Maastricht University

Effects of dietary folate and alcohol intake on promoter methylation in sporadic colorectal cancer: the Netherlands cohort study on diet and cancer

Citation for published version (APA):

van Engeland, M., Weijenberg, M. P., Roemen, G. M. J. M., Brink, M., de Bruine, A. P., Goldbohm, R. A., van den Brandt, P. A., Baylin, S. B., de Goeij, A. F. P. M., & Herman, J. G. (2003). Effects of dietary folate and alcohol intake on promoter methylation in sporadic colorectal cancer: the Netherlands cohort study on diet and cancer. *Cancer Research*, *63*(12), 3133-3137. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=12810640&dopt=Abstract

Document status and date:
Published: 01/01/2003

Document Version:
Publisher's PDF, also known as Version of record

Please check the document version of this publication:

• A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
• The final author version and the galley proof are versions of the publication after peer review.
• The final published version features the final layout of the paper including the volume, issue and page numbers.

Link to publication

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy
If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Download date: 25 Apr. 2024

[CANCER RESEARCH 63, 3133–3137, June 15, 2003]

Effects of Dietary Folate and Alcohol Intake on Promoter Methylation in Sporadic Colorectal Cancer: The Netherlands Cohort Study on Diet and Cancer[1]

Manon van Engeland, Matty P. Weijenberg, Guido M. J. M. Roemen, Mirian Brink, Adriaan P. de Bruïne, R. Alexandra Goldbohm, Piet A. van den Brandt, Stephen B. Baylin, Anton F. P. M. de Goeij, and James G. Herman[2]

The Research Institute GROW, Department of Pathology, University Maastricht, 6200 MD Maastricht, the Netherlands [M. v. E., G. M. J. M. R., A. P. d. B., A. F. P. M. d. G.]; Tumor Biology, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, Maryland 21231 [M. v. E., S. B. B., J. G. H.]; Research Institute NUTRIM, Department of Epidemiology, University Maastricht, 6200 MD Maastricht, the Netherlands [M. P. W., M. B., P. A. v. d. B.]; and TNO Nutrition and Food Research, 3700 AJ Zeist, the Netherlands [R. A. G.]

ABSTRACT

Sporadic colorectal cancer (CRC) is characterized by genetic and epigenetic changes such as regional DNA hypermethylation and global DNA hypomethylation. Epidemiological and animal studies suggest that aberrant DNA methylation is associated with low dietary folate intake, which is aggravated by high alcohol intake. The relationship between promoter methylation of genes involved in CRC carcinogenesis and folate and alcohol intake was investigated. Methylation of the *APC-1A*, *p14^{ARF}*, *p16^{INK4A}*, *hMLH1*, *O^6-MGMT*, and *RASSF1A* promoters was studied using methylation-specific PCR in 122 sporadic CRCs, derived from patients with folate and alcohol intake at either the lower or the higher quintiles of the distribution. Overall, promoter hypermethylation frequencies observed were: 39% for *APC*; 33% for *p14^{ARF}*; 31% for *p16^{INK4A}*; 29% for *hMLH1*; 41% for *O^6-MGMT*; and 20% for *RASSF1A*. For each of the tested genes, the prevalence of promoter hypermethylation was higher in CRCs derived from patients with low folate/high alcohol intake ($n = 61$) when compared with CRCs from patients with high folate/low alcohol intake ($n = 61$), but the differences were not statistically significant. The number of CRCs with at least one gene methylated was higher (84%) in the low folate intake/high alcohol intake group when compared with the high folate intake/low alcohol intake group (70%; $P = 0.085$). Despite the size limitations of this study, these data suggest that folate and alcohol intake may be associated with changes in promoter hypermethylation in CRC.

INTRODUCTION

In addition to hereditary components, known risk factors for CRC[3] are related to lifestyle and environment such as smoking and a Western diet (high meat, energy, and alcohol intake and low fruit, vegetable, and fiber intake; Ref. 1). Although the majority of epidemiological studies point to fruit and vegetable intake as protective for CRC (2–11), the molecular mechanisms for this protection remain to be clarified. It is hypothesized that folate, one of the vitamins mainly found in green leafy vegetables, is in part responsible for the inverse association with CRC risk. The effects of low folate intake are aggravated by high alcohol intake (7), probably because of degradation of folate in the colon by acetaldehyde, the first metabolite of alcohol (12).

Folate, as 5-methyltetrahydrofolate, has a central role in methyl metabolism. It supplies a methyl group to convert homocysteine to methionine, which is then converted to S-adenosylmethionine, the universal methyl donor for methylation of a wide variety of biological substrates such as DNA, RNA, and proteins. Folate deficiency is reported to be associated with the occurrence of point mutations in K-*ras* in colorectal adenomas (13) and carcinomas (14), single and double strand DNA breaks, chromosome breakage (15, 16), and global DNA hypomethylation (17).

Although the overall level of genomic methylation is actually reduced in certain tumor types, including CRCs (18, 19), hypermethylation at several gene promoters has also been reported. It has been hypothesized that global hypomethylation might induce regional *de novo* hypermethylation (20). On the other hand, a recent study by Bariol *et al.* (21) suggests that there is no relationship between global demethylation and regional hypermethylation in CRC.

