BASIC–ALIMENTARY TRACT

The CpG Island Methylator Phenotype and Chromosomal Instability Are Inversely Correlated in Sporadic Colorectal Cancer

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Background & Aims: **The CpG island methylator phenotype (CIMP) is one of the mechanisms involved in colorectal carcinogenesis (CRC). Although CIMP is probably the cause of high-frequency microsatellite instability (MSI-H) sporadic CRCs, its role in microsatellite stable (MSS) tumors is debated. The majority of MSS CRCs demonstrate chromosomal instability (CIN) with frequent loss of heterozygosity (LOH) at key tumor suppressor genes. We hypothesized that the majority of sporadic CRCs without CIN would be associated with CIMP.** ***Methods:*** **We tested 126 sporadic CRCs for MSI and LOH and categorized tumors into MSI, LOH, or MSI−/LOH− subgroups. Methylation status was evaluated using 6 CIMP-related markers (MINT1, MINT2, MINT31, $p16^{INK4\alpha}$, $p14^{ARF}$, and hMLH1) and 6 tumor suppressor genes (PTEN, TIMP3, RUNX3, HIC1, APC, and RARβ2). BRAF V600E mutation analysis was performed using allele-specific polymerase chain reaction and DNA sequencing.** ***Results:*** **We observed frequent methylation at all 12 loci in all CRCs. BRAF V600E mutations correlated with the MSI ($P < .0001$) and MSI−/LOH− ($P = .03$) subgroups. MSI and MSI−/LOH− tumors exhibited more promoter methylation than CRCs with LOH ($P < .0001$). We also found an inverse correlation between the frequencies of methylation and LOH ($\rho = -0.36$; $P < .0001$).** ***Conclusions:*** **The associations between methylation frequencies at CIMP-related markers and MSI or MSI−/LOH− sporadic CRCs suggest that the majority of these tumors evolve through CIMP. These findings suggest that CIN and CIMP represent 2 independent and inversely related mechanisms of genetic and epigenetic instability in sporadic CRCs and confirm that MSI cancers arise as a consequence of CIMP.**

Genomic instability is a key mechanistic component of cancer progression.[1,2] Three mechanisms that increase the diversity of gene expression have been iden-

tified in colorectal cancer (CRC): microsatellite instability (MSI), chromosomal instability (CIN), and CpG island methylator phenotype (CIMP). MSI occurs in approximately 15% of sporadic CRCs and is defined by inactivation of the DNA mismatch repair (MMR) system through acquired hypermethylation of the hMLH1 gene promoter.[3] CIN is present in more than 50% of CRCs and is characterized by aneuploidy and frequent loss of heterozygosity (LOH), facilitating the sequential inactivation of APC, DCC/SMAD4, and p53.[2] As many as 35%–40% of CRC demonstrate CIMP, an epigenetic change causing transcriptional silencing by methylation of cytosine residues at CpG-rich sequences (CpG islands) in the promoter regions of many tumor suppressor genes.[4–6]

Current data indicate that CIMP is an important mechanism of gene inactivation in human carcinogenesis, and there is growing evidence that a number of tumor suppressor genes, including p16, p14, MGMT, and hMLH1, are silenced by promoter methylation in CRC.[7,8] Evidence for CIMP can be found in colorectal adenomas and may be a characteristic feature of the serrated pathway of colorectal tumorigenesis.[9] However, in contrast to MSI and CIN, which are recognized as distinct biologic subtypes of CRC, it is not clear whether CIMP represents a unique mechanistic pathway for colorectal carcinogenesis[10,11] or whether this characteristic occurs through the accumulation of multiple stochastic and random methylation events.[12–14] A key factor in this controversy is the fact that previous investigations have not used uniform methylation detection methods, have utilized different

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methylation targets, and have used arbitrary criteria for defining CIMP.[10,12,13] In addition, most of the previous studies have not used sufficiently large sample sizes to establish convincingly the case for CIMP in CRCs.

Based on current published data, CIMP colorectal tumors are characteristically sporadic (nonfamilial) and have a distinct clinical profile that includes proximal tumor location, female sex, older age, high tumor grade, wild-type *TP53*, higher *BRAF* and *K-Ras* mutations, and frequent MSI.[10,15–18] However, even if MSI tumors were excluded, significant relationships would still be evident with older age, proximal location, and mucinous histology as well as *BRAF* V600E and *K-Ras* mutations.[18,19] CRCs with MSI generally lack *K-Ras* and *TP53* mutations and are associated with a proximal colonic location and a better prognosis than microsatellite stable (MSS) tumors.[20] These associations indicate that sporadic MSI and CIMP tumors share similar biologic features.[6,21]

Sporadic MSI tumors arise as a consequence of *hMLH1* methylation[3] and also show an increased frequency of methylation at other tumor suppressor genes. We previously reported that as many as 35% of all sporadic CRCs lack characteristics of MSI or CIN,[6] and a recent population-based study found high-frequency CIMP in 25% of all MSS tumors.[10] There is a clear need to address the mechanistic basis of CIMP, not only to unify the criteria for "methylation signatures" in various gene promoters, but also to study the relationship between CIMP and other forms of genetic alterations. MSI and CIN are, for the most part, mutually exclusive,[6] and CIMP strongly associates with sporadic MSI CRCs.[3] However, no investigations have determined the relationship between CIMP and CIN. The present study tests the hypothesis that CIN and CIMP are 2 mutually exclusive pathways of genetic and epigenetic instability in sporadic CRCs and that sporadic MSI cancer evolves through the CIMP pathway following epigenetic inactivation of the *hMLH1* gene.

Materials and Methods

Tissue Specimens

The study was performed on a cohort of 126 primary colon cancers, which were obtained from patients with sporadic CRC collected through the Cancer and Leukemia Group B (CALGB)-protocol 9865. Patients signed a protocol-specific informed consent for use of their tissues and institutional review board approval was granted for this study performed on anonymized samples.[6]

Microdissection and DNA Amplification

Serial sections from formalin-fixed, paraffin-embedded, matched normal and neoplastic primary tissues (5 μm) were stained with H&E, and representative normal and tumor regions were identified by microscopic examination. Normal control tissue (nontumor) was obtained from histologically normal mucosa and/or normal lymph nodes.

Genomic DNA was isolated from the paraffin-embedded microdomains removed from the slides by deparaffinizing them in multiple xylene washes. Subsequently, the tissues were hydrated, digested in Proteinase K, and followed by DNA extraction using the QIAamp DNA mini kit (Qiagen, Valencia, CA), per the manufacturer's instructions with some modifications.

