European Journal of Human Genetics (2009) 17, 629 – 635

www.nature.com/ejhg

ARTICLE

MTHFR 677 C > T and 1298 A > C polymorphisms and the age of onset of colorectal cancer in hereditary nonpolyposis colorectal cancer

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Hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome is characterized by inactivating germline mutations in DNA mismatch repair genes resulting in an increased risk of developing an epithelial malignancy. There is considerable variability in disease expression observed in this syndrome, which is thought to be due to a combination of genetic and environmental factors. Alterations in the kinetics of methylene tetrahydrofolate reductase (MTHFR) due to the presence of polymorphisms in the *MTHFR* gene have been associated with an increased risk of colorectal cancer (CRC). Two common single nucleotide polymorphisms (SNPs) located within the *MTHFR* gene, 677 C > T and 1298 A > C, that alter the function of the encoded protein have been the focus of many studies on CRC risk outside the context of an inherited predisposition to disease. In this report, a total of 417 HNPCC participants were genotyped for the 677 C > T and 1298 A > C SNPs to determine whether there exists an association with the age of disease onset of CRC. Genotyping of both SNPs was performed by TaqMan® assay technology. Associations in disease risk were further investigated using Kaplan–Meier survival analysis and Cox hazard regression. The average ages of disease diagnosis were found to be different between individuals harbouring either one of the *MTHFR* polymorphisms. Both Kaplan–Meier and Cox hazard regression analyses revealed a more complex relationship between the two polymorphisms and the age of CRC onset. The Kaplan–Meier survival analysis revealed that compound heterozygotes for the two SNPs developed CRC 10 years later compared with those carrying only wild-type alleles.
European Journal of Human Genetics (2009) **17,** 629–635; doi:10.1038/ejhg.2008.239; published online 21 January 2009

Keywords: HNPCC; colorectal cancer; *MTHFR*; polymorphisms

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Received 28 February 2008; revised 22 October 2008; accepted 13 November 2008; published online 21 January 2009

Introduction

Hereditary non-polyposis colorectal cancer (HNPCC) is characterised by an increased risk of early onset of colorectal cancer (CRC).[1] HNPCC or Lynch syndrome is an autosomal-dominant disorder caused by deleterious germline mutations in DNA mismatch repair (MMR) genes.[1] Deficiencies in MMR activity can result in reduced

recognition and correction of mismatched bases during DNA replication. This results in an increase in genomic instability, which can be visualised in tumour DNA, where there is a loss of MMR activity. This is known as microsatellite instability (MSI), a hallmark of HNPCC tumours.[2] Mutations in the MMR genes *hMLH1* and *hMSH2* account for approximately 60% of HNPCC cases.[3] It has been commonly reported that individuals with HNPCC have an 80% lifetime risk of developing CRC by 70 years of age and this predisposition accounts for somewhere between 2 and 7% of all diagnosed cases. Analyses that are more recent suggest, however, that CRC penetrance has been significantly overestimated, being 47% and 34% for males and females, respectively.[4] The average age of onset of CRC is 44 years of age (as assessed from high-risk families) compared with 64 years in individuals who do not have this genetic predisposition.[5,6] In addition to CRC, there is an increased risk of other epithelial malignancies that include cancers of the endometrium, stomach, ovaries, uroepithelial and biliary tracts, small intestine and brain.[7]

Despite the presence of a germline mutation in a MMR gene being a strong predictor of disease, there is considerable variation in the phenotypic expression in HNPCC patients, in particular the age of CRC onset.[3] This appears to be largely independent of the type or location of MMR mutation, suggesting that genetic or environmental modifying effects influence the age of disease onset.

