Dietary Folate Intake in Combination with MTHFR C677T Genotype and Promoter Methylation of Tumor Suppressor and DNA Repair Genes in Sporadic Colorectal Adenomas

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Abstract

Methylation of the promoter region of tumor suppressor genes is increasingly recognized to play a role in cancer development through silencing of gene transcription. We examined the associations between dietary folate intake, MTHFR C677T genotype, and promoter methylation of six tumor suppressor and DNA repair genes. Patients with colorectal adenoma (*n* = 149) and controls (*n* = 286) with folate intake in the upper or lower tertile with the CC or TT genotype were selected from a case-control study. Methylation-specific PCRs were conducted on colorectal adenoma specimens. The percentages of promoter methylation ranged from 15.7% to 64.2%. In case-case comparisons, folate was inversely associated with promoter methylation, especially among TT homozygotes. Case-control comparisons suggested that folate was not associated with the occurrence of adenomas with promoter methylation, and increased the risk of unmethylated adenomas, especially in TT homozygotes. The interactions between folate and MTHFR genotype were most pronounced for O^6-MGMT: compared with CC homozygotes with low folate intake, the adjusted odds ratios (95% confidence interval) of having a methylated O^6-MGMT promoter were 3.39 (0.82-13.93) for TT homozygotes with low folate intake and 0.37 (0.11-1.29) for TT homozygotes with high folate intake (*P* interaction = 0.02); the odds ratios for the occurrence of adenomas without methylation were 0.57 (0.16-2.11) for TT homozygotes with low folate intake and 3.37 (1.17-9.68) for TT homozygotes with high folate intake (*P* interaction = 0.03). In conclusion, folate intake seems to be inversely associated with promoter methylation in colorectal adenomas in case-case comparisons, and was positively associated with the occurrence of adenomas without promoter methylation in case-control comparisons, especially for TT homozygotes. (Cancer Epidemiol Biomarkers Prev 2007;16(2):327–33)

Introduction

DNA methylation is an important event in gene regulation: it is involved in gene expression, chromatin configuration and structural stability of DNA, binding of transcriptional factors and other proteins, mutations, and imprinting (reviewed in ref. 1). Colorectal neoplasms, both carcinomas and adenomas, show a decreased global DNA methylation level compared with normal tissue (2, 3). Conversely, other studies have shown methylation of the promoter region of specific tumor suppressor genes in colorectal tumors (4), which are increasingly recognized to play an important role in cancer development through silencing of gene transcription (5). Evidence from recent studies suggests that DNA hypomethylation and hypermethylation are independent processes and contribute separately to the process of carcinogenesis (6, 7).

Folate is a vitamin that is essential in DNA metabolism. Deficiency of folate affects purine and pyrimidine synthesis and DNA methylation (8). As 5-methyltetrahydrofolate, folate provides methyl groups for *S*-adenosyl methionine, which is a universal methyl donor in a large number of biological reactions, including the methylation of DNA (9). In the Netherlands Cohort Study on Diet and Cancer, which examined the associations between folate and alcohol intake and promoter methylation of genes involved in colorectal carcinogenesis, it was suggested that the prevalence of promoter hypermethylation was higher in carcinomas from patients with a low folate/high alcohol intake when compared with carcinomas from patients with a high folate/low alcohol intake (10). Kawakami et al. showed that colorectal carcinomas with frequent promoter hypermethylation, derived from Australian patients, have high levels of 5,10-methylenetetrahydrofolate and tetrahydrofolate (11).

An important enzyme in folate metabolism, methylenetetrahydrofolate reductase (MTHFR), catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (9). A common C-to-T substitution in the *MTHFR* gene at nucleotide 677 converts an alanine to valine and is associated with decreased enzyme activity (12). Studies investigating the association between MTHFR C677T genotype and colorectal adenoma risk show nonsignificant relative risks ranging from 0.35 to 2.41 (13-21). However, the MTHFR C677T genotype might modify the association between intake of folate and colorectal adenomas: several studies indicate that the MTHFR TT genotype, in combination with a low folate status, may be a risk factor for colorectal adenomas (14, 16, 17, 22), although some studies do not show an interaction (13, 19, 21). From a Japanese study, it was suggested that the genotypes with low enzymatic activity of MTHFR, which

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consisted of MTHFR 1298 CC, 677 TT, and the combination of 1298 AC and 677 CT, are linked with promoter hypermethylation in proximal colon cancer (23).

