MTHFR, *MTR*, and *MTRR* Polymorphisms in Relation to *p16INK4A* Hypermethylation in Mucosa of Patients with Colorectal Cancer

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We recently analyzed the hypermethylation status of the p16INK4a (p16) gene promoter in normal-appearing mucosa obtained from patients with colorectal cancer. Hypermethylation of *p16* was associated with reduced survival of these patients. In the present study, germ line polymorphisms in the folate- and methyl-associated genes, *methylenetetrahydrofolate reductase* (*MTHFR*), *methionine synthase* (*MTR*) and *methionine synthase reductase* (*MTRR*), were analyzed in the same patient cohort to find a possible link between these genetic variants and *p16* hypermethylation. Genomic DNA was extracted from blood of patients (n = 181) and controls (n = 300). Genotype analyses were run on an ABI PRISM® 7900HT sequence-detection system (Applied Biosystems), using real-time polymerase chain reaction and TaqMan chemistry. The results showed that the genotype distributions of the patient and control groups were similar. No significant differences in cancer-specific or disease-free survival of stage I–III patients according to polymorphic variants were detected, nor were any differences in cancer-specific or disease-free survival detected when patients were subgrouped according to the *MTHFR* or *MTR* genotype groups and dichotomized by *p16* hypermethylation status in mucosa. However, patients with the *MTRR* 66 AA/AG genotypes were found to have a significantly worse cancer-specific survival when the mucosa were positive, compared with negative, for *p16* hypermethylation (hazard ratio 2.7; 95% confidence interval 1.2–6.4; $P = 0.023$). In contrast, there was no difference in survival among patients with the *MTRR* 66 GG genotype stratified by *p16* hypermethylation status. These results indicate a relationship between genetic germ-line variants of the *MTRR* gene and *p16* hypermethylation in mucosa, which may affect the clinical outcome of patients with colorectal cancer.
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INTRODUCTION

The tumor-suppressor gene *p16* codes for a cyclin-dependent kinase inhibitor, p16, that acts like a negative regulator of cell growth and proliferation in the G1 phase of the cell cycle (1). In addition, recent research has implicated p16 as an important regulator of the angiogenic switch (2). In a previous study (3), we analyzed the hypermethylation status of the *p16* gene promoter in macroscopically normal-appearing mucosa derived from 181 patients with colorectal cancer (CRC). The result showed that hypermethylation of *p16* was associated with reduced survival of the patients. In mucosa samples without hypermethylation of *p16* there was a strong correlation between gene expression of reduced folate carrier 1 and folylpolyglutamate synthase (FPGS, EC 6.3.2.17), possibly indicating a balance between uptake and retention of folates. No such correlation was found in mucosa samples displaying *p16* hypermethylation.

Within the cells, folate polyglutamates are converted to 5,10-methylenetetrahydrofolate (methylene-THF), which is required as a methyl donor in the synthesis of dTMP (2′-deoxythymidine-5′-monophosphate) from dUMP (2′-deoxyuridine-5′-monophosphate) (Figure 1). Methylene-THF is also the precursor of metabolically active 5-methyltetrahydrofolate (methyl-THF), which is used in the remethylation of the sulfur-containing amino acid homocysteine to methionine. Endogenous methionine is then catabolized to produce the universal methyl donor S-adenosylmethionine (SAM). The conversion of methylene-THF to methyl-THF is dependent on the enzyme methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20). It is known that the MTHFR variant C677T causes reduced enzyme activity that impairs the folate metabolism, leading to genomic DNA hypomethylation (4) concomitant with gene-specific promoter hypermethylation (5), as is frequently observed in cancer. In addition, reduced MTHFR activity leads to increased plasma levels of

