Evaluation of Markers for CpG Island Methylator Phenotype (CIMP) in Colorectal Cancer by a Large Population-Based Sample

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The CpG island methylator phenotype (CIMP or CIMP-high) with extensive promoter methylation is a distinct phenotype in colorectal cancer. However, a choice of markers for CIMP has been controversial. A recent extensive investigation has selected five methylation markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*) as surrogate markers for epigenomic aberrations in tumor. The use of these markers as a CIMP-specific panel needs to be validated by an independent, large dataset. Using MethyLight assays on 920 colorectal cancers from two large prospective cohort studies, we quantified DNA methylation in eight CIMP-specific markers [the above five plus *CDKN2A* (p16), *CRABP1*, and *MLH1*]. A CIMP-high cutoff was set at $\geq 6/8$ or $\geq 5/8$ methylated promoters, based on tumor distribution and *BRAF/KRAS* mutation frequencies. All but two very specific markers [*MLH1* (98% specific) and *SOCS1* (93% specific)] demonstrated $\geq 85\%$ sensitivity and $\geq 80\%$ specificity, indicating overall good concordance in methylation patterns and good performance of these markers. Based on sensitivity, specificity, and false positives and negatives, the eight markers were ranked in order as: *RUNX3*, *CACNA1G*, *IGF2*, *MLH1*, *NEUROG1*, *CRABP1*, *SOCS1*, and *CDKN2A*. In conclusion, a panel of markers including at least *RUNX3*, *CACNA1G*, *IGF2*, and *MLH1* can serve as a sensitive and specific marker panel for CIMP-high. (*J Mol Diagn* 2007, 9:305–314; DOI: 10.2353/jmoldx.2007.060170)

have been shown to be silenced by promoter methylation in colorectal cancers.^{1,2} In fact, a subset of colorectal cancers have been shown to exhibit promoter methylation in multiple genes, which is referred to as the CpG island methylator phenotype (CIMP).^{2,4–6} CIMP-positive colorectal tumors have a distinct clinical, pathological, and molecular profile, such as associations with proximal tumor location, female sex, mucinous and poor tumor differentiation, microsatellite instability (MSI), and high *BRAF* and low *TP53* mutation rates.^{5,7–13} A link between CIMP-high and JC virus has also been suggested.¹⁴ Promoter CpG island methylation has been shown to occur early in colorectal carcinogenesis.^{15,16} However, not all promoter CpG islands are affected in a similar manner in CIMP-high and non-CIMP-high tumors.¹² Thus, a choice of promoters for a diagnosis of CIMP can substantially influence the features of CIMP-high tumors and may lead to the conclusion that the presence of CIMP as a distinct subtype of colorectal cancer is questionable.^{17,18} Recently, Weisenberger and colleagues¹² have screened 195 CpG islands throughout the human genome (including the classic MINT1, MINT2, and MINT31 loci) and five selected promoter CpG islands (including *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*) that predict epigenomic aberrations in tumor cell and can serve as surrogate markers for CIMP. In contrast, MINT1, MINT2, and MINT31 have been shown to be nonspecific for *BRAF*-mutated CIMP tumors.¹² It is now necessary to validate the use of the CIMP-specific markers using a large number of specimens to avoid any potential bias associated with a small sample size.

In this study using quantitative DNA methylation analysis (MethyLight) and a large number of population-based colorectal cancer specimens, we have evaluated performance characteristics of eight methylation markers including *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1* as well as *CDKN2A* (p16), *CRABP1*, and *MLH1*. The latter three markers have also been selected from screening of the 195 CpG islands to be good markers for CIMP.¹¹ MethyLight assays can reliably distinguish high

Transcriptional inactivation by cytosine methylation at promoter CpG islands of tumor suppressor genes is thought to be an important mechanism in human carcinogenesis.^{1–3} A number of tumor suppressor genes, such as *CDKN2A* (the p16 gene), *MGMT*, and *MLH1*,

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from low levels of DNA methylation, the latter of which likely have little or no biological significance.¹⁹

Materials and Methods

Study Group

We used the databases of two large prospective cohort studies: the Nurses' Health Study ($n = 121,700$ women followed since 1976),^{20–22} and the Health Professionals Follow-Up Study ($n = 51,500$ men followed since 1986).^{22,23} Informed consent was obtained from all participants before inclusion in the cohorts. A subset of the cohort participants developed colorectal cancers during prospective follow-up. Thus, these colorectal cancers represented a population-based, relatively unbiased sample (in contrast to retrospective or single-hospital-based studies). Numerous previous studies on Nurses' Health Study and Health Professionals Follow-Up Study have described baseline characteristics of cohort participants and incident colorectal cancer cases and confirmed that our colorectal cancer cases were well representative as a population-based sample.^{20–23} We collected paraffin-embedded tissue blocks from hospitals where cohort participants with colorectal cancers had undergone resections of primary tumors. We excluded cases when patients were preoperatively treated with radiation and/or chemotherapy. Based on availability of adequate tissue specimens and results, a total of 920 colorectal cancer cases (410 from the men's cohort and 510 from the women's cohort) were included. In only seven cases not included in this study, MethyLight reactions failed (failure rate 7 of $927 = 0.76\%$) despite our attempt for tumor epigenotyping, presumably because of poor quality of the specimens. Most tumors have previously been characterized for MSI, *KRAS* and *BRAF* gene status, and CIMP status.^{11,24,25} However, no study by our group or others has assessed performance characteristics of all of the eight markers examined in this study, using a large number of specimens. Tissue collection and analyses were approved by the Dana-Farber Cancer Institute and Brigham and Women's Hospital Institutional Review Boards.

