COLLEGE of AMERICAN PATHOLOGISTS

Biomarkers in Colorectal Cancer: *BRAF* Testing as a Follow Up to Microsatellite Instability in the Exclusion of Lynch Syndrome

Version: 3.0
Date: Original publication September 2018. Updated July 29, 2021, December 19, 2022

Author
Shannon J. McCall, MD, FCAP, Director of the BioRepository & Precision Pathology Center (BRPC) in the Department of Pathology at Duke University Hospital

Editors
Richard Brown*, MD, FCAP, Peter L. Perrotta, MD, FCAP, Barbara Blond, MBA, Thomas Long, MS
* Denotes senior editor

SYNOPSIS AND RELEVANCE

This module promotes using an approach for evaluating colorectal cancers (CRC) that lack MLH1 and PMS2 protein expression by immunohistochemistry (IHC) or demonstrate microsatellite instability (MSI) by polymerase chain reaction (PCR) testing. A subset of these tumors will test positive for the *BRAF* V600E mutation. This finding is associated with sporadic methylation of *MLH1*, which correlates with sporadic microsatellite instability (MSI). In this subset of patients, it is not recommended to perform germline mutation analysis to exclude Lynch syndrome. Furthermore, it should be noted that *BRAF* V600E mutation analysis may be performed in other contexts and alone is not useful for excluding Lynch syndrome.

Establishing and adhering to algorithms for evaluating CRC can:
1. Ensure that the proper tests and methodologies are used to evaluate patients with CRC.
2. Optimize the utilization of IHC markers and molecular diagnostic tests in CRC.
3. Impact patient care by ensuring that the most clinically useful tests are used to evaluate and guide therapy in patients with CRC.

OBJECTIVES
1. Understand the most recent guidelines for biomarker testing in colorectal cancer.
2. Optimize the selection of tests used by treating or ordering physicians to help categorize colorectal cancers.
3. Recognize the next test to order based on the results of testing for microsatellite instability (MSI) and mismatch repair protein (MMR) testing.

BACKGROUND

Lynch syndrome, otherwise referred to as hereditary nonpolyposis colorectal cancer (HNPCC), is an inherited disorder that increases the risk of several cancers, in particular, colorectal cancer (CRC). It is caused by germline mutations in deoxyribonucleic acid (DNA) mismatch repair (MMR) system genes including *MLH1*, *MSH2*, *MSH6*, and *PMS2*. Mutations in MMR genes prevent proper repair of errors in DNA replication, which results in the formation of abnormal microsatellite fragments. These are short repeated base sequences that can be detected by polymerase chain reaction-based microsatellite instability (MSI) assays. Approximately 3-5% of CRC are attributed to Lynch syndrome.

The two main assays used to test CRC for microsatellite instability are microsatellite instability (MSI) testing which is performed by PCR analysis, and mismatch repair (MMR) protein testing which is performed by immunohisto-chemistry. In the PCR-based approach, the length of known microsatellite regions of DNA is quantified within the tumor. Significant expansion of microsatellite regions confirms microsatellite instability. In the MMR testing, immunohistochemistry for MMR proteins MLH1, MSH2, MSH6 and PMS2 is performed to look for abnormal loss of these proteins within tumor nuclei.

Tumors from patients with Lynch syndrome demonstrate microsatellite instability (MSI). However, MSI is not specific to Lynch syndrome as 15-20% of all CRC tumors will demonstrate MSI. Sporadic CRC with MSI most commonly results from epigenetic silencing of *MLH1,* typically via hypermethylation of *MLH1*. Because *BRAF* V600E mutations have been identified in up to 75% of CRC with epigenetic silencing of *MLH1*, it is unlikely that a CRC-MSI patient with a *BRAF* V600E mutation carries a germline mutation in any of the mismatch repair proteins (ie, has Lynch Syndrome).

Bethesda guidelines used in the past for HNPCC/Lynch syndrome screening recommend testing CRC tumors for MSI in the following situations: patients less than 50 years of age diagnosed with CRC, the presence of a synchronous colorectal or other HNPCC tumor, cancer with MSI-high (MSI-H, now simply MSI) status in a patient less than 60 years old, or a colorectal or HNPCC tumor diagnosed in a first degree relative at age less than 50 years or two or more first degree relatives.

