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A prospective study of methylenetetrahydrofolate reductase and methionine synthase gene polymorphisms, and risk of colorectal adenoma

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We examined the relationship between a functional polymorphism (^{667}C→T, ala→val) of the methylenetetrahydrofolate reductase gene (*MTHFR*) and the risk of colorectal adenomas in the prospective Nurses' Health Study. Among 257 incident polyp cases and 713 controls, the *MTHFR val/val* polymorphism [relative risk (RR) = 1.35, 95% confidence interval (CI) 0.84–2.17] was not significantly associated with risk of adenomas. This lack of association was observed for both small (RR = 1.36, 95% CI 0.76–2.45) and large (RR = 1.32, 95% CI 0.66–2.66) adenomas. Furthermore, there was no significant interaction between this polymorphism and consumption of either folate, methionine or alcohol. We also examined the relationship of a newly identified polymorphism (asp919gly) of the methionine synthase gene (*MS*) with the risk of colorectal adenomas in the same population. The *MS gly/gly* polymorphism was also not significantly associated with risk of colorectal adenomas (RR = 0.66, 95% CI 0.26–1.70). These results, which need to be confirmed in other studies, suggest that the *MTHFR val/val* polymorphism, which has been previously inversely associated with risk of colorectal cancer, plays a role only in a late stage (adenoma→carcinoma) of colorectal tumorigenesis, and/or may protect against malignant transformation in the subset of benign adenomas, which may progress to malignancy.

Introduction

Substantial evidence suggests that risk of developing colorectal carcinoma and adenoma is associated with low-methyl diets that are high in alcohol but low in folate and methionine (1–6). Methylenetetrahydrofolate reductase (MTHFR) is a critical enzyme that regulates the metabolism of folate and, indirectly, methionine. MTHFR irreversibly converts 5,10-methylenetetrahydrofolate, the methyl donor in dTMP synthesis from dUMP, to 5-methyltetrahydrofolate, the primary methyl donor for the remethylation of homocysteine to methionine. A common polymorphism (^{667}C→T, ala→val) in the *MTHFR* gene that locates in chromosome 1p36.3, is associated with decreased enzyme activity and enhanced thermolability of the enzyme (7) as well as an elevation in plasma homocysteine levels (7,8).

In two previous studies (9,10), we observed an inverse association between the *MTHFR val/val* genotype and the risk of colorectal cancer. One possible mechanism for this association is that *val/val* individuals, by sparing intracellular 5,10-methylenetetrahydrofolate, experience reduced uracil misincorporation during DNA synthesis; uracil misincorporation has been shown to cause DNA strand breaks. DNA strand breaks are more frequent in conditions of folate deprivation (11), which has been recently shown to be associated with higher levels of uracil misincorporation in human DNA (12). Alternatively, alterations in levels of 5-methyltetrahydrofolate may alter *S*-adenosylmethionine levels and thus possibly influence DNA methylation patterns: both hypomethylation and hypermethylation of DNA have been shown to be of importance in carcinogenesis (13,14). In this study, we investigated the stage of colorectal tumorigenesis in which the *MTHFR* polymorphism might play a role. We designed a nested case-control study within a cohort study to examine prospectively the association between the *MTHFR* polymorphism and risk of pre-malignant colorectal adenomas.

Another gene on the folate metabolic pathway, methionine synthase (*MS*), was recently cloned (15,16). MS remethylates homocysteine to methionine using a methyl group donated by 5-methyltetrahydrofolate. It is reasonable to predict that genetic variants of MS would have a similar effect to the MTHFR variant leading to abnormalities in DNA methylation and/or DNA synthesis. The most common polymorphism in this gene reported to date (asp919gly) (16,17) alters the amino acid sequence of the protein at a potentially functional site (17); however, no biological function of the polymorphism has been reported. We also examined the association of this *MS* polymorphism and the risk of colorectal adenomas.

