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# Prevalence of Somatic MutL Homolog 1 Promoter Hypermethylation in Lynch Syndrome Colorectal Cancer

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## Abstract

**BACKGROUND:** Colorectal cancers (CRCs) that have microsatellite instability (MSI) and mutL homolog 1 (*MLH1*) immunoloss are observed in 3 clinical scenarios: Lynch syndrome (LS), sporadic MSI CRC, and Lynch-like syndrome (LLS). v-Raf murine sarcoma viral oncogene homolog B1 (*BRAF*) mutational analysis is used to differentiate LS from sporadic MSI CRC. The role of *MLH1* promoter methylation status for the differential diagnosis of these clinical forms is not well established. The objectives of this study were: 1) to analyze *MLH1* promoter methylation

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Additional Supporting Information may be found in the online version of this article.

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in *MLH1*-deficient CRCs by pyrosequencing, and 2) to assess its role in the differential diagnosis of *MLH1*-deficient CRCs.

**METHODS:** In total, 165 CRCs were analyzed, including LS (n = 19), MSI *BRAF*-mutated CRC (n = 37), MSI *BRAF* wild-type CRC (n = 60), and a control group of CRCs without MSI (microsatellite stable [MSS] CRC; n = 49). *MLH1* promoter methylation status was analyzed by pyrosequencing, and the ability of different strategies to identify LS was assessed.

**RESULTS:** The average ± standard deviation methylation in LS (9% ± 7%) was significantly lower than that in MSI *BRAF*-mutated CRC (42% ± 17%; $P < .001$) and in MSI *BRAF* wild-type CRC (25% ± 19%; $P = .002$). Somatic *MLH1* hypermethylation was detected in 3 patients (15.8%) with LS, in 34 patients (91.9%) with MSI *BRAF*-mutated CRC, and in 37 patients (61.7%) with MSI *BRAF* wild-type tumors. Patients with MSI *BRAF* wild-type, unmethylated tumors (ie, LLS) had a stronger family history of CRC than those who had tumors with *MLH1* methylation ($P < .05$). The sensitivity for ruling out LS was 100% for *BRAF* analysis, 84.2% for *MLH1* methylation analysis, and 84.2% for the combination of both analyses.

**CONCLUSIONS:** Somatic *MLH1* promoter methylation occurs in up to 15% of LS CRCs. Somatic *BRAF* analysis is the most sensitive strategy for ruling out LS. Patients who have CRCs with loss of MLH1 protein expression and neither *BRAF* mutation nor *MLH1* methylation resemble patients with LS. *Cancer* 2015;121:1395-404.

### Keywords

Lynch syndrome; colorectal cancer; v-Raf murine sarcoma viral oncogene homolog B1; mutL homolog 1; methylation; microsatellite instability; CpG island methylator phenotype

## INTRODUCTION

Lynch syndrome (LS), an autosomal-dominant, inherited disorder caused by a germline mutation in 1 of the mismatch-repair (MMR) system genes,[1] is the most common form of hereditary colorectal cancer (CRC), accounting for 1% to 5% of all CRCs. The presence of a germline pathogenic mutation in 1 of the MMR genes (mutL homolog 1 [*MLH1*], mutS homolog 2 [*MSH2*], mutS homolog 6 [*MSH6*], postmeiotic segregation increased 2 [*PMS2*], and epithelial cell adhesion molecule [*EPCAM*]) leads to considerably increased risks of CRC, endometrial cancer, and several other types of cancer at an early age. Because of the potential impact of diagnosis on patients and relatives, the identification and characterization of LS has tremendous clinical and scientific relevance.[2]

In patients with LS, the somatic inactivation or “second hit” of the wild-type allele of the affected MMR gene leads to the abnormal function of the MMR gene with the accumulation of errors during DNA replication, especially in repetitive sequences known as microsatellites.[3] Consequently, tumors from patients with LS characteristically demonstrate MMR deficiency, defined as the presence of microsatellite instability (MSI) and/or the loss of MMR protein expression (ie, immunoloss), which are the hallmarks of this disorder.[4]

A diagnosis of LS has the potential to change the natural history of the disease. It has been demonstrated that CRC screening and surveillance can reduce the incidence and

mortality of this tumor.[5] However, an LS diagnosis is challenging in clinical practice, mainly because, in many cases, a high degree of suspicion is required.[6] Currently, studying the proficiency of the MMR genes by either MSI testing or immunohistochemistry is the cornerstone for identifying LS. Germline testing for mutations within the MMR genes in patients who have MMR-deficient tumors is the ultimate gold standard for diagnosing LS. However, MMR deficiency is not a unique feature of LS, and most CRCs with MSI and/or loss of MLH1 expression (which account for up to 15% of all CRCs) are secondary to somatic hypermethylation of the *MLH1* gene promoter, with the subsequent transcriptional silencing of the gene. This situation is associated with the serrated pathway of carcinogenesis, which differs from the traditional adenoma-carcinoma sequence by highly correlated molecular characteristics, including the cytosine-phosphate-guanine (CpG) island methylator phenotype (CIMP), the *BRAF* valine to glutamic acid substitution at codon 600 (*BRAF* V600E mutation), and methylation of the *MLH1* gene promoter.[6] These tumors are more frequent in older patients who have no family history of CRC (so-called “sporadic MSI CRC”),[7] and no genetic counseling is required. In addition, there is a third challenging and frequent subset of patients with MMR-deficient tumors that have recently been defined as Lynch-like syndrome (LLS).[8] LLS usually refers to patients who have MMR-deficient CRC with neither *MLH1* somatic hypermethylation nor germline pathogenic mutation in the MMR genes.[8] The molecular mechanisms of LLS development probably are heterogeneous and are poorly understood. It is plausible that the LLS group includes both patients with sporadic MSI and those with true LS.[9]

