Methylene Tetrahydrofolate Reductase Genotype Modifies the Chemopreventive Effect of Folate in Colorectal Adenoma, but not Colorectal Cancer

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Abstract

Epidemiologic evidence suggests a role for folate, a critical component of the 1-carbon cycle, in colorectal adenoma and cancer pathogenesis. Low folate levels, along with genetic polymorphisms in key enzymes such as methylene tetrahydrofolate reductase (MTHFR), can cause DNA hypomethylation and aberrant CpG methylation, which have been associated with colorectal tumor development. We investigated self-reported folate and alcohol intake alongside possible modifying effects of *MTHFR* 677 C>T and 1298 A>C polymorphisms in UK case-control studies of colorectal adenoma (317 cases, 296 controls) and cancer (500 cases, 742 controls). A significant association between *MTHFR* 1298 and colorectal cancer risk was observed [odds ratio, 1.57; 95% confidence interval (95% CI), 1.05-2.37], which was more pronounced in males (odds ratio, 3.02; 95% CI, 1.63-5.62). Although we found no association between *MTHFR* 677 and colorectal cancer, when data were stratified by sex, an increased risk was seen in females (odds ratio, 1.96; 95% CI, 1.11-3.46) but not in males. High folate intake was associated with a decreased risk for colorectal adenoma (odds ratio, 0.47; 95% CI, 0.30-0.73; P_{trend}, <0.001), which was modified by *MTHFR* 1298 genotype ($P_{interaction}$ = 0.006). However, we found no evidence to support the hypothesis that a high-folate diet protects against colorectal cancer development. Consistent with previous studies, high alcohol intake (≥14 U/wk) was associated with a significantly increased cancer risk (odds ratio, 2.57; 95% CI, 1.81-3.64). Our data suggest that dietary folate intake may be an important determinant for premalignant colorectal disease development but not colorectal cancer, an association that is modified by *MTHFR* genotype. (Cancer Epidemiol Biomarkers Prev 2008;17(9):2421–30)

Introduction

Colorectal cancer accounts for at least 10% of all new cancer diagnoses (1). There is marked geographic variation in colorectal cancer incidence, with a 5- to 10-fold greater incidence observed in Western countries compared with Asia and Africa (1). This observation has led to the hypothesis that environmental factors, most notably those involving diet, may be important determinants of colorectal cancer risk (2).

The role of dietary folate and the influence of genetic variation in folate metabolism has been the focus of many investigations into the etiology of both colorectal adenoma and colorectal cancer. Several epidemiologic studies have reported associations between low folate intake and an increased risk for both colorectal cancer (3, 4) and adenoma (5, 6). In addition, a meta-analysis of seven cohort studies and nine case-control studies examining the association between folate intake and colorectal cancer risk found a significant protective association with dietary folate (7). Folate levels are influenced by excessive alcohol consumption, showed by the high incidence of megaloblastic anemia seen in chronic alcohol users (8). Alcohol acts as a folate antagonist and decreases intestinal folate absorption and hepatic uptake, resulting in increased renal excretion and folate cleavage and reduced folate bioavailability (9). High alcohol intake has previously been associated with increased risk for colorectal cancer (10, 11), with the greatest risk for individuals with high alcohol consumption and a low-folate diet (4, 12). There is also some evidence that excessive alcohol consumption is a risk factor for colorectal adenoma, although data are not consistent (13, 14).

Folate is a critical component of the 1-carbon cycle that regulates DNA synthesis, methylation, and repair (15). Several key enzymes that control the balance between DNA methylation and synthesis have been identified, including MTHFR. MTHFR converts 5,10-methylene tetrahydrofolate (5,10-MeTHF) to 5-methyl tetrahydrofolate, the major circulating form of folate that acts as a methyl donor for the conversion of

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homocysteine to *S*-adenosyl methionine, the universal methyl group donor. Folate deficiency can result in decreased *S*-adenosyl methionine availability, leading to global genomic DNA hypomethylation and aberrant CpG methylation, which impacts gene expression. There is increasing evidence to support a role for altered DNA methylation in the development of colorectal adenomas and cancers (16, 17). In addition, 5,10-MeTHF is required for the conversion of deoxyuridylate to deoxythymidylate for DNA synthesis, and low folate levels can therefore lead to decreased thymidylate levels, resulting in uracil misincorporation during DNA synthesis and increased frequency of DNA strand breaks (18).

Individual variation in MTHFR activity, arising because of a thermolabile form of the enzyme, was first reported by Kang et al (19). Subsequently, common single-nucleotide polymorphisms were identified at nucleotide positions 677C>T (alanine to valine; ref. 20) and 1298A>C (glutamic acid to alanine; ref. 21), which result in decreased catalytic activity (21, 22), thus influencing 5,10-MeTHF and *S*-adenosyl methionine availability. It has previously been reported that both *MTHFR* 677 TT (23, 24) and *MTHFR* 1298 CC (23) genotypes confer protection against colorectal cancer development. However, in individuals with low (or deficient) folate intake or high alcohol consumption, genotype did not have a protective effect (25-27). Similar studies of colorectal adenomas (6, 13, 26) and hyperplastic polyps (6, 28) have shown less consistent results.

We investigated the association between colorectal cancer, dietary folate, and alcohol intake, along with the possible modifying effects of *MTHFR* genotype, in a UK-based case-control study of colorectal cancer patients and controls matched for age, sex, and primary care practice. We extended our analysis to include a second case-control study of colorectal adenoma patients and adenoma-free controls, recruited as part of the UK colorectal cancer fecal occult blood screening pilot study using the same questionnaire as for the cancer study. The primary hypotheses under investigation are that the 677 TT and 1298 CC *MTHFR* genotypes are associated with risk for both colorectal cancer and adenoma, and that these genotypes modify any effect on disease risk associated with the intake of dietary folate and alcohol.

