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Germ-line variants in methyl-group metabolism genes and susceptibility to DNA methylation in human breast cancer

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Abstract. Aberrant CpG island hypermethylation in gene promoter regions may be an important epigenetic event in human neoplasias, including breast cancer. Dietary and genetic factors that alter DNA methylation levels in normal and tumour tissues could therefore influence both the susceptibility to this disease and tumour phenotype, respectively. In the present study of 227 breast cancers, we investigated whether common polymorphisms in 6 key genes involved in methyl group metabolism (thymidylate synthase, methylene tetrahydrofolate reductase, cystathione ß-synthase, DNA methyltransferase 3B, methylene tetrahydrofolate dehydrogenase, and methionine synthase) were associated with major pathological features of this disease or the frequency of CpG island hypermethylation. No associations were observed between any of the polymorphisms and patient age, tumour size, histological grade or patient outcome. However, tumours from patients who were homozygous for the methionine synthase A2756G polymorphism showed strikingly lower estrogen and progesterone hormone receptor concentrations compared to wild-type homozygotes. Moreover, patients who were homozygous for the methylene tetrahydrofolate dehydrogenase G1958A polymorphism showed a significantly higher frequency of tumour CpG island hypermethylation compared to wild-type homozygotes. Our results show that polymorphisms in two genes involved in methyl group metabolism are associated with hormone receptor content and DNA methylation frequency in breast cancer, however these observations are unlikely to be linked.

Introduction

Methylation-induced transcriptional silencing of tumour suppressor genes is a major epigenetic mechanism leading to the inactivation of many important growth control genes during neoplastic development. This occurs via hypermethylation of CpG islands located in the 5'-promoter regions. Some of the genes reported to undergo hypermethylation-associated silencing in breast cancer include *p16*, estrogen receptor (*ER*), *CDH1* and *BRCA1* (1,2). The study of methylated gene targets has helped to elucidate the molecular pathogenesis of breast cancer and may also have clinical value for the early detection of this tumour type.

One of the major unanswered questions is whether germline factors can influence the level of hypermethylation observed in both normal and tumour tissues. In conjunction with environmental factors such as the level of dietary folate intake, this could contribute to the risk of cancer development. An example has been reported for the methionine synthase (*MS*) A2756G variant. Individuals who were homozygous for this polymorphism showed a lower frequency of CpG island hypermethylation in their tumour tissues (3). Indeed, genes that are involved in methyl group metabolism such as *MS* represent good candidates for research into possible links between low-risk genetic factors and cancer. In addition to *MS*, other major genes in this pathway have common polymorphisms with known or suspected functional significance. These include a 28-bp tandem repeat in the enhancer region of thymidylate synthase (*TS*), the C677T polymorphism in methylene tetrahydrofolate reductase (*MTHFR*), a 68-bp insert in cystathione ß-synthase (*CBS*), the G1958A polymorphism in methylene tetrahydrofolate dehydrogenase (*MTHFD1*) and the C46359T polymorphism in DNA methyltransferase (*DNMT3B*). The role of these enzymes in methyl group metabolism is shown in Fig. 1. The *DNMT3B* polymorphism alters enzyme activity (4) and could therefore be expected to directly influence the level of DNA hypermethylation. The other major enzymes involved in methyl group metabolism could influence DNA methylation indirectly by impacting the concentration of the major methyl group donor, S-adenosyl methionine. Studies indicate that polymorphisms in methyl group metabolism genes may contribute to the risk of colorectal cancer, possibly via effects on DNA methylation levels (5-7). Considerably less work has been carried out to establish whether these polymorphisms are linked to breast cancer risk or phenotype.

We recently evaluated a large series of sporadic human breast cancers for hypermethylation of CpG islands in the promoter regions of the *RARß2*, *CDH1*, *ER*, *BRCA1*, *CCND2*, *p16* and *TWIST* genes (8). In the current study, we investigated possible associations between the frequency of methylation at these sites and the presence of germ-line variants in the *TS*, *MTHFR*, *MS*, *CBS*, *MTHFD1* and *DNMT3B*

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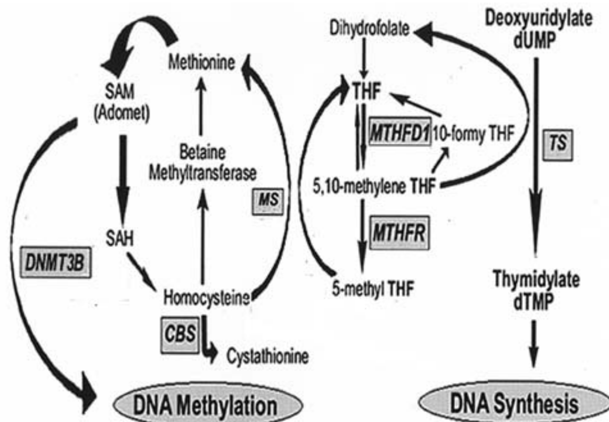