Accordingly, in clinical practice, the differential diagnosis of a patient who has CRC with MSI and loss of MLH1 remains a challenge. The analysis of somatic mutations in the *BRAF* gene is currently the most widely used negative predictor of germline mutations in the MMR genes[10]; therefore, the presence of the *BRAF* V600E mutation should preclude MMR gene analysis. Likewise, *MLH1* promoter methylation analysis has also been proposed to discriminate LS with respect to the sporadic form of MSI CRC. However, previous reports on the evidence of somatic *MLH1* methylation as a second hit in some LS tumors have called into question its performance as a negative predictor of *MLH1* germline mutations.[11] In addition, the current definition of LLS may be biased by the exclusion of potential LS patients who have somatic *MLH1* methylation as a second hit. Accordingly, the role of *MLH1* methylation analysis in this setting is still uncertain. Previous studies have used several different qualitative or semiquantitative methods to analyze *MLH1* methylation,[12] with some variability mainly in the promoter region analyzed.[13] Moreover, whereas pyrosequencing is now considered the gold standard for methylation analysis,[14] the technology has not been assessed in depth in this setting. The objective of the current study was to analyze somatic *MLH1* promoter methylation status in different clinical forms of CRC (ie, LS, MSI *BRAF*-mutated CRC, and MSI *BRAF* wild-type CRC) with loss of MLH1 protein expression by pyrosequencing and to assess its role in the differential diagnosis of *MLH1*-deficient CRCs.

# MATERIALS AND METHODS

## Patient Selection

In total, 165 CRCs that were available as formalin-fixed, paraffin-embedded (FFPE) tissue sections were divided into 4 groups: 1) LS (n = 19), comprised of tumors from patients who had a pathogenic *MLH1* germline mutation; 2) MSI *BRAF*-mutated CRCs (n = 37), comprised of tumors with MLH1 immunoloss associated with somatic *BRAF* mutation, usually known as "sporadic MSI CRC"; 3) MSI *BRAF* wild-type CRCs (n = 60), comprised of tumors with MLH1 immunoloss and an absence of somatic *BRAF* mutation (ie, *BRAF* wild type) in patients who had no pathogenic *MLH1* germline mutation; and 4) a control group of patients who had MMR-proficient tumors (ie, microsatellite stable [MSS] CRC; n = 49) with no MSI that retained expression of MLH1 (Fig. 1). Samples were obtained from patients who attended the high-risk CRC clinic at the Hospital Clinic of Barcelona and from patients who were included in the EPICOLON II study.[15] Within the *MLH1*-LS group, 10 of 19 tumors were obtained from Baylor University Medical Center (Dallas, Tex). Written informed consent was obtained from all participants, and the project obtained institutional review board approval at each participating institution.

## Methods

Demographic, clinical, molecular, and familial characteristics were obtained. Molecular parameters included MSI testing, MMR protein immunostaining, somatic *BRAF* testing, *MLH1* methylation analysis, and *MLH1* germline mutation status in patients who had *BRAF* wild-type tumors.

***MLH1* promoter methylation analysis—**DNA methylation status of the *MLH1* promoter region was established by polymerase chain reaction analysis of bisulfite-modified genomic DNA (EpiTect Bisulfite Kit; Qiagen NV, Venlo, the Netherlands) using pyrosequencing for quantitative methylation analysis (Pyromark Q96ID; Qiagen NV). The primers were designed by using the Pyromark Assay Desing Software package (Qiagen NV) and including 4 CpG sites to analyze the methylation of the promoter. These CpG sites are located in the promoter region associated with loss of protein expression (Supporting Fig. 1; see online supporting information). The primers and polymerase chain reaction conditions that we used are described in Supporting Table 1 (see online supporting information). The mean of all 4 CpG sites analyzed was calculated to obtain a percentage of methylation as previously described.[16] The control group (MMR-proficient tumors) was used to define the cutoff percentage for *MLH1* hypermethylation (mean ± 3 standard deviations).

Somatic *MLH1* methylation analysis was performed in all samples included in the study. Within the subgroup of patients with *BRAF* wild-type and *MLH1*-methylated tumors, we performed an analysis for germline methylation to rule out a constitutional epimutation.[17]

**DNA extraction, MMR deficiency analysis, *MLH1* germline mutation status, and somatic *BRAF* V600E mutation analysis—**The methods used for DNA extraction, MMR deficiency analysis, *MLH1* germline mutation status, and somatic *BRAF*

V600E mutation analysis are detailed in the Supporting Methods (see online supporting information).

**Comparison of *BRAF* and *MLH1* methylation as predictors of MMR gene mutation status—**The performance characteristics of different strategies (ie, sensitivity, specificity, positive predictive value, and negative predictive value) were assessed for their ability to identify LS. More specifically, we compared the use of somatic *BRAF* mutation testing, *MLH1* methylation analysis, and the combination of both techniques together.