In this case-control study, we examined the association between dietary folate intake, MTHFR C677T genotype, their possible interaction, and promoter methylation of six tumor suppressor and DNA repair genes in sporadic colorectal adenomas. The genes that we analyzed were the tumor suppressor genes adenomatous polyposis coli (*APC-1A*), *p14*^{ARF}, *p16*^{INK4A}, Ras association domain family protein 1A (*RASSF1A*), and the DNA repair genes human MutL homologue 1 (*hMLH1*) and O^6-methylguanine-DNA methyltransferase (O^6-*MGMT*). Five of these genes were chosen (*APC-1A*, *p14*^{ARF}, *p16*^{INK4A}, *hMLH1*, and O^6-*MGMT*) because they were markedly methylated in colon cancer in a study examining methylation profiles using a series of 12 genes in 15 major human tumor types (24), whereas *RASSF1A* has been shown to be frequently methylated in colorectal cancer in the previously mentioned Dutch cohort study (25).

Materials and Methods

Study Population. The POLIEP study was a case-control study conducted in the Netherlands to investigate gene-environment interactions and the risk of colorectal adenomas. Participants were recruited among those undergoing endoscopy in 10 outpatient clinics between June 1997 and October 2002. The study design has been described previously (21, 26).

Eligibility criteria were: Dutch speaking, of European origin, ages 18 to 75 years at the time of endoscopy, no hereditary colorectal cancer syndromes (i.e., familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer), no chronic inflammatory bowel disease, no history of colorectal cancer, and no (partial) bowel resection. Response rates varied from 35% to 91% in different outpatient clinics; overall response was 54%. The total study population consisted of 768 cases, defined as those with at least one histologically confirmed colorectal adenoma ever in their life, and 709 controls, defined as those without any colorectal polyp.

Formalin-fixed, paraffin-embedded adenoma tissue was available from 575 cases. From these cases, we selected 164 people in the upper (>212 μg/d) or lower (<183 μg/d) tertile of folate intake with the MTHFR 677 CC or TT genotype. Of these cases, a DNA yield of 15 adenomas (mainly tubular) was too low for methylation-specific PCR (MSP), so we included 149 cases in the analyses (19 low folate and TT genotype, 17 high folate and TT genotype, 57 low folate and CC genotype, and 56 high folate and CC genotype). For case-control comparisons, we also selected controls in the upper or lower tertile of folate intake and with the CC or TT genotype (n = 286; 37 low folate and TT genotype, 15 high folate and TT genotype, 152 low folate and CC genotype, and 82 high folate and CC genotype).

Questionnaires. Participants were asked to fill out self-administered questionnaires according to their habits in the year previous to their colonoscopy or complaints. Dietary intake was assessed with a standardized and validated semiquantitative food-frequency questionnaire that was originally developed for the Dutch cohort of the European Prospective Investigation into Cancer and Nutrition study (27). Subsequently, intake of energy and nutrients was calculated using the Dutch food composition table. Vegetables, bread, and meat contributed to >50% of folate intake. The reproducibility of these products, as assessed with the questionnaire and expressed as Spearman correlation coefficients was 0.76, 0.86, and 0.68 for men, and 0.65, 0.78, and 0.80 for women, respectively. The relative validities compared with 24-h recalls were 0.31, 0.76, and 0.47 for men, and 0.38, 0.78, and 0.70 for women, respectively (27).

To be able to adjust for possibly confounding factors, the participants filled out a general questionnaire on medical history and several lifestyle factors, e.g., smoking, physical activity, and nonsteroidal anti-inflammatory drug use.

MTHFR Genotyping. Blood was drawn by venipuncture and collected in 9 mL EDTA vacutainers (Greiner Bio-One, Kremsmuenster, Austria) and subsequently stored at −20°C until DNA extraction. DNA was isolated from 200 μL whole blood using the QIAamp blood kit (Qiagen, Hilden, Germany), subsequently diluted to a concentration of ~20 ng/μL, and stored at 4°C until analysis.