Testing of CRC for MSI now extends beyond Bethesda criteria. The recent CAP Template for Reporting Results of Biomarker Testing of Specimens from Patients with Carcinoma of the Colon and Rectum highlights the fact that while the specificity of MSI testing for Lynch Syndrome is highest in patients selected by Bethesda criteria, its sensitivity is diminished because a known proportion of CRC arising in Lynch syndrome will not meet these criteria. Furthermore, the value of MSI/PCR and MMR/IHC testing has extended outside of identifying Lynch syndrome. It is now understood that microsatellite instability status carries prognostic and theranostic significance in CRC. Many institutions now consider MSI/PCR and MMR/IHC testing of all CRC as standard of care, despite the fact that a minority of patients with abnormalities in either or both of these assays will have Lynch Syndrome.

Follow-up testing for Lynch syndrome in CRC patients demonstrating MSI by PCR can be dictated by the results of MMR IHC testing, since patients whose tumors have lost nuclear expression of MSH6 and/or MSH2 proteins are likely to have Lynch syndrome, whereas only a minority of patients with IHC abnormalities in MLH1 and/or PMS2 proteins will have Lynch syndrome. This dual-testing approach may be especially useful for identifying patients with germline *MSH6* mutations and loss of nuclear MSH6 protein expression by IHC but who may test negative for MSI by PCR. Follow-up testing for patients with abnormalities of MSH2 and/or MSH6 protein expression should consist of germline mutational analysis by sequencing for Lynch syndrome.

A cost-effective strategy for distinguishing Lynch syndrome tumors from sporadic CRC tumors with MSI can be designed. As indicated above, immunohistochemistry for mismatch repair proteins can either be performed concurrently or following abnormal MSI testing by PCR. Abnormal immunohistochemical testing for MLH1 and/or PMS2 can indicate the need for follow-up testing to risk stratify patients before ordering expensive germline sequencing assays. An evidence-based guideline for molecular biomarkers in the evaluation of colorectal cancer was recently jointly published by the ASCP, CAP, AMP, and ASCO. This guideline contains a summary of the evidence for the appropriate role of *BRAF* V600E testing in colorectal cancer. Identifying true Lynch syndrome patients among those with MLH1 and/or PMS2 expression abnormalities can be facilitated by screening for the *BRAF* V600E mutation, which is seen in three-fourths of sporadic MSI CRC resulting from epigenetic silencing of *MLH1*.

As mentioned above, appropriate *BRAF* V600E mutation testing in CRC extends beyond follow-up of MSI/MMR abnormalities. According to the guideline, the test can reliably provide prognostic information for CRC patients but there is insufficient evidence to support *BRAF* V600E testing as predictive of response to anti-EGFR therapy. Prognostic and theranostic uses of *BRAF* V600E and MSI/MMR testing are beyond the scope of this module.

Additionally, the most common abnormality in sporadic MSI colon cancers is hypermethylation of *MLH1*, therefore finding this abnormality provides strong support for sporadic (non-Lynch) MSI. However, hypermethylation of *MLH1* is not sufficient to distinguish Lynch syndrome from sporadic MSI CRC, as up to 15% of Lynch syndrome CRC patients will demonstrate this finding. Identifying true Lynch syndrome patients among those with MSI by PCR or MLH1 and/or PMS2 protein expression abnormalities may be supported using *BRAF* V600E with or without an *MLH1* hypermethylation assay. For practices that perform only MSI testing via PCR on CRC, current guidelines support testing for *MLH1* hypermethylation and/or *BRAF* V600E mutation in patients with abnormal PCR testing for microsatellite instability whether or not testing via IHC has been performed. The specific choice of tests and their sequence may be influenced by the patient population or individual patient situation; however designing a general algorithmic approach in a multidisciplinary fashion at your institution may identify patients who do not need expensive germline sequencing testing for Lynch syndrome.

