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Clinicopathological risk factor distributions for *MLH1* promoter region methylation in CIMP positive tumors

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Abstract

Background—The CpG Island Methylator Phenotype (CIMP) is a major molecular pathway in colorectal cancer (CRC). Approximately 25% to 60% of CIMP tumors are microsatellite unstable (MSI-H) due to DNA hypermethylation of the *MLH1* gene promoter. Our aim was to determine if the distributions of clinicopathologic factors in CIMP-positive tumors with *MLH1* DNA methylation differed from those in CIMP-positive tumors without DNA methylation of *MLH1*.

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Methods—We assessed the associations between age, sex, tumor-site, MSI status *BRAF* and *KRAS* mutations and family CRC history with *MLH1* methylation status in a large population-based sample of CIMP-positive CRCs defined by a 5-marker panel using unconditional logistic regression to assess the odds of *MLH1* methylation by study variables.

Results—Subjects with CIMP-positive tumors without *MLH1* methylation were significantly younger, more likely to be male, more likely to have distal colon or rectal primaries and the MSI-L phenotype. CIMP-positive *MLH1*-unmethylated tumors were significantly less likely than CIMP-positive *MLH1*-methylated tumors to harbor a *BRAF* V600E mutation and significantly more likely to harbor a *KRAS* mutation. *MLH1* methylation was associated with significantly better overall survival (HR=0.50; 95% Confidence Interval (0.31, 0.82)).

Conclusions—These data suggest that *MLH1* methylation in CIMP-positive tumors is not a completely random event and implies that there are environmental or genetic determinants that modify the probability that *MLH1* will become methylated during CIMP pathogenesis.

Impact—*MLH1* DNA methylation status should be taken into account in etiologic studies.

Keywords

Colorectal cancer; CIMP; *MLH1*; BRAF; KRAS; DNA methylation; survival

Introduction

There are multiple molecular phenotypes of colorectal cancer (CRC) (1). The CpG Island Methylator Phenotype (CIMP) is one of these, present in about 15% of CRCs and characterized by widespread aberrant DNA hypermethylation across the genome of cancer cells (2–6). About 25% to 60% of CIMP-positive tumors, depending on the study population, display high levels of microsatellite instability (MSI-H), mainly due to transcriptional silencing of the *MLH1* DNA mismatch repair gene by somatic DNA methylation of the *MLH1* gene promoter region (4–7). Studies of multiple populations, including the current study population (8, 9), have reported that somatic *MLH1* DNA methylation is associated with several clinicopathological variables including older age, female gender, proximal tumor location and the *BRAF* V600E mutation (reviewed in (10)). These differences have been defined in comparisons of CIMP-positive to CIMP-negative tumors. However, there is much less data on the comparison of *MLH1* methylated to *MLH1* unmethylated tumors within the CIMP-positive tumor subset. We are aware of only one study comparing CIMP-positive *MLH1* unmethylated to CIMP-positive *MLH1* methylated tumors and this small study was limited to MSI-H tumors (11).

DNA methylation of the *MLH1* promoter region, reported in 22% to 49% of CIMP-positive tumors (6, 11–13), is a key event for determining CRC phenotype for two reasons. First, the methylation-induced loss of MLH1 protein expression is likely to be the underlying cause for somatically altered MSI-H tumors, given that the methylation is predominantly associated with loss of its protein expression. Second, the methylation of the *MLH1* promoter almost always occurs in the context of CIMP (5, 6, 14, 15) and, therefore, may also be a consequence of the same pathogenetic mechanisms that are most commonly associated with CIMP. Given this, a key question is whether or not the *MLH1* methylation is

a random event unrelated to the presence of one or more risk factors, for example age or sex, in which case future etiologic studies of CIMP can ignore *MLH1* methylation status. Alternatively, if there are distinct risk factors within CIMP-positive tumors, these studies will need to consider *MLH1* methylation status in the analysis.