To determine the MTHFR C677T polymorphism, we used the PCR/RFLP method described in detail by Frosst et al. (12). PCR was done with internal negative controls. In cases of questionable genotypes through weak visualization of fragments, the genotype was reassessed. To test reproducibility, 20% of the samples were analyzed in duplicate and yielded the same result (100% reproducibility). In addition, we participated in an external quality control program. Results showed a 100% match with the expected MTHFR C677T genotype.

DNA Extraction from Adenoma Tissue. DNA was extracted from formalin-fixed, paraffin-embedded adenoma tissue (10-12, 10-μm-thick sections) using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). Microdissection was done, guided by a H&E-stained 4-μm section, and only those areas containing >60% tumor cells were used. Isolated tissue was incubated overnight at 55°C in 500 μL of cell lysis solution containing 0.5 mg/mL proteinase K (Roche Diagnostics, Mannheim, Germany), followed by 72 h at 37°C. Proteins were removed with the protein precipitation solution according to the manufacturer's protocol. DNA was precipitated with 500 μL of 100% isopropanol at 4°C for 30 min. The pellet was washed with 500 μL of 70% ethanol, air-dried, and subsequently the DNA was rehydrated in 30 μL of DNA hydration solution.

Promoter Methylation Analysis. We determined DNA methylation in the CpG islands of the APC-1A, p14^{ARF}, p16^{INK4A}, hMLH1, O^6-MGMT, and RASSF1A gene promoters by chemical modification of genomic DNA with sodium bisulfite and subsequent MSP assays (10, 28). Sodium bisulfite treatment of 250 ng of DNA was done according to Millar et al. (29), with glycogen used as a carrier. In this reaction, all non-methylated cytosines were converted to uracil, but methylated cytosines (5-methylcytosine) remained as cytosine.

After sodium bisulfite treatment of DNA, we did a MSP assay (10, 28). This method is based on the use of two distinct methylation-specific primer sets for the sequence of interest. The unmethylated primer will only amplify sodium bisulfite–converted DNA in unmethylated condition, whereas the methylated primer is specific for sodium bisulfite–converted methylated DNA. Both the forward and the reverse primers contain one to three CpG dinucleotides in the 3′ region of the primers to accomplish optimal discriminative power between methylated and unmethylated DNA (28). We used MSP primers that were located around the transcription start site and that have been reported, validated, and shown to be associated with gene silencing in cell lines and colorectal cancer (24, 25). Primer sequences and PCR conditions are listed elsewhere (10). Where more primers are listed for one gene, we used the short primers. All PCRs were done with controls for unmethylated alleles (DNA from normal lymphocytes), methylated alleles (normal lymphocyte DNA treated *in vitro* with *Sss*I methyltransferase; New England Biolabs, Beverly, MA), and a negative PCR control without DNA. Ten microliters of each MSP reaction was directly loaded onto 6% denaturing polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. To study the reproducibility of the nested MSP approach, 35% of the specimens were also

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analyzed after bisulfite modification according to the method of Herman et al. (28). Agreement between the two methods was 87% (κ = 0.73). Bisulfite-treated DNA could not be amplified for APC-1A in 2 samples (1.3%), for $p14^{ARF}$ in 8 samples (5.4%), for $p16^{INK4A}$ in 18 samples (12.1%), for hMLH1 in 9 samples (6.0%), and for both O^6-MGMT and RASSF1A in 12 samples (8.1%).

Statistical Analyses. Descriptive statistics of intake of relevant nutrients and other characteristics were computed, according to methylation status (three or more gene promoters methylated compared with less than three gene promoters methylated).

We used logistic regression models, which allow correction for possibly confounding factors, to calculate odds ratios and 95% confidence intervals estimating the relative risk of promoter methylation within adenomas and the relative risk of colorectal adenomas with or without promoter methylation. We examined methylation in each of the six genes separately and concurrent methylation in three or more genes compared with less than three genes. We selected this cutoff point because the median number of methylated genes in our study was three, similar to Bai et al. (30). Those with a low folate intake and MTHFR CC genotype were used as reference.

