Molecular Characterization of MSI-H Colorectal Cancer by *MLHI* Promoter Methylation, Immunohistochemistry, and Mismatch Repair Germline Mutation Screening

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Abstract

Microsatellite instability (MSI) occurs in 10% to 20% of colorectal cancers (CRC) and has been attributed to both *MLH1* promoter hypermethylation and germline mutation in the mismatch repair (MMR) genes. We present results from a large population- and clinic-based study of *MLH1* methylation, immunohistochemistry, and MMR germline mutations that enabled us to (*a*) estimate the prevalence of MMR germline mutations and *MLH1* methylation among MSI-H cases and help us understand if all MSI-H CRC is explained by these mechanisms and (*b*) estimate the associations between *MLH1* methylation and sex, age, and tumor location within the colon. *MLH1* methylation was measured in 1,061 population-based and 172 clinic-based cases of CRC. Overall, we observed *MLH1* methylation in 60% of population-based MSI-H cases and in 13% of clinic-based MSI-H cases. Within the population-based cases with MMR mutation screening and conclusive immunohistochemistry results, we identified a molecular event in MMR in 91% of MSI-H cases: 54% had *MLH1* methylation, 14% had a germline mutation in a MMR gene, and 23% had immunohistochemistry evidence for loss of a MMR protein. We observed a striking age difference, with the prevalence of a MMR germline mutation more than 4-fold lower and the prevalence of *MLH1* methylation more than 4-fold higher in cases diagnosed after the age of 50 years than in cases diagnosed before that age. We also determined that female sex is an independent predictor of *MLH1* methylation within the MSI-H subgroup. These results reinforce the importance of distinguishing between the underlying causes of MSI in studies of etiology and prognosis. (Cancer Epidemiol Biomarkers Prev 2008;17(11):3208–15)

Introduction

Microsatellite instability (MSI-H) is a hallmark feature of Lynch syndrome, which is a rare inherited disorder caused by germline mutation in a mismatch repair (MMR) gene. Although mutations in these genes are highly penetrant, in aggregate, they account for less than 5% of all colorectal cancers (CRC; refs. 1-5). In sporadic colorectal cancers, MSI-H occurs in ~10% to 20% of lesions. A substantial proportion of MSI-H observed in non–Lynch syndrome cases results from hypermethylation of the *MLH1* promoter (6-9). MSI-H has been used as a classification variable for analyses of putative risk factors, gene expression (10), and prognosis (11, 12); however, heterogeneity is likely to exist within the MSI-H subgroup because multiple molecular mechanisms lead to this phenotype.

Previous studies have found that CRC in patients with Lynch syndrome differ from those not associated with Lynch syndrome with regard to tumor and patient characteristics (13, 14). In addition, the good prognosis observed in MSI-H cancer may not be the same in patients with Lynch syndrome and those with MSI-H cancers caused by *MLH1* hypermethylation or other causes (reviewed in ref. 15). Recently, a gene expression signature was proposed to distinguish between so-called

Received 6/3/08; revised 8/21/08; accepted 9/3/08.

Grant support: This work was supported by the National Cancer Institute, NIH, under RFA CA-95-011 and through cooperative agreements with the Australasian Colorectal Cancer Family Registry (U01 CA097735), the University of Southern California Familial Colorectal Neoplasia Collaborative Group (U01 CA074799), the Mayo Clinic Cooperative Family Registry for Colon Cancer Studies (U01 CA074800), the Ontario Registry for Studies of Familial Colorectal Cancer (U01 CA074783), the Seattle Colorectal Cancer Family Registry (U01 CA074794), and the University of Hawaii Colorectal Cancer Family Registry (U01 CA074806) as well as National Cancer Institute T32 CA009142 (J.N. Poynter).

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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doi:10.1158/1055-9965.EPI-08-0512

''sporadic'' MSI-H and Lynch syndrome–associated MSI-H tumors (16).

The prevalence and descriptive characteristics of *MLH1* methylation have been evaluated in previous studies of MSI-H CRC in both population- and clinic-based samples, with evidence that methylation is associated with female gender, proximal tumor location, and older age at diagnosis (17-23); however, most of these studies had a relatively small number of MSI-H cases (range 46-78 cases) and were unable to mutually adjust for these variables. Similar associations with increasing age, female sex, and tumor location in the proximal colon were observed in a study using loss of MLH1 expression as a proxy for *MLH1* methylation (24). In studies that have looked specifically at MSI-H CRC not caused by germline MMR mutations, methylation of *MLH1* was found to explain MSI-H in a majority (83-100%) of these cases (8, 17, 22, 25).

