

CpG Island Methylation in Sporadic Colorectal Cancers and Its Relationship to Microsatellite Instability

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Background & Aims: Methylation of CpG islands is increasingly recognized as an important event in colorectal carcinogenesis. We evaluated the extent of CpG island methylation in 426 sporadic colorectal cancers to define its relationship to microsatellite instability and to describe its clinicopathologic and genetic features. **Methods:** Fresh cancer tissue was obtained from 417 consecutive individuals undergoing curative surgery for sporadic colorectal cancer. Methylation of p16 and hMLH1 promoters was determined by methylation-specific polymerase chain reaction (PCR), whereas methylation at MINT 1, 2, 12, and 31 loci was assessed by bisulfite PCR. Microsatellite instability and K-ras and p53 status were determined using microsatellite PCR, restriction enzyme-mediated PCR, and immunohistochemistry, respectively. **Results:** Individual loci were commonly methylated, but locus-specific phenotypic changes were not seen. CpG island methylation was associated with right-sided location, female sex, and older age, as well as high tumor grade, mucinous type, wild-type P53, microsatellite instability, and K-ras mutations. More than half of tumors showing CpG island methylation were microsatellite stable. Compared with microsatellite unstable cancers, they were more commonly left-sided, had fewer intraepithelial lymphocytes, presented later, and had a worse outcome. **Conclusions:** Colorectal cancers with CpG island methylation have distinct clinicopathologic features and in some cases lead to sporadic microsatellite unstable cancers.

It has long been recognized that the patterns of DNA methylation are grossly perturbed in cancer cells. These changes include global reductions in methylation^{1,2} and regional areas of hypermethylation at CpG islands.³ In recent times, the association between hypermethylation of CpG islands and transcriptional silencing of a number of genes has served to focus attention on this process.^{4–6} Although there is still a great deal of debate surrounding the exact role of methylation in gene silencing, it remains at least “the signature for the mechanisms responsible for loss of gene function.”⁷

In the case of colorectal cancer, there is an indisputable link between biallelic methylation of the hMLH1 promoter and the development of microsatellite instability (MSI).^{8–12} Further studies on the role of CpG island methylation in colorectal cancer have identified certain sites in the genome that are preferentially methylated in tumors (MINT loci), and the term CpG island methylator phenotype (CIMP) has been used to describe tumors in which these MINT loci are commonly methylated.¹³ Importantly, tumors with CIMP were also shown to have methylation of known tumor suppressor genes such as p16¹³ and THBS1¹⁴ as well as the DNA mismatch repair gene hMLH1.¹³ A further finding of the study by Toyota et al. was that methylation of specific MINT loci was only detected in cancer tissue and not in normal mucosa.¹³

Because MSI arises through a process of methylation of the hMLH1 promoter, it is not surprising that a significant overlap between CIMP and sporadic MSI cancers has been reported.^{13,14} Furthermore, a number of studies have clearly indicated that the pattern of DNA methylation in tumors is strongly influenced by age, sex, and anatomic site.^{15–17} In light of these potentially complex interactions, it is not surprising that the clinicopathologic characteristics of tumors arising in association with methylation remain poorly defined.

In this study, we evaluated the frequency and extent of CpG island methylation in a large consecutive series of sporadic colorectal cancers. We chose to examine the methylation status of CpG islands that have previously been reported as correlating most closely with CIMP, namely MINT 1, 2, 12, and 31 as well as p16.¹³ These loci are said to be tumor specific in that they are rarely methylated in normal mucosa, and observations made by us in preliminary experiments supported this. For two

Abbreviations used in this paper: CI, confidence interval; CIMP, CpG island methylator phenotype; MSI, microsatellite instability; MSS, microsatellite stable; OR, odds ratio; PCR, polymerase chain reaction.

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important reasons, we specifically avoided the use of the hMLH1 promoter in the identification of methylated cancers. First, hMLH1 promoter methylation is causally associated with MSI. Thus, its inclusion in a panel of markers defining DNA methylation would compromise the analysis of any relationship between this and MSI. Second, methylation of hMLH1 does not seem to be tumor specific and has often been reported in histologically normal colonic mucosa.¹⁸

In undertaking this study, we sought to better understand the relationship between CpG island methylation and the development of MSI as well as more closely describe the clinicopathologic and genetic features of cancers that show widespread CpG island methylation.

Materials and Methods

Patients and Specimens

After obtaining informed consent, 417 consecutive individuals (with 426 colorectal tumors) undergoing surgical resection of adenocarcinoma of the colon or rectum at St. Vincent's Hospital, Sydney, were enrolled in this prospective study from 1993 to January 2001. Patients with inflammatory bowel disease or a known history of familial adenomatous polyposis or hereditary nonpolyposis coli were excluded from the study.¹⁹ Fresh representative tissue samples (500 μ g) from all tumors and paired normal colonic mucosa from 426 colorectal cancer specimens were immediately frozen at -70°C . The DNA from 9 colorectal tumors from 9 individuals was of insufficient quality or quantity for analysis; therefore, these individuals were also excluded from further analysis. The remaining 417 tumor samples were subject to DNA analysis. Furthermore, 70 paired normal mucosal samples were selected from this group for subsequent evaluation of the tumor specificity of methylation at the chosen sites.

After the collection of tumor material, 12 individuals (13 tumors) were classified as definite or suspected hereditary nonpolyposis coli because they met the following criteria: known germline defect (5 individuals), an MSI cancer showing MSH2 loss by immunostaining (4 individuals), or MSI cancer showing MLH1 loss in the absence of MLH1 promoter methylation (3 individuals). These individuals and their tumors were excluded from further analysis. Therefore, the evaluable population consisted of 396 individuals (175 women and 221 men) with a mean age of 68.7 ± 12.1 years (range, 29–96 years). Women in the study population were older at presentation (mean age, 71.4 ± 12.5 years) than men (mean age, 66.6 ± 11.3 years). These individuals had a total of 404 tumors, of which 71 were TNM stage I (17.6%), 153 were stage II (37.9%), 123 were stage III (30.4%), and 57 were stage IV (14.1%).²⁰ Fresh tumor tissue was collected from 3 individuals with metachronous tumors and 6 individuals with synchronous tumors.