In the present analysis we assessed the correlations between *MLH1* promoter region DNA methylation and age, sex, tumor site and MSI status, as well as *BRAF* and *KRAS* mutations and family history of CRC, in a large population-based sample of CIMP-positive colorectal tumors. We reasoned that if the probability that the *MLH1* promoter-region DNA becomes methylated during CIMP pathogenesis is due solely to a random process within CIMP pathogenesis there will be no differences between the *MLH1* unmethylated and *MLH1* methylated tumors in the genetic or environmental CIMP-associated factors (e.g. age, sex and tumor location) while the different phenotypes (MSS v MSI-H) will affect pathological findings (e.g. infiltrating lymphocytes or signet ring cells) in the two tumor subsets. Finally, we assessed overall survival in CIMP-positive tumors by *MLH1* methylation status, in general and stratified by the presence of *BRAF* and *KRAS* mutations. No tested tumors classified as CIMP-negative were included in the analysis. We did not assess pathologic characteristics of the tumors because it is likely that many of these are consequences rather than causes of the MSI-H phenotype.

Materials and Methods

Subjects

Data for this study were obtained through the Colon Cancer Family Registry (C-CFR), a National Cancer Institute funded registry of CRC cases, family members and population-based controls, which utilized standardized methods for data collection and genotyping. Detailed information about the C-CFR can be found elsewhere (16) and at coloncfr.org. Recruitment at individual C-CFR sites has been described previously (16). Participants were recruited from six C-CFR centers: the University of Southern California (USC) Consortium (Arizona, Colorado, New Hampshire, Minnesota, North Carolina, and Los Angeles, California), the University of Hawaii (Honolulu), Fred Hutchinson Cancer Research Center (FHCRC, Seattle, WA), Mayo Clinic (Rochester, MN), Cancer Care Ontario (Toronto, Canada), and University of Melbourne (Victoria, Australia) using population-based ascertainment strategies. All centers except FHCRC oversampled case probands with first-degree relatives reporting CRC, or CRC case probands diagnosed under age 50 to target families with increased CRC risk. Over 80% of recruited subjects were Caucasian. We used sampling weights that reflected the sampling probability that the case proband was recruited into the registry, accounting for family history, age and race, relative to the base population. First-degree and some second-degree relatives with CRC were also recruited from families with multiple CRC cases. In this study, we included only CRC cases recruited from 1997–2002 (16). Each institution's Institutional Review Board (IRB) approved the study protocol and all subjects signed a written informed consent approved by their IRB. Only subjects who completed the risk factor questionnaire (RFQ) within 5 years of their CRC diagnosis were included.

Tumor blocks

Primary CRC formalin-fixed, paraffin-embedded (FFPE) tissue from the Jeremy Jass Memorial Tissue Bank was collected and processed as previously described (9). Briefly, we requested blocks from all population-based case probands recruited in 1997–2002 as well as their colorectal cancer–affected first-, second-, and third degree relatives. This totaled 3,970 specimens, out of which we received 3,732 (94%) sets of slides. Specifically, we received two unstained 5-mm tissue sections embedded in paraffin from each tumor on positively charged "plus" glass slides without coverslips. Slides were microdissected to enrich for tumor cells and DNA was extracted as described (17). Proteinase K was inactivated by heating at 100° C for 10 minutes. Tissues were randomized before being analyzed.

CIMP determination

The method for CIMP analysis is described in detail in Weisenberger et al. (9): Briefly, all samples were bisulfite converted using the Zymo EZ-96 DNA methylation kit (Zymo Research, Irvine, CA) as specified by the manufacturer. The DNA methylation levels of individual loci were assessed using MethyLight technology as described (9). CIMP status in each sample was determined using a five-gene MethyLight-based signature (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*) described previously (6). All MethyLight CIMP assays were performed using a control reaction specific for Alu repeats as a means of normalizing for input bisulfite DNA amounts. MethyLight data were organized as percent of methylated reference (PMR) value. Genes were considered methylated if the PMR value was ≥ 10. Tumors with methylation in ≥ 3 of these 5 genes were classified as CIMP-positive. Those with 2 or fewer methylated genes were considered CIMP-negative and excluded from this analysis. Out of the 3,732 samples processed, 46 (1.2%) failed the assay. *MLH1* methylation status was determined using the MLH1-M2 MethyLight assay as previously described (6) and classified as methylated for PMR ≥ 10.