The value of *BRAF* V600E mutational analysis in CRC may be increased by:
- Establishing laboratory policies and procedures for assisting clinicians with the appropriate use and interpretation of *BRAF* V600E testing in CRC patients.
- Establishing a microsatellite instability testing strategy for CRC patients that includes *BRAF* V600E mutation testing to segregate MSI or MLH1-deficient tumors.
- Facilitating the evaluation of CRC patients for your health care professionals in a collaborative manner.
- Ensuring that your health information technology services support the decision making of health care providers who utilize *BRAF* V600E testing by optimizing the electronic test ordering and resulting systems.

INSIGHTS

1. While absent MSH6 protein expression by IHC is characteristic of Lynch syndrome, only a small subset of CRC tumors with absent MLH1 protein by IHC will harbor a germline mutation in a mismatch repair protein (ie, germline sequencing for Lynch syndrome is not usually indicated).
2. CRC tumors with absent MLH1 protein by IHC are usually associated with sporadic *MLH1* hypermethylation and the *BRAF* V600E mutation has been frequently detected in these sporadic MSI cases. The BRAF V600E mutation is not associated with Lynch syndrome.
3. *MLH1* hypermethylation assays are not recommended as an initial strategy to distinguish between sporadic MSI CRC and Lynch syndrome because *MLH1* hypermethylation can be seen in up to 15% of Lynch syndrome patients as a secondary finding, however many laboratories find value in combining *MLH1* hypermethylation assays with *BRAF* V600E mutation analysis.
4. *BRAF* V600E mutation analysis either alone or in combination with *MLH1* hypermethylation analysis may be more efficient than germline sequencing tests for detecting Lynch Syndrome-associated mutations. Laboratories should consider using these tests prior to germline analysis of MMR system genes when a CRC has abnormal *MLH1* IHC.

INTERVENTIONS

1. <u>Develop diagnostic algorithms</u>: Consult with pathologists and other physicians in your hospital setting to discuss diagnostic strategies for testing CRC. These algorithms can address what patients should undergo germline testing for Lynch syndrome and what tumors should be tested for *BRAF* V600E with or without *MLH1* hypermethylation.
2. <u>Provide educational information</u>: Educational materials are available from the CAP cancer reporting and biomarker reporting protocols. Educational materials can include scenarios in which *BRAF* V600E mutational analysis is indicated, and germline mutational analysis can be limited to those situations where it is most useful.
3. <u>Screen requests for tumor genetic testing</u>: Requests for tumor genetic testing (eg, *BRAF* V600E mutation testing and/or *MLH1* hypermethylation) can be initiated by the pathologist depending on the results of MSI and IHC testing.
4. <u>Modify test ordering system</u>: Interventions can include making changes to how providers order tumor genetic and germline mutation testing. There are a number of changes that can be made depending on which information system(s) is utilized by your institution.
5. <u>Modify surgical pathology reports</u>: Insert a diagnostic comment within the body of the surgical pathology report indicating *BRAF* and/or *MLH1* hypermethylation testing has been initiated and that the results will guide the need for additional testing.

INTERVENTION ANALYSIS

1. Review the following protocols and templates with the pathologists and other clinical staff as appropriate to your practice setting:
   a. CAP protocol for examining specimens obtained from patients with primary carcinoma of the colon and rectum.
   b. CAP template for reporting results of biomarker testing of specimens from patients with carcinoma of the colon and rectum and genetic mutational analysis.
   c. ASCP/CAP/AMP/ASCO Colorectal cancer biomarker guideline.
2. Prepare an appropriate algorithm for your institution to use when testing CRC tumors. The algorithm that you use may differ depending on availability of tests, oncologist preference, etc.
3. Collect data on how MSI testing and IHC are used to evaluate CRC at your institution (see **Appendix A** for a sample worksheet). Note that the data in Appendix B represents sample data; the point is to determine whether the testing strategy used at the time was optimal or not.
   a. Data collection will also include whether additional testing (eg, germline testing, *BRAF* V600E mutational analysis and/or *MLH1* hypermethylation) was performed and the test results.
   b. These data are most easily retrieved from an appropriate information system (eg, hospital information system, laboratory information system).
   c. The period of data collection (eg, 1 year) will depend on your test volume.
4. Data collection can be repeated after any interventions or educational sessions to determine the impact at your institution.
5. Determine the effectiveness of your interventions by computing the change in the number of *BRAF* V600E and germline mutational analyses ordered after the interventions have been in place for an appropriate amount of time. Because colon cancer cases may be infrequent, and because the follow-up testing plays out over several weeks for any one patient, data collection before and after interventions may need to be 6 months to one year.
6. Value opportunities can be calculated. For example, the impact of performing testing of limited utility may be calculated for germline mutation testing performed on patients whose tumor is positive for the *BRAF* V600E mutation.