Genomic DNA Extraction and Whole Genome Amplification

Genomic DNA was extracted using QIAmp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions as previously described.¹¹ Whole genome amplification of genomic DNA was performed by polymerase chain reaction (PCR) using random 15-mer primers²⁶ for subsequent MSI analysis and *KRAS* and *BRAF* sequencing. Previous studies by us and others showed that whole genome amplification did not significantly affect *KRAS* mutation detection or microsatellite analysis.^{26,27}

Real-Time PCR (MethyLight) for Quantitative DNA Methylation Analysis

Sodium bisulfite treatment on genomic DNA was performed as previously described.¹⁹ For DNA methylation analysis, we typically used one to two tissue sections ($10\ \mu\text{m}$ thick) when large tumor sections were available. Real-time PCR to measure DNA methylation (MethyLight) was performed as previously described.^{28–30} Using ABI 7300 (Applied Biosystems, Foster City, CA) for quantitative real-time PCR, we amplified eight CIMP-specific promoters [*CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOC1*]. *COL2A1* (the collagen 2A1 gene) was used to normalize for the amount of input bisulfite-converted DNA.^{19,30} Primers and probes were previously described as follows: *CACNA1G*, *CRABP1*, and *NEUROG1*^{11,12}; *CDKN2A* and *COL2A1*³⁰; *MLH1*¹⁹; and *IGF2*, *RUNX3*, and *SOC1*.¹² The percentage of methylated reference (PMR, ie, degree of methylation) at a specific locus was calculated by dividing the *GENE:COL2A1* ratio of template amounts in a sample by the *GENE:COL2A1* ratio of template amounts in SssI-treated human genomic DNA (presumably fully methylated) and multiplying this value by 100.³¹ A PMR cutoff value of 4 was based on previously validated data.^{19,30,31} Based on the distribution of PMR values at the *CRABP1* and *IGF2* loci, we raised PMR cutoff to 6 for *CRABP1* and *IGF2*. Precision and performance characteristics of bisulfite conversion and subsequent MethyLight assays have been previously evaluated, and the assays have been validated.¹⁹

MSI Analysis

Methods to determine MSI status have been previously described.³² In addition to the recommended MSI panel consisting of D2S123, D5S346, D17S250, BAT25, and BAT26,³³ we also used BAT40, D18S55, D18S56, D18S67, and D18S487 (ie, 10-marker panel).³² A high degree of MSI (MSI-H) was defined as the presence of instability in $\geq 30\%$ of the markers. A low degree of MSI (MSI-L) was defined as the presence of instability in $< 30\%$ of the markers, and microsatellite stable (MSS) tumors were defined as tumors without an unstable marker. Among 889 tumors with MSI status determined, 131 tumors (15%) were MSI-H.

Sequencing of *KRAS* and *BRAF*

Methods of PCR and sequencing targeted for *KRAS* codons 12 and 13, and *BRAF* codon 600 have been previously described.^{24,26} Among 874 tumors with both *KRAS* and *BRAF* genes sequenced, *KRAS* and *BRAF* mutations were present in 321 tumors (37%) and 116 tumors (13%), respectively.

Statistical Analysis

For statistical analysis, the χ^2 test (or Fisher's exact test for categories with an N value of less than 10) was per-

Table 1. Distribution of Colorectal Cancers According to the Number of Methylated Promoters

	Total no.	Number of methylated promoters										CIMP-low 1 to 5	CIMP-high 6 to 8		
		0 (CIMP-0)	1	2	3	4	5	6	7	8					
All cases	920	431 (47%)	171	86	35	34	27	29	46	61	353 (38%)		136 (15%)	] <i>P</i> = 0.03	
Men	410	194 (47%)	89	41	15	15	13	12	12	19	173 (42%)		43 (10%)		
Women	510	237 (46%)	82	45	20	19	14	17	34	42	180 (35%)		93 (18%)		
MSI-H	131	14 (11%)	12	9	2	0	1	8	30	55	24 (18%)	] <i>P</i> < 0.0001	93 (71%)	] <i>P</i> < 0.0001	
MSI-L	73	34 (47%)	12	7	6	4	4	3	1	2	33 (45%)				6 (8.2%)
MSS	685	368 (54%)	138	69	24	29	22	17	15	3	282 (41%)				35 (5.1%)

formed on categorical data, using the SAS program (version 9.1; SAS Institute, Cary, NC). All P values were two-sided, and statistical significance was set at P values of ≤ 0.05 .

