Genetic Variants of Methyl Metabolizing Enzymes and Epigenetic Regulators: Associations with Promoter CpG Island Hypermethylation in Colorectal Cancer

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Genetic Variants of Methyl Metabolizing Enzymes and Epigenetic Regulators: Associations with Promoter CpG Island Hypermethylation in Colorectal Cancer

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Abstract

Aberrant DNA methylation affects carcinogenesis of colorectal cancer. Folate metabolizing enzymes may influence the bioavailability of methyl groups, whereas DNA and histone methyltransferases are involved in epigenetic regulation of gene expression. We studied associations of genetic variants of folate metabolizing enzymes (*MTHFR*, *MTR*, and *MTRR*), DNA methyltransferase *DNMT3b*, and histone methyltransferases (*EHMT1*, *EHMT2*, and *PRDM2*), with colorectal cancers, with or without the CpG island methylator phenotype (CIMP), *MLH1* hypermethylation, or microsatellite instability. Incidence rate ratios were calculated in case-cohort analyses, with common homozygotes as reference, among 659 cases and 1,736 subcohort members of the Netherlands Cohort Study on diet and cancer (n = 120,852). Men with the *MTHFR 677TT* genotype were at decreased colorectal cancer risk (incidence rate ratio, 0.49; P = 0.01), but the *T* allele was associated with increased risk in women (incidence rate ratio, 1.39; P = 0.02). The *MTR 2756GG* genotype was associated with increased colorectal cancer risk (incidence rate ratio, 1.58; P = 0.04), and inverse associations were observed among women carrying *DNMT3b C→T* (rs406193; incidence rate ratio, 0.72; P = 0.04) or *EHMT2 G→A* (rs535586; incidence rate ratio, 0.76; P = 0.05) polymorphisms. Although significantly correlated (P < 0.001), only 41.5% and 33.3% of CIMP tumors harbored *MLH1* hypermethylation or microsatellite instability, respectively. We observed inverse associations between *MTR A2756G* and CIMP among men (incidence rate ratio, 0.58; P = 0.04), and between *MTRR A66G* and *MLH1* hypermethylation among women (incidence rate ratio, 0.55; P = 0.02). In conclusion, *MTHFR*, *MTR*, *DNMT3b*, and *EHMT2* polymorphisms are associated with colorectal cancer, and rare variants of *MTR* and *MTRR* may reduce promoter hypermethylation. The incomplete overlap between CIMP, *MLH1* hypermethylation, and microsatellite instability indicates that these related "methylation phenotypes" may not be similar and should be investigated separately. (Cancer Epidemiol Biomarkers Prev 2009;18(11):3086–96)

Introduction

Aberrant DNA methylation is an important epigenetic modification that may affect cancer susceptibility (1). Folate metabolizing enzymes are involved in the provision of methyl groups, and the bioavailability of methyl groups is dependent on the activity of these enzymes. Single-nucleotide polymorphisms in these genes have been reported to alter enzymatic activity. For example, rare variants of the *C677T* and *A1298C* polymorphisms in the methylene tetrahydrofolate reductase (*MTHFR*) gene result in reduced enzymatic activity (2, 3) and were inversely associated with colorectal cancer in several observational studies (4, 5). However, it was suggested that subjects having the *MTHFR 677TT* or *1298CC* genotypes were more likely to develop colorectal cancer showing a CpG island hypermethylation phenotype (6, 7) or microsatellite instability (8). Genetic variants of methionine synthase (*MTR*) have been studied less extensively, but the rare *GG* variant of the *MTR A2756G* single-nucleotide polymorphism was suggested to decrease colorectal cancer risk in two studies (9, 10). In contrast, it was suggested that some of the rare genetic variants of methionine synthase reductase (*MTRR*) may be associated with an increased risk for colorectal adenomas and carcinomas (11-13).

Whereas the aforementioned one carbon–metabolizing enzymes are involved in the provision of methyl groups, DNA methyltransferases catalyze the transfer of these methyl groups from S-adenosylmethionine into CpG dinucleotides of DNA. The DNA methyltransferase 3b (*DNMT3b*) is involved in *de novo* methylation (14) and may influence epigenetic regulation of gene expression and cancer cell growth. In this respect, experimental research suggested that *DNMT3b* depletion can reduce aberrant promoter CpG island hypermethylation in

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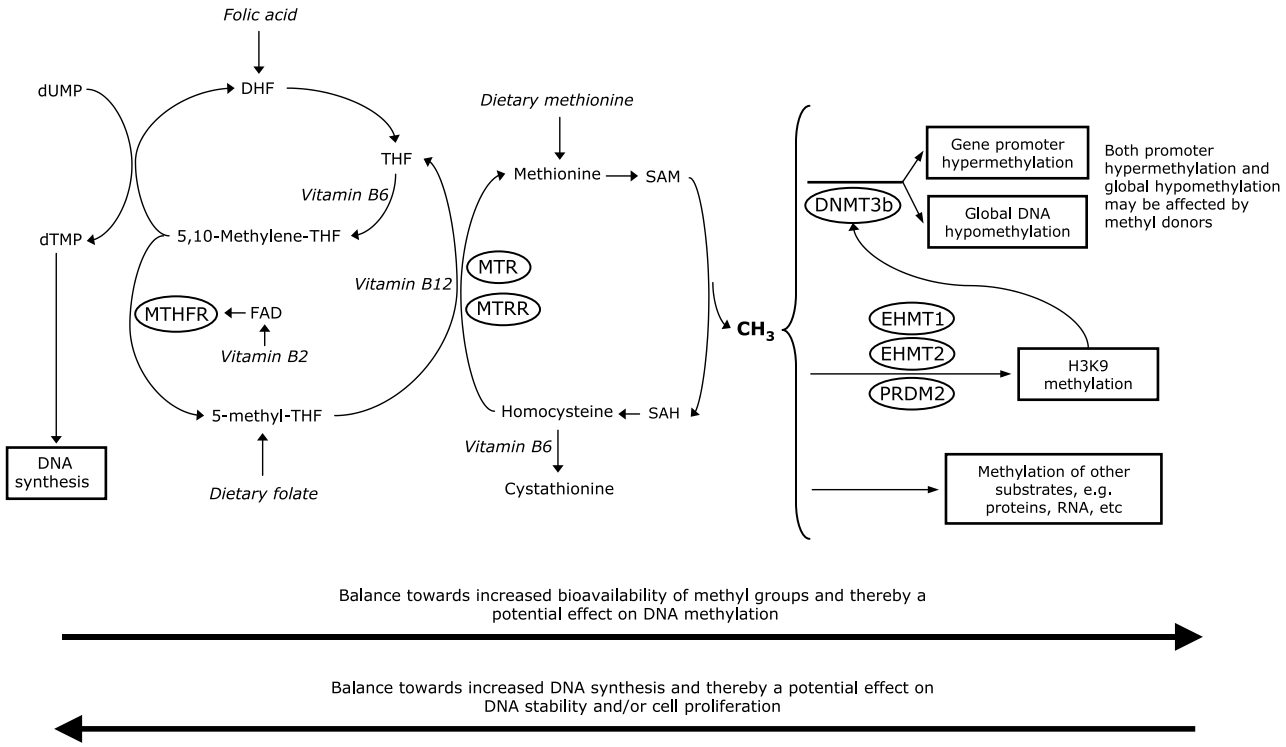

