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Relevance, Pathogenesis, and Testing Algorithm for Mismatch Repair–Defective Colorectal Carcinomas

A Report of the Association for Molecular Pathology

William K. Funkhouser, Jr.,*† Ira M. Lubin,*‡ Federico A. Monzon,*§ Barbara A. Zehnbauer,‡ James P. Evans,¶ Shuji Ogino,‖ and Jan A. Nowak**

From the Mismatch Repair–Defective CRC Working Group of the Association for Molecular Pathology Clinical Practice Committee and the Departments of Pathology and Laboratory Medicine† and Genetics,¶ University of North Carolina, Chapel Hill, North Carolina; the Division of Laboratory Science and Standards,‡ Centers for Disease Control and Prevention, Atlanta, Georgia; the Department of Pathology,§ Methodist Hospital, Houston, Texas; the Department of Pathology, Brigham & Women's Hospital, Harvard Medical School, the Department of Medical Oncology, Dana-Farber Cancer Institute, the Cancer Epidemiology Program, Dana-Farber Cancer Institute and the Harvard Cancer Center,‖ Boston, Massachusetts; and the Northshore University HealthSystem,** Evanston, Illinois*

Loss-of-function defects in DNA mismatch repair (MMR), which manifest as high levels of microsatellite instability (MSI), occur in approximately 15% of all colorectal carcinomas (CRCs). This molecular subset of CRC characterizes patients with better stage-specific prognoses who experience no benefit from 5-fluorouracil chemotherapy. Most MMR-deficient (dMMR) CRCs are sporadic, but 15% to 20% are due to inherited predisposition (Lynch syndrome). High penetrance of CRCs in germline MMR gene mutation carriers emphasizes the importance of accurate diagnosis of Lynch syndrome carriers. Family-based (Amsterdam), patient/family-based (Bethesda), morphology-based, microsatellite-based, and IHC-based screening criteria do not individually detect all germline mutation carriers. These limitations support the use of multiple concurrent tests and the screening of all patients with newly diagnosed CRC. This approach is resource intensive but would increase detection of inherited and *de novo* germline mutations to guide family screening. Although CRC prognosis and prediction of 5-fluorouracil response are similar in both the Lynch and sporadic dMMR subgroups, these subgroups differ significantly with regard to the implications for family members. We recommend that new CRCs should be classified into sporadic MMR-proficient, sporadic dMMR, or Lynch dMMR subgroups. The concurrent use of MSI testing, MMR protein IHC, and *BRAF* c.1799T>A mutation analysis would detect almost all dMMR CRCs, would classify 94% of all new CRCs into these MMR subgroups, and would guide secondary molecular testing of the remainder. *(J Mol Diagn 2012, 14:91-103; DOI: 10.1016/j.jmoldx.2011.11.001)*

Primary colorectal carcinoma (CRC) is a solid tumor that occurs commonly in US adults. In 2011, the American Cancer Society expects approximately 142,000 new cases of CRC. Independent prognostic variables include stage (extent of disease),[1,2] grade (degree of differentiation),[1,2] angiolymphatic invasion,[3] carcinoembryonic antigen level,[3] and DNA mismatch repair (MMR) status.[2,4–6] This article focuses on the relevance, molecular subgroups, and testing strategies for DNA MMR status.

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Standard of practice is not being defined by this article, and there may be alternatives.

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Correspondence: William K. Funkhouser, MD, PhD, Department of Pathology and Laboratory Medicine, CB 7525, Brinkhous-Bullitt Building, University of North Carolina School of Medicine, Chapel Hill, NC 27599-7525. E-mail: bill_funkhouser@med.unc.edu.

Address reprint requests to the Association for Molecular Pathology, c/o Mary Williams, 9650 Rockville Pike, Bethesda, MD 20814-3993. E-mail: mwilliams@asip.org.

Protein heterodimers of MutS homologues (MSH2, MSH6) and of MutL homologues (MLH1, PMS2) are *sine qua non* components of the human multimeric DNA MMR protein complexes that correct strand alignment and base matching errors during DNA replication.[7,8] When any one of these MMR proteins is absent or nonfunctional, the MMR process malfunctions, as reflected by length alterations in microsatellites, ie, microsatellite instability (MSI).[9–11] Therefore, loss-of-function defects in MMR result in error-prone DNA replication and MSI. The *in vitro* effect of this loss is marked—CRC cell lines with defective MLH1 or MSH2 show a three-log increase in the rate of dinucleotide repeat length changes per locus per generation when compared with a MMR-proficient (pMMR) cell line.[12]

With the use of panels of microsatellites to screen CRCs a bimodal distribution of MSI can be observed, with most cases showing <20% or >60% of microsatellites to be unstable.[13] An empirical cutoff at 30% unstable microsatellites has been adopted, resulting in three test results: MSI-high (MSI-H; ≥30% MSI), MSI-low (MSI-L; 0<MSI <30%), and microsatellite stability (MSS; MSI = 0%).[10] With few exceptions (eg, CRCs due to *MSH6* gene mutations), MSI-L cases arise and behave like MSS cases and are considered to be pMMR.[13,14] MSI-H cases correlate with differences in stage at presentation and improved stage-specific prognosis[15,16] and are considered MMR deficient dMMR.[13]

Approximately 15% of CRCs are dMMR, as estimated by MSI-H testing.[17,18] Most of these (12% to 13%) are somatically acquired/sporadic,[16,19] and the remaining 2% to 3% are due to inherited/germline mutation of one allele of an MMR gene.[20] This latter subgroup characterizes CRCs diagnosed in the inherited Lynch (also known as hereditary nonpolyposis colorectal cancer)[21–25] and Muir-Torre[26] syndromes.

