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Mutations in both *KRAS* and *BRAF* may contribute to the methylator phenotype in colon cancer

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Abstract

Background—Colorectal cancers (CRCs) with the CpG island methylator phenotype (CIMP) often associate with epigenetic silencing of h*MLH1* and an activating mutation in the *BRAF* gene. However, the current CIMP criteria are ambiguous, and often result in an underestimation of CIMP frequencies in CRCs. Since *BRAF* and *KRAS* belong to same signaling pathway, we hypothesized that not only mutations in *BRAF*, but mutant *KRAS*, may also associate with CIMP in CRC.

Methods—We determined the methylation status of a panel of 14 markers (7 canonical CIMP-related loci, and 7 new loci), MSI status, and *BRAF/KRAS* mutations in a cohort of 487 colorectal tissues that included both sporadic and Lynch syndrome patients.

Results—Methylation analysis of seven CIMP-related markers revealed that the mean number of methylated loci was highest in *BRAF* mutated CRCs [3.6], versus *KRAS*-mutated [1.2; $P<0.0001$] or *BRAF/KRAS* wild-type tumors [0.7; $P<0.0001$]. However, analyses with seven additional markers showed that the mean number of methylated loci in *BRAF* mutant tumors [4.4] was the same as in *KRAS* mutant CRCs [4.3; $P=0.8610$]. Although sporadic MSI-H tumors had the most average number of methylated markers [8.4], surprisingly Lynch syndrome CRCs also demonstrated frequent methylation [5.1].

Conclusions—CIMP in CRC may result from activating mutations in either *BRAF* or *KRAS*, and the inclusion of additional methylation markers that correlate with mutant *KRAS* may help clarify CIMP in future studies. Additionally, aberrant DNA methylation is a common event not only in sporadic CRC, but also in Lynch syndrome CRCs.

Keywords

BRAF; KRAS; CIMP; colon cancer; methylation

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Introduction

Aberrant promoter hypermethylation associated with transcriptional silencing of multiple tumor suppressor genes has been proposed as a mechanistic component in the evolution of several human cancers [1]. Tumors with a critical degree of aberrant methylation have the CpG Island Methylator Phenotype (CIMP), which was initially described in the colorectal cancer (CRC) [2]. CIMP, microsatellite instability (MSI) and chromosomal instability constitute the three major mechanisms of genomic or epigenetic instability in CRC [3,4]. Since the initial description of CIMP, its role in carcinogenesis has been controversial [3,5–7]. Experimental evidence has consistently supported the presence of CIMP in a subset of CRCs [3,4,8,9], and it has been found that CIMP significantly correlates with a V600E mutation in the *BRAF* gene [10,11]. However, some degree of promoter methylation can be found in normal tissues and virtually all CRCs, so some have argued that CIMP does not constitute a unique pathogenetic pathway [7].

Due to the controversy surrounding CIMP, many laboratories have focused on making a case that CIMP constitutes a discrete group of CRCs through the identification of an optimized panel of CIMP-specific markers [11–13]. Two panels of CIMP-specific markers have been proposed, including those identified by the originators of the concept[12,13], and a second panel recently reported after an extensive evaluation of a very large group of candidate markers [11]. Both panels are highly specific for the identification of CIMP CRCs with *BRAF* V600E mutations [11]. However, there is a lack of consensus on the frequency of CIMP in CRC, because of multiple definitions of CIMP (CIMP+ and CIMP−, CIMP-high and low, etc.), the use of different sets of methylation markers, and differences in diagnostic methodologies. However, the greatest challenge has been to use consistent criteria for CIMP analyses to categorize a cancer positive or negative for this phenotype, and finding a consensus for the definition of CIMP [8,9,14,15].

Nearly all of the approximately 15% of sporadic MSI CRCs come from a CIMP background, caused by the epigenetic silencing of h*MLH1*; however, additional CRCs that are microsatellite stable (MSS) may also be labeled as CIMP depending upon the diagnostic criteria used [4,8,9]. It has been proposed that CIN and CIMP represent two major mechanisms of genomic (or epigenetic) instability in CRC, and that perhaps upto 50% of CRCs might be characterized as having CIMP [4]. Thus, it is important to have a more complete understanding of CIMP, and to reach a consensus on whether this constitutes a unique and unified group of tumors that may evolve through a common pathway.

CIMP determinations using CIMP-related markers have consistently identified clusters of CRCs with MSI and V600E *BRAF* mutations, but rarely *KRAS* mutations [8,9,14,16]. However, when additional methylation loci have been investigated, additional subsets of CRCs have been identified with extensive methylation; these tumors are MSS, and are associated with mutations in the *KRAS* gene [4,17,18]. Although these tumors should be considered CIMP-positive, they have been typically categorized as non-CIMP, because, by definition, analyses using the canonical CIMP criteria considered only those methylation events strictly associated with mutant *BRAF*. Defining the markers in this fashion raises a possible problem of circular reasoning.

BRAF and *KRAS* gene products function in the same serial signaling pathway, and activating mutations in these genes are usually mutually exclusively [10,19]. Interestingly, prior to the more recent discovery of V600E *BRAF* mutations, *KRAS* mutations were proposed as the possible cause of aberrant methylation. It has been shown that fibroblasts transformed by *fos* or *ras*

experience upregulation of DNA methyltransferase (DNMT) expression and consequent global hypermethylation [20].

In light of these considerations, we hypothesized that CIMP in CRC may not be exclusively the result of mutations in *BRAF*, but might be a more general consequence of the upregulation of the RAS-RAF pathway. In the present study, we investigated the relationship between the activating mutations of the *BRAF/KRAS* genes and global hypermethylation using a broad panel of methylation markers that included both the canonical CIMP-related markers, and additional methylation-related loci. We included in our investigation a group of Lynch syndrome-related CRCs, in which there is MSI, but no mutations in *BRAF*. We found that methylation of this broad panel of methylation markers segregates not just with *BRAF*, but also with *KRAS* mutations. Our data provide novel evidence for a potential role of *KRAS* mutations in the evolution of aberrant methylation, and propose a broader panel of methylation markers that may improve our current understanding for the molecular basis of CIMP.

Materials and Methods

Colorectal tissue specimens

We obtained a cohort of 487 colorectal tissues, including 243 cancers, 208 corresponding normal colonic mucosal specimens from patients with CRC (C-N), and 36 normal epithelial tissues from patients without any evidence of neoplasia at colonoscopy (N-N) from Okayama University Hospital, Okayama, Japan and Heidelberg University, Heidelberg, Germany. Of the 243 CRCs, 21 cancers were from Lynch syndrome patients, and had documented germline mutations and associated loss of protein expression of the MMR genes: hMSH2, hMLH1, hPMS2, and hMSH6; the remaining 222 were sporadic CRCs, in which patients did not have any family history of CRC, and had no evidence for polyposis or IBD. Among 222 sporadic CRCs, 184 cases have been previously analyzed for CIMP [17]. Normal mucosal tissues were sampled from distant surgical margins of the CRC resections. All patients provided written informed consent, and the studies were approved by Institutional Review Boards of all institutions involved.