MSI Analysis

Microsatellite analysis of all matched normal and tumor tissues was performed by polymerase chain reaction (PCR) amplification using a panel of 5 National Cancer Institute (NCI)-workshop recommended markers that included 2 mononucleotide (BAT25 and BAT26) and 3 dinucleotide repeat sequences (D2S123, D5S346, and D17S250).[22] PCR was performed using ^{32}P-labeled primers and subsequent electrophoresis on 8% polyacrylamide gels as described previously.[6] Changes in the electrophoretic mobility of DNA amplified by PCR were used to assess MSI. Tumors showing a shift in at least 2 of the 5 markers were classified as high-frequency MSI (MSI-H), in accordance with the international consensus criteria.[22] Low-frequency MSI (MSI-L) was defined as a shift in only 1 of the 5 markers. Tumors that did not show any allelic shifts were classified as MSS. In this study, we grouped MSI-L/MSS tumors together for comparison purposes and for all statistical analyses because both have similar clinical, pathologic, and mutational features and do not differ in clinical outcome.[23]

LOH Analysis

Eight sets of polymorphic microsatellite sequences that are tightly linked to known tumor suppressor genes and DNA MMR genes were used to identify significant allelic losses in the colon cancer specimens. PCR amplification of genomic DNA was performed using ^{32}P-end-labeled primers at microsatellite loci linked to the h*MSH2* locus on 2p16 (D2S123), the h*MLH1* locus on 3p23-21.3 (D3S1029), the *APC* locus on 5q21 (D5S346), and the *p53* locus on 17p13 (D17S250, D17S261) and the *DCC/SMAD2/SMAD4* region on 18q21.3 (D18S64, D18S69, and D18S474). Assessment of LOH (or allelic imbalance) was assigned when a tumor allele showed at least a 50% reduction in the relative intensity of 1 allele in neoplastic tissue compared with the matched normal DNA as described previously.[6] Because the LOH markers utilized in this study have been extensively characterized, we categorized a tumor showing 1 or more LOH events in the 8 markers to have CIN.

BRAF V600E Mutation Analysis

Allele-specific PCR was performed to identify V600E mutations in the *BRAF* gene as described previously.[24] Briefly, 2 sets of different forward primers were utilized to amplify either the wild-type or the mutant alleles of the *BRAF* gene. One of the forward primers

flanked the exon-15 sequence (F1- 5′-TAGGTGATTTT-GGTCTAGCTACAGT-3′) and was used as a positive control to amplify the wild-type as well as the mutant *BRAF*. A second primer with substitution of 2 bases at the 5′-end (F2-5′-GGTGATTTTGGTCTAGCTACAAA-3′) was designed to amplify the mutant *BRAF* sequence only. A common reverse primer (R1-5′-GGCCAAAATTTAAT-CAGTGGA-3′) was used for both reactions. The PCR conditions for both reactions were similar. Hot start reactions were performed using HotStar PCR Mix (Qiagen) with an initial denaturation for 15 minutes at 94°C and subsequent denaturation for 30 seconds at 94°C, annealing for 45 seconds at 52°C, and a final extension for 45 seconds at 72°C. Thirty-five cycles were used to amplify the PCR product with the expected amplicon of 129 base pair. Genomic DNA from HT-29 colon cancer cells was used as a positive control for the detection of *BRAF* mutations. *BRAF* mutation-positive specimens were subsequently subjected to sequencing on an ABI 3100-Avant DNA sequencer (Applied Biosystems Inc., Foster City, CA) for confirmation.

Sodium Bisulfite Modification and Methylation-Specific PCR Assays

Methylation-specific PCR (MSP) was performed on bisulfite-modified DNA templates obtained from human colon cancer tissue materials to study the methylation status of 12 methylation targets. Among these, 9 methylation markers mapped to promoter regions of genes including *hMLH1*, *APC*, $p16^{INK4\alpha}$, $p14^{ARF}$, *TIMP3*, *RUNX3*, *HIC1*, *PTEN*, and *RARβ2*, and the remaining 3 markers amplified methylated in tumor loci (MINT): MINT1, MINT2, and MINT31. Six of these 12 markers (MINT1, MINT2, MINT31, $p14^{ARF}$, $p16^{INK4\alpha}$, and *hMLH1*) have been proposed for identifying cancer-specific methylation, also referred to as CIMP.[25,26] For *hMLH1*, the 5′-region of the gene promoter was investigated for methylation analysis. The primer sequences, PCR conditions, and product sizes for each of the methylation markers analyzed and the specificity of the MSP assays have been described previously.[27–31]

Genomic DNA obtained from paraffin-embedded tissue sections was bisulfite modified to convert all the unmethylated cytosine residues to uracils for subsequent detection of methylated cytosines using methylation-specific primers. MSP assays were performed on the bisulfite-modified DNA using 2 sets of primers specific for amplification of methylated and unmethylated alleles as described previously.[32] Briefly, 0.5–2.0 μg genomic DNA were denatured with NaOH, treated with sodium bisulfite, and subsequently purified using the Wizard DNA Clean-up System (Promega, Madison, WI). Step-down PCR reactions were performed in a 25-μL reaction volume containing 1X PCR buffer (Invitrogen Life Technologies, Carlsbad, CA), 2.5 mmol/L MgCl$_2$, 200 μmol/L dNTPs, 0.5 μmol/L of each PCR primer, 0.75 units of

AmpliTaq polymerase, and approximately 25 ng bisulfite-modified DNA. Reactions were hot started at 95°C for 5 minutes. This was followed by 33 cycles at 95°C for 45 seconds, 57°C for 30 seconds, and 72°C for 30 seconds, followed by a 10-minute extension at 72°C in a PTC 200 DNA Engine Thermocyler (MJ Research, Inc., Waltham, MA). The amplification products were separated on a 3% agarose gel and visualized by ethidium bromide staining and ultraviolet (UV) transillumination.

Human placental DNA (Sigma Chemical Co., St. Louis, MO) treated in vitro with *Sss*I methylase (New England Biolabs Inc., Beverly, MA) was used as a positive control for MSP of methylated alleles, whereas DNA from normal lymphocytes was used as a control for unmethylated alleles. Water was used as a negative PCR control to monitor for contamination.

Statistical Analyses

The relationships among the methylation frequencies at each locus and LOH, MSI, and MSI−/LOH− were assessed for potential associations with a number of clinicopathologic parameters including tumor stage (stages II or III), age at diagnosis of the disease (years), tumor location (proximal, including cecum, right colon, hepatic flexure, and transverse colon; distal, including splenic flexure, left colon, sigmoid colon, and rectosigmoid), differentiation (poor, moderate, or well), nodal status (0, ≥1 and <3, or ≥4), and sex (male or female). Univariate associations of baseline prognostic variables were assessed using the χ^2 test or Fisher exact test.

Differences in the frequency of CIMP-positive tumors (≥3 methylated CIMP markers) between each epigenetic subgroup (MSI vs LOH and MSI−/LOH− vs LOH) were also analyzed with the χ^2 test. In addition, ratios comparing the relative odds of a tumor being CIMP positive (≥3 methylated markers) between epigenetic groups (MSI vs LOH and MSI−/LOH− vs LOH) were calculated with the corresponding 95% confidence interval (CI). To analyze the association between LOH and methylation profiles, we calculated LOH and methylation ratios by dividing the total number of loci showing LOH and/or methylation by the total number of informative cases. The differences between the mean methylation ratios in each subset of CRCs were analyzed by the Wilcoxon test. Correlations between methylation ratios and LOH ratios were analyzed using Spearman rank correlation coefficients (ρ).