Figure 1. Methyl group metabolism in relation to DNA synthesis and methylation pathways. Enzymatic activities for the 6 genes (*TS*, *MTHFR*, *MTHFD1*, *MS*, *CBS*, and *DNMT3B*) investigated in this study are shown. Common variants in these genes were examined for possible associations with phenotype and DNA methylation frequencies in breast cancer.

genes. We also determined whether these polymorphisms were associated with common pathological features of breast cancer or patient outcome from this disease. Our results suggest that certain genetic variants in methyl group metabolism may be associated with phenotypic features of breast cancer, however this does not appear to be mediated through the effects on DNA hypermethylation.

Materials and methods

Breast cancers. Tumour samples from a series of 227 primary invasive breast carcinoma patients undergoing elective surgery at the Sir Charles Gairdner Hospital in Western Australia between 1990 and 1994 were selected for this study. Genomic DNA was extracted from fresh frozen specimens using standard phenol-chloroform extraction procedures. The median age of patients at diagnosis was 59 years (range, 18-93 years) and the median follow-up time was 49 months (range, 2-78 months). Clinical and pathological features of this tumour series have been described earlier (9). Disease-specific survival data were obtained from the Death Registry, Health Department of Western Australia. At the end of the study, 38 (17%) patients had died from disease recurrence. Approximately 92% of node-positive and 23% of node-negative patients received adjuvant systemic therapy comprising either hormone therapy alone or hormone therapy and chemotherapy. Approval for the study was obtained from the University of Western Australia Human Research Ethics Committee.

Genotype analyses. Genotyping for the TS promoter enhancer region (containing double and triple tandem repeats of 28 bp) and the *MTHFR* C677T polymorphisms were carried out using PCR and fluorescent PCR-SSCP, respectively, as described previously by our laboratory (10,11). The 844ins 68-bp polymorphism in *CBS* was determined by PCR and 2% agarose gel electrophoresis as described previously (7). PCR-RFLP was used to genotype single nucleotide polymorphisms (SNPs) in the *DNMT3b* (C46359T), *MTHFD1* (G1958A) and *MS* (A2756G) genes. For the

Table I. Polymorphisms in methyl group metabolism genes and hormone receptor content of breast cancers.

Genotype (n)	ER (fmol/μg) mean ± SD[a]	PR (fmol/μg) mean ± SD[a]
MTHFR C677T		
CC (104)	118±154	106±204
CT (92)	119±168	153±266
TT (19)	137±151	159±182
	NS	NS
TS 28-bp repeat[b]		
3R/3R (55)	110±140	136±248
2R/3R (104)	113±157	131±244
2R/2R (44)	153±197	132±205
	NS	NS
CBS 68-bp insert[c]		
L/S (36)	166±206	161±236
S/S (191)	110±146	129±236
	NS	NS
DNMT3B C46359T		
CC (60)	91±160	105±235
CT (113)	134±166	146±243
TT (49)	119±136	142±221
	NS	NS
MTHFD1 G1958A		
GG (61)	95±130	148±267
AG (113)	117±158	113±194
AA (48)	155±186	167±279
	P=0.062	NS
MS A2756G		
AA (146)	126±150	144±241
AG (60)	126±189	131±247
GG (16)	30±45	50±98
	P<0.001	P=0.005

[a]In each case, the homozygous wild-type group is compared to the homozygous variant group, except for CBS, which was compared to the heterozygous group. [b]3R, triple repeat; 2R, double repeat. [c]L, 68-bp insert; S, no 68-bp insert.

DNMT3b C46359T SNP, the primers 5'-ATGTCTGTC TGCTGTGACAGG-3' (forward) and 5'-TTGTTATTT CGAGTTCGGACC-3' (reverse) were used at an annealing temperature of 54˚C. The resulting 286-bp fragment was digested with *Avr*II (New England BioLabs) at 37˚C overnight followed by electrophoresis of digestion products on 2% agarose gels. PCR primers for the *MTHFD1* G1958A SNP were 5'-CACTCCAGTGTTTGTCCATG-3' (forward) and 5'-GCATCTTGAGAGCCCTGAC-3' (reverse) and used at an annealing temperature of 62˚C. The 330-bp PCR product was digested with *Msp*II (Promega) for 3 h at 37˚C followed by digestion analysis on 2% agarose gels. For the *MS* A2756G SNP, the primers 5'-TGTTCCCAGCTGTTA GATGAAAATC-3' (forward) and 5'-CTCCTTGAGAGA CTCATAATGG-3' (reverse) were used at a 60˚C annealing