Risk factors

We obtained risk factor data (age, sex and family CRC history) from the completed baseline risk factor questionnaire (RFQ) available at coloncfr.org. Age at the time of enrollment was categorized as a three-category variable: ≤ 50, 51–69 and ≥ 70 years. Family history of CRC was self-reported and was considered positive if the case reported CRC in one or more first-degree family members (e.g. parents, siblings or children).

Tumor site

Tumor site was abstracted from pathology reports and/or state or provincial cancer registries and coded using International Classification of Diseases for Oncology (ICD-O), third edition codes. Tumors were labeled as proximal colon if located in the cecum, ascending colon, hepatic flexure, transverse colon and splenic flexure. Tumors were labeled as distal colon if located in the descending colon, sigmoid colon and tumors overlapping the colon and rectum. Tumors were labeled as rectal if located in the rectum or rectosigmoid junction.

KRAS and *BRAF* Mutation testing

DNA from each tumor sample was tested for *BRAF* and *KRAS* mutations. The somatic T>A mutation at nucleotide 1799 causing the p.V600E mutation in *BRAF* was determined using a fluorescent allele-specific PCR assay that amplified a 97bp product for the mutant allele (A1799) and a 94bp product for the wildtype allele (T1799), as previously described (18). Positive controls were run in each experiment and 10% of samples were replicated with 100% concordance. *KRAS* mutation analysis of codons 12 and 13 was performed using direct Sanger sequencing of a 169bp PCR amplified product as previously described (19). The larger amplicon size for *KRAS* analysis compared with *BRAF* V600E contributed to a slightly higher proportion of samples failing to amplify for the *KRAS* assay compared with *BRAF* V600E assay.

MSI testing

MSI was tested using DNA from tumor and matched normal tissue as described in (20) using 10 microsatellite loci (BAT25, BAT26, BAT40, BAT34C4, D5S346, D17S250, ACTC, D18S55, D10S197, and MYCL). Samples were classified as MSI-H if > 30% showed instability, MSS if no markers showed instability and MSI-L otherwise. Tumor classification was based on ≥4 interpretable markers.

MMR gene mutation carriers

Identification of individuals with germline MMR gene mutations was determined for MSI-H tumors as previously described (21, 22). Briefly, all tumors with a missing mismatch repair protein by immunohistochemistry were tested for germline mutations in the corresponding mismatch repair gene. *MLH1*, *MSH2* and *MSH6* mutations were identified using Sanger sequencing or denaturing high performance liquid chromatography (dHPLC), followed by confirmatory DNA sequencing. Large duplication and deletion mutations including those involving *EPCAM*, were detected by Multiplex Ligation Dependent Probe Amplification (MLPA) according to the manufacturer's instructions (MRC Holland, Amsterdam, The Netherlands). *PMS2* mutations were identified as previously described (22, 23) where exons 1–5, 9 and 11–15 were amplified in three long range PCRs followed by nested exon specific PCR/sequencing. The remaining exons (6, 7, 8 and 10) were amplified and sequenced directly from genomic DNA. Large-scale deletions in *PMS2* were detected using the P008-A1 MLPA kit according to manufacturer's specifications (MRC Holland, Amsterdam, The Netherlands). Eight individuals with germline MMR gene mutations were identified in the data set, one of which was CIMP-positive and excluded from the analysis.

Statistical Analyses

All analyses included only tumors tested and classified as CIMP. No tested tumors classified as non-CIMP were included. Contingency tables and Chi-Square analysis were used to assess the prevalence of patient and tumor characteristics in CIMP-positive tumors by *MLH1* methylation status. Unconditional logistic regression was used to compare tumor subsets while mutually controlling for all studied variables except for MSI status, which was too highly associated with *MLH1* methylation to estimate an odds ratio. Pearson correlation coefficients were used to assess correlations between the raw PMR values of the CIMP

markers. All analyses were weighted based on the inverse of the sampling probability that the case proband was recruited into the registry to ensure the numbers represent the entire population of CRC cases at each study site. Subjects were included from all C-CFR sites except Hawaii, because their sampling design precluded this type of weighted analysis. Frequencies are based on the weighted number of tumors in each category.