Differences between Lynch syndrome MSI-H CRC and MSI-H CRC due to DNA methylation or other causes have previously been investigated in studies with a relatively limited numbers of MSI-H cases (13, 17). A large population-based sample of MSI-H cases would permit a more thorough molecular characterization of MSI-H CRC. In the current analysis, we measured *MLH1* methylation, as well as immunohistochemistry and germline mutation in the MMR genes, in 1,222 population-based and 220 clinic-based cases of invasive CRC (including 429 MSI-H cases) collected by the Colon Cancer Family Registry (Colon CFR). We evaluated the molecular characteristics of MSI-H CRC to determine whether all MSI-H tumors can be explained by either germline mutation in one of the MMR genes or *MLH1* methylation. We also evaluated differences in DNA methylation prevalence between population-based and clinic-based cases and confirmed previously reported associations between *MLH1* methylation and descriptive characteristics in MSI-H CRC.

Materials and Methods

Study Population. Participants were recruited for the Colon CFR from six registry centers, including the University of Hawaii (Honolulu, HI), Fred Hutchinson Cancer Research Center (Seattle, WA), Mayo Clinic (Rochester, MN), University of Southern California Consortium (Los Angeles, CA), Cancer Care Ontario (Toronto, Canada), and University of Melbourne (Melbourne, Australia). Families were ascertained through population-based cancer registries (population–based) and high-risk clinics (clinic–based). Some centers recruited all incident cases of CRC whereas others oversampled cases with a family history or early age of onset. Standardized procedures were used to collect epidemiologic data, blood samples, tumor blocks, and pathology reports from cases. Detailed information about the Colon CFR can be found at http://epi.grants.cancer.gov/CFR/ and is reviewed by Newcomb et al. (26).

We obtained informed consent from all participants. The study was approved by the institutional review board(s) at each Colon CFR site.

Classification of Family History. Family history data provided during an interview were used to determine whether individuals met the Amsterdam II criteria (27) and the revised Bethesda guidelines (5), which are guidelines used to identify individuals likely to carry a germline mutation in one of the four known MMR genes (*MLH1, MSH2, MSH6*, and *PMS2*). We attempted to verify family history information by comparing reports from multiple individuals within the same family. When available, medical records, death certificates, pathology reports, and tumor tissues were also used to confirm reported cancer diagnoses.

Microsatellite Instability. MSI was evaluated using a panel of 10 markers (BAT25, BAT26, BAT40, MYCL, D5S346, D17S250, ACTC, D18S55, D10S197, BAT34C4) using standard techniques (28). Results were required for at least four markers to determine MSI status. Tumors were deemed MSI-H if instability was observed at greater than or equal to 30% of markers, MSI-L if greater than 0 and less than 30% of markers were instable, and MSS if all markers were stable. MSI results are available for all cases included in this analysis.

***MLH1* Methylation Assay.** Cases were sampled for *MLH1* methylation testing based on MSI status according to the following strategy: From the population-based series, *MLH1* methylation was measured in all MSI-H and MSI-L cases with sufficient tumor DNA and a random sample of MSS cases. All clinic-based cases with sufficient tumor DNA were also tested.

MLH1 methylation was measured using MethyLight. All DNA samples were randomized and bisulfite converted as previously described (29) with the following exceptions: After bisulfite conversion and loading onto the Qiagen Viral RNA Mini Kit spin columns, each sample was washed with the supplied washed buffers. Desulfonation was done by adding 200 μL of 0.08 mol/L NaOH (in AW1/ethanol wash buffer) to the spin column and incubated for 15 min at room temperature. Afterward, 200 μL of 0.08 mol/L HCl (in AW1/ethanol wash buffer) were added to neutralize the solution, and after a 5-min incubation period, the columns were centrifuged and the filtrate was removed. The desulfonated sample was further washed using supplied wash buffers and eluted as described (29).