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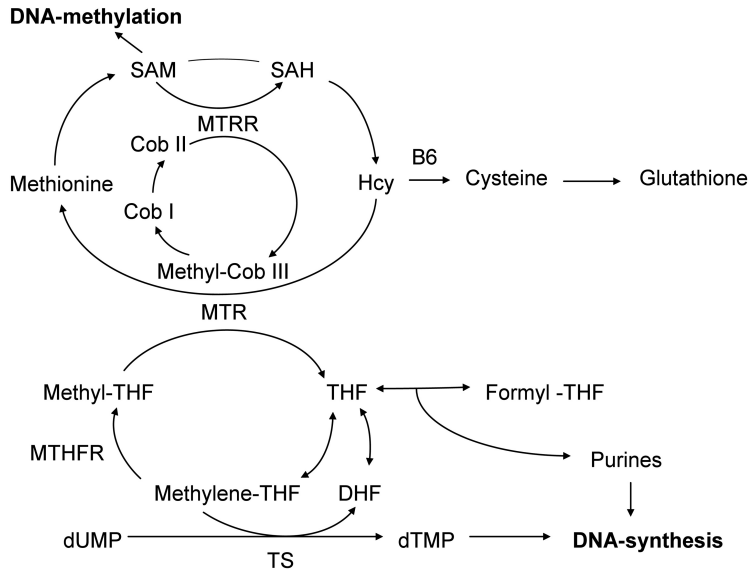

Figure 1. Overview showing the action of the MTHFR, MTR and MTRR enzymes in the folate and methionine metabolism pathways. TS, thymidylate synthase; DHF, dihydrofolate; Formyl-THF, 10-formyltetrahydrofolate; Cob I, cob(I)alamin; Cob II, cob(II)alamin; Methyl-Cob III, methyl cob(III)alamin; SAH, S-adenosylhomocysteine; Hcy, homocysteine; B6, vitamin B6.

homocysteine. Increased levels of homocysteine may promote inflammatory processes by generating reactive oxygen species (ROS) (6). Hence, the metabolite has a crucial role in cellular oxidative stress. Homocysteine may also affect hypo- and hypermethylation of DNA (7). Several case-control studies have shown that the *MTHFR* C677T polymorphism is protective against CRC (8–10), especially among individuals with a folate/methyl-sufficient diet (11,12). However, contradictive results have been found (13–16). Evaluation of the *MTHFR* C677T polymorphism as a predictive marker for CRC patients treated with 5-fluorouracil (5-FU)-based chemotherapy points to a possible link between the *MTHFR* 677 T allele and tumor response (10,17–19). Studies have also shown that the polymorphism might be associated with 5-FU–related cytotoxicity (19,20).

The *MTR* A2756G polymorphism leads to replacement of aspartic acid with glycine in the protein-binding region of methionine synthase (MTR; EC 2.1.1.13) (21). This substitution leads to a less effective enzyme that induces modest reduction of homocysteine (22,23). Paz *et al*. (24) analyzed the association of MTHFR and MTR genetic variants in colorectal, breast and lung cancer patients with 5-methylcytosine content in the genome of the tumors and their normal counterparts, as well as the presence of CpG-island hypermethylation in tumor-suppressor genes, including *p16*. Carriers of the *MTHFR* 677 T allele showed constitutive low levels of 5-methylcytosine in their genomes, and tumors in these patients did not achieve severe degrees of global hypomethylation. Tumors occurring in homozygous carriers of the *MTR* 2756 G allele showed a low number of hypermethylated CpG islands of tumor-suppressor genes. Kang *et al*. (25) recently showed that in uterine cervical cancer patients with the heterozygous genotypes of *MTHFR* and *MTR*, the average number of methylated genes was lower than in patients with the common homozygous genotypes. Occasionally, MTR loses its activity through oxidation of the cofactor cob(I)alamin to cob(II)alamin. The methionine synthase reductase (MTRR; EC 2.1.1.135) enzyme then reactivates MTR in a reaction in which methyl(III)cobalamin is generated (26). The *MTRR* A66G polymorphism leads to a substitution of isoleucine with methionine at codon 22 in the MTRR enzyme (27), resulting in a variant protein exhibiting a 4-fold lower activity compared with the wild-type protein *in vivo* (28). Thus, the *MTRR* 66 G allele should decrease the availability of SAM by reducing the level of active MTR. As a result, DNA hypomethylation may be induced. The *MTR* 2756 GG genotype has been associated with an increased risk of CRC (29), whereas a reduced risk of colorectal adenoma recurrence has been observed in patients heterozygous for the *MTRR* A66G polymorphism (30). The effects of the *MTR* A2756G and *MTRR* A66G variants seem to be influenced by folate intake (9,31).