Figure 1. Folate metabolizing enzymes, DNA methyltransferases, histone methyl transferases, and DNA methylation. Potential targets of methyl groups are DNA, lysines (among which is histone H3 Lys-9), or other substrates, for example, proteins, RNA, etc. DNMT3b activity and promoter CpG island hypermethylation may depend on previous methylation of histone H3 Lys-9. Ovals represent the enzymes, of which single-nucleotide polymorphisms are investigated in this study. dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; DHF, dihydrofolate; THF, tetrahydrofolate; FAD, flavine adenine dinucleotide; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; MTHFR, methylene tetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; DNMT3b, DNA methyltransferase 3b; PRDM2, PR domain 2; EHMT, euchromatine histone methyltransferase.

cancer cells (15-17), whereas DNMT3b overexpression initiated promoter hypermethylation of tumor suppressor genes and the formation of colonic miroadenomas (18). In addition, it was observed that expression of DNMT3b was associated with *p16* and *RASSF1A* promoter methylation in non–small cell lung cancer (19) and with a promoter hypermethylator phenotype in breast cancer (20). Several single-nucleotide polymorphisms of the *DNMT3b* gene have been identified, which may affect catalytic activity of the DNMT3b enzyme. For example, the rare *T* allele of the *DNMT3b C→T* (rs2424913) polymorphism was found to significantly increase *DNTM3b* promoter activity and was associated with an increased risk for lung cancer (21), prostate cancer (22), and colorectal polyps, including colorectal adenomas (23), or with prognosis of head and neck cancer (24). Moreover, subjects with hereditary nonpolyposis colorectal cancer carrying the *TT* genotype developed colorectal cancer at a younger age compared with those homozygous for the wild-type *DNMT3b CC* allele (25). However, the association between the *DNMT3b C→T* (rs2424913) polymorphism and colorectal cancer risk has not previously been investigated.

It has been hypothesized that DNA methyltransferases may only have an effect on the chromatin if histone H3 Lys-9 is first methylated by histone methyltransferases (Fig. 1) and that DNA methylation may thus depend on the activity of histone methyltransferases (26). Experimental research also indicated that methylation of histone H3 Lys-9 and other histones play a critical role in maintaining epigenetic silencing by promoter hypermethylation of genes involved in colorectal cancer (27). The retinoblastoma protein interacting zinc finger gene (*RIZ* or *PRDM2)* is a histone methyltransferase that may act as a tumor suppressor, and *PRDM2* frameshift mutations have been observed in colorectal cancers showing microsatellite instability (28). Genetic variation of *PRDM2* may be hypothesized to affect its gene activity, and single-nucleotide polymorphisms in *PRDM2* were observed to be inversely associated with lung cancer (29). However, an increased risk for breast cancer was suggested (30). Other genes that have been identified as histone methyltransferases are euchromatin histone methyltransferase-1 (*EHMT1*) and -2 (*EHMT2*), and genetic variants of these genes were modestly associated with breast cancer risk in a large case-control study (30). However, the potential impact of genetic variants of *PRDM2, EHMT1*, and *EHMT2* has not previously been studied in relation to colorectal cancer.

The aim of this study was to determine the occurrence of single-nucleotide polymorphisms in genes encoding folate metabolizing enzymes (*MTHFR, MTR*, and *MTRR*), the DNA methyltransferase *DNMT3b*, and histone methyltransferases (*EHMT1, EHMT2*, and *PRDM2*) in a

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large population-based prospective cohort study in the Netherlands. We estimated associations of these single-nucleotide polymorphisms with overall colorectal cancer risk and with colorectal cancers with or without the CpG island methylator phenotype (CIMP), *MLH1* hypermethylation, or microsatellite instability.

Subjects and Methods

Study Population and Tumor Tissue Samples. Tumor material was obtained from incident colorectal cancer patients from the Netherlands cohort study, which has been described in detail elsewhere (31). Briefly, this prospective cohort study was initiated in September 1986 and includes 58,279 men and 62,573 women with the age of 55 to 69 y and free of disease at baseline. The cohort is followed for cancer occurrence by annual record linkage to the Netherlands Cancer Registry and to the Pathologisch Anatomisch Landelijk Geautomatiseerd Archief, a nationwide network and registry of histopathology and cytopathology reports (32, 33). At baseline, participants filled out a self-administered food frequency questionnaire, by which information was obtained about age, sex, family history of colorectal cancer, smoking behavior, and body mass index. A subcohort of 5,000 subjects was randomly selected after baseline exposure measurement to estimate accumulation of person-time in the cohort through biennial follow-up of vital status. Cases with prevalent cancer other than nonmelanoma skin cancer were excluded from this subcohort, which left 4,774 men and women eligible for analysis. Tumor material of the colorectal cancer patients was collected after approval by the ethical review boards of Maastricht University, the Netherlands Cancer Registry, and the Pathologisch Anatomisch Landelijk Geautomatiseerd Archief. During a follow-up period of 7.3 y after baseline, 734 incident colorectal cancer patients were identified who had an available Pathologisch Anatomisch Landelijk Geautomatiseerd Archief report of the lesion, as well as a sufficient amount of isolated DNA.

Collection of Mouth Swabs of Subcohort Members. Subcohort members still alive in December 2000 (n = 3,579) were contacted and asked to collect mouth swabs. Four cotton swabs in a small nonwoven polyethylene envelope were mailed to each subject, including a simple protocol on how to use them. After receipt, the swabs were placed in a falcon tube containing 2.0 mL buffer solution (100 mmol/L NaCl; 10 mmol/L EDTA; 10 mmol/L Tris, pH 8; with 0.2 mg/mL proteinase K and 0.5% w/v SDS). The lysed solution was kept stored at room temperature in the dark. In total, 1,929 subcohort members (54%) returned the mouth swabs with informed consent. The average DNA yield per cotton swab was 0.1 to 10 μg, which corresponded with data from literature (34). DNA could successfully be isolated from 1,829 subcohort members who also had complete follow-up information.