MethyLight analysis of *MLH1* was done as previously reported (30), in which the MLH1-M2 MethyLight reaction was assayed on each sample, and the *ALU* control reaction was used to normalize for bisulfite-converted input DNA (29). We classified samples with a percent of methylated reference (PMR) greater than or equal to 10 as positive for *MLH1* methylation as described (30).

We used the *ALU* control reaction cycle threshold [C(t)] value, an inverse indicator of DNA quantity, as a quality control measure to identify potential false negatives for *MLH1* methylation. The C(t) value represents the PCR cycle in which the fluorescence emitted from the MethyLight TaqMan probe is greater than the background fluorescence signal in the PCR reaction. Because the *ALU* repetitive elements are more common in the genome than a typical single copy gene, the *ALU* control reaction can detect amounts of bisulfite-converted DNA of four orders of magnitude lower than a control reaction directed toward a single-copy gene locus. *MLH1* methylation cannot be accurately determined for samples with minute amounts of bisulfite-converted DNA. In plots using a sliding window of C(t) value, the frequency of *MLH1* methylation decreased as the *ALU* C(t) value increased; no methylation was observed in samples with

an *ALU* C(t) value greater than 27. To determine the optimal *ALU* C(t) value cut point, we evaluated the frequency of *MLH1* methylation using *ALU* C(t) cut points of 20, 22, and 24. We observed a similar *MLH1* DNA methylation prevalence using all three cutpoints (20.5%, 19.7%, and 19.1%, respectively), and we chose to include samples with a C(t) value less than or equal to 24 to retain the largest sample size possible for the analysis while minimizing the potential for false negatives.

Immunohistochemistry. Immunohistochemistry for MLH1, MSH2, MSH6, and PMS2 proteins was done as previously described (31, 32). Immunohistochemistry testing was done on all MSI-H and MSI-L population- and clinic-based samples. Because of the low frequency of absent protein staining in MSS cases (31), some Colon CFR centers did not perform immunohistochemistry testing on all MSS cases. Staining was classified as absent, present, or inconclusive.

MMR Mutation Data. Population-based and clinic-based probands with CRC were tested for mutations in the MMR genes *MSH2, MLH1, MSH6*, and *PMS2*. Mutations in *MSH2* and *MLH1* were detected using a combined approach of denaturing high-pressure liquid chromatography/direct sequencing and multiplex ligation-dependent probe amplification. MMR gene mutation testing for *MSH2* and *MLH1* was conducted for all clinic-based probands, all MSI-H or MSI-L population-based probands, and in a random sample of 300 MSS population-based probands. Our analysis of *MLH1* methylation includes 205 of these randomly selected MSS cases. Direct sequencing was used to detect *MSH6* mutations in cases with absent immunohistochemical staining of MSH6. *PMS2* mutations were evaluated in cases from four of the CFR centers (Australia, Seattle, Mayo, and Ontario) as previously described (33).

For this analysis, we focus on the variants that are considered to have a clearly deleterious effect based on current evidence, specifically those with (*a*) changes known or predicted to truncate protein production, including frameshift and nonsense variants, (*b*) splice site mutations occurring within 2 bp of an intron/exon boundary, and (*c*) missense changes that have been shown to have a deleterious effect.

Molecular Testing Done on Samples Included in Analysis. *MLH1* methylation was measured in 1,222 population-based probands whose tumors were also assessed for MSI. We excluded 161 cases with an *ALU* C(t) value greater than 24, leaving 1,061 cases for this analysis. Of the 1,061 cases included, 374 were MSI-H, 223 were MSI-L, and 464 were MSS. Immunohistochemistry results were available for 719 of these population-based cases, including 317 of 374 MSI-H cases, 205 of 223 MSI-L cases, and 197 of 464 MSS cases. MMR germline mutation status was available for 324 of 374 population-based MSI-H cases, 197 of 223 MSI-L cases, and 205 of 464 MSS cases. In addition, DNA methylation testing was done on 220 clinic-based cases. Forty-eight of these cases were excluded because of the high *ALU* C(t) value, resulting in a sample size of 172 with 55 MSI-H, 12 MSI-L, and 105 MSS cases. Of these 172 cases, 157 (91%) had immunohistochemistry results and 152 (88%) were tested for germline mutation in the MMR genes.

