ORIGINAL ARTICLE

A polymorphism in the methylenetetrahydrofolate reductase gene predisposes to colorectal cancers with microsatellite instability

B Shannon, S Gnanasampanthan, J Beilby, B Iacopetta

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See end of article for authors' affiliations

Correspondence to: Dr B Iacopetta, Department of Surgery, University of Western Australia, 35 Stirling Highway, Crawley 6009, Australia; bjiac@cyllene.uwa.edu.au

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Background: The enzyme methylenetetrahydrofolate reductase (MTHFR) catalyses the formation of folate intermediates that are vital to methylation reactions. A polymorphic variant (TT) has been linked to reduced levels of plasma folate, aberrant DNA methylation in leucocytes, and increased risk of colorectal cancer (CRC) under conditions of low folate intake. The cystathionine beta-synthase (CBS) enzyme reduces homocysteine levels and thus may protect against CRC. The CBS gene has a variant, 844ins68, that has been linked with increased activity. These variants may be involved in the development of the subgroup of CRC displaying aberrant DNA methylation and frequently associated with microsatellite instability (MSI).

Aim: To investigate the frequencies of the TT and 844ins68 genotypes in CRC patients with MSI+ tumours compared with those with MSI– tumours and a control population.

Subjects: Patients with CRC (n=501) and healthy control subjects (n=1207) were studied. CRC cases were classified as MSI+ (n=75) or MSI– (n=426) based on deletions within the BAT-26 mononucleotide repeat.

Methods: Subjects were genotyped for MTHFR using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and PCR-restriction fragment length polymorphism (PCR-RFLP) techniques, and for CBS using PCR.

Results: The MTHFR TT genotype was more frequent in older CRC patients (≥70 y) compared with equivalent aged controls (p=0.03), was associated with a significantly later age of diagnosis in patients with proximal colon tumours (p=0.02), and was almost twice as frequent in MSI+ than in MSI– tumours (p=0.05). Compared with normal controls, the 844ins68 variant of CBS was less frequent in patients with proximal tumours (p=0.02).

Conclusions: The TT genotype of MTHFR is associated with an increased risk of CRC in older populations, possibly due to age related disturbances in folate metabolism. The TT genotype appears to predispose to CRC that is MSI+. This may reflect the involvement of aberrant DNA methylation frequently associated with MSI+. The 844ins68 CBS polymorphism may protect against proximal tumours.

Epidemiological and clinical evidence suggests that folate deficiency may be involved in predisposition to cancer[1] and in particular to colorectal carcinoma (CRC). While the mechanisms linking low folate levels to cancer are not fully understood, one hypothesis involves disruption of the DNA methylation pathway. Folate deficiency leads to a reduced supply of methyl donors resulting in DNA hypomethylation, known to be an early and consistent event in carcinogenesis.[1]

Imbalances in folate and methyl group metabolism may be caused by dietary deficiency or genetic predisposition. Polymorphic variants of key enzymes in these pathways have attracted considerable interest because of the involvement of folate deficiency in a number of disease conditions. In particular, the enzyme methylenetetrahydrofolate reductase (MTHFR) plays a central role in folate metabolism, regulating the flow of folate groups between two important biosynthetic pathways. Its substrate, 5,10-methylenetetrahydrofolate (5,10-methyleneTHF), is an intracellular form of folate required for de novo synthesis of thymidylate and is therefore vital for DNA synthesis. The product of MTHFR activity, 5-methyltetrahydrofolate (5-methylTHF), is the main circulating form of folate in plasma and provides methyl groups for de novo synthesis of methionine and DNA methylation.[1] A common polymorphism in the MTHFR gene involves a C to T substitution at nucleotide 677, converting alanine to valine and resulting in a thermolabile enzyme with reduced activity.[2] Individuals homozygous for the TT variant have approximately 30% of normal enzyme activity and this genotype is associated with lower levels of circulating folate (5-methylTHF), accumulation of 5,10-methyleneTHF, elevated plasma homocysteine levels,[1] and significantly reduced levels of global DNA methylation in peripheral blood leucocytes.[3]