Results

Criteria for CpG Island Methylator Phenotype-High (CIMP-High)

We obtained 920 colorectal cancer specimens and quantified DNA methylation in the eight CIMP-specific gene promoters (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*) by MethyLight technology. All of the eight loci were selected from 195 loci throughout the human genome (including *MINT1*, *MINT2*, *MINT31*, and *THBS1*, which were described in the original CIMP panel⁴), because methylation in these loci was a predictor for CIMP-high and PCR for these loci showed excellent amplification efficiency in methylation-positive samples.^{11,12} Using a smaller number of tumors, we and others have previously validated the use of some combinations of these eight loci for the determination of CIMP-high in colorectal cancer.^{11,12} Among the 920 tumors studied, only rare cases showed PMR within the range of PMR cutoff ± 1 at any locus (data on *CACNA1G*, *CDKN2A*, *CRABP1*, *MLH1*, and *NEUROG1* in the first 460 cases¹¹ and data not shown for all 920 cases). Thus, the methylation status (positive or negative) at each locus could be unequivocally determined for a vast majority of the cases.

Table 1 shows the distributions of the number of methylated promoters (from 0 to 8) in all 920 colorectal cancers. We confirmed the associations between CIMP-high and female sex, and between CIMP-low and male sex as we previously reported using five-marker CIMP panel.²⁴ Table 1 also shows distributions of microsatellite instability-high (MSI-H), MSI-low (MSI-L), and MSS tumors according to the number of methylated promoters. Among 131 MSI-H tumors in this study, we observed a striking bimodal distribution with only one tumor (0.8%) exhibiting 4/8 to 5/8 methylated promoters. Thus, it is reasonable to assume that a cutoff for CIMP-high is either $\geq 6/8$, $\geq 5/8$, or $\geq 4/8$ methylated promoters, at least in MSI-H tumors. Distributions of the number of methylated promoters in MSI-L and MSS tumors did not significantly differ, justifying analysis of the combined MSI-L/MSS category.

BRAF mutations have been shown to be tightly linked to CIMP-high,^{11,12} and *KRAS* mutations have been shown to be more common in CIMP-low tumors than CIMP-high tumors.²⁴ Therefore, we assessed *BRAF* and *KRAS* mutation frequencies according to the number of methylated promoters, to refine the cutoff for CIMP-high. We used *BRAF* and *KRAS* mutations as markers of CIMP-high and CIMP-low, respectively, because no other markers (besides methylation markers) have been shown to separate CIMP-high from CIMP-low as clearly as *BRAF* and *KRAS*.^{12,24} As shown in Figure 1A, *BRAF* mutations were more common in extensively methylated tumors ($\geq 6/8$ methylated promoters) than in tumors with less extensive methylation ($\leq 4/8$ methylated promoters). *KRAS* mutations were less common in tumors with $\geq 6/8$ methylated promoters than in tumors with $\leq 4/8$ methylated promoters. Tumors with 5/8 methylated promoters showed intermediate features between 6/8 methylated tumors and 4/8 methylated tumors. Figure 1B shows a clear separation between MSI-H tumors with $\geq 6/8$ methylated promoters and tumors with $\leq 3/8$ methylated promoters. As shown in Figure 1C, MSI-L/MSS tumors could be separated into CIMP-high ($\geq 6/8$ methylated promoters) and CIMP-low/CIMP-0 ($\leq 4/8$ methylated promoters) categories based on the frequencies of *KRAS* and *BRAF* mutations. Tumors with 5/8 methylated promoters showed intermediate features between 6/8 methylated tumors and 4/8 methylated tumors. Thus, the cutoff for CIMP-high could be narrowed, either $\geq 6/8$ or $\geq 5/8$ methylated promoters, resulting in the CIMP-high frequency of 15% (=136 of 920) or 18% (=163 of 920), respectively, in our large sample.

Assessment of Individual Methylation Markers

To evaluate the performance characteristics of the eight methylation markers, we calculated sensitivity and specificity of each of the individual eight markers among all 920 tumors, with two different cutoffs for CIMP-high ($\geq 6/8$ and $\geq 5/8$ methylated promoters) (Table 2). With either cutoff for CIMP-high, *CRABP1*, *IGF2*, and *NEUROG1* demonstrated very good sensitivity ($\geq 95\%$), whereas *CACNA1G*, *MLH1*, *RUNX3*, and *SOCS1* showed superior specificity ($\geq 90\%$). We did not examine how bimodal was the distribution of tumors according to different marker combinations, as a previous study did to select the best set of markers.¹² This is because bimodal distribution was observed in MSI-H tumors but not in MSI-L/MSS

Figure 1. *BRAF* and *KRAS* mutation frequencies according to number of methylated promoters. **A:** Tumors with $\geq 6/8$ methylated promoters show high *BRAF* mutation rates, whereas tumors with $\leq 5/8$ methylated promoters show high *KRAS* mutation rates. **B:** MSI-H tumors distribute bimodally, and the frequencies of *KRAS* and *BRAF* mutations clearly distinguish CIMP-high tumors from CIMP-low tumors. **C:** MSI-L/MSS tumors can be separated into CIMP-high ($\geq 6/8$ methylated promoters) and CIMP-low/0 ($\leq 4/8$ methylated promoters) based on the frequencies of *BRAF* and *KRAS* mutations. Tumors with $5/8$ methylated promoters reside on the borderline between CIMP-high and CIMP-low.

tumors (Table 1). We rather assessed sensitivity and specificity of each marker, which reflected how concordant methylation patterns were.