Logistic regression models were adjusted for age and sex. Furthermore, we examined if potential confounding factors (i.e., body mass index, physical activity, educational level, smoking, use of nonsteroidal anti-inflammatory drugs, use of multivitamin or B vitamin supplements, indication for colonoscopy, family history of sporadic colorectal cancer, and dietary intake of fat, fiber, alcohol, vitamin B_2, vitamin B_6, vitamin B_{12}, calcium, iron, fruits, and vegetables) were associated both with promoter methylation of any of the six genes and folate intake, and changed the crude estimates by >10% when added to the logistic regression models. The final logistic regression models included the covariates age, sex, body mass index, indication for colonoscopy, and dietary intake of vitamins B_2 and B_6.

All tests of statistical significance were two-sided and the significance level was set at 0.05. We used Statistical Analysis Software (version 8; SAS Institute, Cary, NC) for all analyses.

Results

The observed percentages of promoter methylation were 51.0% (75 of 147) for APC-1A, 61.7% (87 of 141) for $p14^{ARF}$, 54.2% (71 of 131) for $p16^{INK4A}$, 15.7% (22 of 140) for hMLH1, 64.2% (88 of 137) for O^6-MGMT, and 40.9% (56 of 137) for RASSF1A. For analyses examining methylation in three or more genes compared with less than three genes, we left out 11 cases that we could not classify in either group because the DNA could not be amplified for some of the genes. Eighty of 138 (58.0%) patients had three or more methylated gene promoters.

In Table 1, the characteristics of the study population are shown according to methylation status (three or more gene promoters methylated compared with less than three gene promoters methylated). The most striking difference observed was a higher percentage of (tubulo)villous adenomas among those with three or more methylated gene promoters compared with those with less than three methylated gene promoters. Remarkably, no differences were observed in age and sex.

The results of case-case and case-control comparisons evaluating associations between folate intake or MTHFR C677T genotype and the occurrence of adenomas with or without promoter methylation are presented in Table 2. Case-case comparisons showed that there was an inverse association between folate and adenomas with methylation compared with adenomas without methylation. Folate intake was not associated with the occurrence of adenomas with promoter methylation in any of the six genes, but folate intake seemed to be positively associated with adenomas without methylation in the selected genes. The results were consistent for all genes separately and for methylation in less than three genes compared with three or more genes, but none of the associations reached statistical significance. For the MTHFR genotype, case-case comparisons showed no clear pattern. In case-control analyses, there was no difference between the occurrence of adenomas with or without methylation at the six genes: the TT genotype seemed to be slightly positively associated with both subsets of adenomas.

Table 3 shows the interplay between folate and MTHFR C677T genotype in gene promoter methylation. Among the different genes, a consistent pattern was noticeable: within those carrying the CC genotype, folate intake did not seem to be associated with methylation within adenomas, or with adenomas with or without methylation, and within those carrying the TT genotype, there was no clear pattern between folate intake and the occurrence of adenomas with methylation in any of the six genes. However, within TT homozygotes, the association between folate and adenomas with methylation compared with adenomas without methylation was inverse, and folate intake was positively associated with the occurrence of adenomas which are unmethylated in the six genes. These associations were most obvious for O^6-MGMT, in which the interactions were statistically significant. Again, the results were similar when analyzing concurrent methylation in three or more genes compared with less than three genes. The interactions seemed more pronounced among male participants; however, the study did not have enough power for subgroup analyses according to sex (data not shown).

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Table 1. Characteristics of the study population according to methylation status

	Three or more genes methylated (n = 80)	Less than three genes methylated (n = 58)	P
Female (%)	45	47	0.86
Age (y)*	59.2 ± 9.7	58.9 ± 9.6	0.84
Family history of colorectal cancer (% yes)	26	31	0.54
Indication for colonoscopy (% screening)	34	28	0.44
MTHFR C677T genotype (% TT)	24	26	0.78
Histopathology [% (tubulo)villous]	35	21	0.07
Dietary intake			
Energy (kJ/d)*	8,948 ± 2,228	8,582 ± 2,220	0.34
Alcohol (g/d)†	9.8 (1.5; 24.3)	8.9 (0.3; 26.3)	0.73
Vitamin B_2 (mg/d)*	1.67 ± 0.47	1.57 ± 0.55	0.23
Vitamin B_6 (mg/d)*	1.71 ± 0.44	1.61 ± 0.41	0.19
Folate (μg/d)*	205 ± 57	204 ± 61	0.93
Vitamin B_{12} (μg/d)*	5.04 ± 2.34	4.83 ± 2.29	0.59
Supplementary multivitamin use (% yes)	16	19	0.68
Supplementary B vitamin use (% yes)	6	7	0.88

*Mean ± SD.