The relationship between genetic polymorphisms, folate and the *p16* gene has been studied by a few research groups. Kraunz *et al*. (32) found that low dietary intake of folate was associated with *p16* methylation in tumors of head and neck squamous cell carcinoma patients, and that this relationship was modified by the *MTHFR* genotype. Kamiya *et al*. (33) examined the association between the *MTHFR* polymorphism and expression of *p16*. Their results suggested that folate metabolism can affect carcinogenesis through the expression of *p16*. The aim of the present study was to analyze the association of single-nucleotide polymorphisms (SNPs) in the folate- and methyl-associated genes *MTHFR*, *MTR* and *MTRR* to the *p16* promoter hypermethylation status in colorectal mucosa of patients with nonhereditary CRC. The aim was also to estimate the value of the interaction of p16 promoter methylation with the SNPs when predicting clinical outcome.

MATERIALS AND METHODS

Patients and Study Design

Blood and surgically resected specimens from normal-appearing mucosa were initially obtained from 181 patients with nonhereditary colorectal adenocarcinoma diagnosed at the Sahlgrenska

University Hospital/Östra during the period between 1994 and 2004. However, because of noninformative genotyping results, 1 patient was omitted from the study, giving a total of 180 patients. The ethics committee of Göteborg University approved the study and informed consent was obtained from each of the patients. Tissue samples were obtained by surgical resection from areas approximately 10 cm from the primary tumors. All samples were snap frozen in liquid nitrogen immediately after surgical excision and stored at –70°C until use. Surgical records were reviewed for patient sex and age, and pathological records were reviewed for tumor location, differentiation and stage. The mean age of the patients was 69 ± 14 years: 70 ± 15 years for the women and 68 ± 12 years for the men. The primary tumors were graded histopathologically by experienced pathologists as recommended by the World Health Organization (34). Of the primary carcinomas, 4 were highly, 114 were moderately and 62 were poorly differentiated; 70 tumors were of rectal origin and 110 were colonic; 63 of the colonic tumors were right-sided and 47 were left-sided. Malignant tumors were classified according to the TNM (tumor-node-metastasis) staging system (35) into the pathological stages TNM I (n = 10), TNM II (n = 71), TNM III (n = 51) and TNM IV (n = 48). Twenty-nine of the patients underwent presurgical radiotherapy, 26 patients were treated with adjuvant chemotherapy after surgery and 29 underwent palliative treatment. The vast majority of the patients (167 of 180) were subjected to elective surgery whereas 10 were subjected to acute surgery (no information was available for 3 of the patients). The follow-up time of the study was 5 years.

Blood Donors

The control group (n = 300) consisted of white, Swedish blood donors of both sexes from the same geographic area as the study patients. All samples were collected at random and informed consent was obtained from each of the donors.

Determination of *p16* Hypermethylation Status

The hypermethylation status of *p16* was determined using real-time polymerase chain reaction (PCR) as described previously (3).

Genotyping of the *MTHFR*, *MTR* and *MTRR* Genes

Genomic DNA was extracted from fresh-frozen blood using a QIAamp DNA Mini Kit according to the manufacturer instructions (Qiagen, Hilden, Germany). Genotype analyses of the genes *MTHFR* (rs1801133, assay no. C_8714009_10), *MTR* (rs1805087, assay no. C_12005959_10) and *MTRR* (rs1801394, assay no. C_3068176_10) were run on an ABI PRISM® 7900HT sequence-detection system (Applied Biosystems, Foster City, CA, USA) using real-time PCR and TaqMan chemistry. The SNP assays (Applied Biosystems) and the TaqMan PCR master mix (Applied Biosystems) were aliquoted into a 384-well plate using a liquid-handling Biomek FX robot (Beckman Coulter, San Diego, CA, USA). Reactions were characterized by comparing the threshold cycle (C_T) values as described by the manufacturer. All genotype analyses were performed by the Göteborg Genomics Core Facility (Göteborg, Sweden).