Overall Survival

We used Cox proportional hazards regression to evaluate the association between *MLH1* methylation status and overall survival among individuals with CIMP-positive CRC, accounting for sampling weights, using time since diagnosis as the time axis. Survival analyses were adjusted for age, sex, tumor site (proximal vs distal/rectal) and family history of CRC in at least one first-degree relative (yes, no); analyses were further adjusted for study site via stratification of the baseline hazards. Including tumor stage in the model did not modify the results. In addition to this primary analytic model, we conducted sensitivity analyses adjusting for MSI, *BRAF* and *KRAS* mutation status as well as analyses stratifying on these tumor attributes.

For all analyses statistical significance was defined as a p-value ≤ 0.05 in a two-sided test. Statistical analyses for the clinicopathologic variables (age, sex, tumor site, MSI status, family CRC history and *BRAF* and *KRAS* mutations) were performed using SAS 9.3 (SAS institute Inc., Carey, NC) and the survival analysis was conducted using Stata 13 (Stata Corp., College Station, TX).

The processed samples yielded a total of 3,660 CRCs with CIMP classification: 3,544 primary CRCs from case probands and 116 CRCs from affected relatives. Of these primary CRCs, 108 case probands (3.0%) were excluded for having been interviewed more than 5 years after diagnosis, 203 (5.7%) for missing RFQ data, and 104 (2.9%) for missing tumor site data or sampling weights. The final data set included 3,119 (unweighted) primary CRCs from case probands, 411 (unweighted) of which were classified as CIMP-positive and included in this analysis. After applying the sampling weights and deleting one proven Lynch Syndrome subject a total of 786 tumors were included in the analysis.

Results

The overall risk factor distribution and prevalence of DNA methylation for the five CIMP markers and *MLH1* for all CIMP-positive tumors included in this analysis is presented in Table 1. Over 90% of the CIMP-positive study population was older than age 50 and 59% were female. Eighty one percent (81%) of all CIMP-positive tumors were in the proximal colon and approximately half were MSI-H. *BRAF* and *KRAS* mutations were present in 62% and 18% of tumors respectively and 19% of all subjects reported a history of CRC in one or more first degree relatives. *MLH1* was methylated in 51% of CIMP-positive tumors.

We next determined the associations between clinicopathologic factors and *MLH1* methylation status in both univariate and multivariate analyses (Table 2). In univariate analysis, *MLH1* unmethylated tumors occurred significantly more often in those under 50 years of age at diagnosis, in men, and in distal and rectal tumors (all p-values <0.0001).

Individuals with *MLH1* unmethylated tumors were significantly less likely to report a CRC diagnosis in a first-degree relative (P=0.0226). Overall, 7.5% of *MLH1* unmethylated tumors were also MSI-H. In addition, *MLH1* unmethylated tumors were significantly more likely than tumors with *MLH1* methylation to be classified as MSI-L (20.7% compared to 0.25% respectively; P<0.0001). CIMP-positive tumors with *MLH1* methylation were mainly classified as MSI-H (98%), as expected. Tumors without *MLH1* methylation were significantly less likely to have a *BRAF* V600E mutation than *MLH1* methylated tumors (42.3% versus 86.2% respectively; P <0.0001) and were more likely to have a mutation in *KRAS* codon 12 or codon 13 (39.5% versus 2.1% respectively; P<0.0001). Including *MLH1* in the panel defining CIMP status did not change any results (39.5% mutated versus 3.0% mutated respectively). Except for family CRC history, these associations were not altered in a multivariate analysis mutually controlling for all the variables except for MSI status. The odds ratio (OR) for family CRC history was essentially null after multivariate adjustment. There was a low to moderate correlation between the PMR's of all markers with *MLH1* methylation and each other. The correlation coefficients ranged from 0.017 to 0.48.

