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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 10−20% 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: 1) 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 2) estimate the associations between *MLH1* methylation and sex, age, and tumor location within the colon. *MLH1* methylation was measured in 1,061 population- 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 IHC results, we identified a molecular event in MMR in 91% of MSI-H cases: 54% had *MLH1* methylation, 14%

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had a germline mutation in a MMR gene, and 23% had IHC evidence for loss of a MMR protein. We observed a striking age difference, with the prevalence of a MMR germline mutation more than four-fold lower and the prevalence of *MLH1* methylation more than four-fold higher in cases diagnosed after age 50 than in cases diagnosed before age 50. 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.

Keywords

MLH1 methylation; MMR mutation; colorectal cancer

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 five percent of all colorectal cancers (CRC) (1-5). In sporadic colorectal cancers, MSI-H occurs in approximately 10 − 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 patients with Lynch syndrome and those with MSI-H cancers caused by *MLH1* hypermethylation or other causes (reviewed in (15)). Recently, a gene expression signature was proposed to distinguish between so-called “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 (IHC) 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 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

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clinic-based cases and confirmed previously reported associations between *MLH1* methylation and descriptive characteristics in MSI-H CRC.

Methods

Study Population

Participants were recruited for the Colon CFR from six registry centers including the University of Hawaii (Honolulu), Fred Hutchinson Cancer Research Center (Seattle, WA), Mayo Clinic (Rochester, MN), University of Southern California Consortium (Los Angeles), 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 while others over-sampled 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 Amsterdam II Criteria (27) and revised Bethesda guidelines(5), which are guidelines used to identify individuals likely to carry a germline mutation in one of the 4 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 4 markers to determine MSI status. Tumors were deemed MSI-H if instability was observed at $\geq 30\%$ of markers, MSI-L if > 0 and $< 30\%$ 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. Desulphonation was performed by adding 200 µl 0.08M NaOH (in AW1/EtOH wash buffer) to the spin column and incubated for 15 min at room temperature. Afterwards, 200µl of 0.08M HCl (in AW1/EtOH wash buffer) was added to neutralize, and after a 5 min incubation period, the columns were centrifuged and the filtrate removed. The desulphonated sample was further washed using supplied wash buffers and eluted as described (29).

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MethyLight analysis of *MLH1* was performed 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) ≥ 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. Since 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 towards 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 ≤ 24 to retain the largest sample size possible for the analysis while minimizing the potential for false negatives.

Immunohistochemistry

Immunohistochemistry (IHC) for MLH1, MSH2, MSH6, and PMS2 proteins was performed as previously described (31,32). IHC testing was performed 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 IHC testing on all MSS cases. Staining was classified as absent, present or inconclusive.

Mismatch Repair Mutation (MMR) 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 DHPLC/direct sequencing and multiplex ligation dependent probe amplification (MLPA). 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 1) changes known or predicted to truncate protein production including frameshift and nonsense variants, 2) splice site mutations occurring within 2 bp of an intron/exon boundary, and 3) missense changes that have been demonstrated to have a deleterious effect.

Molecular Testing Performed 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 > 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. IHC results were available for 719 of these population-based cases, including 317/374

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MSI-H cases, 205/223 MSI-L cases, and 197/464 MSS cases. MMR germline mutation status was available for 324/374 population-based MSI-H cases, 197/223 MSI-L cases, and 205/464 MSS cases. In addition, DNA methylation testing was performed on 220 clinic-based cases. Forty-eight of these cases were excluded because of 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 IHC 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 (≤ 50 vs. 51−60, 61−70, and > 70 years), sex (male vs. 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 performed using SAS v9.1 (SAS Institute, Cary, N.C.).

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 IHC. IHC results were inconclusive for 3 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 to 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), so 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 location in the right colon were also positively associated with *MLH1* methylation, although location in

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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 (OR=0.19, 95% CI 0.06−0.62) than those not meeting the criteria. The vast majority of our sample was non-Hispanic white (93%), so we did not have power to evaluate differences in *MLH1* methylation by ethnicity.

Figure 1 shows the molecular characteristics of the population-based CRC's. 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 IHC 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% vs. 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%, Figure 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 IHC in MSI-L or MSS cases (Figure 1).

We also repeated the molecular dissection of the MSI-H group after stratification by age. Among MSI-H cases diagnosed before age 50, 69 had complete data for MMR germline mutation screening, *MLH1* methylation and IHC. 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 IHC and 7 (23%) had an unclassified variant in one of the MMR genes.

Among MSI-H cases diagnosed after age 50, 215 had complete data for MMR germline mutation screening, *MLH1* methylation, and IHC. 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. 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 IHC 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 IHC 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 IHC evidence for loss of a MMR protein (Figure 1). The prevalence of *MLH1* methylation and germline MMR mutation differed greatly by age at diagnosis, with cases diagnosed after age 50 having a lower prevalence of germline mutation and a much higher prevalence of *MLH1* methylation than cases diagnosed before age 50.