Statistics

For the statistical analyses, the clinicopathological parameters were categorized as follows: tumor location as right-sided colon, left-sided colon or rectum; tumor stages as TNM stages I–IV; tumor differentiation grade as low/medium or high; patient treatment with adjuvant chemotherapy as yes or no. Cancer-specific survival time was calculated from the date of surgery to the last time of follow-up or to the date of death caused by cancer. Disease-free survival time was calculated from the date of surgery to the last time of follow-up or to the date of recurrence. The statistical significance of the difference in the survival groups was calculated by using the Cox proportional hazard model. Data were analyzed by statistical modeling using the commercial software programs JMP (version 7.0; SAS, Cary, NC, USA) or SPSS (version 16.0) (Chicago, IL, USA). Statistical differences between groups were tested by using the Fisher exact test or the Pearson chi-square test as indicated in the tables. Statistical values of $P \leq 0.05$ were judged as significant.

RESULTS

Distribution of Genotypes in CRC Patients and Healthy Controls

To investigate whether the *MTHFR* C677T, *MTR* A2756G and *MTRR* A66G polymorphisms were predictive for CRC, we compared the genotype frequencies of patients with those of 300 healthy, white blood donors (Table 1). As shown in Table 1, the genotype distributions of the patient group were not significantly different from those of the control group. Furthermore, none of the clinical or pathological parameters (sex, age, tumor location, tumor differentiation grade or tumor stage) were associated with genotypes (data not shown). The *MTHFR* C677T, *MTR* A2756G and *MTRR* A66G genotype distributions were in Hardy-Weinberg equilibrium in both patients and controls.

Association of *p16* Hypermethylation with the *MTHFR*, *MTR* and *MTRR* Genotypes

Table 2 shows the result of the *MTHFR* C677T, *MTR* A2756G and *MTRR* A66G analyses for CRC patients dichotomized by *p16* hypermethylation status in mucosa. As shown, the *MTHFR* 677 TT homozygous genotype was more common among patients with hypermethylated *p16* compared with patients without hypermethylation, 10 of 62 (16.1%) and 8 of 113 (7.1%), respectively, but the difference did not reach significance. No differences were found when the *MTR* and *MTRR* genotype distributions were dichotomized according to p16 hypermethylation status.

Table 1. Distribution of *MTHFR*, *MTR* and *MTRR* genotypes in CRC patients and controls.

Genotype	CRC patients, n (%)[a]	Controls, n (%)[b]	P^c
MTHFR 677			
CC	81 (46.3)	167 (55.9)	
CT	76 (43.4)	107 (35.8)	
TT	18 (10.3)	25 (8.4)	0.13
MTR 2756			
AA	112 (63.6)	193 (64.8)	
AG	55 (31.3)	92 (30.9)	
GG	9 (5.1)	13 (4.3)	0.92
MTRR 66			
AA	22 (12.4)	50 (16.7)	
AG	94 (53.1)	152 (50.8)	
GG	61 (34.5)	97 (32.4)	0.45

[a]Of 180 patient samples, 175, 176 and 177 were informative for the *MTHFR*, *MTR*, and *MTRR* polymorphisms, respectively.
[b]Of 300 control samples, 299, 298 and 299 were informative for the *MTHFR*, *MTR* and *MTRR* polymorphisms, respectively.
[c]*P* by Pearson chi-square test.