Both Lynch and sporadic dMMR subgroups differ in origin but share a final common pathogenesis in terms of loss of MMR protein function/expression and MSI-H.[11,25] Both subgroups have improved stage-specific prognoses,[2,6,15,16,27–31] and neither group derives benefit from 5-fluorouracil (5-FU) chemotherapy[32–36] in contrast to patients with pMMR CRC. Medical oncologists currently use MMR status to guide adjuvant 5-FU therapy decisions for new CRC patients with deep primary tumors without nodal or distant metastases [American Joint Committee on Cancer (AJCC) stage II].[37] Roughly 40% of new CRC patients have nodal metastasis without distant metastasis at presentation (AJCC stage III), and roughly 15% of new CRC patients have distant metastasis at presentation (AJCC stage IV).[38] Although 5-FU is included in common combination chemotherapy regimens for patients with node-positive (stage III) and distant metastatic (stage IV) CRC, dMMR does not currently preclude use of these regimens.[37] Clinical geneticists use MMR status to screen for Lynch syndrome and to counsel probands' unaffected family members.

Understanding the differences in the molecular pathogenesis for these two dMMR subgroups will facilitate the use of molecular diagnostic criteria for each subgroup, allowing logical development of a screening strategy to specifically assign new cases to a subgroup and then guiding clinical management, patient surveillance, and family counseling. Recent emphasis on the detection of all Lynch probands and subclinical carriers has advocated universal testing for MMR defects in all patients with newly diagnosed CRC,[39] with a goal of improved clinical decision-making and treatment outcomes. Such an approach requires a realistic and effective laboratory practice algorithm for diagnostic testing that will detect all dMMR cases and then distinguish Lynch and sporadic dMMR subgroups.

Specific Definition and Pathogenesis of Sporadic dMMR CRC

Sporadic dMMR CRC comprises 12% to 13% of all new cases of CRC[16,18,19,40–44] and can be broadly defined as MSI-H sporadic CRC without germline MMR gene deleterious mutations.

Most sporadic dMMR CRCs are thought to arise in sessile serrated adenomas/polyps (SSA/Ps)[17,45] in the proximal colon of older adults.[40] SSA/P morphologic characteristics are recognizably different from those of conventional adenomatous polyps, which are the precursors for sporadic pMMR and Lynch dMMR CRC.[17,45] SSA/Ps with dysplasia are considered the precursor for sporadic dMMR CRC and show unique molecular features, including *BRAF* c.1799T>A mutation, generalized increase in CpG island methylation (the CpG island methylator phenotype [CIMP]), *MLH1* promoter hypermethylation (PHM), and MSI-H.[17,45]

Like its SSA/P precursor lesion, most invasive sporadic dMMR CRC exhibits MSI-H and loss of function of the MLH1 protein due to CpG island hypermethylation in the *MLH1* gene promoter.[46–49] More than 95% of sporadic dMMR CRC is associated with *MLH1* PHM.[50–52] Reversal of *MLH1* PHM in cell lines with 5′-aza 2′-deoxycytidine leads to rescue of MLH1 protein expression and MSS,[47] implicating *MLH1* PHM as etiologic for this sporadic subgroup of dMMR CRC. *MLH1* PHM is rarely detected in MSS CRC[53] or Lynch CRC.[53,54] Acquired *MLH1* PHM in Lynch syndrome can be the basis for loss of function of the remaining *MLH1* wild-type allele.[49,55,56] Rare cases of germline *MLH1* PHM have been reported.[57,58] Therefore, acquired *MLH1* PHM without germ MMR gene mutation or germline *MLH1* PHM appears to be the basis for development of sporadic dMMR CRC.

MLH1 PHM in sporadic dMMR CRC is explained in most cases by the CIMP, a process of DNA hypermethylation involving multiple gene promoter CpG islands, including *MLH1*.[59–63] Using an eight-locus panel to characterize methylation in CRC produces a bimodal distribution of CIMP, with most tumor samples containing either ≤4 (≤50%) or ≥6 (≥75%) methylated loci [CIMP-high (CIMP-H)].[51] The basis for this increased CpG island methylation may be due to increased DNA methyltransferase activity via mutation or epistatic, transcriptional up-regulation of DNA methyltransferase 3B.[64,65] Approximately 70% of CIMP-H CRCs are sporadic dMMR CRC,[51,66,67] and approximately 85% of sporadic dMMR

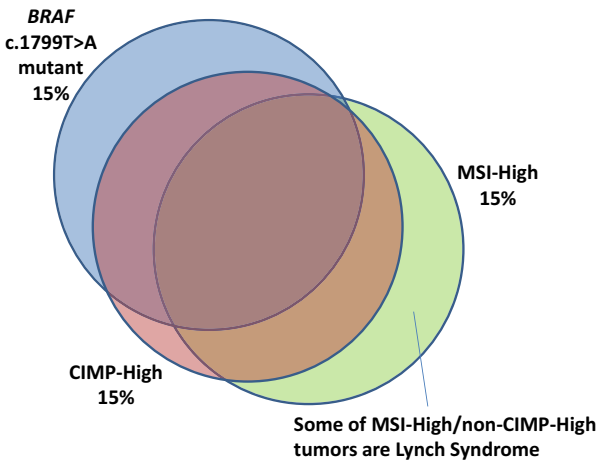
CRCs are associated with CIMP-H.[50–52] CIMP-H cases have a lower risk for CRC-associated death after adjusting for MSI and *BRAF* mutation status.[68]

BRAF gene T>A missense mutation at nucleotide 1799 (c.1799T>A) is found in 60% of the sporadic dMMR CRC subgroup and leads to nonsynonymous amino acid substitution in codon 600 (p.V600E), with constitutive signaling of the BRAF protein.[40,41,68–71] A total of 5% to 10% of pMMR sporadic CRCs[68,72] have the *BRAF* c.1799T>A mutation, but no published cases of Lynch CRC have this mutation.[69,73–76] One case has been found with both BRAF c.1799T>A and a pathogenic germline MMR mutation (Dr. S.N. Thibodeau, personal communication). This specificity of the *BRAF* mutation for non-Lynch CIMP-H CRC comprises a useful testing strategy to identify and subcategorize dMMR CRC.