Sodium bisulfite modification and Combined Bisulfite Restriction Assays (COBRA)

Genomic DNA obtained from colorectal tissues was bisulfite modified to convert all unmethylated cytosine residues to uracils. Bisulfite PCR reactions were carried out and restriction endonuclease digestion was performed on PCR products for methylation analyses of 14 markers (Table 1) [21]. Seven methylation markers were canonical CIMP-related loci, including the *hMLH1*-5' region, *hMLH1*-3' region, $p16^{INK4a}$, $p14^{ARF}$, MINT1, MINT2, and MINT31 [4,5,16]. We then analyzed methylation frequencies at promoter regions of seven additional tumor suppressor genes that have been reported to be frequently methylated in a cancer-specific manner in CRC (*SFRP2*, *RASSF2A*, *MGMT*, *Reprimo*, *3OST2*, *HPP1*, and *APC*) [22–29].

The digested DNA was separated on 3% agarose gels and stained with ethidium bromide. The quantitative methylation levels (ratios of methylated to unmethylated DNA) were determined from the relative intensities of cleaved and non-cleaved PCR products. A marker was considered methylation positive if it showed ≥5% methylation density, and methylation negative if it had <5%, in accordance with canonical CIMP criteria [12].

Microsatellite Analysis

Microsatellite analysis of each tumor tissue was determined using the NCI-workshop panel of recommended markers [30]. Tumors showing a shift in at least one mononucleotide marker and one other marker were classified as MSI-H, and were referred to as MSI cancers throughout

the text. We then analyzed all non-MSI tumors with five additional dinucleotide repeat sequences (D5S107, D8S87, D17S261, D18S35, and D18S58) and one tetranucleotide repeat marker (MYCL1). MSI-Low (MSI-L) was defined as a shift in any of the dinucleotide and/or tetranucleotide markers; tumors which showed no allelic shifts were classified as microsatellite stable (MSS) [31]. We grouped 55 MSI-L and 152 MSS together as non-MSI cancers in this study for comparative purposes because both have similar clinicopathologic, and mutational features.

BRAF and *KRAS* mutation analysis

Direct sequencing was performed to identify *BRAF* V600E mutation and *KRAS* codon 12/13 mutations. Primer sequences for *BRAF* and *KRAS* were: BRAF-F (5′-TGCTTGCTCTGATAGGAAAATGA-3′), BRAF-R (5′-TGGATCCAGACAACTGTTCAAA-3′), KRAS-F (5′-GCCTGCTGAAAATGACTGAA-3′) and KRAS-R (5′-AGAATGGTCCTGCACCAGTAA-3′) that generated fragment lengths of 165 and 167 bp, respectively. PCR products were purified using a QIAquick PCR purification kit (Qiagen) and directly sequenced on an ABI 3100-Avant DNA sequencer.

Statistical analyses

The methylation status of 14 epigenetic markers was analyzed as a categorical variable (methylated: methylation level ≥5%, unmethylated: methylation level <5%). CRCs were divided into subgroups according to MSI/LS or *BRAF/KRAS* mutation status, and the relationship of each epigenetic marker with various clinico-pathological parameters was evaluated using the X^2 test. We used Kruskal-Wallis one way analysis of variance on rank sums to compare overall differences in the average number of methylated loci among subgroups classified by MSI status (sporadic MSI or Lynch syndrome) or *BRAF/KRAS* mutation status. Whenever the Kruskal-Wallis test indicated differences among these subgroups, further pair-wise comparisons for each of the subgroups divided by MSI/Lynch Syndrome or *BRAF/KRAS* mutation status was performed using a non-parametric multiple comparison method using Steel-Dwass test. In order to ascertain the relative risks for methylation, we calculated the odds ratio (OR) of each CRC subgroup according to methylation at each epigenetic marker. A 95% confidence interval (CI) was calculated for each OR. All reported *P* values are 2-sided and a $P<0.05$ was considered statistically significant.

Results

V600E *BRAF* mutations are frequently present in sporadic MSI CRCs, while *KRAS* mutations are exclusively observed in Lynch syndrome and non-MSI CRCs

A total of 487 colorectal tissues, including 243 CRCs, 208 C-Ns, and 36 N-Ns were investigated. All tumors were categorized into subgroups depending upon their MSI status (sporadic or Lynch syndrome), and *BRAF/KRAS* mutations. We found that 36/243 (15%) CRCs were MSI and 207/243 (85%) were non-MSI. Among the 36 MSI tumors, 15 (42%) were sporadic MSI CRCs, while 21 (58%) were from Lynch syndrome patients (which had been oversampled for this analysis). To highlight the differences between Lynch syndrome and sporadic MSI tumors, we categorized all CRCs into three subsets based upon *BRAF/KRAS* mutation status as sporadic MSI, Lynch syndrome and non-MSI cancers. We observed that 8% (20/243) of the CRCs harbored V600E *BRAF* mutations (“*BRAF* mutant”), 33% (80/243) had *KRAS* codon 12 or 13 mutations (“*KRAS* mutant”), and 59% (143/243) CRCs lacked mutations in both *BRAF* and *KRAS* genes (“wild type”).

Table 2 illustrates detailed associations between the CRC groups based upon MSI status (sporadic MSI and Lynch syndrome), and mutation spectrum (*BRAF/KRAS*). We observed that a significant proportion of sporadic MSI CRCs occurred in patients ≥65 years of age (80%),

which was similar to the frequency in non-MSI patients (51%), but as anticipated, the age was significantly lower in Lynch syndrome CRCs (5%, Lynch syndrome vs. sporadic MSI or non-MSI; $P<0.0001$). Sporadic MSI tumors were more frequent in females than males (sporadic MSI, 60%; Lynch syndrome, 19%; Non-MSI, 35%). Also, 92% of sporadic MSI and 69% of Lynch syndrome CRCs were proximally located, in contrast to 30% of non-MSI cancers.

Older patients (≥65 years at diagnosis) frequently harbored *BRAF* (65%) or *KRAS* (56%) mutations, although many lacked mutations in either gene (42%) ($P = 0.0378$ for either mutation vs younger patients). Female patients had significantly more *BRAF* mutations compared to males (*BRAF* mutant, 65%; *KRAS* mutant, 35%; wild type, 32%; $P=0.0147$ for *BRAF* mutant vs. *KRAS* mutant, $P=0.0033$ for *BRAF* mutant vs. Wild type). *BRAF* and *KRAS* mutant CRCs were significantly more common in the proximal colon, compared to Wild type CRCs (*BRAF* mutant, 65%; *KRAS* mutant, 46%; wild type, 26%; $P=0.0013$ for *BRAF* mutant vs. Wild type, $P=0.0047$ for *KARS* mutant vs. Wild type).