Methylene tetrahydrofolate reductase (MTHFR) is an essential enzyme in folate metabolism and subsequently plays a key role in DNA synthesis and methylation.[8] The role of this enzyme is to catalyse the irreversible reaction of 5,10-methyl-tetrahydrofolate (MTHF) to 5-MTHF, which is an integral part of the folate metabolism pathway. 5,10-MTHF is required for DNA synthesis and is in particular involved in uracil incorporation, whereas its product 5-MTHF is the methyl donor for regeneration of methionine from homocysteine for methylation reactions.[9] MTHFR activity can therefore affect levels of both 5,10-MTHF and 5-MTHF, both of which may influence the initiation and growth of tumour cells. Fluctuating amounts of 5,10-MTHF may lead to uracil misincorporation during DNA synthesis resulting in double-strand breaks,[10] whereas inconsistent amounts of 5-MTHF can affect methylation, therefore potentially influencing tumour suppressor or oncogene expression.[8,11]

Two common polymorphisms found within the *MTHFR* gene have recently been the focus of numerous studies on CRC risk.[8,9,12–18] The nucleotide polymorphism 677 C>T (rs1801133) is located within the region coding for the catalytic domain of MTHFR and results in an amino acid substitution from an alanine to a valine at codon position 222 (exon 4).[9,19] The 677 C>T variant has been associated with a reduced enzyme activity.[20,21] This single nucleotide polymorphism (SNP) has been implicated in CRC risk in several CRC patient populations;[17] however, conflicting results remain.[8] The second polymorphism, 1298 A>C (rs1801131), results in an amino acid change from a glutamine to alanine at codon position 429 (exon 7) and is found in a regulatory region of the MTHFR enzyme.[12] This polymorphism is also thought to cause a reduction in MTHFR activity, although its effect is considered to be less than that conferred by the 677 C>T change.[15] Further studies indicate that individuals heterozygous for both SNPs have a 50–60% decrease in MTHFR enzyme activity compared with their wild-type counterparts.[14]

Despite numerous studies examining associations of these two SNPs and CRC risk, there has only been one report that has specifically focused on the potential association of the *MTHFR* variants, 677 C>T and 1298 A>C, with the age of diagnosis of CRC in HNPCC.[18] In a small study by Pande *et al*[18] among 185 *hMSH2* or *hMLH1* mutation carriers, an ~4-year difference in the age of CRC diagnosis was observed in patients harbouring the 677 C>T polymorphism, whereas no effect was observed for the 1298 A>C SNP.

In this study, we investigated whether the 677 C>T and 1298 A>C SNPs in *MTHFR* influence the age of CRC diagnosis in a large group of 417 HNPCC cases, all of which harboured causative mutations in either *hMLH1* or *hMSH2*.

Methods

The sample population consisted of 220 (53%) Australian and 197 (47%) Polish participants. All participants harboured causative mutations in *MLH1* or *MSH2*. Both the Australian and Polish participants were all of North-Western European origin. The Australian patients enrolled in this study were clinic-based and recruited from family cancer clinics from the State of New South Wales. The Polish patients were recruited from the hereditary cancer clinics in and around the city of Szczecin. There were 194 (47%) *MLH1* and 193 (46%) *MSH2* cases with truncating or exon splice site mutations and 30 (7%) missense cases (deemed causative as there was pathogenicity evidence determined by functional studies or segregation analysis reported in the literature). There was no difference in the average age of disease diagnosis in the missense mutation carriers with disease compared with the truncating or exon splice site mutation carriers. Average age of missense mutation carriers was 41 years, whereas that of the nonsense mutation carriers was 43 years. To certify that the missense mutations were deleterious, expression analyses of MSH2, MLH1, PMS2 or MSH6 were performed by immunohistochemistry, and in cases with an ambiguous result, DNA microsatellite testing was undertaken. All tumours associated with missense mutations failed to express the respective protein or showed MSI (data not shown).

Of the 417 participants, 206 (49%) had been diagnosed with CRC as their first tumour. Only patients presenting with CRC as their first tumour were included in this study. The median age of the participants in this study with CRC was 43 years compared with 41 years for those without CRC. The Institutional Ethics Review Boards of the Pomeranian Academy of Medicine and the Hunter New England Health Service approved the study. All participants gave written informed consent for the DNA samples to be used for research into HNPCC. The clinical and demographic characteristics of the study participants are shown in Table 1.