Materials and Methods

Study Populations

Colorectal Cancer Case-Control Study. Full details of the study, including participation rates, have been described previously (29). Briefly, patients between 45 and 80 years old, diagnosed with colorectal cancer from 1997 to 2000, were recruited from hospitals in Dundee, Leeds, and York. For most cases, one sex- and age-matched control was recruited from the same primary care practice as the incident case, with most age matches within 1 year. However, for a minority of cases, multiple controls were selected or no matched control was available. In total, 500 cases and 742 controls were recruited, which included 433 matched case-control pairs. The study was approved by the Tayside Committee for Medical Research Ethics, Leeds Health Authority/St James's and Seacroft University Hospitals Clinical Research Ethics Committee, and the York Research Ethics Committee. Written informed consent was obtained from all study participants.

Colorectal Adenoma Case-Control Study. Colorectal adenoma cases and controls without adenoma were recruited via the Scottish arm of the UK fecal occult blood test–based colorectal cancer screening program. All persons who were 50 to 74 years old and residents of Tayside, Grampian, and Fife were invited to participate in the fecal occult blood test screening program. Individuals with a positive fecal occult blood test and resulting investigative colonoscopy were asked to take part in the study; those who had one or more colorectal adenomas identified by colonoscopy were classified as ''cases'' ($n = 317$), whereas those with no adenomas were classified as ''controls'' ($n = 296$). All individuals with a previous history of cancer or who were diagnosed with colorectal cancer following colonoscopy were excluded from recruitment. Participation rates for cases and controls were >80%. The study was approved by the Tayside Committee on Medical Research Ethics, and written informed consent was obtained from all study participants. Cases and controls were interviewed as soon as possible after colonoscopic examination following a positive fecal occult blood test.

Questionnaire Data Collection. All study participants were interviewed by experienced research interviewers and completed an extensive, 18-page diet and lifestyle

Table 1. Case and control demographics for the colorectal adenoma and colorectal cancer case-control studies

	Colorectal cancer study		Colorectal adenoma study	
	Controls	Cases	Controls	Cases
Total, *n*	742	500	296	317
Sex, *n* (%)				
Male	408 (55.0)	300 (60.0)	174 (58.8)	226 (71.3)
Female	334 (45.0)	200 (40.0)	122 (41.2)	91 (28.7)
Mean age (SD)	67.9 (8.8)	67.3 (8.4)	62.0 (5.6)	62.5 (5.7)
Male	68.2 (8.4)	66.7 (8.3)	62.0 (5.5)	62.7 (5.8)
Female	67.5 (9.3)	68.2 (8.5)	62.0 (5.8)	62.2 (5.7)
Site (primary for cancer), *n* (%)				
Colon only		286 (57.2)		223 (75.1)
Rectum only		214 (42.8)		46 (15.5)
Colon and rectum				28 (9.4)

questionnaire, the Food Frequency and Epidemiology Questionnaire, which was modeled on a questionnaire developed and validated for the European Prospective Investigation into Cancer and Nutrition (30). All participants were asked to estimate the frequency of consumption of 132 food items in the year previous to diagnosis or for controls in the year before interview. The frequency for each item was recorded on a nine-point Likert scale as follows: never or less than one per month, one to three per month, one a week, two to four per week, five to six per week, one a day, two to three per day, four to five per day, and six or more per day. In addition to questions regarding food items, information was also obtained on use of multivitamins and supplements, smoking, and alcohol use.

Folate and Alcohol Intake. We derived folate intake, expressed as micrograms per day from the Food Frequency and Epidemiology Questionnaire; neither folate nor homocysteine levels were measured in the blood. Because alcohol and multivitamins are sources of supplemental folate, four different variables were calculated: (*a*) dietary folate intake alone, (*b*) dietary intake plus alcohol, (*c*) dietary intake plus supplemental folate, and (*d*) dietary folate plus supplemental folate and alcohol. Total folate intake based on each of the four variables was categorized by approximate quartiles based on the control distribution in the colorectal cancer study. The same quartiles were used for both the colorectal cancer and adenoma data sets so that direct comparisons of the effects of folate intake on colorectal

Table 2. Number of colorectal cancer and adenoma cases and controls, odds ratios, and 95% CI for *MTHFR* 677C>T and *MTHFR* 1298A>C, stratified by study