Statistical Analysis. Contingency tables were used to assess the frequency of *MLH1* methylation and germline MMR mutation by MSI status. For the population-based series, a sampling weight was included in the analysis to reflect the probability that a case was recruited to participate in the Colon CFR. Reported percentages are based on the weighted number of individuals in each category. Population- and clinic-based cases were analyzed separately, with the one exception of the comparison of *MLH1* methylation frequency in MSI-H cases ascertained from the two different study samples. Because sampling for inclusion into the *MLH1* methylation analysis was based on MSI status, and very few MSS and MSI-L cases had *MLH1* methylation, analyses of descriptive characteristics were restricted to the MSI-H subset. We evaluated the following descriptive and tumor characteristics: age at diagnosis (less than or equal to 50 years versus 51-60, 61-70, and greater than 70 years),

Table 1. *MLH1* methylation in population and clinic-based cases by MSI status

	MSI-H		MSI-L		MSS	
	Cases (%)	Weighted *n* (%)	Cases (%)	Weighted *n* (%)	Cases (%)	Weighted *n* (%)
Population-based						
MLH1 methylation						
Methylated	206	437 (60)	6	17 (3.1)	6	10 (0.7)
Unmethylated	168	293 (40)	217	518 (97)	458	1,428 (99)
Germline MMR mutation*						
Mutation	59	79 (12)	0	0 (0)	0	0 (0)
No mutation	265	562 (88)	197	494 (100)	205	286 (100)
Untested	50	89 (NA)	26	41 (NA)	259	1,152 (NA)
Clinic-based						
MLH1 methylation						
Methylated	7 (13)		0 (0)		0 (0)	
Unmethylated	48 (87)		12 (100)		105 (100)	
Germline MMR mutation †						
Mutation	33 (70)		3 (25)		2 (2)	
No mutation	14 (30)		9 (75)		91 (98)	
Untested	8 (NA)		0 (NA)		12 (NA)	

Abbreviation: NA, not applicable.
*Fifty MSI-H, 26 MSI-L, and 259 MSS population-based cases were not tested for germline MMR mutations.
† Eight MSI-H and 12 MSS clinic-based cases were not tested for germline MMR mutations.

Table 2. Descriptive characteristics of *MLH1* methylation in 313 MSI-H population-based cases with IHC data

	Methylated*		Unmethylated †						P ‡
			Loss of MLH1		Loss of other MMR		No evidence of MMR loss		
	Cases	Weighted n (%)	Cases	Weighted n (%)	Cases	Weighted n (%)	Cases	Weighted n (%)	
Sex									
Males	44	119 (34)	25	69 (70)	35	48 (48)	16	42 (67)	
Females	125	229 (66)	26	29 (30)	32	52 (52)	10	21 (33)	0.002
Age group									
≤50	9	16 (5)	22	25 (26)	27	34 (34)	11	29 (46)	
51-60	26	51 (15)	14	17 (17)	20	31 (30)	6	9 (15)	
61-70	81	155 (45)	8	45 (46)	15	20 (20)	8	24 (38)	
>70	53	126 (36)	7	10 (10)	5	16 (16)	1	1 (2)	<0.0001
Site§									
Right colon	155	318 (92)	41	58 (84)	45	58 (65)	17	40 (63)	
Left colon	9	19 (6)	6	8 (12)	12	22 (25)	5	8 (13)	
Rectum	4	10 (2.3)	3	3 (4)	7	9 (10)	4	15 (24)	0.0003
Amsterdam II									
Yes	6	11 (3)	10	12 (13)	17	24 (24)	1	1 (2)	
No	163	338 (97)	41	85 (87)	50	76 (76)	25	62 (98)	<0.0001
Bethesda									
Yes	81	160 (46)	39	45 (47)	50	72 (72)	20	45 (71)	
No	88	189 (54)	12	52 (53)	17	28 (28)	6	18 (29)	0.13

NOTE: Sixty-one cases with incomplete immunohistochemistry data were excluded.
*Four cases with *MLH1* methylation and no loss of MLH1 expression were included in this category.
†The unmethylated group includes tumors with a germline mutation and tumors with no detected germline mutation.
‡P value for χ^2 test of heterogeneity.
§Five cases with unspecified tumor location were excluded.

sex (male versus female), and tumor location (right colon, left colon, and rectum). We also evaluated differences in *MLH1* methylation by Amsterdam II criteria and revised Bethesda guidelines. Contingency table methods were used to evaluate differences in characteristics of MSI-H cases with *MLH1* methylation. Logistic regression was used to estimate adjusted associations between descriptive characteristics and *MLH1* methylation within MSI-H colon cancers. All statistical analyses were done using SAS v9.1 (SAS Institute).