Importantly, *BRAF* mutations were detected at high frequencies in sporadic MSI tumors (67%), were seldom present in non-MSI tumors (5%), and did not occur in Lynch syndrome patients (0%). On the other hand, *KRAS* mutations were never present in sporadic MSI cancers (0%), while 33% of Lynch syndrome and 35% of non-MSI CRC exhibited *KRAS* mutations.

Aberrant DNA hypermethylation is rare in normal colon, but is frequently observed in CRC

We investigated the methylation status at fourteen methylation-related loci using quantitative COBRA in the total cohort of 487 colorectal tissues. We observed that 97% of CRCs (236/243), 46% of C-Ns (96/208) and 19% of N-Ns (7/36) showed evidence for methylation at ≥1 markers (Figure 1A). Methylation levels in C-N and N-N tissues were quite low (<5% methylation) in comparison to that observed in CRCs, suggesting the cancer-specificity of various methylation markers (Figure 1B). Furthermore, the proportion of tissues with methylation at ≥2 loci was significantly higher in CRCs (90%, 219/243) than in C-Ns (14%, 29/208) or N-Ns (3%, 1/36; $P<0.0001$).

As shown in Table 3, the overall frequency of promoter hypermethylation at each gene/locus was highly variable: *SFRP2* (63%), *RASSF2A* (68%), *MGMT* (24%), *Reprimo* (24%), *3OST2* (82%), *HPP1* (69%), *APC* (29%), *hMLH1*-5' region (22%), *hMLH1*-3' region (4%), $p16^{INK4a}$ (17%), $p14^{ARF}$ (10%), MINT1 (12%), MINT2 (21%) and MINT31 (26%).

A distinct subset of markers is methylated in sporadic MSI versus Lynch syndrome CRCs

We correlated the methylation status at individual epigenetic markers in the MSI tumors (Table 3). When methylation features were compared between sporadic MSI and Lynch syndrome tumors, as expected, sporadic MSI tumors were more frequently methylated at most of the markers, the exceptions being: MINT31, *SFRP2*, and *APC*. Interestingly, aberrant methylation at these three loci was more frequent in Lynch syndrome tumors. MINT31, which is one of the canonical CIMP markers, was methylated at equal frequencies in Lynch syndrome CRCs (62%) as in sporadic MSI (60%). Of potential importance for understanding the genesis of tumors in Lynch syndrome, methylation at the *APC* promoter was more frequently observed in Lynch syndrome CRCs (48%) in comparison to both sporadic MSI (27%) and non-MSI (27%) tumors.

Hypermethylation frequency at individual markers is related to *BRAF* and *KRAS* mutational status in CRC

We interrogated the relationship between methylation frequencies at each of the 14 epigenetic markers and the presence or absence of *BRAF/KRAS* mutations in the total cohort of CRCs (Table 3). We observed that the six classical CIMP-related markers (*hMLH1*-5' region,

hMLH1-3' region, $p16^{INK4a}$, MINT1, MINT2, and MINT31) along with *Reprimo* were significantly more frequently methylated in *BRAF* mutant CRCs compared to the other subgroups. However, we found that methylation frequencies at the other five markers (*SFRP2*, *RASSF2A*, *MGMT*, *3OST2*, and *HPP1*) were almost same or relatively higher in CRCs harboring *KRAS* mutations. At the same time, *APC* methylation frequencies were comparable in all three subsets (25% in *BRAF* mutants, 29% in *KRAS* mutants, and 29% in wild types).

Epigenetic alterations can be interpreted in the context of the mutational spectrum of the *KRAS-BRAF* genes

We estimated the odds ratios (OR) for methylation at each marker among subgroups of CRCs segregated by sporadic MSI/Lynch syndrome and *BRAF/KRAS* mutation status (Figure 2 and Supplemental Table). When ORs were calculated for sporadic MSI vs Lynch syndrome CRCs, we observed that methylation of all seven canonical CIMP markers, plus *Reprimo*, showed a positive risk for methylation in sporadic MSI tumors (Figure 2A). Contrariwise, methylation of MINT31 was significantly associated with Lynch syndrome cancers (OR, 5.74; 95%CI, 2.29–15.2). None of the other non-canonical epigenetic markers showed any risk associations based upon MSI and/or Lynch syndrome status.

We then examined ORs for methylation at each marker among the subgroups of CRCs categorized by *BRAF/KRAS* mutation status (Figure 2B). The ORs of the six canonical markers (*hMLH1*-5' region, *hMLH1*-3' region, $p16^{INK4a}$, MINT1, MINT2, and MINT31), together with Reprimo were significantly higher in *BRAF* mutant CRCs, while five of the seven new markers (*SFRP2*, *RASSF2A*, *MGMT*, *3OST2*, and *HPP1*) showed significantly higher ORs in *KRAS* mutant CRCs. Although the OR of $p14^{ARF}$ was the highest for *BRAF* mutant cancers, it was not significantly different compared to other two subgroups. Similarly, the OR for *APC* methylation was similar among various subsets (OR for *BRAF* mutant, 0.81; for *KRAS* mutant, 1.00; for wild type, 1.07). This analysis permitted us to categorize the epigenetic markers into three distinct subsets: *BRAF*-related markers (*hMLH1*-5' region, *hMLH1*-3' region, $p16^{INK4a}$, MINT1, MINT2, MINT31, and *Reprimo*); *KRAS*-related markers (*SFRP2*, *RASSF2A*, *MGMT*, *3OST2*, and *HPP1*); and markers independent of the *BRAF/KRAS* mutations ($p14^{ARF}$ and *APC*).

Aberrant DNA methylation is more frequent in Lynch syndrome CRCs than in non-MSI CRCs

Both Lynch syndrome CRCs and tumors with methylated h*MLH1* promoters have the MSI phenotype, but they arise through different pathways. To better understand the differences between these types of CRCs, we determined the average numbers of methylated loci in each subgroup of CRCs (Figure 3A and Table 4). When methylation data were utilized using all 14 epigenetic markers, the average number of methylated loci was highest in sporadic MSI CRCs (8.4; 95%CI, 6.0–10.2), followed by Lynch syndrome CRCs (5.1; 95%CI, 4.1–6.2), and was least in non-MSI CRCs (4.4; 95%CI, 4.1–4.8). Not surprisingly, when the data were analyzed using only the canonical CIMP markers (*hMLH1*-5' region, *hMLH1*-3' region, $p16^{INK4a}$, $p14^{ARF}$, MINT1, MINT2, and MINT31), the average number of methylated loci was significantly higher in sporadic MSI CRCs (3.6; 95%CI, 2.3–4.9), compared to Lynch syndrome (1.5; 95%CI, 1.0–2.0), or non-MSI subgroups (0.9; 95%CI, 0.7–1.0; $P<0.0001$). However, interestingly, when the data were analyzed only using the seven additional methylation markers, the differences in average number of methylated loci between Lynch syndrome and non-MSI tumors became much smaller (sporadic MSI [4.8; 95%CI, 4.1–5.5], Lynch syndrome [3.7; 95%CI, 3.0–4.4], non-MSI [3.6; 95%CI, 3.3–3.8]).