	Colorectal cancer				Colorectal adenoma			
	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)	*P*	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)	*P*
MTHFR 677	592	490			296	308		
CC	271 (45.8)	238 (48.6)	1.00		130 (43.9)	135 (43.8)	1.00	
CT	272 (46.0)	199 (40.6)	0.83 (0.65-1.07)	0.16	139 (47.0)	132 (42.9)	0.91 (0.65-1.28)	0.61
TT	49 (8.3)	53 (10.8)	1.23 (0.80-1.89)	0.34	27 (9.1)	41 (13.3)	1.46 (0.85-2.51)	0.17
Overall test				0.13				0.23
Overall trend			0.99 (0.83-1.19)	0.95			1.10 (0.87-1.40)	0.43
CC/CT vs TT	543 (91.7)	437 (89.2)	1.34 (0.89-2.02)	0.16	269 (90.9)	267 (86.7)	1.53 (0.91-2.56)	0.11
Males	322	297			174	221		
CC	147 (45.7)	159 (53.5)	1.00		68 (39.1)	99 (44.8)	1.00	
CT	150 (46.6)	116 (39.1)	0.71 (0.51-0.99)	0.05	90 (51.7)	93 (42.1)	0.71 (0.47-1.08)	0.11
TT	25 (7.8)	22 (7.4)	0.81 (0.44-1.51)	0.51	16 (9.2)	29 (13.1)	1.24 (0.63-2.47)	0.53
Overall test				0.14				0.13
Overall trend			0.81 (0.63-1.04)	0.10			0.96 (0.71-1.29)	0.79
CC/CT vs TT	297 (92.2)	275 (92.6)	0.95 (0.52-1.72)	0.87	158 (9.2)	192 (86.9)	1.49 (0.78-2.84)	0.23
Females	270	193			122	87		
CC	124 (45.9)	79 (40.9)	1.00		62 (50.8)	36 (41.4)	1.00	
CT	122 (45.2)	83 (43.0)	1.07 (0.72-1.59)	0.75	49 (40.2)	39 (44.8)	1.37 (0.76-2.47)	0.29
TT	24 (8.9)	31 (16.1)	2.03 (1.11-3.71)	0.02	11 (9.0)	12 (13.8)	1.88 (0.75-4.69)	0.17
Overall test				0.06				0.32
Overall trend			1.31 (0.99-1.72)	0.06			1.37 (0.91-2.07)	0.13
CC/CT vs TT	246 (91.1)	162 (83.9)	1.96 (1.11-3.46)	0.02	111 (91.0)	75 (86.2)	1.61 (0.68-3.85)	0.28
MTHFR 1298	593	488			296	308		
AA	288 (48.6)	231 (47.3)	1.00		140 (47.3)	155 (50.3)	1.00	
AC	259 (43.7)	200 (41.0)	0.96 (0.75-1.24)	0.77	130 (43.9)	124 (40.3)	0.86 (0.62-1.21)	0.39
CC	46 (7.8)	57 (11.7)	1.54 (1.01-2.36)	0.05	26 (8.8)	29 (9.4)	1.01 (0.57-1.79)	0.98
Overall test				0.09				0.66
Overall trend			1.13 (0.94-1.35)	0.20			0.94 (0.74-1.21)	0.65
AA/AC vs CC	547 (92.2)	431 (88.3)	1.57 (1.05-2.37)	0.03	270 (91.2)	279 (90.6)	1.08 (0.62-1.88)	0.79
Males	323	296			174	221		
AA	158 (48.9)	125 (42.2)	1.00		85 (48.9)	113 (51.1)	1.00	
AC	150 (46.4)	133 (44.9)	1.12 (0.80-1.56)	0.50	78 (44.8)	84 (38.0)	0.81 (0.53-1.23)	0.32
CC	15 (4.6)	38 (12.8)	3.20 (1.68-6.09)	<0.001	11 (6.3)	24 (10.9)	1.64 (0.76-3.53)	0.21
Overall test				0.001				0.18
Overall trend			1.45 (1.13-1.86)	0.004			1.06 (0.78-1.43)	0.73
AA/AC vs CC	308 (95.4)	258 (87.2)	3.02 (1.63-5.62)	<0.001	163 (93.7)	197 (89.1)	1.81 (0.86-3.80)	0.12
Females	270	192			122	87		
AA	130 (48.2)	106 (55.2)	1.00		55 (45.1)	42 (48.3)	1.00	
AC	109 (40.4)	67 (34.9)	0.75 (0.51-1.12)	0.16	52 (42.6)	40 (46.0)	1.01 (0.57-1.79)	0.98
CC	31 (11.5)	19 (9.9)	0.75 (0.40-1.41)	0.37	15 (12.3)	5 (5.8)	0.44 (0.15-1.30)	0.14
Overall test				0.33				0.28
Overall trend			0.83 (0.63-1.09)	0.18			0.79 (0.52-1.22)	0.29
AA/AC vs CC	239 (88.5)	173 (90.1)	0.85 (0.46-1.55)	0.59	107 (87.7)	82 (94.3)	0.44 (0.15-1.25)	0.12

NOTE: Cancer study: likelihood ratio test for interaction between sex and *MTHFR 677* using the trend model (likelihood ratio $\chi^2(1) = 6.31$; $P = 0.01$). Adenoma study: likelihood ratio test for interaction between sex and *MTHFR 677* using the trend model (likelihood ratio $\chi^2(1) = 1.89$; $P = 0.17$). Cancer study: likelihood ratio test for interaction between sex and *MTHFR* 1298 using the trend model (likelihood ratio $\chi^2(1) = 8.78$; $P = 0.003$). Adenoma study: likelihood ratio test for interaction between sex and *MTHFR* 1298 using the trend model (likelihood ratio $\chi^2(1) = 1.14$; $P = 0.29$).
Abbreviation: OR, odds ratio.

Table 3. Alcohol and folate intake, sex- and age-adjusted odds ratios, and 95% CI, stratified by study

	Colorectal cancer				Colorectal adenoma			
	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)	*P*	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)	*P*
Alcohol intake*	738	484			296	317		
0	294 (39.8)	140 (28.9)	1.00		75 (25.3)	65 (20.5)	1.00	
1-5	155 (21.0)	79 (16.3)	1.09 (0.78-1.53)	0.62	51 (17.2)	54 (17.0)	1.28 (0.77-2.14)	0.35
6-13	140 (19.0)	101 (20.9)	1.60 (1.13-2.25)	0.007	64 (21.6)	67 (21.1)	1.00 (0.61-1.66)	0.99
≥14	149 (20.2)	164 (33.9)	2.57 (1.81-3.64)	<0.001	106 (35.8)	131 (41.3)	1.05 (0.64-1.71)	0.86
Overall trend			1.36 (1.22-1.53)	<0.001			1.00 (0.85-1.18)	0.98
Folate intake†	734	468			296	317		
≤276.07	184 (25.1)	117 (25.0)	1.00		75 (25.3)	112 (35.3)	1.00	
276.08-325.70	182 (24.8)	103 (22.0)	0.90 (0.64-1.26)	0.53	57 (19.3)	76 (24.0)	0.89 (0.57-1.41)	0.63
325.71-397.86	185 (25.2)	124 (26.5)	1.06 (0.77-1.47)	0.73	79 (26.7)	70 (22.1)	0.61 (0.39-0.95)	0.03
≥397.87	183 (24.9)	124 (26.5)	1.08 (0.78-1.50)	0.64	85 (28.7)	59 (18.6)	0.47 (0.30-0.73)	0.001
Overall trend			1.04 (0.94-1.15)	0.46			0.77 (0.67-0.89)	<0.001

*Alcohol consumption at 40 y of age (U/wk), with limits based on quartiles derived from population controls.
†Folate intake derived from nutrient analysis including folate supplements but excluded folate contributed from alcohol.

cancer and adenoma risk could be made. We use estimated dietary folate, including from supplements (but excluding alcohol), as the primary measure of folate in this analysis but have repeated all analyses for all measures.