To investigate whether altered DNA methylation is associated with folate and alcohol intake, we examined promoter methylation of genes that have been reported to be involved and methylated in CRC carcinogenesis, *i.e.*, *APC-1A*, *p14^{ARF}*, *p16^{INK4A}*, *hMLH1*, *O^6-MGMT*, and *RASSF1A* (22, 23). This was done using an optimized MSP method. This study was performed with 122 CRC archival specimens derived from incident cases who participate in the prospective NLCS (24). Patients with low folate intake in addition to high alcohol intake at baseline ($n = 61$) were compared with patients with high folate intake in addition to low alcohol intake ($n = 61$) with respect to the methylation status of a series of gene promoters.

MATERIALS AND METHODS

Study Population and Sample Procurement. The paraffin-embedded CRC samples used in this study were derived from patients who participate in NLCS. This study started in September 1986 and included 58,279 men and 62,573 women (55–69 years at baseline). At baseline, the cohort members completed a mailed, self-administered food frequency questionnaire on dietary habits and other risk factors for cancer. The study design has been described in detail elsewhere (24). Follow-up for incident cancer is established annually by computerized record linkage with all cancer registries in the Netherlands and with PALGA, a nationwide pathology database (25). Since 1989, the coverage of PALGA is 100% (26). Record linkage covering the period from 1989 up to the end of 1993 (7.3 years follow-up, with exclusion of the first 2.3 years of follow-up) resulted in 819 eligible incident, histologically confirmed CRC patients. After approval by the Medical Ethical Committee of the Maastricht University and PALGA, tissue samples were collected from 54 pathology laboratories throughout the Netherlands. Tumor tissue sample collection was started in August 1999 and was completed in December 2001.

For this pilot study, 61 CRCs of patients with high methyl donor intake [high folate intake ($\geq$215 μg/day) in addition to extremely low alcohol intake (0–4 g/day)] and 61 CRCs of patients with low methyl donor intake [folate intake ($<$215 μg/day) in addition to high alcohol intake ($\geq$5 g/day)] were selected (for selection criteria and definitions; Table 1). Among the selected patients were 68 males and 54 females (58–76 years of age at time of diagnosis), 30 Dukes A, 39 Dukes B, 31 Dukes C, and 15 Dukes D CRCs. Dukes stage was not reported in 7 cases. The distribution of the location of the tumors was colon ($n = 84$), rectosigmoid ($n = 14$), and rectum ($n = 24$). The

Received 11/5/02; accepted 4/14/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the Dutch Cancer Society and the René Vogels Foundation.

[2] To whom requests for reprints should be addressed, at Tumor Biology, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Room 543, 1650 Orleans Street, Baltimore, MD 21231. Phone: (410) 955-8506; Fax: (410) 614-9884; E-mail: hermanji@jhmi.edu.

[3] The abbreviations used are: CRC, colorectal cancer; APC, adenomatous polyposis coli; hMLH1, human mut-L homologue; O^6-MGMT, O^6-methylguanine DNA methyl transferase; NLCS, Netherlands Cohort Study on Diet and Cancer; MSP, methylation-specific PCR; OR, odds ratio; CI, confidence interval; SNP, single nucleotide polymorphism; PALGA, Pathologisch Anatomisch Landelijk Geautomatiseerd Archief.

Table 1 *Selection of CRC patients based on extreme intakes of folate and alcohol intake (quintile distribution)*

Methyl donor group (dichotome)	Quintile combination[a]	Methyl donor category	Folate intake (μg/day)	Alcohol intake (g/day)	*n*
Low folate/high alcohol ($n = 61$)	F1/A5	1	0–157	≥30	7
	F1/A4	2	0–157	15–29	14
	F1/A3	3	0–157	5–14	15
	F2/A5	4	158–186	≥30	16
	F2/A4	5	158–186	15–29	6
	F3/A5	6	187–215	≥30	3
High folate/low alcohol group ($n = 61$)	F4/A1	7	215–255	0	19
	F5/A2	8	≥255	0.1–4	24
	F5/A1	9	≥255	0	18

[a] Quintiles of folate (F) and alcohol (A) intake; *i.e.*, F1/A5 is first quintile of folate and fifth quintile of alcohol.

122 patients in this study were representative for the complete group of eligible CRC patients with respect to age, gender, Dukes stage, and tumor location distribution.

After identification by a pathologist (A. P. d. B.), genomic DNA of tumor cells was microdissected and extracted using proteinase K (Qiagen) and the Puregene DNA Isolation Kit (Gentra Systems) and stored at 4°C. Part of this set of samples has previously been characterized for *RASSF1A* promoter methylation (23).

Food Frequency Questionnaire. At the start of the NLCS in 1986, all participants completed a food frequency questionnaire on daily food consumption and potential confounders (*e.g.*, smoking, occupation, physical activity, family history of cancer, drug use). The dietary section of the questionnaire, a 150-item semiquantitative food frequency questionnaire, concentrated on habitual consumption of food and beverages during the year preceding the start of the study. The questionnaire was validated against a 9-day dietary record. Daily mean nutrient intakes are calculated using the computerized Dutch food composition table. Folate intake was calculated from newly established liquid chromatography data for foods (27).