To ascertain the relative risk of a tumor harboring a specific genetic alteration based on the methylation status for any given CIMP-related marker, we calculated the odds ratio (OR) for methylation in each of the subgroups for each marker. A 95% CI was calculated for each OR. An OR = 1.00 indicates that the odds of a tumor being MSI, LOH, or MSI−/LOH− are the same whether the given promoter target is methylated or not. However, for a given CRC subgroup (eg,

Table 1. The Overall Relationships Among Genetic and Epigenetic Factors and Clinicopathologic Features in Sporadic CRCs

	Cohort % (number)	MSI % (number)	MSI−/ LOH− % (number)	LOH % (number)	P value
Age (y)	(123)				
<50	16 (20)	14 (3)	11 (5)	21 (12)	
51–64	44 (54)	41 (9)	55 (24)	37 (21)	.41[a]
>64	40 (49)	45 (10)	34 (15)	42 (24)	
Sex	(123)				
Male	50 (61)	50 (11)	52 (23)	47 (27)	.92[a]
Female	50 (62)	50 (11)	48 (21)	53 (30)	
Location	(119)				
Proximal	47 (56)	59 (13)	53 (23)	37 (20)	.14[a]
Distal	53 (63)	41 (9)	47 (20)	63 (34)	
Tumor stage	(123)				
II	24 (30)	45 (10)	27 (12)	14 (8)	.01[a]
III	76 (93)	55 (12)	73 (32)	86 (49)	
Lymph nodes	(123)				
0	24 (30)	45 (10)	27 (12)	14 (8)	
1–3	54 (66)	36 (8)	50 (22)	63 (36)	.06[a]
>4	22 (27)	18 (4)	23 (10)	23 (13)	
Differentiation	(122)				
Well	11 (13)	14 (3)	14 (6)	7 (4)	
Moderate	68 (83)	41 (9)	68 (30)	79 (44)	.02[b]
Poor	21 (26)	45 (10)	18 (8)	14 (8)	

NOTE. Of 126 CRCs, 123 cases had available clinicopathologic information.

[a]P value were calculated by the χ^2 test.

[b]P value was calculated by Fisher exact test.

MSI), an OR value less than 1.00 for a specific marker indicates that the odds of MSI are less when that marker is methylated. Similarly, an OR value of >1.00 would indicate higher odds of MSI when that marker is methylated. All reported P values are 2-sided, and P < .05 was considered statistically significant.

Results

Sporadic CRCs (n = 126) were divided into 3 categories based on MSI and CIN determination. The first subgroup was MSI-H CRCs (n = 24; referred to as MSI). Among this subgroup, 4 tumors demonstrated overlap with LOH but were categorized along with MSI CRCs because MSI is likely the predominant mechanism of genetic instability in these cancers. The second subgroup comprised CRCs that did not demonstrate evidence for MSI-H or LOH and were categorized as MSI−/ LOH− (n = 45). The remaining 57 tumors belonged to the third subset of CRCs, which had LOH (implying CIN) and are referred to as "LOH." Among the 126 CRCs analyzed for methylation using the 12 markers, informative data were obtained for all cases, although, in some instances, certain methylation loci did not amplify in a PCR reaction despite multiple attempts. Statistical analyses were based on the actual number of informative cancers in each instance.

Tumor Characteristics

Of 126 CRCs, clinicopathologic information was available from 123 cases. The study cohort contained 30 stage II and 93 stage III CRCs. Among the 3 subsets of CRCs, stage III tumors were significantly higher in non-MSI groups (MSI−/LOH− and LOH) compared with MSI cancers (P = .01). MSI CRCs were significantly more poorly differentiated compared with the other 2 groups (P = .02; Table 1). Although not significant, tumors in the MSI and MSI−/LOH− subsets were more frequently proximally located compared with LOH tumors. Similarly, in comparison with the other 2 subsets of tumors, more of the MSI cancers did not have lymph node metastases (P = .06). No significant differences were observed for age and sex among any of the subgroups.

CIMP Is Frequently Present in the Majority of MSI and MSI−/LOH− CRCs

Using MSP, we examined the methylation status of 126 sporadic CRCs using 6 CIMP-related markers (MINT1, MINT2, MINT31, p14[ARF], p16[INK4α], and hMLH1) and promoter region methylation of 6 additional tumor suppressor genes implicated in colon carcinogenesis (APC, PTEN, TIMP3, RUNX3, HIC1, and RARβ2). Detailed results of the MSP analyses are presented in Figure 1, and the frequency of CIMP-positive CRCs is summarized in Table 2. There is a lack of consensus regarding which methylation markers consistently define CIMP[12,13,15,33]; however, the primary aim of the this study was not to make any specific recommendations in this regard. Instead, we investigated the ability of 6 CIMP-related markers and an equal number of additional methylation loci to determine whether tumors with these CIMP-related markers also had other forms of genetic instability. To obtain a quantitative measure of CIMP, we analyzed our data using relatively conservative criteria[16] and categorized a tumor as CIMP-positive if ≥3 of 6 markers were methylated. Using such criteria, we observed that significantly more MSI (18 of 24) and MSI−/LOH− (16 of 45) CRCs were CIMP positive compared with LOH tumors (5 of 57), which were unlikely to show this degree of methylation (P = .001 between the MSI−/ LOH− and LOH subgroups). The odds ratios for methylation in each subset of CRC were as follows: 11.6 (95% CI: 4.29–35.4) for MSI tumors, 1.39 (95% CI: 0.63–3.03) for MSI−/LOH− tumors, and 0.13 (95% CI: 0.05–0.32) for tumors with LOH. These data indicate that nearly all of the CIMP-positive CRCs are represented in the MSI or MSI-/LOH- subgroups and that 49% (34 of 69) of CRCs are CIMP positive after the exclusion of LOH CRCs.

V600E BRAF Mutation Strongly Correlates With CIMP-Positive Tumors

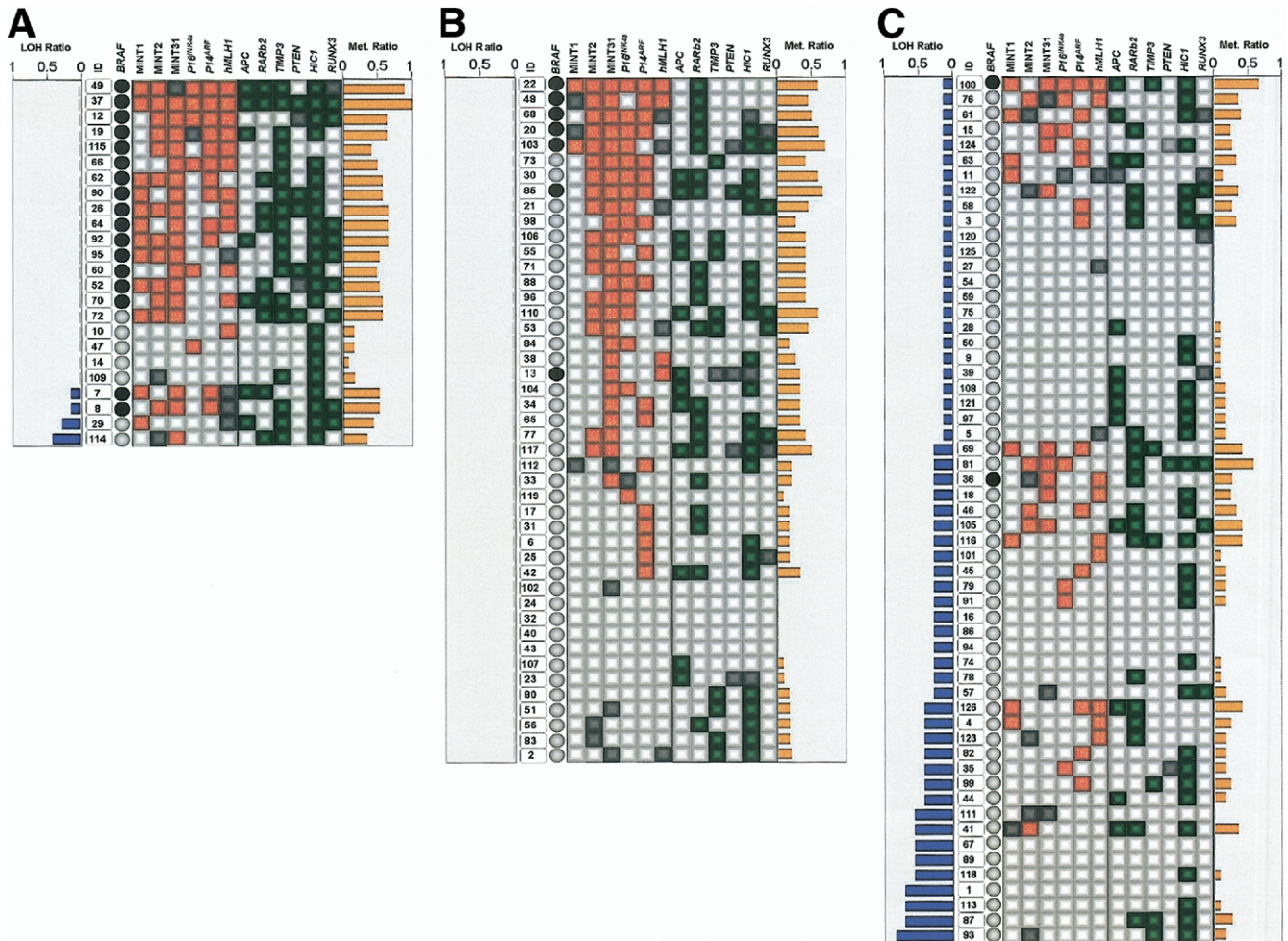A specific mutation of BRAF (V600E) that is commonly found in poorly differentiated mucinous