Genotyping Analyses. Nine fragments containing the *MTHFR, MTR, MTRR, DNMT3b, EHMT1, EHMT2*, and *PRDM2* single-nucleotide polymorphisms were amplified using multiplex PCR amplification and single-base extension reactions, as described previously by Knaapen et al. (35). Genomic DNA (50 ng) was added to 1× PCR buffer (Invitrogen), 1.75 mmol/L $MgCl_2$ (Invitrogen), 0.4 μmol/L dNTPs (Amersham Bioscience), 100 nmol/L of each primer (Eurogentec), and 0.25 U Platinum Taq (Invitrogen) in a final volume of 10 μL. PCR conditions were as follows: 3 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 62°C and 30 sec at 72°C, and a final extension for 5 min at 72°C and 4 min at 20°C. To degrade excess PCR primers and dNTPs, 5 μL multiplex PCR product was incubated with 2 μL EXO-SAP IT (Amersham Bioscience) at 37°C for 15 min, followed by 80°C for 15 min to deactivate the enzyme. The multiplex single-base extension reaction was done using a SNaPShot multiplex kit, as described by the manufacturer (Applied Biosystems). single-base extension primers were designed to bind immediately adjacent 5′ to the Single-nucleotide polymorphism of interest with a template-specific part of 20 to 33 bp and a temperature of 60°C. rs numbers of the single-nucleotide polymorphisms and primer sequences are shown in Supplementary Table S1. During thermal cycling, the primers are extended at their 3′ end with a single dideoxyribonucleoside triphosphate labeled with a distinct fluorophore, revealing the genotype of the single-nucleotide polymorphism. Single-base extension was done using 25 cycles of 96°C for 10 s and 60°C for 30 s. Following cycling, the reaction was treated with 1 U Shrimp Alkaline Phosphatase (Amersham Bioscience) at 37°C for 1 h to degrade the unincorporated dideoxynucleotide triphosphates, followed by enzyme deactivation at 75°C for 15 min. One microliter of single-base extension product was mixed with 13 μL of Hi-Di formamide (Applied Biosystems) and 0.4 μL of Genescan-120 size standard (Applied Biosystems), subsequently denatured at 95°C for 5 min, and then analyzed on an ABI Prism 3100 genetic analyzer using Genemapper Analysis software (version 4.0).

To validate the genotype data, we sequenced every fragment containing a specific single-nucleotide polymorphism in a subset of 30 samples, including 10 colorectal cancer cases, 10 female, and 10 male subcohort members with mouth swabs. Sequencing was done using the BigDye Terminater v1.1 cycle sequencing kit, following the manufacturer's recommendations using the ABI 3700 genetic analyzer. The sequencing results were similar to the SNaPShot results for all but one (99.6%) of the nine single-nucleotide polymorphisms within these 30 samples. Reproducibility of the SNaPShot analysis was established by subjecting 93 samples, composed of 31 colorectal cancer cases, 31 female, and 31 male subcohort members with mouth swabs, twice to the complete SNaPShot analysis procedure, from multiplex PCR of genomic DNA to the genetic analysis of the samples. We observed that the analyses could be reproduced in 99.5% of these cases.

Promoter Methylation Analyses. The CIMP was defined by promoter hypermethylation of at least three of five methylation markers (*CACNA1G, IGF2, NEUROG1, RUNX3*, and *SOCS1*), as suggested by Weisenberger et al. (36). DNA methylation in the CpG islands of these five CIMP markers and of the *MLH1* gene was determined by Methylation Specific PCR (37). Bisulfite modification was carried out on 500 ng DNA using a commercially available kit (Zymo Research). To facilitate Methylation Specific PCR analysis on DNA retrieved from formalin-fixed, paraffin-embedded tissue, DNA was first amplified with flanking PCR primers that amplify bisulfite modified DNA but do not preferentially amplify methylated or unmethylated DNA. The resulting fragment was used as a template for

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Table 1. Prevalence of genotypes of subcohort members and colorectal cancer patients in the Netherlands cohort study

Gene and SNP	dbSNP number	Genotype (MAF)	Men and women		Men		Women	
			Subcohort (HWE)*	CRC cases	Subcohort (HWE)*	CRC cases	Subcohort (HWE)*	CRC cases
MTHFR C677T	rs1801133	*CC*	876 (48.9 %)	318 (46.2 %)	409 (45.4%)	179 (46.8%)	467 (52.3%)	139 (45.3%)
		CT	750 (41.8 %)	320 (46.4 %)	405 (44.9%)	184 (48.2%)	345 (38.7%)	136 (44.3%)
		TT	167 (9.3 %)	51 (7.4 %)	87 (9.7%)	19 (5.0%)†	80 (9.0%)	32 (10.4%)
		(0.30)	(0.72)		(0.36)		(0.16)	
MTHFR A1298C	rs1801131	*AA*	735 (41.6%)	299 (43.7%)	345 (39.3%)	167 (43.8%)	390 (43.9%)	132 (43.6%)
		AC	774 (43.8%)	275 (40.2%)	423 (48.2%)	166 (43.6%)	351 (39.5%)	109 (36.0%)
		CC	258 (14.6%)	110 (16.1%)	110 (12.5%)	48 (12.6%)	148 (16.6%)	62 (20.4%)
		(0.37)	(0.02)		(0.26)		(<0.001)	
MTR A2756G	rs1805087	*AA*	1190 (65.9%)	449 (64.5%)	589 (65.1%)	257 (66.4%)	601 (66.8%)	192 (62.1%)
		AG	543 (30.1%)	204 (29.3%)	281 (31.0%)	108 (27.9%)	262 (29.1%)	96 (31.1%)
		GG	72 (4.0%)	43 (6.2%)	35 (3.9%)	22 (5.7%)	37 (4.1%)	21 (6.8%)
		(0.19)	(0.31)		(0.84)		(0.22)	
MTRR A66G	rs1801394	*AA*	367 (20.4%)	136 (19.5%)	193 (21.4%)	65 (16.7%)	174 (19.4%)	71 (23.0%)
		AG	833 (46.4%)	338 (48.3%)	399 (44.2%)	196 (50.2%)	434 (48.5%)	142 (45.9%)
		GG	597 (33.2%)	225 (32.2%)	310 (34.4%)	129 (33.1%)	287 (32.1%)	96 (31.1%)
		(0.56)	(0.01)		(0.003)		(0.67)	
DNMT3b C→T‡	rs2424913	*CC*	597 (32.9%)	240 (34.1%)	303 (33.4%)	132 (33.9%)	294 (32.5%)	108 (34.4%)
		CT	895 (49.5%)	348 (49.5%)	449 (49.6%)	184 (47.3%)	446 (49.4%)	164 (52.2%)
		TT	318 (17.6%)	115 (16.4%)	154 (17.0%)	73 (18.8%)	164 (18.2%)	42 (13.4%)
		(0.42)	(0.58)		(0.57)		(0.82)	
DNMT3b C→T‡	rs406193	*CC*	1331 (74.3%)	528 (75.6%)	686 (76.7%)	291 (74.6%)	645 (71.9%)	237 (76.9%)
		CT	415 (23.1%)	152 (21.8%)	190 (21.2%)	88 (22.6%)	225 (25.1%)	64 (20.8%)
		TT	46 (2.6%)	18 (2.6%)	19 (2.1%)	11 (2.8%)	27 (3.0%)	7 (2.3%)
		(0.14)	(0.05)		(0.18)		(0.18)	
EHMT1 G→A‡	rs4634736	*GG*	1444 (80.6%)	568 (81.7%)	730 (81.4%)	320 (83.1%)	714 (79.8%)	248 (80.0%)
		GA	334 (18.6%)	121 (17.4%)	163 (18.2%)	64 (16.6%)	171 (19.1%)	57 (18.4%)
		AA	14 (0.8%)	6 (0.9%)	4 (0.4%)	1 (0.3%)	10 (1.1%)	5 (1.6%)
		(0.10)	(0.27)		(0.11)		(0.95)	
EHMT2 G→A	rs535586	*GG*	755 (42.2%)	297 (42.9%)	390 (43.3%)	155 (40.3%)	365 (41.2%)	142 (46.3%)
		GA	810 (45.3%)	311 (44.9%)	414 (45.9%)	185 (48.0%)	396 (44.6%)	126 (41.0%)
		AA	223 (12.5%)	84 (12.2%)	97 (10.8%)	45 (11.7%)	126 (14.2%)	39 (12.7%)
		(0.35)	(0.80)		(0.41)		(0.27)	
PRDM2 G→A‡	rs2235515	*GG*	1065 (59.0%)	377 (55.7%)	527 (58.5%)	210 (56.0%)	538 (59.6%)	167 (55.3%)
		GA	640 (35.5%)	262 (38.7%)	320 (35.5%)	141 (37.6%)	320 (35.4%)	121 (40.1%)
		AA	99 (5.5%)	38 (5.6%)	54 (6.0%)	24 (6.4%)	45 (5.0%)	14 (4.6%)
		(0.23)	(0.82)		(0.56)		(0.77)	