Family histories of colorectal carcinoma and other malignancies were obtained by interviewing patients or their next of

kin. Treatment with chemotherapy and/or radiotherapy was recorded for each patient, and follow-up was undertaken for 5 years or until death. Cancer recurrence dates and causes of death were verified by death certificates, medical records, or the treating physician.

Histopathologic Analysis of Tumors

For all tumors, the histopathologic type, stage, and size of the tumor were determined independently by a histopathologist within the Department of Anatomical Pathology at St. Vincent's Hospital. The tumor grade, extent of mucin production, and tumor growth pattern as well as the presence of a Crohn's-like inflammatory infiltrate, intraepithelial lymphocytes, or peritumoral lymphocytes were determined prospectively without knowledge of the mismatch repair status. Tumors in which $<10\%$ of cells formed glands were classified as high grade (poorly differentiated), whereas those containing more than 50% extracellular mucin were classified as mucinous.²¹ The tumor growth pattern was interpreted as either infiltrative or expansile, as per previously published criteria.²² The extent of peritumoral and Crohn's-like lymphoid reactions was classified according to the method of Jass et al.²² Intraepithelial lymphocytes were identified by light microscopy on H&E sections as cells with the morphology of lymphocytes, seen wholly within tumor epithelium. They were classified as conspicuous when more than 30 were present per 10 high-power fields.

DNA Extraction and Bisulfite Modification

For preparation of DNA from fresh tissues, the frozen tissue was macerated in 500 μ L ice-cold lysis buffer, 10 mmol/L Tris-HCl, pH 7.8, 1 mmol/L EDTA, 100 mmol/L NaCl, 1% sodium dodecyl sulfate, and 500 μ g/mL proteinase K using a sterile Eppendorf homogenizer. After incubation overnight with shaking at 50°C , DNA was extracted with phenol/chloroform and precipitated with ethanol. Bisulfite modification of DNA was performed by the incubation of 2 μ g DNA in 50 μ L 0.2 mol/L NaOH at 37°C for 10 minutes and then again for 20 hours at 50°C in the presence of 0.5 mmol/L hydroquinone and 2.6 mol/L sodium bisulfite.²³ DNA was resin purified, treated with 0.3 mol/L NaOH for 5 minutes at room temperature, and precipitated.

Methylation-Specific Polymerase Chain Reaction: Detection of Promoter Methylation in p16 and hMLH1

Analysis of hMLH1 was performed as previously described.⁹ For p16, the polymerase chain reaction (PCR) reaction mix (20 μ L) contained 100 ng bisulfite-modified DNA in FastStart reaction buffer (Roche Diagnostics GmbH, Mannheim, Germany), 2 mmol/L MgCl_2 , 200 μ mol/L deoxynucleoside triphosphate, 1.25 U FastStart Taq DNA polymerase (Roche Diagnostics GmbH), and 0.8 μ mol/L of each primer.²³ The primers used for the amplification of unmethylated DNA were 5'-TTATTAGAGGGTGGGGTGGATTGT-3' (sense)

and 5'-CAACCCCAAACCACAACCATAA-3' (antisense) and for methylated DNA were 5'-TTATTAGAGGGTGGGGCG-GATCGC-3' (sense) and 5'-GACCCCGAACCGCGACCG-TAA-3' (antisense). For the unmethylated primers, the reactions were incubated initially at 95°C for 5 minutes and then for 10 cycles at 95°C for 30 seconds, 73°C touchdown 1°C per cycle for 30 seconds, 72°C for 30 seconds, then 25 cycles of 95°C for 30 seconds, 63°C for 30 seconds, and 72°C for 30 seconds before a final extension at 72°C for 4 minutes. For the methylated primers, the following conditions were used: 1 cycle at 95°C for 5 minutes; 10 cycles at 95°C for 30 seconds, 78°C touchdown 1°C per cycle for 30 seconds; 25 cycles at 95°C for 30 seconds, 68°C for 30 seconds, and 72°C for 30 seconds before a final extension at 72°C for 4 minutes. All reactions were performed using a Perkin Elmer Gene Amp PCR System 9600 (Perkin Elmer, Norwalk, CT). All PCR amplicons were analyzed by electrophoresis on a 10% polyacrylamide gel, and each PCR run included separate reactions with template from the p16 methylated control colorectal cell lines SW48 or T84 and normal peripheral blood lymphocytes as well as controls with no template. The expected size of PCR products was 150 base pairs and 151 base pairs for methylated and unmethylated reactions, respectively. The analysis of hMLH1 and p16 methylation was performed separately by 2 of the investigators without knowledge of other PCR results or tumor details.

Bisulfite PCR and Restriction Enzyme Analysis

The methylation status of MINT 1, 2, 12, and 31 was determined using primers and conditions as previously described.^{13,24} An additional primer set (5'-TTYGTTTATTA-GAGTATTTTYGGTGG-3' [sense] and 5'-CCRTACRC-CTTCTACAATTAAC-3' [antisense]) was used for the analysis of MINT2 to improve the specificity of this reaction.

Each PCR reaction mix (20 µL) contained 50 ng bisulfite-modified DNA in FastStart reaction buffer (Roche Diagnostics GmbH), 2.5 mmol/L MgCl₂, 200 µmol/L deoxynucleoside triphosphate, 1.25 U FastStart Taq DNA polymerase (Roche Diagnostics GmbH), and 0.74 µmol/L of each primer. For MINT 1, 2, and 31, the reactions were incubated at 95°C for 5 minutes and then for 35 cycles at 95°C for 30 seconds, 55°C for 15 seconds, 72°C for 30 seconds, and finally 72°C for 4 minutes. For MINT12, the following conditions were used: 1 cycle at 95°C for 5 minutes followed by a 45-cycle touchdown protocol of 95°C for 30 seconds, 68°C (3 cycles), 56°C (4 cycles), 54°C (5 cycles), and 52°C (33 cycles) for 15 seconds each and 72°C for 30 seconds; this protocol was followed by a final cycle at 72°C for 4 minutes. All PCR amplicons were analyzed by electrophoresis before restriction digestion with the relevant enzymes. MINT 1 and 2 amplicons (5 µL) were digested at 65°C for 4 hours with 20 U *TaqI* (MBI, Fermentas, Lithuania), MINT12 amplicons with 10 U *TaiI* (MBI) at 65°C for 4 hours, and MINT31 amplicons with 20 U *BstUI* (New England Biolabs, Boston, MA) for 4 hours at 60°C. Restriction digests were performed according to the manufacturer's in-

structions and were analyzed by electrophoresis on either 10% polyacrylamide or 2.5% agarose using ethidium bromide staining and visualization with UV illumination.

