Colorectal Cancer and the Methylenetetrahydrofolate Reductase 677C → T and Methionine Synthase 2756A → G Polymorphisms: A Study of 2,168 Case-Control Pairs from the JANUS Cohort

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Abstract

Polymorphisms in genes involved in the metabolism of folate and methyl groups have been implicated with risk of colorectal cancer. We evaluated the relation between the polymorphisms 677C → T of the methylenetetrahydrofolate reductase (*MTHFR*) and 2756A → G of the methionine synthase (*MTR*) genes and risk of colorectal cancer. From the Norwegian JANUS cohort of 309,000 subjects, 2,179 cases were identified and a similar number of controls were selected. The controls were matched for age, gender, time, and place of serum donation. Genotypes were obtained from 2,168 case-control pairs by real-time PCR of serum samples. Risk of colorectal cancer was estimated with conditional and unconditional logistic regression. Median age at diagnosis was 60 years and mean follow-up 13 years. The odds ratio for MTHFR TT versus CC was 0.73 [95% confidence interval (95% CI), 0.58-0.92] and for MTR GG versus AA was 0.65 (95% CI, 0.47-0.90). No interaction between the polymorphisms was found. Relative risk estimates were similar for men and women, and for young and old age at diagnosis. For the MTR GG genotype, risk reduction was observed at the two most distal sites (sigmoideum and rectum) only (P = 0.003). The folate marker, serum total homocysteine (tHcy), was measured in 1,837 subjects. Odds ratio for the upper versus the lower tertile of tHcy was 1.32 (95% CI, 1.04-1.68). No significant effect modification by tHcy levels was detected for either polymorphism. In summary, we found significantly reduced risk of colorectal cancer in subjects with the MTHFR 677 TT and MTR 2756 GG genotypes. No interaction between the polymorphisms, or of either polymorphism with tHcy, was detected. (Cancer Epidemiol Biomarkers Prev 2004;13(12):2175–80)

Introduction

The 677C → T (ala222val) polymorphism of the methylenetetrahydrofolate reductase (*MTHFR*) gene has been associated with risk of common diseases, including cancer (1). The enzyme encoded by the gene is involved in the metabolism of folate coenzyme forms and thus in nucleotide synthesis and modification (methylation) of DNA. The variant enzyme is associated with reduced (30%) enzyme activity and increased thermolability (1).

The first study of colorectal cancer and MTHFR 677C → T was published in 1996 (2). This, and several subsequent (3-6) studies reported a reduced risk associated with the 677 TT genotype; however, most main effects were not statistically significant. Analyses of interaction of the variant with folate, methionine, alcohol, B6, and other nutrients showed that the TT genotype was generally most protective in conjunction with a methyl-replete diet and healthy lifestyle, whereas risk reduction was abolished in combination with low B vitamin/methionine status and/or high alcohol intake (3, 5). These findings, however, were not repeated in a recent large case-control study (7). There have also been reports on no, or slightly increased risk of colorectal cancer associated with the TT genotype (8-11), and risk associations with other genotype or nutrient combinations (e.g., CC and low folate; ref. 12). Two studies reported age-related risk of colorectal cancer for the TT genotype (11, 13). A review of articles on MTHFR 677C → T polymorphism and colorectal cancer has recently been published by Little et al. (14).

Another polymorphism related to folate metabolism, the methionine synthase (MTR) 2756A → G (asp919gly), has been described recently. The two enzymes, MTR and MTHFR, are closely related, as 5-methyltetrahydrofolate, the product of the MTHFR reaction, is a substrate for MTR. MTR, in turn, catalyzes the conversion of homocysteine to methionine (Fig. 1). The MTR 2756A → G, unlike the MTHFR 677C → T polymorphism, is not associated with elevated homocysteine (15) or low circulating folate (16). The MTR GG genotype was associated with reduced risk of adenomas (17) and cancer in the

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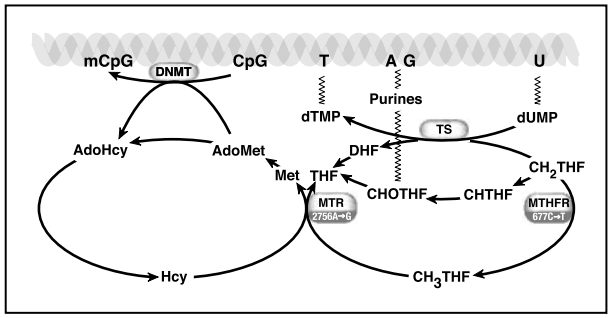