Abbreviations: SNP, single-nucleotide polymorphism; MAF, minor allele frequency among subcohort members (men and women combined); HWE, Hardy-Weinberg equilibrium; CRC, colorectal cancer.
*P value for test for Hardy-Weinberg equilibrium based on the distribution of genotypes among subcohort members.
†χ^2 Test; $P < 0.05$ for the difference in genotype frequencies between colorectal cancer cases and subcohort members who did not develop colorectal cancer.
‡Single-nucleotide polymorphism occurring in an intron of the gene.

the Methylation Specific PCR reaction (38, 39). All PCRs were done with controls for unmethylated alleles (DNA from normal lymphocytes), methylated alleles (normal lymphocyte DNA treated *in vitro* with SssI methyltransferase, New England Biolabs), and a control without DNA. Ten microliters of each Methylation Specific PCR reaction was directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. The Methylation Specific PCR analyses were successful in 81%, 79%, 79%, 90%, 83%, and 93% of the 734 patients for *CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1*, and *MLH1*, respectively.

Microsatellite Instability. Microsatellite instability was determined by a pentaplex PCR, using the microsatellite instability markers *BAT-26, BAT-25, NR-21, NR-22,* and *NR-24,* as described in detail by Suraweera et al. (40). Microsatellite instability analyses were successful in 662 (90%) of the 734 available samples.

Statistical Analyses. Data analyses were conducted overall and for men and women separately. The prevalence of the genotypes and minor allele frequencies of the single-nucleotide polymorphisms were calculated for subcohort members and colorectal cancer cases. χ^2 Tests were used to test differences in prevalence between colorectal cancer cases and subcohort members who did not develop colorectal cancer. The Hardy-Weinberg equilibrium was tested using a P value threshold of <0.05 among subcohort members to evaluate whether, for each single-nucleotide polymorphism, the individual alleles combined into the observed genotypes in a random manner. Although this subcohort was a random sample of the total cohort and thus selection would not be expected, the DNA samples were collected from individuals still alive several years after baseline. If survival of these subjects was due to presence of specific genotypes, this may bias the estimated associations. However, because genetic status is unknown of the remaining subcohort members, it is impossible to compare survival between genotypes, and we therefore alternatively compared mean age of patients between genotypes of each gene.

The overlap between the three methylation endpoints, that is, CIMP, *MLH1* hypermethylation, and microsatellite instability, was compared and tested by χ^2 tests. Cox proportional hazards regression models were used to estimate age-adjusted incidence rate ratios and 95%

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confidence intervals (95% CI), taking homozygotes of common alleles as reference category in all analyses. Analyses were done overall and stratified by CIMP status, *MLH1* hypermethylation, and microsatellite instability, allowing us to compare the effects between the recently defined CIMP phenotype (36), the "classic" methylation marker *MLH1*, and microsatellite instability, and to account for a potential incomplete overlap that

Table 2. Associations of single-nucleotide polymorphisms in folate-metabolizing enzymes and epigenetic regulators with colorectal cancer risk

Gene and SNP	dbSNP number	Genotype	Men and women			Men			Women			Interaction with sex
			*n**	RR (95% CI)†	*P*	*n*	RR (95% CI)‡	*P*	*n*	RR (95% CI)‡	*P*	*P*
MTHFR C677T	rs1801133	*CC*	318	1.00	179	1.00	139	1.00				
		CT	320	1.23 (1.02-1.50)	0.04	184	1.10 (0.84-1.43)	0.49	136	1.43 (1.07-1.91)	0.02	
		TT	51	0.80 (0.56-1.15)	0.23	19	0.49 (0.28-0.85)	0.01	32	1.26 (0.78-2.05)	0.34	0.03
		CT + TT vs *CC*	371	1.15 (0.95-1.39)	0.15	203	0.98 (0.76-1.27)	0.89	168	1.39 (1.06-1.83)	0.02	
MTHFR A1298C	rs1801131	*AA*	299	1.00	167	1.00	132	1.00				
		AC	275	0.89 (0.72-1.09)	0.26	166	0.81 (0.62-1.07)	0.14	109	1.00 (0.73-1.35)	0.98	
		CC	110	1.05 (0.79-1.38)	0.74	48	0.89 (0.59-1.35)	0.58	62	1.23 (0.85-1.78)	0.28	0.42
		AC + CC vs *AA*	385	0.93 (0.77-1.12)	0.45	214	0.83 (0.64-1.07)	0.15	171	1.07 (0.81-1.41)	0.64	
MTR A2756G	rs1805087	*AA*	449	1.00	257	1.00	192	1.00				
		AG	204	1.03 (0.84-1.27)	0.77	108	0.95 (0.72-1.26)	0.73	96	1.14 (0.85-1.54)	0.38	
		GG	43	1.58 (1.03-2.43)	0.04	22	1.45 (0.79-2.66)	0.23	21	1.76 (0.97-3.21)	0.06	0.64
		AG + GG vs *AA*	247	1.10 (0.90-1.33)	0.35	130	1.01 (0.77-1.32)	0.94	117	1.22 (0.92-1.62)	0.17	
MTRR A66G	rs1801394	*AA*	136	1.00	65	1.00	71	1.00				
		AG	338	1.08 (0.85-1.39)	0.53	196	1.36 (0.96-1.92)	0.09	142	0.83 (0.59-1.18)	0.31	
		GG	225	1.03 (0.79-1.34)	0.83	129	1.23 (0.85-1.78)	0.27	96	0.85 (0.58-1.24)	0.39	0.15
		AG + GG vs *AA*	563	1.06 (0.84-1.34)	0.62	325	1.30 (0.94-1.81)	0.11	238	0.84 (0.60-1.16)	0.29	
DNMT3b C→T	rs2424913	*CC*	240	1.00	132	1.00	108	1.00				
		CT	348	0.95 (0.78-1.17)	0.66	184	0.91 (0.68-1.20)	0.50	164	1.01 (0.75-1.36)	0.94	
		TT	115	0.90 (0.68-1.19)	0.46	73	1.00 (0.69-1.45)	0.99	42	0.76 (0.50-1.16)	0.20	0.37
		CT + TT vs *CC*	463	0.94 (0.77-1.14)	0.54	257	0.93 (0.71-1.22)	0.60	206	0.95 (0.71-1.26)	0.72	
DNMT3b C→T	rs406193	*CC*	528	1.00	291	1.00	237	1.00				
		CT	152	0.91 (0.73-1.14)	0.43	88	1.13 (0.83-1.54)	0.43	64	0.71 (0.51-0.99)	0.05	
		TT	18	1.05 (0.59-1.87)	0.87	11	1.32 (0.59-2.95)	0.50	7	—	—	0.10
		CT + TT vs *CC*	170	0.93 (0.75-1.15)	0.49	99	1.15 (0.86-1.54)	0.36	71	0.72 (0.52-0.99)	0.04	
EHMT1 G→A	rs4634736	*GG*	568	1.00	320	1.00	248	1.00				
		GA	121	0.93 (0.73-1.18)	0.55	64	0.92 (0.66-1.28)	0.62	57	0.95 (0.67-1.34)	0.76	
		AA	6	—	—	1	—	—	5	—	—	0.41
		GA + AA vs *GG*	127	0.93 (0.73-1.18)	0.55	65	0.89 (0.64-1.24)	0.49	62	0.98 (0.70-1.37)	0.90	
EHMT2 G→A	rs535586	*GG*	297	1.00	155	1.00	142	1.00				
		GA	311	0.92 (0.76-1.13)	0.44	185	1.10 (0.84-1.44)	0.48	126	0.74 (0.55-0.99)	0.05	
		AA	84	1.01 (0.74-1.37)	0.95	45	1.17 (0.76-1.80)	0.47	39	0.84 (0.55-1.27)	0.44	0.13
		GA + AA vs *GG*	395	0.94 (0.78-1.14)	0.53	230	1.12 (0.86-1.44)	0.41	165	0.76 (0.58-1.01)	0.05	
PRDM2 G→A	rs2235515	*GG*	377	1.00	210	1.00	167	1.00				
		GA	262	1.14 (0.93-1.38)	0.20	141	1.09 (0.83-1.43)	0.54	121	1.20 (0.90-1.60)	0.21	
		AA	38	1.09 (0.72-1.66)	0.67	24	1.02 (0.60-1.75)	0.93	14	1.20 (0.63-2.29)	0.58	0.85
		GA + AA vs *GG*	300	1.13 (0.94-1.37)	0.20	165	1.08 (0.83-1.40)	0.57	135	1.20 (0.91-1.58)	0.20	