Study participants were asked to report how many alcoholic drinks (measured as pints of beer or cider; glasses of wine, sherry, or other fortified wine; and single measures of spirits) they consumed during an average week at 40 years of age. Responses were converted into units of alcohol consumed each week, and levels of exposure were categorized in the same way as the folate data by approximate quartiles based upon colorectal cancer control distributions.

Sample Collection, DNA Extraction, and Molecular Genetic Analysis. Peripheral blood samples were collected from all study participants in EDTA tubes and stored at -20°C before analysis. Genomic DNA was extracted using a QIAamp 96 DNA blood kit (Qiagen) according to the manufacturer's instructions and stored in aliquots at 4°C. Following DNA extraction, samples were robotically replica-plated in a series of 96-well daughter plates that were used for all subsequent genotyping analysis. Analysis of the *MTHFR* 677 C>T (rs1801133) and *MTHFR* 1298 A>C (rs1801131) polymorphisms was carried out using preformatted Taqman drug metabolizing genotyping assays for allelic discrimination (Applied Biosystems), and end-point fluorescence was read on an ABI PRISM 7700 sequence detection system and analyzed using Sequence Detector software (version 1.7a). For quality control purposes, we included on each 96-well plate previously analyzed samples representative of each genotype where the genotype had been verified by sequencing, along with multiple no-template control samples. In addition, 1% of samples (cases and controls) were selected at random for repeat analysis. Overall, the failure rate was <5%.

Statistical Analysis. All analyses were carried out using the statistical analysis software Stata (Stata Statistical Software: Release 7.0).

Evidence against Hardy-Weinberg equilibrium was tested in controls using a χ^2 goodness-of-fit test. Linkage disequilibrium between the two polymorphisms was measured using Lewontin *D'* measure (http://www-gene.cimr.cam.ac.uk/clayton/). Haplotype frequencies were estimated using the estimation-maximization algorithm, and differences in frequencies between cases and controls were assessed using the likelihood ratio test.

Folate and alcohol consumption and *MTHFR* genotypes were compared between cases and controls in the colorectal adenoma and colorectal cancer case-control studies using χ^2 tests and logistic regression, including tests for trend in risk with genotype or quartile of exposure. Analyses were conducted as unadjusted and adjusted for age or age and sex. Effect sizes are presented as odds ratios with 95% confidence intervals (95% CI). Analysis of matched pairs using conditional logistic regression was additionally done in the cancer case-control study and reported in the Results, where notable differences to the adjusted results were observed. In the adenoma study, there were 28 individuals who had adenomas in both the colon and rectum; when data were stratified by subsite, individuals who had multiple adenoma sites were included in both strata.

We examined the association of each of the four defined variables for folate intake, described previously, with risk for colorectal adenoma and colorectal cancer. Interactions were investigated between alcohol and folate intake and *MTHFR* 677 and 1298 genotypes by doing stratified unadjusted and adjusted analyses on cases and controls and comparing risk estimates across strata. Differences in risk estimates were formally tested using the likelihood ratio test by comparing logistic regression models with and without an interaction term. For analyses involving interactions with *MTHFR* genotypes, the quartiles used for folate and alcohol consumption were collapsed into tertiles to overcome low homozygote variant numbers. The middle quartiles of the folate and alcohol data were combined in both the colorectal adenoma and colorectal cancer analyses.

Results

Demographics for the colorectal adenoma and colorectal cancer case-control studies are summarized in Table 1. Among those subjects who participated in the colorectal cancer study, 490 cases and 592 controls were genotyped for *MTHFR* polymorphisms, including 433 matched pairs; for the adenoma study, 308 cases and 296 controls were genotyped. There were no differences with respect

Table 4. Effects of alcohol and folate intake according to *MTHFR* 677 C>T genotype for the colorectal cancer and adenoma study

	Colorectal cancer					
	CC/CT			TT		
	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)*	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)*
Alcohol intake †	540	421		48	53	
0	215 (39.8)	115 (27.3)	1.00	20 (41.7)	23 (43.4)	1.00
1-13	215 (39.8)	157 (37.3)	1.37 (1.00-1.88)	21 (43.8)	16 (30.2)	0.53 (0.20-1.44)
≥14	110 (20.4)	149 (35.4)	2.55 (1.73-3.76)	7 (14.6)	14 (26.4)	1.63 (0.41-6.55)
Overall trend			1.58 (1.30-1.92)			1.07 (0.56-2.07)
Likelihood ratio, χ^2 (*df*) ‡			1.15 (1), $P = 0.28$			
Folate intake§	536	407		48	51	
≤276.07	140 (26.1)	101 (24.8)	1.00	9 (18.9)	15 (29.4)	1.00
276.08-397.86	261 (48.7)	196 (48.2)	1.04 (0.76-1.43)	24 (50.0)	25 (49.0)	0.83 (0.29-2.39)
≥397.87	135 (25.2)	110 (27.0)	1.15 (0.80-1.65)	15 (31.3)	11 (21.6)	0.65 (0.19-2.18)
Overall trend			1.05 (0.93-1.18)			0.87 (0.59-1.28)
Likelihood ratio, χ^2 (*df*) ‡			2.31 (1), $P = 0.13$			

NOTE: Adjusted odds ratios and 95% CI.
Abbreviation: *df*, degrees of freedom.
*Adjusted by sex and age.
†Alcohol consumption (U/wk) at 40 y of age, with limits based on quartiles derived from population controls.
‡Test of interaction between alcohol consumption and MTHFR 677 using the likelihood ratio test based on the trend model.
§Folate intake derived from nutrient analysis, including folate supplements but excluding folate from alcohol.

to age and sex between cases and controls with and without genotyping data available.