Abbreviations: CI, confidence interval; LRT, likelihood ratio test; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; NHS, Nurses' Health Study; RR, relative risk.

Materials and methods

Subjects

The Nurses' Health Study (NHS) began in 1976 when 121 700 married, registered female nurses between ages of 30 and 55 from 11 US states completed a mailed questionnaire on risk factors for cancer and coronary heart disease. The cohort is followed by questionnaire every 2 years and self-reported diagnoses of colorectal adenomas are confirmed by medical record review. In the NHS cohort, >90% of the adenomas were diagnosed in individuals who had had an endoscopic procedure for screening or for unrelated gastrointestinal conditions. In 1980, the questionnaire included a comprehensive food frequency questionnaire that assessed diet during the previous year. In 1989–1990, we collected blood specimens from 32 826 women, among whom 257 cases of first incident distal or proximal colorectal adenoma were subsequently identified through June 1, 1994. Each case was matched to a control who had not been diagnosed with colorectal adenomas, who was born in the same year, and who had a sigmoidoscopy in the interval since blood sampling. In additional analyses, we included 463 cohort members who had been genotyped as controls in a breast cancer case-control study (18) among which 214 had a history of sigmoidoscopy, of whom seven individuals were diagnosed with adenomas. After excluding these seven

Table I. Relation of *MTHFR* and *MS* genotypes to colorectal adenomas among 257 cases and 713 controls in a prospective case-control study nested in the Nurses' Health Study I

Gene	Genotype	Controls		All cases		Small adenomas		Large adenomas		RR[a] all (95% CI)	RR[a] small (95% CI)	RR[a] large (95% CI)
		n	(%)	*n*	(%)	*n*	(%)	*n*	(%)			
MTHFR	*val/val*	66	(9.3)	30	(11.7)	17	(11.6)	11	(11.5)	1.35 (0.84–2.17)	1.36 (0.76–2.45)	1.32 (0.66–2.66)
	val/ala	324	(45.0)	126	(48.6)	71	(50.0)	48	(50.0)]	1.00	1.00	1.00
	ala/ala	323	(45.3)	102	(39.7)	58	(39.7)	37	(38.5)]	(ref.)	(ref.)	(ref.)
MS	*gly/gly*	21	(2.9)	6	(2.3)	3	(2.1)	3	(3.1)	0.66 (0.26–1.70)	0.61 (0.17–2.11)	0.96 (0.27–3.42)
	gly/asp	236	(33.1)	85	(33.1)	56	(38.4)	24	(25.0)]	1.00	1.00	1.00
	asp/asp	456	(64.0)	166	(64.6)	87	(59.6)	69	(71.9)]	(ref.)	(ref.)	(ref.)

[a]Logistic regression controlling for age, family history, smoking status, body mass index and intakes of folate, methionine, alcohol, fiber and saturated fat. *Val/ala* and *ala/ala* were combined as reference for *MTHFR* and *gly/asp* and *asp/asp* as reference for *MS*.

individuals, 456 additional controls were available for a total of 713 combined control subjects.

Genotyping

Genotyping for *MTHFR* was described elsewhere (9). In brief, two primers were designed from the cDNA sequence to generate a 198 bp fragment. The primer sequences are 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ and 5′-AGGACGGTGCGGTGAGAGTG-3′.

Amplification was performed using initial denaturation at 95°C for 2 min followed by 29 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s with a final extension at 72°C for 10 min. PCR product was digested with *HinfI* and size-fractionated on 6% polyacrylamide gels.

Genotyping for *MS* was carried out using a modification of the method of Leclerc *et al*. (16). In brief, primers with the sequences 5′-GAACTAGAAGA-CAGAAATTCTCTA-3′ and 5′-CATGGAAGAATATCAAGATATTAGA-3′ were used in a PCR reaction. Amplification was performed using initial denaturation at 95°C for 2 min followed by 30 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 30 s with a final extension of 72°C for 10 min. The PCR buffer consisted of 50 mM KCl, 10 mM Tris, pH 9.0, 0.1% Triton X and 4 mM $MgCl_2$. PCR product was digested with *Hae*III and size-fractionated on 6% polyacrylamide gels. Ten percent quality control samples were included. Laboratory personnel were blind to case-control and quality control status.