APPENDIX A: SAMPLE DATA COLLECTION WORKSHEET

Medical Record Number	Patient Age	Date	MSI Testing Results	IHC Results	*BRAF* V600E Testing Appropriate?	*BRAF* V600E Testing Result?	*MLH1* Hypermethyl-ation Testing Appropriate?	*MLH1* Hypermethyl-ation Assay Result?	Germline Analysis (sequencing any MMR gene or *EPCAM*) Indicated?	Germline Analysis (sequencing any MMR gene or *EPCAM*) Performed?
1234567	50	1/1/2021	MSI	Loss of MLH1	Yes	Positive	Yes	Positive	No	Yes
1234578	45	1/5/2021	MSS	Intact	No	Not done	No	Not done	No	No
1234589	40	1/5/2021	MSI	Loss of MSH2	No	Not done	No	Not done	Yes	Yes
1234596	60	1/10/2021	MSI	Loss of MSH2	No	Not done	No	Not done	Yes	Yes
1234636	70	1/20/2020	MSS	N/A	No	Not done	No	Not done	No	Yes
1234563	50	1/22/2020	MSI	Loss of MLH1	Yes	Not done	Yes	Positive	Yes, if *BRAF* V600E mutation negative	Yes
1234565	45	1/28/2020	MSI	Loss of MSH6	No	Not done	No	Not done	Yes	Yes
Total Tests Performed:					2	1	2	2	4	6

QUESTION 1 OBJECTIVE
Understand the most recent national guidelines for biomarker testing in colorectal cancer.

QUESTION 1
A 45-year-old patient has been newly diagnosed with early stage colorectal cancer. Which of the following tests should be routinely performed in this scenario?
A. HER2 expression analysis
B. Mismatch repair testing (MSI and IHC)
C. Next generation sequencing "hot spot" panel
D. *PTEN* mutation analysis
E. *RAS* mutation analysis

The correct answer is B. The CAP Colorectal Cancer Biomarker Reporting Guidelines indicate that mismatch repair testing is important for detecting Lynch syndrome.
A is incorrect. Analysis of HER2 protein expression is useful in breast and gastric cancers, not in CRC.
C is incorrect. Next generation sequencing panels may help direct systemic chemotherapeutic options in patients with later stage CRC and may not be indicated at the time of initial diagnosis in an earlier stage patient.
D is incorrect. *PTEN* mutations resulting in loss of expression may have a role in directing systemic chemotherapy in later stage patients.
E is incorrect. *KRAS* mutations have been associated with lack of response to EGFR targeted therapies used in later stage disease. This testing may not be indicated at the time of initial diagnosis in an earlier stage patient.

REFERENCE
Bartley AN, Hamilton SR, Alsabeh R, et al. Members of the Cancer Biomarker Reporting Workgroup, College of American Pathologists. Template for reporting results of biomarker testing of specimens from patients with carcinoma of the colon and rectum. *Arch Pathol Lab Med.* 2014;138(2):166-170. https://doi.org/10.5858/arpa.2013-0231-CP

QUESTION 2 OBJECTIVE
Optimize the selection of tests used by clinicians to help categorize colorectal cancers such as MSS, MSI-sporadic, or MSI-Lynch syndrome.