SNP genotyping

Genotyping of the *MTHFR* 677 C>T and 1298 A>C SNPs was performed on the ABI PRISM® 7500 Real-Time (RT) PCR System (PE Applied Biosystems, Foster City, CA, USA), using primers and probes from Assay-by-Demand (Applied Biosystems) for 677 C>T (rs1801133, assay ID: 526886) and 1298 A>C (rs1801131, assay ID: 526686). The assay was performed under universal conditions, with each reaction containing 50 ng DNA, 0.125 μl 40× Assay Mix and 2.5 μl TaqMan® Universal PCR master mix made to a final volume of 5 μl with sterile water. Thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 50 cycles of 92°C for 15 s and 60°C for 1 min. After the PCR reaction, plates were scanned by the ABI PRISM® 7500 PCR system to determine genotypes by allelic discrimination. Genotyping accuracy was assessed by repeating 10% of the DNA samples, which were randomly selected from both the control and affected patient populations. Concordance of 100% was observed between both genotypes for all samples.

Table 1 Demographics and genetic traits of HNPCC study participants according CRC status

	CRC (n = *206*)	*No CRC* (n = *211*)	*Total* (n = *417*)
Gender			
Male	83	73	156 (37.4%)
Female	123	138	261 (62.6%)
Age of onset (years)			
Mean (SD)	42.7 (11.4)	41.1 (15.2)	41.9 (13.3)
Median	43	41	42
Range	16–78	17–95	16–95
MMR mutation			
Truncation	188	199	387 (92.8%)
Missense	18	12	30 (7.2%)
MMR gene mutation			
MLH1	104	120	224 (53.7%)
MSH2	102	91	193 (46.3%)
MTHFR 677 C>T			
CC	105	101	206 (49.4%)
CT	83	91	174 (41.7%)
TT	18	19	37 (8.9%)
MTHFR 1298 A>C			
AA	92	86	178 (42.7%)
AC	89	98	187 (44.8%)
CC	25	27	52 (12.5%)

CRC, colorectal cancer; HNPCC, hereditary non-polyposis colorectal cancer; MMR, mismatch repair.

Statistical analysis

Differences in the average age of disease onset in the polymorphism carriers compared with patients wild type for both polymorphisms were determined using the Student's *t*-test.

The assessment of any association between *MTHFR* genotypes and the age of CRC onset was carried out by the survival analysis methods of Kaplan–Meier and Cox hazard regression modelling. Kaplan–Meier survival curves were used to plot the proportion of participants who were cancer free *versus* the patient age of diagnosis of CRC in relation to *MTHFR* genotype. This univariate survival analysis method used the Wilcoxon test, which emphasises observations from early diagnosis, the log-rank test, which gives more weight to later ages, and finally the Tarone–Ware test, which is an intermediate of the two previous tests. In cases where nonsignificant results were found for all three tests, only the log rank test was stated. Cox proportional hazard regression models were used to test significant findings found by Kaplan–Meier and to generate hazard ratios (HRs) and 95% confidence intervals (CIs) in a multivariate analysis taking into account MMR mutation, family member status and gender. Age of diagnosis was defined as patient age at the time of CRC diagnosis. For unaffected participants, age was based on the date of birth and disease-free status at last follow-up, being treated as censored in the analysis. All statistics were set at a significance level of $P \leq 0.05$. The statistical analysis in this study was carried out using Intercooled Stata 8.0 (Stata Corp., College Station, TX, USA).

Additional survival analysis was performed using haplotype pairs rather than SNPs as the predictor variable of interest. The haplotype analysis was implemented using a stochastic EM algorithm in a Cox proportion hazards regression framework.[22] Additional predictor variables included in the model were gender, MMR mutation type (missense or truncation/deletion) and MMR gene mutation. The model was fitted using THESIAS software.

Results

The *MTHFR* genotypes were determined for 677 C>T and 1298 A>C by RT-PCR. Both SNPs were found to be in the Hardy–Weinberg equilibrium in both the Polish and Australian participants. There was no significant difference in allele and genotype frequencies between the Australian

and Polish participants, which allowed for pooling of the genotyping results. The allele and genotype distributions by CRC status, mutation and gender are shown in Table 2. Using Lewontin statistics, the two SNPs were found to be in linkage disequilibrium with a *D* score of 0.96.