Table 3 shows the association between *MLH1* methylation and overall survival in multivariate analyses. Patients with CIMP-positive, *MLH1* DNA methylated tumors were 50% less likely to die during the observation period of up to 15 years in univariate analysis (Hazard Ratio (HR) = 0.50 (95% Confidence Interval (CI) = 0.31, 0.82)). This association was no longer significant after adjustment for MSI status, but the point estimate did not change materially. Adjustment for *BRAF* or *KRAS* mutation status also did not substantially change the estimates. In stratified analyses, the HR associated with *MLH1* methylated status was significantly decreased only for cases with a mutated *BRAF* (HR=0.41 (95% CI=0.22, 0.77) or a wild-type *KRAS* (HR=0.46 (95% CI=0.25, 0.84)).

Discussion

In this large population-based sample *MLH1* methylation in CIMP-positive tumors was associated with the characteristics commonly observed for MSI-H and CIMP-high tumors relative to non-CIMP tumors (10). CIMP positive *MLH1*-unmethylated tumors were more prevalent in men, and significantly more common in younger age groups and in distal tumors than CIMP positive, *MLH1*-methylated tumors. The prevalence of *BRAF* and *KRAS* mutations also differed significantly between the *MLH1* unmethylated and *MLH1* methylated tumors.

Overall, our results are consistent with those of other studies. Kim et al. observed a highly significant difference in the sex ratio in individuals with CIMP-positive, *MLH1* methylated tumors (55% female) compared to individuals with CIMP-positive tumors where *MLH1* was unmethylated (9% female), in a Korean population (11). An increase in the prevalence of distal tumors among those without *MLH1* methylation compared to those with *MLH1* methylation was also seen in that study, as well as a significantly lower prevalence of *BRAF* mutation and higher prevalence of *KRAS* mutations in unmethylated tumors, as we observed here. However, all tumors were MSI-H in that study and the prevalence of Lynch Syndrome in the small set of CIMP-positive *MLH1* unmethylated tumors was not provided. Similar differences were observed in studies comparing CIMP/MSI-H tumors (as a proxy for *MLH1*

DNA methylation) to non-CIMP tumors (4, 7, 24) and studies that assessed risk factors for *MLH1* DNA methylation in CIMP-positive tumors compared to non-CIMP tumors (12, 25).

The different age distributions in subjects with *MLH1* unmethylated tumors compared to those with a *MLH1* methylated tumor suggests a role for age-associated epigenetic drift as a factor promoting *MLH1* methylation in our study population (26, 27). A positive association between age and *MLH1* DNA methylation has been reported (28, 29). Looking only at CIMP-positive *MLH1* methylated or CIMP-positive MSI-H tumors, an association between *MLH1* methylation or MSI-H and older age was observed in multiple studies (4, 7, 11, 30) supporting this conclusion.

A preponderance of females in CIMP-positive tumors is not a universal finding. Several studies report a higher CIMP prevalence in male subjects (13, 31–34) as we observed for the CIMP-positive, *MLH1* unmethylated tumors in our population. It may be significant that four of these five studies were of Asian populations. Additionally, in a previous analysis of this population, we observed that several risk factors for a CIMP positive tumor were significantly associated with a CIMP-positive tumor only among females (9). Taken together these data suggest that female sex is a proxy for more biologically relevant exposures that vary between populations.

Several previous studies of potential CIMP markers have reported that there may be more than one type of CIMP-positive tumor (35–40). In these studies, using significantly more marker genes, tumors designated as CIMP could be further differentiated statistically into a CIMP-Low/CIMP2/Intermediate (IME) or low methylation (LME) group and a CIMP-High/CIMP1/High methylation (HME) group with different marker gene sets. Tumors classified as CIMP-Low were significantly more likely to be MSS and have a *KRAS* mutation in these populations compared to tumors classified as CIMP-High. This is similar to what we observed in our data after stratifying on *MLH1* methylation alone, even though all tumors used in this analysis were classified as CIMP-positive using a marker panel for what is variably called CIMP-H (35, 37, 39), CIMP1 (38) or HME (36, 40). We note that *MLH1* methylation status was not a criterion for establishing CIMP status in the current and other analyses (35, 38, 39). However, redefining CIMP status using a 6 marker panel that included *MLH1* methylation did not change any of the observed associations. Future studies are required to assess directly whether *MLH1* methylation is associated with CIMP-Low or if different types of CIMP tumors must be identified using separate marker panels.