The causal pathogenic relationship between CIMP and *BRAF* c.1799T>A mutation is unclear. Both alterations may be detected in SSA/Ps,[66,77,78] the precursor lesions of most sporadic dMMR CRC.[45] Population-based sample data show that *BRAF* c 1799T>A mutation status affects CIMP status,[79] suggesting roles for *BRAF* mutation and mitogen-activated protein kinase pathway activation in the development of CIMP. However, introduction of mutant *BRAF* c.1799T>A into CRC cell lines does not lead to CIMP[80]; admittedly, established cancer cell lines do not recapitulate the carcinogenesis process within the tumor microenvironment. Alternatively, CIMP-mediated silencing of proapoptotic genes, such as *IGFBP7,* may precede and allow survival of clones with the *BRAF* c.1799T>A mutation.[78,80]

CIMP, *BRAF* c.1799T>A mutation, *MLH1* PHM, and MSI-H frequently occur together (Figure 1). MSI-H CRCs are more likely than MSS/MSI-L CRCs to be CIMP-H.[18] Conversely, CIMP-H CRCs are more likely than non-CIMP-H CRCs to be MSI-H, have the *BRAF* c.1799T>A mutation, and show *MLH1* PHM.[18,40,81] Most CIMP-H CRCs contain the *BRAF* c.1799T>A mutant allele, regardless of MSI status.[18,51,68] These data suggest that most sporadic dMMR CRCs due to *MLH1* PHM and resultant MSI-H constitute a large subset within CIMP-H CRC.

One hypothesis is that CIMP-H and *BRAF* c.1799T>A (p.V600E) lead to *MLH1* PHM in some serrated adenomas, with subsequent loss of function of *MLH1*, development of MSI-H, and development of clonal CRC. Stringent molecular diagnostic criteria for this sporadic dMMR CRC subgroup might be cases with MSI-H, CIMP-H, *MLH1* PHM, MLH1 protein loss, and *BRAF* c.1799T>A (p.V600E) mutation. The challenge is to craft a testing algorithm that will identify almost all patients with dMMR CRC and also distinguish the sporadic dMMR CRC subgroup from the Lynch syndrome subgroup.



The probability of:	Within each of these subtypes of colorectal cancer: CIMP-High	BRAF c.1799T>A	MSI-High	MLH1 PHM
CIMP-High		70%	70%	85%
BRAF c.1799T>A	70%		50%	60%
MSI-High	70%	50%		100%
MLH1 PHM	70%	50%	75-80%	

Figure 1. The relationship of the CIMP-H, *BRAF* 1799T>A (p.V600E), and MSI-H variables in new colorectal carcinomas. Each variable is seen in approximately 15% of new CRC cases, and there is significant overlap among the variables, detailed in the subjacent table. All cases with *MLH1* PHM are MSI-H, but only 75% to 80% of cases with MSI-H show *MLH1* PHM.

Specific Definition and Pathogenesis of Inherited dMMR CRC (Lynch Syndrome)

Heritable dMMR CRC (Lynch syndrome) comprises approximately 2.5% of all new cases of CRC[24,25,35,82–84] and is currently defined as due to a germline MMR gene deleterious mutation.[85] Mutations include not only MMR gene point mutations[25] but also large germline deletions involving *MSH2* or *MLH1,*[86–88] germline deletions of the *EPCAM* (TACSTD1) gene upstream of *MSH2*,[58] and germline *MLH1* PHM.[57,58] Age distribution is unimodal, with a mode at the age of 45 to 50 years but with a range of 25 to 70 years.[89] Penetrance for CRC is estimated to be 80% by the age of 80 years[90] but may be lower in female carriers[91] and dependent on the underlying mutation.[92] These data indicate a benefit for early identification and regular surveillance of mutation carriers, contingent on effective intervention, therapy, and treatment.

Estimates and confidence intervals of the proportions of Lynch CRC cases due to germline mutation of each of these four MMR genes (weighted proportions: *MLH1,* 32%; *MSH2,* 39%; *MSH6,* 10% to 14%; *PMS2,* 15%[85]) are confounded by incomplete testing of all four MMR genes in most studies and by skewing in some ethnic groups with high-frequency founder mutations.[93] The remaining normal second allele might be somatically deleted,[94] mutated, or hypermethylated.[49,55,56] Biallelic loss of function of an MMR gene product in Lynch syndrome frequently is associated with same-locus MMR protein loss and MSI-H.[95] Lynch dMMR CRCs rarely retain MMR protein immunoreactivity when a deleterious missense mutation is present.[96] Sensitivity of immunohistochemistry (IHC) for the presence of a mutation in a given MMR gene is 81%

for *MLH1*, 88% for *MSH2*, and 76% for *MSH6*.[85] IHC has a mediocre to substantial interobserver κ statistic of 0.49 to 0.79, which varies by expertise of the pathologist, demonstrating the need for strict scoring criteria to assure quality.[97] Sensitivity of MSI-H for germline mutations in MMR genes is 89% to 92% for *MLH1* mutations, 90% to 93% for *MSH2* mutations, 25% to 76% for *MSH6* mutations, and 67% for *PMS2* mutations.[85,98]