MTHFR genotypes and the average age of disease onset

A comparison of the average age of disease diagnosis revealed a significant difference ($P=0.008$) in the age of disease diagnosis in individuals who harboured one or more polymorphisms in *MTHFR* (43.72 years) compared with those CRC cases who did not harbour either SNP (37.62 years).

Association of the 677 C>T and 1298 A>C polymorphisms with CRC penetrance

The Kaplan–Meier survival analysis was undertaken to determine more precisely the relationship between the two polymorphisms and disease expression. No statistically significant association between the 677 C>T genotypes and the age of CRC onset (log rank, $P=0.57$) was detected (see Figure 1a). Also when the heterozygotes and variant homozygotes were compared with wild-type homozygotes no association was observed (test, $P=0.41$) (Figure 1b). Analysis of the *hMLH1* and *hMSH2* mutation carriers alone failed to reveal any association (log rank, $P=0.65$ and log rank, $P=0.86$, respectively). Subdivision of the group by gender also revealed no associations (males: Log rank test, $P=0.76$; females: Log rank test, $P=0.52$).

Genotyping of the *MTHFR* 1298 A>C SNP revealed similar results (Figure 2a). No significant difference in the age of onset was found for the individual 1298 A>C genotypes (log rank, $P=0.13$), *hMLH1* (log rank, $P=0.17$), *hMSH2* (log rank, $P=0.1$), nor by subdividing by gender, males (log rank, $P=0.79$) or females (log rank, $P=0.11$). When the heterozygotes and variant homozygotes were compared with wild-type homozygotes, however, a protective trend for 1298 A>C polymorphism was observed (Figure 2b). A significant association in two of the three tests was found (log rank, $P=0.045$ and the Tarone–Ware, $P=0.044$). The Wilcoxon test, which emphasises cases at an early age of diagnosis, showed a trend in the same direction ($P=0.053$). Overall, the Kaplan–Meier analysis suggested that the median age of diagnosis was 50 years of age for the heterozygous/homozygous variant 1298 A>C allele carriers compared with 47 years of age for the wild-type carriers. Multivariate Cox hazard regression modelling, including the variables MMR mutation, gender and

Table 2 *MTHFR* allele frequencies for 677 C>T and 1298 A>C for the study cohort

MTHFR 677 C>T	*CC*	*CT*	*TT*	*Any T allele*
All participants (n = *417*)	*206 (49.4%)*	*174 (41.7%)*	*37 (8.8%)*	*211 (50.6%)*
Allele frequency	*0.703*		*0.297*	
CRC status				
CRC+ (*n* = 206)	105 (51%)	83 (40.3%)	18 (8.7%)	101 (49%)
CRC− (*n* = 211)	101(47.9%)	91 (43.1%)	19 (9.0%)	110 (52.1%)
Mutation				
MLH1 (*n* = 224)	105(46.7%)	95 (42.4%)	24 (10.7%)	119 (53.1%)
MSH2 (*n* = 193)	101(52.6%)	79 (40.7%)	13 (6.7%)	92 (47.6%)
Gender				
Male (*n* = 156)	72 (46.2%)	70 (44.9%)	14 (8.9%)	84 (53.8%)
Female (*n* = 261)	134(51.3%)	104 (40%)	23 (8.7%)	127 (48.7%)
MTHFR 1298 A>C	*AA*	*AC*	*CC*	*Any C allele*
All participants (n = *417*)	*178(42.5%)*	*187 (44.9%)*	*52 (12.6%)*	*239 (57.5%)*
Allele frequency	*0.650*		*0.350*	
CRC status				
CRC+ (*n* = 206)	92 (44.7%)	89 (43.2%)	25 (12.1%)	114 (55.3%)
CRC− (*n* = 211)	86 (40.8%)	98 (46.4%)	27 (12.8%)	125 (59.2%)
Mutation				
MLH1 (*n* = 224)	94 (41.8%)	103 (46.2%)	27 (12%)	131 (58.2%)
MSH2 (*n* = 193)	84 (43.5%)	84 (43.5%)	25 (13.0%)	109 (56.5%)
Gender				
Male (*n* = 156)	69 (44.2%)	67 (43%)	20 (12.8%)	87 (55.8%)
Female (*n* = 261)	109 (41.8%)	120 (46%)	32 (12.2%)	152 (58.2%)

CRC, colorectal cancer.

