Folate Metabolism Polymorphisms Influence Risk of Colorectal Adenoma Recurrence

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Abstract

Folate intake is inversely related to risk of developing colorectal neoplasia. Associations between risk of colorectal neoplasia and polymorphisms in genes coding for enzymes involved in folate metabolism have also been reported, suggesting a relationship between genotype and development of colorectal neoplasia. To further investigate the effects of folate metabolism genotypes on colorectal neoplasia, we genotyped 546 patients participating in a randomized controlled trial of folate supplementation for the prevention of colorectal adenoma recurrence. A significantly reduced risk of recurrence was observed in patients heterozygous for the *MTRR* A66G polymorphism [relative risk (RR), 0.64; 95% confidence interval (95% CI), 0.46-0.90] or heterozygous for the *MTHFR* A1298C polymorphism (RR, 0.71; 95% CI, 0.52-0.97). Furthermore, a significant reduction in recurrence risk was seen in *MTRR* A66G heterozygotes who received folate supplements but not in those who did not receive folate. Patients heterozygous for the *MTHFR* C677T polymorphism had a nonsignificant risk reduction (RR, 0.92; 95% CI, 0.69-1.23), as did patients with one or two variant alleles for the *MTR* A2756G polymorphism (RR, 0.82; 95% CI, 0.60-1.12). No influence on recurrence risk was observed for the *TSER*, *TSER* 3R G>C, and *TS* 1494del6 variants. These findings provide additional support for the hypothesis that germ line variants in folate metabolism genes influence the development of colorectal adenomas. (Cancer Epidemiol Biomarkers Prev 2006;15(9):1607–13)

Introduction

An inverse association between dietary folate intake and risk of development of both colorectal adenoma (CRA) and colorectal carcinoma (CRC) has been documented in a number of epidemiologic studies (1, 2). Aberrations in both DNA methylation and DNA synthesis and repair play a major role in carcinogenesis, and folate coenzymes, acting as acceptors or donors of one-carbon units, influence both these processes (3, 4). DNA methylation is dependent on *S*-adenosylmethionine, and dietary folate depletion can diminish *S*-adenosylmethionine pools whereas folate deficiency has been reported to result in uracil misincorporation in DNA (5, 6). Either or both of these mechanisms may underlie the benefits of increased folate intake in prevention of colorectal neoplasia development.

The enzymes 5,10-methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), methionine synthase reductase (MTRR), and thymidylate synthetase (TS) play important and interrelated roles in folate metabolism (Fig. 1). The MTHFR enzyme occupies a pivitol position, catalyzing the irreversible conversion of 5,10-methylene-THF to 5-methyl-THF. The MTHFR substrate, 5,10-methylene-THF, is used by TS in the methylation of dUMP to dTMP, which is the sole *de novo* source of thymidine required for DNA synthesis and repair. The MTHFR product, 5-methyl-THF, is the methyl group donor for the remethylation of homocysteine to methionine catalyzed by MTR in a reaction dependent on vitamin B_{12} as an intermediate methyl carrier (7). MTR may become inactive due to oxidation of its vitamin B_{12} cofactor and restoration of MTR activity is dependent on reductive remethylation of vitamin B_{12} by MTRR (8).

The genes coding for MTHFR, MTR, MTRR, and TS enzymes are polymorphic; in some cases, the genetic variants have been shown to affect the function of the expressed proteins (9, 10). The effects of folate metabolism variants on risk of developing CRA and CRC have been investigated in a number of case-control studies and their interactions with dietary folate have also been reported (11, 12). Variant alleles of the *MTHFR* C677T and A1298C polymorphisms generate an enzyme with reduced activity compared with wild-type and have been associated with reduced CRC and CRA risk (9, 10, 12-16). *MTR* A2756G heterozygote and homozygous variant genotypes are associated with reduced plasma homocysteine levels, and *MTRR* A66G variant alleles generate an enzyme with lower affinity for *MTR* (17, 18). *MTRR* 66GG homozygotes have been reported to be at increased risk of CRC, whereas reports of associations of the *MTR* A2756G polymorphism and colorectal neoplasia are inconsistent (12, 19, 20).

Polymorphisms in the *TS* 5′ untranslated region enhancer region (*TSER*) and 3′ untranslated region have been reported to correlate with altered TS expression. The *TSER* contains a 28-bp tandem repeat sequence, and individuals carrying alleles with three repeats (3R) have higher TS mRNA levels than individuals carrying alleles with two repeats (2R; ref. 21). An additional polymorphism mapping to the second repeat sequence of the 3R allele, *TSER* 3R G>C, may also affect TS expression with 3RC alleles being associated with comparable expression to 2R alleles (22, 23). Expression of TS may be further influenced by a 6-bp deletion at position 1,494 in the 3′ untranslated region. The −6bp allele has been reported to result in low TS mRNA stability and low TS expression compared with the wild-type allele (24). Variant alleles

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Note: List of members of the UKCAP Consortium is available on request.