The critical relevance of diagnosing patients as having Lynch syndrome relates to patient follow-up and family testing. The proband is at increased risk for secondary carcinomas in the colon and at risk for other Lynch-associated primary neoplasms.[90] For inherited mutations, unaffected siblings from the same parents have a 50% chance of being carriers. Carriers in the family should be identified for genetic counseling regarding the fivefold to sixfold increased risk of carcinoma inherent in Lynch syndrome[85,99] and the benefits of enhanced routine surveillance for Lynch-associated malignant neoplasms.[99–101] Seven studies reviewed by the Evaluation of Genomic Applications in Practice and Prevention Working Group (EGAPP)[85] suggest that roughly half of family members approached avail themselves of counseling opportunities, and 95% of those counseled avail themselves of recommended MMR gene mutation testing. Most identified carriers (53% to 100%) in these seven studies agreed to recommended early and follow-up surveillance colonoscopies.[85]

Final Common Pathogenesis of dMMR CRC

Both inherited (Lynch) and sporadic subgroups of dMMR CRC share several features. By definition, they lose function of both allelic gene products for one or more MMR proteins, and they usually lose immunoreactivity for the affected MMR protein in paraffin IHC.[25,95,96] When either MLH1 or MSH2 is not expressed, the heterodimer partner protein (PMS2 for MLH1 and MSH6 for MSH2) is also not expressed.[25] The converse is not true; when either PMS2 or MSH6 is not expressed (without *MLH1* or *MSH2* gene alteration), the heterodimer partner proteins (MLH1 for PMS2 and MSH2 for MSH6) are still expressed,[102] possibly due to alternate heterodimer partners that can substitute for PMS2 and MSH6. As with MSI-H, improved stage-specific survival and absence of 5-FU response is associated with loss of MMR protein expression.[30]

MSI-H presumably contributes to the pathogenesis of dMMR CRC via involvement of microsatellites in coding regions of tumor suppressor or gatekeeper genes, such as *TGFBR2* and *BAX*.[103, 104] MSI-H can be found in the dysplastic serrated adenoma/polyp precursor of sporadic dMMR CRC[105] and in the adenomatous polyp precursor of Lynch dMMR CRC.[106] Progression to invasive CRC may be faster in these dMMR CRC precursors.[45]

Lynch and sporadic subgroups of dMMR CRC can show unique morphologic characteristics compared with pMMR CRC.[17] Many MSI-H CRCs show statistically significant increases in tumor-infiltrating lymphocytes, Crohn's-like reaction, and mucin production and a significant decrease in intraglandular neutrophil-rich ("dirty") necrosis.[107,108] The tumor-infiltrating lymphocytes may accumulate in response to neopeptides generated by frameshift mutations in coding sequences.[109,110]

Lynch and sporadic subgroups of dMMR CRC also share different clinical outcomes when compared with pMMR CRC, including lower stage at initial diagnosis[16,68] and improved stage-specific survival.[2,6,15,16,27–31] Multivariate analyses have found that MSI-H and CIMP-H are good prognostic variables but that the *BRAF* c.1799T>A mutation is a poor prognostic variable.[68,111,112] Poor prognosis associated with CIMP-H in previous studies may have been due to the confounding effects of the *BRAF* mutation.[68,112–114] MSI-H CRCs are associated with absence of response to 5-FU therapy,[32–36] guiding current medical oncology management of patients with AJCC stage II CRC.

Detection of dMMR CRC

Sporadic dMMR CRC

Recognition of the improved prognosis and the absence of response to 5-FU therapy justify a testing strategy for the detection of the sporadic dMMR subgroup of CRC. Assay performance assessment is challenging without a diagnostic "gold standard" reference method. Given an expected 142,000 new CRC cases in the United States in 2011, an estimated 17,750 patients (12.5% of the total) will present with sporadic dMMR CRC.

At the clinical and morphologic level, sporadic dMMR CRC frequently presents in the proximal aspect of the colon in older patients, is more common in women, and shows an expanding growth pattern, mucinous features, tumor-infiltrating lymphocytes, and absence of intraglandular neutrophil-rich ("dirty") necrosis.[71,115] Sporadic dMMR CRC can be predicted using the presence of any three of these factors, with a sensitivity of 98% and specificity of 48%.[71] A similar study found that MSI-H CRC can be predicted using the presence of any one of seven factors (old age, proximal site, and five morphologic factors) with a sensitivity of 92% and specificity of 46%.[108]

At the IHC and genetic level, MLH1 protein loss was found in 93 of 97 sporadic dMMR CRC cases (96%), and MSI-H was found in 96 of these 97 cases (99%).[25,116] Importantly, the two methods complemented each other because all *MLH1* PHM cases were identified by one of the two methods (97 of 97, 100%) (Table 1[25,49,116–119]). This finding implies that a comprehensive strategy for detection of sporadic dMMR CRC should use both MSI and MMR protein IHC testing.