Each PCR run and restriction digest included separate reactions with template from the positive control cell line DLD1. As above, each reaction was interpreted separately by 2 of the investigators without knowledge of other PCR results or details for that tumor and was classified as methylated, unmethylated, or not assessable. A final result was determined only when both observers were in agreement. In the case of discordant interpretation, analysis was repeated on 2 further occasions; if concordance was not obtained, the result was considered nonassessable at that locus.

There were 25 tumors in which amplification was not achievable at 1 of 5 loci. In 12 of these cases, methylation at the 4 assessable loci was either entirely absent (10) or present at all loci (2), and these were considered as 0% or 100% methylated for the purposes of quantitative analysis in this study. Likewise, the 9 cases in which methylation was found at only 1 of 4 loci were considered to show methylation at 20% of loci, whereas the 4 cases with methylation at 3 of 4 loci were considered to show 80% methylation. No cases showed methylation at 2 of 4 loci.

Analysis of Microsatellite Status and K-ras

The microsatellite status of each tumor was determined by amplifying DNA from tumor and paired normal samples in a 10-µL volume containing 100 ng DNA, 200 µmol/L deoxynucleoside triphosphate, 3.0 mmol/L MgCl₂, 0.27 µmol/L of each primer, 0.25 U Tth polymerase in a buffer of 16.6 mmol/L (NH₄)₂SO₄, 0.45% Triton X-100, 0.2 mg/mL gelatin, and 67 mmol/L Tris-HCl, pH 8.8 (Biotech International Ltd., Perth, Western Australia).²⁵ The reactions were incubated at 95°C for 2 minutes, followed by 30 cycles at 95°C for 45 seconds, 57°C for 45 seconds, and 72°C for 1 minute. The primers used in this study were Bar25, Bar26, D5S346, D2S123, and D17S250.²⁶ Products were run on an ABI 377 sequencer and analyzed using Genescan and Genotyper software (ABI, Foster City, CA). Two observers (R.W. and E.M.) performed the analysis of MSI, and the results were reported without knowledge of pathologic or immunohistochemical status. A tumor sample was considered to show MSI if 2 or more of the markers showed instability. When one or none of the markers showed instability, the tumor was designated as microsatellite stable (MSS).

Mutations at the first and second bases of codon 12 of the *K-ras* gene were detected using REMS-PCR (restriction endonuclease-mediated selective polymerase chain reaction) as previously described.²⁷

Immunohistochemical Analysis of p53, hMLH1, and hMSH2 Protein

Detection of mismatch repair enzymes was performed on 4-µm paraffin sections of tumor tissue as previously described²⁵ using monoclonal anti-human hMLH1 and hMSH2 antibodies (Pharmingen, San Diego, CA). Staining was con-

sidered assessable when nuclear staining was seen in either stromal or germinal follicle lymphocytes or in normal epithelial cells in the base of the crypts. A complete absence of staining of tumor cells for one of the mismatch repair proteins in the presence of staining for the other protein was considered indicative of absence of expression. For the identification of accumulation of p53 within tumor cells, paraffin sections of tumor tissue were subjected to immunohistochemical analysis of p53 as previously described²⁸ using the mouse anti-human p53 antibody DO7 (DAKO, Dakopatts, Denmark). Tumor was considered to show accumulation of p53 protein when more than 20% of tumor cells showed nuclear staining of moderate to high intensity, in the absence of staining in the stromal cells and normal epithelium. All immunohistochemical analysis was performed with the investigator blinded to the clinical parameters of the tumor.

Statistical Analysis

Categorical variables were compared using the χ^2 test or the Fisher exact test as appropriate. In the comparison with MSI status, Student *t* test was used for analysis of patient age. Tumor methylation was considered as an interval variable (0–5). Because it showed a skewed distribution, nonparametric statistical methods were used to analyze data. Specifically, the Wilcoxon rank sum test was used to compare extent of methylation with categorical variables, whereas the Spearman ρ test was used to determine the relationship between extent of methylation and age. Logistic regression analysis was used to examine the relationship between tumor progression and degree of methylation. In this analysis, tumor stage was considered as a dichotomous variable (early, stages I and II; advanced, stages III and IV) and the odds ratio (OR) and 95% confidence intervals (CIs) were determined for a variety of factors. Survival was measured from the date of resection of colorectal cancer until either death, the completion of 5 years of follow-up, or the censor date (January 31, 2001). Individuals dying from causes other than cancer were censored at the date of death. For the 284 individuals alive at the time of the last follow-up, the median follow-up period was 30.9 months (range, 0.9–60 months). Survival curves were prepared according to the method of Kaplan and Meier, and statistical significance between curves was tested using the log-rank test. Univariate survival analysis was performed by Cox proportional hazards regression model, and the relative risk and its 95% CI were assessed for each factor. A multivariate analysis was used to determine which factors were independent prognostic factors for survival. A probability value of <0.05 was considered significant. All data were analyzed using SPSS statistical software version 9.0 (SPSS Inc., Chicago, IL).

Results

Assessment of CpG Island Methylation

The typical results of PCR analysis of bisulfite-treated DNA for methylation of MINT loci as well as p16 are shown in Figure 1.

The frequency of methylation at these loci in 404 colorectal tumors is given in Table 1. Methylation was assessable in more than 99% of cases for most loci. In the case of MINT2, initial analysis using published primers¹³ resulted in a relatively high frequency of nonassessable cases (28%). Because of this, a revised primer set was designed as described, and this decreased the rate of nonassessable cases at this locus to 5% (21 of 404). Results obtained with the new primer set were concordant with those assessable results obtained with the initial MINT2 primer set (data not shown).

To confirm that the methylation loci were indeed tumor specific, analysis of methylation in paired normal mucosa was performed from 70 cases in this study, including 26 cases with methylation at more than 2 loci. There was evidence of methylation in only 2 of these normal mucosal samples and then at only 1 locus (MINT12 in each case). In the corresponding 70 tumor samples from these cases, the frequency of methylation at each locus was as follows: MINT1-27, 39%; MINT2-24, 34%; MINT12-28, 40%; MINT31-16, 23%; p16-26, 37%.