### Statistical Analysis

All data were analyzed using the software packages SPSS (version 18.0; SPSS Inc., Chicago, Ill) and GraphPad Prism (version 4.0; GraphPad Software, Inc., San Diego, Calif). Quantitative variables were analyzed using the Student *t* test, the Wilcoxon test (for nonparametric paired analyses), and the Mann-Whitney *U* test (for nonpaired analyses). Qualitative variables were analyzed using either the chi-square test or the Fisher test. Two-sided *P* values < .05 were regarded as significant.

## RESULTS

Clinicopathologic features of the patients included in the study are summarized in Table 1. The patients with LS, as expected, were younger than the other 2 groups of patients with *MLH1*-deficient tumors (mean age: LS vs MSI *BRAF*-mutated CRC, 44.9 years vs 73.6 years [$P < .0001$]; LS vs MSI *BRAF* wild-type CRC, 44.9 years vs 67.3 years [$P = .0001$]). Patients with MSI *BRAF* wild-type CRCs were younger than patients in the MSI *BRAF*-mutated group (mean age: 67.3 years vs 73.6 years, respectively; $P = .005$). Compared with the patients who had MSS tumors, the 3 groups with *MLH1*-deficient tumors were associated with a high frequency of proximal tumors ($P = .0001$). Patients with LS displayed an overall stronger family history of CRC and LS-related tumors compared with the other groups (overall, $P < .004$).

### MLH1 *Methylation Analysis in* MLH1*-Deficient CRCs*

We performed *MLH1* methylation analysis using a new pyrosequencing assay optimized for FFPE tissues. The assay analyzes the methylation status of 4 CpG sites located in the specific functional region that drives transcriptional silencing of the gene (Supporting Fig. 1; see online supporting information). For further analysis, we used the average methylation levels of the 4 CpG sites analyzed.

The mean ± standard deviation *MLH1* promoter methylation was 7.6% ± 2.5% in the MSS group, 9.1% ± 7.1% in the LS group, 41.9% ± 17.3% in the MSI *BRAF*-mutated group, and 25.3% ± 19.5% in the MSI *BRAF* wild-type group. The level of methylation was significantly lower in the LS group compared with the MSI *BRAF*-mutated and MSI *BRAF* wild-type groups ($P < .0001$ and $P = .002$, respectively) (Fig. 2, Supporting Table 2; see online supporting information).

Next, we analyzed the methylation results as a categorical variable using the mean level of *MLH1* methylation in the MSS group as the cutoff for *MLH1* hypermethylation (mean

± 3 standard deviations, which corresponds to 15%). By using the 15% cutoff, all MSS tumors were unmethylated at the *MLH1* promoter, methylation was detected in almost all MSI *BRAF*-mutated CRCs (34 of 37 tumors; 91.9%) and in most MSI *BRAF* wild-type CRCs (37 of 60 tumors; 61.7%), but methylation also was detected in a small number of LS samples (3 of 19 tumors; 15.8%). The differences between the 3 groups were statistically significant (Fig. 3, Supporting Table 2; see online supporting information).

Overall, these results indicate that pyrosequencing allows a quantitative and precise analysis of the methylation levels of the *MLH1* promoter in FFPE samples of CRC. It is noteworthy that 15.8% of LS tumors displayed *MLH1* methylation. Although *MLH1* methylation is almost universal in MSI *BRAF*-mutated tumors, 62% of MSI *BRAF* wild-type tumors display *MLH1* promoter hypermethylation.

## Patients With MSI BRAF Wild-Type CRCs Without MLH1 Methylation Have Clinical Features Suggestive of LS

To further characterize the MSI *BRAF* wild-type group, we compared its clinicopathologic features according to the somatic *MLH1* methylation status (Table 2). Overall, patients with MSI *BRAF* wild-type CRC and *MLH1*-unmethylated tumors (so-called LLS) displayed a stronger family history of neoplasia. It is worth noting that patients who had unmethylated MSI *BRAF* wild-type CRCs had a stronger family history of CRC than those who had methylated tumors (ie, a first-degree or second-degree relative with CRC: 30.4% vs 8.1%, respectively; $P = .05$) and also had relatives diagnosed at younger ages (aged <50 years; 21.7% vs 2.7%, respectively; $P = .05$). We also observed a stronger family history of LS-related tumors, although the difference was not statistically significant (first-degree or second-degree relative aged <50 years with LS-related tumor; 26.1% vs 10.8%, respectively; $P = .235$). No differences in sex or CRC features were observed.

To rule out a constitutional *MLH1* epimutation within the subgroup of MSI *BRAF* wild-type CRC with *MLH1*-methylated tumors, we carried out an analysis of *MLH1* methylation in the germline (blood DNA), but no constitutional *MLH1* epimutation was identified. Overall, these results indicate that MSI *BRAF* wild-type CRCs occur in 2 clinically different scenarios, depending on their *MLH1* methylation status: 1) an LLS subgroup includes patients who have MSI *BRAF* wild-type CRC with neither a germline *MLH1* mutation nor somatic *MLH1* hypermethylation (n = 23); and 2) a sporadic MSI *BRAF* wild-type subgroup, comprised of patients who have *BRAF* wild-type tumors with somatic *MLH1* methylation and no germline *MLH1* mutations (n = 37). These results suggest that at least some patients with LLS may carry other germline mutations that would escape detection using standard screening methods.