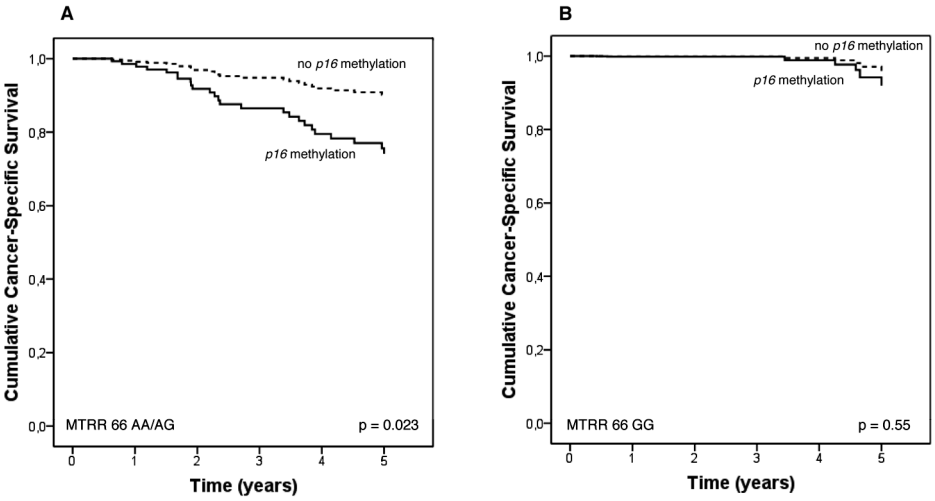

Figure 2. Multivariate Cox model showing cancer-specific survival of stage I–III patients subgrouped according to *MTRR* genotypes and dichotomized *p16* hypermethylation status in mucosa. (A) As shown, patients with the *MTRR* 66 AA/AG genotypes had a significantly worse survival ($P = 0.023$) when the mucosa was positive for *p16* hypermethylation (n = 59) compared with when the mucosa was negative for *p16* hypermethylation (n = 30). (B) In contrast, there was no difference in survival among patients with the *MTRR* 66 GG genotype stratified by p16 hypermethylation status ($P = .55$; n = 24 and n = 16 in respective groups).

MTHFR, *MTR* and *MTRR* Genotypes and Survival of Stage I–III Patients Dichotomized by *p16* Hypermethylation in Mucosa

The clinical follow-up time of the patients was extended in the present study compared with the previous one, and at the time of this report all patients had been followed for at least 5 years. Because of this, the survival data were reanalyzed to examine whether *p16* hypermethylation in mucosa was still a risk factor for cancer-specific and disease-free survival of patients with stage I–III disease. As before, we used a multivariate Cox regression analysis model adjusted for known risk factors such as sex, age, tumor stage, tumor differentiation grade and tumor location. The model was also adjusted for whether or not the patients had been treated with radiotherapy prior to surgery and/or adjuvant chemotherapy after surgery. The results showed that the *p16* hypermethylation status was prognostic (hazard ratio [HR] 2.6, 95% confidence interval [CI] 1.2–5.7; $P = 0.013$] and HR 2.2, 95% CI 1.1–4.4; $P = 0.033$) for cancer-specific and disease-free survival, respectively.

Because of the low representation of individuals having the *MTHFR* 677 TT, *MTR* 2756 GG and *MTRR* 66 AA homozygous genotypes, each of these groups was pooled with the corresponding group of heterozygotes before survival analysis. No significant differences in cancer-specific or disease-free survival of stage I–III patients associated with the *MTHFR*, *MTR* and *MTRR* genotype variants could be detected. Nor were any differences in cancer-specific or disease-free survival detected when patients were subgrouped according to the *MTHFR* or *MTR* genotype groups and dichotomized by *p16* hypermethylation status in mucosa. However, patients with the *MTRR* 66 AA/AG genotype had a significantly worse cancer-specific survival (HR 2.7, 95% CI 1.2–6.4; $P = 0.023$) (Figure 2A) and disease-free survival (HR 2.3, 95% CI 1.0–5.0; $P = 0.044$) when the *p16* gene was hypermethylated in mucosa. As shown in Table 3, there was no difference in the distribution of patients having the *MTRR* AA/AG genotypes according to tumor stage, tumor differentiation grade or therapy conditions after dichotomization by *p16* hypermethylation status. No differences in cancer-specific (Figure 2B) or disease-free survival according to *p16* hypermethylation status were observed among patients having the *MTRR* 66 GG genotype.

DISCUSSION

The major mechanism of *p16* gene inactivation associated with development of CRC seems to be promoter-region methylation (36). Recently, Belshaw *et al*. (37) showed that multinomial logistic regression models based on the CpG island methylation profiles from normal mucosa could be used to distinguish cancer patients from noncancer patients and that p16 was one of the most informative variables. As previously reported by us (3), the hypermethylation status of the *p16* gene promoter in mucosa obtained 10 cm from the tumor site was associated with outcome of the CRC patients. In the present study, the same patient cohort was analyzed regarding the possible link between genetic germ-line polymorphisms

Table 2. Distribution of *MTHFR*, *MTR* and *MTRR* genotypes in the CRC patients dichotomized by *p16* hypermethylation status in mucosa.