The relationship between the MTHFR polymorphism and CRC is unclear. Several case control studies have shown a 40–50% reduction in CRC risk for individuals with the TT genotype compared with those with the CC or CT genotype but the protective effect appeared to be dependent on having an adequate dietary folate intake.[4] This dietary interaction was more pronounced for patients who were diagnosed at an older age or in whom the tumours were located in the proximal colon.[5] Other studies suggest that the TT genotype may actually increase the risk of CRC in men with high alcohol consumption[6] or with poor dietary folate intake.[7] Together these results suggest that the risk of CRC conferred by the TT

Abbreviations: MTHFR, methylenetetrahydrofolate reductase; CBS, cystathionine beta-synthase; CRC, colorectal cancer; MSI, microsatellite instability; 5,10-methyleneTHF, 5,10-methylenetetrahydrofolate; 5-methylTHF, 5-methyltetrahydrofolate; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism; OR, odds ratio; HR, hazard ratio.

genotype of MTHFR may differ between individuals depending on age, sex, and dietary habits.

Polymorphic variants of other enzymes involved in folate and methyl group metabolism have not yet been well characterised. Cystathionine beta-synthase (CBS) irreversibly removes homocysteine from the methionine cycle by transsulfuration to cystathionine,[8] a pathway that may become more important when folate supply is limited. Plasma homocysteine levels increase under conditions of folate deficiency and are considered to be a highly sensitive indicator of cellular folate depletion.[1] Increased plasma homocysteine has been linked to DNA hypomethylation in lymphocytes through conversion to S-adenosylhomocysteine, a potent inhibitor of DNA methyltransferase.[8] The 844ins68 variant of CBS has a 68 base pair insertion, resulting in an enzyme that may have increased activity since it has been associated with decreased levels of plasma homocysteine.[9]

A growing body of evidence suggests that CRC can progress along separate pathways with distinct molecular, phenotypic, and clinical characteristics.[10] One such pathway may involve the recently described methylator phenotype, characterised by frequent hypermethylation of CpG islands[11] that often leads to transcriptional silencing. Aberrant methylation of the DNA repair gene *hMLH1*[12] gives rise to the majority of sporadic CRC with the microsatellite instability positive (MSI+) phenotype. In the present study we investigated the hypothesis that CRC patients with MSI+ tumours have an increased frequency of the TT MTHFR genotype and a decreased frequency of the 844ins68 CBS genotype. Our reasoning was that individuals with these genotypes may be, respectively, predisposed to or protected from CRC characterised by aberrant DNA methylation. Although folate deficiency is linked specifically to DNA hypomethylation,[1] CRC can demonstrate both global DNA hypomethylation and simultaneous focal hypermethylation of CpG islands.[13] Our hypothesis assumes that altered folate/methyl group metabolism may play a role in both of the above forms of methylation abnormality.

METHODS

Colorectal cancer cases and controls

Frozen or routinely processed tumour samples were obtained from patients undergoing surgery for CRC at Sir Charles Gairdner Hospital, Western Australia, between 1985 and 1998. Of 501 tumour samples successfully genotyped for MTHFR, 75 were selected because they were previously characterised as MSI+ based on deletions in the BAT-26 mononucleotide repeat.[14] All MSI+ tumours originated in the proximal colon while the remaining 426 MSI− tumours originated in the proximal (n=203) or distal (n=223) colon. Only Dukes' stage B or C tumours were included in the study. Of these 501 tumour samples, 449 were successfully genotyped for the CBS gene. The control population comprised 1207 healthy individuals (599 males and 608 females), aged 20–92 years, from the Western Australian population. The majority (1090) had previously been genotyped for MTHFR as part of a separate study into cardiovascular diseases.[15] A smaller subgroup (n=155) of this population was genotyped as normal controls for the CBS gene.

MTHFR and CBS genotyping

DNA from CRC and control cases was genotyped for MTHFR using an adapted silver stain polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method[16] or by PCR-restriction fragment length polymorphism with the *HinfI* restriction enzyme.[2] MTHFR primer sequences were: 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ and 5′-CCTCACCTG GATGGGAAAGATCC-3′ (annealing temperature 60°C).