## Somatic BRAF Analysis Is the Most Sensitive Strategy for Ruling Out an LS Diagnosis

To determine the most sensitive strategy for the identification of LS among patients with *MLH1*-deficient CRC, we compared the performance characteristics of the different approaches (somatic *BRAF* analysis followed by germline testing in the absence of a mutation, *MLH1* methylation analysis followed by germline testing in the absence *MLH1*

methylation, or a combination of both *BRAF* and *MLH1* methylation analysis followed by germline testing in the absence of both *BRAF* mutation and *MLH1* methylation) (Table 3).

The sensitivity and specificity for the diagnosis of a patient with an *MLH1* germline mutation (LS) were 100% and 38.1%, respectively, for *BRAF* analysis, 84.2% and 73.2%, respectively, for *MLH1* methylation analysis, and 84.2% and 76.3%, respectively, for both techniques combined. The number of patients requiring germline genetic analysis was 79 (68.1%) for *BRAF* analysis and 42 (36.2%) for *MLH1* methylation analysis. According to these results, the most sensitive strategy for LS diagnosis in CRC patients who have MLH1 immunoloss is determining the presence of a somatic *BRAF* mutation. Combining the 2 techniques did not add any benefit to the diagnosis.

## DISCUSSION

The diagnosis of LS is challenging. Evidence suggests that the most sensitive strategy for the identification of LS carriers among patients with CRC is performing MSI testing and/or immunostaining in all patients[18] followed by germline testing in those with MMR-deficient tumors. Although LS is the most common inherited form of CRC, most tumors with MMR deficiency are in the context of sporadic MSI CRCs because of the epigenetic silencing of *MLH1* caused by promoter hypermethylation. Sporadic MSI CRCs are thought to evolve through the serrated pathway of carcinogenesis, which closely correlates with the CIMP phenotype and *BRAF* mutations. Patients with LLS are those who display tumor MMR deficiency not associated with the serrated pathway of carcinogenesis but in whom germline testing does not identify a pathogenic mutation.[8] The differential diagnosis of these 3 clinical scenarios is decisive in clinical practice to offer adequate genetic counseling. Tumor *BRAF* mutation status is a strong negative predictor of LS; accordingly, *BRAF* testing is routinely used for the differential diagnosis between sporadic MSI CRCs and LS.[19] However, evidence analyzing *MLH1* promoter methylation as a negative predictor of LS is highly heterogeneous.[13] In this study, we demonstrated the role of *MLH1* promoter methylation status in the differential diagnosis of *MLH1*-deficient tumors using pyrosequencing, which is considered the gold standard for methylation analysis. It is noteworthy that 15% of patients with LS displayed somatic *MLH1* hypermethylation, raising the possibility that *MLH1* promoter methylation is the “second hit” in a considerable number of carriers.[20] In addition, we observed that patients with MSI *BRAF* wild-type CRCs were clinically heterogeneous, including those with LLS and sporadic MSI *BRAF* wild-type CRC. Finally, we demonstrated that tumor *BRAF* testing is the most sensitive strategy for ruling out an LS diagnosis among patients with *MLH1*-deficient CRCs, although it is less specific than somatic *MLH1* methylation analysis.

Somatic *BRAF* V600E mutation and *MLH1* methylation have been studied extensively as predictive markers of MMR gene mutation status.[6,7,21] It has been demonstrated that *BRAF* mutational analysis is the strongest negative predictor of a germline *MLH1* mutation in *MLH1*-deficient tumors. Previous studies indicated that *BRAF* status as a selection criterion for referring patients to germline genetic testing was an effective strategy for reducing costs and effort.[10,22] However, *BRAF* mutations occasionally may be identified in MMR gene mutation carriers. A recent literature review reported a frequency of 1.4% for *BRAF*-

mutated tumors in LS carriers.[22] Because *BRAF* mutations are so rare in patients with LS, the possibility that these are false-positive results, or even the presence of a sporadic MSI tumor as a phenocopy in a patient with LS, cannot be ruled out. Regarding *MLH1* methylation analysis as a negative predictor of germline MMR mutations, there has been great variability the results, depending on the promoter region analyzed and the techniques used.[12] Evidence supports the finding that methylation of the *MLH1* promoter C-region is associated with loss of tumor MLH1 expression.[13] In a recent literature review, methylation of the *MLH1* promoter C-region was reported in 5% of tumors from *MLH1* mutation carriers.[22] These data suggest that inactivation of the *MLH1* wild-type allele by methylation as a second hit in patients with LS is a frequent event and that a considerable proportion of *MLH1* mutation carriers may be undiagnosed when depending on *MLH1* methylation alone for triaging LS. In our study, by using a robust and precise technique for methylation analysis, we observed that a small but meaningful proportion of patients with LS may carry somatic *MLH1* methylation, likely as a second hit. This result clearly argues against the use of *MLH1* methylation analysis as a negative predictor of an *MLH1* germline mutation. In that sense, our study demonstrates that somatic *BRAF* status is the most sensitive strategy for ruling out an *MLH1* germline mutation among *MLH1*-deficient tumors, because it performs better than methylation analysis.