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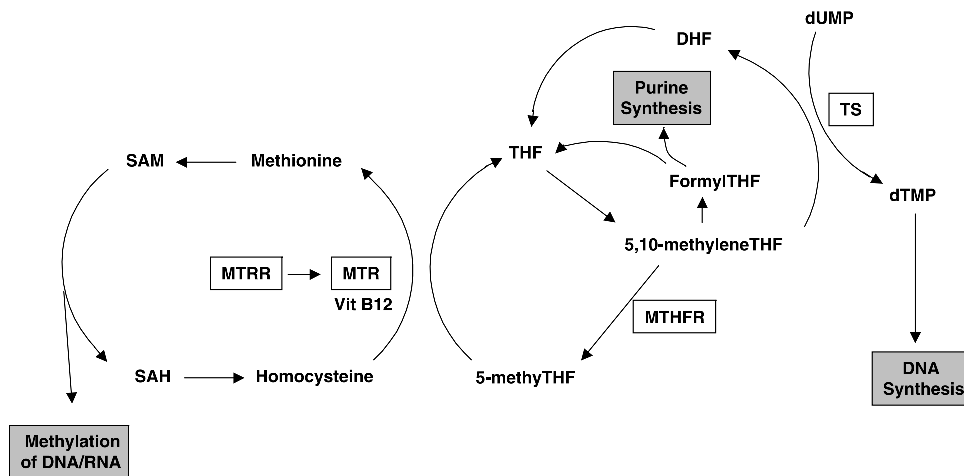

Figure 1. Schematic representation of folate metabolism. Enzymes with polymorphisms investigated in this study are boxed. *THF,* tetrahydrofolate; *DHF,* dihydrofolate; *dTMP,* deoxythymidine monophoshate; *SAM, S*-adenosylmethionine; *SAH, S*-adenosylhomocysteine.

conferring reduced TS expression have been found to be associated with reduced CRC risk and interact with folate and alcohol intake in determining CRA risk (25-27).

To date, studies investigating the role of folate metabolism variants in colorectal neoplasia have been restricted to case-control and cohort studies of CRA and CRC risk. We aimed to further examine this relationship by doing a comprehensive analysis of polymorphisms in key folate metabolism genes using DNA from patients participating in a large randomized controlled intervention trial for the prevention of CRA recurrence.

Materials and Methods

Trial Participants. The United Kingdom Colorectal Adenoma Prevention (UKCAP) trial is a multicenter, double-blind, randomized controlled trial of folate and aspirin for the prevention of CRA recurrence. The details of the trial have previously been published (28). Briefly, eligible patients had one or more histologically confirmed CRA removed at full colonoscopy in the 6 months before enrollment and were not taking folate supplements or regular aspirin or non-aspirin nonsteroidal anti-inflammatory drugs. Patients were randomized to four intervention groups: folate alone (500 μg daily), aspirin alone (300 mg daily), both folate and aspirin, and double placebo. Compliance with trial medication was assessed by direct questioning at 4-monthly follow-up outpatient appointments.

After 3 years, or earlier if symptoms dictated, adenoma recurrence was assessed by full colonoscopic examination. The primary end point was histologically confirmed CRA or CRC. Between 1997 and 2001, 945 patients were recruited, of which 942 were eligible and were randomized. Information on results of follow-up colonoscopy (at 3 years or earlier) was available for 853 patients. Blood samples for extraction of germ line DNA were collected from 546 patients. Not all patients from the original trial could be included in this molecular subprotocol as some could not be contacted and others did not consent to DNA analysis. All patients included in the genotyping analyses were of Caucasian ethnicity.

Informed consent for the study was obtained from all participants and the study was carried out with ethical review board approval in accordance with the tenets of the declaration of Helsinki.

Data Collection. Dedicated interviewers conducted face-to-face interviews to obtain lifestyle and medical information, including family history data from all trial participants. Patients also completed a food frequency questionnaire thrice during the trial period (at 4, 16, and 28 months). The questionnaire was an adaptation of the Willett food frequency questionnaire. Nutrient intake was determined using a nutrient conversion database.

Polymorphism Selection. Candidate polymorphisms were selected for genotyping on the basis of a *priori* evidence for functional effects on expressed proteins and an influence on folate metabolism and colorectal neoplasia risk. Database and literature searches were done to establish a hierarchy of polymorphisms likely to influence adenoma recurrence and interact with folate supplementation in determining recurrence risk. The polymorphisms selected were *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, *MTRR* A66G, *TSER, TSER* 3R G>C, and *TS* 1494del6.