QUESTION 2
All of the following are reasons to perform both microsatellite instability (MSI) testing by PCR and mismatch repair protein testing (MMR) by immunohistochemistry for MLH1, MSH2, MSH6, and PMS2 EXCEPT:
A. Some missense mutations lead to nonfunctional mismatch repair proteins that can still be detected by immunohistochemistry.
B. There is improved sensitivity for Lynch syndrome when both tests are performed together.
C. Patients with germline *MSH6* mutations can be microsatellite stable (MSS) by PCR.
D. Genetic mutations causing MSI phenotype outside of *MLH1, MSH2, MSH6,* and *PMS2* have not been reported.

The correct answer is D. Deletion mutations in *EPCAM* have also been associated with microsatellite instability and Lynch syndrome.
A is incorrect. 'A' is a true statement.
B is incorrect. 'B' is a true statement.
C is incorrect. 'C' is a true statement.

REFERENCE
Bartley AN, Hamilton SR, Alsabeh R, et al. Template for reporting results of biomarker testing of specimens from patients with carcinoma of the colon and rectum. *Arch Pathol Lab Med.* 2014;138 (2):166-170. https://doi.org/10.5858/arpa.2013-0231-CP

QUESTION 3 OBJECTIVE
Recognize next test to order when the tumor demonstrates MSI by PCR and immunohistochemistry for mismatch repair proteins is abnormal.

QUESTION 3
A 50-year-old woman has been recently diagnosed with colorectal cancer. Microsatellite instability (MSI/PCR) testing reveals the tumor is MSI. The appropriate tumor immunohistochemical studies (MMR/IHC) are also performed. Patient germline testing for mutations in mismatch repair genes is the NEXT indicated test for all of the following patterns EXCEPT:
A. Loss of MLH1 and/or PMS2 expression
B. Dual loss of MSH2 and MSH6 expression
C. Isolated loss of MSH2 expression

D. Isolated loss of MSH6 expression

The correct answer is A. Abnormalities in MLH1 and PMS2 expression can be due to Lynch syndrome, or they can be associated with somatic *MLH1* hypermethylation (epigenetic silencing). Testing for *BRAF* V600E mutation is the next indicated test for this pattern.

B is incorrect. This pattern is associated with Lynch Syndrome. Germline testing is indicated.

C is incorrect. This pattern is associated with Lynch Syndrome. Germline testing is indicated.

D is incorrect. This pattern is associated with Lynch Syndrome. Germline testing is indicated.

REFERENCES

1. Bartley AN, Hamilton SR, Alsabeh R, et al. Template for reporting results of biomarker testing of specimens from patients with carcinoma of the colon and rectum. *Arch Pathol Lab Med.* 2014;138 (2):166-170. https://doi.org/10.5858/arpa.2013-0231-CP
2. Kato A, Sato N, Sugawara T, et al. Isolated loss of PMS2 immunohistochemical expression is frequently caused by heterogenous *MLH1* promoter hypermethylation in Lynch syndrome screening for endometrial cancer patients. *Am J Surg Pathol.* 2016;40(6):770-776. doi:10.1097/PAS.0000000000000606

QUESTION 4 OBJECTIVE

Recognize the information that can be gained from *BRAF* V600E testing of colorectal cancer.

QUESTION 4

Which of the following best describes the prognosis and/or therapeutic implications of colorectal cancers harboring the *BRAF* V600E mutation?

A. Are associated with longer progression-free and overall survival
B. Comprise about 30% of colorectal cancers
C. Do not have germline mutations in *MSH2* and are only rarely associated with *MLH1* mutations.
D. Have a more robust response to epidermal growth factor receptor (EGFR)-targeted therapies such as cetuximab and panitumumab
E. Usually also have mutations in *KRAS*.

The correct answer is C. Colorectal cancers in patients with germline mutations of *MSH2* have been shown to not harbor the *BRAF* V600E mutation. Only rare colorectal cancers with germline mutations of *MLH1* will harbor the BRAF V600E mutation. This is the reason testing for *BRAF* V600E can be performed in MSI colorectal cancers with abnormal MLH1 immunohistochemistry – it dramatically reduces the likelihood that germline mutations are a cause of the IHC abnormality. Further information can be gained by adding an *MLH1* hypermethylation assay.