To compare performance characteristics of individual markers, we evaluated two features of individual markers: the sum of sensitivity and specificity (the larger, the better) and the sum of the numbers of false-positive cases and false-negative cases (the smaller, the better) (Table 3). Although sensitivity or specificity is important in a setting where there are some characteristic samples (eg, sensitivity is important when testing only MSI-H tumors), the latter (the number of false-positive and false-negative cases) is important in a relatively unbiased clinical setting because it directly reflects the number of misdiagnosed cases. We assigned rank order numbers for individual markers according to the sum of sensitivity and specificity from the largest to the smallest. We also assigned rank order numbers for individual markers according to the sum of false-positive/-negative cases from the smallest to

Figure 2. Sensitivity, specificity, and cross-panel classification error rate against panel 8. Panel 1 (*RUNX3* only) through panel 7 contain incrementing numbers of markers, adding one by one from *CACNA1G*, *IGF2*, *MLH1*, *NEUROG1*, *CRABP1*, and *SOCS1*. Panel 8 contains all eight markers including *CDKN2A*. **A:** Specificity generally increases with an increasing number of markers. Sensitivity depends on the number of markers and a CIMP-high cutoff. **B:** The classification error rate decreases with an increasing number of markers.

the largest. The sum of these rank order numbers for both CIMP-high cutoffs ($\geq 6/8$ and $\geq 5/8$ methylated promoters) was an overall score for each marker (smaller, the better) (Table 3). We admit that there is no established method to evaluate methylation markers using a large sample; however, our method took into account the parameters commonly assessed for other clinical laboratory markers. As a result, *RUNX3* seemed to be the best marker, followed by *CACNA1G* and *IGF2*. *MLH1* ranked fourth by virtue of its best specificity (98%). *NEUROG1*, *CRABP1*, *SOCS1*, and *CDKN2A* ranked fifth, sixth, seventh, and eighth, respectively.

To avoid confounding effect of each marker on diagnosing CIMP status, sensitivity and specificity were also determined for each marker with CIMP status determined by seven markers excluding the marker that is being evaluated (Table 4). For instance, to assess sensitivity and specificity of *CACNA1G*, CIMP status was determined by seven markers excluding *CACNA1G*. In this way, we could avoid confounding effect of *CACNA1G* status on diagnosing the CIMP status by the eight markers including *CACNA1G*. For this analysis, CIMP-high was defined as the presence of $\geq 5/7$ methylated promoters. As in Table 4, each marker still exhibited good sensitivity and/or specificity for the diagnosis of CIMP-high, indicating good overall concordance of methylation status in the eight markers. The eighth ranked marker *CDKN2A* demonstrated 85% sensitivity and 81% specificity. Table 5 shows the methylation frequency of each marker in colorectal cancers with a specific *BRAF/KRAS* status. The methylation frequencies of the eight markers were high in the *BRAF*-mutated tumors and low in the

Table 2. Sensitivity and Specificity of Each Marker for Diagnosis of CIMP-High

Marker	Total no.	CIMP-high (cutoff $\geq 6/8$ markers methylated)		CIMP-high (cutoff $\geq 5/8$ markers methylated)	
		Positive (sensitivity)*	Negative (specificity) [‡]	Positive (sensitivity)*	Negative (specificity) [‡]
	920	136 (15%) [†]	784	163 (18%) [†]	757
<i>CACNA1G</i>					
(+)	199	133 (98%)*	66	145 (89%)*	54
(-)	721	3	718 (92%) [‡]	18	703 (93%) [‡]
<i>CDKN2A</i>					
(+)	273	123 (90%)*	150	142 (87%)*	131
(-)	647	13	634 (81%) [‡]	21	626 (83%) [‡]
<i>CRABP1</i>					
(+)	291	135 (99%)*	156	161 (99%)*	130
(-)	629	1	628 (80%) [‡]	2	627 (83%) [‡]
<i>IGF2</i>					
(+)	216	132 (97%)*	84	157 (96%)*	59
(-)	704	4	700 (89%) [‡]	6	698 (92%) [‡]
<i>MLH1</i>					
(+)	115	98 (72%)*	17	101 (62%)*	14
(-)	805	38	767 (98%) [‡]	62	743 (98%) [‡]
<i>NEUROG1</i>					
(+)	271	133 (98%)*	138	155 (95%)*	116
(-)	649	3	646 (82%) [‡]	8	641 (85%) [‡]
<i>RUNX3</i>					
(+)	183	132 (97%)*	51	152 (93%)*	31
(-)	737	4	733 (93%) [‡]	11	726 (96%) [‡]
<i>SOCS1</i>					
(+)	155	98 (72%)*	57	106 (65%)*	49
(-)	765	38	727 (93%) [‡]	57	708 (94%) [‡]

*Sensitivity of each marker is defined as the number of CIMP-high cases positive for a given marker divided by the number of all CIMP-high cases.