Genotyping. Constitutional DNA was extracted from EDTA venous blood samples using a standard salt extraction procedure and quantified with PicoGreen (Invitrogen, Paisley, United Kingdom). *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *MTRR* A66G genotypes were generated using TaqMan technology implemented on an ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Genotyping PCR reactions contained 6.25 μL of ABI TaqMan PCR Master Mix, 0.16 μL of ABI SNP assay-by-design master mix containing 900 nmol/L forward primer, 900 nmol/L reverse primer, 200 nmol/L VIC-labeled MGB probe, and 200 nmol/L FAM-labeled MGB probe, 100 ng of template DNA, and double-distilled water to a final volume of 12.5 μL.

The *TSER* and *TS* 1494del6 polymorphisms were analyzed using fluorescent size discrimination on an ABI 3100 genetic analyzer (Applied Biosystems). A previously described PCR-RFLP assay was used to generate the *TSER* 3R G>C polymorphism genotypes (22). Briefly, amplified fragments were digested with *Hae*III restriction enzyme (New England Biolabs, Hitchin, United Kingdom) and the digestion products separated by electrophoresis on a 4% agarose gel. *Hae*III digestion of the 3RG fragment produced bands of 11, 27, 28, 44, 47, and 66 bp, whereas digestion of the 3RC fragment produced bands of 11, 27, 44, 47, and 94 bp. *TSER* genotypes were then divided into high (3RG/3RG, 3RG/3RC, and 3RG/2R) and low (3RC/3RC, 3RC/2R, and 2R/2R) TS expression groups (22).

ABI Prism 7900HT Sequence Detection System (version 2.1) and Genotyper software (version 3.7; Applied Biosystems) were used for TaqMan and fluorescent size discrimination genotyping analyses. Genotyping assays for each polymorphism were validated using control samples of known homozygote wild-type, heterozygote, and homozygote variant genotypes generated by direct sequencing. Unblinded control samples were included on each sample plate and were

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correctly genotyped by the SDS or Genotyper software on 100% of occasions. Positive call rates for TaqMan and Genescan analyses were 99% and 98%, respectively. Samples that were called by the TaqMan software or Genotyper software were not repeated whereas samples that were not called were genotyped by direct sequencing. For the *TSER* 3R G>C RFLP, both unblinded and blinded control samples were included on all gels and were correctly genotyped on all occasions. Laboratory staff employed in genotyping were blinded to clinical outcome. Details of all PCR primer sequences and reaction conditions are available on request.

Statistical Analysis. The χ^2 and t tests were used to compare variables between the whole UKCAP trial population and genotyped patients. Genotype frequencies were tested for departure from Hardy-Weinberg equilibrium using the χ^2 test. The relationship between genotype and risk of colorectal neoplasia recurrence was assessed by means of relative risks (RR) and 95% confidence intervals (95% CI), calculated using Poisson regression with robust error variances. Both unadjusted and adjusted RRs were calculated. Variables for sex and interval between entry and follow-up colonoscopy were included in adjusted analyses because these two variables were found to significantly influence recurrence risk. Because both models yielded very similar results in all conditions, only adjusted RRs are presented. The likelihood ratio test was used to explore interactions between variables with respect to recurrence risk by comparing models with and without a multiplicative term for the two variables.

Stratification according to folate treatment was done by combining patients who received either folate alone or folate and aspirin to form a ''folate-treated'' group. Patients who received either aspirin alone or double placebo were combined to form a ''non-folate-treated'' group.

Information from individual patients' food frequency questionnaires was averaged. To remove incorrectly completed questionnaires and patients whose diet was inadequately captured by the questionnaire, patients with the highest and lowest 2.5% of energy intakes were excluded, leaving 495 patients in the dietary folate analyses. Energy-adjusted tertile cut points were used to designate low (lowest tertile), medium (middle tertile), and high (highest tertile) dietary folate intakes. To investigate the combined effects of dietary folate intake and folate treatment on recurrence risk, a composite variable, ''folate-index,'' was generated. Dietary folate intake was designated as ''low'' (lowest tertile) or ''adequate'' (middle and highest tertiles), and these subgroups were further divided into folate-treated and non-folate-treated. The folate index variable was defined as 1, low dietary folate/non-folate-treated; 2, adequate dietary folate/non-folate-treated; 3, low dietary folate/folate treated; and 4, adequate dietary folate/folate treated.

Our study had 90% power to detect the main effect of genotype on recurrence risk, assuming a variant allele frequency of 0.4, RR of recurrence of 0.5 associated with presence of the variant allele in a dominant model, and a significance level of 0.05 (two sided). The study had 35% power to detect an interaction between genotype and folate treatment in determining recurrence risk, assuming a RR of recurrence of 1.08 associated with folate treatment (as observed in this study), a RR of 0.5 for presence of variant allele, and a significance level of 0.05 (two sided).