Regarding patients with LLS, a recent report indicated that >50% of MMR-deficient tumors that previously had tested negative for germline MMR gene mutations carried 2 somatic events in either *MLH1* or *MSH2* (mutation or loss of heterozygosity).[9] These results suggest a nonhereditary cause for a proportion of individuals with LLS, consistent with observations that relatives of patients with LLS are at lower risk of CRC than relatives of patients with LS.[8] LLS constitutes 1 of the biggest challenges in clinical practice, because relatives of patients with LLS are advised to undergo the same screening as patients with LS (including colonoscopy every 1–2 years starting at age 25 years and screening for extracolonic cancers). Our results indicate that, like MSI *BRAF*-mutated CRCs, most MSI *BRAF* wild-type tumors (61.7%) characteristically exhibit somatic *MLH1* methylation, suggesting a sporadic origin (ie, sporadic MSI *BRAF* wild-type tumors). However, the subgroup of MSI *BRAF* wild-type tumors without somatic *MLH1* methylation (ie, LLS) exhibit clinical features suggestive of LS (ie, a stronger family history of CRC). This finding suggests that MSI *BRAF* wild-type tumors constitute a heterogeneous group, probably comprised of patients who have sporadic MSI CRCs and patients with unidentified LS. On the basis of our results, in patients with *MLH1*-deficient tumors who have wild-type *BRAF* and no germline *MLH1* mutation, *MLH1* methylation analysis may play a role in identifying patients whose relatives may benefit from preventive strategies. This hypothesis is in agreement with the results from a recent meta-analysis, which studied the correlation of *BRAF* mutation and *MLH1* methylation with MMR germline mutation,[22] in which 75% of MSI *BRAF* wild-type tumors displayed *MLH1* methylation when patients were not selected for family history, whereas this percentage dropped to 13% when only familial cases were considered.

The strengths of this study are, first, that we analyzed both *BRAF* status and *MLH1* methylation in all samples. Second, we designed a new and optimized pyrosequencing assay for methylation analysis of the *MLH1* promoter that is suitable for FFPE samples. Previous

studies[12,23–25] have used different techniques for methylation analysis (methylation-specific multiplex ligation-dependent probe amplification, MethyLight, methylation-specific polymerase chain reaction analysis), but not pyrosequencing, which is currently considered the gold standard for methylation analysis. Third, to our knowledge, this is the first study to include not only sporadic MSI CRCs and LS tumors, but also a large group of patients with MSI *BRAF* wild-type tumors without MMR germline mutations. Finally, we ruled out constitutional *MLH1* epimutation[26] in those patients who had somatic methylation and no germinal mutation. Nevertheless, we are aware of some limitations. Our analysis of the performance of different strategies for diagnosing LS was based on a selected population of patients; accordingly, the resulting values may not reflect those from a population-based cohort with *MLH1*-deficient tumors. However, we believe that our results contribute to a better understanding of the role of *MLH1* methylation in *MLH1*-deficient CRCs.

In conclusion, we report that up to 15% of *MLH1*-deficient tumors from patients with LS demonstrate somatic *MLH1* methylation, probably as a second hit. Therefore, a meaningful proportion of *MLH1* mutation carriers could be undiagnosed when relying on *MLH1* methylation for germline genetic testing. *BRAF* mutational status constitutes the most sensitive strategy for ruling out LS among *MLH1*-deficient CRCs. Finally, somatic *MLH1* methylation studies can be valuable for family risk assessment in patients who have MSI-*BRAF* wild-type tumors without MMR germline mutations.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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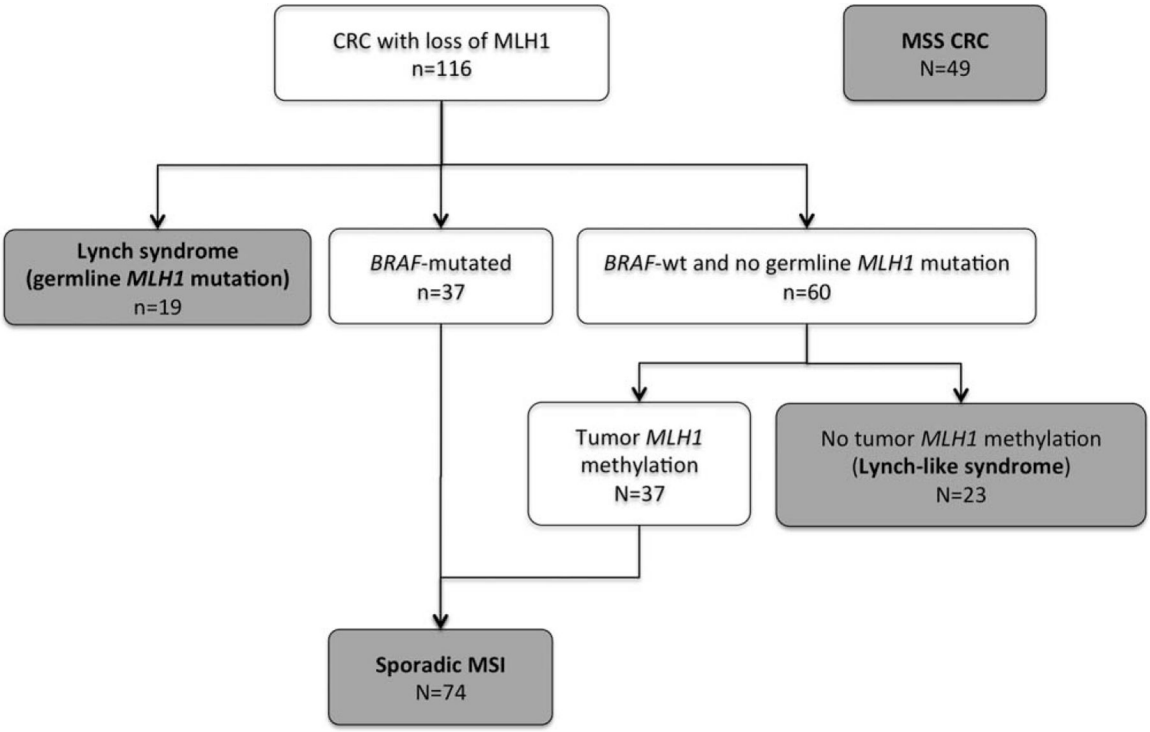