Figure 1. Biochemical pathways involving MTHFR and MTR, relation to DNA synthesis, integrity, and epigenetic modification (methylation). CG dinucleotide sequences in DNA (CpG) may be methylated by DNA methyltransferases (*DNMT*) with *S*-adenosylmethionine (*AdoMet*) as methyl donor. The methyl groups are, in part, provided via 5,10-methylenetetrahydrofolate (CH_2THF) through reduction by MTHFR to methyltetrahydrofolate (CH_3THF) and remethylation of homocysteine (*Hcy*) to methionine (*Met*) by MTR. 10-Formyl tetrahydrofolate (*CHOTHF*) is used in the synthesis of the purines A and G, whereas 5,10-methylenetetrahydrofolate is used in the methylation of dUMP to dTMP by thymidylate synthase (*TS*). Occasionally, uracil may be incorporated into DNA instead of thymidine.

colorectum (16), but the associations were not statistically significant. Others have found no association with colorectal cancer (6, 18). Significant associations with risk, however, have been reported for lymphomas (19, 20), but not for other neoplasias (21, 22).

The JANUS biobank contains serum collected from individuals participating in the Norwegian health screening programs and from blood donors. Subjects (n = 2,179) with colorectal cancer were identified in this cohort and included as cases in the present study. Serum samples were genotyped for the MTHFR 677C → T and MTR 2756A → G single nucleotide polymorphisms.

Materials and Methods

Subjects. The JANUS participants were recruited from several sources: Red Cross blood donors (men and women, ages 20-65, collected in 1973-1991), the Oslo Study (men only, ages 40-49, collected in 1972-73), and through population-based national health screening programs for cardiovascular disease risk factors (all counties, men and women, collected from 1974-1991). Most subjects recruited through the health screening programs were 35 to 49 years old, but also older individuals (65-67 years) were recruited from two counties in 1989 to 1990. The participation in the Oslo Study was 65% (23) and in the first health screening program (1974-78) 88% (24). Participation declined through the 1980s to 60% to 70%.

Serum specimens were stored at −25°C. At the time of case identification, the biobank contained samples from 309,000 individuals. Data on body mass index and smoking were obtained for subjects participating in the health screenings. Details on serum and data collection have been published previously (25). Information on the JANUS serum bank can be retrieved at http://www.kreft.no/dt_main_allatonce.asp?gid=2300.

From this cohort, 2,179 subjects with colorectal cancer diagnosed from January 1973 to September 2001 were identified through the Norwegian Cancer Registry. The reporting of colorectal cancer cases to the Norwegian Cancer Registry in the study period is regarded as close to complete (26). Cases had adenocarcinoma with origin in colon or rectum as verified by histologic examination of the primary tumor. Subjects with other tumors, either before or after the time of diagnosis, were also eligible. Among the cases, 32% had localized disease, 43% had disease with regional spread, and 22% had disease with distant spread. The status of the remaining 3% was unknown. Controls were selected at random from the risk set for each case using a density-sampling scheme and matched for sex, age (±6 months), place (county), and date (±6 months) of blood sampling. The case-control ratio was 1:1. The study protocol was approved by The Regional Committee for Medical Research Ethics of Western Norway. We compared the colorectal cancer cases in our study to all cases reported to the Cancer Registry during the years 1971 to 2001. In the national series, 49.8% were men and mean age was 69.9 years at diagnosis.

Genotyping. DNA from trace amounts of leukocytes in 50 μL serum was extracted using the GenoM-96 automatic DNA purification system (Qiagen, Darmstadt, Germany). The standard 50 μL protocol for whole blood was used, and DNA was eluted in 100 μL of a buffer containing 1 mmol/L Tris-HCl and 0.1 mmol/L EDTA (pH 8.0).

