Dietary Factors and Microsatellite Instability in Sporadic Colon Carcinomas

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Abstract

Microsatellite instability (MSI) occurs in 10–20% of the sporadic colon carcinomas and appears to be primarily due to alterations in *hMLH1* and *hMSH2*. Little is known about the role of diet in MSI-related colon carcinogenesis. We used data from a Dutch population-based case-control study on sporadic colon carcinomas (184 cases and 259 controls) to evaluate associations between dietary factors previously reported as being associated with colon cancer risk and MSI, hMLH1 expression, and *hMLH1* hypermethylation. Red meat intake was significantly differently related to microsatellite instability-high (MSI-H) tumors compared with microsatellite instability-low/microsatellite stable (MSI-L/MSS) [odds ratio (OR), 0.3; 95% confidence interval (CI), 0.1–0.9]. It was inversely associated with MSI-H tumors when compared with the population-based controls (OR, 0.5; 95% CI, 0.2–1.2) and positively associated with MSI-L/MSS tumors (OR, 1.5; 95% CI, 0.9–2.6). A positive association was observed for alcohol intake with MSI-H tumors (OR, 1.9; 95% CI, 0.8–4.7). Fruit consumption seemed to especially decrease the risk of MSI-H tumors with hypermethylated *hMLH1* (Methyl$^+$ tumors) [Methyl$^+$ *versus* controls: OR = 0.4 and 95% CI = 0.2–0.9; MSI-H tumors without hypermethylated *hMLH1* (Methyl$^-$ tumors) *versus* controls, OR = 1.2 and 95% CI = 0.8–1.7; Methyl$^+$ *versus* Methyl$^-$ tumors, OR = 0.2 and 95% CI = 0.1–0.9]. Most other evaluated dietary factors were not distinctively associated with a specific MSI or *hMLH1* methylation status. Our data suggest that red meat consumption may enhance the development of MSI-L/MSS carcinomas in particular, whereas alcohol intake appears to increase the risk of MSI-H tumors. Fruit consumption may especially decrease the risk of MSI-H carcinomas exhibiting epigenetically silenced *hMLH1*.

Introduction

Approximately 10–20% of the sporadic colon carcinomas and most colon tumors associated with the hereditary nonpolyposis colorectal cancer syndrome are characterized by MSI[5] (1–5). MSI is a hallmark of DNA MMR deficiency that in turn appears to be primarily due to inherited and/or acquired alterations in the MMR genes *hMLH1* and *hMSH2*. The presence of MSI correlates well with the absence of either hMLH1 or hMSH2 (6–8). In sporadic colon carcinomas, loss of hMLH1 expression is frequently the result of hypermethylation of the promoter region of *hMLH1*, whereas loss of hMSH2 expression seems to occur through genetic mutations only (9–11).

Diet has been repeatedly implicated in the etiology of colon cancer, and certain dietary factors, especially those previously reported to be associated with colon cancer risk, may well specifically influence the development of microsatellite unstable colon carcinomas. Most colon cancers exhibit MSI or CIN, another type of genetic instability, but not both (12). This suggests different molecular pathways to colon cancer that may reflect different environmental exposures (13). Supporting this idea, Bardelli *et al.* (14) demonstrated that exposure to the alkylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanide produced tumor cells characterized by MSI, whereas exposure to the bulky-adduct-forming agent 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine produced tumor cells characterized by CIN.

Thus far, few epidemiological studies have examined associations between diet and MSI, and knowledge about the role of dietary factors in MSI-related sporadic colon carcinogenesis is limited. Slattery *et al.* (15) reported a positive association between long-term alcohol consumption and occurrence of MSI. The only other epidemiological study on dietary factors and occurrence of MSI in colon carcinomas published to date reported a positive association between well-done red meat consumption and MSI (16). Associations between dietary factors and MMR protein expression or *hMLH1* promoter hypermethylation have, to our knowledge, not been examined previously.

In this study, we evaluate associations between dietary factors and the occurrence of MSI, as determined with the Bethesda reference panel markers (17), in a Dutch population-based case-control study on sporadic colon carcinomas. To further explore the relationship between diet and the presence

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[5] The abbreviations used are: MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSI-L/MSS, microsatellite instability-low/microsatellite stable; MMR, mismatch repair; CIN, chromosomal instability; OR, odds ratio; CI, confidence interval; TNM, tumor-node-metastasis.

of MSI, we additionally assess associations with MMR protein expression and *hMLH1* promoter hypermethylation.