A is incorrect. Colorectal cancers harboring *BRAF* mutations are associated with shorter progression-free and overall survival. This is important because *BRAF* mutation testing is appropriate in MSS CRC to provide this additional prognostic information.

B is incorrect. *BRAF*-mutated colorectal cancers make up about 10% of all colorectal cancers.

D is incorrect. Colorectal cancers harboring the *BRAF* V600E mutation may have a limited response to cetuximab and panitumumab; this evidence is not yet conclusive according to the 2017 CAP/ASCO guideline for CRC biomarkers.

E is incorrect. *KRAS* and *BRAF* mutations are almost entirely mutually exclusive in colorectal cancer.

REFERENCES

1. Sepulveda AR, Hamilton SR, Allegra CJ, et al. Molecular biomarkers for the evaluation of colorectal cancer: guideline from the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology. *Arch Pathol Lab Med.* 2017;141(5):625-757. https://doi.org/10.5858/arpa.2016-0554-CP
2. Gonsalves WI, Mahoney MR, Sargent DJ, et al. Patient and tumor characteristics and *BRAF* and *KRAS* mutations in colon cancer, NCCTG/Alliance N0147. *J Natl Cancer Inst.* 2014;106(7). https://doi.org/10.1093/jnci/dju106
3. Bartley AN, Hamilton SR, Alsabeh R, et al. Template for reporting results of biomarker testing of specimens from patients with carcinoma of the colon and rectum. *Arch Pathol Lab Med*. 2014;138 (2):166-170. https://doi.org/10.5858/arpa.2013-0231-CP

MODULE REFERENCES

1. Umar A, Boland CR, Terdiman JP, et al. Revised Bethesda guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. *J Natl Cancer Inst.* 2004; 96(4):261-268. doi:10.1093/jnci/djh034
2. Wang L, Cunningham JM, Winters JL, et al. *BRAF* mutations in colon cancer are not likely attributable to defective DNA mismatch repair. *Cancer Res.* 2003; 63(17):5209-5212.
3. Bartley AN, Hamilton SR, Alsabeh R, et al. Template for reporting results of biomarker testing of specimens from patients with carcinoma of the colon and rectum. *Arch Pathol Lab Med.* 2014;138 (2):166-170.

https://doi.org/10.5858/arpa.2013-0231-CP

4. Sepulveda AR, Hamilton SR, Allegra CJ, et al. Molecular biomarkers for the evaluation of colorectal cancer: guideline from the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology. *Arch Pathol Lab Med.* 2017;141(5):625-657. https://doi.org/10.5858/arpa.2016-0554-CP

5. Moreira L, Balaguer F, Lindor N, et al. Identification of Lynch syndrome among patients with colorectal cancer. *JAMA.* 2012;308(15):1555-1565. doi:10.1001/jama.2012.13088

6. Moreira L, Munoz J, Cuatrecasas M, et al. Prevalence of somatic mutl homolog 1 promoter hypermethylation in Lynch syndrome colorectal cancer. *Cancer.* 2015;121(9):1395-1404. https://doi.org/10.1002/cncr.29190.

7. Domingo E, Niessen RC, Oliveira C, et al. *BRAF*-V600E is not involved in the colorectal tumorigenesis of HNPCC in patients with functional *MLH1* and MSH2 genes. *Oncogene.* 2005;24(24):3995-3998. doi:10.1038/sj.onc.1208569

8. Bessa X, Balleste B, Andreu M, et al. A prospective, multicenter, population-based study of BRAF mutational analysis for Lynch syndrome screening. *Clin Gastroenterol Hepatol.* 2008;6(2):206-214. doi:10.1016/j.cgh.2007.10.011

9. Kato A, Sato N, Sugawara T, et al. Isolated loss of PMS2 immunohistochemical expression is frequently caused by heterogeneous *MLH1* promoter hypermethylation in Lynch syndrome screening for endometrial cancer patients. *Am J Surg Pathol.* 2016;40(6):770-776. doi:10.1097/PAS.0000000000000606