The Influence of *MTHFR* Genotype on Colorectal Adenoma and Cancer Incidence. *MTHFR* 677C>T and 1298A>C genotype distributions are summarized in Table 2. In both studies, control genotype frequencies for the *MTHFR* 677 C>T and 1298 A>C single-nucleotide polymorphisms were in Hardy-Weinberg equilibrium and were comparable to those previously reported in Caucasian populations (23, 24, 31). In addition, genotype frequencies were comparable between centers (data not shown).

Colorectal Cancer Case-Control Study. There were no significant case-control differences in *MTHFR* 677 genotype frequencies in the colorectal cancer study (Table 2). Furthermore, when data were stratified by subsite, there were no significant case-control differences for either colon ($P = 0.29$) or rectal ($P = 0.19$) sites (data not shown). However, when stratified by sex, homozygosity for the variant allele (*MTHFR 677* TT) was associated with a significantly increased risk in females (odds ratio, 1.96; 95% CI, 1.11-3.46; $P = 0.02$), and the presence of a single copy of the variant allele was associated with a marginally significant decreased risk for colorectal cancer in males (odds ratio, 0.71; 95% CI, 0.51-0.99). The overall trend for increasing number of variant alleles in males or females was not significant. Using the trend model, the likelihood ratio test for interaction indicated that there was a possible interaction between *MTHFR* 677 and sex [$\chi^2(1) = 6.31$; $P = 0.01$].

We observed significant differences in *MTHFR* 1298 genotype frequencies between cases and controls in the colorectal cancer study when comparing CC homozygotes with AA homozygotes and heterozygotes combined (odds ratio, 1.57; 95% CI, 1.05-2.37; Table 2). When we stratified by sex, the association was seen only in males and not in females, with the risk increasing with the number of copies of the variant allele ($P_{trend} = 0.004$) such that male homozygote variants had the highest risk for colorectal cancer (odds ratio, 3.20; 95% CI, 1.68-6.09). A significant interaction between *MTHFR* 1298 and sex ($P = 0.003$) was observed.

Stratification by subsite from the matched analysis (data not shown) showed a significantly increased risk was observed for colon cancer when the CC genotype was compared with AA/AC (odds ratio, 2.38; 95% CI, 1.25-4.56). The effect from the unmatched analysis was smaller but still approached significance ($P = 0.07$; data not shown). No significant differences were observed for rectal cancer in the matched analysis (odds ratio, 1.36; 95% CI, 0.68-2.71).

The *MTHFR* polymorphisms were in strong linkage disequilibrium ($D' = 0.98$). Of the four possible haplotypes, the 677T/1298C haplotype was rarely observed (estimated frequency of 0.2% compared with expected frequency of 10% under linkage equilibrium). There was no significant difference in haplotype frequencies between cases and controls ($P = 0.44$).

Colorectal Adenoma Case-Control Study. There was no significant case-control difference in the distribution of the *MTHFR* 677 and 1298 genotypes in the adenoma case-control study (Table 2). In contrast to the colorectal cancer study, we did not observe any significant sex-related differences in disease susceptibility for *MTHFR* 677 or 1298; the strongest suggested association involved the CC 1298 genotype in males, as in the cancer study, but this did not approach significance ($P = 0.21$).

Alcohol and Folate Intake. Table 3 summarizes reported alcohol intake at age 40 years for the colorectal adenoma and cancer case-control studies. The results for controls between the two studies show variation, notably the proportion of participants reporting nondrinker status; investigation of the prevalence of drinking by year of birth shows a steady increase, most notable in the cancer study, of increasing drinking for those with a later date of birth (data not shown). The discrepancy in

Table 4. Effects of alcohol and folate intake according to *MTHFR* 677 C>T genotype for the colorectal cancer and adenoma study (Cont'd)

Colorectal adenoma					
CC/CT			TT		
Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)*	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)*
269	267		27	41	
65 (24.2)	54 (20.2)	1.00	10 (37.0)	8 (19.5)	1.00
105 (39.0)	104 (39.0)	1.14 (0.72-1.80)	10 (37.0)	14 (34.2)	1.88 (0.47-7.47)
99 (36.8)	109 (40.8)	0.99 (0.59-1.66)	7 (25.9)	19 (46.3)	3.83 (0.77-19.2)
		0.99 (0.76-1.28)			1.96 (0.88-4.38)
		1.45 (1), P = 0.23			
269	267		27	41	
71 (26.4)	96 (36.0)	1.00	4 (14.8)	12 (29.3)	1.00
122 (45.4)	117 (43.8)	0.71 (0.48-1.08)	14 (51.9)	24 (58.5)	0.54 (0.14-2.04)
76 (28.3)	54 (20.2)	0.53 (0.33-0.85)	9 (33.3)	5 (12.2)	0.19 (0.04-0.91)
		0.81 (0.70-0.95)			0.57 (0.35-0.95)
		1.71 (1), P = 0.19			

Table 3 then reflects the broader range of years of birth among the cancer cases. In the age- and sex-adjusted analysis, alcohol consumption was associated with increased risk for colorectal cancer; odd ratios for individuals drinking an average of 6 to 13 U and >14 U/wk were 1.60 (95% CI, 1.13-2.25) and 2.57 (95% CI, 1.81-3.64), respectively, compared with nondrinkers. Similar increased risks were seen in both males (odds ratio, 2.52; 95% CI, 1.64-3.87) and females (odds ratio, 1.69; 95% CI, 0.70-4.07) for ≥14 U/wk. When data were stratified by cancer subsite, increased risk was associated with the highest two quartiles of reported alcohol intake for both colon (6-13 U: odds ratio, 1.57; 95% CI, 1.04-2.35; ≥14 U: odds ratio, 2.75; 95% CI, 1.58-4.79) and rectal cancer (6-13 U: odds ratio, 1.65; 95% CI, 1.02-2.68; ≥14 U: odds ratio, 2.88; 95% CI, 1.79-4.63).