Furthermore, the average numbers of methylated loci were consistently higher for Lynch syndrome CRCs compared with non-MSI CRCs (Table 4 and Figure 3A). More specifically, Lynch syndrome CRCs showed significantly higher methylation when data were analyzed from

seven CIMP canonical markers (1.5 versus 0.9; *P*=.0230). Although Lynch syndrome cancers were more frequently methylated than non-MSI cancers, these data did not reach significance when comparisons were drawn from 14 unselected epigenetic markers (5.1 versus 4.4; *P*=0.2782), or the seven additional markers (3.7 versus 3.6; *P*=0.9977).

Comparable methylation frequencies occur in *BRAF* and *KRAS* mutant CRCs analyzed using the additional epigenetic markers

We then determined whether the average numbers of methylated loci were influenced by the presence of *BRAF* and *KRAS* mutations in CRC (Figure 3B and Table 4). When we used data from all 14 markers, we observed that the average number of methylated loci was highest in *BRAF* mutated tumors (8.0; 95%CI, 6. 5–9. 5), followed by *KRAS* mutants (5. 5; 95%CI, 5.0–6.0) and the lowest in CRCs with wild-type *BRAF* and *KRAS* genes (3.9; 95%CI, 3.5–4.3; *P*<0.0001). Similar findings were observed when data were analyzed from the seven canonical CIMP markers alone (*BRAF* mutant [3.55; 95%CI, 2.7–4.4], *KRAS* mutant [1.2; 95%CI, 0.9–1.5], wild type [0.7; 95%CI, 0.6–0. 9]; *P*<0.0001).

Interestingly, when the data were analyzed using the seven additional markers, the average number of methylated loci in *KRAS* mutated cancers was no different than that in *BRAF* mutant CRCs (*BRAF* mutant [4.4; 95%CI, 3.7–5.1], *KRAS* mutant [4.3; 95%CI, 4.0–4.6]; *P*=0.8610), and average number of methylated loci in both of these subgroups was significantly higher than in the tumors lacking mutations in both *BRAF* and *KRAS* [3.2; 95%CI, 2.9–3. 5];*BRAF* vs Wild type, *P*<0.01; *KRAS* vs Wild type, *P*<0.0001).

Discussion

This study investigates the relationship between mutational activation in the *RAS-RAF* signaling pathway and global hypermethylation using a panel of epigenetic markers in a cohort of 487 colorectal tissues. More specifically, we asked whether CIMP in the colon is exclusively correlated with *BRAF* mutations, or whether it may also associate with mutant *KRAS*, since both genes are members of the same signaling pathway. Additionally, we asked whether CIMP is primarily a disease of older individuals with sporadic MSI CRCs, or whether Lynch syndrome CRCs may also have molecular features consistent with CIMP. We have provided evidence that aberrant hypermethylation of various tumor suppressor genes and related loci not only associate with mutant *BRAF*, but also with mutant *KRAS*, and that activation of the KRAS-BRAF pathway induces aberrant promoter methylation in multiple genes. Additionally, we found that Lynch syndrome CRCs have frequent methylation, challenging the supposition that CIMP is exclusively a molecular characteristic of sporadic CRCs.

Although the CIMP concept was first proposed in CRC almost a decade ago [2], only recently has evidence supported its existence in a specific subset of sporadic CRCs [3,4,8,9]. In spite of this, the molecular basis of CIMP remains unclear and is a matter of active investigation. Currently, CIMP CRCs are characterized using a panel of markers that were selected, in part, through their association with the V600E *BRAF* mutation [11–13]. Although the canonical CIMP-related markers are highly specific, this panel may not be completely adequate, as there is no consensus on the frequency of CIMP in CRC [8,9]. Part of the problem may be attributed to differences in methodologies for measuring methylation in each laboratory, but central to this issue is a lack of consensus criteria and definitions for CIMP in CRC [8,9,14,15]. Moreover, some studies have reported data in which a subset of CRCs have intermediate levels of aberrant DNA methylation defined as CIMP-low, as they failed to meet the more restrictive criteria for CIMP [14,32–35]. This has led others to challenge the methylator phenotype as a discrete “pathway” in colorectal carcinogenesis.

Herein, we present data that clearly suggest that CIMP-associated aberrant methylation observed in CRCs may not be limited to those with V600E *BRAF* mutations. Rather, CIMP determination appears to be dependent upon the choice of methylation markers. When data are analyzed using canonical CIMP-related markers, one can identify a distinct cluster of CRCs that are strongly associated with mutant *BRAF* [14,33]. However, this tight clustering of CRC disappears when additional epigenetic markers are analyzed. We demonstrate that when data are evaluated from canonical markers, *BRAF* mutant CRCs have the highest average number of methylated loci. However, when additional methylation markers are analyzed, both *BRAF* and *KRAS* mutated cancers show similar degrees of methylation, and methylation levels in these two subgroups are significantly higher than in CRCs that are wild-type for these genes.

In comparison to the classical CIMP markers that strongly associate with mutant *BRAF*, data analysis with non-canonical methylation markers in our study resulted in loss of the typical bimodal distribution observed for CIMP-positive and negative tumors. However, these data are biologically relevant since a significant amount of aberrant methylation was associated with mutant *KRAS*. Additionally, *when* data were analyzed from only non-CIMP markers, *KRAS* mutated and wild type CRCs were statistically distinguishable from each other (Table 4). Similar suggestions have been made in previous studies [11,36]. It is difficult to directly compare our panel of non-canonical CIMP markers with the markers interrogated in the previous studies [11,36]. A common feature among these studies is the suggestion that a significant amount of methylation positively associates with mutant *KRAS* in the colon. Since *KRAS* and *BRAF* belong to the same growth signaling pathway, these data argue that activating mutations in either of these genes may have equivalent effects in mediating aberrant DNA methylation. This brings the RAS/RAF story full circle, as it was initially found that *KRAS* mutations were responsible for upregulating DNA methylation and a methylator phenotype in vitro [20]. In support of this, a recent study has clearly shown that Ras-mediated epigenetic silencing occurs through a specific, but complex pathway, involving components that are essential for maintaining a fully transformed phenotype in a fibroblast cell line [37].

It has been proposed that CIMP CRCs that harbor *BRAF* mutations and demonstrate MSI may originate through a unique pathway that includes the progression of sessile serrated polyps to MSI CRCs [38]. It has been suggested that V600E *BRAF* mutations are present in sessile serrated polyps and serrated aberrant crypt foci (ACF), whereas, *KRAS* mutations are more highly associated with non-serrated polyps and ACFs [32,39]. Interestingly, 90% of ACF with *BRAF* mutations were found to be MSS [39]. Approximately 70% of sporadic MSI-H CRCs exhibit *BRAF* mutations and a majority of sporadic MSI-H CRCs are caused by extensive hypermethylation of the *hMLH1* gene [17,32]. Our data and that from others suggest that aberrant DNA methylation may be induced by upregulation of the RAS-RAF pathway [37], and that genetic alteration in *BRAF* or *KRAS* might be an earlier event that precedes aberrant DNA methylation.