Figure 1. Frequent hypermethylation at multiple loci in MSI and MSI-/LOH- sporadic CRCs. The Figure provides a detailed data profile from 126 CRCs analyzed for methylation status at 12 methylation loci, frequency of *BRAF* mutations, MSI status, and LOH incidence. MSI status of each tumor was evaluated using standard markers, and the mean LOH ratios (*blue horizontal bars*) were determined for each tumor through analysis of 10 LOH markers tightly linked to 5q, 17p, and 18q loci. *BRAF V600E* mutation was investigated using allele-specific PCR and a *filled dark circle* represents the mutation-positive CRCs. Methylation status was analyzed using MSP, and data are presented for 6 CIMP-related markers including MINT1, MINT2, MINT31, *p16INK4α*, *p14ARF*, and *hMLH1* (shown as *red boxes*) and 6 additional markers corresponding to promoter regions of key tumor suppressor genes: *APC*, *RARβ2*, *TIMP3*, *PTEN*, *HIC1*, and *RUNX3* (depicted in *green*). For the gene-specific MSP data, the *filled boxes* represent a methylated gene promoter, *black boxes* illustrate failed PCR amplification, and *empty boxes* indicate an unmethylated promoter. The *final column* on the right depicts the mean methylation ratios of each tumor (*orange horizontal bars*), based on all 12 markers. (*A*) Methylation profiles in MSI CRCs (n = 24). Extensive methylation is observed in CIMP-related and additional markers. This pattern is also reflected by the high mean methylation ratios in these tumors suggesting CIMP in these cancers. *BRAF V600E* mutation was present in 71% (17/24) of these tumors. Only 4 cases had any LOH events. (*B*) Methylation profiles in the MSI-/LOH- subset of CRCs (n = 45). Frequent methylation was observed in CIMP-related markers in the majority of these tumors. BRAF V600E mutation was confined to CRCs with a high frequency of methylation. (*C*) Methylation profiles in the subset of CRCs demonstrating LOH (n = 57). The samples are sorted based on increasing LOH ratios. As shown, there is substantially less methylation in these tumors. More importantly, methylation at the CIMP-related markers was even less frequent, and only 3% (2 of 57) of cases showed *BRAF* mutations.

CRCs has been associated with CIMP.[10,11] We determined the presence of V600E mutations in the 3 subgroups of CRCs. As depicted in Tables 3 and 4 and Figure 1, a total of 26 of 126 (21%) CRCs harbored *BRAF* mutations. Among these, a significant correlation was observed between *BRAF* mutation and MSI (70.8%; 17 of 24; $P < .0001$ for MSI vs MSI−) and MSI−/LOH− (15.5%; 7 of 45; $P = .03$ for MSI−/ LOH− vs MSI−/LOH+), whereas LOH tumors had an

inverse correlation with *BRAF* mutations (3.5%; 2 of 57; $P < .0001$ for LOH+ vs LOH−).

Methylation of CIMP-Related Markers Distinguishes CIMP From CIN

We performed an analysis using 6 CIMP-related markers and a similar number of additional methylation markers to determine the ability of various markers to distinguish CIMP from other genetic alterations in a

Table 2. Overall Frequency of Epigenetic Alterations in Various Subsets of Sporadic CRCs

	% (number) of CIMP+ (≥3/6 markers)	Odds ratio for CIMP (95% CI)	P value[a] (vs MSI−/ LOH−)
MSI−/LOH− (n = 45)	36 (16)	1.39 (0.63–3.03)	N/A
MSI (n = 24)	75 (18)	11.6 (4.29–35.4)	.002
LOH (n = 57)	9 (5)	0.13 (0.05–0.32)	.001

[a]P values were calculated by the χ^2 test.

cohort of sporadic CRCs. The frequency of methylation at each marker in the total cohort of all CRCs is summarized in Table 3. We observed that methylation of at least 1 locus was present in 110 of 126 (87.3%) of all CRCs. The frequency of promoter methylation by gene was as follows: MINT1 (18%), MINT2 (30%), MINT31 (45%), *HIC1* (62%), *p14ARF* (33%), *RARβ2* (38%), *TIMP3* (26%), *APC* (25%), *hMLH1* (22%), *p16INK4α* (24%), *RUNX3* (16%), and *PTEN* (6%).

When the tumors were segregated based on the designated patterns of genomic or epigenetic alterations, we found that MSI cancers were frequently methylated at

majority of the markers, and 75% (9 of 12) of these markers demonstrated a significantly higher frequency of methylation ($P = .05$ to $P < .0001$) compared with non-MSI CRCs (Table 3 and Figure 1A). Among these 9 markers, 5 were CIMP-related markers (MINT1, MINT2, MINT31, *p14ARF*, and *hMLH1*). As shown in Table 5, the estimated odds ratios indicate an association between MSI (OR values range from 1.12 to 17.2) and the frequency of methylation. Figure 2A illustrates the relationship between MSI and methylation of each marker with the OR values plotted in descending order. This Figure suggests no obvious segregation in the odds of MSI between the 2 sets of markers (CIMP related and the additional markers) because most markers demonstrated a strong association between MSI and methylation. Interestingly, among the CIMP-related markers, methylation of *hMLH1* had the strongest association (OR, 9.35), followed by MINT1, MINT31, MINT2, *p14ARF*, and *p16INK4α*, confirming that *hMLH1* methylation is a key event in the genesis of sporadic MSI CRCs.