[†]Prevalence of CIMP-high.[‡]Specificity of each marker is defined as the number of non-CIMP-high cases negative for a given marker divided by the number of all non-CIMP-high cases.

BRAF/KRAS wild-type tumors and the *KRAS*-mutated tumors.

Comparison of CIMP Panels

We assessed various combinations of markers from the eight methylation markers as CIMP-specific marker panel. Some combinations have previously been described.^{11,12} These eight markers were selected based on screening of 195 loci that were distributed throughout the human genome, and methylation in these markers were considered to reflect the global epigenomic status of cancer cells.^{11,12} All of the eight markers showed sensitivities $>60\%$ and specificities $\geq 80\%$ for CIMP-high (Tables 2 and 4). Then, we could consider that a classi-

fication based on all of the eight markers was more accurate in reflecting true CIMP status (global epigenomic status) than any other marker combinations. Thus, we designated the eight-marker panel (panel 8) as the gold standard. *BRAF* and *KRAS* mutation frequency spectra indicated that a separation between CIMP-high and CIMP-low by the five markers of Weisenberger and colleagues¹² was not perfect with more borderline cases (40 cases showing 3/5 methylated promoters, 43% *KRAS*-mutated, and 28% *BRAF*-mutated) and that, although still not perfect, CIMP panel 8 served as a superior gold standard panel (with 27 cases showing 5/8 methylated promoters, 44% *KRAS*-mutated, and 33% *BRAF*-mutated). The overall high sensitivities and specificities of the eight markers indicated that methylation of

Table 3. Assessment of Individual Methylation Markers

Marker	Overall ranking	Sum of four rank order numbers	Rank for the sum of sensitivity and specificity		Rank for false-positive and -negative cases	
			CIMP-high cutoff			
			≥6/8	≥5/8	≥6/8	≥5/8
<i>RUNX3</i>	1	4.5	1	1	1.5*	1
<i>CACNA1G</i>	2	11	2	3	3	3
<i>IGF2</i>	2	11	3	2	4	2
<i>MLH1</i>	4	19.5	7	7	1.5*	4
<i>NEUROG1</i>	5	21	4	5	6	6
<i>CRABP1</i>	6	23	5	4	7	7
<i>SOCS1</i>	7	26	8	8	5	5
<i>CDKN2A</i>	8	28	6	6	8	8

*Average of rank order numbers 1 and 2.

Table 4. Sensitivity and Specificity of Each Marker for Diagnosis of CIMP-High (Determined by Seven Markers Excluding Each Marker to Be Evaluated)

Marker	Total no.	CIMP-high (cutoff $\geq 5/7$ markers methylated)	
		Positive (sensitivity)*	Negative (specificity)†
<i>CACNA1G</i>			
(+)	199	133 (88%)*	66
(-)	721	18	703 (91%)†
<i>CDKN2A</i>			
(+)	273	123 (85%)*	150
(-)	647	21	626 (81%)†
<i>CRABP1</i>			
(+)	291	135 (99%)*	156
(-)	629	2	627 (80%)†
<i>IGF2</i>			
(+)	216	132 (96%)*	84
(-)	704	6	698 (89%)†
<i>MLH1</i>			
(+)	115	98 (61%)*	17
(-)	805	62	743 (98%)†
<i>NEUROG1</i>			
(+)	271	133 (94%)*	138
(-)	649	8	641 (82%)†
<i>RUNX3</i>			
(+)	183	132 (92%)*	51
(-)	737	11	726 (93%)†
<i>SOCS1</i>			
(+)	155	98 (63%)*	57
(-)	765	57	708 (93%)†

Note that CIMP-high is determined by seven makers excluding each marker to be evaluated to assess sensitivity and specificity without confounding the effect of the particular marker to be evaluated. For example, to assess sensitivity and specificity of *CACNA1G*, CIMP-high is determined by seven markers excluding *CACNA1G*. Sensitivity and/or specificity of each marker is still quite good.

*Sensitivity of each marker is defined as the number of CIMP-high cases positive for a given marker divided by the number of all CIMP-high cases.

†Specificity of each marker is defined as the number of non-CIMP-high cases negative for a given marker divided by the number of all non-CIMP-high cases.

these markers was reasonably linked and any of these markers could serve as a good surrogate marker for the CIMP status. We further attempted to find the best combination of a relatively small number of markers for an accurate classification of the CIMP status.

We compared three combinations of markers, which were designated as follows: panel 8, panel 5A (*RUNX3*, *CACNA1G*, *IGF2*, *NEUROG1*, and *SOCS1*) described by

Weisenberger and colleagues,¹² and panel 5B (*CACNA1G*, *MLH1*, *NEUROG1*, *CRABP1*, and *CDKN2A*) described by Ogino and colleagues,¹¹ and panel 4 (including the four best markers; *RUNX3*, *CACNA1G*, *IGF2*, and *MLH1*). For each CIMP panel, we examined the *BRAF* and *KRAS* mutation frequencies according to the number of methylated promoters (as in Figure 1) to determine a cutoff for CIMP-high (data not shown). In general, CIMP-high was defined as >65 to 70% of promoters methylated.