In this study we noticed a positive association of RASSF2A methylation with mutant *KRAS*, which contradicts an earlier report [23]. However, the study by Hesson and colleagues [23] involved a small subset of tissues (8 adenomas, and 33 carcinomas). A subsequent study analyzed a cohort of 140 CRCs, and reported that 67% of CRCs with RASSF2 methylation displayed *BRAF* or *KRAS* mutations (P=0.0009), and showed that inactivation of RASSF2 enhanced *KRAS*-induced oncogenic transformation [25]. Our present data are in agreement with this recent study, and we feel confident that our data are reliable, and that RASSF2A methylation positively associates with mutant *KRAS* in the colon.

CIMP predominantly occurs in older individuals with sporadic MSI CRCs, but it has not been rigorously investigated in Lynch syndrome [32,40]. In this study, we observed that sporadic MSI CRCs had the highest degree of aberrant methylation regardless of the epigenetic markers

analyzed [32]. However, unexpectedly, we observed that Lynch syndrome patients, who were much younger, had higher degree of methylation than that of non-MSI CRCs, particularly when data were analyzed from seven canonical CIMP markers (P=0.023). It is possible that the higher degree of methylation observed in Lynch syndrome may be due, in part, to methylation events associated with the frequent *KRAS* mutations.

In the past decade, the efforts of several labs have been focused upon the molecular mechanisms of aberrant DNA methylation, but our current understanding into these processes is limited. Although this study does not provide a conclusive explanation for the specific processes that control DNA methylation, but our data provide indirect evidence that highlight the importance of *KRAS*-associated methylation events. We also found that aberrant DNA methylation is a much more common event in Lynch syndrome patients than was previously presumed. We speculate that genetic alterations in the *BRAF* and *KRAS* oncogenes is an early event in the evolution of a methylator phenotype, and that regulation of aberrant DNA methylation may be located downstream in the RAS-RAF signaling pathway. Finally, considering the paucity of information on the causes of CIMP, in the future studies it may be prudent to interpret aberrant DNA methylation in the context of mutations in both *BRAF* and *KRAS* genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

CIMP, CpG island methylator phenotype; C-N, normal colorectal tissue from a patient with colorectal cancer; COBRA, combined bisulfite restriction assay; CRC, colorectal cancer; MSI, microsatellite instability; MSI-H, MSI-high; MSI-L, MSI-low; MSS, microsatellite stable; N-N, colorectal tissue from a normal patient (without colorectal neoplasia).

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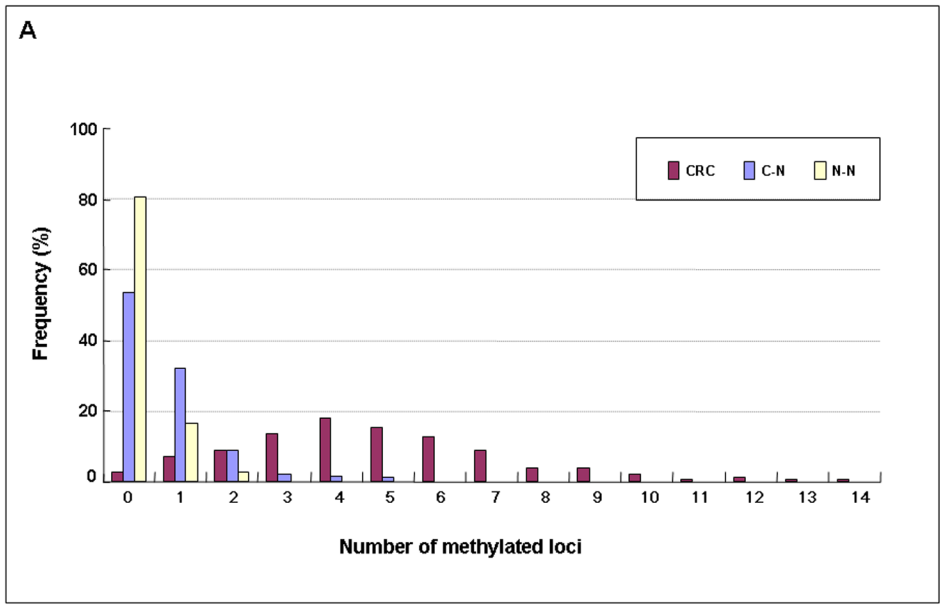

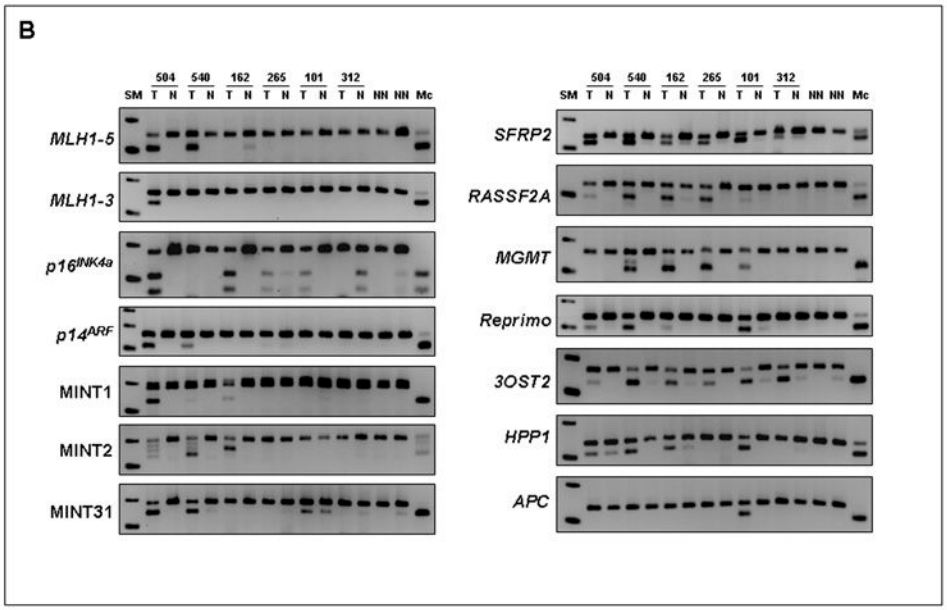

Figure 1. Frequent hypermethylation at multiple loci in CRCs

A) Distribution of all colorectal tissue specimens based upon the number of methylated promoter loci. CRC denotes colorectal cancer; C-N denotes corresponding normal colonic epithelium; N-N denotes normal colonic epithelium without neoplasia at colonoscopy.