Materials and Methods

Study Population. A population-based case-control study on diet and colon cancer was conducted in the Netherlands between 1989 and 1993. Details were described previously (18). In short, cases ($n = 204$) were women and men newly diagnosed with first primary incident colon carcinoma. They were recruited in regional hospitals in the Netherlands and invited to participate by their medical specialists within 3 months of diagnosis. Sixty percent of those invited agreed to participate. Controls ($n = 259$), frequency-matched to the cases by age (5-year intervals), sex, region, and degree of urbanization, were randomly recruited by the general practitioners of the cases. Of the controls invited, 57% agreed to participate. All subjects were Dutch-speaking Caucasians, up to 75 years old at time of diagnosis, mentally competent to complete the interview, and had no known personal history of cancer, familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer, ulcerative colitis, or Crohn's disease. Except for a more favorable Dukes' stage in cases, participants did not differ importantly from nonparticipants. Formalin-fixed, paraffin-embedded colon tumor tissue, collected before the start of chemo- or radiotherapy, was available from 185 cases; normal tissue (that is, tumor-free colon tissue) was available from 159 cases. In 2000, it became known that one of the cases exhibited a germ-line mutation in one of the MMR genes. This case was excluded from the analyses, leaving a final number of 184 cases.

Data Collection. Usual dietary habits were assessed by an interview-based questionnaire. The questionnaire covered the complete dietary pattern. The interval between diagnosis and interview was, for cases, 3–6 months. The consumption frequency per month in the preceding year (for cases, the year preceding diagnosis or symptoms), number of months during which the item was used, number of portions per consumption, and portion sizes of 289 food items were collected. Average daily intake of nutrients was calculated using the Dutch National Food Table (19). The interviewing of cases and controls was balanced over seasons to account for seasonal fluctuations in food patterns. During the interview, information was also obtained on current and previous smoking habits, aspirin and nonsteroidal anti-inflammatory drug use, family history of colorectal cancer, and personal medical history. Information on the location of the tumors was obtained from pathological reports. TNM tumor stage was determined by re-evaluation of the information in the pathological reports.

DNA Extraction. Both tumor and normal DNA were extracted from formalin-fixed, paraffin-embedded tissue as described elsewhere (20). Microdissection was performed, and for tumor DNA, only those areas containing >60% tumor cells were used; normal DNA was isolated from tumor-free colon tissue.

MSI. Paired tumor and normal DNA were analyzed for MSI with the five Bethesda reference panel markers (17): *BAT25*; *BAT26*; *D5S346*; *D2S123*; and *D17S250*. When matching normal DNA was not available ($n = 25$; *BAT25/26*-only group), only *BAT25* and *BAT26* were checked for instability. Methods have been discussed in detail elsewhere (21). Tumors were classified as MSI-H if two or more markers showed instability and as MSI-L/MSS if one or none of the markers examined showed instability (17).

Immunohistochemical Analysis. All tumors were subjected to immunohistochemical analysis to determine hMLH1 and hMSH2 expression. Immunohistochemical staining was performed on 4-μm sections of formalin-fixed, paraffin-embedded tissue using standard procedures. After deparaffinization and rehydration, endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide for 30 min. Antigen retrieval was accomplished by boiling for 10 min in 1 mM EDTA (pH 8.0) for hMLH1 and in 10 mM citrate buffer (pH 6.0) for hMSH2. Nonspecific antibody binding was prevented by preincubating the sections with 10% normal horse serum in 1% BSA/PBS for 10 min. Subsequently, sections were incubated overnight at 4°C with monoclonal antibodies against human hMLH1 (clone G168-15; dilution 1:100; BD PharMingen International/Becton Dickinson) or hMSH2 (clone GB12; dilution 1:40; Oncogene Research Products). Antibody binding was detected using the DAKO Envision+ System (DAKO Corp.) for hMLH1 and the Vectastain ABC KIT (Brunschwig, Amsterdam, the Netherlands) for hMSH2. Diaminobenzidine was used for visualization, and sections were counterstained with hematoxylin. Staining was evaluated using normal cells as internal control. Loss of expression was recorded when nuclear staining was present in surrounding normal cells and absent in tumor cells.