For *MTHFR*, we combined the *val/ala* and *ala/ala* genotypes into a single reference category, because of the overlapping enzymatic activity between *val/ala* and *ala/ala* genotypes, and the fact that plasma homocysteine levels are only slightly higher among *ala/val* heterozygotes than *ala/ala* homozygotes, but substantially higher among *val/val* homozygotes (19). For *MS*, we also compared *gly/gly* genotype with *gly/asp* and *asp/asp* genotypes combined.

Statistical analysis

We calculated the odds ratios to estimate the relative risks (RRs) that were used throughout the paper, and 95% confidence intervals (95% CIs) for the association of genotypes with colorectal adenomas using conditional logistic regression models. We used unconditional logistic regression in analyses including additional controls from the breast cancer case-control study. We assessed effect modification of the relation of folate, methionine and alcohol consumption (each categorized into three groups based on the distribution in controls) and risk of adenomas by *MTHFR* genotype using a likelihood ratio test (LRT) to compare the goodness-of-fit of the model with the interaction term (genotype*exposure), with the reduced model containing only indicator variables of the main effects of genotype and exposure (i.e. without the interaction term). Linkage between *MTHFR* and *MS* genotypes was determined using the EH program (New York State Psychiatric Institute and Columbia University, New York) and interaction between the *MTHFR* and *MS* variant genotypes was calculated using the Wald test. All analyses were performed using SAS 6.0 (SAS Institute) unless specified otherwise.

Results

Among the 257 matched controls who underwent sigmoidoscopy, the frequencies of *MTHFR val/val*, *val/ala* and *ala/ala* genotypes were 9.7, 45.1 and 45.1%, similar to that of the 456 added controls from a breast cancer study (9.0, 45.6 and 45.4%, respectively); thus, we combined the control groups in most analyses. The frequencies of the combined 713 controls were 9.3, 45.4 and 45.3% for *val/val*, *val/ala* and *ala/ala* genotypes, respectively (Table I). Because of overlapping enzyme activity (19) as well as similar distributions of *ala/ala* and *val/ala* genotypes between cases and controls, we combined the two genotypes as a single reference group to increase statistical power. The frequency of the *MTHFR val/val* genotype among 257 cases (11.7%) was not statistically different from that among 713 controls (9.3%), with a multivariate-adjusted RR of 1.35 (95% CI 0.84–2.17). Restricting our analyses to the 257 matched pairs did not materially alter the estimate of RR but resulted in a wider CI (RR = 1.40, 95% CI 0.77–2.53). When stratified by the size of the adenomas [small (<10 mm) versus large (⩾10 mm)], we observed little difference in the association with *MTHFR* genotype between small adenomas (RR = 1.36, 95% CI 0.76–2.45) and large adenomas (RR = 1.32, 95% CI 0.66–2.66) using all controls.

Similar to the data from the overall cohort (2), we observed inverse associations between risk of adenomas and consumption of folate and methionine; however, the associations were not significant most likely due to insufficient sample size. In contrast to the overall cohort, little indication of a positive association between alcohol intake and polyp risk was present in this nested case-control study. It is possible that since fewer women in this study (33%) consumed >5.7 g/day alcohol (the cut-off point for the highest tertile of alcohol consumption in this study) compared with the overall cohort (45%), we had less power to detect a moderate increase in risk.