CpG Island Methylation at Specific Loci

For all tumors examined, the rate of methylation at individual loci was between 25% and 29%, with the exception of MINT31, which had a relatively low rate of positivity (14%). Table 1 shows the relationships between the presence of methylation at individual loci and a range of clinicopathologic features of individuals and their tumors. A number of these features were significantly associated with methylation at each of the different loci examined in this study. Specifically, methylation at each locus was more common in older individuals and in women. Furthermore, irrespective of the locus analyzed, methylation was associated with right-sidedness, mucinous tumor type, prominent intraepithelial lymphocytes, and MSI (Table 1). In contrast, mutations of *K-ras* were more common in tumors showing methylation at MINT2 ($P = 0.027$) but were not significantly increased in tumors showing methylation at other loci. Finally, there was no difference in the frequency of p53 accumulation in tumors showing methylation at any of the loci tested.

Clinicopathologic and Genetic Features of Tumors Showing Widespread CpG Island Methylation

A histogram showing the frequency of methylation at all loci for different cancers is shown in Figure 2. It is apparent that the frequency of methylation in the tumors examined in this study shows a continuous rather

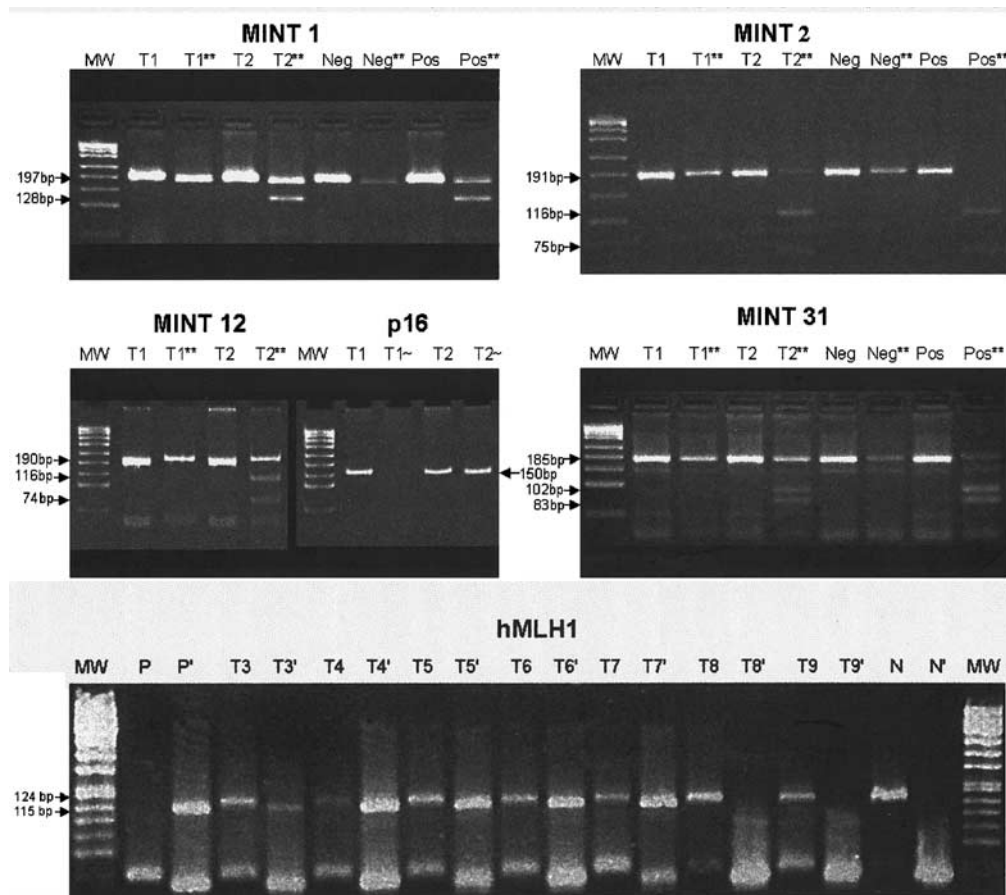


Figure 1. Analysis of methylation of hMLH1, p16, and the MINT loci in colorectal tumors. For p16 and hMLH1 promoter analysis, bisulfite-modified tumor DNA was amplified in separate reactions using primers specific for unmethylated or methylated template (methylation-specific PCR). Reactions using methylation-specific primers are designated with ~ for p16 and * for hMLH1. Tumor samples T3–T7 show methylation of the hMLH1 promoter, whereas T8 and T9 are unmethylated. T2 is methylated at p16, whereas T1 is not. Note that because each tumor sample contains some normal DNA, unmethylated primers will always produce an amplicon. For analysis of methylation at MINT loci, bisulfite-modified DNA was first PCR amplified using appropriate loci-specific primers, and then the amplicons were digested with restriction enzymes that only cut amplicons generated from methylated template (MINT 1 and 2, *TaqI*; MINT 12, *TaqI*; MINT 31, *BstUI*). Analysis of each MINT locus is shown for unmethylated (T1) and methylated (T2) tumors. In each case, amplicons are shown before and after digestion (** indicates digestion). In all cases, T1 is resistant to digestion, and the product size is unaltered (T1 vs. T1**). In contrast, amplicons from tumor T2 digest to produce smaller products of expected size (T2 vs. T2**) at all MINT loci. Note that not all the amplicons digest, because each sample contains some normal (unmethylated) tissue. Positive control methylated DNA was obtained from DLD1 colon cancer cell lines (Pos) for MINTs 1, 2, 12, and 31, from T84 cells (Pos) for p16, and from SW48 cells (P) for hMLH1. DNA from human peripheral blood lymphocytes was used as a negative control for all reactions (Neg or N). Molecular weight marker (MW) is ϕ X174/*HinfI* for hMLH1 and pUC19/*MspI* for all other analyses.

than bimodal distribution. For this reason, nonparametric methods were used to determine the relationship between a range of clinicopathologic features and the extent of methylation at all loci examined in this study. On this basis, the presence of CpG island methylation correlated with age (Spearman ρ , 0.205; $P < 0.001$) and was associated with female sex ($P < 0.001$). It was also significantly associated with high tumor grade ($P = 0.02$), right-sided location ($P < 0.001$), mucinous phenotype ($P < 0.001$), the presence of intraepithelial lymphocytes ($P < 0.001$), and a Crohn's-like infiltrate ($P = 0.02$), wild-type p53 status ($P = 0.002$), mutant *K-ras* ($P = 0.03$), and MSI ($P < 0.001$).