Methylation Analysis. A PCR-based *Hpa*II-*Msp*I restriction enzyme assay was used to determine hypermethylation of the promoter region of *hMLH1* in MSI-H tumors. The region analyzed, −593 to −312, includes four *Hpa*II/*Msp*I sites (at positions −339, −345, −525, and −565, relative to the transcription start site; GenBank accession number U83845.1). Tumor DNA was digested, in separate reaction tubes, with *Hpa*II (Roche) and *Msp*I (Roche). Additionally a "digest" was performed with H_2O instead of restriction enzyme ("undigested"). The digests (total volume, 10 μl) contained 40 ng of DNA, 1 μl of *Hpa*II or *Msp*I or H_2O, and 1 μl of SuRE/Cut Buffer L (Roche). All samples were incubated overnight at 37°C. Subsequently, the samples were subjected to PCR, which was performed in a total volume of 50 μl containing 5 μl of PCR buffer II (Applied Biosystems), 10 pmol of forward primer (sequence, 5′-GACCAGGCACAGGGCCCCATCGC) and reverse primer (sequence, 5′-ATATCCAGCCAATAGGAGCAGAGATG), 0.3 unit of AmpliTaq Gold (Applied Biosystems), and the 10 μl of digested DNA. PCR reaction conditions were as follows: 10 min at 94°C; followed by 38 cycles of 40 s at 92°C, 45 s at 58°C, and 1 min at 72°C; followed by 5 min at 72°C. The PCR products were loaded (undigested, *Hpa*II, and *Msp*I) on an ethidium bromide-stained 2% agarose gel, with PCR products originating from the same tumor in adjacent lanes. Tumors were scored positive for *hMLH1* promoter hypermethylation if PCR product was present in the undigested lane and *Hpa*II-treated lane, but not in the *Msp*I-treated lane; tumors were scored negative if PCR product was present in the undigested lane only. Note that this method does not reveal whether only one or both *hMLH1* alleles are hypermethylated. The upstream region of *hMSH2* was not examined for hypermethylation because *hMSH2* does not seem to be prone to hypermethylation-associated inactivation (10).

Statistical Analysis. The distribution of MSI, hMLH1, and hMSH2 expression and *hMLH1* promoter hypermethylation in the colon carcinomas was determined. Differences in tumor characteristics between MSI-H and MSI-L/MSS cases were assessed using *t* tests for continuous variables and χ^2 tests for categorical variables; $P < 0.05$ was considered significant. The categorization of the dietary factors in tertiles and the interquartile ranges (Q_3 to Q_1; used to quantify associations on a continuous scale) were based on the distribution of intake in the

Table 1 Characteristics of the study population

	Controls (n = 259)	Cases		P^a
		MSI-H (n = 40; 22%)	MSI-L/MSS (n = 144; 78%)	
Age (in yrs, mean ± SD)	61.8 ± 10.0	61.9 ± 10.1	61.8 ± 10.4	0.97
Sex (% women)	47.5	45.0	45.8	0.93
Family history of colorectal cancer (%)	11.5	16.7	21.1	0.56
Body mass index (kg/m^2, mean ± SD)	26.0 ± 3.8	27.3 ± 5.4	25.5 ± 4.0	0.06
Ever smoked (%)	69.1	67.5	70.4	0.72
Dietary factors (g/day, mean ± SD)				
Vegetables	207.9 ± 124.2	168.4 ± 75.4	170.6 ± 82.1	0.88
Fruit	230.8 ± 180.6	214.7 ± 167.1	214.6 ± 160.6	0.996
Red meat	73.9 ± 34.1	69.2 ± 38.0	81.1 ± 35.2	0.07
Fish	18.2 ± 21.2	20.2 ± 26.7	22.3 ± 33.8	0.72
Dairy products	268.2 ± 244.3	351.0 ± 337.4	322.5 ± 330.1	0.63
Alcohol[b]	13.7 ± 17.6	18.7 ± 23.6	15.9 ± 25.3	0.53
Dietary fiber[b]	28.8 ± 8.3	27.6 ± 10.0	27.0 ± 8.3	0.70
Calcium[b] (mg/day)	1254.3 ± 406.3	1263.4 ± 436.9	1275.0 ± 424.4	0.88
Vitamin C[b] (mg/day)	117.5 ± 53.8	93.5 ± 42.9	105.5 ± 53.8	0.19
Total energy intake (kJ/day)	9362 ± 2844	10783 ± 3762	10197 ± 3053	0.31
Tumor characteristics				
Tumor location (% proximal)	n.a.[c]	67.5	34.7	<0.01
TNM stage (% I/II)	n.a.	75.0	58.3	0.07