B) The figure illustrates COBRA data for seven canonical CIMP-related markers (*hMLH1*-5' region, *hMLH1*-3' region, $p16^{INK4a}$, $p14^{ARF}$, MINT1, MINT2 and MINT31) and the seven new markers (*SFRP2*, *RASSF2A*, *MGMT*, *Reprimo*, *3OST2*, *HPP1* and *APC*) in CRCs (T), corresponding normal colonic epithelium (N) and normal colonic epithelium without neoplasia on colonoscopy (NN). Mc indicates *SssI* methylase-treated control human genomic DNA. PCR conditions of p16, MINT1, MINT2, and MINT31 were described previously by Rashid et al.,[35]; and for that of p14 by Shen et al.,[15]

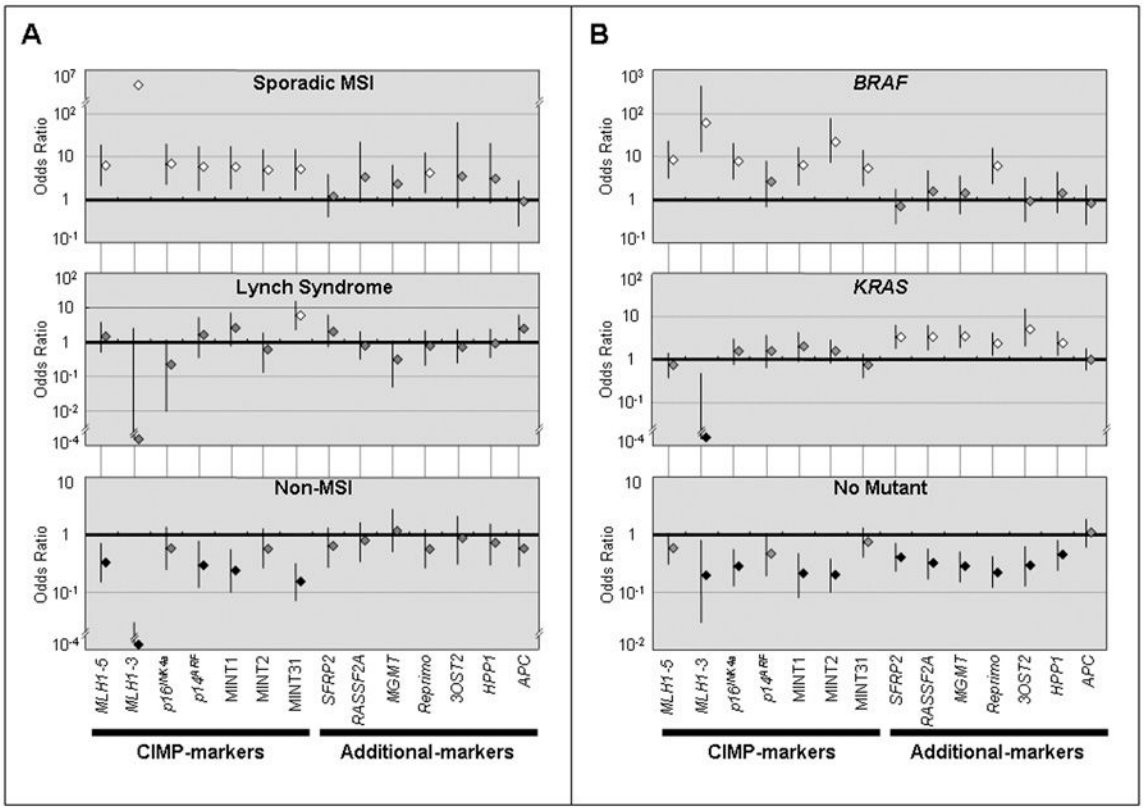

Figure 2. The odds ratio (OR) for methylation at each epigenetic marker in subgroups of CRCs categorized by MSI status (sporadic or Lynch syndrome) (panel A), or *BRAF/KRAS* mutational status (panel B)

A) The upper, middle, and bottom panels illustrate the ORs for sporadic MSI tumors (MSI), Lynch syndrome CRCs, and non-MSI CRCs, respectively.

B) The upper, middle, and bottom panels illustrate the ORs for *BRAF* mutant CRCs (*BRAF*), *KRAS* mutant CRCs (*KRAS*), and wild type (both wild type) CRCs, respectively.

The vertical bars depict the 95% CIs for this ratio. An odds ratio (OR) >1.0 for a given marker is represented by a white square and suggests a positive association with that subgroup (>1.0), while an OR <1.0 represents a negative risk and is shown as black squares. An OR for an epigenetic marker that does not show any positive or negative associations is shown in gray. All odds ratios are presented in a log scale.

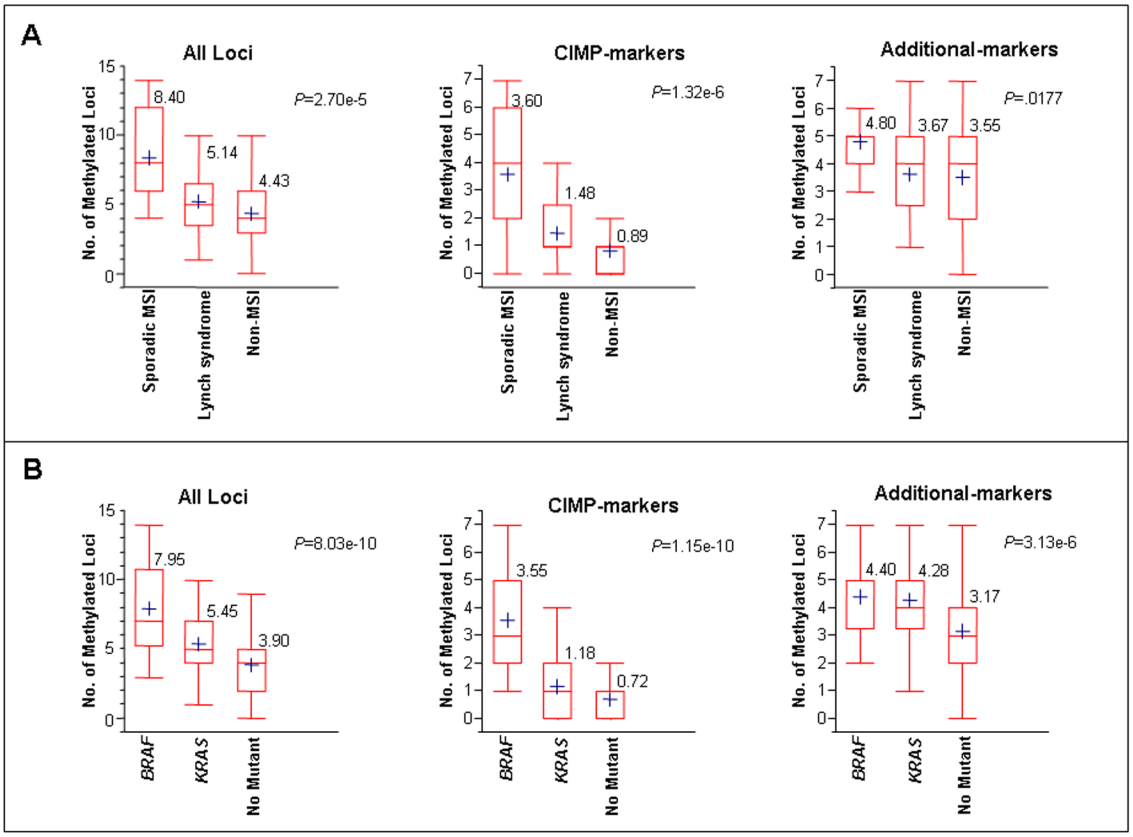

Figure 3. Average numbers of methylated loci in various subgroups of CRCs categorized by MSI status (panel A) or *BRAF/KRAS* mutation status (panel B)