†Median (25th percentile; 75th percentile).

Table 2. Odds ratios (95% confidence intervals) for the association between dietary intake of folate or MTHFR C677T genotype and gene promoter methylation

Promoter methylation	Dietary folate intake*		MTHFR C677T genotype	
	<183 μg/d	>212 μg/d	CC	TT
APC-1A				
No. methylated/not methylated/controls	40/35/189	35/37/97	61/51/234	14/21/52
Methylated versus not methylated	1 (ref.)	0.73 (0.33-1.62)	1 (ref.)	0.56 (0.25-1.25)
Methylated versus controls	1 (ref.)	0.99 (0.53-1.87)	1 (ref.)	1.10 (0.55-2.18)
Not methylated versus controls	1 (ref.)	1.34 (0.70-2.57)	1 (ref.)	1.95 (1.04-3.68)
$p14^{ARF}$				
No. methylated/not methylated/controls	44/29/189	43/25/97	68/40/234	19/14/52
Methylated versus not methylated	1 (ref.)	0.86 (0.37-1.97)	1 (ref.)	0.87 (0.38-1.96)
Methylated versus controls	1 (ref.)	0.99 (0.53-1.83)	1 (ref.)	1.35 (0.72-2.54)
Not methylated versus controls	1 (ref.)	1.20 (0.59-2.46)	1 (ref.)	1.71 (0.84-3.50)
$p16^{INK4A}$				
No. methylated/not methylated/controls	36/30/189	35/30/97	55/45/234	16/15/52
Methylated versus not methylated	1 (ref.)	0.54 (0.22-1.32)	1 (ref.)	1.08 (0.46-2.52)
Methylated versus controls	1 (ref.)	0.90 (0.47-1.74)	1 (ref.)	1.40 (0.72-2.75)
Not methylated versus controls	1 (ref.)	1.53 (0.76-3.10)	1 (ref.)	1.56 (0.78-3.13)
hMLH1				
No. methylated/not methylated/controls	11/59/189	11/59/97	18/87/234	4/31/52
Methylated versus not methylated	1 (ref.)	0.79 (0.25-2.45)	1 (ref.)	0.64 (0.20-2.11)
Methylated versus controls	1 (ref.)	0.98 (0.35-2.81)	1 (ref.)	1.10 (0.34-3.50)
Not methylated versus controls	1 (ref.)	1.21 (0.70-2.09)	1 (ref.)	1.65 (0.96-2.85)
O^6-MGMT				
No. methylated/not methylated/controls	47/22/189	41/27/97	66/37/234	22/12/52
Methylated versus not methylated	1 (ref.)	0.62 (0.26-1.49)	1 (ref.)	1.00 (0.44-2.30)
Methylated versus controls	1 (ref.)	0.99 (0.54-1.82)	1 (ref.)	1.64 (0.90-3.01)
Not methylated versus controls	1 (ref.)	1.55 (0.73-3.28)	1 (ref.)	1.50 (0.71-3.18)
RASSF1A				
No. methylated/not methylated/controls	27/42/189	29/39/97	40/62/234	16/19/52
Methylated versus not methylated	1 (ref.)	0.81 (0.35-1.91)	1 (ref.)	1.55 (0.69-3.50)
Methylated versus controls	1 (ref.)	1.04 (0.51-2.12)	1 (ref.)	1.95 (0.98-3.89)
Not methylated versus controls	1 (ref.)	1.28 (0.68-2.41)	1 (ref.)	1.43 (0.76-2.71)
At least three genes methylated				
No. ≥3 methylated/<3 methylated/controls	40/29/189	40/29/97	61/43/234	19/15/52
≥3 methylated versus <3 methylated	1 (ref.)	0.66 (0.29-1.54)	1 (ref.)	1.00 (0.44-2.24)
≥3 methylated versus controls	1 (ref.)	1.01 (0.54-1.89)	1 (ref.)	1.52 (0.81-2.88)
<3 methylated versus controls	1 (ref.)	1.50 (0.74-3.05)	1 (ref.)	1.66 (0.83-3.33)