We also investigated whether the association between risk of colorectal adenomas and consumption of folate, methionine and alcohol was modified by *MTHFR* genotype. In Table II, we show RRs and 95% CIs for *val/val* individuals compared with *val/ala* and *ala/ala* individuals within three strata of dietary folate and methionine intake as well as alcohol consumption. We did not observe any significant interaction between the *MTHFR val/val* polymorphism and consumptions of folate, methionine and alcohol (all $P_{\text{interaction}} \geqslant 0.13$). There was no significant difference in the association of folate and methionine intake and risk of adenomas among women with a *val/val* genotype compared with women with *ala/ala* or *val/ala* genotypes. A significantly increased risk associated with *val/val* genotype was observed only among non-drinkers. The U-shaped association across three alcohol consumption levels as well as a lack of a plausible biological explanation suggests that this positive association may be a chance finding.

In addition, we examined the association of the newly identified *MS* asp919gly polymorphism and risk of colorectal adenomas. The frequencies of *gly/gly*, *gly/asp*, and *asp/asp* genotypes were 2.9, 33.1 and 64.0% for the 713 combined controls and 2.3, 33.1 and 64.6% for the cases (Table I). The

Table II. Relationship of folate, methionine and alcohol to risk of colorectal adenomas stratified by *MTHFR* genotype in the Nurse's Health Study I

Strata		Consumption level[a]		
		Low	Medium	High
Folate				
All women	Case	89	72	76
	Control	213	205	215
	RR[b]	1.00	0.84	0.84
	(95% Cl)	(ref.)	(0.58–1.21)	(0.58–1.20)
ala/ala or val/ala	Case	79	65	65
	Control	189	190	197
	RR	1.00	0.81	0.78
	(95% Cl)	(ref.)	(0.55–1.20)	(0.53–1.15)
val/val	Case	10	7	10
	Control	24	14	18
	RR	0.99	1.21	1.31
	(95% Cl)	(0.45–2.16)	(0.47–3.10)	0.58–2.96
Methionine				
All women	Case	91	79	67
	Control	211	207	215
	RR	1.00	0.89	0.73
	(95% Cl)	(ref.)	(0.62–1.28)	(0.50–1.05)
ala/ala or val/ala	Case	78	70	61
	Control	188	188	200
	RR	1.00	0.90	0.74
	(95% Cl)	(ref.)	(0.62–1.37)	(0.50–1.09)
val/val	Case	13	8	6
	Control	23	19	14
	RR	1.36	1.03	1.03
	(95% Cl)	(0.66–2.82)	(0.43–2.44)	(0.38–2.78)
Alcohol				
All women	Case	75	82	80
	Control	207	209	217
	RR	1.00	1.10	1.03
	(95% Cl)	(ref.)	(0.76–1.59)	(0.71–1.49)
ala/ala or val/ala	Case	61	77	71
	Control	189	183	204
	RR	1.00	1.33	1.09
	(95% Cl)	(ref.)	(0.89–1.97)	(0.74–1.62)
val/val	Case	14	5	8
	Control	18	25	13
	RR	2.43	0.62	1.91
	(95% Cl)	(1.14–5.19)	(0.23–1.70)	(0.76–4.84)

$P_{interaction}$ = 0.25 for folate, 0.17 for methionine, 0.13 for alcohol.
[a]Cut-off points for low and high categories are: folate ⩽310 μg/day, ⩾508 μg/day;
[b]Age-adjusted RR.

genotype frequencies were in Hardy–Weinberg equilibrium in both cases and controls. Because of the similar genotype distribution among cases and controls, we combined *gly/asp* and *asp/asp* as a single reference group to increase statistical power. Risk of colorectal adenomas among *gly/gly* individuals was not significantly different from that of *gly/asp* and *asp/asp* individuals (RR = 0.66, 95% CI 0.26–1.70). Restricting our analyses to the 257 matched pairs did not materially alter the estimate of RR but resulted in a wider CI (RR = 0.67, 95% CI 0.21–2.19). When stratified sizes of adenomas, *gly/gly* individuals were at a slightly lower risk of small adenomas (RR = 0.61, 95% CI 0.17–2.11) than that of large adenomas (RR = 0.96, 95% CI 0.27–3.42) using all controls; however, the difference was not significant.