In the adenoma case-control study, there was no association between risk for adenoma and increasing alcohol consumption. Furthermore, no difference was observed when data were stratified by sex or adenoma subsite (data not shown).

Folate intake, summarized in Table 3, was derived from nutrient analysis of the Food Frequency and Epidemiology Questionnaire and included supplement intake but excluded folate from alcohol. We used this variable in our analysis because it provided a comprehensive estimate of an individual's overall folate intake and removed the potential for any observed associations attributable to alcohol intake, which itself is associated with risk for colorectal adenoma and cancer. No significant case-control differences in folate intake in the colorectal cancer study were observed (Table 3). Data were comparable for males and females and did not differ by cancer subsite. In contrast, however, a decreased risk for colorectal adenomas was observed with increasing folate intake (P_{trend} <0.001) such that the highest quartile of folate intake (>397.87 μg/d) was associated with a significantly decreased risk (odds ratio, 0.47; 95% CI, 0.30-0.73). In addition, based on suggestions that there may be interactions between folate and smoking and the knowledge that smoking is a risk factor for colorectal adenoma, data were adjusted by smoking status. However, no difference in the results was observed when we adjusted by ever versus never smoked (data not shown).

For the cancer analysis, the exclusion of folate from supplement did not modify the results (data not shown). When alcohol was included among the sources of folate, there was an association between folate intake and risk for colorectal cancer, but only in the highest intake group (data not shown), with high intake being associated with increased risk for cancer. There was limited variation in the adenoma case-control study when the other measures of folate were considered.

Furthermore, we also stratified data according to the recommended daily allowance of folate (≥200 μg/d). In the colorectal cancer study, there was no significant difference between the number of cases (n = 447, 95.5%) and controls (n = 705, 96.0%) that equaled or exceeded the recommended daily intake of folate (odds ratio, 0.88; 95% CI, 0.50-1.57). Conversely, with respect to the adenoma study, we observed a statistically significant difference between the number of cases (n = 290, 91.5%) and controls (n = 283, 95.6%) that consumed ≥200 μg/d of folate (odds ratio, 0.48; 95% CI, 0.24-0.95; P < 0.05).

Modifying Effects of *MTHFR* Genotype on Alcohol and Folate Intake. We investigated the potential modifying effects of the two *MTHFR* single-nucleotide polymorphisms on alcohol and folate intake for both the colorectal adenoma and cancer case-control studies (Tables 4 and 5). There was no significant evidence of interaction between alcohol consumption and *MTHFR* 677C>T genotype in either the colorectal adenoma or cancer case-control study (Table 4). Likewise, no evidence of interaction was observed for *MTHFR* 1298A>C and alcohol in the adenoma study (Table 5). However, a significant interaction was seen in the colorectal cancer study between *MTHFR* 1298A>C and alcohol (P = 0.02) such that those individuals with a high alcohol intake and homozygotes for the *MTHFR* 1298 C allele were at a significantly higher risk for cancer than those individuals with a high alcohol consumption that were either AA or AC for the *MTHFR* 1298 polymorphism.

We found no evidence for an interaction between folate intake and either the *MTHFR* 677 (P = 0.13) or 1298 single-nucleotide polymorphisms (P = 0.14) in the

Table 5. Effects of alcohol and folate intake according to *MTHFR* 1298A>C genotype for the colorectal cancer and adenoma study

	Colorectal cancer					
	AA/AC			CC		
	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)*	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)*
Alcohol intake †	543	417		46	55	
0	209 (38.5)	124 (29.7)	1.00	24 (52.2)	12 (21.8)	1.00
1-13	221 (40.7)	150 (36.0)	1.19 (0.87-1.63)	17 (37.0)	23 (41.8)	1.90 (0.69-5.20)
≥14	113 (20.8)	143 (34.3)	2.39 (1.62-3.53)	5 (10.9)	20 (36.4)	3.97 (1.00-15.77)
Overall trend			1.51 (1.25-1.84)			1.97 (1.01-3.87)
Likelihood ratio, χ^2 (*df*) ‡			5.09 (1), $P = 0.02$			
Folate intake§	539	406		46	51	
≤276.07	133 (24.7)	104 (25.6)	1.00	16 (34.8)	11 (21.6)	1.00
276.08-397.86	264 (49.0)	196 (48.3)	0.96 (0.70-1.31)	21 (45.7)	25 (49.0)	1.85 (0.65-5.25)
≥397.87	142 (26.4)	106 (26.1)	0.96 (0.67-1.38)	9 (19.6)	15 (29.4)	3.09 (0.89-10.71)
Overall trend			0.99 (0.88-1.11)			1.43 (0.95-2.15)
Likelihood ratio, χ^2 (*df*) ‡			2.23 (1), $P = 0.14$			

NOTE: Adjusted odds ratios and 95% CI.
*Adjusted by sex and age.
† Alcohol consumption (U/wk) at 40 years of age, with limits based on quartiles derived from population controls.
‡ Test of interaction between alcohol consumption and MTHFR 1298 using the likelihood ratio test based on the trend model.
§Folate intake derived from nutrient analysis, including folate supplements but excluding folate from alcohol.

colorectal cancer case-control study. However, there was a significant interaction between folate intake and *MTHFR* 1298 genotype in the adenoma case-control study ($P = 0.006$; Table 5), where the combination of the AA/AC genotype, along with high folate intake, was significantly associated with a protective effect (odds ratio, 0.39; 95% CI, 0.24-0.62). Conversely, *MTHFR* 1298 CC homozygotes with high folate intake showed no such protective effect (odds ratio, 3.44; 95% CI, 0.72-16.54). There was no evidence for interaction between *MTHFR* 677 genotype and folate intake in the adenoma case-control study, although high folate intake was protective for both the CC/CT (odds ratio, 0.53; 95% CI, 0.33-0.85) and TT (odds ratio, 0.19; 95% CI, 0.04-0.91) genotypes (Table 4).