[a] MSI-H *versus* MSI-L/MSS, t test for continuous variables and χ^2 test for categorical variables.
[b] Adjusted for total energy intake by regression analysis, for women and men separately.
[c] n.a., not applicable.

control population. Energy-adjusted nutrient intake was computed separately for women and men as the residual from the regression model with total energy as the independent variable and absolute nutrient intake as the dependent variable. Subsequently, the mean nutrient intake was added to each residual (22). Case-control comparisons, comparing cases with a specific tumor status (*e.g.*, MSI-H) with the population-based controls, were conducted to estimate the relative risk of developing carcinomas with this particular status. In addition, case-case comparisons were conducted to evaluate heterogeneity in dietary risk factors for the different tumor subsets. ORs and corresponding 95% CIs were calculated using multiple logistic regression models. Linear trend was assessed using the tertile medians as continuous variables in multiple logistic regression models. All analyses were adjusted for age (years, continuously), sex, body mass index (kg/m^2, continuously) and total energy intake (kJ/day, continuously). Alcohol intake was additionally adjusted for cigarette smoking (never, ever). Additional adjustment for TNM stage, tumor location, cigarette smoking, and other dietary factors did not change the estimates significantly (that is, not more than 10%). All analyses were performed with the use of the SAS statistical software package (SAS version 8.0; SAS Institute Inc., Cary, NC).

Results

Forty of the 184 (22%) colon carcinomas included in this study were MSI-H. All other tumors showed either instability in one (n = 20; 11%) or in none (n = 124; 67%) of the markers and were classified as MSI-L/MSS. In 10 of the tumors for which matching normal tissue was not available, *BAT25* and *BAT26* were both unstable; both markers were stable in the other 15 tumors. Characteristics of the study population are presented in Table 1. No statistically significant differences between cases with MSI-H tumors and cases with MSI-L/MSS tumors were observed. However, body mass index seemed higher and meat intake seemed lower among participants with MSI-H tumors. Proximal tumors (that is, those located in caecum, ascending colon, hepatic flexure, or transverse colon) and TNM stage I/II tumors were more common, but the difference of the latter was statistically nonsignificant, in the MSI-H subset.

Table 2 shows results of case-control and case-case comparisons conducted to assess associations between the various dietary factors and MSI-H tumors and MSI-L/MSS tumors. Vegetable intake was inversely associated with MSI-H tumors as well as MSI-L/MSS tumors when the two tumor subsets were separately compared with the population-based controls. Red meat consumption was inversely, but statistically nonsignificantly, associated with MSI-H tumors and positively, but again statistically nonsignificantly, associated with MSI-L/MSS tumors. Interestingly, case-case comparison showed that red meat consumption was significantly differently related to MSI-H tumors than to MSI-L/MSS tumors. Additional adjustment for vegetable and fruit intake and cigarette smoking did not change the observed associations for red meat consumption significantly. Vitamin C intake showed association patterns similar to those observed for vegetable consumption. Although statistically nonsignificant, alcohol intake seemed to increase the risk of MSI-H tumors in particular (Table 2).

To gain insight in the underlying cause(s) of MSI, expression of hMLH1 and hMSH2 was determined in all tumors by immunohistochemistry, and all MSI-H tumors were examined for hypermethylation of the promoter region of *hMLH1*. Twenty-six of the MSI-H tumors showed absence of hMLH1 expression (MLH1^{neg} tumors); six showed absence of hMSH2 expression (MSH2^{neg} tumors); none of the tumors showed absence of both proteins; and, hMLH1 and hMSH2 were both present in all MSI-L/MSS tumors (Table 3). Twenty MSI-H tumors exhibited *hMLH1* promoter hypermethylation; hMSH2 was present in all hypermethylated tumors; hMLH1 was present in three hypermethylated tumors (Table 3). The *hMLH1* promoter methylation status of three MSI-H tumors could not be determined due to PCR failure. Two of these tumors (including one MSI-H tumor of the *BAT25/26*-only group) showed absence of hMLH1 expression; hMLH1 and hMSH2 were both present in the other

Table 2 Dietary factors and MSI[a] in sporadic colon carcinomas: case-control and case-case comparisons