Methylation was frequently observed at several markers in the 45 tumors that were MSI−/LOH−. However, a statistically significant association was observed only for the CIMP-related markers, including MINT2 ($P < .001$),

Table 3. The Relationship Between Methylation Frequencies of Epigenetic Markers and BRAF Mutations in Sporadic CRCs Demonstrating MSI and CIN

			All CRCs % (number) (n = 126)	MSI status % (number)			CIN status % (number)		
				MSI (n = 24)	MSI-L/MSS (n = 102)	P value[a]	LOH (+) (n = 61)	LOH (−) (n = 65)	P value[a]
Genetic marker	*BRAF*	Mutant	21 (26)	71 (17)	9 (9)	<.0001	7 (4)	34 (22)	.0001
	V600E	Wt	79 (100)	29 (7)	91 (93)		93 (57)	66 (43)	
CIMP-related markers	MINT1	M	18 (22)	50 (12)	10 (10)	<.0001	17 (10)	19 (12)	.7
		U	82 (100)	50 (12)	90 (88)		83 (50)	81 (50)	
	MINT2	M	30 (35)	59 (13)	23 (22)	.001	11 (6)	47 (29)	<.0001
		U	70 (81)	41 (9)	77 (72)		89 (48)	53 (33)	
	MINT31	M	45 (53)	78 (18)	37 (35)	.0004	21 (12)	68 (41)	<.0001
		U	55 (64)	22 (5)	63 (59)		79 (45)	32 (19)	
	p16INK4α	M	24 (30)	26 (6)	24 (24)	.83	12 (7)	37 (23)	.001
		U	76 (93)	74 (17)	76 (76)		88 (53)	63 (40)	
	p14ARF	M	33 (42)	50 (12)	29 (30)	.05	23 (14)	43 (28)	.02
		U	67 (84)	50 (12)	71 (72)		77 (47)	57 (37)	
	hMLH1	M	22 (25)	60 (12)	14 (13)	<.0001	16 (9)	27 (16)	.17
		U	78 (89)	40 (8)	86 (81)		84 (46)	73 (43)	
Additional epigenetic markers	*APC*	M	25 (31)	29 (7)	24 (24)	.58	23 (14)	26 (17)	.72
		U	75 (94)	71 (17)	76 (77)		77 (46)	74 (48)	
	RARβ2	M	38 (48)	42 (10)	37 (38)	.69	33 (20)	43 (28)	.23
		U	62 (78)	58 (14)	63 (64)		67 (41)	57 (37)	
	TIMP3	M	26 (33)	75 (18)	15 (15)	<.0001	15 (9)	38 (24)	.004
		U	74 (92)	25 (6)	85 (86)		85 (52)	62 (40)	
	PTEN	M	6 (7)	23 (5)	2 (2)	.0003	2 (1)	10 (6)	.05
		U	94 (109)	77 (17)	98 (92)		98 (57)	90 (52)	
	HIC1	M	62 (76)	92 (22)	55 (54)	.0009	57 (35)	67 (41)	.26
		U	38 (46)	8 (2)	45 (44)		43 (26)	33 (20)	
	RUNX3	M	16 (19)	39 (9)	11 (10)	.0008	12 (7)	20 (12)	.28
		U	84 (99)	61 (14)	89 (85)		88 (50)	80 (49)	

Wt, wild type; M, methylated; U, unmethylated.
[a]P values were based on the χ^2 test.

Table 4. The Relationship Between Epigenetic Alterations and BRAF Mutations in MSS Sporadic CRCs Without Evidence for CIN

			Total % (number) (n = 102)	MSS CRCs % (number)		P value[a]
				LOH (−) (n = 45)	LOH (+) (n = 57)	
Genetic marker	*BRAF V600E*	Mutant	9 (9)	16 (7)	4 (2)	.03
		Wt	91 (93)	84 (38)	96 (55)	
CIMP-related markers	MINT1	M	10 (10)	5 (2)	14 (8)	.12
		U	90 (88)	95 (40)	86 (48)	
	MINT2	M	23 (22)	40 (17)	10 (5)	<.001
		U	77 (72)	60 (26)	90 (46)	
	MINT31	M	37 (35)	63 (26)	17 (9)	<.0001
		U	63 (59)	37 (15)	83 (44)	
	$p16^{INK4\alpha}$	M	24 (24)	39 (17)	13 (7)	.002
		U	76 (76)	61 (27)	88 (49)	
	$p14^{ARF}$	M	29 (30)	40 (18)	21 (12)	.04
		U	71 (72)	60 (27)	79 (45)	
	hMLH1	M	14 (13)	10 (4)	17 (9)	.35
		U	86 (81)	90 (36)	83 (45)	
Additional epigenetic markers	*APC*	M	24 (24)	27 (12)	21 (12)	.54
		U	76 (77)	73 (33)	79 (44)	
	RARβ2	M	38 (37)	44 (20)	32 (18)	.18
		U	62 (63)	56 (25)	68 (39)	
	TIMP3	M	15 (15)	20 (9)	11 (6)	.16
		U	85 (86)	80 (35)	89 (51)	
	PTEN	M	2 (2)	3 (1)	2 (1)	.83
		U	98 (92)	98 (39)	98 (53)	
	HIC1	M	55 (54)	56 (23)	54 (31)	.87
		U	45 (44)	44 (18)	46 (26)	
	RUNX3	M	11 (10)	12 (5)	9 (5)	.7
		U	89 (85)	88 (37)	91 (48)	

Wt, wild type; M, methylated; U, unmethylated.
[a]P values were based on the χ^2 test.

MINT31 ($P < .0001$), $p16^{INK4\alpha}$ ($P = .002$), and $p14^{ARF}$ ($P = .04$) (Table 4 and Figure 1B). Of note, these same 4 CIMP-related markers showed a significant inverse association between methylation and LOH. A distinctive distribution of ORs measuring the association between MSI−/LOH− and methylation of the individual markers was observed (Table 5 and Figure 2B). We found that, unlike MSI, where all 12 markers had an OR >1.0, only 6 of the 12 markers demonstrated an OR >1.0 for MSI−/LOH−. Among the 6 markers, 4 were CIMP related, including $p16^{INK4\alpha}$ (OR, 3.20; 95% CI: 1.37-7.48), $p14^{ARF}$ (OR, 1.58; 95% CI: 0.74–3.40), MINT31 (OR, 3.15; 95% CI: 1.43–6.93), and MINT2 (OR, 2.00; 95% CI: 0.89–4.49).

By contrast, the overall incidence of methylation was significantly lower in LOH tumors compared with tu-

Table 5. Odds Ratios for Methylation vs Nonmethylation in Various Subsets of CRCs for Each of the Epigenetic Markers

	Gene markers	MSI OR[a] (95% CI)	MSI−/LOH− OR[a] (95% CI)	LOH OR[a] (95% CI)
CIMP-related markers	MINT1	8.80 (3.13–24.7)	0.15 (0.03–0.68)	0.83 (0.33–2.10)
	MINT2	4.73 (1.78–12.5)	2.00 (0.89–4.49)	0.14 (0.05–0.38)
	MINT31	6.07 (2.07–17.8)	3.15 (1.43–6.93)	0.12 (0.05–0.29)
	$p16^{INK4\alpha}$	1.12 (0.40–3.15)	3.20 (1.37–7.48)	0.23 (0.09–0.59)
	$p14^{ARF}$	2.40 (0.97–5.94)	1.58 (0.74–3.40)	0.39 (0.18–0.85)
	hMLH1	9.35 (3.21–27.2)	0.28 (0.09–0.89)	0.53 (0.21–1.31)
Other epigenetic markers	*APC*	1.32 (0.49–3.56)	1.17 (0.51–2.70)	0.86 (0.38–1.94)
	RARβ2	1.20 (0.49–2.97)	1.51 (0.72–3.19)	0.64 (0.31–1.33)
	TIMP3	17.2 (5.87–50.4)	0.61 (0.25–1.46)	0.29 (0.12–0.69)
	PTEN	13.5 (2.42–75.5)	0.30 (0.03–2.58)	0.15 (0.02–1.31)
	HIC1	8.96 (2.00–40.2)	0.68 (0.31–1.46)	0.66 (0.31–1.37)
	RUNX3	5.46 (1.89–15.8)	0.60 (0.20–1.80)	0.57 (0.21–1.57)