The joint effects of alcohol and folate were examined in the colorectal cancer and adenoma studies, and we observed no interaction between folate and alcohol intake for either cancer or adenoma.

Discussion

The role of diet in the pathogenesis of colorectal cancer has been the focus of many epidemiologic investigations, and there is consistent evidence that specific dietary factors, including high meat intake, confer an increased risk for colorectal cancer (32) and possibly adenoma (33, 34). However, there is less consistent evidence for a role for folate and the influence of genetic variation in enzymes involved in folate metabolism.

Our findings suggest that dietary folate protects against the development of colorectal adenomas but does not influence progression to more advanced colorectal disease. This finding is consistent with the results of several previous studies (5, 14). Dietary folate may protect against adenoma formation either by preserving normal patterns of DNA methylation and/or preventing uracil misincorporation. Because aberrant DNA methylation is thought to be an early event in colorectal tumor development, it is possible, therefore, that folate protects against adenoma development by maintaining adequate levels of *S*-adenosyl methionine, which acts as a methyl donor for normal DNA methylation (35). However, both adenoma cases and controls had a positive fecal occult blood test, which may indicate underlying gastrointestinal conditions other than colorectal adenomas, such as hemorrhoids, anal fissures, or inflammatory bowel disease, which may be indicative of, or related to a less healthy diet, and thus, our association may have been underestimated. Nevertheless, the controls from the adenoma and cancer study have very similar characteristics with respect to the sex distribution, 677 and 1298 genotype distributions, and folate intake. The major difference between the adenoma and cancer controls was in relation to alcohol consumption, with a greater proportion of adenoma controls in the highest quarter that is in part due to the geographic, age, and period of birth differences between the two series (data not shown). However, a greater proportion of the adenoma cases were also in the highest quarter compared with the cancer cases.

In contrast to our findings and those of others (36), high folate intake has previously been reported to be protective against colorectal cancer development (3, 4). The reasons for these discrepancies are not clear but may be attributable to the fact that folate levels are known to differ between world populations, especially following the mandatory fortification of foods with folic acid in the late 1990s in several countries, including the United States. Thus, although folate intake has significantly increased in these countries, a substantial proportion of the northern European population do not consume the recommended daily amount of folic acid (37). In previous studies investigating associations between folate intake and colorectal cancer risk, median folate intake has been reported to be almost 400 μg/d (38), yet only ~25% of cases and controls in our colorectal cancer study consumed more than this amount. However, using the UK folate recommended daily allowance of 200 μg/d,

Table 5. Effects of alcohol and folate intake according to *MTHFR* 1298A>C genotype for the colorectal cancer and adenoma study (Cont'd)

Colorectal adenoma					
AA/AC			CC		
Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)*	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)*
270	279		26	29	
66 (24.4)	55 (19.7)	1.00	9 (34.6)	7 (24.1)	1.00
103 (38.2)	109 (39.1)	1.19 (0.76-1.89)	12 (46.2)	9 (31.0)	0.94 (0.22-3.97)
101 (37.4)	115 (41.2)	1.08 (0.65-1.82)	5 (19.2)	13 (44.8)	1.97 (0.41-9.52)
		1.03 (0.80-1.34)			1.40 (0.64-3.06)
		1.91 (1), $P = 0.17$			
270	279		26	29	
65 (24.1)	102 (36.5)	1.00	10 (38.5)	6 (20.7)	1.00
126 (46.7)	130 (46.6)	0.66 (0.44-0.99)	10 (38.5)	11 (37.9)	2.33 (0.53-10.32)
79 (29.2)	47 (16.8)	0.39 (0.24-0.62)	6 (23.0)	12 (41.4)	3.44 (0.72-16.54)
		0.73 (0.62-0.86)			1.45 (0.87-2.41)
			7.55 (1), $P = 0.006$		

>95% of colorectal cancer study participants (cases and controls) reported that their daily intake was in excess of this, and we observed no difference in intake between cases and controls. With respect to colorectal adenoma, however, we observed a statistically significant difference between the numbers or cases and controls that met the UK recommended daily allowance. It is biologically plausible that the amount of folate required to modify the risk for developing colorectal adenoma is lower than that required to influence colorectal cancer risk. Therefore, it is possible that, although folate intake in the UK diet is sufficiently high enough to modify risk for colorectal adenoma, it is not sufficient to influence cancer risk.

It has also been reported that Food Frequency Questionnaires validated for folate intake show poor correlation between RBC folate levels and dietary folate intake (39, 40). Although this highlights the limitations of folate intake assessment by Food Frequency and Epidemiology Questionnaire, we have used the same method of assessment of folate intake to compare risk in independent case control studies of colorectal adenoma and cancer patients. Furthermore, there is evidence from high-performance liquid chromatography analysis of fruits and vegetables that food composition tables do not accurately reflect the true levels of folate content in food (41), such that actual levels are <25% lower than estimated from national tables. Consequently, the number of individuals reaching their recommended daily allowance is likely to be lower than reported.