The MTHFR 677C → T and MTR 2756A → G variants were determined using real-time PCR with 5′ exonuclease (Taqman) probes according to Ulvik and Ueland (27). Genotyping was done twice. In the first run, 7 μL serum were dried in the wells of the PCR plate (AB-0900, ABgene, Epsom, United Kingdom) before real-time PCR as described in ref. (27). In the second run, we added DNA (20 μL) purified from serum as described above. PCR reactions were started by overlaying dried samples with 40 μL of PCR mastermix. Probes and primers were the same as described (27) except the 677T probe sequence, which was changed to 5′-TGAT-GAAATCGACTCCCG-3′.

Discrepant calls were less than 5%, and such samples were subject to repeated analyses. Altogether, we obtained results from 4,349 (MTHFR) and 4,360 (MTR) out of 4,373 subjects.

Determination of Total Homocysteine. Homocysteine was derivatized by ethyl-chloroformate and analyzed by gas chromatography-mass spectrometry as described (28). The coefficient of variation was 3%. Measurements were only possible in 1,837 serum samples. The remaining samples had been collected into tubes containing iodoacetate, which formed adducts with homocysteine and other thiols, and made reliable determination of homocysteine impossible.

Statistical Analysis. Odds ratios (ORs) and 95% confidence intervals (95% CIs) of colorectal cancer comparing variant genotypes with the wild type (CC

Table 1. Characteristics of study population

	Cases	Controls
All (*N*)	2,168	2,168
Blood donors	180	180
Oslo study, men only	465	465
Cardiovascular screening programs	1,523	1,523
Gender, male (%)	63.5	63.5
Age at sampling (y), *n* (%)		
<39	206 (9.4)	206 (9.4)
39-49	1,601 (73.5)	1,601 (73.5)
>49	372 (17.1)	372 (17.1)
Age at diagnosis (y), *n* (%)		
<55	767 (35.2)	
55-64	641 (29.4)	
>64	771 (35.4)	
Smoking (*n* = 3,854), %		
Current	43.5	46.6
Former	25.2	21.8
Ever	68.7	68.4
Body mass index (*n* = 2,860), mean (range)		
Males	25.6 (16.7-41.3)	25.2 (17.7-36.3)
Females	24.9 (16.5-45.0)	24.9 (15.6-42.8)

and AA) were computed using conditional logistic regression stratified on each case-control set. Information about smoking and body mass index was available for 3,854 and 2,860 subjects, respectively. In all main analyses, these variables changed the risk estimates for the genotypes negligibly and were not included as adjustment variables in the presented results.

Effect modification of the estimated ORs by sex, age, and tumor localization was assessed by the statistical significance of product terms in the logistic regression models. As an illustration, the test for difference in genotype effect in distal versus proximal tumors was carried out by assessing the significance of adding a product term of genotype (TT versus CT/TT or GG versus AG/AA) and an indicator of location (distal = 1, proximal = 0) to a model containing a main genotype term. For the assessment of possible effect modification by tertiles of homocysteine levels, the conditional approach was not suited, and we used unconditional logistic regression with adjustment for the matching variables (age, gender, time, and place of serum donation).

Analyses were done with SAS for Windows (version 8.2). Two-sided $P < 0.05$ was considered statistically significant.

Results

Included in the study were 2168 cases and the same number of age- and sex matched controls. Men constituted 63.5% of the cases, and the median age at diagnosis was 61 years for men and 56 years for women. The mean follow-up was 13 years. A summary of characteristics of cases and controls is presented in Table 1.

The associations between the MTHFR and MTR genotypes and colorectal cancer were assessed separately for men and women, and for young (<65 years) and older age at diagnosis (≥65 years; Table 2). Overall, the OR for the MTHFR TT versus CC genotype was 0.73 (95% CI,

Table 2. Relative risk of colorectal cancer by MTHFR 677C → and MTR 2756A → G genotypes and according to gender and age at diagnosis