	ORs (95% CIs)[b]				
	T1	T2	T3	P^{trend}	Continuous[c]
Vegetables (g/day)	≤166	166–223	>223		/106
No. MSI-H/MSS/controls	20/70/87	12/45/87	8/29/85		40/144/259
All cases *vs.* controls	1.0	0.6 (0.4–1.0)	0.4 (0.2–0.6)	<0.01	0.6 (0.5–0.8)
MSI-H *vs.* controls	1.0	0.5 (0.2–1.2)	0.4 (0.1–0.9)	0.02	0.6 (0.4–0.9)
MSS *vs.* controls	1.0	0.7 (0.4–1.1)	0.4 (0.2–0.7)	<0.01	0.6 (0.5–0.8)
MSI-H *vs.* MSS	1.0	0.9 (0.4–2.0)	0.8 (0.3–2.2)	0.67	0.9 (0.6–1.5)
Fruit (g/day)	≤142	142–269	≥269		/164
No. MSI-H/MSS/controls	15/51/87	15/49/85	10/44/87		40/144/259
All cases *vs.* controls	1.0	0.9 (0.6–1.5)	0.7 (0.5–1.2)	0.22	0.9 (0.7–1.0)
MSI-H *vs.* controls	1.0	1.0 (0.4–2.3)	0.6 (0.2–1.4)	0.21	0.8 (0.6–1.2)
MSS *vs.* controls	1.0	0.9 (0.6–1.5)	0.8 (0.5–1.3)	0.40	0.9 (0.7–1.1)
MSI-H *vs.* MSS	1.0	1.1 (0.5–2.6)	0.7 (0.3–1.8)	0.47	0.9 (0.7–1.4)
Red meat (g/day)	<58	58–87	≥87		/43
No. MSI-H/MSS/controls	16/33/85	11/56/87	13/55/87		40/144/259
All cases *vs.* controls	1.0	1.3 (0.8–2.1)	1.2 (0.7–1.9)	0.60	1.1 (0.8–1.4)
MSI-H *vs.* controls	1.0	0.6 (0.3–1.5)	0.5 (0.2–1.2)	0.11	0.6 (0.4–1.0)
MSS *vs.* controls	1.0	1.6 (1.0–2.8)	1.5 (0.9–2.6)	0.19	1.2 (0.9–1.6)
MSI-H *vs.* MSS	1.0	0.3 (0.1–0.8)	0.3 (0.1–0.9)	0.03	0.5 (0.3–0.9)
Fish (g/day)	<7	7–19	>19		/20
No. MSI-H/MSS/controls	15/43/82	9/41/91	16/60/86		40/144/259
All cases *vs.* controls	1.0	0.8 (0.5–1.3)	1.1 (0.7–1.7)	0.62	1.1 (0.9–1.2)
MSI-H *vs.* controls	1.0	0.6 (0.2–1.5)	0.9 (0.4–1.9)	0.84	1.0 (0.7–1.3)
MSS *vs.* controls	1.0	0.8 (0.5–1.4)	1.2 (0.7–2.0)	0.43	1.1 (0.9–1.3)
MSI-H *vs.* MSS	1.0	0.6 (0.2–1.6)	0.6 (0.3–1.4)	0.27	0.9 (0.7–1.1)
Dairy products (g/day)	≤117	117–305	>305		/287
No. MSI-H/MSS/controls	12/45/87	12/48/86	16/51/86		40/144/259
All cases *vs.* controls	1.0	1.0 (0.6–1.6)	1.0 (0.6–1.6)	0.87	1.1 (0.9–1.4)
MSI-H *vs.* controls	1.0	0.9 (0.4–2.3)	1.1 (0.5–2.7)	0.70	1.2 (0.8–1.7)
MSS *vs.* controls	1.0	1.0 (0.6–1.6)	0.9 (0.5–1.6)	0.77	1.1 (0.9–1.4)
MSI-H *vs.* MSS	1.0	1.0 (0.4–2.5)	1.3 (0.5–3.2)	0.54	1.1 (0.8–1.5)
Alcohol[d] (g/day)	<3.8	3.8–12.9	>12.9		/18.4
No. MSI-H/MSS/controls	12/57/86	10/31/87	18/56/86		40/144/259
All cases *vs.* controls	1.0	0.8 (0.5–1.3)	1.2 (0.7–1.9)	0.30	1.1 (1.0–1.4)
MSI-H *vs.* controls	1.0	1.2 (0.5–3.2)	1.9 (0.8–4.7)	0.13	1.2 (0.9–1.7)
MSS *vs.* controls	1.0	0.7 (0.4–1.2)	1.0 (0.6–1.8)	0.49	1.1 (0.9–1.4)
MSI-H *vs.* MSS	1.0	1.7 (0.6–4.6)	1.7 (0.7–4.1)	0.36	1.1 (0.9–1.4)
Dietary fiber[d] (g/day)	<25	25–31	>31		/10.3
No. MSI-H/MSS/controls	17/69/86	11/32/87	12/43/86		40/144/259
All cases *vs.* controls	1.0	0.5 (0.3–0.8)	0.6 (0.4–1.0)	0.03	0.7 (0.6–1.0)
MSI-H *vs.* controls	1.0	0.8 (0.3–1.8)	0.7 (0.3–1.7)	0.48	0.8 (0.5–1.3)
MSS *vs.* controls	1.0	0.5 (0.3–0.8)	0.6 (0.3–1.0)	0.03	0.7 (0.5–0.9)
MSI-H *vs.* MSS	1.0	1.6 (0.6–3.9)	1.2 (0.5–2.9)	0.71	1.1 (0.7–1.8)
Calcium[d] (mg/day)	≤1062	1062–1358	>1358		/458.3
No. MSI-H/MSS/controls	12/48/87	13/35/86	15/61/86		40/144/259
All cases *vs.* controls	1.0	0.9 (0.6–1.5)	1.3 (0.8–2.1)	0.22	1.0 (0.8–1.3)
MSI-H *vs.* controls	1.0	1.4 (0.6–3.4)	1.5 (0.6–3.5)	0.42	1.1 (0.7–1.5)
MSS *vs.* controls	1.0	0.8 (0.5–1.4)	1.3 (0.8–2.2)	0.24	1.0 (0.8–1.3)
MSI-H *vs.* MSS	1.0	1.8 (0.7–4.5)	1.1 (0.4–2.6)	0.99	1.0 (0.7–1.5)
Vitamin C[d] (mg/day)	≤89	89–133	>133		/68.5
No. MSI-H/MSS/controls	17/62/87	18/49/86	5/33/86		40/144/259
All cases *vs.* controls	1.0	0.9 (0.5–1.4)	0.5 (0.3–0.8)	<0.01	0.7 (0.5–0.9)
MSI-H *vs.* controls	1.0	1.2 (0.5–2.5)	0.3 (0.1–0.8)	0.03	0.5 (0.3–0.8)
MSS *vs.* controls	1.0	0.8 (0.5–1.3)	0.5 (0.3–0.9)	0.02	0.7 (0.6–1.0)
MSI-H *vs.* MSS	1.0	1.3 (0.6–2.9)	0.5 (0.2–1.6)	0.38	0.7 (0.4–1.2)