An example of assessment of cross-panel classification errors is shown in Table 6. Tumors were classified as CIMP-high or non-CIMP-high, by one panel (eg, panel 5A) versus the other panel (eg, panel 8). We counted the number of tumors for which classifications (CIMP-high versus non-CIMP-high) were discordant by the two panels. In the example of panel 5A versus panel 8 (Table 6), the cross-panel classification error rate was $13/920 = 1.4\%$. Table 7 shows the cross-panel classification error rates for all pairwise comparisons of the four CIMP panels (8, 5A, 5B, and 4). Remarkably, no pairwise comparison showed the error rate greater than 3.2%, implying that, for a vast majority of tumors, it made no difference to determine the CIMP status by any of these panels. Even panel 4 with only a half of the number of markers in panel 8 showed an excellent concordance rate (98%) with panel 8.

We next examined sensitivity, specificity, and cross-panel classification error rate (with panel 8 as the gold standard) of each CIMP panel with an incrementing number of markers from panel 1 (*RUNX3* only) to panel 7, adding markers one by one, in the order of: *CACNA1G*, *IGF2*, *MLH1*, *NEUROG1*, *CRABP1*, and *SOCS1* (Figure 2). CIMP-high was defined as follows, based on the *BRAF* and *KRAS* mutation frequencies (data not shown): 2/2 methylated promoters for panel 2, 3/3 methylated promoters for panel 3, $\geq 3/4$ methylated promoters for panel 4, $\geq 4/5$ methylated promoters for panel 5, $\geq 5/6$ methylated promoters for panel 6, $\geq 5/7$ methylated promoters for panel 7, and $\geq 6/8$ methylated promoters for panel 8. There was a trend toward higher specificity with a larger number of markers. Sensitivity depended on both CIMP-high cutoff and the number of markers. Panel 4 showed excellent sensitivity and specificity, which were almost as high as those by panel 7. With regard to the classification error rates, there was a trend toward a lower error rate

Table 5. Frequency of Methylation Positivity in Each Marker in Colorectal Cancer According to *BRAF* and *KRAS* Status

Marker	Overall		<i>BRAF</i> (-) <i>KRAS</i> (-)		<i>BRAF</i> (+) <i>KRAS</i> (-)		<i>BRAF</i> (-) <i>KRAS</i> (+)		<i>BRAF</i> (+) <i>KRAS</i> (+)	
	n	%	n	%	n	%	n	%	n	%
Total	874		443		110		315		6	
<i>CACNA1G</i>	191	22	59	13	81	74	49	16	2	33
<i>CDKN2A</i>	263	30	94	21	87	79	79	25	3	50
<i>CRABP1</i>	277	32	98	22	95	86	81	26	3	50
<i>IGF2</i>	208	24	56	13	89	81	61	19	2	33
<i>MLH1</i>	110	13	39	8.8	62	56	7	2.2	2	33
<i>NEUROG1</i>	258	30	80	18	97	88	79	25	2	33
<i>RUNX3</i>	177	20	44	9.9	90	82	40	13	3	50
<i>SOCS1</i>	147	17	56	13	55	50	34	11	2	33

Table 6. Assessment of Cross-Panel Classification Error in Two CIMP Marker Panels (An Example)

		Number of methylated promoters (panel 8)									Total
		0	1	2	3	4	5	6	7	8	
Number of methylated promoters (panel 5A)*	0	431	90	14	2						537
	1		81	57	11						149
	2			15	19	21					55
	3				3	11	21	5			40
	4					2	6	21	21		50
	5							3	25	61	89
Total		431	171	86	35	34	27	29	46	61	920

CIMP-high is defined as ≥ 4 methylated promoters in the five-marker panel and ≥ 6 methylated promoters in the eight-marker panel, based on the *BRAF* and *KRAS* mutation rates. The numbers of cases shaded in gray represent cases with discordant CIMP status by the two panels (CIMP-high by one panel and CIMP-low by the other panel). The error rate comparing these two panels is calculated as $(2 + 6 + 5)/920 = 1.4\%$.

*This five-marker panel (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*) was described by Weisenberger et al.¹²

with a larger number of markers. The error rate by panel 4 was 2.0%. Thus, it seemed that panel 4 was almost as good as panel 8, which included twice as many markers.

Finally, we examined clinicopathological features including tumor location (right-sided versus left-sided), tumor grade (poorly differentiated versus well to moderately differentiated), and mucinous features (with $\geq 50\%$ mucinous component) in relation to combined MSI/CIMP status determined by either CIMP panel 8 or panel 4 (Figure 3). Both MSI-H and CIMP-high (determined by either panel) were correlated with proximal (right) location, poor differentiation, and mucinous tumors, and there was no substantial difference between classifications by panel 8 and panel 4.