	All subjects			Men			Women		
	Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)
MTHFR genotype									
CC	1,103	1,092	1	710	695	1	393	397	1
CT	899	886	1.01 (0.89-1.15)	560	562	0.98 (0.84-1.15)	339	324	1.06 (0.86-1.30)
TT	157	212	0.73 (0.58-0.92)	102	133	0.76 (0.57-1.01)	55	79	0.69 (0.46-1.02)
Age <65 y									
CC	706	714	1	430	435	1	276	279	1
CT	602	576	1.07 (0.92-1.25)	354	340	1.07 (0.87-1.31)	248	236	1.07 (0.84-1.37)
TT	83	127	0.66 (0.48-0.90)	56	81	0.70 (0.48-1.02)	27	46	0.58 (0.33-1.00)
Age ≥65 y									
CC	397	376	1	280	260	1	117	116	1
CT	297	310	0.91 (0.73-1.13)	206	222	0.85 (0.66-1.11)	91	88	1.03 (0.70-1.53)
TT	74	85	0.83 (0.58-1.18)	46	52	0.83 (0.54-1.28)	28	33	0.83 (0.45-1.52)
MTR genotype									
AA	1,457	1,402	1	937	895	1	510	504	1
AG	647	693	0.90 (0.79-1.02)	396	436	0.86 (0.73-1.02)	245	254	0.96 (0.78-1.18)
GG	64	97	0.65 (0.47-0.90)	35	57	0.58 (0.38-0.89)	29	40	0.73 (0.44-1.19)
Age <65 y									
AA	933	918	1	580	558	1	353	360	1
AG	404	437	0.91 (0.78-1.07)	231	263	0.85 (0.68-1.05)	173	174	1.01 (0.78-1.29)
GG	49	61	0.79 (0.54-1.17)	26	34	0.72 (0.43-1.23)	23	27	0.88 (0.49-1.57)
Age ≥65 y									
AA	514	481	1	357	337	1	157	144	1
AG	237	253	0.87 (0.70-1.09)	165	173	0.88 (0.67-1.15)	72	80	0.86 (0.58-1.26)
GG	15	36	0.40 (0.22-0.73)	9	23	0.37 (0.17-0.81)	6	13	0.44 (0.17-1.17)

NOTE: Analyzed by conditional logistic regression stratified by case-control sets.

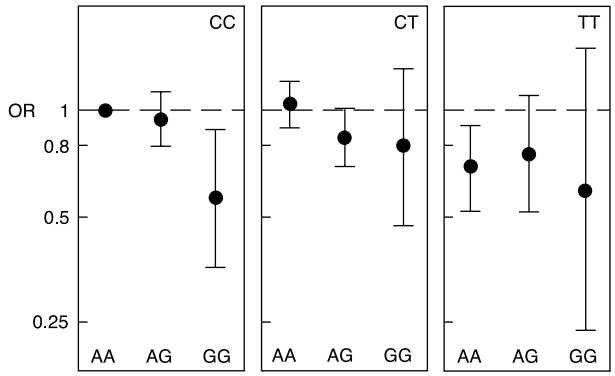

Figure 2. Combined effects of the MTHFR and MTR genotypes. Genotype combinations are grouped to show the effect of MTR genotypes within each MTHFR genotype category. *Bars,* 95% CIs.

0.58-0.92) and for MTR GG versus AA genotype 0.65 (95% CI, 0.47-0.90). No dose-response effect was evident for the *MTHFR* T-allele (OR, 1.01; 95% CI, 0.89-1.15 for CT versus CC). For MTR, the risk of the heterozygote variant was intermediate (OR 0.90; 95% CI, 0.79-1.02 for AG versus AA). The risk estimates were similar for men and women and similar according to age at diagnosis.

We determined the combined effects of the MTHFR 677C → T and MTR 2756A → G polymorphisms. Only a marginally reduced risk was observed for the combination of MTHFR TT and MTR GG compared with MTHFR TT or MTR GG alone. The lowest risk was found for the combination MTHFR CC/MTR GG. However, the small number of subjects in the combined groups resulted in wide 95% CIs of the risk estimates (Fig. 2). A test for interaction between the two polymorphisms was nonsignificant (P = 0.59).

