation and repair of DNA. Functional genetic variants in the methylene tetrahydrofolate reductase (MTHFR) and thymidylate synthase (TS) genes may be risk factors for breast cancer because of their central roles in cellular folate metabolism. Patients and Methods: The MTHFR C677T and TS tandem repeat polymorphisms were investigated in a case-control study of 339 women with breast cancer for possible associations with the risk of this disease, tumor phenotype and patient survival. Results: The MTHFR and TS polymorphisms were not associated with a significantly increased risk of breast cancer. No associations were observed with any pathological or molecular feature and neither polymorphism was associated with survival from this disease. Conclusion: The common MTHFR C677T and TS enhancer region polymorphisms were not risk factors for breast cancer in this patient cohort, nor were they associated with phenotypic features or with prognosis.*

Epidemiological, animal model and human intervention studies support the concept that a high level of dietary folate intake is associated with a decreased risk for several common cancer types including breast cancer (1-3). Folate has a central role in one-carbon metabolism and is a critical coenzyme for both nucleotide synthesis and methylation reactions. The 5,10-methylene tetrahydrofolate reductase (*MTHFR*) and thymidylate synthase (TS) genes play key roles in this one-carbon metabolism. The activity of *MTHFR* determines the cellular availability of 5,10-methylenetetrahydrofolate used for thymidylate and purine synthesis, as well as 5-methyltetrahydrofolate used for methylation reactions including that of DNA. *TS* catalyzes the conversion of dUMP to dTMP, the rate-limiting step for DNA synthesis in mammalian cells. Folate insufficiency has been associated with an increased rate of uracil misincorporation into DNA, leading in turn to an increased rate of chromosomal damage and cancer risk (4). Disruption of the folate pathway can also alter the normal patterns of DNA methylation and lead to changes in DNA stability and expression (5), both of which are critical to the process of carcinogenesis.

A common polymorphism in the coding region of *MTHFR* (C677T, alanine to valine) markedly reduces the activity of this enzyme (6). Approximately 10-20% of individuals in Caucasian populations are homozygous for the T allele. This variant has been proposed as a low risk genetic factor for several cancer types including colorectal, lung and esophageal (1). The *MTHFR* C677T polymorphism has been associated with hypomethylation of peripheral blood cell DNA in normal individuals (7) and with low levels of folate intermediates in colorectal tumors (8), thus demonstrating direct links with biochemical markers of cellular folate metabolism in normal and neoplastic tissues.

A tandem repeat polymorphism comprising triple (3R) or double (2R) repeats of a 28 bp sequence has been identified in the enhancer region of the TS promoter (9). Gastrointestinal cancer patients who are homozygous for the triple repeat (3R/3R) show 2-4-fold higher levels of *TS* activity compared to double repeat homozygous (2R/2R) or heterozygous (2R/3R) patients (10). The *TS* 3R/3R genotype was recently shown to be a risk factor for colorectal cancer (11) but a protective factor for acute adult lymphocytic leukaemia (12).

Several studies have investigated the *MTHFR* C677T polymorphism as a risk factor for breast cancer (3, 13-19). These reported significantly higher frequencies of TT homozygotes in bilateral (13), early onset (15) and premenopausal (17,18) breast cancer patients. The TT

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genotype in association with low dietary folate intake was also found to be a risk factor when compared to individuals with the CC genotype and having a high folate intake (19). In another study of 141 breast cancer cases and 109 age-matched controls, increased serum folate concentrations were associated with reduced risks of breast cancer for the overall group and for individuals with the *MTHFR* 677 CC homozygous wildtype or CT heterozygous genotypes (3). A relatively large case-control study found no associations however between *MTHFR* C677T genotype and either the risk of breast cancer or the clinicopathological features of this disease (16). There have been no published reports to date on possible associations between the TS enhancer region polymorphism and breast cancer risk.

In previous studies we reported significant associations between various phenotypic features of breast cancer and polymorphisms in the *TP53* and *p21* (20), luteinizing hormone receptor (21) and *IL-6* (22) genes. The aim of the current study was to investigate whether the common and functionally important *MTHFR* C677T and *TS* enhancer region polymorphisms involved in methyl group metabolism are associated with the risk of breast cancer and with phenotypic features of this disease including patient survival.

Patients and Methods

Subjects. The study included 339 consecutive cases of primary breast carcinoma diagnosed between 1990 to 1994 at the Sir Charles Gairdner Hospital in Perth, Australia. Genomic DNA was extracted from surgically resected frozen tumor specimens using standard procedures. The median age at surgery was 58 years (range, 18-92 years) and the median follow-up time was 87 months (range, 2-116 months). At the end of the study 57 patients (17%) had died from disease recurrence. Clinical and pathological features of this tumor series were described earlier (23). The majority of subjects (>95%) were of Caucasian descent. Age-matched controls to within 10 years were chosen from a local, healthy population. Ethics approval for the study was obtained from the University of Western Australia Human Research Ethics Committee.