Questionnaire data of all cases and the subcohort are key-entered twice and processed in a manner blinded with respect to case/subcohort status to minimize observer bias in coding and interpreting the data. Both procedures make use of a highly standardized protocol. Subjects with incomplete or inconsistent dietary data are excluded from data analysis, according to criteria described in detail elsewhere (28). Patients with low folate intake in addition to high alcohol intake were defined as the low methyl donor group, whereas patients with high folate intake in addition to low alcohol intake were defined as the high methyl donor group (Table 1). Dietary factors adjusted for in data analyses are chosen on the basis of a previous analysis on the association between folate and CRC (2).

Promoter Methylation Analysis. DNA methylation in the CpG islands of the *APC-1A*, *p14^{ARF}*, *p16^{INK4a}*, *hMLH1*, *O^6-MGMT*, and *RASSF1A* gene promoters was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent MSP as described in detail elsewhere (29). In brief, 1 μg of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega), again treated with NaOH, precipitated with ethanol, and resuspended in H_2O.

To facilitate MSP 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 the MSP reaction.

All PCRs were performed with controls for unmethylated alleles (DNA from normal lymphocytes), methylated alleles [normal lymphocyte DNA treated *in vitro* with *Sss*I methyltransferase (New England Biolabs)], and a control without DNA. Primer sequences and PCR conditions are described in Table 2. Ten μl of each MSP reaction were directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. To study the reproducibility of the nested MSP approach, duplicate CRC specimens were analyzed. Reproducibility was 95%.

Data Analysis. Frequencies of promoter methylation of specific genes and frequencies and means of other variables in patients with high and low methyl donor intake were computed. Differences in frequencies of variables were tested using the χ^2 test, and differences in mean levels of variables were tested using the *t* test. Logistic regression analyses were performed to test whether there was a difference in methyl donor intake (low *versus* high) between CRCs with at least one gene methylated *versus* no genes methylated while adjusting for potential confounders. Because nine categories of methyl-donor intake were distinguished (categories 1 through 9 in Table 1), although only based on extreme levels of intake, logistic regression analyses were also performed including this variable instead of the dichotomous intake variable in the model. A *P* of 0.05 was considered to be statistically significant. All tests of statistical significance were two-sided. Data analysis was done using SPSS software (version 9.0).

RESULTS

To enable MSP analysis on DNA retrieved from formalin-fixed, paraffin-embedded tumor tissue, nested MSP was performed. Nested

Table 2 *MSP primer sequences and PCR conditions*

Primer set	Sense primer	Antisense primer	Amp Size (bp)	Annealing temperature (°C)	No. of PCR cycles
APC-1A flank	5′-TGGGYGGGGTTTTGTGTTTTATT-3′	5′-TACRCCCACACCCAACCAATC-3′	136	56	35
APC-1A U[a]	5′-GTGTTTTATTGTGGAGTGTGGGTT-3′	5′-CCAATCAACAAACTCCCAACAA-3′	108	60	25
APC-1A M	5′-TATTGCGGAGTGCGGGTC-3′	5′-TCGACGAACTCCCGACGA-3′	98	60	25
p14^{ARF} flank	5′-TTTAGTTTGTAGTTAAGGGGGTAGGAG-3′	5′-CRCTACCCACTCCCCCRTAAACC-3′	207	56	35
p14^{ARF} flank short	5′-GYGTTGTTTATTTTTGGTGTTAAAGG-3′	5′-AAATATAAACCACRAAAACCCTCACT-3′	152	56	35
p14^{ARF} U	5′-TTTTTGGTGTTAAAGGGTGGTGTAGT-3′	5′-CACAAAAACCCTCACTCACAACAA-3′	132	60	25
p14^{ARF} M	5′-GTGTTAAAGGGCGGCGTAGC-3′	5′-AAAACCCTCACTCGCGACGA-3′	122	60	25
p16^{INK4a} flank	5′-AGAAAGAGGAGGGGTTGGTTGG-3′	5′-ACRCCCRCACCTCCTCTACC-3′	193	56	35
p16^{INK4a} flank short	5′-GGGTTGGTTGGTTATTAGAGGGT-3′	5′-RACCRTAACCAACCAATCAACC-3′	148	56	35
p16^{INK4a} U	5′-TTATTAGAGGGTGGGGTGGATTGT-3′	5′-CAACCCCAAACCACAACCATAA-3′	151	60	30
p16^{INK4a} U short	5′-GTTGGTTATTAGAGGGTGGGGTGGATTGT-3′	5′-AACCAAAAACTCCATACTACTCCCCACCA-3′	124	62	25
p16^{INK4a} M	5′-TTATTAGAGGGTGGGGCGGATCGC-3′	5′-GACCCCGAACCGCGACCGTAA-3′	150	60	30
p16^{INK4a} M short	5′-TTATTAGAGGGTGGGGCGGATCGC-3′	5′-GAAAACTCCATACTACTCCCCGCCG-3′	115	62	25
O^6-MGMT flank	5′-GYGTTTYGGATATGTTGGGATAGTT-3′	5′-AAACTCCRCACTCTTCCRAAAAC-3′	135	56	35
O^6-MGMT U	5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′	5′-AACTCCACACTCTTCCAAAAACAAAACA-3′	93	60	30
O^6-MGMT M	5′-TTTCGACGTTCGTAGGTTTTCGC-3′	5′-GCACTCTTCCGAAAACGAAACG-3′	81	60	30
hMLH1 flank	5′-GGAGTGAAGGAGGTTAYGGGTAAGT-3′	5′-AAAAACRATAAAACCCTATACCTAATCTATC-3′	182	56	35
hMLH1 flank short	5′-TTTTGAYGTAGAYGTTTTATTAGGGT-3′	5′-AAAACRATAAAACCCTATACCTAATCTATC-3′	154	56	35
hMLH1 U	5′-TTTTGATGTAGATGTTTTATTAGGGTTGT-3′	5′-ACCACCTCATCATAACTACCCACA-3′	124	60	35
hMLH1 U short	5′-TGTGTGTTTGTTGTTTGTTATATATTGTTT-3′	5′-ACCACCTCATCATAACTACCCACA-3′	98	60	35
hMLH1 M	5′-ACGTAGACGTTTTATTAGGGTCGC-3′	5′-CCTCATCGTAACTACCCGCG-3′	115	60	35
hMLH1 M short	5′-GTTCGTCGTTCGTTATATATCGTTC-3′	5′-CCTCATCGTAACTACCCGCG-3′	89	60	35
RASSF1A flank	5′-GTTTAGTTTGGATTTTGGGGGAG-3′	5′-CCCRCAACTCAATAAACTCAAACTC-3′	144	56	35
RASSF1A U	5′-GGGGTTTGTTTTGTGGTTTTGTTT-3′	5′-AACATAACCCAATTAAACCCATACTTCA-3′	81	60	30
RASSF1A M	5′-GGGTTCGTTTTGTGGTTTCGTTC-3′	5′-TAACCCGATTAAACCCGTACTTCG-3′	76	60	30