Our findings relating to the potential modifying effects of *MTHFR* genotype differ to those previously published, where inverse associations have been reported for the influence of both *MTHFR* 677 and 1298 polymorphisms on colorectal adenoma and cancer risk (23, 24, 31). We observed no association between *MTHFR* 677 and colorectal cancer risk (odds ratio, 1.34; 95% CI, 0.89-2.02), although a significant positive association was seen in females (odds ratio, 1.96; 95% CI, 1.11-3.46). Only one study has previously reported an increased risk for colorectal cancer associated with this polymorphism, but the finding was not significant and was based on small numbers ($n = 74$; ref. 42). We also found an increased risk for colorectal cancer with the *MTHFR* 1298 polymorphism (odds ratio, 1.57; 95% CI, 1.05-2.37), which when the data were stratified by sex, was only evident in males (odds ratio, 3.02; 95% CI, 1.63-5.62). Although differences between males and females have been reported in relation to *MTHFR* 677 genotypes and lung cancer risk (43) and *MTHFR* 677 and 1298 genotypes and schizophrenia (44), there have been no previous reports of sex-specific associations with colorectal cancer risk. It is therefore more likely that our observation is predominantly attributable to differences in homozygote variant frequencies between male and female controls; the same differences were also seen among adenoma study controls.

The reasons for differences in the findings of previous studies with respect to associations between *MTHFR* polymorphisms and disease risk are unclear but may again reflect differences in circulating folate levels between populations. It is known that *MTHFR* genotypes with lower enzyme activity favor increased availability of the nonmethylated form of folate (5,10-MeTHF) for DNA synthesis and decreased levels of 5-methyl tetrahydrofolate for DNA methylation. If adequate levels of folate are available, even if MTHFR activity is low, there is sufficient conversion of 5-methyl tetrahydrofolate for DNA methylation while still shunting 5,10-MeTHF toward the synthesis of dUMP to dTMP. Thus, the functional effect of these polymorphisms may be influenced by folate availability, which in turn, may have a bearing on disease risk. Indeed, it has previously been shown that associations of polymorphisms in genes involved in the folate pathway with colorectal cancer risk may be modified by folate levels (31, 45). Previous studies have investigated the interaction between *MTHFR* polymorphisms and folate intake in colorectal adenoma and colorectal cancer based on the hypothesis that high folate intake combined with the *MTHFR* 677 TT genotype would result in decreased risk for disease (3, 25, 26, 38, 46). However, we found no evidence of an interaction between *MTHFR* genotypes and folate in our colorectal cancer case-control study. With respect to colorectal adenoma, our observation that *MTHFR* 677 TT homozygotes with the highest folate intake were at decreased risk for colorectal adenoma development (Table 5) is consistent with previous studies based on plasma folate levels (46, 47), whereas

the results from Food Frequency and Epidemiology Questionnaire have been variable, with some studies finding suggestions of decreased risk with increased folate intake (26). We also found evidence of a non-significant decreased risk for adenoma with CC and CT genotypes of a similar magnitude to the plasma folate studies, and in keeping with these studies, the risk was greatest with the TT genotype. Furthermore, we found evidence of an interaction between *MTHFR* 1298 genotype and folate intake in our adenoma patients ($P = 0.006$). This finding is in contrast to studies of plasma folate where the TT genotype seems to be protective rather than a risk factor.

Risk for colorectal disease has also been associated with high levels of alcohol consumption, and our finding of an increased risk for colorectal cancer in individuals who reported drinking more than 6 U/wk is comparable to data from previous studies (3, 10, 25, 46). Alcohol has a number of properties that may account for this association, including acting as a folate antagonist and influencing folate availability by affecting its absorption, metabolism, and excretion; inducing reactive oxygen species; altering DNA methylation; and acting as a cocarcinogen. In addition, because alcohol has considerable energy value, high consumption of alcohol will impact on nutritional status, leading not only to primary malnutrition but also, importantly, for colorectal cancer risk malabsorption of nutrients such as folate. Furthermore, genetic variation in the enzymes that catalyze the breakdown and excretion of alcohol and thus influence acetaldehyde levels may also be important because acetaldehyde is responsible for many of the detrimental effects of alcohol, including its ''anti-folate'' activity. Unlike previous studies, we saw no evidence of an interaction between *MTHFR* 677 genotype and alcohol intake in either the colorectal adenoma or cancer study, although our finding for colorectal adenoma is comparable to a previous UK study where no interaction was observed with either of the *MTHFR* polymorphisms (48). Interestingly, we did observe a significant interaction in the colorectal cancer study between *MTHFR* 1298A>C and alcohol, with a similar, although not significant, association seen in the adenoma study. However, these observations are complicated by several factors; controls in both studies with the *MTHFR* 1298 CC genotype were less likely to be heavy drinkers, more female controls were 1298CC homozygotes compared with males, and >90% of controls in the highest alcohol intake quartile (≥14 units) were males.

Data from animal studies support the hypothesis that folate deficiency is an early-acting risk factor in colorectal disease pathogenesis (49). However, it is the precise timing of folate deficiency that seems to be a critical determinant of disease progression because carcinogenesis can also be increased by folate supplementation and protected against by folate deficiency (50, 51), which is in contrast to our findings. Recent data from a randomized control trial of folate supplementation for the prevention of new colorectal adenomas in individuals with a previous history of adenomas suggested that those individuals assigned to the folate arm of the trial had an increased risk for advanced lesions and were more likely to have a greater number of them (52). In addition, data from a randomized control trial of folate with B vitamins for vascular disease suggested that folate was associated with an increased risk for colorectal cancer (53). Taken together, these data, along with the findings from epidemiologic studies, emphasize the need for a greater understanding of the effects of folate on colorectal tumor initiation, progression, and recurrence.

In summary, our data confirm previously reported associations between high levels of alcohol intake and colorectal cancer risk along with a protective role for dietary folate in the development of premalignant colorectal adenoma. With respect to the primary hypotheses under investigation, we observed no association between the *MTHFR* 677 polymorphism and colorectal cancer or adenoma risk nor any interaction with alcohol or dietary folate. However, the *MTHFR* 1298 CC genotype was associated with colorectal cancer risk, and a statistically significant interaction with alcohol intake was seen. Although this genotype was not associated with overall risk for adenoma, a statistically significant interaction with dietary folate intake was observed such that disease risk in association with high folate intake only decreased in those with AA or AC genotypes and actually increased in those with the CC genotype.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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