[a] The subset MSI-L/MSS is, for brevity, called MSS in this table.
[b] Adjusted for age, sex, body mass index, and total energy intake. Alcohol is additionally adjusted for cigarette smoking. Trend was assessed using the median values of the tertiles as continuous variables.
[c] Per interquartile range (Q_3 to Q_1).
[d] Adjusted for total energy intake by regression analysis, for women and men separately.

tumor. Furthermore, 6 of the 10 MSI-H tumors of the *BAT25/26*-only group showed absence of hMLH1 expression; hMLH1 and hMSH2 were both present in the other 4 tumors. Four of the MSI-H tumors of the *BAT25/26*-only group exhibited *hMLH1* promoter hypermethylation, hMLH1 was present in one of these tumors. MSI-H tumors with hypermethylated *hMLH1*and hMLH1 not present were classified as $Methyl^+$ tumors (*i.e.*, $Methyl^+$ tumors are a subset of the $MLH1^{neg}$ tumors; $n = 17$); MSI-H tumors without hypermethylated *hMLH1* were classified as $Methyl^-$ tumors ($n = 17$).

Table 3 Expression of hMLH1 and hMSH2 and *hMLH1* promoter methylation status[a] *N* (%)

	All tumors (n = 184)	MSI-H (n = 40)	MSI-L/MSS (n = 144)	Hypermethylation	
				Yes (n = 20)	No (n = 17)
hMLH1 absent	26 (14)	26 (65)	0	17 (85)	7 (41)
hMSH2 absent	6 (3)	6 (15)	0	0	6 (35)
hMLH1 and hMSH2 present	152 (83)	8 (20)	144 (100)	3 (15)	4 (24)

[a] The *hMLH1* promoter methylation status of three MSI-H tumors could not be determined.