Relationship Between CpG Island Methylation and MSI

A total of 43 tumors (10.7%) from 42 individuals in this study showed MSI, and these tumors had the typical features of sporadic MSI cancers.^{29–31} When compared with MSS tumors, they were found with increased frequency in older individuals (mean, 75.6 ± 8.8 years vs. 67.9 ± 12.2 years; $P < 0.001$) and in women (76% vs. 40%; $P < 0.001$). The MSI tumors were more likely to be right-sided ($P < 0.001$), mucinous ($P < 0.001$), and high grade ($P < 0.001$) and to have intraepithelial lymphocytes ($P < 0.001$), Crohn's infiltrate ($P < 0.001$),

Table 1. Characteristics of Individuals and the Phenotypic Features of Cancers Showing CpG Island Methylation at Specific Loci

	MINT1			MINT2			MINT12			MINT31			p16		
	M	UM	P	M	UM	P	M	UM	P	M	UM	P	M	UM	P
Individuals															
No. (%)	97 (25)	299 (76)		107 (29)	168 (72)		114 (29)	281 (71)		55 (14)	338 (86)		100 (25)	296 (75)	
Mean age (yr) ± 1 SD	71.5 ± 12.1	67.7 ± 12.1	0.008	71.5 ± 12.5	67.4 ± 11.9	0.003	71.1 ± 11.7	67.5 ± 12.2	0.007	72.9 ± 11.0	67.9 ± 12.2	0.004	72.2 ± 11.7	67.6 ± 12.1	0.001
P value															
Sex (%)															
F	14	30		15	28		16	28		8	36		16	28	
M	10	46	0.001	13	44	0.013	13	43	0.002	6	50	0.047	9	47	<0.001
P value															
Tumors															
No. (%)	97 (24)	307 (76)		109 (28)	274 (72)		114 (28)	289 (72)		57 (14)	344 (86)		101 (25)	303 (75)	
Side (%)															
Right	14	22		15	21		15	21		9	27		16	20	
Left	10	53	<0.001	13	51	<0.001	13	51	<0.001	5	59	0.001	9	54	<0.001
P value															
Grade ^a (%)															
High	6	7		5	8		4	9		3	10		5	8	
Low	18	69	<0.001	23	64	0.049	24	63	NS	11	76	0.02	20	67	0.003
P value															
Mucinous (%)															
Yes	8	9		8	9		7	10		6	11		7	10	
No	16	67	<0.001	21	63	<0.001	21	62	<0.012	8	75	<0.001	18	65	0.001
P value															
IEL (%)															
Increased	10	11		9	12		9	11		6	14		9	11	
Normal	15	65	<0.001	20	59	0.005	19	61	<0.001	8	72	<0.001	16	63	<0.001
P value															
p53 (%)															
Increased	11	44		13	43		14	42		5	50		12	43	
Normal	14	31	0.023	16	28	0.008	15	29	0.03	9	36	0.001	13	32	NS
P value															
K-ras (%)															
Mutant	7	18		10	15		10	16		4	21		7	18	
Wild	19	56	NS	20	55	0.027	21	54	NS	11	65	NS	19	56	NS
P value															
MSI (%)															
Present	9	2		8	3		7	3		7	4		7	4	
Absent	16	74	<0.001	21	69	<0.001	21	69	<0.001	8	82	<0.001	18	71	<0.001
P value															

M, methylated; UM, unmethylated; IEL, intraepithelial lymphocytes; p53, immunohistochemically detectable P53 protein; NS, not significant.
^aHigh, high-grade (poorly differentiated) tumor; low, all tumors not of high grade, including both well and moderately differentiated lesions.

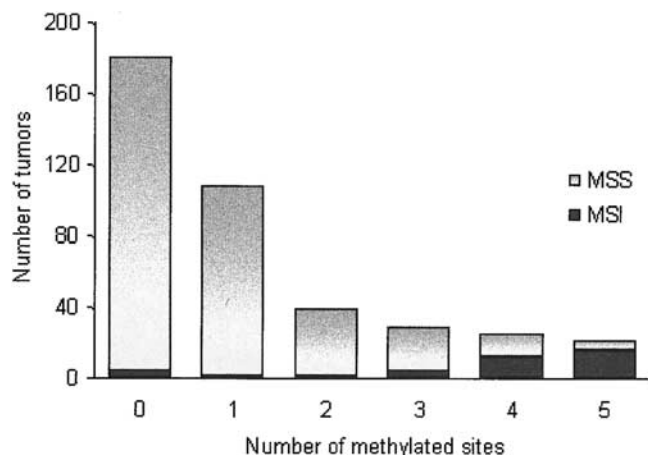


Figure 2. Distribution of CpG island methylation in sporadic colorectal cancer. The histogram shows the total number of methylated loci in MSS and MSI tumors. It is apparent that methylation at the 5 loci examined in this study showed a continuous distribution, with 181 of the 404 tumors (45%) unmethylated at all 5 loci and 115 tumors (28%) methylated at more than 1 locus.

peritumoral lymphocytes ($P = 0.002$), and wild-type p53 ($P < 0.001$). Although *K-ras* mutations were less frequent in MSI than MSS tumors, this difference was not significant (15.8% vs. 26.5%; $P = 0.15$).

Of these 43 MSI tumors, 34 (79%) showed CpG island methylation at more than 2 loci (Figure 2). On the other hand, absence of methylation at any locus was seen in only 5 (12%) of MSI tumors. The MSI tumors without methylation were not significantly different in terms of clinicopathologic and genetic features from their methylated counterparts (Table 2).

CpG Island Methylation in MSS Tumors

A total of 361 tumors were MSS. In contrast to MSI tumors, most MSS tumors showed only low levels of

CpG island methylation, with only 42 (12%) showing methylation at more than 2 loci (Figure 2). Nevertheless, when considered in absolute terms, MSS tumors accounted for more than half of all cancers showing methylation at more than 2 loci.