Results

In the population-based series, *MLH1* methylation was observed in 60% of the MSI-H tumors, 3.1% of MSI-L tumors, and 0.7% of MSS tumors (Table 1). In the clinic-based series, the prevalence of *MLH1* methylation in MSI-H tumors (13%) was much lower than in the population-based series ($P < 0.0001$). *MLH1* methylation was not observed in any clinic-based MSI-L or MSS tumors. In the population-based MSI-L and MSS cases with *MLH1* methylation, we did not detect loss of MLH1 protein expression by immunohistochemistry. Immunohistochemistry results were inconclusive for three MSS cases with *MLH1* methylation. We compared the PMR value in the tumors with *MLH1* methylation across categories of MSI status, and we observed a lower median PMR value in the MSS tumors with *MLH1* methylation (median PMR = 19, range 12-30) compared with the MSI-H (median PMR = 47, range 10-128) and MSI-L (median PMR = 40, range 17-73) tumors with *MLH1* methylation.

Germline MMR mutations were identified in 12% of population-based MSI-H cases (Table 1). We did not detect any germline MMR mutations in MSI-L or MSS population-based cases. In the clinic-based series, germline MMR mutations were detected in 70% of MSI-H cases, 25% of MSI-L cases, and 2% of MSS cases. *MLH1* methylation was detected in one population-based case with a germline mutation in *MSH2* and in one clinic-based case with a germline mutation in *MLH1*.

MLH1 methylation was rarely observed in rectal tumors (Table 2); thus, we assessed age at diagnosis, sex, tumor location, and Lynch syndrome family history classification as independent predictors of DNA methylation only among population-based MSI-H colon cancers. Tumors with unspecified location within the colon were also excluded from this analysis. Older age at diagnosis was the strongest predictor of *MLH1* methylation after mutual adjustment for the other variables we evaluated (Table 3). Female sex and tumor

Table 3. Predictors of MLH1 methylation in population-based MSI-H colon cancer (345 cases)

	OR (95% CI)*	Adjusted OR (95% CI) †
Female sex	2.63 (1.38-5.02)	2.93 (1.36-6.29)
Age group		
51-60 vs ≤50	4.75 (1.56-14.5)	2.69 (0.75-9.61)
61-70 vs ≤50	18.0 (6.16-52.3)	9.92 (2.85-34.5)
>70 vs ≤50	23.8 (6.73-84.0)	13.2 (3.26-53.4)
P value trend	<0.0001	<0.0001
Right colon (vs left)	4.52 (1.77-11.5)	5.72 (0.88-37.4)
Amsterdam II	0.14 (0.05-0.39)	0.19 (0.06-0.62)
Bethesda criteria	0.31 (0.15-0.65)	0.62 (0.27-1.45)

NOTE: Eleven MSI-H cases with missing tumor location and 18 MSI-H cases with rectal cancer were excluded.
Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.
*All models included sampling weights and were adjusted for CFR center.
†Model included sampling weights and was adjusted for CFR center and the other covariates in the table.