Statistical analyses were undertaken using STATA, version 7.0 (Stata Corporation, College Station, TX). All tests were two sided and $P < 0.05$ was considered significant.

Results

There were no significant differences in age, sex, intervention, time interval between entry and follow-up colonoscopy, or outcome between the whole UKCAP trial population and patients included in the genotyping analysis (Table 1). Of the 546 genotyped patients, 130 (23.8%) had ≥1 CRA detected at follow-up colonoscopy and 7 (1.3%) had a CRC detected. Seventy patients (12.8%) had advanced colorectal neoplasia detected, defined as CRAs with villous or tubulovillous features, size ≥1 cm, severe dysplasia, or CRC.

In the 546 patients included in the genotyping analysis, neither folate nor aspirin treatment significantly influenced risk of colorectal neoplasia (CRA or CRC) recurrence [RR, 1.08 (95% CI, 0.82-1.42) and RR, 0.92 (95% CI, 0.70-1.21), respectively]. These results were not significantly different from those of the main trial (28). In the genotyped subgroup, male patients were at a significantly increased risk of recurrence compared with females (RR, 1.58; 95% CI, 1.17-2.14) whereas a positive family history of colorectal neoplasia did not have a significant influence. Cigarette smoking (ever versus never) did not influence recurrence risk.

Genotype frequencies for all seven polymorphisms were in Hardy-Weinberg equilibrium. Recurrence risk in patients with heterozygous and homozygous variant genotypes, both individually and combined, was compared with that in patients with homozygous wild-type genotype for each variant (Table 2). An influence of *MTRR* A66G genotype on risk of colorectal neoplasia recurrence was observed. Patients with AG genotype had a significantly reduced risk for recurrence compared with patients with AA genotype (RR, 0.64; 95% CI, 0.46-0.90). Patients with GG genotype also had a reduced risk, albeit nonsignificantly (RR, 0.83; 95% CI, 0.59-1.16). Compared with patients with AA genotype, patients with either one or two G alleles were at significantly reduced risk of recurrence (RR, 0.71; 95% CI, 0.53-0.96). When the analysis was restricted to advanced colorectal neoplasia recurrence, the effect of *MTRR* A66G genotype seemed to be enhanced. Compared with patients with AA genotype, patients with either one or two G alleles had a RR of 0.55 (95% CI, 0.36-0.86).

MTHFR A1298C genotype also significantly influenced recurrence risk. Patients with AC genotype had a reduced risk compared with those with AA genotype (RR, 0.71; 95% CI, 0.52-0.97). Patients with CC genotype, however, were at nonsignificant increased risk. A similar pattern of risk was

Table 1. Comparison of the total UKCAP trial population and patients genotyped in this study

Variable	UKCAP trial population	Genotyped patients
n	853	546
Age*		
Mean (y)	57.5	57.3
SD	9.3	9.3
Sex		
Male	477 (56%)	289 (53%)
Female	376 (44%)	256 (47%)
Intervention		
Folate alone	215 (25.2%)	144 (26.4%)
Aspirin alone	217 (25.4%)	131 (24.0%)
Folate and aspirin	217 (25.4%)	135 (24.7%)
Double placebo	204 (23.9%)	136 (24.9%)
Colonoscopy interval †		
Mean (mo)	40.3	40.7
Range (mo)	2-79	6-74
Outcome ‡		
Adenoma	207 (24.2%)	130 (23.8%)
Carcinoma	11 (1.3%)	7 (1.3%)
Advanced neoplasia	104 (12.2%)	70 (12.8%)

*Age at entry colonoscopy.
†Interval between entry and follow-up colonoscopy.
‡Follow-up colonoscopy outcome definitions were adenoma, histologically confirmed CRA; carcinoma, histologically confirmed CRC; advanced neoplasia, CRA ≥1 cm diameter, villous or tubulovillous histology, severe dysplasia, or CRC.

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Table 2. Risk of detection of any colorectal neoplasia and advanced neoplasia at follow-up colonoscopy according to folate metabolism genotypes