TS and MTHFR genotyping. PCR amplification of the TS promoter enhancer region containing double and triple tandem repeats was performed using forward (5' AAAAGGCGCGCGGAAG GGGTCCT 3') and reverse (5' TCCGAGCCGGCCACAGGCAT 3') primers. Reactions were carried out in 15 μL volumes comprising 1 μL of DNA preparation, 0.2U of Taq Polymerase (Qiagen, Australia), primers at a final concentration of 0.4 μM, DMSO at a final concentration of 10%, Mg^{2+} at a final concentration of 3 mM and 5x reaction mix (BioTech International, Perth) containing nucleotides and buffer. A total of 32 PCR cycles were carried out (94˚C for 40 sec, 62˚C for 40 sec and 72˚C for 1 min) following hot-start at 94˚C for 5 min during which time DNA was added. PCR product containing triple repeats (144 bp size) was distinguished from that containing double repeats (116 bp) by electrophoretic separation on 3% agarose gels with appropriate standard markers. Patients who were homozygous for the triple repeat (3R/3R) displayed only the larger PCR product, those homozygous for the double repeat (2R/2R) displayed only the smaller PCR product, while heterozygous individuals (2R/3R) showed both the larger and smaller PCR products.

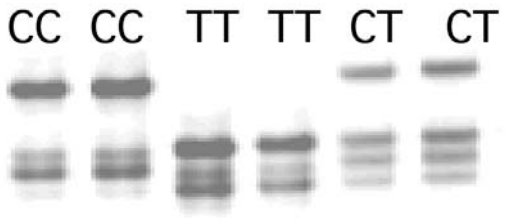

Figure 1. *Genotyping for the MTHFR C677T polymorphism using fluorescent PCR-SSCP technique. CC wild-type homozyote, CT heterozygote and TT homozygous variant are shown.*

Genotyping for the *MTHFR* C677T polymorphism was carried out using fluorescent PCR-SSCP as described previously (24). The HEX-labelled fluorescent primers used for PCR were: 5'-TGAAGGAGAAGGTGTCTGCGGGA and 5'-CCTCACCTGG ATGGGAAAGATCC (forward and reverse, respectively). The annealing temperature used was 62˚C and the 145 bp size PCR product (2 μl) was denatured in 4 μl of formamide loading buffer before electrophoretic separation as single stranded DNA on non-denaturing, 10% polyacrylamide gels. Gels were run at 1200V using the Corbett Research GS2000 real time DNA fragment analyzer as recommended by the manufacturer (Corbett Research, Australia). Three distinct banding patterns corresponding to the two homozygous (CC, TT) and one heterozygous (CT) genotypes were clearly visible. A small number of samples were tested by PCR-RFLP in order to identify the correct banding pattern for each genotype. Successful genotyping for the TS enhancer region and *MTHFR* C677T polymorphisms was achieved for 323 and 334 breast cancer cases, respectively.

Statistical analysis. The chi-square test was used to determine associations between the presence of *TS* enhancer region or *MTHFR* C677T polymorphisms and various clinicopathological features of the breast tumors, as well as for independence of the alleles (Hardy-Weinberg equilibrium). Patient survival for different genotype groups was examined using Cox regression analysis. All tests were two-tailed, and statistical significance was assumed at $p<0.05$. Analyses were performed using the SPSS statistical software package (Chicago, IL, USA).

Results

An example of the results obtained using fluorescence SSCP for genotyping of the *MTHFR* C677T polymorphism is shown in Figure 1. Allele frequencies for this polymorphism were 0.33 for controls and 0.29 for breast cancers, while for the TS enhancer region polymorphism (3R) they were 0.55 for controls and 0.53 for cases. Both the *TS* and *MTHFR* genetic variants were in Hardy-Weinberg equilibrium in both populations. Frequencies for *TS* 3R/3R and *MTHFR* TT homozygous individuals in this breast cancer series were 27% and 8% respectively (Table I). Neither genetic variant

Table I. *TS enhancer region and MTHFR genotype distributions in breast cancers and controls*

	TS Enhancer Region (%)				MTHFR C677T (%)				
	Total	2R/2R	2R/3R	3R/3R	Total	CC	CT	TT	CT/TT
Controls	345	76 (22)	161 (47)	108 (31)	551	242 (44)	259 (47)	50 (9)	309 (56)
Breast Cancer	323	70 (22)	167 (52)	86 (27)	334	166 (50)	141 (42)	27 (8)	168 (50)
OR		1.00	1.13	0.86		1.00	0.79	0.79	0.79
95% CI			0.76-1.66	0.56-1.33			0.60-1.06	0.47-1.31	0.60-1.04
P			0.55	0.51			0.11	0.36	0.094

was present at a significantly different frequency to that observed in an age-matched control population, although a trend ($p=0.094$) was observed for a protective effect in the combined *MTHFR* CT/TT group compared to wild-type CC individuals (OR=0.79, 95% CI 0.60-1.04).