In the subset (n = 1,837) with total homocysteine (tHcy) measurements, the mean tHcy concentration was lower in women (11.0 mol/L) compared with men (12.7 mol/L) and higher in individuals with the TT (14.0 mol/L) compared with the CC variant (11.7 mol/L) of the MTHFR polymorphism. tHcy did not vary by MTR genotype. ORs for colorectal cancer comparing the different combinations of sex-specific tertiles of tHcy and genotypes are shown in Table 3. tHcy was significantly associated with increased risk. OR for the upper versus lower tertile was 1.32 (CI, 1.04 - 1.68; P for trend = 0.02). However, our data indicated no effect modification of tHcy levels on the risk associated with the MTHFR polymorphism. For MTR, our analysis suggested a stronger protective effect of the variant genotype in individuals with low tHcy levels compared with those in the upper tHcy tertile. The result in the middle tHcy tertile was intermediate. A test for effect modification between the MTR genotype and tHcy, however, was nonsignificant (P = 0.42).

Table 4 shows the relationship between genotypes and cancer at different anatomic subsites. For MTR GG, there was reduced risk at the two most distal sites (sigmoideum and rectum), but a slightly increased risk at the more proximal sites (P = 0.003 for difference between distal and proximal sites). For the MTHFR polymorphism, there were no significant differences in risk according to tumor location.

Discussion

We have studied associations between the MTHFR 677C → T and MTR 2756A → G polymorphisms and the risk of colorectal cancer in the large Norwegian JANUS cohort. For both polymorphisms, the homozygous variant was associated with a significant risk reduction of more than 25%. For the MTR 2756A → G, but not for the MTHFR 677C → T polymorphism, the risk for heterozygotes was intermediate. Analysis of the combined effect of MTHFR and MTR genotypes showed no significant interaction.

This is the largest study published to date on risk of colorectal cancer and single nucleotide polymorphisms related to folate and methionine metabolism. The cohort consisted mainly of subjects participating in health screening programs in Norway. Cases and controls were matched for gender and closely matched for age, place, and time of serum donation. The study population was relatively homogenous and characterized by low levels of migration. The large number of cases and controls allowed for more precise estimation of effects in subgroups than was possible in previous studies. The

Table 3. Relative risk of colorectal cancer by genotype at different strata of tHcy*

Genotype			Sex-specific tertiles of tHcy					
	Overall		Tertile 1		Tertile 2		Tertile 3	
	Cases/control	OR (95% CI)	Cases/control	OR (95% CI)	Cases/control	OR (95% CI)	Cases/control	OR (95% CI)
MTHFR 677								
CC	466/486	1	158/192	1	169/156	1.37 (1.00-1.87)	139/138	1.32 (0.95-1.84)
CT	369/359	1.06 (0.87-1.29)	106/118	1.09 (0.77-1.53)	124/125	1.25 (0.90-1.75)	139/116	1.51 (1.08-2.12)
TT	73/75	1.01 (0.71-1.44)	16/19	1.03 (0.51-2.10)	19/16	1.51 (0.75-3.06)	38/40	1.21 (0.73-1.99)
MTR 2756								
AA	611/599	1	185/215	1	205/184	1.34 (1.01-1.79)	221/200	1.37 (1.03-1.83)
AG	271/285	0.95 (0.78-1.17)	90/101	1.06 (0.75-1.50)	96/98	1.22 (0.86-1.74)	85/86	1.23 (0.85-1.79)
GG	27/37	0.71 (0.43-1.19)	6/13	0.54 (0.20-1.47)	12/15	0.94 (0.43-2.08)	9/9	1.24 (0.48-3.23)

NOTE: Analyzed by unconditional logistic regression with adjustment for matching variables.
*tHcy measurements available only for 909 cases and 921 controls.

Table 4. Relative risk of colorectal cancer by genotype at subsites of colorectum

Genotype	Caecum/ascendens		Transversum/descendens		Sigmoideum		Rectum	
	Cases/control	OR (95% CI)	Cases/control	OR (95% CI)	Cases/control	OR (95% CI)	Cases/control	OR (95% CI)
MTHFR 677								
CC	210/209	1	145/140	1	301/309	1	411/396	1
CT	150/157	0.95 (0.71-1.29)	135/127	1.04 (0.73-1.48)	245/233	1.10 (0.87-1.41)	341/345	0.96 (0.78-1.18)
TT	38/36	1.09 (0.62-1.91)	21/38	0.57 (0.32-1.01)	46/60	0.80 (0.52-1.23)	52/73	0.69 (0.47-1.02)
MTR 2756								
AA	262/264	1	191/183	1	403/383	1	544/528	1
AG	119/127	0.95 (0.70-1.29)	93/107	0.85 (0.61-1.18)	177/184	0.92 (0.72-1.19)	236/251	0.92 (0.74-1.14)
GG	16/11	1.47 (0.66-3.30)	17/15	1.06 (0.52-2.15)	10/35	0.26 (0.12-0.54)	21/33	0.64 (0.37-1.12)

NOTE: Analyzed by conditional logistic regression stratified by case-control sets.

colorectal cancer patients in our cohort were diagnosed at a relatively young age (mean 60 years, compared with the population-based mean of 70 years at diagnosis). Therefore, our results may not be representative for individuals who are diagnosed with colorectal cancer late in life.