Within the MSS tumor group, methylation was associated with increased age (Spearman ρ 0.11; $P = 0.04$), right-sided tumor location ($P < 0.001$), and mucinous phenotype ($P = 0.004$). It also occurred more commonly in women, although this did not reach statistical significance ($P = 0.06$).

However, and in contrast to methylated MSI tumors, methylation in the MSS tumor group was not associated with high tumor grade or the presence of any pattern of inflammatory cell infiltrate (intraepithelial, peritumoral, or Crohn's-like). Also, it was noted that MSS methylated tumors show an anatomic bimodal distribution, with the greatest number of tumors in this group occurring in either the rectum or ascending colon (Figure 3).

Finally, and again unlike the case with MSI tumors, methylation in MSS tumors was associated with mutant *K-ras* ($P = 0.004$) but not with accumulation of p53 protein ($P = 0.18$). For descriptive purposes, tumors were grouped into 4 subsets based on microsatellite status and the number of methylated loci, and their clinicopathologic features are shown in Table 2.

Analysis of methylation at the hMLH1 promoter was performed on a subset of 239 tumors in this study, and typical results are shown in Figure 1. Overall, hMLH1 methylation was seen in 33% of cancers and was strongly associated with methylation of the 5 other loci examined ($P < 0.001$). hMLH1 promoter methylation was seen in 35 of 38 MSI tumors (92%) and was associated with definite immunohistochemical loss of MLH1 protein ex-

Table 2. Individual Characteristics and Phenotypic Features of Cancers Grouped According to CIMP and Microsatellite Status

	CIMP+/MSS	CIMP-/MSS	CIMP+/MSI	CIMP-/MSI
No. of cancers	42	317	34	9
No. of people	42	312	34	8
Age (yr)	70.7 ± 12.8	67.4 ± 12.2	76.8 ± 8.3	70.7 ± 9.5
Female sex (%)	25 (60)	116 (37)	28 (82)	4 (50)
Right-sided (%)	26 (62)	85 (27)	28 (82)	8 (89)
Tumor stage (% I:II:III:IV)	2:13:16:11 (5:31:38:26)	60:117:99:41 (19:37:31:13)	6:18:7:3 (18:53:21:9)	2:5:1:1 (22:56:11:11)
High tumor grade (%)	9 (21)	26 (8)	10 (29)	7 (78)
Mucinous type (%)	12 (29)	38 (12)	16 (47)	2 (22)
Intraepithelial lymphocytes (%)	7 (18)	40 (13)	24 (75)	6 (75)
Peritumoral lymphocytes (%)	8 (20)	63 (21)	14 (44)	3 (38)
Crohn's-like infiltrate (%)	11 (28)	33 (11)	3 (38)	12 (38)
p53 accumulation (%)	23/42 (55)	181/307 (59)	6/33 (18)	4/8 (50)
Mutant <i>K-ras</i> (%)	17/40 (43)	66/273 (24)	6/30 (17)	0/8 (0)

NOTE. In this table, CIMP+ tumors are defined as having methylation at more than 2 of 5 loci.

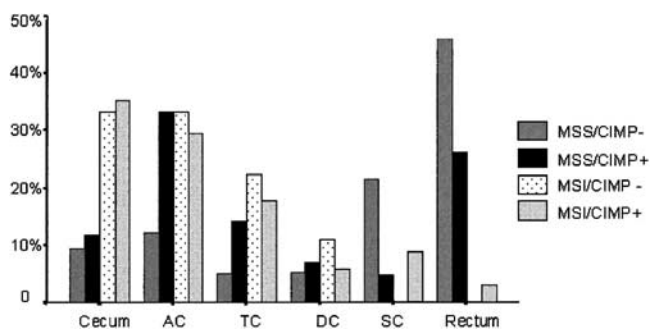


Figure 3. Anatomic distribution of MSS and MSI tumors according to methylation. Tumors were classified into 1 of 4 groups based on their microsatellite and CpG island methylation status, with CIMP+ defined as methylation at more than 2 of 5 loci. For each of the 4 groups, the anatomic distribution of cancers is shown as a percentage of all cancers in that group (MSS/CIMP-, n = 317; MSS/CIMP+, n = 42; MSI/CIMP-, n = 9; MSI/CIMP+, n = 34). AC, ascending colon; TC, transverse colon; DC, descending colon; SC, sigmoid colon.

pression in 27 of these 35 cases. Furthermore, MLH1 methylation was seen in 45 of 201 MSS tumors (22%), including 17 tumors (8%) that showed no evidence of methylation at any other locus. All 45 of these cases showed normal immunostaining for hMLH1.

Tumor Stage as a Function of Microsatellite and Methylation Status

The stage distribution of tumors when classified according to CIMP and microsatellite status is shown in Table 2. It is apparent that tumor stage varies considerably within each group. To analyze this further, tumors were grouped as either early (stage I/II) or advanced (stage III/IV) lesions, and the relationship between stage and a variety of factors was determined using logistic regression analysis (Table 3). Variables significantly related to higher tumor stage in a univariate analysis were microsatellite stability, absence of intraepithelial lymphocytes, and left-sided location (Table 3). A multivariate analysis showed that methylation of tumors was

associated with a higher tumor stage, whereas right-sided tumors and those with prominent intraepithelial lymphocytes were of lower stage at presentation.

Disease Outcomes

Univariate analysis showed that a higher tumor stage (OR, 6.43; 95% CI, 4.6–9.01; *P* < 0.001) and male sex (OR, 1.68; 95% CI, 1.08–2.62; *P* = 0.02) were associated with a worse outcome, whereas the presence of intraepithelial leukocytes (OR, 0.43; 95% CI, 0.22–0.82; *P* = 0.01) was associated with improved survival. However, neither methylation nor MSI was prognostically significant in univariate (OR, 1.7; 95% CI, 0.6–2.5; *P* = 0.50; and OR, 0.7; 95% CI, 0.3–1.4; *P* = 0.23, respectively) or multivariate analyses (OR, 2.0; 95% CI, 0.9–4.6; *P* = 0.09; and OR, 0.4; 95% CI, 0.2–1.1; *P* = 0.08, respectively).

However, examination of survival curves indicated that MSI cancers were associated with an improved outcome (Figure 4), whereas within the MSS cancer group, increasing methylation was associated with a trend to decreased survival. This latter trend was most marked in MSS tumors showing methylation at more than 3 loci (Figure 4; *P* = 0.006, log-rank test).