Discussion

We conducted this study to evaluate comprehensively the performance characteristics of CpG island methylation markers that have been shown to be useful for the determination of CIMP status. Weisenberger and colleagues¹² have recently conducted an extensive investigation to select methylation markers that are specific for CIMP and can be used as surrogate markers for epigenomic aberrations in tumor cell. The proposed promoter panel needs to be validated by a large independent

dataset to confirm acceptable sensitivities and specificities of markers. Our resource of a large number of colorectal cancer specimens obtained from two large prospective cohorts has enabled us to test the five markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*) selected from 195 loci by Weisenberger and colleagues¹² as well as three other markers [*CDKN2A* (p16), *CRABP1*, and *MLH1*] that were also selected by the initial screening of the 195 loci.¹¹ The large number of tumors provided a sufficient

Table 7. Cross-Panel Classification Error Rate in 920 Colorectal Cancers

	Panel 8*	Panel 5A [†]	Panel 5B [‡]	Panel 4 [§]
Panel 8*		13 (1.4%)	15 (1.6%)	18 (2.0%)
Panel 5A [†]	13 (1.4%) [¶]		28 (3.0%)	21 (2.3%)
Panel 5B [‡]	15 (1.6%) [¶]	28 (3.0%)		29 (3.2%)
Panel 4 [§]	18 (2.0%) [¶]	21 (2.3%)	29 (3.2%)	

For each panel, the threshold distinguishing CIMP-high from CIMP-low samples was determined based on the frequencies of *BRAF* and *KRAS* mutations.

*Panel 8 includes *RUNX3*, *CACNA1G*, *IGF2*, *MLH1*, *NEUROG1*, *CRABP1*, *SOCS1*, and *CDKN2A* (CIMP-high cutoff, $\geq 6/8$ methylated promoter).

[†]Panel 5A includes *RUNX3*, *CACNA1G*, *IGF2*, *NEUROG1*, and *SOCS1* (CIMP-high cutoff, $\geq 4/5$ methylated promoter).

[‡]Panel 5B includes *CACNA1G*, *MLH1*, *NEUROG1*, *CRABP1*, and *CDKN2A* (CIMP-high cutoff, $\geq 4/5$ methylated promoter).

[§]Panel 4 includes *RUNX3*, *CACNA1G*, *IGF2*, and *MLH1* (CIMP-high cutoff, $\geq 3/4$ methylated promoter).

[¶]Differences in the error rates against panel 8 were not statistically significant (all pairwise comparisons, $P > 0.26$).

Figure 3. Frequencies of right-sided tumors (A), poorly differentiated tumors (B), and mucinous tumors (C) in various MSI/CIMP subtypes of colorectal cancer. Gray and open bar graphs indicate frequencies of each feature in MSI/CIMP subtypes determined by CIMP panel 4 and CIMP panel 8, respectively. Note that there were no substantial differences in the features examined (anatomical location, tumor grade, or mucinous features) between classifications determined by CIMP panel 4 and panel 8.

power to examine each category of tumors with a specific number of methylated promoters (eg, 4/8, 5/8, or 6/8 methylated promoters). As the gold standard CIMP panel, we used an expanded panel of the eight methylation markers (making 920 cases $\times$ 8 markers = 7360 methylation data points), which include the five markers proposed by Weisenberger and colleagues.¹² It is reasonable to include more markers sensitive and specific for CIMP-high in a gold standard CIMP-specific marker panel. *BRAF* and *KRAS* mutation frequency spectra indicated that CIMP panel 8 served as a superior gold standard CIMP panel to the five markers (CIMP panel 5A).

We have shown that all of the eight methylation markers evaluated exhibit good sensitivity and specificity for overall CIMP status and that the best individual marker to predict the CIMP status is *RUNX3*, followed by *CACNA1G*, *IGF2*, *MLH1*, *NEUROG1*, *CRABP1*, *SOC1*, and last, *CDKN2A*. *CDKN2A* still exhibits more than 80% sensitivity and specificity for the prediction of CIMP status determined by the other seven markers. The four best markers we proposed are slightly different from the five markers proposed by Weisenberger and colleagues,¹² presumably because of a difference in the sources and sizes of samples; however, we emphasize that all of the eight markers tested have shown good concordance of methylation patterns. Lower sensitivity of *SOC1* and lower specificity of *NEUROG1* (compared with *RUNX3*, *CACNA1G*, and *IGF2*) have previously been shown by Weisenberger and colleagues (see Figures 4 and 5 in Weisenberger et al¹²). However, various marker combinations in CIMP panels do not misclassify substantial numbers of tumors, especially when *RUNX3* is included in a CIMP panel. The validity of the eight markers was also shown by a striking bimodal distribution of MSI-H tumors according to the number of methylated markers (Table 1 and Figure 1). However, we failed to observe such bimodality in MSI-L/MSS tumors. In addition, although we could separate CIMP-high from CIMP-low by the frequencies of *BRAF* and *KRAS* mutations, the difference between CIMP-high and CIMP-low in MSI-L/MSS tumors was not as clear-cut as in MSI-H tumors (Figure 1). It remains to be seen whether there exists a different set of markers that can even more clearly separate the rare MSS CIMP-high subtype (~5% of all colorectal cancers) from the MSS CIMP-low subtype.