The average number of methylated loci in each subset was calculated by all fourteen markers (all Loci), seven canonical CIMP markers (CIMP-related) or the seven additional markers (additional markers). In the box-plot diagrams, the horizontal line within each box represents the median; the limits of each box are the inter-quartile ranges, the whiskers are the maximum and minimum values, and the blue cross within each box depicts the mean value. The numbers above each box denotes the mean number of methylated loci. The *P* values above the square panels were based on Kruskal-Wallis one way analysis of variance on ranks, and represents the statistical differences in average methylation among all three subsets (sporadic MSI, Lynch syndrome and non-MSI) of CRCs. Statistical differences among any two individual groups are shown as pair-wise comparisons in Table 4.

Table 1

Primer sequences, Restriction endonuclease and PCR conditions for COBRA

Locus	Primer Sequence	Product Size (Restriction Enzyme)	Temperature (Degrees in Celsius)
hMLH1-5	F; 5′YGGGTAAGTYGTTTTGAYGTAGA3′ R; 5′ATACCTAATCTATCRCCRCCTCATC3′	148 (*Hha*I)	59 (5), 57 (10), 55 (30)
hMLH1-3	F; 5′GGGAGGGAYGAAGAGATTTAGT3′ R; 5′ACCTTCAACCAATCACCTCAAT3′	160 (*Rsa*I)	59 (5), 57 (10), 55 (30)
$p16^{INK4a}$	F; 5′GGTTTTGGYGAGGGTTGTTT3′ R; 5′ACCCTATCCCTCAAATCCTCTAAAA3′	181 (*Taq*I)	58 (3), 56 (7), 54 (15), 52 (20)[#]
$p14^{ARF}$	F; 5′TTTYGGGGYGGAGATGGGT3′ R; 5′ATCACCAAAAACCTACRCACCATATTC3′	160 (*Taq*I)	60 (45)[##]
MINT1	F; 5′GGGTTGGAGAGTAGGGGAGTT3′ R; 5′CCATCTAAAATTACCTCRATAACTTA3′	199 (*Taq*I)	55 (45)[#]
MINT2	F; 5′YGTTATGATTTTTTTGTTTAGTTAAT3′ R; 5′TACACCAACTACCCAACTACCTC3′	203 (TaqI)	60 (3), 58 (7), 56 (15), 54 (20)[#]
MINT31	F; 5′GAYGGYGTAGTAGTTATTTTGTT3′ R; 5′CATCACCACCCCTCACTTTAC3′	185 (*HpyCH4*IV)	58 (3), 56 (7), 54 (15), 52 (20)[#]
SFRP2	F; 5′GGTTGTTAGTTTTTYGGGGTTT3′ R; 5′AACCAAAACCCTACAACATCRT3′	148 (*Hha*I)	59 (5), 57 (10), 55 (30)
RASSF2A	F; 5′TTGGGGAGGGTTTGATAGTTT3′ R; 5′CRCACCCTACRCCCCTCTAAAA3′	131 (*Taq*I)	62 (5), 60 (10), 58 (30)
MGMT	F; 5′GTTTTTAGAAYGTTTTGYGTTT3′ R; 5′CCTACAAAACCACTCRAAACTA3′	145 (*BstU*I)	53 (10), 50 (35)
Reprimo	F; 5′GGGTTGGTTTAGTTTYGTTAAGTTT3′ R; 5′TAAAAATTTCCCAAAAACCTCTCC3′	138 (*Taq*I)	60 (15), 58 (30)
3OST2	F; 5′TTTGGTTAGTAGTTTTIGGAGAAGA3′ R; 5′CCCTATAAACCATAACTCCATAAACC3′	171 (*Taq*I)	60 (5), 58 (10), 56 (30)
HPP1	F; 5′TGTTTAGTAGTTYGTTGTTYGGTTT3′ R; 5′AACCCTCGCAAAATATCCAAC3′	137 (*Nru*I)	58 (5), 56 (10), 54 (30)
APC	F; 5′GGTTTTGTGTTTTATTGYGGAGTG3′ R; 5′CACCAATACAACCACATATCIATCAC3′	156 (*Taq*I)	60 (45)

[#]PCR conditions described previously by Rashid et al., [35]

[##]Shen et al., [15]

Table 2

Associations between clinico-pathological data, MSI status and mutation spectrum of the Ras pathway genes in all CRC patients

		MSI status -% (n)				*BRAF/KRAS* mutation status -% (n)			
		Sporadic (n=15)	**MSI Lynch Syndrome (n=21)**	**Non-MSI (n=207)**	***P* value**	***BRAF* mutant (n=20)**	***KRAS* mutant (n=80)**	**Wild type (n=143)**	***P* value**
Age	≥65	80 (12)	5(1)	51 (105)	<0.0001[a]	65 (13)	56 (45)	42 (60)	0.4782[d]
	<65	20 (3)	95 (20)	49 (102)	0.0283[b]	35 (7)	44 (35)	58 (83)	0.0523[e]
					<0.0001[c]				0.0403[f]
Gender	Female	60 (9)	19 (4)	35 (73)	0.0117[a]	65 (13)	35 (28)	32 (45)	0.0147[d]
	Male	40 (6)	81 (17)	65 (134)	0.0553[b]	35 (7)	65 (52)	69 (98)	0.0033[e]
					0.1343[c]				0.5899[f]
Location	Proximal	92 (12)	69 (11)	30 (60)	0.0912[a]	65 (11)	46 (36)	26 (36)	0.1507[d]
	Distal	8 (1)	31 (5)	70 (143)	<0.0001[b]	35 (6)	54 (42)	74 (101)	0.0013[e]
	Not known	(2)	(5)	(4)	0.0079[c]	(3)	(2)	(6)	0.0047[f]
Stage	I–II	67 (8)	67 (2)	40 (83)	1.000[a]	50 (10)	39 (29)	43 (54)	0.3600[d]
	III–IV	33 (4)	33 (1)	60 (123)	0.0717[b]	50 (10)	61 (46)	57 (72)	0.5498[e]
	Not known	(3)	(18)	(1)	0.3558[c]	(0)	(5)	(17)	0.5595[f]
***BRAF/KRAS* Mutation Status**	*BRAF* mutant	67 (10)	0(0)	5(10)	<0.0001[a]	-	-	-	
	KRAS mutant	0(0)	33 (7)	35 (73)	<0.0001[b]	-	-	-	
	Both wild type	33 (5)	67 (14)	60 (124)	0.5546[c]	-	-	-	

Abbreviations: MSI, microsatellite instability; *BRAF* mutant, CRCs with *BRAF V600E* mutation; *KRAS* mutant, CRCs with *KRAS* mutations; Wild type, CRCs with neither *BRAF* nor *KRAS* mutations.