Given sporadic dMMR CRC, the *BRAF* c.1799T>A mutation is expected in 60% of cases. If this mutation is present, then Lynch syndrome is virtually excluded.[69,73–76] Therefore, the presence of *BRAF* c.1799T>A in MSI-H CRC supports a diagnosis of sporadic dMMR CRC. The absence of this mutation only increases the likelihood of Lynch syndrome, a diagnosis that still re-

Table 1. Agreement and Complementarity of IHC Loss and MSI-H in Series of CRCs Stratified by Etiology

No. of cases	Cohort studied	Detection using IHC loss only, No. (%)	Detection using MSI-H only, No. (%)	Detection using both IHC and MSI, No. (%)	Reference
		Probable Sporadic dMMR CRC Subgroup			
68	Absence of MLH1 or MSH2 mutations	3 of 9 (MLH1)	8 of 9	9 of 9 (complementary)	117
46	MSI-H, MLH1 PHM	30 of 36 (MLH1)	36 of 36 (selected for MSI-H)	36 of 36	49
257	MLH1 PHM Absence of MLH1 or MSH2 mutations	36 of 36 (MLH1)	36 of 36	36 of 36	25
1066	Any MSI, MLH1 PHM Absence of MLH1 or MSH2 mutations	57 of 61 (MLH1)	60 of 61	61 of 61 (complementary)	116
1978	Revised Bethesda criteria (+) Absence of MLH1 or MSH2 mutations	70 of 80 (MLH1)	73 of 80	80 of 80 (complementary)	118
	Total	196 of 222 (88)	177 of 186 (95)	186 of 186 (100)	
		Definite Lynch dMMR CRC Subgroup			
68	MLH1 or MSH2 mutations	5 of 6 (MLH1, MSH2)	5 of 6	6 of 6 (complementary)	117
257	MLH1 or MSH2 mutations	5 of 5 (MLH1, MSH2)	5 of 5	5 of 5	25
1066	MLH1, MSH2, MSH6, or PMS2 mutations	21 of 23 (MLH1, MSH2, MSH6)	21 of 23	23 of 23 (complementary)	116
1978	MLH1 or MSH2 mutations	11 of 11 (MLH1, MSH2)	10 of 11	11 of 11	118
	Total	42 of 45 (93)	41 of 45 (91)	45 of 45 (100)	
		dMMR CRC (Indeterminate Subgroup)			
3821	Multicenter colon cancer family registry	667 of 751 (89) (MLH1, MSH2, MSH6, PMS2)	749 of 751 (99.7)	751 of 751 (100) (complementary)	119

quires clinical and family histories and confirmatory sequencing/deletion testing.

Lynch Syndrome

With the implications of a diagnosis of Lynch syndrome for at-risk family members, biomarkers have been sought at the family history, patient history, morphologic, and molecular levels. None of these approaches detects all tumors with Lynch syndrome germline abnormalities, leading to the proposal to screen all patients with newly diagnosed CRC for heritable mutations.[39] With 142,000 new CRC cases expected in the United States in 2011, an estimated 3550 proband patients (2.5% of the total) will present with Lynch syndrome CRC. Table 2[83, 108] summarizes the data on Lynch detection methods.

Amsterdam II screening criteria[120] are based on family history of Lynch-associated carcinomas and the identification of CRC in one person younger than 50 years. These criteria have a sensitivity of 42% to 50% and a specificity of 97% to 98% for the detection of associated MMR gene mutations.[83,121] Thus, use of Amsterdam II criteria alone would miss the diagnosis of at least 50% of new Lynch syndrome patients (Table 2[83,108]).

Revised Bethesda criteria[122] are based on family history of Lynch-associated carcinoma, patient age at diagnosis, MSI-H histologic findings, and history of other Lynch-associated carcinomas. One series using these criteria showed a sensitivity of 95% and a specificity of 38% in the detection of underlying MMR gene mutations.[83] Thus, revised Bethesda criteria alone would miss the diagnosis of 5% of new Lynch syndrome patients

Table 2. Test Performance in Detection of Lynch Syndrome

Test	Sensitivity, %	Specificity, %	Estimated Lynch probands missed (of 3550), No. (%)
Amsterdam II criteria	42–50	97–98	1780–2060 (50–58)
Revised Bethesda criteria	95	38	180 (5)
Barnetson et al[83]	95	14	180 (5)
Greenson et al[108]	92		280 (8)
MSI	89 (MLH1) 90 (MHS2) 76 (MSH6)		11–355 (0.3–10)
IHC	81 (MLH1) 88 (MHS2) 76 (MSH6)		390–425 (11–12)
Sequencing	99.5	99.9	0 (0)

(Table 2[83,108]). In practice, this number is likely higher because of the common absence of a detailed family history of cancer in many patients.

Novel models of univariate clinical predictors of MMR gene mutation status have been combined into a weighted equation to estimate the probability of an underlying MMR gene mutation.[83] The variables included age, sex, tumor location, presence of synchronous or metachronous tumors, a first-degree relative with CRC, and a first-degree relative with endometrial carcinoma. These criteria provided a sensitivity of 95% with a specificity of 14% for the detection of underlying MMR gene mutations. This two-step approach alone would miss the diagnosis of 5% of new Lynch syndrome cases (Table 2[83]).

A morphology-based study of all MSI-H cases determined sensitivities of 53% to 70% for individual age, site, and morphologic features, with a sensitivity of 92% when any single feature was present.[108] These morphologic criteria alone would miss the diagnosis of 8% of new Lynch syndrome cases (Table 2[108]).