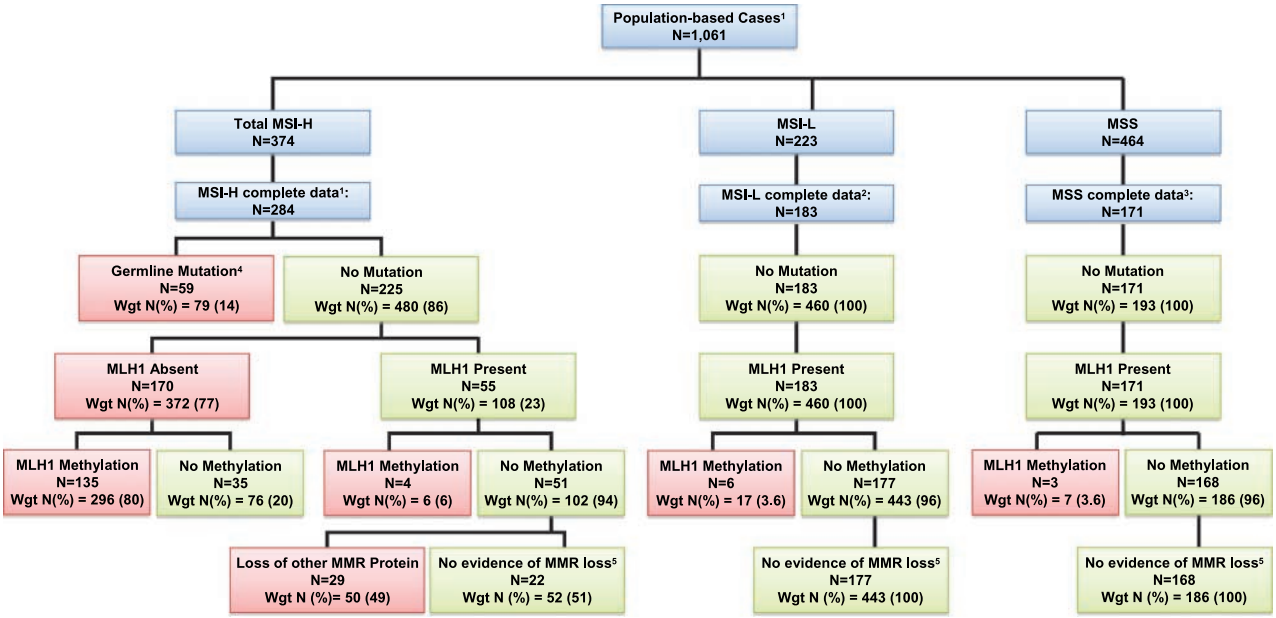

Figure 1. MMR mutation status, methylation status, and immunohistochemistry results for population-based CRC. *Blue boxes,* initial sample set; *red boxes,* samples where an alteration in MMR function was observed; *green boxes,* no detected alteration in MMR function. *1,* 50 MSI-H cases with no results from MMR testing and 40 additional MSI-H cases with incomplete results for immunohistochemistry were excluded. *2,* 26 MSI-L cases with no results from MMR testing and 14 additional MSI-L cases with incomplete results for immunohistochemistry were excluded. *3,* 259 MSS cases with no results from MMR testing and 34 additional MSS cases with incomplete results for immunohistochemistry were excluded. *4, MLH1* methylation was detected in a tumor from one individual with a germline mutation in MSH2. *5,* MMR loss = loss of MLH1, MSH2, or MSH6.

location in the right colon were also positively associated with *MLH1* methylation, although location in the right colon was not statistically significant in the adjusted model. Cases who fulfilled the Amsterdam II criteria were significantly less likely to have *MLH1* methylation (odds ratio, 0.19; 95% confidence interval, 0.06-0.62) than those not meeting the criteria. The vast majority of our samples were non-Hispanic white (93%); thus, we did not have power to evaluate differences in *MLH1* methylation by ethnicity.

Figure 1 shows the molecular characteristics of the population-based CRCs. Our main interest was to determine how many MSI-H tumors could apparently be explained by either mutation in one of the MMR genes or *MLH1* methylation. After restricting to MSI-H cases with germline mutation screening and conclusive immunohistochemistry results (n = 284), the frequency of germline mutation in one of the MMR genes (*MSH2, MLH1, MSH6,* or *PMS2*) was slightly higher than in the overall sample of MSI-H cases (14% versus 12%). As expected, we observed loss of MLH1 expression in a majority of MSI-H cases without a known germline mutation (i.e., sporadic MSI-H CRC), and we detected *MLH1* methylation in a majority of these cases (80%, Fig. 1). Among MSI-H tumors without loss of MLH1 expression, *MLH1* methylation was very rare (6%). In the four MSI-H tumors with DNA methylation and no loss of MLH1 expression, the median PMR value was 29 (range 23-34). This is qualitatively lower than the median PMR value for the tumors with DNA methylation and loss of MLH1 expression; however, the limited number of tumors with DNA methylation and no loss of MLH1 expression did not permit meaningful statistical comparison between these groups. We did not observe germline MMR mutations or loss of any MMR protein by immunohistochemistry in MSI-L or MSS cases (Fig. 1).