[a]Odds ratios were calculated for the odds of MSI (n = 24) vs other (n = 102). LOH positive (n = 61) vs other (n = 63), and MSI−/LOH− (n = 45) vs other (n = 81).
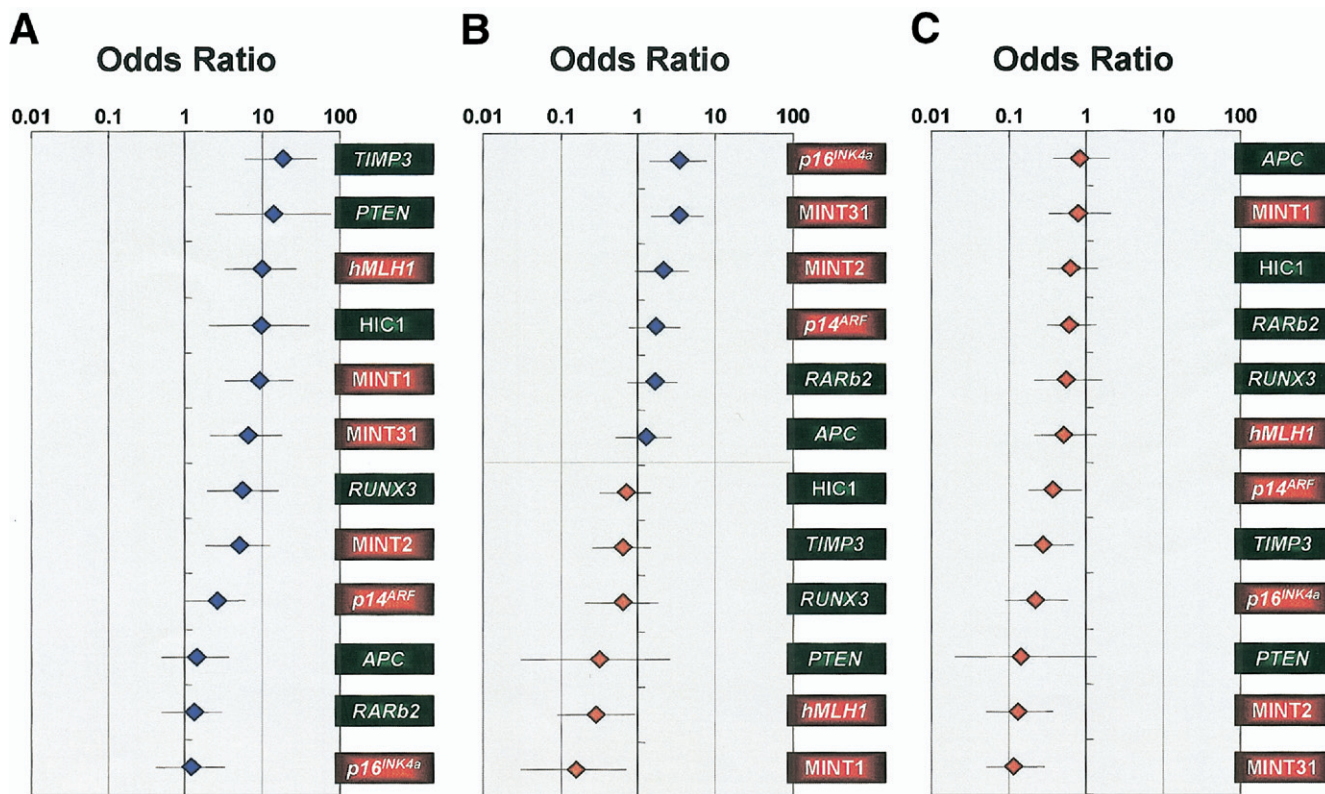
Figure 2. Frequency of methylation at various markers allows discrimination between genetic and epigenetic forms of genomic instability. The Figure illustrates the odds ratios in MSI, LOH, and MSI-/LOH- subsets of CRCs for methylation vs nonmethylation in each of the CIMP-related markers (shown in *red*) and additional markers (*green*). *Squares* represent the estimated odds ratio for each marker. The *horizontal bars* depict the 95% CI for this ratio. An odds ratio >1.0 represents a positive association with methylation of the marker (*blue*), whereas values <1.0 indicates a negative risk (*red*). All odds ratios are presented in a log scale. (*A*) Odds ratios for MSI cancers at each marker. As shown, most of the markers show a positive association between MSI CRCs and methylation; *APC*, *RARβ2*, and *p16*^{INK4α} are exceptions. (*B*) Odds ratios for MSI-/LOH- tumors at each marker. Six of the 12 markers demonstrated a positive risk of MSI-/LOH- with methylation. These results suggest that the 6 CIMP-related markers (*p16*^{INK4α}, MINT31, MINT2, *p14*^{ARF}, *RARβ2*, and *APC*) may be able to identify CIMP tumors from an unselected cohort of sporadic CRCs. (*C*) Odds ratios for LOH CRCs at each marker. As illustrated, a negative risk between LOH CRCs and methylation is seen with each marker in this category and is significant for *p14*^{ARF}, *TIMP3*, *p16*^{INK4α}, *PTEN*, MINT2, and MINT31.

mors that were LOH-negative (Table 3 and Figure 1C). None of the 12 methylation loci had a positive correlation with LOH, and an inverse association between methylation and LOH existed for all the markers (based on OR <1.0 [see Table 5 and Figure 2C]). This inverse correlation was statistically significant for 4 of the 6 CIMP-related markers, including MINT2 (*P* < .0001), MINT31 (*P* < .0001), *p16*^{INK4α} (*P* = .001), and *p14*^{ARF} (*P* = .02), and for 2 of the other markers, *TIMP3* (*P* = .004) and *PTEN* (*P* = .05). These results suggest that tumors demonstrating methylation of these markers are less likely to have LOH.

CIMP Overlaps With Sporadic MSI but Correlates Inversely With LOH in Sporadic CRCs

Next, we examined the degree to which CIMP defined a subset of CRCs distinct from MSI and CIN tumors. Our data showed that the mean methylation ratios in MSI and MSI-/LOH- tumors were significantly higher than in tumors of the LOH group, irrespec-

tive of the total number of methylation markers analyzed (Figure 3A). Methylation of 4 of the CIMP-related markers (*p16*^{INK4α}, MINT31, MINT2, and *p14*^{ARF}) was strongly associated with MSI-/LOH- CRCs. To better define CIMP, we individually analyzed the mean methylation ratios of all 12 markers, the 6 CIMP-related markers, and the 4 selected CIMP-related markers, to assess the predictive value for CIMP. The mean methylation ratio of the MSI-/LOH- subgroup was significantly higher using only the 4 CIMP-related markers (see Figure 3A), and there was overlap between the CRCs and the MSI group (*P* = .26), and a simultaneous distinct separation from the LOH tumors (*P* < .0001).