Considering that both MTHFR and MS participate in folate metabolism, we examined whether these two enzymes are linked or function synergistically. We found no evidence of linkage between these *MTHFR* and *MS* genotypes, and there was no significant interaction between these genotypes and adenoma occurrence ($P_{interaction}$ = 0.73).

Discussion

This is the first prospective study to examine the relationship between the *MTHFR val/val* genotype and risk of colorectal adenomas. We did not observe any material association, nor did we observe an interaction between the *MTHFR val/val* polymorphism and consumption of folate, methionine and alcohol. The inherent limitation of our study, like most studies on gene–environment interactions, is the relatively poor power to test interactions as well as main effects when the variant frequency is relatively low. Thus, it is possible that a modest association could exist but was not detected because of the limited size of the study.

A low methyl (low folate, low methionine and high alcohol) diet has been positively associated with colorectal cancer as well as pre-malignant adenomas (1,2). In two previous studies (9,10), we observed that the *MTHFR val/val* genotype was inversely associated with colorectal cancer risk, and that this inverse association was abolished among those with low folate and methionine intake, or high alcohol consumption. This suggests that the benefit of the *MTHFR val/val* genotype can be offset by a methyl-deficient diet. The *val/val* individuals, whose MTHFR enzyme is less efficient in converting 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, may be less prone to dTMP stress and in turn to colorectal carcinogenesis.

Thus, absence of an inverse association of the *MTHFR val/val* genotype with colorectal adenomas in this study is somewhat puzzling and unexpected. It is possible that the *MTHFR val/val* polymorphism is only protective in a subset of colorectal adenomas that have potential to progress to malignant tumors through the mechanism discussed above. It has been shown that the cumulative incidence of colorectal cancer among patients with an adenoma 10 mm or larger was only 10% over 15 years of follow-up (20), indicating that only a fraction of adenomas would undergo metastatic progression to cancer. If the benefit of the *MTHFR val/val* genotype was limited to a small subset of pre-malignant adenomas, an overall association with adenomas would be difficult to detect.

A related explanation is that the MTHFR may play a role only in a late stage of colorectal tumorigenesis (adenoma→carcinoma). In a recent study by Blount *et al*. (12), folate deficiency was associated with massive misincorporation of uracil into DNA and subsequent chromosomal breaks in humans, presumably because of deficient methylation of dUMP to dTMP. Failure to repair chromosomal breaks may lead to genomic instability (21), which is a hallmark of progression to invasive cancer and metastatic progression of tumors (22). We speculate that as adenomas progress to carcinoma, colorectal epithelium cells divide faster and are more prone to nucleotide pool imbalances. In particular, dUMP may replace dTMP, the limiting nucleotide for DNA synthesis, and its misincorporation into DNA may result in strand break-induced genomic instability. If the *MTHFR val/val* polymorphism makes cells less susceptible to nucleotide pool imbalance, it may be less critical in the early stage of tumorigenesis when tumor cells divide less often.

We did not observe any association between *MS gly/gly* genotype and risk of colorectal adenomas. The overall allele frequency of the *gly* allele was 19%, consistent with previous

reports (15% in ref. 16; 20% in ref. 17). The Asp919gly polymorphism is the only highly prevalent polymorphism identified in the *MS* gene so far. This polymorphism lies in the protein binding region that is involved in MS activity (17); however, no biological functions of this polymorphism have been established. A larger study is needed to confirm these observations, and to examine whether the genotype is associated with colorectal cancer.

In conclusion, we did not observe an association of the *MTHFR* and *MS* polymorphisms with risk of colorectal adenomas in this prospective study, nor did we observe significant interactions between the *MTHFR* polymorphism and consumption of folate and methionine. These results, which need to be confirmed in other studies, suggest that the benefit of the *MTHFR val/val* genotype is either associated with the small subset of colorectal adenomas that proceed to malignant transformation, or it acts only in a later stage of colorectal tumorigenesis.

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