NOTE: Adjusted for age, sex, body mass index, indication for colonoscopy, and dietary intake of vitamin B_2 and vitamin B_6.
*Adjusted for total energy intake, according to Willett and Stampfer (45).

Discussion

In this observational study, we examined whether dietary folate intake and MTHFR C677T genotype were associated with promoter methylation of tumor suppressor genes and DNA repair genes in colorectal adenoma specimens. In case-case comparisons, folate was inversely associated with adenomas with methylation compared with adenomas without methylation. This association was most pronounced among those carrying the MTHFR TT genotype and was consistent for distinct genes. In case-control comparisons, folate intake was not associated with the occurrence of adenomas with promoter methylation in the genes under study, but seemed to be positively associated with adenomas without methylation in the selected genes. Again, this association was more pronounced among TT homozygotes. Although most of the associations were not statistically significant, the results were consistent for all six separate genes and for methylation in three or more genes compared with less than three genes, which strengthens the results. The MTHFR TT genotype was slightly positively associated with both adenomas with and without methylation, but was not statistically significant.

Most other studies examining promoter methylation in sporadic adenomas found frequencies of methylation of individual genes that were either lower (31-35) or in the same range as in our study (6, 30, 36-38). However, comparing our results with other studies is difficult for several reasons: different studies examine different genes, and background exposures and characteristics of adenomas (for example tubular/villous) may vary between studies. We selected participants from a Dutch case-control study, in which folate intake was positively associated with adenoma occurrence (21). Dietary folate intake in this case-control study was relatively low (mean, 195 μg/d). This may partly account for the relatively high percentages of promoter methylation in our study. Differences in adenoma characteristics may explain a further part of the relatively high methylation frequencies that we found. In our study, villous adenomas showed higher frequencies of promoter methylation than tubular adenomas. Furthermore, 15 of 164 adenomas (9.1%), which comprised mainly tubular adenomas, could not be analyzed due to insufficient DNA yield. Two studies that observed lower frequencies of methylation included only adenoma samples that showed low-grade dysplasia (33), or only tubular adenomas (34), whereas Kim et al. (39) found higher frequencies of methylation in advanced adenomas compared with nonadvanced adenomas (39). Conversely, other studies indicate similar methylation patterns in both advanced and nonadvanced adenomas (6, 30). We also examined promoter methylation in normal-appearing rectal biopsies from patients with adenoma that participated in an intervention trial, using the same method.[5] Percentages of methylation were appreciably lower than in adenomas for all genes except for APC.

[5] Van den Donk M, Pellis L, Crott JW, et al. A randomized, placebo-controlled intervention study on the effects of folic acid and vitamin B12 on promoter methylation and uracil incorporation in rectal mucosa DNA among MTHFR C677T genotypes.

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To our knowledge, we are the first to investigate the role of folate, MTHFR C677T genotype, and their interplay in promoter methylation in colorectal adenomas. In colorectal cancer, however, a few studies looked at the association between folate or MTHFR C677T genotype and promoter methylation. van Engeland et al. (10) studied promoter methylation of the same six genes in the Netherlands Cohort Study on Diet and Cancer. The prevalence of promoter methylation was higher in carcinomas from patients with low methyl donor intake when compared with carcinomas from patients with high methyl donor intake (based primarily on folate intake), although the differences were not statistically significant (10). This is in accordance with our adenoma results. However, in a case-control study from the U.S., no associations were found between dietary folate, vitamins B_6 and B_{12}, methionine, and alcohol, and the CpG island methylator phenotype status of colorectal carcinomas (40). Kawakami et al. (11) examined the associations between the folate intermediates 5,10-methylenetetrahydrofolate and tetrahydrofolate in colorectal carcinomas from Australian patients, polymorphisms including MTHFR C677T, and methylation of gene promoters. They showed that colorectal carcinomas with frequent promoter methylation have high levels of folate intermediates in tissue. However, similar to our findings, they did not find a relationship between MTHFR C677T polymorphism and gene promoter methylation (11). The interplay between folate and MTHFR C677T was not studied. In a study by Paz et al. (41), the MTHFR C677T genotype was also not related to the frequency of promoter methylation in patients with cancer. Folate intake was not taken into account (41).