Abbreviation: RR, incidence rate ratio.
*Number of cases; subgroups of ≤10 cases were considered too small for precise estimates and were therefore omitted from the table.
†Adjusted for age and sex.
‡Adjusted for age.

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Table 3. Frequency of colorectal cancers harboring CIMP and overlap with *MLH1* promoter hypermethylation and microsatellite instability

	Frequency of molecular phenotype, *n* (%)	CIMP		*P**
		CIMP+	CIMP–	
CIMP				
CIMP+	167 (27.7)	—	—	—
CIMP–	436 (72.3)	—	—	
MLH1 promoter methylation				
Methylated	152 (22.1)	68 (41.5)	59 (14.0)	<0.001
Unmethylated	534 (77.9)	96 (58.5)	363 (86.0)	
MSI				
MSI	84 (12.7)	52 (33.3)	17 (4.2)	<0.001
MSS	578 (87.3)	104 (67.7)	390 (95.8)	

Abbreviations: CIMP+; three or more of five CIMP markers methylated; CIMP-, zero to two of five CIMP markers methylated; MSI, microsatellite instability; MSS, Microsatellite Stable tumors.
*χ^2 Test.

may exist between related molecular phenotypes (41). Because two single-nucleotide polymorphisms were determined for *MTHFR* and *DNMT3b*, we estimated incidence rate ratios for combinations of genotypes within these genes. In addition, incidence rate ratios for combinations of genotypes per functional group (that is, based on the number of rare alleles in any of the folate-metabolizing enzymes *MTHFR, MTR*, and *MTRR*, or in any of the histone methyltransferases *EHMT1, EHMT2*, and *PRDM2*) or the combination of all the studied genes were estimated. SEs of the incidence rate ratios were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort (42). The proportional hazards assumption was tested using the scaled Schoenfeld residuals (43). Multivariate-adjusted analyses were additionally conducted, including the covariates dietary folate, vitamin B2, vitamin B6, methionine, alcohol, energy intake, family history of colorectal cancer, smoking behavior, and body mass index. Interactions with sex were tested for each of the single-nucleotide polymorphisms. Statistical analyses were done with the Stata statistical software package (version 10).

Results

The overall and gender-specific prevalences of genotypes were calculated and compared between subcohort members and colorectal cancer cases. We observed that the rare *MTHFR 677TT* variant occurred more often in subcohort members than in cases and that this difference was present among men (prevalences in subcohort and cases were 9.7% and 5.0%, respectively). Prevalences of other genotypes did not significantly differ between subcohort members and cases (Table 1). The distribution of the *MTHFR A1298C, MTRR A66G,* and *DNMT3b C→T* (rs406193) genotypes deviated from the Hardy-Weinberg equilibrium ($P < 0.05$). However, mean age was similar between genotypes of any of the single-nucleotide polymorphisms, suggesting that there was no survivorship effect due to genetic status.

Overall, subjects with the heterozygous *MTHFR 677CT* genotype were at modestly increased colorectal cancer risk compared with participants with the common *CC* genotype (incidence rate ratio, 1.23; $P = 0.04$; Table 2). Similarly, positive associations existed among women with this genotype (incidence rate ratio, 1.43; $P = 0.02$) and for the occurrence of the rare *T* allele (that is, the combination of the *CT* and *TT* genotypes compared with *CC*; incidence rate ratio, 1.39; $P = 0.02$). Conversely, we observed that men homozygous for the *MTHFR 677TT* genotype were at reduced colorectal cancer risk (incidence rate ratio, 0.49; $P = 0.01$). There was a significant interaction between sex and *MTHFR C677T* genotypes ($P = 0.03$). Whereas *MTHFR A1298C* was not associated with colorectal cancer, we observed that the *MTR 2756GG* genotype was associated with increased risk for colorectal cancer (incidence rate ratio, 1.58; $P = 0.04$), particularly among women (incidence rate ratio, 1.76; $P = 0.06$). Among men, the *MTR 2756GG* genotype was associated with a nonsignificant increase of colorectal cancer risk (incidence rate ratio, 1.45; $P = 0.23$). *MTRR A66G* genotypes were not associated with colorectal cancer.

Among women, compared with common homozygotes, the rare alleles of *DNMT3b C→T* (rs406193) and *EHMT2 G→A* (rs535586) were associated with decreased colorectal cancer risk [incidence rate ratio, 0.72 ($P = 0.04$) and 0.76 ($P = 0.05$), respectively], but such associations were not observed among men. No associations were observed between individual genotypes of the remaining genes and overall colorectal cancer risk. Combining *MTHFR* and *DNMT3b* genotypes, genotypes

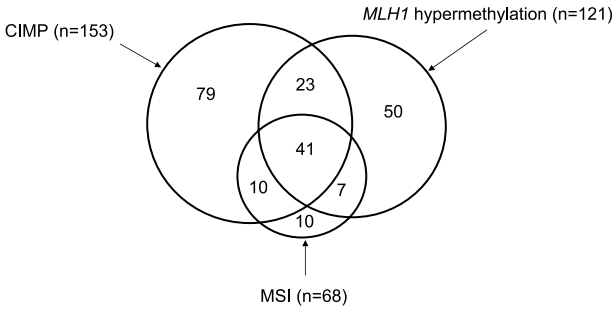

Figure 2. Overlap between CIMP, *MLH1* hypermethylation, and microsatellite instability in colorectal cancers, showing at least one of these three aberrations. In total, there were $n = 271$ colorectal cancers harboring at least one aberration. Numbers are based on colorectal cancers with complete analyses of all three molecular characteristics ($n = 547$ colorectal cancers). The sizes of the different areas in this figure may not exactly reflect the numbers of the applicable subsets.