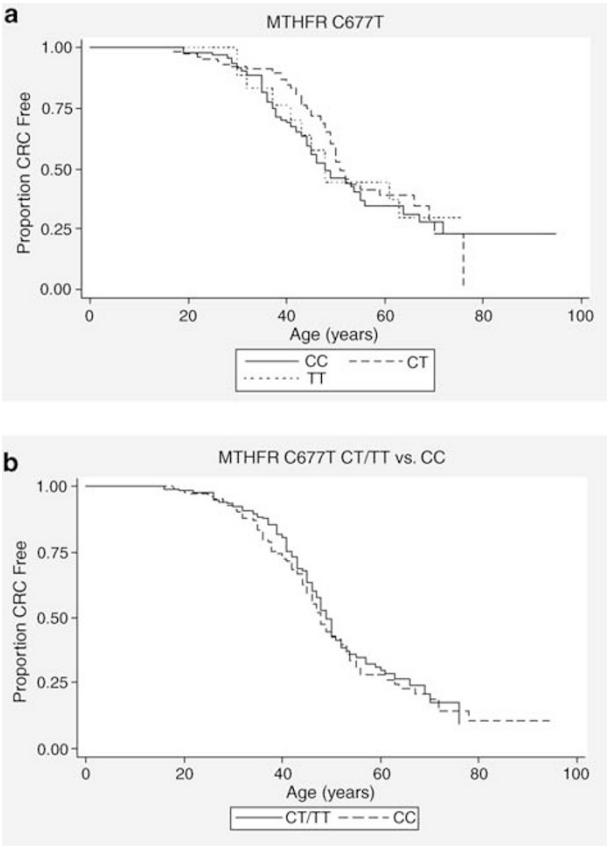

Figure 1 Kaplan–Meier survival curves for *MTHFR* 677 C>T representing time to age of CRC by genotype. (**a**) By wild type (CC), heterozygote (CT) and homozygote (TT) genotypes. (**b**) By combined heterozygote/homozygote variant (CT/TT) *versus* wild type (CC).

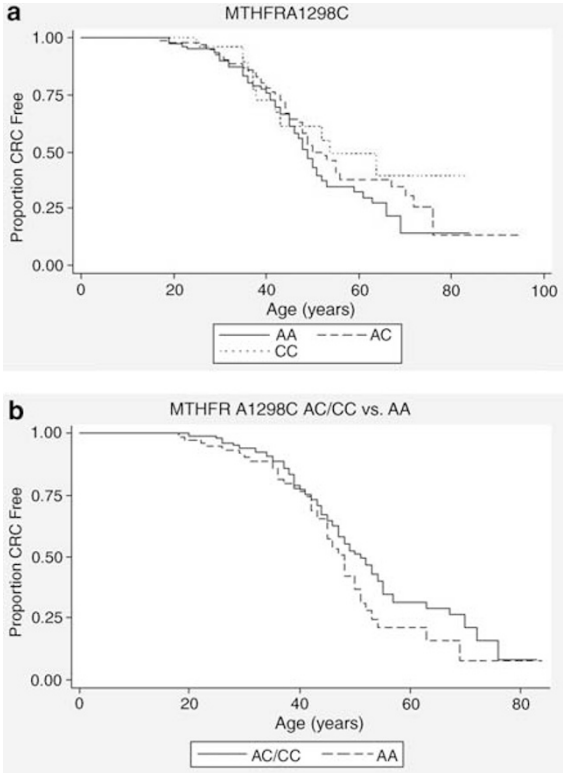

Figure 2 Kaplan–Meier survival curves for *MTHFR* 1298 A>C representing time to age of CRC by genotype. (**a**) By wild type (AA), heterozygote (AC) and homozygote (CC) genotypes. (**b**) By combined heterozygote/homozygote variant (AC/CC) *versus* wild type (AA).