Discussion

This study considers the frequency and extent of CpG island methylation in a large consecutive group of sporadic colorectal cancers and provides information on the features of tumors showing this phenomenon as well as the interaction between this event and the development of MSI.

Patterns of CpG Island Methylation in Sporadic Colorectal Cancer

In the present study, we found that the frequency of CpG island methylation was distributed in a contin-

Table 3. Tumor Stage in Relation to Degree of Methylation and Microsatellite Status

Risk factor	Stage I/II (n = 224)	Stage III/IV (n = 180)	Univariate analysis OR (95% CI) <i>P</i> (trend)	Multivariate analysis OR (95% CI) <i>P</i>
Methylation	NA	NA	1.22 (0.6–2.4) <i>P</i> = 0.55	2.95 (1.2–7.3) <i>P</i> = 0.019
MSI	13.9%	6.7%	0.45 (0.2–0.9) <i>P</i> = 0.023	0.58 (0.2–1.5) <i>P</i> = 0.26
IEL present	27.1%	12.1%	0.37 (0.2–0.7) <i>P</i> = 0.0004	0.34 (0.2–0.7) <i>P</i> = 0.003
Right-sidedness	41.5%	30.0%	0.60 (0.4–0.9) <i>P</i> = 0.02	0.60 (0.4–1.0) <i>P</i> = 0.04

NOTE. Logistic regression analysis was used to calculate the relationship between tumor stage and the degree of methylation at the 5 loci examined (methylation), the presence of MSI, the presence of intraepithelial lymphocytes (IEL), and right-sidedness. The OR and 95% CI are presented for univariate and multivariate analysis, together with the *P* trend value in the case of methylation. NA, not applicable.

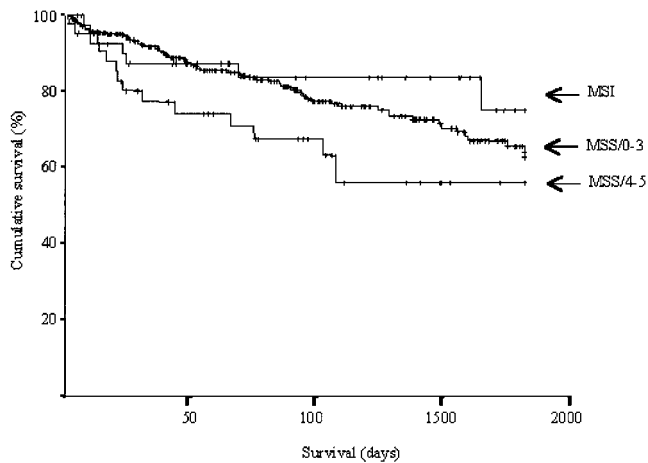


Figure 4. Overall survival according to microsatellite methylation status. Kaplan-Meier plot showing cumulative survival for individuals with MSI tumors as well as those individuals with MSS tumors that were methylated at either more than 3 of 5 loci (MSS/4-5) or at <4 of 5 loci (MSS/0-3). The differences between the 3 groups were statistically significant ($P = 0.006$, log-rank test).

uous fashion across all the tumors examined. This contrasts with the bimodal distribution reported by Toyota et al., in which tumors methylated at more than 2 of 7 loci (mean, 5.1 loci per tumor) were clearly distinguishable from largely unmethylated tumors (mean, 0.3 loci per tumor).^{13,32} In considering this apparent discrepancy, it is noteworthy that MSI tumors in the current study were much more likely to show high levels of methylation than MSS tumors (Figure 2). Furthermore, whereas MSI tumors accounted for 10.6% of cases in the current study, they represented 32%¹³ and 22%³² of cases in the 2 unselected series reported by Toyota et al. It is therefore possible that the higher proportion of MSI tumors may have led to an appearance of bimodality in the series reported previously by Toyota et al.

These issues notwithstanding, our data provide further evidence that methylation of CpG islands is a common event in many colorectal cancers and can be extensive in some tumors.^{13,32} It also confirms observations made by others that methylation of loci used in this study is specific for tumors.^{13,32}

Because the distribution of methylation frequency in the current series of cancers was not bimodal, the classification of any particular tumor as CIMP was necessarily arbitrary. Based on a bimodal distribution, Toyota et al. found that CIMP tumors represented 58%¹³ and 51%³² of 2 small series of unselected colorectal cancers. If similar criteria were applied to the current study (namely, an arbitrary cutoff at more than 1 of 5 loci methylated), then CIMP tumors would represent 28% of all tumors. In part, the observed difference in frequency of CIMP tumors may relate to methodological issues and

particularly to the use of qualitative rather than quantitative criteria for the interpretation of gel-based methylation assays.³³ However, with the exception of MINT31, rates of positivity at individual loci in the present study were similar to those found by other investigators.¹³ It is perhaps more likely that the difference in frequency of CIMP tumors is largely attributable to the differences in populations studied and in particular to different proportions of MSI cancers in each study group.

CIMP

Only limited information is available in the literature regarding the clinicopathologic properties of tumors showing CpG island methylation. Toyota et al. reported that CIMP tumors were more likely to occur in the proximal colon but were not associated with age, sex, or tumor stage.^{13,32} In the current study, we found that methylation was strongly associated with the occurrence of tumors in the elderly, in women, and in the right colon. The tumors themselves were more likely to be of high grade and of a mucinous type. These are, of course, the clinicopathologic features classically attributed to sporadic MSI cancers^{25,29-31}; thus, it was not surprising that both MSI and the absence of p53 mutations were also strongly associated with the presence of CpG island methylation. Interestingly, studies of p16 in colorectal cancer have also assigned many of these characteristics to tumors showing silencing of this gene through gene methylation.¹⁵ It is possible that observations made previously on p16 methylation and on sporadic MSI may in fact largely reflect the phenotype of tumors characterized by widespread CpG island methylation. The observation that p16 methylation correlates with MSI¹⁴ serves to reinforce the point that these observable phenomena may be underpinned by a predisposing event, namely the widespread and random methylation of CpG islands within the genome of colonic epithelial cells.