[a] U, unmethylated DNA specific primers; M, methylated DNA specific primers.

Table 3 *Prevalence of promoter methylation, clinicopathological parameters and other patient characteristics for the total number of CRCs and for the high and low methyl donor intake groups*

	Total (n = 122)	Methyl donor intake		P
		High (n = 61)	Low (n = 61)	
Methylation				
Promoter methylation				
APC	47/122 (39%)	20/61 (33%)	27/61 (44%)	0.193
p14^{ARF}	40/120 (33%)	17/60 (28%)	23/60 (38%)	0.245
p16^{INK4}	37/119 (31%)	18/59 (31%)	19/60 (32%)	0.891
hMLH1	35/122 (29%)	17/61 (28%)	18/61 (30%)	0.841
O^6-MGMT	50/121 (41%)	22/61 (36%)	28/60 (47%)	0.236
RASSF1A	24/122 (20%)	9/61 (15%)	15/61 (25%)	0.172
Number of genes methylated				
0	28/122 (23%)	18/61 (30%)	10/61 (16%)	
1	25/122 (20%)	12/61 (20%)	13/61 (21%)	
2	29/122 (24%)	13/61 (21%)	16/61 (26%)	
3	18/122 (15%)	8/61 (13%)	10/61 (16%)	
4	16/122 (13%)	9/61 (15%)	7/61 (12%)	
5	4/122 (3%)	1/61 (2%)	3/61 (5%)	
6	2/122 (2%)	0/61 (0%)	2/61 (3%)	0.411
At least 1 gene methylated	94/122 (77%)	43/61 (70%)	51/61 (84%)	0.085
Clinicopathological parameters				
Dukes stage[a]				
A	30 (26%)	17 (29%)	13 (23%)	
B	39 (34%)	18 (30%)	21 (37%)	
C	31 (27%)	13 (22%)	18 (32%)	
D	15 (13%)	11 (19%)	4 (7%)	0.19
Location of tumor				
Colon	84 (69%)	45 (74%)	39 (64%)	
Rectosigmoid	14 (11%)	4 (7%)	10 (16%)	
Rectum	24 (20%)	12 (20%)	12 (20%)	0.223
Proximal location (yes)[b]	75 (62%)	36 (60%)	39 (64%)	0.498
Other patient characteristics				
Age at diagnosis (yr)	67.4	66.7	68.1	0.086
Gender (male)	68 (56%)	27 (44%)	41 (67%)	0.011
Family history of CRC (yes)	19 (16%)	9 (15%)	10 (16%)	0.803
Dietary factors				
Energy intake (kcal)	1951	2105	1797	0.002
Fiber intake (g)	27	32.9	21.2	0.019
Vitamin C intake (mg)	107	135.1	79	0.001
Iron intake (mg)	12.7	14.6	10.8	0.022

[a] For 7 cases, Dukes stage is not reported.
[b] For 1 case, proximal/distal location was not determined.