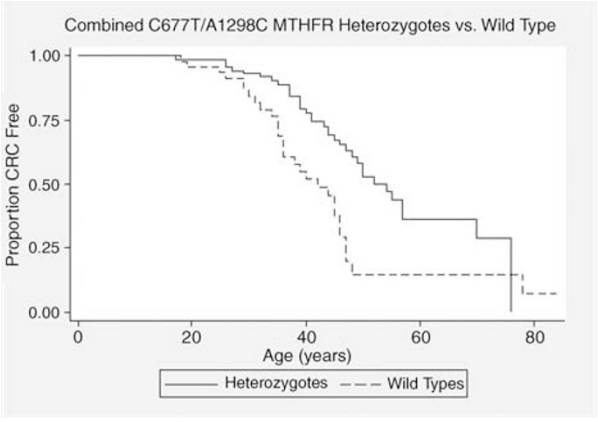

Figure 3 Combined Kaplan–Meier survival analysis for *MTHFR* 677 C>T and 1298 A>C. Plots comparing time with CRC onset by combined heterozygote (CT/AC) *versus* combined wild type (CC/AA).

family, was then used to determine the significance of the Kaplan–Meier analysis. Without the family variable, the association retained its borderline significance (HR: 0.79, 95% CI: 0.6–1.0, $P=0.073$), but when the family variable was included into this model the final outcome was non-significant ($P=0.11$). Despite this statistically nonsignificant result, the trend for wild-type carriers of 1298 A>C to develop CRC at an earlier age still remained.

Next, a joint analysis was performed between heterozygote forms of *MTHFR* 677 C>T (CT/TT) and 1298 A>C (AC/CC) compared with the wild types (CC/AA) for both SNPs. A strong association between the age of CRC onset and the combined *MTHFR* genotypes was detected (Figure 3). The log rank ($P=0.001$), Wilcoxon ($P=0.0008$), and Tarone–Ware ($P=0.0005$) tests were all highly significant with the median age of CRC onset 10 years later (52 years of age) in the participants heterozygous for both SNPs compared with those carrying both wild-type genotypes (42 years of age). This result was substantiated using Cox modelling where the variables of MMR mutation, gender and family were included. A significant *P*-value was obtained ($P=0.007$; HR: 0.47; 95% CI: 0.27–0.81) among the 133 patients who carried both heterozygous or both wild-type genotypes. Owing to the absence of patients carrying both homozygous variant genotypes, these were not assessed.

The haplotype analysis verified the results of the heterozygote forms of *MTHFR* 677 and 1298 compared with the wild types (CC/AA). The frequency of the AC, CC, AT and CT haplotypes were 36, 34, 29 and 1%, respectively. Compared with the AC haplotype, the HRs for the AT and CC haplotypes were similar and statistically significant with estimates of 0.70 (0.55, 0.90) for the AT haplotype and 0.66 (0.52, 0.84) for the CC haplotype. The HR for the CT haplotype was 1.06 (0.44–2.56).

Analysis of *hMSH2* mutation carriers alone carrying the combined 677 C>T/1298 A>C (CT/AC) genotype compared with those with only the wild-type alleles revealed a significant difference in the age of disease onset similar to that identified in the combined study population (log rank: $P=0.05$; Wilcoxon test: $P=0.037$; and Tarone–Ware test: $P=0.032$). Similarly, differences in the age of CRC onset were also observed for *hMLH1* mutation carriers (log rank: $P=0.005$; Wilcoxon test: $P=0.007$; and Tarone–Ware: $P=0.005$).

Discussion

MTHFR polymorphisms have been the focus of many studies and in particular for investigations into CRC where fluctuations in folate levels caused by 677 C>T and 1298 A>C variants potentially lead to an altered risk of cancer by subsequent variation of the deoxynucleotide pool.[8,9,12–18]

The results from this study are highly suggestive of a protective effect against CRC development in HNPCC individuals who harbour the heterozygote genotypes of 677 C>T and 1298 A>C in *MTHFR* compared with those carrying only wild-type alleles. Overall, a significant effect was observed for the patients who carry 677 C>T SNP (CT or TT) regarding the average age of disease onset compared with those who did not carry this SNP ($P=0.0279$). These findings are consistent with several studies that have shown the 677 C>T change to be associated with a reduced risk of bowel cancer in unselected CRC populations and in the study by Pande *et al*,[18] which reported a similar effect with the TT genotype being associated with later ages of CRC onset.