Establishing that a tumor has an MSI-H phenotype has important prognostic value (41) and may also be predictive of treatment response (42). Data from a meta-analysis concluded that the MSI-H phenotype, due mainly to *MLH1* methylation, is associated with a better prognosis than tumors with the MSS phenotype (41) so identifying the two different types of CIMP-positive tumors, those in which the *MLH1* promoter region DNA is not methylated (MSS), and those with *MLH1* methylation (MSI-H) may be of clinical relevance. In a recent meta-analysis CIMP was associated with a bad prognosis in both MSI-H and MSS tumors (43). In our data, using only CIMP-positive tumors, overall survival was decreased in those without *MLH1* methylation compared to those with *MLH1* methylation. This is consistent with the close association between *MLH1* methylation and MSI-H and the reduction in

significance after controlling for MSI status. However, in our population, the better prognosis was limited to subjects with a tumor that was *KRAS*-wildtype, most of whom also had tumors that were *BRAF*-mutated. The presence of the *BRAF* V600E mutation was associated with a poor prognosis overall in a meta-analysis (44) while data from several studies suggest that the poor prognosis may be ameliorated by the presence of MSI-H (45–49). In one of those studies survival was significantly better in MSI-H, *BRAF* mutated tumors relative to MSS, *BRAF* wild type tumors (46). Reaching a consensus about the modifying effect of *BRAF* mutation on prognosis in CIMP-positive *MLH1* methylated tumors will require further study.

The strengths of our study include the facts that it is the largest study to date of CIMP-positive CRC tumors and that we utilized a population-based sample weighted for age, race and family history so that our results can be generalized. We limited the current analysis to CIMP positive tumors so that unmeasured causal factors were equally relevant for both unmethylated and methylated tumors and there can be no confounding by differential effects of unmeasured exposures relevant mainly to non-CIMP tumors. Our study also has some weaknesses. Although we used a set of well-characterized markers to define CIMP status (6) an eight marker CIMP panel has been described (3) which may have eliminated some tumors from this analysis had it been used instead (50). Alternatively, some CIMP tumors in the parent data set may not have been identified as CIMP using our marker panel and so were wrongly excluded from the analysis. Additionally, it is possible that some *MLH1* unmethylated tumors should have been classified as CIMP-Lo as discussed above. It is unclear how this misclassification may have biased the results. Studies using genome-wide methylation techniques to improve CIMP classification, which we were unable to do, may decrease this type of misclassification in the future. Our assay for *MLH1* methylation was limited to 8 CpG sites in the previously described C region of the promoter (51) which might have caused us to misclassify some methylated tumors as unmethylated, biasing our results toward null values. We were unable to control for MSI status in our multiple regression analysis due to the small number of *MLH1*-methylated MSS tumors so there may be residual confounding by this variable in the multivariate OR's. It is unclear how this may have biased our adjusted OR's. *KRAS* mutation data was missing for 16% of tumors, although approximately equally in *MLH1* methylated (15.2%) and unmethylated subsets (17%), and we only looked at *KRAS* mutations in codons 12 and 13. Thus, our data for *KRAS* mutations may be biased toward the null value. Finally, we were not able to assess or control for the association between *MLH1* methylation and the *MLH1* -93GA genotype (rs1800734) because only about 25% of the study population was genotyped for that SNP.