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per functional group, or genotypes of all studied genes did not reveal any clear associations with colorectal cancer. Multivariate-adjusted analyses resulted in similar findings compared with age-adjusted analyses (data not shown).

In total, 167 (27.7%) of the 603 colorectal cancers with available CIMP analyses showed the CIMP phenotype (Table 3; Fig. 2), with no significant difference between men and women (data not shown). As reported previously for *MLH1* hypermethylation, microsatellite instability, and *BRAF* mutations (41), the overlap between CIMP, *MLH1* hypermethylation, and microsatellite instability was also incomplete. Although statistically significant ($P_{\chi 2} < 0.001$), 41.5% of the colorectal cancers with CIMP showed *MLH1* methylation, whereas only 33.3% harbored microsatellite instability.

MTHFR C677T polymorphisms were not associated with any of the methylation endpoints among men (Table 4). However, we observed that women carrying the rare T allele of *MTHFR C677T* were at significantly increased risk for developing a tumor without CIMP (CIMP-; incidence rate ratio, 1.40; $P = 0.04$), without *MLH1* hypermethylation (incidence rate ratio, 1.49; $P = 0.01$), or without microsatellite instability (incidence rate ratio, 1.36; $P = 0.04$) but also those with CIMP (CIMP+; incidence rate ratio, 1.65; $P = 0.04$; Table 5). Among men, the rare *C* allele of the *MTHFR A1298C* polymorphism was inversely associated with colorectal cancers without CIMP (incidence rate ratio, 0.72; $P = 0.03$). Inverse associations were also observed between *MTR A2756G* and CIMP+ among men (incidence rate ratio, 0.58; $P = 0.04$) and between *MTRR A66G* and *MLH1* hypermethylation among women (incidence rate ratio, 0.55; $P = 0.02$).

Among women, the rare *T* allele of *DNMT3b C→T* (rs406193) was associated with decreased risks for colorectal cancers without CIMP (incidence rate ratio, 0.67; $P = 0.04$), colorectal cancers without microsatellite instability (incidence rate ratio, 0.70; $P = 0.04$), and a nonsignificant decreased risk for colorectal cancers without *MLH1* hypermethylation (incidence rate ratio, 0.73; $P = 0.09$). However, although not statistically significant, incidence rate ratios were also decreased for colorectal cancers harboring CIMP or *MLH1* hypermethylation. Similarly, the rare A allele of *EHMT2 G→A* (rs535586) was inversely associated colorectal cancers without *MLH1* methylation (incidence rate ratio, 0.73), colorectal cancers without microsatellite instability (incidence rate ratio, 0.73), or nonsignificantly with colorectal cancers without CIMP (incidence rate ratio, 0.75) in women. Conversely, we observed a positive association (incidence rate ratio, 1.99) of *EHMT2 G→A* with *MLH1* hypermethylation among men.

Discussion

We studied associations between single-nucleotide polymorphisms in folate metabolizing enzymes, a DNA methyltransferase, and histone methyltransferases with colorectal cancer risk, accounting for related "methylation phenotypes" in a large prospective cohort study in the

Table 4. Associations of single-nucleotide polymorphisms in folate metabolizing enzymes and epigenetic regulators with colorectal cancer risk according to methylation status of the tumor among men

Gene and SNP	Genotype	CIMP				*MLH1* promoter hypermethylation				MSI			
		CIMP+		CIMP−		Methylated		Unmethylated		MSI		No MSI	
		*n**	RR (95% CI)†	*n*	RR (95% CI)†	*n*	RR (95% CI)†	*n*	RR (95% CI)†	*n*	RR (95% CI)†	*n*	RR (95% CI)†
MTHFR C677T (rs1801133)‡	*CC*	43	Ref.	103	Ref.	32	Ref.	143	Ref.	21	Ref.	145	Ref.
	CT + TT	40	0.81 (0.51-1.28)	135	1.14 (0.84-1.54)	41	1.11 (0.68-1.81)	153	0.93 (0.70-1.22)	20	0.83 (0.44-1.58)	166	0.99 (0.75-1.30)
MTHFR A1298C (rs1801131)	*AA*	30	Ref.	112	Ref.	32	Ref.	132	Ref.	16	Ref.	139	Ref.
	AC + CC	54	1.16 (0.72-1.87)	125	0.72 (0.53-0.97)	40	0.81 (0.49-1.32)	165	0.81 (0.61-1.07)	24	0.97 (0.50-1.88)	173	0.80 (0.61-1.06)
MTR A2756G (rs1805087)	*AA*	66	Ref.	153	Ref.	49	Ref.	199	Ref.	25	Ref.	209	Ref.
	AG + GG	19	0.58 (0.34-0.99)	87	1.14 (0.83-1.55)	24	0.98 (0.58-1.65)	100	1.01 (0.75-1.35)	16	1.31 (0.68-2.51)	107	1.02 (0.77-1.36)
MTRR A66G (rs1801394)	*AA*	10	Ref.	45	Ref.	12	Ref.	49	Ref.	5	Ref.	56	Ref.
	AG + GG	75	—	197	1.14 (0.79-1.66)	61	1.32 (0.69-2.52)	252	1.34 (0.93-1.92)	36	—	262	1.22 (0.87-1.72)
DNMT3b C→T (rs2424913)	*CC*	29	Ref.	81	Ref.	31	Ref.	95	Ref.	17	Ref.	101	Ref.
	CT + TT	56	0.92 (0.60-1.49)	161	0.95 (0.69-1.30)	42	0.65 (0.39-1.06)	205	1.03 (0.77-1.38)	24	0.66 (0.35-1.28)	216	1.03 (0.77-1.36)
DNMT3b C→T (rs406193)	*CC*	59	Ref.	182	Ref.	54	Ref.	223	Ref.	30	Ref.	239	Ref.
	CT + TT	26	1.49 (0.90-2.45)	60	1.11 (0.79-1.57)	19	1.19 (0.68-2.08)	78	1.18 (0.86-1.62)	11	1.25 (0.61-2.56)	79	1.12 (0.82-1.53)
EHMT1 G→A (rs4634736)	*GG*	74	Ref.	197	Ref.	57	Ref.	253	Ref.	36	Ref.	259	Ref.
	GA + AA	11	0.65 (0.34-1.26)	43	0.96 (0.65-1.40)	16	1.23 (0.69-2.22)	46	0.84 (0.58-1.21)	4	—	57	0.96 (0.68-1.36)
EHMT2 G→A (rs535586)	*GG*	34	Ref.	98	Ref.	20	Ref.	130	Ref.	15	Ref.	124	Ref.
	GA + AA	50	1.11 (0.69-1.76)	143	1.10 (0.81-1.48)	53	1.99 (1.16-3.41)	168	0.97 (0.73-1.28)	26	1.30 (0.67-2.51)	190	1.15 (0.88-1.52)
PRDM2 G→A (rs2235515)	*GG*	46	Ref.	126	Ref.	41	Ref.	160	Ref.	47	Ref.	166	Ref.
	GA + AA	36	1.07 (0.67-1.71)	109	1.19 (0.88-1.60)	31	1.04 (0.63-1.71)	130	1.11 (0.84-1.47)	34	0.73 (0.37-1.44)	140	1.16 (0.88-1.52)

Abbreviation: Ref., reference.
*Number of cases; subgroups of ≤10 cases were considered too small for precise estimates and were therefore omitted from the table.
†Age-adjusted incidence rate ratios (95% CI).
‡dbSNP number.