MSP analysis could easily be performed for *APC-1A*, *O^6-MGMT*, and *RASSF1A*, genes for which the designed MSP amplicons are relatively small, *i.e.*, <165 bp (see Table 2). However, using the flanking MSP primers originally designed for *p14^{ARF}*, *p16^{INK4a}*, and *hMLH1* (30), the success rate for amplification decreased with increasing size of the MSP amplicons. Bisulfite-treated DNA could not be amplified for *p14^{ARF}* (207 bp) in 18% (22 of 121), for *p16^{INK4a}* (193 bp) in 12% (14 of 120), and for *hMLH1* (182 bp) in 16% (20 of 123) of CRC cases. To increase the MSP success rate, MSP primers for these promoter regions were redesigned to obtain shorter amplicons (<155 bp). Using these primer sets, a subgroup of CRC cases (n = 52), which did and which did not amplify using the original MSP primer sets, were amplified using the redesigned MSP primers. The MSP success rate increased to 94% (49 of 52) for *p14^{ARF}*, 90% (47 of 52) for *p16^{INK4a}*, and 100% (52 of 52) for *hMLH1*. The concordance between the original and the redesigned short primer sets was tested and was found to be 94% (31 of 33) for *p16^{INK4a}*, 94% (30 of 32) for *hMLH1*, and 93% (25 of 27) for *p14^{ARF}*. Because of the rate of concordance between the different primer sets, data using both sets of primers were pooled. Genes that showed methylation using only one of both primer sets were considered as methylated.

Overall, promoter hypermethylation frequencies observed were: 39% (47 of 122) for *APC-1A*; 33% (40 of 120) for *p14^{ARF}*; 31% (37 of 119) for *p16^{INK4A}*; 29% (35 of 122) for *hMLH1*; 41% (50 of 121) for *O^6-MGMT*; and 20% (24 of 122) for *RASSF1A* (Table 3). Representative examples of the MSP reactions are shown in Fig. 1.

For all genes, the prevalence of promoter hypermethylation was higher in CRCs derived from patients with low methyl donor intake when compared with CRCs from patients with high methyl donor intake, however, none of these individual differences reached statistical significance. The percentage of CRCs with at least one gene methylated *versus* no gene methylated was higher (84%) in the low methyl donor intake group when compared with the adequate/high methyl donor intake group (70%; P = 0.085; Table 3).

Dukes stage and location of the tumor did not differ between the two groups of patients (Table 3). Age at diagnosis was higher in patients with low methyl donor intake. There were significantly more males in the low methyl donor intake group (67%) when compared with the high methyl donor intake group (44%; P = 0.011; Table 3). Factors previously shown to be associated with folate intake, *i.e.*, energy-, fiber-, vitamin C-, and iron intake (2), were all significantly lower in patients with low methyl donor intake compared with patients with adequate/high methyl donor intake (Table 3).

Table 4 summarizes the Dukes stage, location of the tumor, age at diagnosis and energy-, fiber-, vitamin C-, and iron intake, none of which are associated with promoter methylation. Family history of cancer frequency was higher (25%) in patients who had none of the six studied genes methylated, compared with patients with at least one gene methylated (13%), but the difference did not reach statistical significance (Table 4). Table 4 also shows that gender is significantly associated with promoter methylation (P = 0.046). Sixty-one percent of patients were male in the group with at least one gene methylated *versus* 39% in the group with no genes methylated (Table 4). Caution is warranted in interpretation because these results are based on a selection of extreme intakes.

The OR for promoter methylation of at least one gene methylated

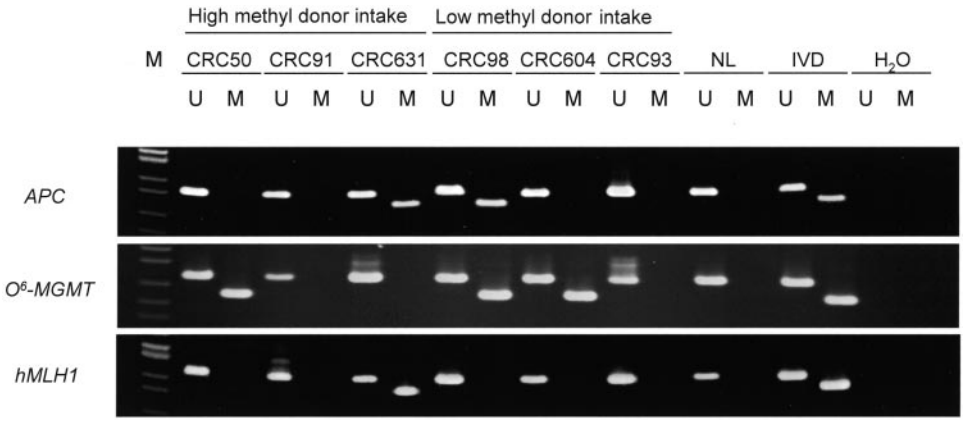

Fig. 1. Representative examples of *APC-1A*-, O^6*-MGMT*-, and *hMLH1*-nested MSP reactions of six primary CRCs and controls. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated alleles; the presence of product in those lanes marked M indicates the presence of methylated alleles. All CRCs include amplification with the U primer set, probably a result of the presence of normal, contaminating tissue. Normal lymphocytes (NL) and *in vitro* methylated DNA (IVD) were used as negative and positive controls for *APC-1A*, O^6*-MGMT*, and *hMLH1* promoter methylation, respectively. The H_2O control was included in the flanking PCR and subsequently in the MSP reaction.

was 2.13 (95% CI, 0.89–5.11) for patients with low methyl donor intake *versus* high methyl donor intake. Adjustment for age, sex, energy-, fiber-, vitamin C-, and iron intake and family history of cancer revealed an OR of 1.82 (95% CI, 0.49–6.78). When the methyl donor intake variable with nine categories was modeled in a similar way, a positive association with promoter methylation of at least one gene was still observed, although not statistically significant (unadjusted OR, 1.14, $P = 0.124$; adjusted OR, 1.12, $P = 0.420$).