Genotyping for CBS involved analysis of PCR product size by electrophoresis on 3% agarose gels. Primer sequences for CBS were: 5′-CGCCCTCTGCAGATCATTGG-3′ and 5′-CCTTCCA CCTCGTAGGTTGTC-3′ (annealing temperature 55°C).

PCR reactions were carried out in a volume of 12.5 µl containing 1×polymerisation buffer (Biotech International, Perth, Australia), 0.4 µM of each primer, 2.5 mM Mg^{2+}, and 0.5 U *Taq* polymerase (Qiagen, Melbourne, Australia). Reactions were "hot started" by addition of DNA at 94°C prior to commencement of cycling (32 cycles of 30 seconds at 94°C, 40 seconds at annealing temperature and 40 seconds at 72°C, followed by a final 10 minute extension at 72°C). For SSCP, 10 µl of PCR product were mixed with an equal volume of loading buffer, heated at 95°C for five minutes, and then run at 200 V for three hours on 8 cm length 18% non-denaturing polyacrylamide gels. Silver staining was performed as described previously.[16] The SSCP banding pattern for each MTHFR genotype (CC, CT, and TT) was confirmed for six representative cases by *Hinf*I digestion of the PCR product, as described previously.[2]

Statistical analysis

Statistical comparisons were performed using the SPSS (Chicago, Illinois, USA) software package. Associations were determined with the χ^2 test for independence using the Pearson statistic or Fisher's exact test when expected frequencies fell below five. Age differences between subgroups were compared by the Mann-Whitney U test. Multivariate analysis to identify predictors of MSI status was performed using a logistic regression model with a backwards stepwise removal of factors based on the likelihood ratio test. Cox's proportional hazards univariate survival analysis was used to calculate hazard ratios (HR) and 95% confidence intervals (CI). Multivariate survival analysis was performed using a Cox regression model with stepwise forward selection of independent variables based on the likelihood ratio. All p values given are two tailed with $p<0.05$ taken as statistically significant.

RESULTS

MTHFR genotype frequencies in CRC and controls

The MTHFR TT genotype frequency in 1207 control subjects from Western Australia was 9%. A slightly higher frequency of 11% was observed in 501 CRC patients (table 1). The frequency of each MTHFR genotype in case and control subgroups is also shown in table 1. Because several studies have reported reduced frequency of the TT genotype in older age groups,[17–19] both CRC and control groups were divided into ⩾70 and <70 year age groups. The median age of CRC patients (70 years) was chosen for this division. Results from males and females were combined as no sex differences in MTHFR genotype frequencies were observed (data not shown). Similar to previous studies,[17–19] a trend for reduced frequency of the TT genotype was observed in older control individuals (p=0.08). The proportion of TT individuals was significantly elevated in CRC patients diagnosed at ⩾70 years compared with the equivalent aged controls (12% *v* 7%; p=0.03), suggesting this genotype was a risk factor for CRC in older populations. Younger CRC and control groups showed no difference in TT genotype frequency. CRC patients with an MSI+ tumour were more than twice as likely to have the TT genotype compared with control individuals of similar age (16% *v* 7%; p=0.03).

MTHFR genotype and age of CRC diagnosis

The associations between MTHFR genotype, tumour site, MSI status, and patient age at diagnosis are shown in table 2. For both MSI+ and MSI− tumours originating in the proximal colon, TT genotype was associated with a later age of diagnosis compared with patients with the CC genotype. This was not seen for tumours originating in the distal colon where age at diagnosis was similar for both the CC and TT genotypes. TT individuals with tumours in the proximal colon developed cancer at a significantly later age compared with those with distal tumours (median ages 74 and 67 years, respectively;