Although the definition of MSI status in CRC has been standardized,[22] determination of LOH and CIMP is more complex, and these are dynamic processes. It is therefore likely that increasing the total number of LOH or CIMP markers analyzed in a specific scenario may increase the apparent frequency of LOH and CIMP. To control for this, we analyzed our data by plotting the mean LOH vs
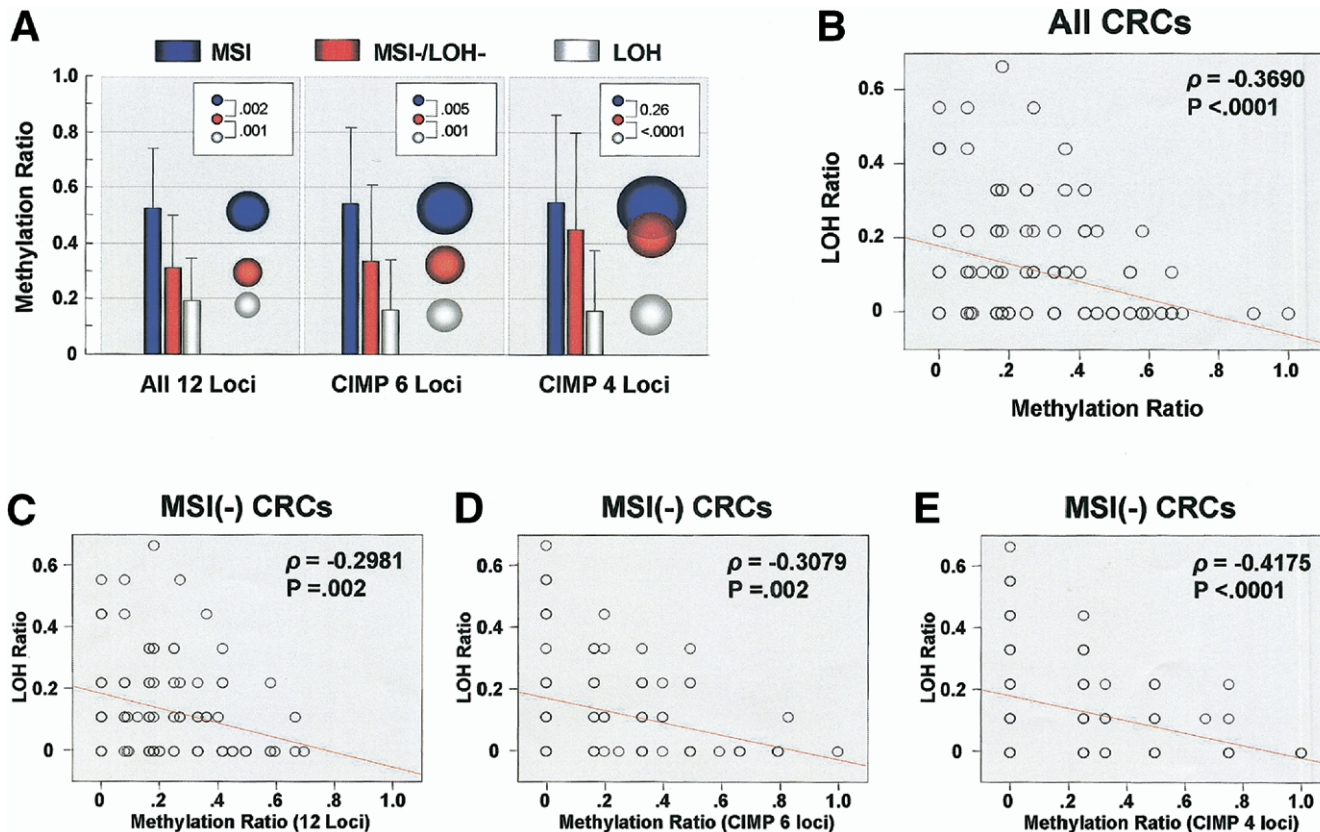
Figure 3. Sporadic CRCs with extensive methylator phenotype inversely correlate with LOH. This Figure illustrates the utility of selected markers for identifying CIMP and the relationship between CIMP and LOH in sporadic CRCs. For these analyses, we compared the mean LOH vs the methylation ratios in each tumor. (*A*) Mean methylation ratios were determined based on the number of markers methylated in the 3 subsets of CRCs. The *3 vertical columns* represent the mean methylation ratios in MSI (*blue*), MSI-/LOH- (*red*), and LOH (*white*) CRCs. The *error bars* denote the SD. The *filled circles* (color matched with *vertical columns*) represent the 95% CI of the mean methylation ratios. The *rectangular boxes* in the *upper panels* represent the pairwise correlation between the mean methylation ratios in each subset of CRCs; the *P* values were calculated by the Wilcoxon test. As shown in the *3 panels*, analysis of the methylation ratios using only 4 CIMP-related markers in comparison with either 12 or 6 CIMP-related markers clearly demonstrates a significant positive association for MSI and MSI-/LOH- tumors but a clear segregation and inverse correlation for LOH CRCs. (*B*) The relationship between methylation ratios calculated using all 12 methylation markers was compared with LOH ratios in the total cohort of 126 CRCs. As shown, an inverse correlation was observed ($\rho = -0.3690$; $P < .0001$) between the incidence of methylation and LOH. These results suggested that increasing either the number of methylation markers or LOH markers would have no effect on the relationship between 2 phenotypes, which are mutually exclusive. Because sporadic MSI tumors closely relate to CIMP, underscoring the relationship between CIMP and LOH, we excluded all MSI cases and reanalyzed the data to show the relationship between the methylation ratios in MSI-negative cases and the LOH burden in these tumors. (*C*) The relationship between mean LOH and methylation ratios obtained using all 12 markers. As indicated, even following exclusion of MSI cases, a significant inverse correlation between methylation and LOH was evident ($\rho = -0.2981$; $P = .002$). (*D*) The relationship between LOH and methylation incidence using 6 CIMP-related markers in MSI-negative cases. A significant inverse relationship was observed ($\rho = -0.3079$; $P = .002$), and there was no added gain using only the CIMP-related markers, compared with using all 12 markers. (*E*) The relationship between LOH and the methylation ratios of the 4 CIMP-related markers in MSI-negative CRCs. Interestingly, the inverse association between LOH and methylation was more prounounced when CIMP data were compared using 4 markers ($\rho = -0.4175$; versus $\rho = -0.2981$ with 12 markers or $\rho = -0.3079$ with 6 markers), suggesting that these selected markers will help identify high-frequency methylated CRCs.

the methylation ratios. This approach should reveal associations between the methylator phenotype and CIN and compensate for changes in the total number of LOH or methylation events (Figure 3B–E). As shown in Figure 3B, differences between the mean methylation ratios from the 12 markers vs the LOH ratios for all CRCs were highly significant (Spearman rank correlation coefficient, $\rho = -0.36$; $P < .0001$). Additionally, analyses of methylation and LOH ratios of all MSS CRCs demonstrated significant inverse correlations between methylation and LOH events. As depicted in Figure 3C–E, the inverse association was strongest when the data were analyzed using only 4 selected CIMP-related markers ($\rho = -0.41$; $P < .0001$), rather than all 12 markers ($\rho = -0.29$; $P = .002$) or the original 6 CIMP-related markers ($\rho = -0.30$; $P = .002$). Taken together, these data show that a significant excess of promoter methylation is present in the sporadic MSI and MSI−/LOH− tumors.

Discussion

Previously, we characterized a large cohort of sporadic CRCs by determining whether they exhibited MSI or CIN and found that ~35% of tumors lacked charac-

teristics of either of these.[6] In addition, we demonstrated that sporadic MSI CRCs were distinct from those with CIN, a finding that has been confirmed by others.[34] These results led us to hypothesize that CIMP is a mechanism of tumor promoter gene silencing in CRCs lacking CIN or MSI.