In conclusion, our analysis suggests that there are significant differences between CIMP-positive tumors with *MLH1* DNA methylation and CIMP-positive tumors without such methylation for variables classically associated with CIMP. The differences included exposures (e.g. age, sex and tumor site) which cannot be consequences of the CIMP phenotype. These results, in the context of data from other populations, are consistent with the hypothesis that *MLH1* methylation in CIMP-positive tumors is not a completely random event and implies that there are environmental or genetic determinants that modify the probability that *MLH1* will become methylated during CIMP pathogenesis. This suggests

that etiologic studies of the CIMP pathway may need to stratify on *MLH1* methylation status.

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Table 1

Characteristics of the study population with CIMP-positive[1] cancers

	Weighted N[2]	Prevalence (%)
Age		
<50	52	6.6
51–69	406	51.6
≥ 70	329	41.8
Sex		
Male	322	41.0
Female	464	59.0
Tumor Site[3]		
Proximal	636	80.9
Distal	83	10.5
Rectal	68	8.6
MSI status[4]		
MSS	280	35.6
MSI-L	81	10.3
MSI-H	424	53.9
Missing	2	0.25
BRAF mutation		
Present[5]	490	62.3
Absent	274	34.8
Missing	22	2.9
KRAS mutation		
Present[6]	138	17.6
Absent	520	66.2
Missing	128	16.3
Family History[7]		
No	632	80.3
Yes	149	18.9
Missing	6	0.76
N methylated:		
CACNA1G	619	78.7
IGF2	737	93.8
NEUROG1	781	99.4
RUNX3	693	88.2
SOCS1	429	54.6

	Weighted N[2]	Prevalence (%)
MLH1	402	51.2

[1] Defined as a PMR ≥10 for at least 3 of 5 genes: *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*.

[2] As defined in the text the sampling weights are the inverse of the sampling fraction which corrected for the oversampling of case probands by age, race and family history. N's are rounded to the nearest whole number.

[3] Tumors were labeled as ascending colon if located in the cecum through the splenic flexure. Tumors were labeled as distal colon if located in the descending colon through the sigmoid colon and included tumors 'overlapping the colon and rectum.' Tumors were labeled as rectal if located in the rectum or rectosigmoid junction.

[4] MSI status was determined using 10 microsatellites as described in the text.

[5] Presence of the *BRAF* V600E mutation.

[6] Presence of either a codon 12 or codon 13 *KRAS* mutation.

[7] Defined as a Self-reported history of CRC in at least one 1st degree relative.

Table 2

Associations between *MLH1* methylation and selected variables in CIMP-positive[1] colorectal cancer

	Methylated[2] Weighted N[3] (%)	**Not Methylated[2] Weighted N (%)**	**Univariate P-value (Chi Square)**	**OR (95% CI)[4] for *MLH1* methylation**	**P-value for OR**
Age					
<50	8 (1.9)	45 (11.6)		1.0	
51–69	191 (47.4)	215 (55.9)	<0.0001	5.85 (2.29, 15.0)	0.0002
≥ 70	204 (50.7)	125 (32.5)		5.97 (2.32, 15.4)	0.0002
Sex					
Male	98 (24.3)	229 (58.4)	<0.0001	1.0	
Female	305 (75.7)	160 (41.6)		2.76 (1.85, 4.11)	<0.0001
Tumor Site[5]					
Proximal	369 (91.7)	267 (69.5)		3.20 (1.35, 7.60)	0.0084
Distal	22 (5.4)	61 (15.9)	<0.0001	0.85 (0.29, 2.53)	0.7713
Rectal	12 (2.9)	56 (14.6)		1.0	
MSI status[6]					
MSS	5 (1.17)	275 (71.7)			
MSI-L	1 (0.25)	80 (20.7)	<0.0001	Infinite[6]	-
MSI-H	395 (98.6)	29 (7.51)	<0.0001		
BRAF mutation					
Present[7]	327 (86.2)	162 (42.3)		2.50 (1.53, 4.08)	
Absent	52 (13.8)	221 (57.7)	<0.0001	1.0	0.0002
KRAS mutation					
Present[8]	7 (2.1)	131 (39.5)		0.10 (0.04, 0.24)	
Absent	319 (97.9)	201 (60.5)	<0.0001	1.0	<0.0001
Family History[9]					

	Methylated[2] Weighted N[3] (%)	Not Methylated[2] Weighted N (%)	Univariate P-value (Chi Square)	OR (95% CI)[4] for *MLH1* methylation	P-value for OR
No	311 (78.0)	321 (84.0)		1.0	
Yes	88 (22.0)	61 (16.0)	0.0266	1.04 (0.64, 1.69)	0.8859

[1] Defined as a % methylated reference – see text (PMR) was ≥10 for at least 3 of 5 genes: *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*.