Variant	Genotype	Any colorectal neoplasia		Advanced colorectal neoplasia*	
		Detected/not detected	RR† (95% CI)	Detected/not detected	RR† (95% CI)
MTHFR A2756G	CC	66/178	Reference	34/210	Reference
	CT	56/188	0.92 (0.69-1.23)	30/214	1.00 (0.65-1.55)
	TT	15/43	1.18 (0.73-1.89)	6/52	0.98 (0.43-2.25)
	CT/TT	71/231	0.97 (0.73-1.27)	36/266	1.00 (0.66-1.52)
MTHFR A1298C	AA	75/193	Reference	35/233	Reference
	AC	43/184	0.71 (0.52-0.97)‡	24/203	0.85 (0.53-1.37)
	CC	19/32	1.19 (0.83-1.71)	11/40	1.43 (0.79-2.57)
	AC/CC	62/216	0.81 (0.62-1.06)	35/243	0.98 (0.64-1.49)
MTR A2756G	AA	101/280	Reference	48/333	Reference
	AG	34/114	0.88 (0.64-1.19)	22/126	1.19 (0.77-1.83)
	GG	2/15	0.41 (0.12-1.39)	0/17	
	AG/GG	36/129	0.82 (0.61-1.12)	22/143	1.06 (0.68-1.64)
MTRR A66G	AA	38/79	Reference	23/94	Reference
	AG	54/212	0.64 (0.46-0.90)§	26/240	0.51 (0.31-0.85)§
	GG	45/118	0.83 (0.59-1.16)	21/142	0.64 (0.38-1.06)
	AG/GG	99/330	0.71 (0.53-0.96)‡	47/382	0.55 (0.36-0.86)§
TSER	3R/3R	38/112	Reference	21/129	Reference
	3R/2R	63/205	0.92 (0.67-1.27)	33/235	0.86 (0.53-1.40)
	2R/2R	34/89	1.08 (0.74-1.55)	16/107	0.90 (0.50-1.62)
	3R/2R and 2R/2R	97/294	0.97 (0.72-1.31)	49/342	0.87 (0.56-1.37)
TS high/low∥	High	55/153	Reference	29/179	Reference
	Low	80/253	0.96 (0.73-1.27)	41/292	0.93 (0.61-1.42)
TS 1494del6	wt/wt	68/204	Reference	36/236	Reference
	wt/−6bp	59/179	0.98 (0.74-1.31)	28/210	0.92 (0.59-1.43)
	−6bp/−6bp	10/26	1.03 (0.60-1.77)	6/36	1.19 (0.60-2.37)
	wt/−6bp and −6bp/−6bp	69/205	0.99 (0.75-1.30)	34/246	0.96 (0.63-1.45)

*Advanced colorectal neoplasia was defined as CRAs with villous or tubulovillous features, size ≥1 cm, severe dysplasia, or CRC.
†RR and 95% CI adjusted for sex and interval between entry and follow-up colonoscopy.
‡$P < 0.05$.
§$P = 0.01$.
∥High and low TS expression genotypes based on *TSER* and *TSER* 3R G>C polymorphisms (high: 3RG/3RG, 3RG/3RC, 3RG/2R; low: 3RC/3RC, 3RC/2R, 2R/2R).

observed for the *MTHFR* C677T genotype, with heterozygotes being at nonsignificant reduced risk, whereas TT homozygotes were at increased risk. Patients heterozygous for the *MTR* A2756G polymorphism were at reduced recurrence risk, as were those with GG genotype, although the number of homozygous variant individuals was small.

TSER and *TS* 1494del6 genotypes did not influence recurrence risk and further stratification of *TSER* genotype using the 3R G>C SNP genotype did not materially alter recurrence risk.

In the genotyped patients, mean daily dietary folate intake was 310 μg/d (range, 82-674 μg/d); 88% of subjects had daily intakes above the European Community recommended daily allowance of 200 μg/d and 18% had daily intakes above the U.S. recommended daily allowance of 400 μg/d. Thus, 500 μg/d folate supplementation was an appropriate dose for this population and would have achieved a folate-replete status in most subjects.

We investigated the combined effects of genotype and folate treatment on recurrence by calculating RRs after stratification by folate treatment (Table 3). *MTRR* A66G heterozygote genotype significantly reduced recurrence risk in patients treated with folate (RR, 0.53; 95% CI, 0.34-0.83) but not in those who did not receive folate (RR, 0.80; 95% CI, 0.47-1.37). The influence of *MTHFR* A1298C genotype was also most apparent in patients treated with folate, in whom the heterozygote genotype conferred a significantly reduced recurrence risk. Similarly, variant *MTR* genotypes reduced risk only in folate-treated patients. The differences in effects of the polymorphisms in folate-treated and non-folate-treated patients should be interpreted with caution, however, as there were no significant interactions between genotype and folate treatment.

Following stratification by folate treatment and consideration of only advanced colorectal neoplasia recurrence, a similar pattern was observed with significantly reduced recurrence risks in folate-treated subjects only (Table 4). Specifically, folate-treated patients with *MTRR* A66G heterozygote and homozygote variant genotypes had significantly reduced risks of advanced neoplasia recurrence [RR, 0.49 (95% CI, 0.25-0.96) and RR, 0.39 (95% CI, 0.19-0.82), respectively], whereas those who did not receive folate had nonsignificantly reduced risks. Patient numbers in the folate treatment stratified advanced neoplasia recurrence analyses were small, however, and thus power for detection of interactions was low and these results should be treated with caution.