The differences in the average ages of disease onset indicate that there is a protective effect conferred by the presence of one or more polymorphisms in the *MTHFR* gene. This type of analysis does not, however, provide any information regarding age-specific differences that may become stronger or weaker as patients get older. To address this issue, a more specific measure of the effects of the polymorphism(s) is required. This has been achieved by examining where the greatest protective effects of the polymorphisms exist.

The *MTHFR* 1298 A>C SNP has also been found to have a similar protective effect in sporadic cases of CRC and our findings also indicate a protective effect in HNPCC as homozygote and heterozygote carriers together had an average age of disease onset of 44.32 years compared with non-carriers whose average age was 37.62 years ($P=0.009$). This is in contrast to Pande *et al*,[18] who did not observe any significant association in the age of disease onset in HNPCC patient with this SNP.

MTHFR has a fundamental position in folate metabolism catalysing the irreversible reaction of 5,10-MTHF to 5-MTHF. A decrease in 5-MTHF may lead to a reduction in DNA methylation, whereas 5,10-MTHF is required for thymidine synthesis,[10] emphasising the significance of MTHFR enzyme activity for these two important biochemical pathways. It has been shown earlier that individuals who carry the heterozygote forms 677 C>T and 1298 A>C have a 50–60% decrease in MTHFR activity compared with those with only wild-type alleles.[14] A reduction in MTHFR activity leads to greater quantities of its substrate 5,10-MTHF, required for DNA synthesis, and thereby reduces the availability of uracil. Misincorporation of uracil during DNA synthesis may result in double-strand breaks during DNA excision repair.[10] The increased pool of 5,10-MTHF pushes the folate metabolism towards DNA synthesis, in turn reducing the pool of uracil. Reduced amounts of uracil may also reduce the overall risk of uracil misincorporation owing to its limited availability. For individuals with MMR deficiency, the effect of reduced MTHFR enzyme activity may be a substantial advantage as uracil misincorporation could be particularly deleterious in conjunction with an impaired DNA repair pathway. The subsequent lower levels of 5-MTHF may also be beneficial due to a potential reduction in DNA methylation. Hypermethylation of the promoter of tumour suppressor or MMR genes may lead to gene silencing, therefore a reduction in methylation through decreased MTHFR activity could lead to less likelihood of this silencing occurring. Despite the risk of oncogene activation, a decreased level of DNA methylation may help maintain the integrity of the active allele in MMR-deficient individuals as seen in HNPCC cases.

This notion of methylation levels and uracil misincorporation being linked to age of CRC onset in HNPCC patients is shared by Pande *et al*;[18] however, our findings differ quite considerably. Our results are based on a larger sample size (419 *versus* 185), thereby providing more robust statistical support. The claim by Pande *et al*[18] of an earlier disease onset age for *hMLH1* mutation carriers harbouring only the wild-type allele *MTHFR* (C677C) could not be substantiated in our study. In the study by Pande *et al*,[18] the association observed was based on relatively few observations and it remains likely that this represents a type 1 statistical error rather than a true association.

The main limitation of our study is that we were unable to include any dietary or lifestyle factors, in particular levels of folate. Folate has been shown to be strongly linked to CRC in many studies,[23] with low levels of folate found to

increase risk by hypomethylation and the misincorporation of uracil. In contrast to this, however, folate deficiency in rapidly dividing cells as found in a tumour could lead to inefficient DNA synthesis and eventual apoptosis of the developing cancer. In this case, increased folate levels would promote malignant growths.[15] Evidently, folate status also influences MTHFR enzyme activity and would therefore be extremely useful information in future studies.

In conclusion, our findings indicate significant evidence for the combined *MTHFR* 677 C>T and 1298 A>C effect in CRC onset age for HNPCC cases. We speculate that this effect is because of the substantial influence of both these SNPs in folate metabolism, with both heterozygote forms required to make a significant impact on disease expression.

Acknowledgements

We acknowledge the support to the University of Newcastle, the Hunter Medical Research Institute and the Centre for Information Based Medicine in funding this study. We also thank the participants in the study for allowing their DNA to be used for further research into this disease.

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