We also repeated the molecular dissection of the MSI-H group after stratification by age. Among the MSI-H cases diagnosed before age 50 years, 69 had complete data for MMR germline mutation screening, *MLH1* methylation, and immunohistochemistry. Thirty-two of these cases (39%) had a detected MMR germline mutation, 7 cases (14%) had *MLH1* methylation, and 30 cases (47%) could not be explained by either of these mechanisms. Among the 47% of cases with no detected MMR germline mutation or *MLH1* methylation, 21 (51%) had evidence for loss of a MMR protein by immunohistochemistry and 7 (23%) had an unclassified variant in one of the MMR genes.

Among MSI-H cases diagnosed after age 50 years, 215 had complete data for MMR germline mutation screening, *MLH1* methylation, and immunohistochemistry. The prevalence of MMR germline mutations (8.6%) was much lower and the prevalence of *MLH1* methylation (63%) was much higher in these cases compared with cases diagnosed before age 50 years. In these older MSI-H cases, 56 (28%) of MSI-H cases could not be explained by MMR germline mutation or *MLH1* methylation, 78% of these cases had evidence for loss of a MMR protein by immunohistochemistry, and 5% had an unclassified variant in one of the MMR genes.

Discussion

The main objective of this analysis was to perform molecular characterization of the MSI-H phenotype within a large series of population-based CRC. When we restricted the population-based series to cases with MMR germline mutation testing and complete immunohistochemistry results, we identified a molecular event in MMR in 91% of MSI-H cases: 54% had *MLH1* methylation, 14% had a germline mutation in a MMR gene (*MSH2*, *MLH1*, *MSH6*, or *PMS2*), and 23% had isolated immunohistochemistry evidence for loss of a MMR protein (Fig. 1). The prevalence of *MLH1* methylation and germline MMR mutation differed greatly by age at diagnosis, with cases diagnosed after age 50 years having a lower prevalence of germline mutation and a much higher prevalence of *MLH1* methylation than cases diagnosed before age 50 years.

MLH1 methylation was observed more frequently in population-based tumors than in clinic-based MSI-H tumors. This can be explained by the higher frequency of clinic-based MSI-H cases with a MMR germline mutation (Table 1). In addition, the MSI-H cases in the population-based series were diagnosed at an older age than the MSI-H cases in the clinic-based series (median age 63 years, range 22-75 years versus median age 44 years, range 19-77 years, respectively). The low frequency of *MLH1* methylation in the clinic-based sample has clinical implications. Our data suggest that *MLH1* methylation may explain MSI-H CRC in the absence of a detected germline mutation in some, but not all, of these cases. The low frequency of *MLH1* methylation in clinic-based cases with a germline mutation also suggests that germline mutation and methylation are largely independent mechanisms for inactivation of *MLH1* and that the remaining wild-type allele in most Lynch syndrome cases is not typically inactivated by DNA methylation.

Previous studies have reported that *MLH1* methylation is associated with older age at diagnosis and female sex (18, 20, 23, 34). In addition, MSI-H tumors in general (35-37), as well as tumors with *MLH1* methylation, are more likely to be located within the proximal colon (23, 38, 39). Our large sample of population-based MSI-H cases allowed us to evaluate independent associations between these descriptive characteristics and *MLH1* methylation within the MSI-H subgroup. We observed statistically significant positive associations for female sex and older age at diagnosis in the multivariable adjusted model and a statistically significant inverse association with Amsterdam II criteria (Table 3). Although we have not measured CpG island methylator phenotype (CIMP) in these samples to date, a previous report found that most sporadic MSI-H were CIMP positive because *MLH1* was methylated in these samples (30). CIMP can occur in the context of both MSI-H and MSS CRC, with different molecular alterations distinguishing the two groups of CIMP-positive tumors (21). These data suggest that the MSI-H tumors with *MLH1* methylation are likely CIMP positive and the associations we have observed between *MLH1* methylation and descriptive characteristics may also apply to CIMP-positive MSI-H CRC.