Further possible interactions between dietary folate intake and genotype in colorectal neoplasia recurrence were explored by comparing the recurrence risks associated with variant genotypes within the three tertiles of dietary folate intake and following combination of dietary folate intake and folate-treatment into a single variable. No significant interactions were observed.

The effects of combined ''high-risk'' genotypes on recurrence risk were also explored. Subjects with double heterozygote genotype for the *MTHFR* A1298C and *MTRR* A66G polymorphisms had a markedly reduced recurrence risk compared with subjects with double homozygous wild-type genotype (RR, 0.38; 95% CI, 0.22-0.67). Including folate treatment in this analysis did not result in a further risk reduction, although numbers were small and power inevitably limited.

Discussion

Our findings suggest that folate metabolism polymorphisms play a role in determining risk of CRA recurrence. Specifically, patients with heterozygote *MTRR* A66G and *MTHFR* A1298C genotypes had a reduced recurrence risk. In addition to these statistically significant findings, we also observed

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nonsignificant influences of polymorphisms in the *MTHFR* and *MTR* genes. Our study had a modest sample size, and these influences may reach significance in a larger study. Collectively, these data support evidence from previous case-control and cohort studies indicating that folate metabolism variants influence development of colorectal neoplasia.

The largest effect on recurrence risk was seen with *MTRR* A66G variant genotypes. Whereas the functional effects of this variant have not been fully established, *in vitro* experiments suggest that variant MTRR enzyme restores MTR activity less efficiently than wild-type, and the *MTRR* A66G genotype has been shown to influence plasma homocysteine level in humans (18, 29). Coupled with the observation that individuals with GG genotype are at increased risk of neural tube defects, a condition known to be associated with low folate levels, these data provide circumstantial evidence that this *MTRR* variant is functional (30).

The influence of *MTRR* A66G genotypes on risk of colorectal neoplasia has previously been reported in two case-control studies of CRC (12, 20). Both found that risks were elevated in GG homozygotes. Whereas this contrasts with our data suggesting a protective effect of this genotype on CRA recurrence, it is entirely conceivable that genetic variants such as *MTRR* A66G may have different effects on risks of colorectal neoplasia development and recurrence. Studies of folate intake in animal models provide evidence that the timing of deficiency or supplementation is important in the development of colorectal neoplasia (31, 32). Folate supplementation reduced the development and progression of intestinal tumors if introduced before the establishment of neoplastic foci. Paradoxically, when introduced after foci were established, supplementation had an adverse effect promoting the development of tumors. These data are compatible with a model in which folate supplementation has a role in preventing early development of colorectal neoplasia, but can promote the growth of established neoplastic lesions (33, 34). Recently, support for this model has come from a study in humans, where folate treatment in patients who had CRA removed at colonoscopy led to an increased risk of CRA recurrence (35). Thus, in a similar manner to folate supplementation, it is possible that polymorphisms such as *MTRR* A66G, which are thought to have an effect through differential folate metabolism, may also have opposing effects on different stages of colorectal neoplasia development.

Because the effects of variant *MTRR* A66G are likely to be mediated by altered MTR activity, variants of the *MTR* gene itself would also be expected to influence recurrence risk. In our study, individuals with one or two variant *MTR* A2756G alleles had a reduced recurrence risk, albeit nonsignificantly, and this influence was also enhanced in patients treated with folate.

We observed a significantly reduced risk of recurrence in patients heterozygous for the *MTHFR* A1298C variant, and heterozygosity for the *MTHFR* C677T variant also conferred a nonsignificant reduced risk. Interestingly, however, patients with homozygous variant genotypes for either *MTHFR* polymorphism were at increased recurrence risk. Functional studies have reported that both heterozygous and homozygous variant genotypes for either polymorphism result in reductions in enzyme activity compared with wild-type and might therefore be expected to have a similar effect on recurrence risk (10, 36). Although conflicting associations for heterozygote and homozygote variant genotypes have also been reported in case-control studies (37-39), they are difficult to explain biologically, and it may be that our observation of a reduced risk in *MTHFR* A1298C heterozygotes was due to chance. The reduced recurrence risk associated with the *MTRR* A66G polymorphism was also only significant in heterozygote

Table 3. Risk of detection of any colorectal neoplasia by genotype and folate treatment