Table 1 Frequency (n (%)) of methylenetetrahydrofolate reductase (MTHFR) genotypes (CC, CT, and TT) in control subjects and in colorectal cancer (CRC) patient subgroups

	n	CC (%)	CT (%)	TT (%)	p Value
Controls	1207	533 (44)	560 (47)	114 (9)	
CRC cases	501	249 (50)	197 (39)	55 (11)	0.70
Controls <70 y	980	425 (43)	456 (47)	99 (10)	
Controls ≥70 y	227	108 (48)	104 (46)	15 (7)	0.08
CRC cases <70 y	242	135 (55)	84 (35)	23 (10)	
CRC cases ≥70 y	259	114 (44)	113 (44)	32 (12)	0.10
Controls <70 y	980	425 (43)	456 (47)	99 (10)	
Cases <70 y	242	135 (55)	84 (35)	23 (10)	0.25
Controls ≥70y	227	108 (48)	104 (46)	15 (7)	
Cases ≥70 y	259	114 (44)	113 (44)	32 (12)	0.03
Controls ≥60 y*	467	204 (44)	229 (49)	34 (7)	
MSI+ cases	75	28 (37)	35 (47)	12 (16)	0.03

p values for χ^2 test comparing proportions of CC and TT between groups.
*The ≥60 year group was used as age matched controls for microsatellite instability positive (MSI+) cases as 80% of these patients were aged 60 years or more.

Table 3 Association of methylenetetrahydrofolate reductase (MTHFR) genotypes with clinicopathological features in colorectal cancer (CRC)

	n	CC (%)	CT (%)	TT (%)	p Value
Sex					
Male	231	115 (50)	96 (41)	20 (9)	
Female	270	134 (50)	101 (37)	35 (13)	0.19
Site					
Distal	223	120 (54)	79 (35)	24 (11)	
Proximal	278	129 (46)	118 (43)	31 (11)	0.54
Stage					
Dukes' B	186	94 (50)	64 (35)	28 (15)	
Dukes' C	315	155 (49)	133 (42)	27 (9)	0.07
Grade					
Moderate/well	431	210 (49)	174 (40)	47 (11)	
Poor	48	29 (61)	15 (31)	4 (8)	0.38
MSI status					
–	203	101 (50)	83 (41)	19 (9)	
+	75	28 (37)	35 (47)	12 (16)	0.05
p53 mutation					
–	303	148 (49)	116 (38)	39 (13)	
+	153	73 (48)	66 (43)	14 (9)	0.35
K-ras mutation					
–	180	86 (48)	77 (43)	17 (9)	
+	81	51 (63)	23 (28)	7 (9)	0.44

Values are number (%).
MSI, microsatellite instability.
p values for χ^2 test comparing proportions of CC and TT between groups.

p=0.03). In contrast, no difference in age at diagnosis between proximal and distal CRC was observed for CC individuals.

MTHFR genotype and clinicopathological features of CRC

No significant associations were observed between the TT genotype and clinical features such as patient sex, tumour site, or tumour grade, or with the presence of p53 or K-ras gene mutations. A trend was observed for the TT genotype to be more frequent in patients with Dukes' B stage disease compared with stage C (p=0.07) (table 3). The TT genotype was more frequent in patients with MSI+ tumours compared with those with MSI– tumours (p=0.05; table 3). Multivariate analysis including the covariates of patient age, sex, tumour location, and MTHFR genotype showed that only tumour site (odds ratio (OR)=15.7 (95% CI 4.7–52.6); p<0.0001) and MTHFR genotype (OR=1.5 (95% CI 1.02–2.3); p=0.04) were significant predictors of the MSI+ phenotype. Inclusion of CBS genotype as a covariate in this model resulted in the MTHFR genotype no longer being significant (OR=1.4 (95% CI 0.9–2.3); p=0.11), presumably due to reduced sample size. CBS genotype in this model was not an independent predictor of MSI+.

Survival analysis was carried out on non-adjuvant treated patients only (n=365) to avoid the problem of mixed treatment groups. Cox's univariate analysis revealed that Dukes' stage C was a significant indicator of poor survival (HR=3.8 (95% CI 2.5–5.8); p<0.0001) whereas the TT genotype was a significant factor of improved survival (HR=0.77 (95% CI 0.6–0.99); p=0.04). In multivariate analysis, MTHFR genotype was not an independent predictor of survival and only Dukes' stage remained significant. There were insufficient numbers of TT patients (n=5) treated with chemotherapy to determine the influence of this genotype on the survival benefit from treatment.