The present study used a panel of markers to define CIMP in a large cohort of CRCs for which MSI and CIN status was known. Our results indicate that (1) CIMP is frequently present both in MSI tumors, and in MSI-L/MSS CRCs, and involves as many as half of all sporadic CRCs; (2) the 6 originally proposed CIMP-related markers are highly specific for identifying tumors with the methylator phenotype; and (3) CIMP represents a distinct subtype of sporadic CRCs that is inversely associated with CIN. Collectively, these results suggest that CIN and CIMP constitute 2 major and mutually exclusive pathways of tumor evolution in sporadic CRCs. According to this model, CRCs develop by loss of multiple tumor suppressors, and these defects occur by allelic loss in tumors with CIN, by inability to repair single nucleotide mismatches and instability at microsatellite sequences in tumors with MSI, and by methylation-associated silencing in tumors with CIMP. Because methylation of the *hMLH1* promoter is the cause of MSI in nearly all sporadic CRCs,[3] tumors that are MSI constitute a subset of tumors with CIMP. CRCs in Lynch syndrome make up a minority of all MSI cancers and evolve differently.

The overall frequency of DNA methylation in normal tissues increases with age. CIMP, however, is defined by a set of methylation-specific markers that are consistently methylated in tumors to a much greater extent than in normal intestinal mucosa from elderly individuals. Although many studies support a role for CIMP in pathogenesis of sporadic CRC,[7,10,11] markedly different frequencies of this characteristic are reported,[11,15,33,35] with a lack of agreement among different investigators, likely because of differences in methodology and criteria used to define the CIMP phenotype. The work performed here provides a framework for identifying CIMP by placing methylation-specific changes in the context of MSI and CIN in a relatively large group of sporadic CRCs. The overall frequency of genetic and epigenetic alterations examined in this study is similar to those reported previously. In this context, we observed that, although methylation of the 5′-region of *hMLH1* was strongly associated with MSI CRCs, some proportion of non-MSI cancers were also methylated. Similar observations were made previously when it was suggested that 3′-region methylation of *hMLH1* primarily associates with MSI CRCs, whereas 5′-region methylation may be present in non-MSI cancers, and extensive methylation of these regions is required for transcriptional silencing.[17,18,30] When we defined CIMP as being present in tumors exhibiting

positivity in ≥50% of a panel of CIMP-related markers,[16] we found that approximately half of sporadic CRCs met this standard. This figure is larger than in other studies in which the frequency of CIMP was approximately 20%–30%.[10]

Investigators interested in CIMP have identified a specific set of methylation markers that allows identification of the methylator phenotype.[7,10,15,16] In this study, we confirmed that the 6 conventional CIMP-related markers (MINT1, MINT2, MINT31, *p16INK4α*, *p14ARF*, and *hMLH1*) differentiated between tumors with MSI and LOH and that examination of promoter methylation at other tumor suppressor loci did not significantly impact this distinction. Interestingly, only 4 of the conventional CIMP-related markers (MINT2, MINT31, *p16INK4α*, and *p14ARF*) were associated with MSI−/LOH− tumor status. Additionally, the methylation status at these 4 markers showed overlap between MSI−/LOH− and MSI CRCs but clearly distinguished this group of tumors from the MSI−/LOH+ neoplasms, suggesting that the first 2 subsets of tumors collectively represent CIMP and inversely correlate with CIN.

Although many recent studies have utilized MSP assays to detect CIMP,[10,13] it has been suggested that MSP may not be an ideal methodology to detect CIMP.[7] If the MSP assay conditions are not sufficiently stringent, one may overestimate methylation frequencies for a given target gene. However, in this study, we took precautions in designing and performing MSP assays using highly stringent conditions and further confirmed the reproducibility and specificity of MSP assays by ensuring methylation detection only in the tumor tissues and not in the DNA from normal colonic epithelium.

Although the published studies are small, it has been suggested that there are phenotypic characteristics common to CRCs with CIMP. These include the features associated with MSI, such as proximal location and poor differentiation, and changes not seen in MSI tumors, such as a high rate of mutations in *BRAF* and *K-Ras* MSI-L/MSS and CIMP+ tumors, may have a particularly poor prognosis.[36] Mutations in the *BRAF* and *K-Ras* genes are common in sporadic CRC but are mutually exclusive in these neoplasms.[10,18] In this study, we observed a significant association between MSI and V600E *BRAF* mutations, which has been shown to link tightly with promoter methylation of *hMLH1* in MSI CRCs.[18] We observed a similar but somewhat weaker correlation between *BRAF* mutations and MSI−/LOH− as well, but these mutations were not associated with LOH. Although we did not perform *K-Ras* mutation analysis in this study, our data suggest that MSI−/LOH− cancers may harbor more *K-Ras* mutations.[16] An explanation for why one mutation occurs as opposed to the other has been elusive.

In conclusion, until a clear etiology for CIMP is identified, we cannot know for certain whether CIMP is an acquired defect with a primary etiology or whether this abnormal pattern of promoter methylation is a random process that is selected for in tumor cells. The former concept is supported by our observation that CIMP is present not only in MSI tumors but in tumors that lack CIN and MSI characteristics.

Appendix

The following institutions participated in the study:

Institution name	Location	Principal Investigator	Grant No.
CALGB Statistical Office	Durham, NC	Stephen George, PhD	Supported by CA33601
Dana Farber Cancer Institute	Boston, MA	George P. Canellos, MD	Supported by CA32291
Dartmouth Medical School–Norris Cotton Cancer Center	Lebanon, NH	Marc Ernstoff, MD	Supported by CA04326
Massachusetts General Hospital	Boston, MA	Michael L. Grossbard, MD	Supported by CA12449
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Roswell Park Cancer Institute	Buffalo, NY	Ellis Levine, MD	Supported by CA02599
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SUNY Upstate Medical University	Syracuse, NY	Stephen L. Graziano, MD	Supported by CA21060
The Ohio State University	Columbus, OH	Clara D. Bloomfield, MD	Supported by CA77658
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University of California at San Francisco	San Francisco, CA	Alan Venook, MD	Supported by CA60138
University of Chicago Medical Center	Chicago, IL	Gini Fleming, MD	Supported by CA41287
University of Illinois at Chicago	Chicago, IL	David Gustin, MD	Supported by CA74811
University of Iowa	Iowa City, IA	Gerald Clamon, MD	Supported by CA47642
University of Maryland Cancer Center	Baltimore, MD	David Van Echo, MD	Supported by CA31983
University of Massachusetts Medical Center	Worcester, MA	Mary Ellen Taplin, MD	Supported by CA37135
University of Minnesota	Minneapolis, MN	Bruce A. Peterson, MD	Supported by CA16450
University of Missouri/Ellis Fischel Cancer Center	Columbia, MO	Michael C. Perry, MD	Supported by CA12046
University of North Carolina at Chapel Hill	Chapel Hill, NC	Thomas C. Shea, MD	Supported by CA47559
University of Tennessee Memphis	Memphis, TN	Harvey B. Niell, MD	Supported by CA47555
Wake Forest University School of Medicine	Winston-Salem, NC	David D. Hurd, MD	Supported by CA03927
Walter Reed Army Medical Center	Washington, DC	John C. Byrd, MD	Supported by CA26806

BASIC–ALIMENTARY TRACT

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