Variant	Genotype	Intervention				$P_{interaction}$
		Folate-treated		Non-folate-treated		
		Colorectal neoplasia detected/not detected	RR* (95% CI)	Colorectal neoplasia detected/not detected	RR* (95% CI)	
MTHFR C677T	CC	41/87	Reference	25/91	Reference	0.30
	CT	23/96	0.71 (0.46-1.10)	33/92	1.21 (0.80-1.85)	
	TT	10/22	1.27 (0.71-2.29)	5/21	1.03 (0.46-2.27)	
	CT/TT	33/118	0.82 (0.56-1.21)	38/113	1.19 (0.79-1.79)	
MTHFR A1298C	AA	43/94	Reference	32/99	Reference	0.42
	AC	19/94	0.56 (0.35-0.88) †	24/90	0.89 (0.57-1.39)	
	CC	12/27	1.00 (0.64-1.56)	7/15	1.57 (0.83-2.96)	
	AC/CC	31/121	0.67 (0.47-0.97) †	31/105	0.99 (0.65-1.49)	
MTR A2756G	AA	56/135	Reference	45/145	Reference	0.75
	AG	17/61	0.74 (0.48-1.15)	17/53	1.00 (0.64-1.57)	
	GG	1/9	0.35 (0.06-2.02)	1/6	0.45 (0.09-2.21)	
	AG/GG	18/70	0.70 (0.45-1.08)	18/59	0.94 (0.61-1.46)	
MTRR A66G	AA	23/41	Reference	15/38	Reference	0.47
	AG	30/109	0.53 (0.34-0.83) ‡	24/103	0.80 (0.47-1.37)	
	GG	21/55	0.65 (0.41-1.03)	24/63	1.08 (0.65-1.79)	
	AG/GG	51/164	0.57 (0.38-0.86) ‡	48/166	0.92 (0.58-1.47)	
TSER	3R/3R	24/59	Reference	14/53	Reference	0.71
	3R/2R	31/101	0.81 (0.54-1.21)	32/104	1.10 (0.65-1.85)	
	2R/2R	17/43	1.06 (0.64-1.78)	17/46	1.15 (0.66-2.02)	
	3R/2R and 2R/2R	48/144	0.88 (0.60-1.29)	49/150	1.12 (0.69-1.81)	
TS high/low§	High	31/81	Reference	24/72	Reference	0.81
	Low	41/122	1.03 (0.71-1.51)	39/131	0.89 (0.59-1.35)	
TS 1494del6	wt/wt	39/98	Reference	29/106	Reference	0.71
	wt/−6bp	30/90	0.87 (0.59-1.28)	29/89	1.14 (0.76-1.72)	
	−6bp/−6bp	5/17	0.77 (0.35-1.69)	5/9	1.40 (0.67-2.92)	
	wt/−6bp and −6bp/−6bp	35/107	0.86 (0.59-1.24)	34/98	1.17 (0.79-1.74)	

*RR and 95% CI adjusted for sex and interval between entry and follow-up colonoscopy.
†$P < 0.05$.
‡$P < 0.01$.
§High and low TS expression genotypes based on *TSER* and *TSER* 3R G>C polymorphisms (high: 3RG/3RG, 3RG/3RC, 3RG/2R; low: 3RC/3RC, 3RC/2R, 2R/2R).

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Table 4. Risk of detection of advanced colorectal neoplasia by genotype and folate treatment

Variant	Genotype	Intervention				$P_{interaction}$
		Folate-treated		Non-folate-treated		
		Advanced colorectal neoplasia* detected/not detected	RR† (95% CI)	Advanced colorectal neoplasia* detected/not detected	RR† (95% CI)	
MTHFR C677T	CC	20/108	Reference	14/102	Reference	0.95
	CT	15/104	1.04 (0.56-1.92)	15/110	1.02 (0.53-1.95)	
	TT	3/29	0.89 (0.28-2.81)	3/23	1.16 (0.35-3.88)	
	CT/TT	18/133	1.01 (0.56-1.83)	18/133	1.04 (0.56-1.94)	
MTHFR A1298C	AA	19/118	Reference	16/115	Reference	0.89
	AC	12/101	0.81 (0.42-1.54)	12/102	0.86 (0.42-1.76)	
	CC	7/22	1.21 (0.56-2.59)	4/18	1.80 (0.72-4.49)	
	AC/CC	19/123	0.92 (0.52-1.61)	16/120	0.99 (0.52-1.90)	
MTR A2756G	AA	28/163	Reference	20/170	Reference	0.57
	AG	10/68	0.90 (0.49-1.66)	12/58	1.53 (0.81-2.89)	
	GG	0/10		0/7		
	AG/GG	10/78	0.80 (0.43-1.50)	12/65	1.35 (0.70-2.59)	
MTRR A66G	AA	13/51	Reference	10/43	Reference	0.32
	AG	17/122	0.49 (0.25-0.96)‡	9/118	0.45 (0.20-1.03)	
	GG	8/68	0.39 (0.19-0.82)§	13/74	0.84 (0.41-1.73)	
	AG/GG	25/190	0.45 (0.24-0.84)§	22/192	0.62 (0.32-1.22)	
TSER	3R/3R	14/69	Reference	7/60	Reference	0.42
	3R/2R	15/117	0.66 (0.35-1.25)	18/118	1.19 (0.55-2.61)	
	2R/2R	9/51	0.98 (0.46-2.18)	7/56	0.93 (0.36-2.35)	
	3R/2R and 2R/2R	24/168	0.76 (0.42-1.36)	25/174	1.10 (0.53-2.32)	
TS high/low‖	High	17/95	Reference	12/84	Reference	0.96
	Low	21/142	0.98 (0.56-1.79)	20/150	0.89 (0.47-1.69)	
TS 1494del6	wt/wt	22/115	Reference	14/121	Reference	0.35
	wt/−6bp	14/106	0.74 (0.40-1.38)	14/104	1.20 (0.64-2.27)	
	−6bp/−6bp	2/20	0.57 (0.18-1.82)	4/10	2.30 (0.95-5.57)	
	wt/−6bp and −6bp/−6bp	16/126	0.72 (0.40-1.29)	18/114	1.35 (0.74-2.45)	