CBS polymorphism frequencies in CRC cases and controls

The frequencies of the 844ins68 CBS genotype in normal control subjects and CRC subgroups are shown in table 4. These data refer to individuals heterozygous for the insert as no polymorphic homozygotes were detected. The 844ins68 variant was found at reduced frequency in patients who developed proximal tumours compared with normal control subjects, suggestive of a protective effect against CRC at this site. Although a reduction was seen in both the MSI+ and MSI– groups, this was significant only in the latter group, presumably due to the small number of MSI+ cases. No

Table 2 Median age at diagnosis for colorectal cancer (CRC) patient subgroups according to methylenetetrahydrofolate reductase (MTHFR) genotype

		Age (y) at diagnosis			
	n	CC	CT	TT	p Value*
MSI+ proximal	75	67 (34–89)	72 (38–89)	78 (56–91)	0.04
MSI– proximal	203	68 (34–92)	73 (49–100)	74 (51–91) p=0.5†	0.06
MSI– distal	223	67 (32–89)	69 (39–91)	67 (32–81) p=0.03‡	0.46

Values are median (range).
MSI+, microsatellite instability positive.
*p values for U test comparing median ages of CC versus TT.
†p value for U test comparing median ages of TT between MSI+ and MSI– proximal CRCs.
‡p value for U test comparing median ages of TT between MSI– CRCs from the proximal and distal colon.

Table 4 Frequency of CBS 844ins68 heterozygotes in control subjects and in colorectal cancer (CRC) patient subgroups

	n	CBS 844ins68 (%)	p Value
Normal controls	155	15 (9.7)	
Total CRC	449	23 (5)	0.05
Total proximal CRC	248	9 (3.6)	0.02
MSI+ proximal	64	3 (4.7)	0.20
MSI– proximal	184	6 (3.3)	0.03
MSI– distal	201	14 (7)	0.50

Values are number (%).
MSI, microsatellite instability.
p values for χ^2 test comparing proportion of CBS 844ins68 heterozygotes in each CRC group to the normal control group.

associations with patient age, sex, or survival were observed for the CBS variant (data not shown).

DISCUSSION

A plethora of epidemiological evidence implicates folate in carcinogenesis,[1 20] mainly because of its roles in DNA synthesis and methylation. Polymorphic variants of the genes involved in folate and methyl group metabolism have the potential to affect DNA methylation levels and therefore the risk of CRC. In particular, the TT genotype of MTHFR is associated with raised homocyteine levels and altered DNA methylation in lymphocytes.[1 3] The 844ins68 genotype of CBS is associated with lowered homocysteine levels[9] and thus may protect against altered DNA methylation. An as yet poorly characterised group of CRC displays aberrant DNA methylation involving global hypomethylation as well as simultaneous focal hypermethylation of CpG islands.[13] The majority of sporadic MSI+ CRC belong to this so-called methylator phenotype.[11] In the present study we investigated the frequency of MTHFR and CBS variants in a large number of previously characterised CRC patients.[14] Using MSI+ as a surrogate marker for aberrant DNA methylation, we investigated the hypothesis that CRC patients with MSI+ tumours have an increased frequency of the TT MTHFR genotype and a decreased frequency of the 844ins68 CBS genotype compared with patients with MSI– tumours or control individuals.

The frequency of 844ins68 heterozygotes in our control group (9.7%) was slightly lower than the 12% previously reported for an Australian population.[9] Similarly, the TT genotype frequency observed in the control group (9.4%) was slightly lower than the 12% generally reported for Caucasian and Asian populations.[4] In accordance with several other studies of older (>80 years) Dutch,[17] Japanese,[18] and French[19] populations, we found a reduced TT genotype frequency in control individuals older than 70 years of age (table 1). In males it has been suggested this may be due to increased mortality from cancer[17] but no details on the type of cancer were available. Our results indicate that this increased mortality may be due in part to CRC. Although the Japanese and Dutch studies found that older male populations in particular had reduced TT frequencies, we found no evidence of sex differences in the current study.