Review of data for MSI testing in Lynch syndrome detection estimated 89% sensitivity for *MLH1* mutation detection, 90% sensitivity for *MSH2* mutation detection, and 76% sensitivity for *MSH6* mutation detection.[85] Data from the Colorectal Family Registry indicated that only 0.3% of dMMR CRC showed MMR protein loss without MSI-H.[119] Assuming that most of these data pertained to Lynch syndrome patients, MSI criteria alone would miss the diagnosis of 0.3% to 10% new Lynch syndrome patients (Table 2).[83,108]

Review of data for IHC testing in Lynch syndrome detection estimated 81% sensitivity for *MLH1* mutation detection, 88% sensitivity for *MSH2* mutation detection, and 76% sensitivity for *MSH6* mutation detection.[85] Colorectal Family Registry data found that 11% of dMMR CRC showed MSI-H without MMR protein loss.[119] Assuming that most of these Colorectal Family Registry data pertained to Lynch syndrome patients, IHC criteria alone would fail to detect 11% to 12% of new Lynch syndrome patients (Table 2).[83,108] Receiver operating characteristic curves show similar areas under the curves for MSI and IHC testing.[95]

High-throughput sequencing-by-synthesis chemical tests are reducing the cost of targeted resequencing for known substitutions, insertions, and deletions.[123] Sequencing of germline DNA to detect germline mutations in MMR genes is estimated to show a sensitivity of 99.5% and a specificity of 99.96%.[124]

Although neither MSI-H nor IHC for MMR proteins has a sensitivity of 100% for detection of germline MMR gene mutations, the two assays together are complementary and would have identified 100% of the 45 Lynch cases in a set of four CRC series describing a total of 3369 patients (Table 1[25,49,116–119]).

The incomplete sensitivity of any single testing strategy emphasizes that these tests should not be used alone or even as single initial screening tests in a multitest algorithm. Failure to diagnose Lynch syndrome in CRC patients would preclude recognition and clinical care of multiple presymptomatic family members who are also at risk, amplifying the clinical and public health impact of screening insensitivity.[39] Half of the affected first-degree relatives of patients with Lynch syndrome are expected to be mutation carriers; thus, the actual number of missed patient diagnoses (defined as carriers) may be as high as three to eight times the number of symptomatic probands.[85,116] These are strong arguments in support of universal testing and detection based on immunophenotypic and molecular diagnostic criteria.

Algorithmic Strategies to Detect and Subset dMMR CRC (Sporadic and Lynch)

Lynch and sporadic dMMR CRC should be diagnosed in all patients to ensure accurate prognosis, treatment, and risk assessment for relatives. Clinical presentation, family history, tumor morphologic features, IHC, and MSI are not 100% sensitive; therefore, a better testing algorithm is needed to identify dMMR CRC cases and to accurately assign these cases to Lynch and sporadic subgroups of dMMR CRC.

Current knowledge allows some rules for creation of a practical test algorithm. Almost all dMMR CRC will be detected by the combination of MSI and IHC testing[25,49,116–119] (Table 1). In the presence of dMMR, the additional loss of protein expression of MSH2/MSH6, MSH6 alone, or PMS2 alone increases the likelihood of Lynch syndrome. On the other hand, the concomitant incidence of dMMR, CIMP-H, and *MLH1* PHM supports a diagnosis of sporadic dMMR CRC. Detection of the *BRAF* c.1799T>A mutation serves to exclude the diagnosis of Lynch syndrome.

We propose that the MMR screening algorithm include parallel testing for MSI, *BRAF* c.1799T>A mutation, and IHC for the four MMR proteins. Figure 1 illustrates the interrelatedness of these characteristics in a Venn diagram and a table of covariation probabilities. Figure 2 illustrates the proposed algorithm.

Use of this algorithm should allow MMR subgroup assignment for most cases (Figure 2). If the CRC is MSS with normal IHC, then it is pMMR. If the CRC is MSI-H or MSI-L and IHC shows only MSH6 or PMS2 loss, then the likelihood of Lynch syndrome increases, and *MSH6* or *PMS2* gene sequencing, respectively, is indicated. If the CRC is MSI-H and IHC shows MSH2 and MSH6 loss, then the likelihood of Lynch syndrome increases, and *MSH2* sequencing/deletion testing is indicated. If the CRC is MSI-H, IHC shows MLH1 loss, and the *BRAF* c.1799T>A mutation is present, then it is highly likely sporadic dMMR CRC. Only with the combination of MSI-H, loss of MLH1 immunoreactivity, and absence of the *BRAF* mutation is there substantial uncertainty, and the likelihood of Lynch syndrome versus sporadic dMMR CRC may vary according to the clinical scenario. On the basis of a 5:1 ratio of sporadic dMMR cases to new Lynch cases and a *BRAF* c.1799T>A mutation sensitivity of 60% and specificity of 100% for the sporadic dMMR subgroup, Bayes theorem estimates that 74% of these remaining unassigned cases will be sporadic dMMR. In this circumstance, CIMP testing, *MLH1* PHM testing, and/or *MLH1* germline sequenc-