**Figure 1.**

This flow chart is a schematic representation of the classification of the mutL homolog 1 (*MLH1*)-deficient colorectal carcinomas (CRCs) that were included in the current study based on an analysis of both somatic v-Raf murine sarcoma viral oncogene homolog B1 (*BRAF*) mutational status and *MLH1* methylation. Gray boxes indicate the final diagnosis based on molecular analysis. MSS indicates microsatellite stability; *BRAF*, v-Raf murine sarcoma viral oncogene homolog B1; wt, wild type; MSI, microsatellite instability.

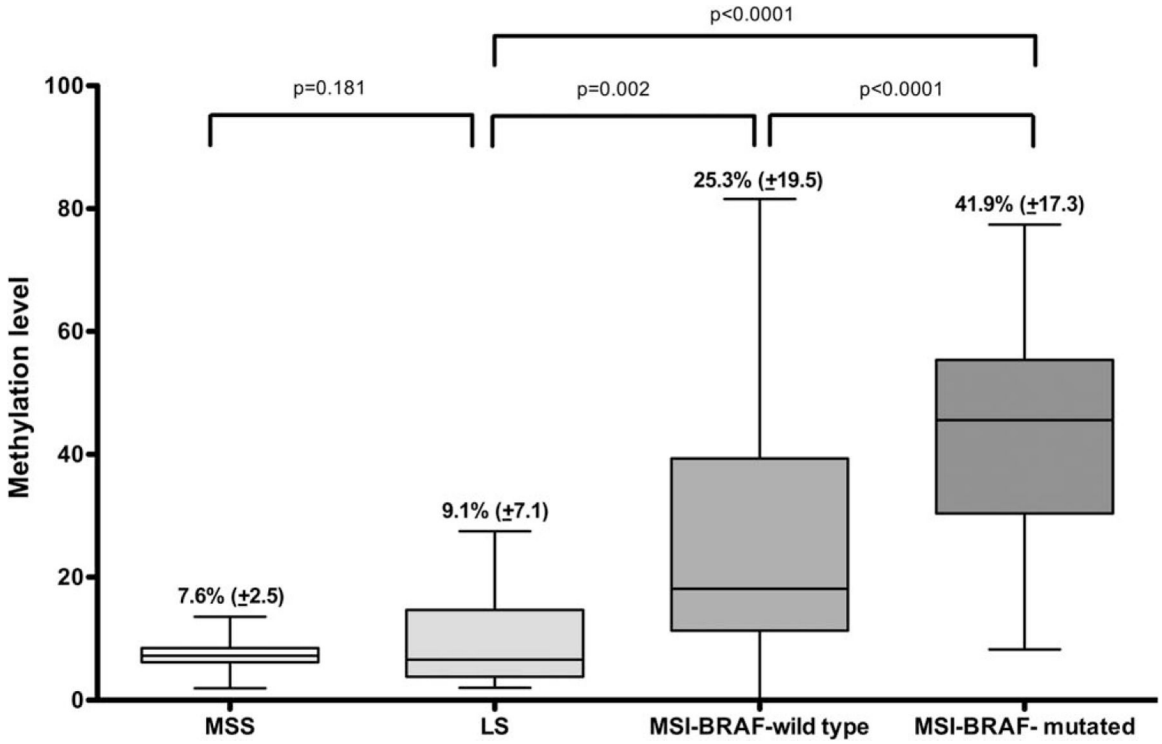


**Figure 2.**

MutL homolog 1 (*MLH1*) methylation results are illustrated for the 4 study groups. Box-and-whisker plots show the median methylation level expressed as a percentage (horizontal lines), the 25th and 75th percentiles (boxes), and the maximum and minimum levels (whiskers). Means and standard deviations also are indicated, and statistical comparisons between groups are depicted. MSS indicates microsatellite stability; LS, Lynch syndrome; MSI, microsatellite instability; *BRAF*, v-Raf murine sarcoma viral oncogene homolog B1.