[2] *MLH1* was defined as methylated if the PMR was ≥ 10.

[3] As defined in the text the sampling weights are the inverse of the sampling fraction which corrected for the oversampling of case probands by age, race and family history. N's are rounded to the nearest whole number.

[4] The odds ratio (OR) was estimated using unconditional logistic regression for odds of *MLH1* methylation controlling for all other variables in the table except MSI status which effects could not be estimated due to small cell-specific numbers in the methylated-MSS group resulting in an estimate of infinity.

[5] Tumors were labeled as ascending colon if located in the cecum through the splenic flexure. Tumors were labeled as distal colon if located in the descending colon through the sigmoid colon and included tumors 'overlapping the colon and rectum.' Tumors were labeled as rectal if located in the rectum or rectosigmoid junction.

[6] MSI status was determined using 10 microsatellites as described in the text.

[7] Presence of the *BRAF* V600E mutation.

[8] Presence of either a codon 12 or codon 13 *KRAS* mutation.

[9] Defined as a Self-reported history of CRC in at least one 1st degree relative.

Table 3

Association of *MLH1* methylation[1] in CIMP-positive[2] CRC's with overall survival[3] after CRC diagnosis[*]

Analysis	Unweighted N(deaths)	Weighted N (deaths)	HR (95% CI)
Overall Survival (baseline analysis[4])	390 (162)		
MLH1 unmethylated	188 (92)	371 (168)	
MLH1 methylated	202 (70)	374 (118)	0.50 (0.31, 0.82)
Additionally controlling for MSI status			0.47 (0.20, 1.08)
Additionally controlling for *BRAF*[5] mutation status			0.49 (0.28, 0.86)
Additionally controlling for *KRAS*[6] mutation status			0.50 (0.28, 0.87)
Additionally controlling for joint *BRAF* and *KRAS* status			0.51 (0.28–0.91)
In *BRAF* mutated cases	250 (97)	465 (179)	0.41 (0.22, 0.77)
In *BRAF* wild-type cases	136 (62)	269 (103)	0.88 (0.39–1.99)
Missing *BRAF* status	4 (3)		
In *KRAS* mutated cases	64 (28)	137 (52)	1.35 (0.29, 6.33)
In *KRAS* wild-type cases	265 (113)	499 (193)	0.46 (0.25, 0.84)
Missing *KRAS* status	61 (21)		
BRAF mutated/*KRAS* wildtype	209 (87)	392 (153)	0.41 (0.21, 0.79)
BRAF wildtype/*KRAS* mutated	63 (28)	135 (52)	3.60 (0.98, 13.23)
BRAF wildtype/*KRAS* wildtype	55 (25)	106 (39)	0.35 (0.13, 0.91)

[*] Excludes 21 cases with missing information on date of diagnosis or death.

[1] MLH1 methylation was defined as a PMR ≥ 10 using MethyLight analysis as defined in the text.

[2] Defined as a PMR ≥10 for at least 3 of 5 genes: *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*.

[3] Median follow-up from time since diagnosis was 10 years (range 5 months – 15 years). Overall survival was estimated using a Cox proportional hazards regression.

[4] Baseline analysis adjusted for age, sex, tumor site (proximal vs. distal/rectal) and family history of CRC in at least one 1st degree relative (yes, no). Stratification factors were as identified in column one of the table.

[5] Presence of the *BRAF* V600E mutation.

[6] Presence of either a codon 12 or codon 13 *KRAS* mutation.