Our results suggest that, whereas folate does not seem to be involved in the occurrence of adenomas with methylated promoter sites, high folate intake is associated with an increased occurrence of adenomas with unmethylated promoter sites of these tumor suppressor and DNA repair genes. This, in turn, is associated with the increased transcriptional activity of these genes (5) and could be interpreted such that high folate induces protective responses. However, without having insight into the effects on proliferation and other cell turnover variables as well as on methylation at other sites, this cannot be concluded. Only a more extensive understanding of the regulation of methylation in relation to gene expression, cell turnover, and carcinogenesis will allow us to fully interpret our findings.

The results are in accordance with our case-control study examining the association between folate intake and colorectal adenoma risk (21). In that study, we found that folate intake was positively associated with colorectal adenomas, especially for those with low vitamin B_2 intake. Due to the small number of participants in the current study, it was not possible to perform analyses stratified for vitamin B_2 intake. Furthermore, it was not possible to stratify for alcohol intake. However, in our case-control study, although alcohol was an independent risk factor (26), it did not modify or confound the association between folate intake and adenoma occurrence (21), hence, we think that alcohol intake does not influence our results markedly.

Table 3. Odds ratios (95% confidence intervals) for the interplay between dietary folate intake and MTHFR C677T genotype in gene promoter methylation

Promoter methylation	MTHFR CC genotype		MTHFR TT genotype		*P* interaction
	Folate intake* <183 μg/d	Folate intake* >212 μg/d	Folate intake* <183 μg/d	Folate intake* >212 μg/d	
APC-1A					
No. methylated/not methylated/controls	30/26/152	31/25/82	10/9/37	4/12/15	
Methylated versus not methylated	1 (ref.)	0.94 (0.39-2.27)	0.88 (0.29-2.64)	0.30 (0.08-1.12)	0.25
Methylated versus controls	1 (ref.)	1.07 (0.54-2.14)	1.24 (0.54-2.85)	0.91 (0.26-3.24)	0.62
Not methylated versus controls	1 (ref.)	1.04 (0.49-2.22)	1.33 (0.55-3.22)	3.25 (1.25-8.45)	0.20
$p14^{ARF}$					
No. methylated/not methylated/controls	32/23/152	36/17/82	12/6/37	7/8/15	
Methylated versus not methylated	1 (ref.)	1.20 (0.48-3.00)	1.70 (0.53-5.48)	0.49 (0.14-1.68)	0.10
Methylated versus controls	1 (ref.)	1.03 (0.52-2.04)	1.40 (0.63-3.11)	1.32 (0.45-3.83)	0.89
Not methylated versus controls	1 (ref.)	0.87 (0.38-1.99)	1.00 (0.37-3.74)	3.07 (1.05-8.99)	0.09
$p16^{INK4A}$					
No. methylated/not methylated/controls	27/21/152	28/24/82	9/9/37	7/6/15	
Methylated versus not methylated	1 (ref.)	0.48 (0.18-1.28)	0.88 (0.28-2.76)	0.72 (0.19-2.84)	0.55
Methylated versus controls	1 (ref.)	0.85 (0.41-1.76)	1.21 (0.50-2.92)	1.46 (0.49-4.33)	0.62
Not methylated versus controls	1 (ref.)	1.62 (0.73-3.60)	1.69 (0.68-4.16)	2.44 (0.77-7.76)	0.88
hMLH1					
No. methylated/not methylated/controls	8/44/152	10/43/82	3/15/37	1/16/15	
Methylated versus not methylated	1 (ref.)	1.13 (0.33-3.89)	1.32 (0.29-5.96)	0.26 (0.03-2.52)	0.19
Methylated versus controls	1 (ref.)	1.15 (0.36-3.71)	1.41 (0.34-5.83)	0.77 (0.09-6.91)	0.56
Not methylated versus controls	1 (ref.)	1.04 (0.56-1.92)	1.25 (0.61-2.60)	2.56 (1.07-6.15)	0.24
O^6-MGMT					
No. methylated/not methylated/controls	32/19/152	34/18/82	15/3/37	7/9/15	
Methylated versus not methylated	1 (ref.)	1.00 (0.38-2.63)	3.39 (0.82-13.93)	0.37 (0.11-1.29)	0.02
Methylated versus controls	1 (ref.)	1.09 (0.55-2.15)	1.78 (0.84-3.75)	1.54 (0.53-4.53)	0.73
Not methylated versus controls	1 (ref.)	0.99 (0.42-2.34)	0.57 (0.16-2.11)	3.37 (1.17-9.68)	0.03
RASSF1A					
No. methylated/not methylated/controls	17/34/152	23/28/82	10/8/37	6/11/15	
Methylated versus not methylated	1 (ref.)	1.15 (0.44-3.03)	3.53 (1.08-11.50)	0.78 (0.22-2.76)	0.06
Methylated versus controls	1 (ref.)	1.16 (0.51-2.64)	2.09 (0.86-5.12)	2.07 (0.64-6.63)	0.83
Not methylated versus controls	1 (ref.)	1.01 (0.50-2.05)	0.92 (0.38-2.25)	2.54 (0.96-6.72)	0.13
At least three genes methylated					
No. ≥3 methylated/<3 methylated/controls	28/23/152	33/20/82	12/6/37	7/9/15	
≥3 methylated versus <3 methylated	1 (ref.)	0.95 (0.37-2.42)	2.17 (0.66-7.11)	0.43 (0.13-1.49)	0.07
≥3 methylated versus controls	1 (ref.)	1.07 (0.53-2.16)	1.60 (0.72-3.59)	1.52 (0.52-4.45)	0.86
<3 methylated versus controls	1 (ref.)	1.13 (0.50-2.53)	1.01 (0.37-2.77)	3.40 (1.19-9.68)	0.14