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Genotype (n)	Methylated sites (mean ± SD)	P-value
MTHFR C677T		
CC (89)	3.1±1.3	
CT (81)	3.2±1.2	
TT (17)	3.1±1.2	NS
TS 28-bp repeat		
3R/3R (46)	2.9±1.5	
2R/3R (89)	3.2±1.2	
2R/2R (39)	3.4±1.2	0.090[a]
CBS 68-bp insert		
L/S (31)	3.0±1.3	
S/S (162)	3.2±1.3	NS
DNMT3B C46359T		
CC (53)	3.1±1.4	
CT (100)	3.1±1.1	
TT (40)	3.3±1.5	NS
MTHFD1 G1958A		
GG (50)	2.8±1.2	
GA (99)	3.2±1.3	
AA (44)	3.4±1.1	0.013[b]
MS A2756G		
AA (133)	3.2±1.3	
GA (47)	3.0±1.2	
GG (13)	3.5±1.4	NS

[a]3R/3R vs. 2R/2R; [b]AA vs GG.

temperature. The 100-bp PCR product was digested with *Hae*III (Promega) at 37˚C for 3 h before separation on 2.5% agarose gels.

Analysis of gene methylation status. Hypermethylation at CpG islands in the 5' promoter regions of the *RARβ2*, *CDH1*, *ER*, *BRCA1*, *CCND2*, *p16* and *TWIST* genes was previously evaluated in 193 tumours from this breast cancer series using the PCR-MSP technique (8). The frequency of methylation ranged from 11% for *CCND2* to 84% for ER.

Statistical analysis. The Chi-square test was used to evaluate possible associations between genetic variants in *CBS*, *DNMT3B*, *MTHFD1* and *MS* and various clinicopathological features. The Student's t-test was used to evaluate differences in hormone receptor content or gene methylation frequencies between different genotype groups. Patient survival was examined using Cox regression analysis. All tests were two-tailed and statistical significance was assumed at P<0.05. The SPSS statistical software package (Chicago, IL, USA) was used throughout.

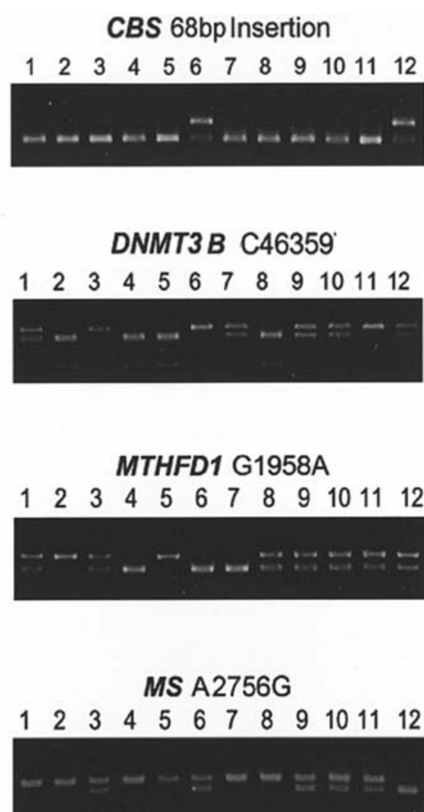

Figure 2. Representative examples of genotype analysis for the *CBS*, *DNMT3B*, *MTHFD1* and *MS* genes. Lanes 6 and 12 show heterozygotes for the 68-bp insert polymorphism in *CBS*. Lanes 2, 4, 5 and 8 show homozygotes (TT) for the C46359T polymorphism in *DNMT3B*. Lanes 2 and 5 show homozygotes (AA) for the G1958A polymorphism in *MTHFD1*. Lane 12 shows a homozygote (GG) for the A2756G polymorphism in *MS*.

Results

Representative genotype analyses for *CBS*, *DNMT3B*, *MTHFD1* and *MS* are shown in Fig. 2. The variant allele frequencies were: *CBS* (68-bp insert, 0.08), *DNMT3B* C46359T (T allele, 0.47), *MTHFD1* G1958A (A allele, 0.47) and *MS* A2756G (G allele, 0.20). All polymorphisms were in Hardy-Weinberg equilibrium. We have previously reported a lack of association between the conventional pathological features for this breast cancer series and both the *TS* 28-bp tandem repeat polymorphism and the *MTHFR* C677T polymorphism (10). In the present study, no significant associations were observed between polymorphisms in the *CBS*, *DNMT3B*, *MTHFD1* and *MS* genes and the clinicopathological features of patient age, tumour size, nodal status, histological grade or type. Breast cancers from patients who were homozygous for the *MS* A2756G polymorphism contained significantly lower concentrations of both estrogen and progesterone hormone receptors (Table I). A trend was also observed for higher estrogen receptor concentrations in patients who were homozygous for the *MTHFD1* G1958A polymorphism. No associations were observed between any of the common variants in methyl group metabolizing genes and patient survival from breast cancer.