DISCUSSION

A better understanding of the causality of CRC can be established by combining epidemiology and research on molecular mechanisms. Here, this approach was used to study whether dietary intake of folate and alcohol is associated with hypermethylation of tumor suppressor- and DNA repair genes in CRC specimens derived from the NLCS. Nested MSP analysis was optimized, and as a result, almost all CRC specimens, irrespective of the quality of the DNA, could be analyzed for hypermethylation in the promoter regions of *APC-1A*, $p14^{ARF}$, $p16^{INK4A}$, *hMLH1*, O^6*-MGMT*, and *RASSF1A*. This optimized nested MSP approach enables high throughput promoter methylation analysis in archival, formalin-fixed and paraffin-embedded tissues for molecular epidemiology studies.

For $p14^{ARF}$, $p16^{INK4A}$, and O^6*-MGMT*, the overall prevalence of promoter methylation, *i.e.*, 33, 31, and 41%, respectively, are in the range of the prevalence reported for CRC (22, 31, 32). For *RASSF1A*, the prevalence of promoter methylation was similar to the frequency reported on a larger series of CRCs from the same cohort (23). For *APC-1A* and *hMLH1*, the overall prevalence is higher than reported thus far (30, 33). This difference might be because the CRC material used in this study is obtained from patients from a different geographic area (the Netherlands) compared with the patient material used in the other studies (United States).

Table 4 *Clinicopathological parameters and other patient characteristics in CRCs with no genes versus at least one gene methylated*

	No genes methylated ($n = 28$)	At least one gene methylated ($n = 94$)	P
Clinicopathological parameters			
Dukes stage			
A	7/26 (27%)	23/89 (26%)	
B	10/26 (39%)	29/89 (33%)	
C	4/26 (15%)	27/89 (30%)	
D	5/26 (19%)	10/89 (11%)	0.412
Location of tumor			
Colon	19/28 (68%)	65/94 (69%)	
Rectosigmoid	2/28 (7%)	11/94 (12%)	
Rectum	7/28 (25%)	18/94 (19%)	0.675
Proximal location (yes)	19/28 (68%)	56/93 (60%)	0.465
Other patient characteristics			
Age at diagnosis (yr)	67.8	67.3	0.581
Gender (male)	11/28 (39%)	57/94 (61%)	0.046
Family history of cancer (yes)	7/28 (25%)	12/94 (13%)	0.117
Dietary factors			
Energy intake (kcal)	1937	1955	0.854
Fiber intake (g)	28.6	26.6	0.274
Vitamin C intake (mg)	115	104	0.339
Iron intake (mg)	13.2	12.6	0.368

Although not significant, our results indicate that methyl donor deficiency is associated with overall methylation of multiple genes, an effect which is stronger than the effect on specific promoter regions itself. The association, although not statistically significant, between the nine different categories of methyl donor intake and promoter methylation of at least one gene supports this. However, these results have to be interpreted with caution because only extreme categories were used, and intermediate categories of methyl donor intake in this pilot study were omitted. In addition, stratification of patients was done primarily on folate intake and secondarily on alcohol intake. Therefore, the conclusions of this study are based primarily on folate intake. Other dietary factors of importance for methyl donor intake (*e.g.*, vitamins B6 and B12) were not accounted for in this selection of patients.

Increasing the power of the study, including CRCs from patients with the complete spectrum of folate and alcohol intake and analysis of the effect of intake of other methyl donors such as vitamins B6 and B12, will reveal whether the observed effects of methyl donor deficiency is stable. In addition, it is necessary to study whether there is effect modification by alcohol (as suggested with overall CRC in the First National Health and Nutrition Examination Survey study; Ref. 7) in the complete group of CRCs derived from the NLCS. Another drawback of this study is that only case-case and not case-cohort analyses were performed. Knowledge on global hypomethylation status, which is hypothesized to occur previously to regional hypermethylation, would also be interesting to analyze with respect to promoter hypermethylation. In addition, it is possible that the observed effect of folate deficiency on promoter methylation will be stronger after stratification for functionally important SNPs in genes involved in folate metabolism. Methylenetetrahydrafolate reductase, methionine synthase, and methionine synthase reductase are interesting enzymes involved in the generation of S-adenosylmethionine, the primary methyl donor for DNA methylation reactions. All three enzymes have been reported to have common SNPs, which are associated with enhanced thermolability and decreased enzyme activity. The effect of the SNPs on colorectal and other cancer risks also seems to be dependent on methionine, folate, vitamins B6 and B12, and alcohol intake (34–40).

In conclusion, despite its limited size, this study suggests that methyl donor intake is associated with an increased frequency of promoter hypermethylation of genes involved in CRC carcinogenesis.

ACKNOWLEDGMENTS

We thank Marco M. M. Pachen for technical assistance and Jasper E. Manning and Kam-Wing Jair for helpful suggestions. We also thank Sacha H. M. van de Crommert, Willy van Dijk, Marijke I. G. Moll, Jolanda J. H. Nelissen, Conny W. C. de Zwart, Harry P. L. van Montfort, and Ruud J. G. C. Schmeitz for data input and data management.