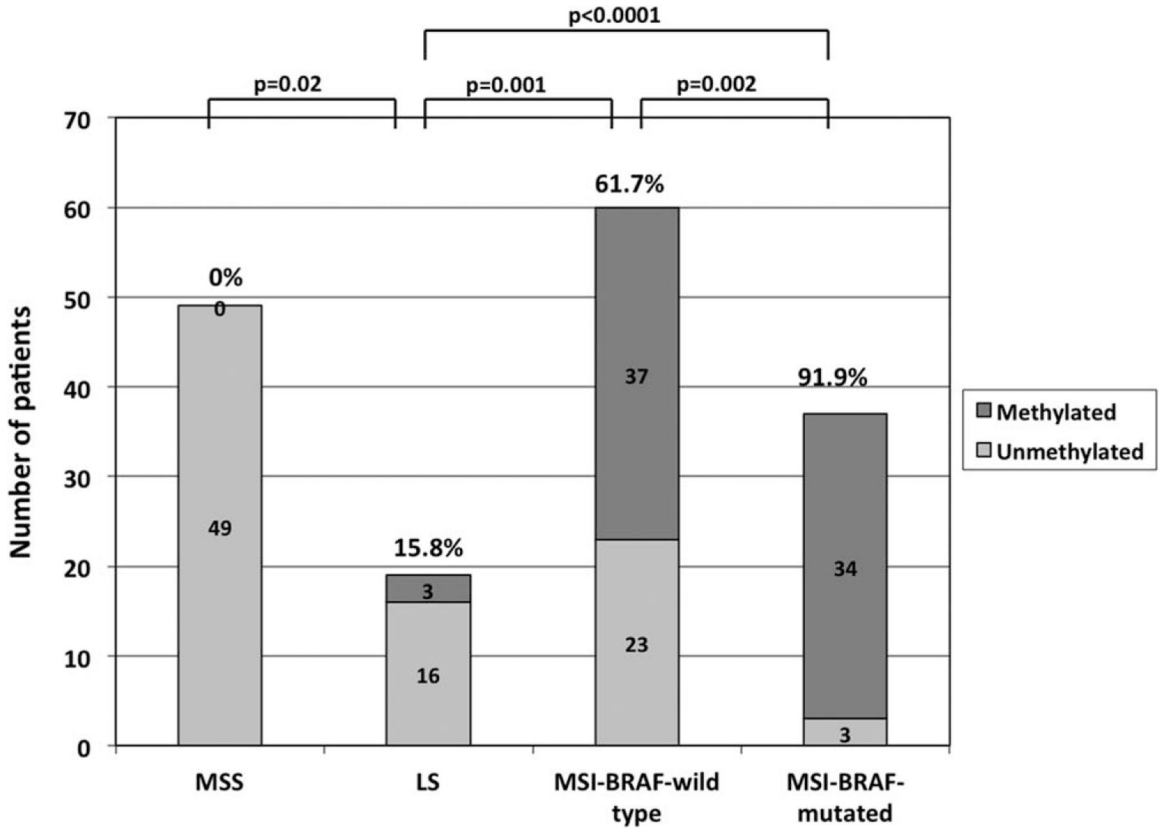


**Figure 3.**

The distribution of mutL homolog 1 (*MLH1*) somatic hypermethylation is illustrated for the 4 study groups. For each group, the dark gray bars indicate methylated tumors. The percentages of *MLH1*-methylated tumors are indicated, and statistical comparisons between the groups are depicted. MSS indicates microsatellite stability; LS, Lynch syndrome; MSI, microsatellite instability; *BRAF*, v-Raf murine sarcoma viral oncogene homolog B1.

**TABLE 1.**

Clinicopathologic Features of Patients Included in this Study

| Feature | No. of Patients (%) | | | | *P* | |
|---|---|---|---|---|---|---|
| | LS, n = 19 | MSI *BRAF* Mutated, n = 37 | MSI *BRAF* Wild Type, n = 60 | MSS, n = 49 | LS vs MSI *BRAF* Mutated[a] | LS vs MSI *BRAF* Wild Type[a] |
| Age: Mean ± SD, y | 44.9 ± 15.7 | 73.6 ± 8.5 | 67.3 ± 15.1 | 73.8 ± 10.1 | **<.0001** | **.0001** |
| Men | 9 (47.4) | 14 (37.8) | 29 (48.3) | 25 (51) | .689 | .849 |
| Proximal location[b] | 12/18 (66.7) | 34 (91.9) | 45 (75) | 17/48 (35.4) | **.047** | .692 |
| Synchronic CRC | 2 (10.5) | 3 (8.1) | 2 (3.3) | 1 (2) | .845 | .518 |
| Poor differentiation | 3/17 (17.6) | 9 (26.5) | 12 (21.4) | 3/44 (6.8) | .726 | .996 |
| Mucinous CRC | 8 (42.1) | 15 (40.5) | 15 (26.8) | 7/47 (14.9) | .861 | .335 |
| Tumor-infiltrating lymphocytes | 3/14 (21.4) | 12/32 (37.5) | 17 (36.9) | 24/47 (49) | .466 | .450 |
| FDR or SDR with CRC | 14 (73.6) | 4 (10.8) | 10 (16.7) | 0 (0) | **<.0001** | **<.0001** |
| FDR or SDR with CRC aged <50 y | 10 (52.6) | 1 (2.7) | 6 (10) | 0 (0) | **<.0001** | **.0002** |
| FDR with CRC | 9 (47.4) | 2 (5.4) | 8 (13.3) | 0 (0) | **.0007** | **.004** |
| FDR with CRC aged <50 y | 8 (42.1) | 1 (2.7) | 5 (8.3) | 0 (0) | **.0006** | **.001** |
| FDR or SDR with LS-related tumor[c] | 11 (57.9) | 13 (35.1) | 20 (33.3) | 0 (0) | .178 | 0.1 |
| FDR or SDR with LS-related tumor aged <50 y | 7 (36.8) | 3 (8.1) | 10 (16.7) | 0 (0) | **.022** | .122 |
| Revised Bethesda guidelines | 17 (89.5) | 4 (10.8) | 22 (36.7) | 4 (8.2) | **<.0001** | **.0002** |
| Amsterdam I criteria | 10 (52.6) | 1 (2.7) | 3 (5) | 0 (0) | **<.0001** | **<.0001** |
| Amsterdam II criteria | 10 (52.6) | 1 (2.7) | 4 (6.7) | 0 (0) | **<.0001** | **<.0001** |
| MSI | 15/15 (100) | 37 (100) | 55/58 (94.8) | 0 (0) | – | .865 |

Abbreviations: *BRAF*, v-Raf murine sarcoma viral oncogene homolog B1; CRC, colorectal carcinoma; FDR, first-degree relative; LS, Lynch syndrome; MSI, microsatellite instability; MSS, microsatellite stability; SD, standard deviation; SDR, second-degree relative.