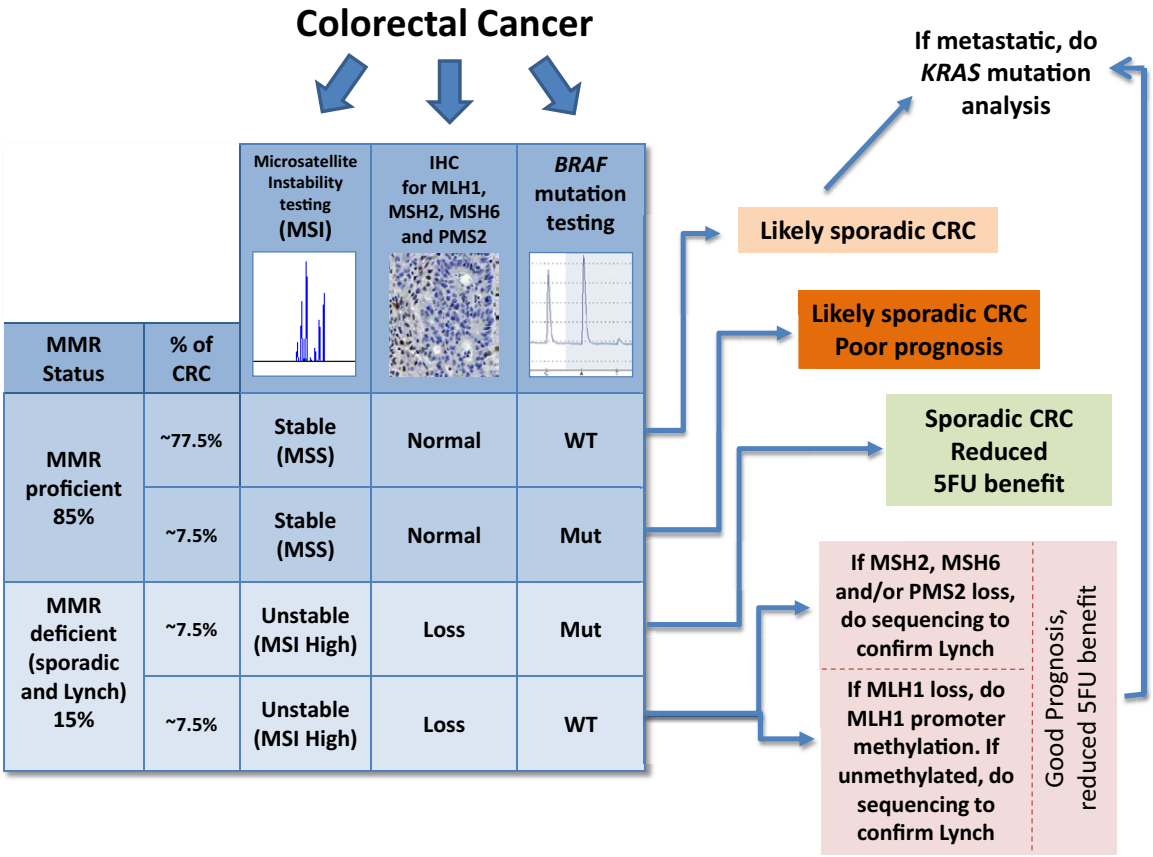


Figure 2. The proposed testing strategy and the possible test outcomes, downstream additional testing, subgroup assignment, prognosis, and prediction of therapeutic response.

ing/deletion testing should be performed. CIMP-H and somatic *MLH1* PHM would support a diagnosis of sporadic dMMR CRC, and presence of an *MLH1* germline mutation, deletion, or hypermethylation would support a diagnosis of Lynch syndrome.

Alternative Screening Algorithms

National organizations have recommended various testing algorithms to maximize detection of inherited MMR gene mutations in patients with Lynch syndrome (summarized in Table 3). The National Comprehensive Cancer Network recommends use of Amsterdam or revised Bethesda criteria as the initial screening step.[125] This approach would miss the diagnosis of 5% to 58% of new Lynch syndrome cases, as well as most sporadic dMMR CRC cases. EGAPP estimated detection rates and costs of testing using four different testing strategies: i) MMR gene sequencing/deletion testing on all probands; ii) MSI testing, followed by MMR gene sequencing/deletion testing on all MSI-H cases; iii) IHC testing, with protein loss guiding targeted MMR gene sequencing/deletion testing; and iv) IHC, with *BRAF* c.1799T>A testing of cases with MLH1 protein loss.[85] Each of these would fail to detect all dMMR CRC. The first approach could identify most Lynch cases but not the sporadic dMMR CRC cases. The second, third, and fourth approaches would fail to classify some Lynch and 11% to 100% of sporadic dMMR CRC cases. The first strategy is the only one to capture all Lynch cases but could cost seven times as much as the fourth strategy. A similar comparison of four strategies, each starting with a single test, was recently published by the Centers for Disease Control and Prevention,[122] with similar limitations to the EGAPP model. The (IHC→sequencing) strategy and (IHC+/− *BRAF* c.1799T>A →sequencing) strategy were more cost-effective for diagnosis of Lynch syndrome probands and carriers. However, 11% to 12% of Lynch cases would not be diagnosed due to the absence of MSI testing to identify MSI-H tumors with normal IHC in Lynch syndrome patients (Table 1[25,49,116–119]). The American College of Gastroenterology recommends initial classification by the revised Bethesda criteria, followed by MSI testing and/or IHC.[126] This approach would miss 5% of new Lynch cases and an unknown percentage of sporadic dMMR CRC cases.