MLH1 methylation was observed more frequently in population-based than 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

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were diagnosed at an older age than the MSI-H cases in the clinic-based series (median age 63, range 22−75 vs. median age 44, range 19−77, 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: 1) false negative results for either *MLH1* methylation or MMR germline mutation; 2) somatic inactivation in one of the known MMR genes; or 3) 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 1−2% 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 1). 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.

While the majority of *MLH1* methylation was observed in MSI-H cases, we did observe *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 IHC data for 3 MSS cases. We observed a lower PMR value in the MSS tumors with *MLH1* methylation compared to the MSI-H tumors with *MLH1* methylation. In addition, we observed *MLH1* methylation in 4 MSI-H cases with no observed loss of MLH1 expression (Figure 1). One plausible explanation for these findings is mono-allelic *MLH1* methylation. MethyLight is a quantitative assay and a previous study has demonstrated that this technique is capable of distinguishing between monoallelic and bi-allelic DNA methylation (41).

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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, IHC 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 compared to cases diagnosed after age 50 (39% vs. 9%, respectively) while the prevalence of *MLH1* methylation was much lower in cases diagnosed before age 50 than in cases diagnosed after age 50 (14% vs. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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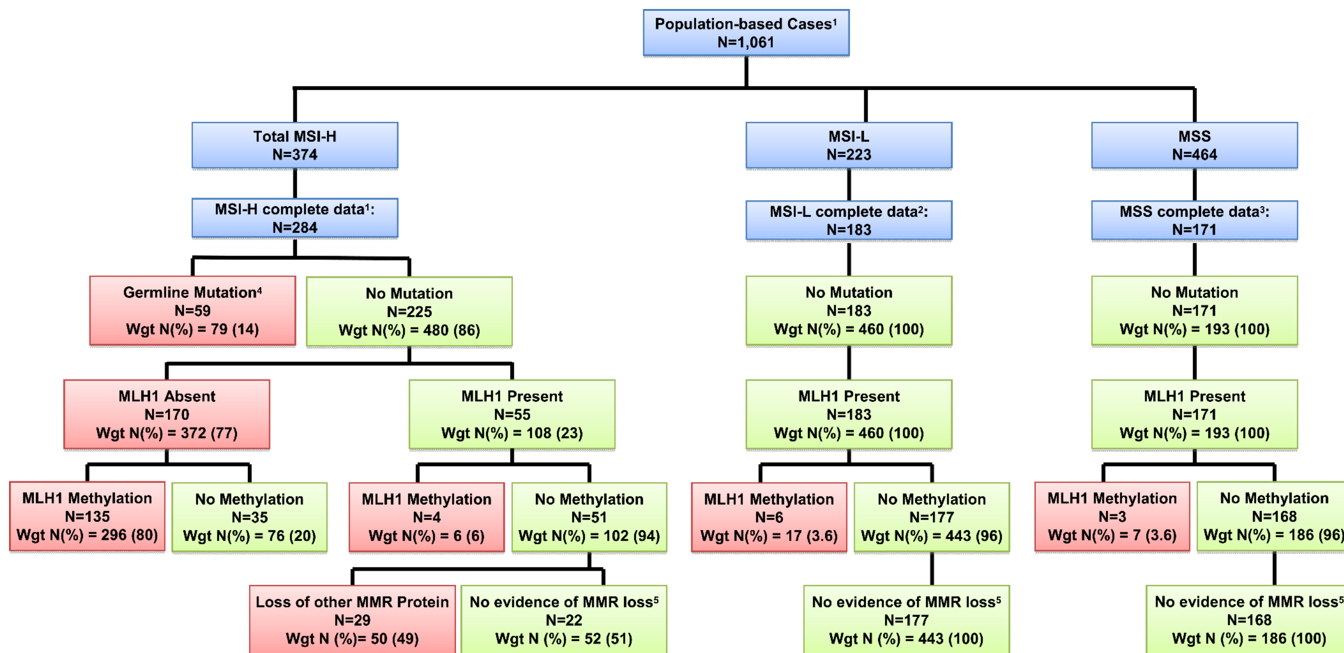

Figure 1. MMR mutation status, methylation status, and IHC results for population-based CRC

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

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Table 1

MLH1 Methylation in Population and Clinic-based Cases by MSI Status

	MSI-H			MSI-L			MSS		
		Weighted			Weighted			Weighted	
	Cases	**N**	**%**	**Cases**	**N**	**%**	**Cases**	**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	1428	99
Germline MMR Mutation[1]									
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	1152	NA
	Clinic-based								
***MLH1* Methylation**									
Methylated	7	NA	13	0	NA	0	0	NA	0
Unmethylated	48	NA	87	12	NA	100	105	NA	100
Germline MMR Mutation[2]									
Mutation	33	NA	70	3	NA	25	2	NA	2
No Mutation	14	NA	30	9	NA	75	91	NA	98
Untested	8	NA	NA	0	NA	NA	12	NA	NA

[1] 50 MSI-H, 26 MSI-L, and 259 MSS population-based cases were not tested for germline MMR mutations

[2] 8 MSI-H and 12 MSS clinic-based cases were not tested for germline MMR mutations