Biological Significance of Methylation at Individual Loci

A clear conclusion that can be drawn from this study is that methylation at any one of the loci examined rarely alters tumor phenotype or behavior. This is evident in the striking similarity in phenotype of tumors showing methylation at any of the 5 loci examined in this study. The finding provides strong indirect support for the hypothesis that methylation at individual loci, rather than being central to tumor development, simply reflects a broader and probably stochastic process of CpG island methylation. In this view, the progressive methylation of CpG islands probably relates to their shared structural characteristics and serves as a biomarker of a

global process of increased methylation. Data in the current study provide only indirect information on the cause of this process of CpG island methylation. Clearly the process is much more commonly seen in the elderly, in women, and in the right colon, although the reasons for these associations are not apparent. Identification of precursor lesions, if they exist, may assist in better understanding the cause of this process. We have previously described the development of MSI in hyperplastic polyps.¹² By inference, it is possible that these lesions may arise from or are susceptible to a process of CpG island methylation.

CpG Island Methylation and MSI

CpG island methylation is known to cause sporadic MSI through methylation of the hMLH1 promoter. Not surprisingly, Toyota et al. found a strong correlation between CIMP and both the presence of hMLH1 methylation and MSI.^{13,32} Interestingly, however, they found that only half of all cancers and none of the adenomas with CpG island methylation showed hMLH1 methylation. Taken together, these findings suggest that CpG island methylation may precede the development of MSI and indeed may occur in its absence in many tumors. An aim of the current study was to explore the relationship between these 2 phenomena and in particular to identify the extent to which tumors show CpG island methylation in the absence of MSI.

Several observations in this study support the premise that MSI cancers arise from a larger group of tumors in which the process of CpG island methylation is already established.

As previously discussed and shown in Table 2, this study has highlighted the striking similarities between all tumors showing CpG island methylation, irrespective of their microsatellite status. Furthermore, within the group of tumors characterized by CpG island methylation, there were distinct and substantive subsets showing microsatellite stability or instability. Using a cutoff of methylation at more than 2 loci, we found that only 34 of the 76 cancers (45%) showing CpG island methylation were also microsatellite unstable. Interestingly, the likelihood of MSI within a tumor increased with the extent of methylation at individual loci. Thus, 2% of the tumors with methylation at only 1 of 5 loci were MSI, but this percentage increased progressively to reach 73% of tumors with methylation at all 5 loci (Figure 2). This observation is consistent with a stochastic process of tumor methylation and a gradually increasing probability of biallelic MLH1 methylation, as manifest by the presence of MSI.

Finally, there were only a small number ($n = 5$) of MSI tumors in the current study that showed no CpG island methylation. These cases may represent unrecognized germline mutations, despite the stringent criteria used in this study to reduce this possibility. Alternatively, these cases may represent tumors with a tendency toward methylation but in which hMLH1 methylation has occurred before methylation at other loci has reached a level detectable in the assay system used in this study. Toyota et al. previously noted that 25% of MSI cancers did not show CpG island methylation at more than 3 of 7 loci,¹³ although it is not clear how many of these tumors were from individuals with unrecognized germline mismatch repair defects.

The present study also highlighted several pathologic and genetic differences between MSS and MSI tumors showing CpG island methylation. These included a more left-sided distribution of methylated MSS lesions within the bowel (Figure 3), decreased intraepithelial and peritumoral lymphocytic infiltration, significantly higher rates of *K-ras* mutation, and higher rates of p53 accumulation (Table 2). These observed differences provide a number of potential insights into the specific effects of MSI on the progression of tumors already showing CpG island methylation.

It is apparent that increased numbers of intraepithelial lymphocytes, long regarded as a hallmark of MSI cancers, are indeed very specific to this process and presumably arise as a direct result of the development of MSI rather than from the indirect effects of widespread promoter methylation. This is of course supported by previous observations made in the setting of hereditary loss of mismatch repair genes.

The finding of lower rates of *K-ras* and p53 accumulation in the MSI subgroup of CpG island methylated tumors is perhaps not surprising. These observations have been made previously with respect to MSI cancers^{29,34} and presumably indicate that these genetic changes provide no selection advantage for lesions that have already developed MSI. Perhaps more surprisingly, we found that heavily methylated MSS tumors had significantly higher rates of *K-ras* mutation than those seen in the overall sample (43% vs. 24%). These findings are similar to those of Toyota et al., who found that *K-ras* mutations were more frequent in cancers showing CpG island methylation.³² It is not immediately apparent why methylation-prone lesions that fail to develop MSI should have a higher rate of *K-ras* mutations than that seen in nonmethylated lesions. One possibility is that CpG island methylation is responsible, directly or indirectly, for the development of mutations of this gene.

Whitehall et al. have recently shown that methylation-induced silencing of the O6-MGMT gene is associated with increased *K-ras* mutations.³⁵ Certainly, methylation of genes involved in maintaining wild-type *K-ras* status or repairing mutations of that gene may offer a plausible explanation for the high rate of *K-ras* mutations in MSS methylated tumors. Interestingly, *K-ras* mutations have long been recognized as occurring most frequently in villous adenomas and serrated adenomas, lesions that fall within the gambit of the “serrated neoplasia pathway.” This provides a further tentative link between this pathway and the phenomenon of global CpG island methylation, even in the absence of MSI.

Disease Outcomes

No previous studies have considered the influence of widespread CpG island methylation on disease outcome. In the current study, individuals with heavily methylated tumors that were MSS had a significantly worse outcome compared with those individuals with methylated and microsatellite unstable tumors. They also had a poorer outcome than individuals with non-methylated MSS tumors and indeed compared with all other individuals in the study. In large part, these differences in outcome reflected the fact that methylated MSS tumors were of later stage at presentation. We are unable to determine whether the methylated MSS tumor type is of more advanced stage because of an inherent biological aggressiveness or if tumors of advanced stage are simply more likely to have become methylated. However, the recognition of these 2 prognostically distinct groups of methylated cancers may help to explain the finding of cancers that on clinicopathologic grounds show many of the features of sporadic MSI tumors yet do not show hMLH1 protein loss or MSI and that are often well advanced at diagnosis.

In summary, this study builds on the concept of CpG island methylation as a mechanism of tumor progression in colorectal cancer and identifies this event in a subset of sporadic cancers. It provides support to the hypothesis that sporadic MSI arises in a subset of these methylation-prone lesions. It also emphasizes that tumor progression can occur in the absence of MSI to produce MSS but that heavily methylated tumors have their own relatively distinct clinicopathologic and genetic features.

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