Clinical investigations have published algorithms to detect Lynch syndrome probands[84,122,127,128] (summarized in Table 3). Each has been optimized to detect germline mutations but may also assign cases to the sporadic dMMR subgroup. The strategy of Lindor et al[127] has simultaneous MSI and IHC testing of patients who also have at least one of the Bethesda criteria. Those tumors that have MSI-H and/or IHC loss are triaged to MMR gene sequencing, whereas MLH-1 IHC loss/normal

Table 3. Expected Classification of CRC into dMMR Subgroups

Screening test	Correctly assigned to sporadic dMMR subgroup (12.5% in this subgroup*)	Correctly assigned to Lynch dMMR subgroup (2.5% in this subgroup*)	Correctly assigned to a dMMR subgroup (15% in this subgroup*)
Amsterdam II criteria[120] only	0.0	1.2	1.2
Revised Bethesda criteria[122] only	0.0	2.4	2.4
Morphologic analysis only	0.0	0.0	0.0
MSI testing only	0.0	0.0	0.0
MMR IHC only	0.0	1.7	1.7
BRAF c.1799T>A test only	0.0	0.0	0.0
NCCN[125] (Amsterdam II or revised Bethesda criteria screening first)	Unknown	1.1–2.4	1.1–2.4
EGAPP[85] model 1 (MMR gene sequencing/deletion)	0.0	2.5	2.5
EGAPP model 2 (MSI, then MMR gene sequencing/deletion if MSI-H)	0.0	2.5	2.5
EGAPP model 3 (IHC first, then MMR gene sequencing if protein lost)	11.1	2.2	13.3
EGAPP model 4 (IHC first; then *BRAF* if MLH1 lost)	7.5	1.7	9.2
American College of Gastroenterology[126] (revised Bethesda criteria screening first, then MSI or IHC)	Unknown	2.4	>2.4
Lindor et al[127] (revised Bethesda criteria screening, then MSI and IHC, then MMR gene sequencing, then *BRAF* if wild-type MLH1)	Unknown	2.5	>2.5
Vasen et al[84] (revised Bethesda criteria screening first, then IHC or MSI)	Unknown	2.5	>2.5
Gatalica and Torlakovic[128] (MSI first, then *BRAF,* then IHC, then gene sequencing)	12.5	2.2	14.7
Concurrent MSI and MMR IHC	0.0	1.7	1.7
Concurrent MSI, MMR IHC, and *BRAF*	7.5	1.7	9.2
Concurrent MSI, MMR IHC, and *BRAF* with follow-up sequencing as needed	12.5	2.5	15.0

*Expected percentage of total CRC in each dMMR subgroup.
NCCN, National Comprehensive Cancer Network.

DNA sequence cases are referred for *MLH1* PHM or *BRAF* c.1799 T>A mutation testing. Detection of both Lynch and sporadic dMMR CRC is facilitated but requires initial clinical stratification, three serial tests with pathologist evaluations, and summary decisions to assign all cases into molecular subgroups. The strategy of Vasen et al[84] begins with the Bethesda criteria, reflecting high-risk patients to IHC only and low-risk patients to either IHC or MSI. IHC loss or MSI-H or MSI-L prompts resequencing for germline MMR mutations. This approach would fail to detect sporadic dMMR cases, as well as 11% of Lynch syndrome cases. The strategy of Gatalica and Torlakovic[128] begins with MSI testing; MSI-H cases then progress to *BRAF* c.1799T>A mutation testing, and *BRAF* mutation-negative tumors proceed to IHC testing. All Lynch and sporadic dMMR CRC could be recognized except for the 10% to 14% of Lynch syndrome cases that are MSH6 mutant/MSI-L, but four serial tests with pathologist evaluations/triage decisions would be necessary.

Universal testing of new CRC patients is predicted to be relatively cost-effective, particularly when detection of carrier status for first- and second-degree relatives of the proband are included.[124] Modeling of four molecular testing strategies estimates that the lowest incremental cost-effectiveness ratio (net cost per life-year saved) would be obtained using a strategy with IHC and *BRAF* mutation testing, followed by sequencing of the MMR gene with a loss of protein expression detected by IHC (*MLH1* would be sequenced only when *BRAF* sequence was normal).[124] Modeling of both molecular and clinical strategies estimates that an MSI, IHC, and *BRAF* mutation testing strategy would be the most cost-effective.[129]

Summary of Recommendations

We propose that parallel MSI, MMR protein IHC, and *BRAF* c.1799T>A mutation testing be performed at the time of a new diagnosis of CRC. This would permit as-

signment to pMMR, sporadic dMMR, and suspicious Lynch dMMR subgroups for approximately 94% of CRC cases, with only the MSI-H, MLH1-lost, and *BRAF* wild-type cases (5% to 6% of total CRC) unassigned (Figure 2 and Table 3). This strategy extends the CDC model with the highest cost-effectiveness of initial IHC with or without *BRAF*,[124] and also identifies the estimated 11% of patients with Lynch syndrome who are MSI-H and IHC immunoreactive.

Our recommended approach would maximize diagnostic information using three tests currently available in most local/regional laboratories and would triage the unassigned 6% of the cases to referral laboratories doing high volumes of hypermethylation, sequencing, and deletion testing for resolution of subgroup assignment. An additional 1.7% of cases (those assigned to the Lynch syndrome subgroup) would also be referred to define the germline mutation/deletion involved. Our approach may be cost-effective, but further study is needed to demonstrate this. Our expectation is that the cost of testing will be less than the cost of delayed diagnosis and absent surveillance of Lynch carriers. At the clinical level, clinical geneticists will work up and counsel patients with dMMR CRC, as well as unaffected family members of Lynch syndrome probands. Medical oncologists will be able to make prompt therapeutic decisions for their patients with stage II CRC. Gastroenterologists will be able to define appropriate follow-up intervals for patients based on polyp morphologic findings and CRC MMR subgroup. The end result will be improved diagnostic accuracy regarding CRC molecular subgroup assignment, appropriate therapy guided by CRC molecular subgroup, appropriate genetic counseling for patients with germline MMR mutations, and appropriate counseling and screening of unaffected family members of patients with Lynch syndrome for the proband's known germline MMR mutation.

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