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Table 5. Associations of single-nucleotide polymorphisms in folate-metabolizing enzymes and epigenetic regulators with colorectal cancer risk according to methylation status of the tumor among women

Gene and SNP	Genotype	CIMP				*MLH1* promoter hypermethylation				MSI			
		CIMP+		CIMP−		Methylated		Unmethylated		MSI		No MSI	
		*n**	RR (95% CI)†	*n*	RR (95% CI)	*n*	RR (95% CI)	*n*	RR (95% CI)	*n*	RR (95% CI)	*n*	RR (95% CI)
MTHFR C677T (rs1801133)‡	*CC*	33	Ref.	82	Ref.	41	Ref.	94	Ref.	18	Ref.	115	Ref.
	CT+TT	47	1.65 (1.02-2.65)	100	1.40 (1.01-1.94)	37	1.03 (0.64-1.65)	121	1.49 (1.09-2.03)	23	1.49 (0.78-2.84)	136	1.36 (1.02-1.82)
MTHFR A1298C (rs1801131)	*AA*	34	Ref.	80	Ref.	32	Ref.	92	Ref.	22	Ref.	102	Ref.
	AC+CC	46	1.12 (0.70-1.80)	99	1.01 (0.73-1.41)	45	1.15 (0.71-1.85)	120	1.08 (0.79-1.48)	19	0.73 (0.38-1.39)	146	1.18 (0.87-1.59)
MTR A2756G (rs1805087)	*AA*	54	Ref.	115	Ref.	47	Ref.	137	Ref.	25	Ref.	156	Ref.
	AG+GG	26	0.96 (0.59-1.58)	70	1.22 (0.87-1.71)	31	1.32 (0.82-2.13)	60	1.17 (0.85-2.61)	16	1.28 (0.67-2.45)	98	1.26 (0.93-1.70)
MTRR A66G (rs1801394)	*AA*	16	Ref.	46	Ref.	24	Ref.	44	Ref.	11	Ref.	58	Ref.
	AG+GG	64	1.00 (0.56-1.79)	139	0.75 (0.51-1.11)	53	0.55 (0.33-0.92)	252	0.99 (0.68-1.46)	29	0.66 (0.32-1.37)	196	0.84 (0.59-1.20)
DNMT3b C→T (rs2424913)	*CC*	23	Ref.	66	Ref.	23	Ref.	80	Ref.	10	Ref.	92	Ref.
	CT+TT	57	1.24 (0.74-2.06)	121	0.91 (0.65-1.27)	55	1.18 (0.71-1.97)	141	0.88 (0.64-1.21)	31	—	165	0.89 (0.66-1.21)
DNMT3b C→T (rs406193)	*CC*	61	Ref.	145	Ref.	60	Ref.	167	Ref.	30	Ref.	196	Ref.
	CT+TT	19	0.74 (0.43-1.29)	40	0.67 (0.45-0.98)	17	0.69 (0.39-1.21)	51	0.73 (0.51-1.05)	10	—	57	0.70 (0.50-0.98)
EHMT1 G→A (rs4634736)	*GG*	69	Ref.	142	Ref.	62	Ref.	175	Ref.	37	Ref.	199	Ref.
	GA+AA	11	0.62 (0.32-1.21)	43	1.19 (0.81-1.75)	15	1.01 (0.56-1.82)	44	0.98 (0.67-1.44)	3	—	56	1.10 (0.78-1.57)
EHMT2 G→A (rs535586)	*GG*	35	Ref.	86	Ref.	35	Ref.	102	Ref.	15	Ref.	119	Ref.
	GA+AA	44	0.82 (0.51-1.32)	143	0.75 (0.54-1.04)	42	0.80 (0.50-1.28)	114	0.73 (0.54-1.00)	25	1.07 (0.56-2.07)	132	0.73 (0.54-0.98)
PRDM2 G→A (rs2235515)	*GG*	49	Ref.	99	Ref.	39	Ref.	122	Ref.	21	Ref.	137	Ref.
	GA+AA	31	0.94 (0.58-1.51)	109	1.22 (0.87-1.69)	37	1.41 (0.88-2.26)	91	1.11 (0.81-1.51)	20	1.41 (0.75-2.66)	111	1.20 (0.90-1.61)

*Number of cases; subgroups of ≤10 cases were considered too small for precise estimates and were therefore omitted from the table.
†Age-adjusted incidence rate ratios (95% CI).
‡dbSNP number.

Netherlands. We observed that *MTHFR C677T* may have opposite effects in men and women and that *MTR A2756G* potentially increases colorectal cancer risk. In addition, genetic variants of *DNMT3b* and *EHMT2* may reduce colorectal cancer risk among women. Rare variants of *MTR* and *MTRR* were inversely associated with CIMP in men and *MLH1* hypermethylation in women, respectively, possibly by lowering methyl group availability. Although methylation-associated characteristics were significantly related, the overlap between CIMP and *MLH1* hypermethylation or microsatellite instability was limited.

The *MTHFR C677T* and *A1298C* polymorphisms have previously been investigated. Rare variants of these polymorphisms were generally inversely associated with colorectal cancer risk (4, 5, 12, 44, 45) or with colorectal adenoma recurrence (46). Conversely, associations with increased colorectal cancer risk (47-49), colorectal adenoma risk (50), or increased risk for colorectal adenoma recurrence (51, 52) have been observed as well. Analyses were stratified by sex in some studies, and positive associations with colorectal cancer were reported among men for *MTHFR C677T* (47) and *A1298C* (48, 50). In addition, similar to our study, the rare *T* allele of the *MTHFR C677T* polymorphism has been observed to be associated with increased colorectal cancer risk among women but not among men (48). *MTHFR* polymorphisms may reduce enzymatic activity and thereby decrease the pool of 5-methyltetrahydrofolate. The resulting lower availability of methyl groups may lead to global DNA hypomethylation (53). It may also be associated with increased promoter hypermethylation in colorectal cancer (7), with CIMP (6), or with microsatellite instability (8, 54, 55), which is highly correlated with CIMP in colorectal cancer (36). Although these two opposite methylation patterns may develop independently of one another, global DNA hypomethylation and gene promoter hypermethylation are observed concurrently in colorectal cancer (56-58). However, it is currently unknown to what extent alterations in methyl group metabolism influence this imbalance in the distribution of cytosine methylation, and neither do we know to what extent this influences colorectal carcinogenesis. Moreover, methyl groups may have different targets, for example, DNA, RNA, or proteins (Fig. 1), and the relative contribution of methyl groups to either of these targets is unknown.