Several major studies have reported a significantly reduced risk of developing CRC in TT individuals with adequate dietary folate intake,[5–7] suggestive of a protective effect of this genotype. We did not find a lower TT frequency in CRC patients less than 70 years of age and in contrast reported a significant 1.7-fold increase in TT frequency in older (≥70) patients compared with age matched controls, suggestive of predisposition to CRC. The reduced CRC risk reported by previous studies appeared to be influenced by diet as the TT genotype afforded no protective effect[5] or was associated with an increased risk[6 7] in patients with low folate due to poor dietary intake or to high alcohol consumption. Dietary information was not available in the present study, precluding analysis of this variable.

The importance of adequate dietary folate intake for reducing CRC risk in TT individuals appears to be more pronounced in those diagnosed at an older age or with tumours arising in the proximal colon.[5] Possibly related to these observations, we found that TT was associated with a significantly older age of CRC onset in the proximal colon only (table 2). These results suggest that the TT genotype may predispose to tumours with the methylator phenotype as patients with MSI+ tumours showing methylation of *hMLH1* are, on average, almost 20 years older than MSI+ patients without methylation of this gene.[21] Both the MSI+[10] and methylator phenotypes[11 22] occur more frequently in the proximal colon. The TT genotype of MTHFR is linked to elevated levels of plasma homocysteine[1] and disturbed DNA methylation,[3] both of which have been shown to increase with patient age.[11 19 23] For individuals with age related increases in homocysteine levels and DNA methylation abnormality, the TT genotype may place an additional burden making them more susceptible to development of late onset CRC of the methylator phenotype.

Increased TT genotype frequency has also been reported in patients with inflammatory bowel disease,[24 25] a condition associated with raised homocysteine levels,[24] defects in folate absorption, and an increased risk of CRC.[1] Other conditions associated with raised plasma homocysteine concentration include cigarette smoking and low oestrogen levels,[26] both recently shown to increase the risk of MSI+ CRC.[27–29] Although speculative, the combined evidence suggests that TT genotype may become an additional risk factor for CRC when methyl group metabolism is disturbed by conditions such as poor dietary intake, inflammatory bowel disease, old age, and possibly also by smoking and low oestrogen levels. The current study indicates that CRC in TT individuals is more likely to develop along the MSI+ pathway of carcinogenesis. As the majority of sporadic MSI+ tumours arise through methylation of the *hMLH1* gene,[11] we propose that the association observed here between TT genotype and MSI+ (table 3) may in fact be a reflection of altered DNA methylation that is itself related to disturbed folate/methyl group metabolism.

The frequency of CBS genotypic variants in CRC has not previously been reported. We found that patients with proximal tumours have a significantly reduced frequency of the CBS 833ins68 heterozygous genotype compared with control subjects (table 4), suggestive of a protective effect against proximal colon tumours. As discussed above, both the MSI+ and methylator phenotypes occur more frequently in the proximal colon. The reduction in 844ins68 frequency did not reach significance for the MSI+ tumour group but this may be due to the very low numbers and requires confirmation in a larger study. As this variant has been associated with reduced homocysteine levels,[9] its lower frequency in the proximal tumour group may indicate a protective effect against CRC of the methylator phentoype.

In summary, we have confirmed previous findings suggesting that the TT variant of MTHFR is associated with increased mortality in older populations. Our results suggest this may involve predisposition to CRC and especially to those tumours developing along the MSI+/methylator phenotype pathway of carcinogenesis. We also report evidence suggesting that the 844ins68 variant of CBS may protect against CRC of the MSI+/methylator phenotype. The MSI+ phenotype was used in the current study as a surrogate marker of aberrant methylation. Further studies that directly identify the methylator phenotype will be required to confirm the relationships between the TT and 844ins68 genotypes and aberrant DNA methylation in CRC. Given that folate intermediates are involved in the mechanism of fluorouracil based chemotherapies,[30] it will also be interesting in future research to determine whether genotypic variants that influence folate metabolism have predictive value for this treatment.

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Authors' affiliations

B Shannon, S Gnanasampanthan, B Iacopetta, Department of Surgery, University of Western Australia, Nedlands 6009, Australia
J Beilby, Clinical Biochemistry, Pathcentre, Sir Charles Gairdner Hospital, Nedlands 6009, Australia

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