Genotype	*p16* Hypermethylation status		
	Negative, n (%)	Positive, n (%)	P^a
MTHFR 677			
CC	56 (49.6)	25 (40.3)	
CT	49 (43.4)	27 (43.6)	
TT	8 (7.0)	10 (16.1)	0.14
MTR 2756			
AA	66 (59.5)	46 (70.8)	
AG	39 (35.1)	16 (24.6)	
GG	6 (5.4)	3 (4.6)	0.31
MTRR 66			
AA	15 (13.3)	7 (10.9)	
AG	58 (51.3)	36 (56.3)	
GG	40 (35.4)	21 (32.8)	0.80

[a]*P* by Pearson chi-square test.

Table 3. Distribution of patients having the *MTRR* 66 AA/AG genotypes according to tumor stage, tumor differentiation grade, and therapy conditions, after dichotomization by p16 hypermethylation status

	No p16 hypermethylation, n (%)	p16 Hypermethylation, n (%)	P^a
Tumor stage			
I + II	35 (59)	19 (63)	
III	24 (41)	11 (37)	0.82
Tumor differentiation grade			
Low/medium	40 (68)	20 (67)	
High	19 (32)	10 (33)	1.0
Adjuvant chemotherapy			
No	47 (80)	26 (87)	
Yes	12 (20)	4 (13)	0.56
Presurgical radiotherapy			
No	49 (83)	25 (83)	
Yes	10 (17)	5 (17)	1.0

[a]*P* by Fisher exact test.

of the genes *MTHFR*, *MTR* and *MTRR* and the hypermethylation status of *p16*.

MTHFR, MTR and MTRR are important enzymes in the folate and methyl pathways and each enzyme has polymorphic variants that might be associated with risk of CRC. However, the data showed that the genotype distributions of these genes did not differ significantly between patient and control groups, indicating that none of the genetic variants had influenced the cancer susceptibility. There appeared to be no link between any of the polymorphic variants and the *p16* methylation status, because no significant differences according to genotype distributions in patients subgrouped by *p16* hypermethylation status could be found. Because the clinical follow-up time of the patients was extended in the present study compared with the previous one, we reanalyzed the survival data. As before, *p16* hypermethylation was found to be an independent prognostic marker for cancer-specific survival. Analysis of the whole group of stage I–III patients showed that none of the genetic variants *MTHFR* C677T, *MTR* A2756G or *MTRR* A66G were related to cancer-specific or disease-free survival. However, patients with the *MTRR* 66 AA/AG genotype had a significantly worse cancer-specific survival if the *p16* gene was hypermethylated in mucosa. In contrast, there was no difference in survival related to *p16* hypermethylation among patients with the *MTRR* 66 GG genotype.

The reason for the particularly unfavorable outcome of patients having the combination of the *MTRR* 66 AA/AG genotype and *p16* hypermethylation in mucosa might be linked to a state of chronic inflammation and oxidative stress. Chen *et al*. found that a number of inflammation markers were altered in mucosa of patients with CRC compared with controls (38). These alterations could be found in mucosa several centimeters from the tumor site. It is known that a long-standing chronic inflammation in the colorectal epithelium will lead to oxidative stress (39), a condition considered to be one of the major sources of carcinogenesis. Growing evidence supports a role of ROS-induced generation of oxidative stress in epigenetic changes such as hypo- and hypermethylation (40). Interestingly, inactivation of *p16* by homozygous deletion or methylation at the promoter region seems to be a common molecular mechanism in oxidative stress–induced carcinogenesis (41,42). Romanenko *et al*. (43) presented data suggesting that oxidative stress–induced methylation and inactivation of *p16* by persistent chronic exposure to ionizing radiation could be one of the major pathways responsible for malignant progression of renal cell carcinomas. Likewise, the results of Belinsky *et al*. (44) supported a possible role for oxidative stress, inflammation and *p16* methylation in the etiology of human lung cancer. The link between *p16* and oxidative stress was also demonstrated by Govindarajan *et al*. (45), who analyzed the mechanisms behind the appearance of soft-tissue sarcomas at sites of foreign material implantations. Malignant fibrous histiocytomas arose in mice after implantation of nickel sulfide and all tumors showed hypermethylation of *p16*. The authors concluded that the oxidative stress that was induced caused loss of p16. Jang *et al*. (46) showed that nontumorous tissue adjacent to gastric cancers displayed a close association among the grade of chronic inflammation and *p16* gene promoter hypermethylation.