We next examined whether polymorphisms in the methyl group metabolism genes were associated with the frequency of CpG island promoter methylation in 7 genes (*RARβ2*, *CDH1*, *ER*, *BRCA1*, *CCND2*, *p16* and *TWIST*) previously

evaluated for methylation by PCR-MSP (8). Patients who were homozygous for the *TS* 28-bp double tandem repeat (2R/2R) showed a trend for more frequent promoter methylation in their breast cancers, while patients who were homozygous for the *MTHFD1* G1958A polymorphism showed a significantly higher frequency of methylation (Table II). None of the individual methylated CpG sites showed a significant association with any of the polymorphisms.

Discussion

Studies on colorectal cancer indicate that polymorphisms in key genes involved in methyl group and folate metabolism are likely to be associated with the risk for this tumour type (5-7), presumably via effects on DNA methylation or synthesis (Fig. 1). Contradictory reports have been published for the risk of breast cancer associated with the *MTHFR* C677T polymorphism, with various studies suggesting protection (12), no association (10,13,14) or increased risk (15). Patient age, dietary folate intake and hormone replacement therapy were all found to be confounding factors in these studies. *In vitro* and clinical studies showing that *TS* and *MTHFR* polymorphisms are associated with sensitivity to the chemotherapy agent 5-fluorouracil (16,17) also support the contention that germ-line variants in methyl group metabolism play an important role in determining tumour biology.

We recently evaluated the CpG island methylation status of 7 tumour suppressor genes in the current breast cancer series (8). This allowed us in the present study to investigate whether common polymorphisms in 6 key genes involved in methyl group metabolism are associated with the frequency of DNA hypermethylation in breast cancer. In agreement with Paz *et al* (3), we found no evidence to suggest that the *MTHFR* C677T or *CBS* 68-bp insert polymorphisms were associated with CpG island hypermethylation in tumour DNA (Table II). In contrast to that group, however, the *MS* A2756G polymorphism failed to show an association with methylation frequency in our study. This discrepancy could be due to the different tumour types examined and low number of GG homozygotes in both the Paz *et al* (n=5) and current (n=16) studies. Our results are also in agreement with another study in cervical cancer, which found no significant association between the *MTHFR* C677T or *MS* A2756G polymorphisms and the frequency of gene methylation (18).

The only polymorphism found to show a significant association with the frequency of methylation in breast cancer was *MTHFD1* G1958A (Table II). It is not immediately obvious why this variant would be associated with CpG island methylation frequency, other than through a possible link with the concentration of intracellular folate intermediates. In support of this, *in vitro* studies in colorectal and gastric cell lines have shown a link between *MTHFD1* expression and sensitivity to 5-fluorouracil (19,20). Further research is required, however, to evaluate DNA methylation and intracellular folate levels in relation to the *MTHFD1* G1958A polymorphism in normal and tumour tissues.

DNMT3B is involved in the maintenance of DNA methylation, and hence the functional C46359T polymorphism in this gene (4) might be expected to show an association with increased frequency of CpG island promoter methylation in cancer. However, we found no evidence to support this in the current study of breast cancer (Table II). A previous study reported a protective effect of the T allele against breast cancer (21), whereas another found it was associated with increased incidence of lung cancer (4). Although we did not compare T allele frequencies between breast cancer and control individuals, our work suggests that any possible link between the *DNMT3B* polymorphism and risk for this disease is unlikely to be mediated through alterations in CpG island promoter methylation. Another study found that *DNMT3B* expression levels demonstrated strong prognostic value in breast cancer (22). We found no evidence of prognostic significance for the *DNMT3B* C46359T polymorphism in the current study, suggesting this genetic variant may not be an important regulator of gene expression levels in breast cancer tissues.

An intriguing result from this study was the strong association between homozygosity for the *MS* A2756G polymorphism and low hormone receptor content (Table I). This was the only significant association observed between any of the common polymorphisms in methyl group metabolism genes and clinicopathological features of breast cancer, including patient outcome. Methionine synthase converts homocysteine to methionine, a precursor for the universal methyl group donor, S-adenosyl methionine. The *MS* A2756G polymorphism was not associated with more frequent methylation of the *ER* gene (result not shown), and is therefore unlikely to provide an explanation for the association with lower ER concentration. Further studies carried out in independent tumour series are required to confirm the association between the *MS* 2756 GG genotype and low hormone receptor content observed here in breast cancer.

In conclusion, our study has shown that polymorphisms in two genes involved in methyl group metabolism, *MS* A2756G and *MTHFD1* G1958A, are associated with hormone receptor status and DNA methylation frequency, respectively, in breast cancer. Additional studies in this area should aim to quantitatively evaluate the methylation levels in DNA from normal tissues of individuals with known folate intake and genotype status.

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