Although, in our study, *MTHFR C677T* tended to increase overall colorectal cancer risk among women, this effect could not be explained by a distinct positive association with CIMP only. Among men on the other hand, the *MTHFR 677TT* genotype was associated with reduced overall colorectal cancer risk, and the *C* allele of *MTHFR A1298C* was inversely associated with colorectal cancers without CIMP. An increased pool of 5,10-methylenene tetrahydrofolate by MTHFR polymorphisms may optimize DNA synthesis and lower the incorporation of uracil into DNA (59). However, this may have dual consequences for carcinogenesis because increased synthesis and stability of DNA possibly protects against carcinogenesis but may also increase cell proliferation in tumors. Nonetheless, we hypothesize that MTHFR may act as a switch being able to shift the balance between DNA methylation

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and DNA synthesis (Fig. 1), both of which may have distinct consequences for carcinogenesis. Our observations suggest that *MTHFR* polymorphisms, albeit in different directions, may influence colorectal cancer risk in men and women but that a change in promoter hypermethylation, as measured by CIMP or *MLH1* hypermethylation, may not be the primary contributor to carcinogenesis in these individuals.

Among subjects in our study population, the *MTR 2756GG* genotype was associated with increased colorectal cancer risk. Genetic variants of *MTR* were not associated with colorectal cancer in a number of previous studies (12, 60, 61). Modest risk reductions among *MTR 2756GG* homozygotes were found in one colorectal adenoma study (9) and in one colorectal cancer study (10), whereas also nonsignificant increased risks were observed between this single-nucleotide polymorphism and colorectal adenoma (11) or colorectal cancer (13). Rare variants of *MTR* were associated with lower plasma homocysteine concentrations (62), suggesting lower catalytic activity of the MTR enzyme and lower availability of methyl groups. Interestingly, we observed that the *MTR A2756G* single-nucleotide polymorphism was associated with reduced risk for colorectal cancers with CIMP among men.

It was previously observed that rare variants of the *MTRR A66G* single-nucleotide polymorphism may be inversely associated with colorectal adenoma recurrence (46) but with increased colorectal cancer risk (13). In addition, other single-nucleotide polymorphisms in *MTRR* were associated with an increased risk for colorectal adenoma (11) and colorectal cancer (12). We did not observe an association with overall colorectal cancer risk in our study, but the *G* allele of the *MTRR A66G* polymorphism was associated with reduced risk for colorectal cancers with *MLH1* hypermethylation among women. The *MTRR A66G* single-nucleotide polymorphism may also be associated with reduced catalytic activity of the MTRR enzyme (63). Our data suggest that this possibly results in decreased promoter hypermethylation in colorectal cancer similar to *MTR A2756G*, which is involved in the same metabolic conversion in the one-carbon metabolism.

The CIMP phenotype has not uniquely been defined because various sets of promoters have previously been used with different cutoff values (64). To investigate an optimal set of markers for CIMP, Weisenberger et al. (36) screened 195 CpG island methylation markers and proposed a robust new panel of five markers to define CIMP in colorectal cancer. This new set has been validated successfully in a large group of incident colorectal cancer patients (65), and we have used these new markers in our study. However, we have shown that the overlap between CIMP and *MLH1* or microsatellite instability may be incomplete. It has also been suggested that CIMP may consist of three molecular subtypes based on presence or absence of microsatellite instability, *BRAF, KRAS*, or *p53* mutations (66), but these characteristics are not mutually exclusive. In addition, we previously observed incomplete overlap between *MLH1* hypermethylation or expression, microsatellite instability, and *BRAF* mutations (41). The current study suggests that lower enzymatic activity of folate metabolizing enzymes *MTR* and *MTRR* is inversely associated with either CIMP or *MLH1* hypermethylation. In view of these observations, it remains important to investigate associations with CIMP and other methylation endpoints separately.

The *DNMT3b C→T* (rs2424913) single-nucleotide polymorphism was not associated with colorectal cancer risk. However, an increased risk for colorectal cancer could be expected because this polymorphism increases enzymatic activity of DNMT3b (21), and DNMT3b overexpression was associated with increased promoter hypermethylation in different types of cancers (15-17, 19, 20). Moreover, positive associations were observed with colorectal polyps and early onset of colorectal cancer in hereditary nonpolyposis colorectal cancer patients (23, 25) and several other cancers (21, 22, 24). Other genetic variants of *DNMT3b* that have been identified are the *-283T→C* and *-579G→T* polymorphisms, of which the latter was associated with reduced colorectal cancer risk (67). In addition, lung cancer risk was reduced in individuals carrying rare alleles of each of these single-nucleotide polymorphisms (68), which was contrary to the increased lung cancer risk associated with *DNMT3b C→T* (rs2424913) observed by Shen et al. (21). Interestingly, enzymatic activity of DNMT3b was decreased when the rare allele of *-283T→C* was present (68). Apparently, different polymorphisms in *DNMT3b* may have opposite effects and individuals may be more susceptible to carcinogenesis with higher DNMT3b enzymatic activity (21-23, 25), whereas decreased activity may protect against carcinogenesis (67, 68). Whereas a similar but weak inverse association was previously observed with breast cancer for the *DNMT3b C→T* (rs406193) polymorphism (30), we observed an inverse association with colorectal cancer among women. However, this protective effect was not substantially different between colorectal cancers with or without CIMP, *MLH1* hypermethylation, or microsatellite instability.

For histone methyltransferases, we observed that the rare A allele of *EHMT2 G→A* (rs535586) was inversely associated with colorectal cancer among women. A significant positive association was observed with breast cancer, although the incidence rate ratio was small for that particular polymorphism (30). These observations seem contradictive but may nonetheless suggest that this *EHMT2* polymorphism affects carcinogenesis of different cancer types. Importantly, it is currently unknown to which extent single-nucleotide polymorphisms in histone methyltransferases such as *EHMT2* lead to altered enzymatic activity. Obviously, the exact role of *EHMT2* in colorectal carcinogenesis needs further investigation.

This is the first prospective cohort study reporting associations between genetic variants of DNA methyltransferases and histone methyltransferases with colorectal cancer risk, suggesting the importance of such epigenetic regulators in the carcinogenesis of colorectal cancer. The analyses were stratified for gender and molecular phenotypes of the tumor. Therefore, several tests were done for each single-nucleotide polymorphism, which may have increased the danger of reporting chance findings. It could also be argued that some of these subgroups should preferably have been larger. For these reasons, caution must be taken in drawing definite conclusions, and the findings should be replicated in future research.

In conclusion, genetic variants of methyl metabolism enzymes and/or epigenetic regulators may affect colorectal carcinogenesis, and our observations suggest that reduced enzymatic activity of some folate metabolizing enzymes may result in decreased gene promoter hypermethylation in colorectal cancer. Hence, it would

be interesting to study whether methyl donor intake affects promoter hypermethylation and the potential modifying effect of polymorphisms in folate metabolizing enzymes, DNA methyltransferases, and histone methyltransferases. We observed that the overlap of CpG island hypermethylation markers and associated characteristics may be incomplete, indicating that these characteristics should be considered separately when studying potential causes of a methylation phenotype in colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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