All *P* values were calculated by the X^2 test.

[a] *P* values were calculated between Sporadic MSI vs. Lynch syndrome

[b] *P* values were calculated between Sporadic MSI vs. Non-MSI cancers

[c] *P* values were calculated between Lynch syndrome vs. Non-MSI

[d] *P* values were calculated between BRAF mutant vs. KRAS mutant

[e] *P* values were calculated between BRAF mutant vs. Wild Type

[f] *P* values were calculated between KRAS mutant vs. Wild Type

Table 3

Frequency of DNA methylation at each epigenetic marker in CRCs and its association with MSI status or mutation spectrum of the *BRAF/KRAS* genes

Epigenetic Marker	Total - %(no.) (n=243)	MSI status -% (n)				*BRAF/KRAS* mutation status -% (n)			
		MSI							
		Sporadic (n=15)	Lynch syndrome (n=21)	Non-MSI (n=207)	*P* value	*BRAF* mutant (n=20)	*KRAS* mutant (n=80)	Wild Type (n=143)	*P* value
***hMLH1*-5**	22 (54)	60 (9)	29 (6)	19 (39)	0.0593[a] 0.0002[b] 0.2857[c]	65 (13)	19 (15)	18 (26)	<0.0001[d] <0.0001[e] 0.9163[f]
***hMLH1*-3**	4(9)	60 (9)	0 (0)	0 (0)	<0.0001[a] <0.0001[b] NC[c]	35 (7)	0 (0)	1 (2)	<0.0001[d] <0.0001[b] 0.2880[f]
***P16*^{INK4a}**	17 (42)	53 (8)	5 (1)	16 (33)	0.0009[a] 0.0003[b] 0.1705[c]	55 (11)	21 (17)	10 (14)	0.0026[d] <0.0001[e] 0.0177[f]
***P14*^{ARF}**	10 (24)	33 (5)	14 (3)	8(16)	0.1753[a] 0.0011[b] 0.3003[c]	20 (4)	13 (10)	7(10)	0.3873[d] 0.0518[e] 0.1675[f]
MINT1	12 (30)	40 (6)	24 (5)	9 (19)	0.2985[a] 0.0003[b] 0.0374[c]	40 (8)	18 (14)	6 (8)	0.0298[d] <0.0001[e] 0.0042[f]
MINT2	21 (52)	53 (8)	14 (3)	20 (41)	0.0122[a] 0.0025[b] 0.5413[c]	80 (16)	26 (21)	11 (15)	<0.0001[d] <0.0001[e] 0.0022[f]
MINT31	26 (62)	60 (9)	62 (13)	19 (40)	0.9080[a] 0.0002[b] <0.0001[c]	60 (12)	21 (17)	23 (33)	0.0006[d] 0.0005[e] 0.7537[f]
SFRP2	63 (154)	67 (10)	76 (16)	62 (128)	0.5294[a] 0.7095[b] 0.1938[c]	55 (11)	80 (64)	55 (79)	0.0209[d] 0.9836[e] 0.0002[f]
RASSF2A	68 (164)	87 (13)	62 (13)	67 (138)	0.1020[a] 0.1088[b] 0.6602[c]	75 (15)	83 (66)	58 (83)	0.4444[d] 0.1469[e] 0.0002[f]
MGMT	24 (59)	40 (6)	10 (2)	25 (51)	0.0301[a] 0.1884[b] 0.1182[c]	30 (6)	40 (32)	15 (21)	0.4099[d] 0.0844[e] <0.0001[f]
Reprimo	24 (57)	53 (8)	19 (4)	22 (45)	0.0314[a] 0.0056[b]	60 (12)	34 (27)	13 (18)	0.0313[d] <0.0001[e]

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Epigenetic Marker	Total - %(no.) (n=243)	MSI status -% (n)				*BRAF/KRAS* mutation status -% (n)			
		MSI							
		Sporadic (n=15)	Lynch syndrome (n=21)	Non-MSI (n=207)	*P* value	*BRAF* mutant (n=20)	*KRAS* mutant (n=80)	Wild Type (n=143)	*P* value
					0.7748[c]				0.0002[f]
3OST2	82 (198)	93 (14)	76 (16)	81 (168)	0.1736[a] 0.2362[b] 0.5825[c]	80 (16)	94 (75)	75 (107)	0.0546[d] 0.6145[e] 0.0005[f]
HPP1	69 (168)	87 (13)	67 (14)	68 (141)	0.1719[a] 0.1323[b] 0.8921[c]	75 (15)	80 (64)	62 (89)	0.6234[d] 0.2660[e] 0.0061[f]
APC	29 (70)	27 (4)	48 (10)	27 (56)	0.2036[a] 0.9740[b] 0.0477[c]	25 (5)	29 (23)	29 (42)	0.7383 [d] 0.6861[e] 0.9221[f]

All *P* values were calculated by the X^2 test.

[a] *P* values were calculated between Sporadic MSI vs. Lynch syndrome

[b] *P* values were calculated between Sporadic MSI vs. Non-MSI cancers

[c] *P* values were calculated between Lynch syndrome vs. Non-MSI

[d] *P* values were calculated between BRAF mutant vs. KRAS mutant

[e] *P* values were calculated between BRAF mutant vs. Wild Type

[f] *P* values were calculated between KRAS mutant vs. Wild Type

Table 4

Pair-wise comparisons of methylation levels for various epigenetic markers with different subgroups of CRCs segregated based upon their MSI status and *BRAF/KRAS* mutation status

Subset of Markers	MSI status Pairwise Comparison	*P* value	*BRAF/KRAS* mutation status Pairwise Comparison	*P* value
All Markers	Sporadic MSI vs. Lynch Syndrome	0.0101	*BRAF* vs. *KRAS*	0.0042
	Sporadic MSI vs. Non-MSI	4.22e-05	*BRAF* vs. Wild type	2.56e-06
	Lynch syndrome vs. Non-MSI	0.2782	*KRAS* vs. Wild type	1.40e-05
CIMP Markers	Sporadic MSI vs. Lynch Syndrome	1.23e-02	*BRAF* vs. *KRAS*	3.44e-06
	Sporadic MSI vs. Non-MSI	1.46e-05	*BRAF* vs. Wild type	1.05e-06
	Lynch syndrome vs. Non-MSI	0.0230	*KRAS* vs. Wild type	0.0353
Additional Markers	Sporadic MSI vs. Lynch Syndrome	0.0509	*BRAF* vs. *KRAS*	0.8610
	Sporadic MSI vs. Non-MSI	0.0124	*BRAF* vs. Wild type	0.00883
	Lynch syndrome vs. Non-MSI	0.9977	*KRAS* vs. Wild type	1.95e-05

P values were based on Steel-Dwass test.