During the state of chronic inflammation, DNA damage and mutations are induced by increased production of ROS and electrophiles. Consequently, the need for folates used in DNA synthesis and repair will be increased. Because folates are very susceptible to oxidation, an increased oxidative degradation of folates may become relevant under oxidative

stress conditions. Thus, oxidative stress could represent an endogenous reason for folate deficiency, even when the dietary intake is within the recommended range (47). The relationship between promoter methylation of genes involved in colorectal carcinogenesis, including *p16*, and folate was recently investigated (48). The results showed that low folate/high alcohol intake was associated with increased gene promoter hypermethylation in CRC. In our previously published study (3), *FPGS* gene expression (used as a surrogate marker for folate) was shown to be lower in mucosa with *p16* hypermethylation, compared with mucosa without it. The difference did not reach significance ($P = 0.079$), however, possibly due to the limited number of samples that could be analyzed.

Folates are needed in the elimination of homocysteine via both the transmethylation and the transsulfuration pathways (49). Under physiological conditions, the strongest risk factor for hyperhomocysteinemia seems to be low serum folate levels (7). In cases of increased oxidative stress, however, the homocysteine level may not reflect folate deficiency but rather a deficiency in vitamin B_{12} (cobalamin) (50). Markedly-elevated concentrations of homocysteine have been found in colonic mucosa of inflammatory bowel disease patients (51,52). In patients with Crohn disease the *MTRR* A66G polymorphism was shown to be associated with oxidant stress and disease activity, whereas vitamin B_{12} and the *MTHFR* 677 TT genotype were the main determinants of hyperhomocysteinemia (53). The *MTRR* 66 AA genotype was a significant independent predictor of Crohn disease risk, and patients with this genotype had significantly higher levels of the oxidative stress marker, superoxide dismutase. Thus, it is possible that CRC patients having the *MTRR* 66 AA variant are more predisposed to oxidative stress.

Although the relatively small number of cases included in the present study leads to a limited statistical power, especially for subgroup analyses, the results indicate an association of the *MTRR* AA/AG genotype with clinical outcome that deserves further study in a larger patient cohort. A larger study would also give the opportunity to analyze how interactions between the *MTHFR* C677T, *MTR* A2756G and *MTRR* A66G variants may affect *p16* hypermethylation. In addition, there are other rare *MTHFR*, *MTR* and *MTRR* polymorphisms (9) that would be of interest to analyze in an extended study. The *MTHFR* A1298C variant, for instance, may be a possible risk factor for CRC, although the results published so far are highly controversial (10,30,54–56). The evaluation of the polymorphism as a predictor of clinical response to 5-FU–based chemotherapy has also led to inconclusive results (10,18,57,58). Because of these inconsistent results, the polymorphism was not analyzed in the present study.

Some of the discrepancies that have been noted regarding the effects of genotypes are probably caused by differences among study populations relating to the average intake of dietary factors. Interactions between genotype and folate may only be detected in patients with high or low plasma folate levels (11,12,55) or in patients receiving folate supplements (30). Thus, the lack of analysis of micronutrients in the tissue samples is a limitation of the present study. However, large populations are required to examine gene–nutrient interactions using traditional epidemiological methods.

In conclusion, our results indicate that patients with the *MTRR* 66 AA/AG genotypes have significantly worse cancer-specific survival when the mucosa is positive for *p16* hypermethylation. The reason for the particularly unfavorable outcome of patients having the combination of the *MTRR* 66 AA/AG genotype and *p16* hypermethylation in mucosa might be linked to a state of oxidative stress, which seems to be associated with this specific genotype as well as with *p16* methylation. Further studies are needed in order to analyze whether oxidative stress in macroscopically normal-appearing mucosa of patients with CRC leads to low folate/vitamin B_{12} levels and aberrant DNA methylation that could increase the risk of recurrent disease as well as a worse cancer-specific survival.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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