*Advanced colorectal neoplasia was defined as CRAs with villous or tubulovillous features, size ≥1 cm, severe dysplasia, or CRC.
†RR and 95% CI adjusted for sex and interval between entry and follow-up colonoscopy.
‡$P < 0.05$.
§$P = 0.01$.
‖High and low TS expression genotypes based on *TSER* and *TSER* 3R G>C polymorphisms (high: 3RG/3RG, 3RG/3RC, 3RG/2R; low: 3RC/3RC, 3RC/2R, 2R/2R).

subjects. However, homozygous variant subjects also showed a reduced recurrence risk, albeit nonsignificantly, and in folate-treated individuals this reduced risk was on the borderline of statistical significance.

The finding of an enhancement of the effect of variant folate metabolism genotypes, and in particular the *MTRR* A66G genotype, in folate-treated patients is intriguing. These observations are consistent with a previous case-control study in which carriers of variant *MTHFR* C677T genotypes had a reduced CRC risk only if they were folate replete (13). Collectively, the results of this study are consistent with reduced adenoma recurrence in subjects with reduced conversion of homocysteine to methionine, due to either reduced MTR enzyme activity or reduced activity upstream at the MTHFR enzyme, but only in subjects who are folate replete. It is possible that a folate-replete status overcomes the relative inefficiency of the methionine synthesis pathway in these subjects while the benefits of increased 5,10-methylene-THF for DNA synthesis remain. The results of folate stratification must be interpreted with caution, however, because the interactions between treatment group and genotype were not significant. Stratifying patients into folate-treated and non-folate-treated groups in our study inevitably led to a reduction in power, and larger studies are required to adequately investigate interactions between genotype and folate intervention.

Our finding of significant effects of variant genotypes on recurrence risk when only advanced lesions were considered is important. It is likely that even with meticulous colonoscopy technique, a proportion of small adenomas may be missed, both at initial and follow-up colonoscopy (40). This may result in incorrect estimation of recurrence rates. Consideration of only lesions ≥1 cm in size should reduce the number of missed lesions and would therefore be expected to increase the size of any genuine associations. Such an effect was observed in our study, in particular for the risk reduction associated with the *MTRR* A66G variant.

Of the variants included in this study, the *MTHFR* C677T and *TS* polymorphisms have the most convincing evidence from case-control studies for an influence in determining risk of CRA and CRC. Although an influence of *MTHFR* C677T genotype on CRA recurrence risk is suggested in our study, these nonsignificant findings may indicate a genuine lack of effect in this setting rather than simply insufficient power, and *TS* polymorphisms did not influence recurrence risk. Patients who have been found at colonoscopy to have CRA are at an increased risk of both further CRA and CRC compared with the general population (41, 42). It is conceivable that at the time of colonoscopic removal of incident CRA, further unidentified neoplastic foci may already exist. By investigating CRA recurrence rather than incidence, a more advanced stage of the adenoma-carcinoma sequence may be under scrutiny. Thus, variant genotypes that have a protective effect in the early development of CRA may not influence the progression of already existing neoplastic foci that could underlie a significant proportion of the recurrences seen in this setting. Variant genotypes may also exert their influences over longer periods of time than the median 41-month colonoscopy interval in this study. Longer follow-up may increase the proportion of recurrences observed that represent new neoplastic foci, rather than foci that were already present at the time of detection of the incident adenoma.

This is the first study to investigate the effects of folate metabolism polymorphisms on CRA recurrence, and as such, any findings need to be confirmed in further studies. However,

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the influences of these variants on CRA recurrence risk observed in our study lend further weight to the accumulating evidence for their role in the development of colorectal neoplasia.

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