[a] Statistically significant *P* values are indicated in bold.

[b] These are tumors located proximal to the splenic flexure.

[c] LS-related tumors include those located in endometrium, stomach, ovaries, urinary tract, small intestine, pancreas, bile ducts, brain, or sebaceous glands.

**TABLE 2.**

Clinicopathologic Features of Patients With Microsatellite-Instable, *BRAF* Wild-Type Colorectal Carcinoma Without MutL Homolog 1 (*MLH1*) Germline Mutation According to *MLH1* Methylation Status

| Feature | *MLH1* Methylated, n = 37 | *MLH1* Unmethylated, ie, LLS, n = 23 | *P* [a] |
|---|---|---|---|
| Age: Mean ± SD, y | 68.5 ± 15.1 | 65.3 ± 15.2 | .948 |
| Men | 16 (43.2) | 13 (56.5) | .462 |
| Proximal location[b] | 28 (75.7) | 17 (73.9) | .878 |
| Synchronic CRC | 1 (2.7) | 1 (4.3) | .693 |
| Poor differentiation | 6/33 (18.2) | 6 (26.1) | .705 |
| Mucinous CRC | 9/35 (25.7) | 6/21 (28.6) | .937 |
| Tumor-infiltrating lymphocytes | 8 (21.6) | 9 (39.1) | .242 |
| Metachronic Lynch-related tumor[c] | 1 (2.7) | 2 (8.7) | .669 |
| FDR or SDR with CRC | 43 (8.1) | 7 (30.4) | **.05** |
| FDR or SDR with CRC aged <50 y | 1 (2.7) | 5 (21.7) | **.05** |
| FDR with CRC | 2 (5.4) | 6 (26.1) | **.05** |
| FDR with CRC aged <50 y | 1 (2.7) | 4 (17.4) | .128 |
| FDR or SDR with LS-related tumor | 10 (27) | 10 (43.5) | .301 |
| FDR or SDR with LS-related tumor aged <50 y | 4 (10.8) | 6 (26.1) | .235 |
| Revised Bethesda guidelines | 14 (37.8) | 8 (34.8) | .970 |
| Amsterdam I criteria | 1 (2.7) | 2 (8.7) | .669 |
| Amsterdam II criteria | 1 (2.7) | 3 (13) | .303 |
| MSI | 35/36 (97.2) | 20/22 (90.9) | .658 |
| *MLH1* methylation: Mean ± SD, % | 35.63 ± 18 | 8.68 ± 3.67 | < **.0001** |

Abbreviations: CRC, colorectal carcinoma; FDR, first-degree relative; LLS, Lynch-like syndrome; LS, Lynch syndrome; MSI, microsatellite instability; MSS, microsatellite stability; SD, standard deviation; SDR, second-degree relative.

[a] Statistically significant *P* values are indicated in bold.

[b] These are tumors located proximal to the splenic flexure.

[c] LS-related tumors include those located in endometrium, stomach, ovaries, urinary tract, small intestine, pancreas, bile ducts, brain, or sebaceous glands.

**TABLE 3.**

Performance Characteristics of the Different Molecular Approaches for the Identification of Lynch Syndrome Among Patients With MutL Homolog 1-Deficient Colorectal Cancer

| Molecular Strategy | No. of Evaluable Probands | No. of Probands Requiring Germline MMR Gene Analysis (%) | Germline MMR Gene Mutation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Sensitivity | | Specificity | | PPV | | NPV | |
| | | | No. (%) | 95% CI, % | No. (%) | 95% CI, % | No. (%) | 95% CI, % | No. (%) | 95% CI, % |
| *BRAF* wild type | 116 | 79 (68.1) | 19/19 (100) | 97.3–100 | 37/97 (38.1) | 27.9–48.3 | 19/79 (24) | 13.9–34.1 | 37/37 (100) | 98.6–100 |
| *MLH1* unmethylated | 116 | 42 (36.2) | 16/19 (84.2) | 65.1–100 | 71/97 (73.2) | 63.8–82.5 | 16/42 (38.1) | 22.2–53.9 | 71/74 (95.9) | 90.7–100 |
| *BRA*F wild type and *MLH1* unmethylated | 116 | 39 (33.6) | 16/19 (84.2) | 65.1–100 | 74/97 (76.3) | 67.3–85.2 | 16/39 (41) | 24.3–57.7 | 74/77 (96.1) | 91.1–100 |

Abbreviations: *BRAF*, v-Raf murine sarcoma viral oncogene homolog B1; CI, confidence interval; *MLH1*, mutL homolog 1; MMR, mismatch repair; NPV, negative predictive value; PPV, positive predictive value.