Weisenberger and colleagues¹² examined 195 CpG islands throughout the human genome (including *MINT1*, *MINT2*, *MINT31*, and *THBS1*, which were described by Toyota and colleagues⁴) and five selected markers including *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOC1*. *CDKN2A*, *CRABP1*, and *MLH1* were also shown to be good predictors for CIMP status by the initial screening of the 195 loci.¹¹ Using a limited number of samples (40 MSS tumors and 10 sporadic MSI-H tumors), Weisenberger and colleagues¹² compared the five markers with the classic methylation markers including *CDKN2A*, *MINT1*, *MINT2*, *MINT31*, and *MLH1*. Their data have shown that methylation in *CDKN2A* and *MLH1* is correlated well with *BRAF* mutations as the new five markers, whereas methylation in *MINT1*, *MINT2*, and *MINT31* is not specific for *BRAF*-mutated CIMP tumors (see Figures 4

and 5 in Weisenberger and colleagues¹²). That is why *MINT1*, *MINT2*, and *MINT31* were excluded from the current study. Nonetheless, we emphasize that our data do not necessarily indicate that these MINT markers or other CpG islands are inappropriate for assessment of CIMP in colorectal cancer, because it remains a possibility that a difference in primer designs and PCR conditions may substantially change sensitivity and specificity of a particular marker for the detection of CIMP in colorectal cancer.

We propose the use of (at least) four markers, including *RUNX3*, *CACNA1G*, *IGF2*, and *MLH1*, as a cost-effective CIMP-specific promoter panel. Some investigators have suggested that *MLH1* may be eliminated from a CIMP panel because the CIMP status of MSS tumors is primarily determined by methylation markers other than *MLH1*.⁸ However, in our previous study including *MLH1* in the CIMP promoter panel, there were very similar *BRAF* mutation frequencies in CIMP-high versus non-CIMP-high tumors, regardless of MSI status.¹¹ Thus, we cannot justify exclusion of *MLH1* from a CIMP panel solely because of its tight association with MSI phenotype. In the current study, *MLH1* proved to be the most specific marker for CIMP-high (ie, 98% specificity). Besides an important role in our proposed CIMP-specific panel, *MLH1* methylation testing is clinically useful because most, although not all, MSI-H tumors in Lynch syndrome (a major form of hereditary nonpolyposis colorectal cancer, HNPCC) show no evidence of *MLH1* methylation.^{34,35} Thus, *MLH1* methylation positivity would favor against a diagnosis of Lynch syndrome, in which hereditary colorectal cancer is typically caused by germline and somatic mutations in one of mismatch repair genes including *MSH2*, *MLH1*, *MSH6*, and *PMS2*.^{34,35} However, we emphasize that *MLH1* methylation is not a diagnostic test for Lynch syndrome because not all MSI-H tumors without *MLH1* methylation are related to Lynch syndrome and because *MLH1* methylation positivity cannot completely exclude the possibility of Lynch syndrome.

MSI-H CIMP-low/0 colorectal cancer is an interesting subtype that warrants discussion. As discussed above, most colorectal cancers arising in a background of Lynch syndrome/HNPCC exhibit MSI-H, but no evidence of *MLH1* methylation or CIMP-high. Thus, most HNPCCs belong to the MSI-H CIMP-low/0 group. However, HNPCCs probably constitute only a minority in the MSI-H CIMP-low/0 group because the population frequency of Lynch syndrome/HNPCC among all colorectal cancers is estimated to be 1 to 3%,³⁵ and the population frequency of MSI-H CIMP-low/0 colorectal cancers is estimated to be 4.3% (=38 of 889, Table 1). These data imply the presence of MSI-H CIMP-low/0 tumors unrelated to Lynch syndrome/HNPCC, in contrast to Weisenberger and colleagues¹² who state that MSI-H tumors arise either through CIMP pathway or in a background of Lynch syndrome/HNPCC. There is probably a different pathway (CIMP-low/0 unrelated to HNPCC) to MSI-H tumors.

We have previously shown that CIMP-low is associated with male sex and *KRAS* mutations.²⁴ In the current study, we have confirmed that these associations still persist with increases in both the number of cases and the number of methylation markers in a CIMP panel.

However, differences between CIMP-low (1/8 to 5/8 methylated promoters) and CIMP-0 (0/8 methylated promoters) are still not large. Further studies are necessary to assess whether CIMP-low represents a distinct phenotype in colorectal cancer and, if the hypothesis is true, to determine reasonably good markers for the detection of CIMP-low. We recognize that the markers we have chosen are sensitive and specific for the detection of CIMP-high, rather than the identification of CIMP-low.

In summary, we have evaluated CIMP-specific DNA methylation markers and criteria for CIMP-high by a large population-based colorectal cancer sample. Our findings indicate that all of the eight markers evaluated are reasonably good surrogate markers to determine the CIMP status and that (at least) four markers including *RUNX3*, *CACNA1G*, *IGF2*, and *MLH1* constitute a sensitive and specific CIMP panel for the purpose of research and clinical use.

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