This is the first study to show a significantly reduced risk of colorectal cancer associated with the MTR 2756 GG genotype. The risk estimate is similar in magnitude to that obtained in two previous cohort studies (16) but different from the risk estimates close to one obtained in two case-control studies (6, 20).

A meta-analysis of 14 previous studies of MTHFR 677 TT genotype and risk of colorectal cancer showed considerable variation between estimates, but overall a moderately decreased risk for the TT relative to the CC genotype (14).

Shannon et al. (11) showed that the MTHFR TT genotype conferred increased risk of colorectal cancer in subjects older but not younger than 70 years. A similar finding of age-related risk was reported in a study of all cancers (13). We found a nonsignificant difference in ORs when dividing the cancer cases into ≥65 versus <65 years at diagnosis. The ORs tended to be higher in the high age groups and lowest in the group of young females, which had a high percentage of cases diagnosed at ≤54 years.

High folate intake and/or blood levels has been associated with reduced risk of colorectal cancer (29, 30). Moreover, the MTHFR 677C → T polymorphism is known to affect blood folate levels (1). Chen et al. (31) suggested a possible interaction between the MTHFR 677C → T polymorphism and folate status. They observed that the TT genotype was protective in folate-replete subjects, whereas the combination of TT and low folate status conferred no protection, or even increased risk (31). The finding of decreased risk for MTHFR TT combined with high folate status was repeated in some (5, 6) but not in all subsequent reports (7, 12, 32). No study has shown a significant effect modification by folate levels of the association between 677C → T MTHFR polymorphism and colorectal cancer risk. We had no data on folate intake, and serum folate could not be determined due to degradation during prolonged storage. We therefore measured in a subset (n = 1837) of the samples serum tHcy known to be elevated in folate-deficient subjects (33). The finding of a significantly increased colorectal cancer risk at higher tHcy levels confirms previous reports (16, 34). We observed no effect modification of the MTHFR cancer association by tHcy levels. Thus, our results do not support the existence of an interaction between the MTHFR polymorphism and folate status. However, our evidence is only indicative, as tHcy is known to be influenced by other factors than folate status, including B12 status and kidney function.

The lack of an overall effect of the TT genotype among those with tHcy measurements was unexpected. We could not find any differences in characteristics between those with and those without tHcy measurements. We therefore ascribe this finding to chance.

We analyzed risk associated with the MTHFR and MTR genotypes according to different anatomic subsites of the colorectum. For MTR GG, risk was lower at distal (rectum and sigmoideum) than at proximal (remaining colon) locations. Paz et al. (35) found that the MTR GG genotype was associated with reduced prevalence of hypermethylated promoters of tumor suppressor genes. If confirmed by others, this may explain the protective effect of MTR GG, as tumor progression is thought to depend on deactivation of both alleles of one or several tumor suppression genes. Deactivation commonly occurs by hypermethylation of one allele and through (partial) chromosome loss for the other allele (36). Because chromosomal instability is more frequent in the distal than in the proximal colon (37), this might explain the stronger protective effect in the distal versus the proximal subsites. A subset of proximal tumors, however, is associated with hypermethylation of DNA repair genes. Thus, reduced risk for the MTR GG genotype would also be expected at these sites. Further research will be required to elucidate the mechanisms by which MTR GG may be protective against cancer in the colorectum.

In conclusion, we found significantly reduced risk, of 27% and 35%, respectively, of colorectal cancer in subjects with the MTHFR 677 TT and MTR 2756 GG genotypes. No interaction between the polymorphisms, or of either polymorphism with the functional B vitamin marker tHcy, was apparent.

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