Several reports have suggested the *MTHFR* TT homozygote frequency is higher in early onset (15) or premenopausal (17,18) breast cancer patients. In the present study, however, patients aged <40 years (n=54, 16% of cases) or <50 years (n=120, 35% of cases) at diagnosis showed no significant differences in TT genotype frequency compared to age-matched controls. In fact, the mean age at diagnosis of *MTHFR* TT breast cancer patients was identical to that of *MTHFR* CC patients (58.1 yrs). Neither the TS 3R/3R nor *MTHFR* TT genotypes were associated with any of the major clinicopathological features of breast cancer (nodal involvement, tumor size, tumor type, histological grade, steroid receptor status, ploidy) or with the molecular features of erbB2 amplification and *TP53* mutation (results not shown). The TS 3R/3R genotype showed no association with the *MTHFR* C677T genotype.

Cox univariate analysis revealed no significant differences in overall survival between the *MTHFR* CC and combined CT/TT patient groups (RR=1.06, 95%CI: 0.63-1.78) or between the TS 3R/3R and 2R/2R patient groups (RR=1.22, 95%CI: 0.78-1.91).

Discussion

There is increasing evidence to suggest that genetic variation in modifier genes such as *CYP19*, *GSTP1* and *TP53* are associated with the risk of breast cancer (25). Candidate modifier genes for breast cancer are likely to be involved in DNA damage repair, carcinogen metabolism and steroid metabolism. In addition to being risk factors for the development of this disease, common genetic variants having functional significance for gene expression or activity level may also influence tumor phenotype. This could include clinical, pathological and molecular features, as well as the toxicity and response to adjuvant treatments. We have previously shown significant associations in this breast cancer series between polymorphisms in the *p21* gene and the risk and phenotype of this disease (20), the *LH* receptor gene and age of diagnosis (21), and the *IL-6* gene with phenotype (22). In the same cohort however we found no evidence to link a functional polymorphism in the *Cyclin D1* gene with either the risk or phenotype of breast cancer (26).

In the current study we investigated whether polymorphisms in two genes with key roles in DNA synthesis and methylation are associated with breast cancer risk or phenotype. Although several studies have examined for possible links between the *MTHFR* C677T polymorphism and breast cancer (3, 13-19), this is the first to report on the TS enhancer region polymorphism. In contrast to earlier workers (15,17,18), we found no association between the *MTHFR* C677T polymorphism and age of breast cancer diagnosis. Our results support those of a large case-control study that found no association between this polymorphism and either the risk or phenotype of breast cancer (16).

The *MTHFR* C677T polymorphism is associated with global hypomethylation of normal blood cell DNA (7) and with lower levels of the folate intermediates tetrahydrofolate and 5,10-methylene tetrahydrofolate in colorectal tumors (8). If these findings apply to other normal and tumor tissues, the *MTHFR* C677T polymorphism could be expected to influence the risk of cancer development and the resulting phenotype. The results of the present study do not support a role for this polymorphism in breast cancer risk or clinicopathological phenotype, however, suggesting that it may not influence the methylation status of normal breast tissue DNA or the level of folate intermediates in normal and neoplastic breast epithelial cells. This does not exclude the possibility that dietary factors such as folate and alcohol intake in combination with the *MTHFR* C677T polymorphism might alter DNA methylation and synthesis in normal breast tissues. Genetic variants in other genes involved in folate

metabolism such as methionine synthase and methylene tetrahydrofolate dehydrogenase could also play a role in breast cancer risk and phenotype.

Similar to the *MTHFR* C677T polymorphism, the TS enhancer region polymorphism showed no associations with either the risk (Table I) or phenotype of breast cancer. The TS 3R/3R genotype has been linked to higher enzyme activity levels in gastrointestinal tumors (10) and in a relatively small series of colorectal cancers we recently showed a trend for association with lower tissue concentrations of folate intermediates (8). Both the *MTHFR* C677T (27, 28) and TS enhancer region (29) polymorphisms have been implicated in the chemosensitivity of breast and colorectal cancers to 5-fluorouracil. The *MTHFR* C677T polymorphism has also been associated with increased toxicity to cyclophosphamide/methotrexate/fluorouracil chemotherapy in breast cancer (30). These observations highlight the biological and clinical importance of the *MTHFR* and TS polymorphisms with respect to response and toxicity to chemotherapy, despite their apparent lack of association with breast cancer risk or phenotype found in the present study.

In conclusion, our data from an Australian population suggests that common, functional polymorphisms in two critical genes involved in DNA synthesis and methylation are not risk factors for breast cancer and do not determine the phenotype or prognosis of this disease. Further studies are required to determine whether the *MTHFR* C677T and TS enhancer region polymorphisms influence the level of DNA methylation or intracellular folate intermediates in normal tissues or in neoplastic breast tissues. Such associations might explain reported links between these genetic variants and the response and toxicity to antifolate chemotherapy.

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