REFERENCES

1. Potter, J. D. Colorectal cancer: molecules and populations. J. Natl. Cancer Inst. (Bethesda), *91:* 916–932, 1999.
2. Konings, E. J., Goldbohm, R. A., Brants, H. A., Saris, W. H., and van den Brandt, P. A. Intake of dietary folate vitamers and risk of colorectal carcinoma: results from the Netherlands Cohort Study. Cancer (Phila.), *95:* 1421–1433, 2002.
3. Steinmetz, K. A., and Potter, J. D. Vegetables, fruit, and cancer prevention: a review. J. Am. Diet. Assoc., *96:* 1027–1039, 1996.
4. Freudenheim, J. L., Graham, S., Marshall, J. R., Haughey, B. P., Cholewinski, S., and Wilkinson, G. Folate intake and carcinogenesis of the colon and rectum. Int. J. Epidemiol., *20:* 368–374, 1991.
5. Giovannucci, E., Stampfer, M. J., Colditz, G. A., Hunter, D. J., Fuchs, C., Rosner, B. A., Speizer, F. E., and Willett, W. C. Multivitamin use, folate, and colon cancer in women in the Nurses' Health Study. Ann. Intern. Med., *129:* 517–524, 1998.
6. Fuchs, C. S., Willett, W. C., Colditz, G. A., Hunter, D. J., Stampfer, M. J., Speizer, F. E., and Giovannucci, E. L. The influence of folate and multivitamin use on the familial risk of colon cancer in women. Cancer Epidemiol. Biomark. Prev., *11:* 227–234, 2002.
7. Su, L. J., and Arab, L. Nutritional status of folate and colon cancer risk: evidence from NHANES I epidemiologic follow-up study. Ann. Epidemiol., *11:* 65–72, 2001.
8. Benito, E., Stiggelbout, A., Bosch, F. X., Obrador, A., Kaldor, J., Mulet, M., and Munoz, N. Nutritional factors in colorectal cancer risk: a case-control study in Majorca. Int. J. Cancer, *49:* 161–167, 1991.
9. Meyer, F., and White, E. Alcohol and nutrients in relation to colon cancer in middle-aged adults. Am. J. Epidemiol., *138:* 225–236, 1993.
10. Ferraroni, M., La Vecchia, C., D'Avanzo, B., Negri, E., Franceschi, S., and Decarli, A. Selected micronutrient intake and the risk of colorectal cancer. Br. J. Cancer, *70:* 1150–1155, 1994.
11. Boutron-Ruault, M. C., Senesse, P., Faivre, J., Couillault, C., and Belghiti, C. Folate and alcohol intakes: related or independent roles in the adenoma-carcinoma sequence? Nutr. Cancer, *26:* 337–346, 1996.
12. Homann, N., Tillonen, J., and Salaspuro, M. Microbially produced acetaldehyde from ethanol may increase the risk of colon cancer via folate deficiency. Int. J. Cancer, *86:* 169–173, 2000.
13. Martinez, M. E., Maltzman, T., Marshall, J. R., Einspahr, J., Reid, M. E., Sampliner, R., Ahnen, D. J., Hamilton, S. R., and Alberts, D. S. Risk factors for Ki-ras protooncogene mutation in sporadic colorectal adenomas. Cancer Res., *59:* 5181–5185, 1999.
14. Slattery, M. L., Curtin, K., Anderson, K., Ma, K. N., Edwards, S., Leppert, M., Potter, J., Schaffer, D., and Samowitz, W. S. Associations between dietary intake and Ki-ras mutations in colon tumors: a population-based study. Cancer Res., *60:* 6935–6941, 2000.
15. Fenech, M. The role of folic acid and Vitamin B12 in genomic stability of human cells. Mutat. Res., *475:* 57–67, 2001.
16. Duthie, S. J. Folic acid deficiency and cancer: mechanisms of DNA instability. Br. Med. Bull., *55:* 578–592, 1999.
17. Duthie, S. J., Narayanan, S., Blum, S., Pirie, L., and Brand, G. M. Folate deficiency *in vitro* induces uracil misincorporation and DNA hypomethylation and inhibits DNA excision repair in immortalized normal human colon epithelial cells. Nutr. Cancer, *37:* 245–251, 2000.
18. Feinberg, A. P., Gehrke, C. W., Kuo, K. C., and Ehrlich, M. Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res., *48:* 1159–1161, 1988.
19. Feinberg, A. P., and Vogelstein, B. Alterations in DNA methylation in human colon neoplasia. Semin. Surg. Oncol., *3:* 149–151, 1987.
20. Warnecke, P. M., and Bestor, T. H. Cytosine methylation and human cancer. Curr. Opin. Oncol., *12:* 68–73, 2000.
21. Bariol, C., Suter, C., Cheong, K., Ku, S. L., Meagher, A., Hawkins, N., and Ward, R. The relationship between hypomethylation and CpG island methylation in colorectal neoplasia. Am. J. Pathol., *162:* 1361–1371, 2003.
22. Esteller, M., Corn, P. G., Baylin, S. B., and Herman, J. G. A gene hypermethylation profile of human cancer. Cancer Res., *61:* 3225–3229, 2001.