Possible explanations for MSI in the group with no detected *MLH1* methylation or MMR germline mutation include (*a*) false-negative results for either *MLH1* methylation or MMR germline mutation; (*b*) somatic inactivation in one of the known MMR genes; or (*c*) some other method of inactivation of mismatch repair. False negatives for *MLH1* methylation or MMR germline mutation are unlikely to explain all of these cases in this population-based series as *MLH1* methylation was rarely observed in cases without loss of MLH1 and MMR germline mutations are estimated to occur in only 1% to 2% of CRC (2, 4). We detected variants of uncertain biological significance in our MMR germline mutation screening and it is also possible that some of these variants may be functional. Among the cases without a clearly deleterious MMR germline mutation, unclassified variants were observed in 4% of cases with loss of MLH1 protein and no DNA methylation, 4% of cases with loss of one of the other MMR genes, and 24% of cases with no detected loss of MMR function (Supplementary Table S1). Somatic mutations have been reported in MSI-H CRC in previous studies (17, 22) and this may explain MSI in some of these cases. Additionally, a germline polymorphism in the *MLH1* promoter has been reported to be associated with risk of MSI-H CRC (40). Such sequence variants, particularly when homozygous, may also offer an explanation for a portion of the remainder of MSI-H CRC. We did not perform screening for this polymorphism.

Although the majority of *MLH1* methylation was observed in MSI-H cases, we observed *MLH1* methylation in 3.1% of MSI-L and 0.7% of MSS cases in the population-based series with no resulting loss of MLH1 expression, although we did not have MLH1 immunohistochemistry data for three MSS cases. We observed a lower PMR value in the MSS tumors with *MLH1* methylation compared with the MSI-H tumors with *MLH1* methylation. In addition, we observed *MLH1* methylation in four MSI-H cases with no observed loss of MLH1 expression (Fig. 1). One plausible explanation for these findings is monoallelic *MLH1* methylation. MethyLight is a quantitative assay and a previous study has shown that this technique is capable of distinguishing between monoallelic and biallelic DNA methylation (41).

This study has several limitations. Because the prevalence of *MLH1* methylation decreased with increasing *ALU* C(t) value, it is likely that we have some samples with undetected *MLH1* methylation in the unmethylated category. However, there were no statistically significant differences in *ALU* C(t) value by age, gender, or tumor location within the MSI-H category (data not shown), suggesting that the percentage of false negatives should not differ within these groups. Undetected carriers of MMR germline mutations may exist in our study population because we did not test all individuals for *MSH6* and *PMS2* mutations. In addition, immunohistochemistry results for MSH6 and PMS2 were not available for all cases. This study also has several strengths, including the largest sample size to date of tumors with both MSI and *MLH1* methylation status, systematically collected epidemiologic data and tumor characteristics, and inclusion of both population- and clinic-based families.

In summary, we observed *MLH1* methylation in 60% of population-based MSI-H tumors and 13% of clinic-based MSI-H colorectal tumors. As expected, the

prevalence of germline mutation in one of the MMR genes was higher in cases diagnosed before age 50 years compared with cases diagnosed after age 50 years (39% versus 9%, respectively) whereas the prevalence of *MLH1* methylation was much lower in cases diagnosed before age 50 years than in cases diagnosed after age 50 years (14% versus 63%). Within population-based MSI-H colon cancer, we were able to establish that older age at diagnosis and female sex are independent predictors of *MLH1* methylation and that a great majority of MSI-H CRC could be explained by either germline mutation within one of the MMR genes or *MLH1* methylation. However, there was a subset of cases where the MSI-H phenotype could apparently not be explained by either of these mechanisms. Further research will be required to better understand the MSI-H phenotype in these cases.

Disclosure of Potential Conflicts of Interest

P.W. Laird: Epigenomics AG Speakers Bureau/Honoraria, TherEpi Corp. Ownership interest. The other authors disclosed no potential conflicts of interest.

Acknowledgments

We thank the CFR study coordinators and data managers who helped prepare the data set for these analyses (Maggie Angelakos, Terrilea Burnett, Helen Chen, Darshana Daftary, Pat Harmon, Heide Miller-Pakvasa, Douglas Snazel, Terry Teitsch, and Allyson Templeton) as well as the participants in the Colon CFR who have generously donated their time for this project.

The content of the manuscript does not necessarily reflect the views or policies of the National Cancer Institute or any of the collaborating centers in the CFRs, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government or the CFR.

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