NOTE: Adjusted for age, sex, body mass index, indication for colonoscopy, and dietary intake of vitamin B_2 and vitamin B_6.
*Adjusted for total energy intake, according to Willett and Stampfer (45).

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It would be interesting to study the influence of polymorphisms in other genes that are involved in the one-carbon metabolism on promoter methylation. However, in the current study, we selected the participants based on the MTHFR C677T genotype and folate intake. Therefore, there is not enough power to take other polymorphisms into account. Another interesting biomarker to study would be global DNA hypomethylation. This is not possible within the scope of the present study because of the scarcity of adenoma tissue.

The use of a food-frequency questionnaire may not be the most accurate way to assess folate status. Most food-frequency questionnaires that were validated for folate intake show poor correlation between erythrocyte folate and dietary folate intake (42, 43). The European Prospective Investigation into Cancer and Nutrition questionnaire that we used showed a positive, although poor correlation between plasma folate and dietary folate intake (44). We were not able to assess erythrocyte folate or plasma folate in the current study. We focused on dietary folate intake because supplement use is not common in the Netherlands and supplementary folic acid intake was not assessed in this study. Adjusting the logistic models for supplementary multivitamin or B vitamin use did not change the results.

Cases were asked to recall their dietary habits from the past, which may have led to misclassification. However, systematic errors in dietary recall are less likely to bias results from case-case comparisons as misclassification will presumably be at random. Therefore, if this had an influence on our results, it would have attenuated them.

In summary, although the findings should be considered exploratory, this study suggests that folate intake is inversely associated with promoter methylation within colorectal adenomas, especially in those with the TT genotype. Case-control comparisons revealed that folate intake was positively associated with the occurrence of adenomas without promoter methylation in selected genes, but not with the occurrence of adenomas with promoter methylation, an effect that is most explicit in TT homozygotes. Despite the small number of participants, results from separate genes point in the same direction and some interactions between folate intake and MTHFR C677T genotype reached statistical significance. Therefore, we believe that our results justify conducting studies with larger sample sizes.

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