23. van Engeland, M., Roemen, G. M., Brink, M., Pachen, M. M., Weijenberg, M. P., de Bruïne, A. P., Arends, J-W., van den Brandt, P. A., de Goeij, A. F., and Herman, J. G. K-ras mutations and RASSF1A promoter methylation in colorectal cancer. Oncogene, *21:* 3792–3795, 2002.
24. van den Brandt, P. A., Goldbohm, R. A., van 't Veer, P., Volovics, A., Hermus, R. J., and Sturmans, F. A large-scale prospective cohort study on diet and cancer in the Netherlands. J. Clin. Epidemiol., *43:* 285–295, 1990.
25. Van den Brandt, P. A., Schouten, L. J., Goldbohm, R. A., Dorant, E., and Hunen, P. M. Development of a record linkage protocol for use in the Dutch Cancer Registry for Epidemiological Research. Int. J. Epidemiol., *19:* 553–558, 1990.
26. Goldbohm, R. A., van den Brandt, P. A., and Dorant, E. Estimation of the coverage of Dutch municipalities by cancer registries and PALGA based on hospital discharge data. Tijdschr. Soc. Gezondheidsz., *72:* 80–84, 1994.
27. Konings, E. J., Roomans, H. H., Dorant, E., Goldbohm, R. A., Saris, W. H., and van den Brandt, P. A. Folate intake of the Dutch population according to newly established liquid chromatography data for foods. Am. J. Clin. Nutr., *73:* 765–776, 2001.
28. Goldbohm, R. A., van den Brandt, P. A., Brants, H. A., van't Veer, P., Al, M., Sturmans, F., and Hermus, R. J. Validation of a dietary questionnaire used in a large-scale prospective cohort study on diet and cancer. Eur. J. Clin. Nutr., *48:* 253–265, 1994.
29. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA, *93:* 9821–9826, 1996.
30. Herman, J. G., Umar, A., Polyak, K., Graff, J. R., Ahuja, N., Issa, J. P., Markowitz, S., Willson, J. K., Hamilton, S. R., Kinzler, K. W., Kane, M. F., Kolodner, R. D., Vogelstein, B., Kunkel, T. A., and Baylin, S. B. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc. Natl. Acad. Sci. USA, *95:* 6870–6875, 1998.
31. Esteller, M., Tortola, S., Toyota, M., Capella, G., Peinado, M. A., Baylin, S. B., and Herman, J. G. Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. Cancer Res., *60:* 129–133, 2000.
32. Esteller, M., Hamilton, S. R., Burger, P. C., Baylin, S. B., and Herman, J. G. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res., *59:* 793–797, 1999.
33. Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M. A., Gonzalez, S., Tarafa, G., Sidransky, D., Meltzer, S. J., Baylin, S. B., and Herman, J. G. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res., *60:* 4366–4371, 2000.
34. Ma, J., Stampfer, M. J., Giovannucci, E., Artigas, C., Hunter, D. J., Fuchs, C., Willett, W. C., Selhub, J., Hennekens, C. H., and Rozen, R. Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. Cancer Res., *57:* 1098–1102, 1997.
35. Slattery, M. L., Potter, J. D., Samowitz, W., Schaffer, D., and Leppert, M. Methylenetetrahydrofolate reductase, diet, and risk of colon cancer. Cancer Epidemiol. Biomark. Prev., *8:* 513–518, 1999.
36. Ulrich, C. M., Kampman, E., Bigler, J., Schwartz, S. M., Chen, C., Bostick, R., Fosdick, L., Beresford, S. A., Yasui, Y., and Potter, J. D. Colorectal adenomas and the C677T MTHFR polymorphism: evidence for gene-environment interaction? Cancer Epidemiol. Biomark. Prev., *8:* 659–668, 1999.
37. Levine, A. J., Siegmund, K. D., Ervin, C. M., Diep, A., Lee, E. R., Frankl, H. D., and Haile, R. W. The methylenetetrahydrofolate reductase 677C→T polymorphism and distal colorectal adenoma risk. Cancer Epidemiol. Biomark. Prev., *9:* 657–663, 2000.
38. Ulvik, A., Evensen, E. T., Lien, E. A., Hoff, G., Vollset, S. E., Majak, B. M., and Ueland, P. M. Smoking, folate and methylenetetrahydrofolate reductase status as interactive determinants of adenomatous and hyperplastic polyps of colorectum. Am. J. Med. Genet., *101:* 246–254, 2001.
39. Ma, J., Stampfer, M. J., Christensen, B., Giovannucci, E., Hunter, D. J., Chen, J., Willett, W. C., Selhub, J., Hennekens, C. H., Gravel, R., and Rozen, R. A polymorphism of the methionine synthase gene: association with plasma folate, vitamin B12, homocyst(e)ine, and colorectal cancer risk. Cancer Epidemiol. Biomark. Prev., *8:* 825–829, 1999.
40. Matsuo, K., Suzuki, R., Hamajima, N., Ogura, M., Kagami, Y., Taji, H., Kondoh, E., Maeda, S., Asakura, S., Kaba, S., Nakamura, S., Seto, M., Morishima, Y., and Tajima, K. Association between polymorphisms of folate- and methionine- metabolizing enzymes and susceptibility to malignant lymphoma. Blood, *97:* 3205–3209, 2001.