Subsequently, we assessed associations (quantified on a continuous scale) between the various dietary factors and absence of hMLH1 expression and *hMLH1* promoter methylation status. Associations observed with $MLH1^{neg}$ tumors generally did not differ significantly from those observed with MSI-H tumors (data not shown). We were unable to separately evaluate associations with absence of hMSH2 expression because only six tumors in our study population exhibited this phenotype. Fruit consumption was inversely associated with $Methyl^+$ tumors and positively associated with $Methyl^-$ tumors when the two subsets were separately compared with the population-based controls [$Methyl^+$, OR, 0.4 (95% CI, 0.2–0.9); $Methyl^-$, OR, 1.2 (95% CI, 0.8–1.7); per 164 g/day]. Case-case comparison showed that fruit consumption was significantly differently related to $Methyl^+$ than to $Methyl^-$ tumors [$Methyl^+$ *versus* $Methyl^-$, OR, 0.2 (95% CI, 0.1–0.9)]. Vegetable consumption and red meat intake were both inversely associated with $Methyl^+$ tumors as well as $Methyl^-$ tumors [vegetables (per 106 g/day): $Methyl^+$ *versus* controls, OR, 0.5 (95% CI, 0.3–1.0); $Methyl^-$ *versus* controls, OR, 0.6 (95% CI, 0.3–1.1). Red meat (per 43 g/day): $Methyl^+$ *versus* controls, OR, 0.4 (95% CI, 0.2-.09); $Methyl^-$ *versus* controls, OR, 0.5 (95% CI, 0.3–1.0)]. They were, like most other evaluated dietary factors, not specifically associated with $Methyl^+$ tumors or $Methyl^-$ tumors but seemed to affect both pathways to MSI-H tumors equally (data not shown).

Discussion

In this study, we evaluated associations between dietary factors previously reported to be associated with colon cancer risk and occurrence of MSI, hMLH1 expression, and *hMLH1* promoter hypermethylation in sporadic colon carcinomas. Red meat intake was significantly differently related to MSI-H tumors as compared with MSI-L/MSS tumors. A positive association was observed with MSI-L/MSS tumors, whereas an inverse association was observed with MSI-H tumors. Alcohol intake seemed to increase the risk of MSI-H tumors in particular. Interestingly, fruit consumption was significantly differently related to $Methyl^+$ tumors compared with $Methyl^-$ tumors, suggesting differences in the role fruit intake plays in the etiology of these two distinct MSI-H colon carcinoma subsets. Vegetable consumption lowered the risk of MSI-H tumors as well as MSI-L/MSS tumors but, like most other evaluated dietary factors, was not distinctively associated with a specific MSI or *hMLH1* promoter methylation status.

Overall, 22% of the 184 sporadic colon carcinomas were MSI-H. This is consistent with frequencies reported previously (5, 7, 15). MSI-H tumors were more common in the *BAT25/26*-only group than in the group for which matching normal tissue was available. However, this is probably due to chance, and we do not expect that our decision to use only *BAT25* and *BAT26* tumor results when matching normal tissue was not available has resulted in extensive misclassification or led to serious misinterpretation of our results. Six of the 10 MSI-H tumors of the *BAT25/26*-only group also showed loss of hMLH1 expression. Inclusion of the four tumors that expressed hMLH1 as well hMSH2 in the MSI-L/MSS group instead of the MSI-H group did not change the observed associations significantly (data not shown).

Immunohistochemistry showed that most (65%) MSI-H tumors in our study population had a *hMLH1*-associated etiology. In 17 of the 24 (71%) $MLH1^{neg}$ tumors in which promoter methylation status could be determined, *hMLH1* was inactivated by promoter hypermethylation. This is in line with what has been reported for sporadic colon cancers by others (5, 9–11, 23). MSI-H as determined with the Bethesda reference panel had a 100% sensitivity for identifying colon tumors with hMLH1 or hMSH2 loss of expression. HMLH1 and hMSH2 were both present in eight of the MSI-H tumors. Possibly these tumors expressed altered, nonfunctional hMLH1 or hMSH2 protein that could be detected by immunohistochemistry (24), or the MSI may have been the result of alterations in one of the other MMR genes. With regard to the three MSI-H tumors with hMLH1 and hMSH2 expression that showed hypermethylation of the *hMLH1* promoter, hypermethylation possibly affected only one of the two *hMLH1* alleles in these tumors, or it affected some CpG sites but left other sites, whose methylation might be necessary for inactivation of *hMLH1*, intact (25).

As in any retrospective case-control study, the possibility of information and selection bias is an important concern. Cases and controls were asked to recall their diets from the past, and differential recall is possible. However, because cases are unaware, for instance, of the MSI status of their tumors, systematic errors in recall are less likely to bias results from case-case comparisons. Our cases were relatively healthy. That is, the frequency of TNM stage I/II tumors among the cases was relatively high (62%), compared with the frequency reported by the Dutch Cancer Registry (51%; Ref. 26).

In this study, red meat intake was significantly differently related to MSI-H tumors as compared with MSI-L/MSS tumors. It increased the risk of MSI-L/MSS tumors, whereas an inverse association was observed with MSI-H tumors. Red meat prepared at high temperatures is a major source of heterocyclic amines. Heterocyclic amines are bulky-adduct-forming agents. They are mutagenic and carcinogenic in animals (27), and those present in red meat have been found to be associated with increased risk of colorectal cancer in humans (28). A possible explanation, admittedly speculative, for the observed associations with red meat intake is that genes involved in the pathway that results in MSI-L/MSS tumors, *e.g.*, *APC* (29, 30), are more susceptible to mutations caused by red meat consumption than *hMLH1* and *hMSH2*, and/or that "red meat" mutations in MSI-L/MSS pathway-related genes exert a higher selective growth advantage. In addition, most MSI-L/MSS tumors probably exhibit CIN (12); thus, viewing red meat consumption as

a surrogate marker for exposure to heterocyclic amines, our results are in line with the observations of Bardelli *et al.* (14).

Previously, Slattery *et al.* (15) observed no associations between red meat intake and MSI status of colon carcinomas in a large population-based case-control study. Wu *et al.* (16) reported a positive association between well-done red meat consumption and MSI-H tumors in a case-only study but did not observe a significant association with red meat intake in general. The dissimilarities with our results might be caused by differences in meat cooking methods and/or differences in the composition of the study populations, *e.g.*, in the frequency of participants with a positive family history of colorectal cancer, or chance.

None of the other dietary factors evaluated in this study was significantly differently related to MSI-H tumors as compared with MSI-L/MSS tumors. Consistent with Slattery *et al.* (15), alcohol intake did seem more positively associated with MSI-H tumors than with MSI-L/MSS tumors. In our study, however, the associations were statistically nonsignificant. This may be due to the smaller size of our study population.

This is, to our knowledge, the first study that has evaluated associations between dietary factors and *hMLH1* promoter hypermethylation in colon carcinomas. Fruit consumption was significantly differently related to Methyl$^+$ tumors compared with Methyl$^-$ tumors. It decreased the risk of Methyl$^+$ tumors, whereas a positive association was observed with Methyl$^-$ tumors. This suggests, assuming that epigenetically silenced *hMLH1* and genetically inactivated hMLH1 or hMSH2 exert the same selective advantage for MSI-H tumor formation, that fruits or their constituents are specifically involved in the prevention of *hMLH1* promoter hypermethylation. The exact mechanisms responsible for silencing of specific genes by promoter hypermethylation are not yet clear. However, fruits may be interrelated with DNA methylation through involvement in the supply of methyl groups and/or through involvement in processes that modify utilization of methyl groups, *e.g.*, regulation of DNA methyltransferase activity.

To conclude, our data suggest that, in subjects not suspected of carrying an inherited mutation in one of the MMR genes, red meat consumption may promote the development of MSI-L/MSS carcinomas in particular, whereas alcohol intake appears to increase the risk of MSI-H tumors. Fruit consumption may especially decrease the risk of MSI-H carcinomas exhibiting epigenetically silenced *hMLH1*. Our study population was relatively small, and, hence, the statistical power of the study was relatively low. This may explain why only a few statistically significant associations were observed. Although all evaluated dietary factors were previously reported to be associated with colon cancer risk, it should additionally be noted that multiple comparisons might lead to chance findings. Thus, confirmation of our results by other studies is necessary. Nonetheless, the observed relation between fruit consumption and *hMLH1* promoter methylation status is intriguing and calls for further investigation. Epigenetic events such as *hMLH1* promoter hypermethylation are, by definition, susceptible to change. Elucidation of the mechanisms through which dietary factors influence (gene-specific) epigenetic events may prove useful for the development of effective dietary intervention strategies for colon cancer prevention.

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Dietary Factors and Microsatellite Instability in Sporadic Colon Carcinomas

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