Molecular Diagnostics of Colorectal Cancer

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Context.—Of all gastrointestinal tract epithelial malignancies, molecular diagnostics has impacted colorectal cancer the most. Molecular testing can detect sporadic and inherited colorectal cancers that arise through the microsatellite instability pathway and can determine the efficacy of targeted drug therapy.

Objectives.—To review the microsatellite instability pathway of colorectal carcinoma carcinogenesis and to demonstrate the diagnostic utility of molecular testing in the detection of patients with Lynch syndrome, an inherited disorder of this pathway. Also, to review the significance of detection of KRAS and BRAF gene mutations in predicting the response to anti–epidermal growth factor receptor therapies.

Data Sources.—This article is based on original publications and review articles that are accessible through the PubMed biomedical database (US National Library of Medicine).

Conclusions.—In modern pathology practice, molecular testing is a standard tool that is used to diagnose an inherited colorectal cancer predisposition syndrome (Lynch syndrome) and to help predict outcome and response to therapy for patients with advanced colorectal cancer.

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Advances in molecular medicine have brought the field of oncologic pathology to a new level. Pathologists have abundant ancillary tools at their disposal not only to aid in the diagnosis but also to help predict outcome and response to therapy. Although molecular diagnostics is rapidly expanding to include all epithelial neoplasms of the gastrointestinal tract, clinical testing has progressed the furthest in colorectal cancer (CRC) and as such, the molecular diagnostics of CRC will be the topic of this review. Molecular tests that are currently in use, including microsatellite instability (MSI) testing to detect inheritable disease, and KRAS and BRAF mutational analysis to predict response to therapy, will be discussed.

MICROSATELLITE INSTABILITY

Colorectal cancer encompasses a complex disease process and should no longer be viewed as one disease. Colorectal cancer can be sporadic or inherited and can arise through more than one molecular pathway (Figure 1). Most CRCs are sporadic and arise through the chromosomal instability (CIN) pathway resulting in loss of heterozygosity and acquired loss of various genes such as APC, TP53, and DCC; KRAS mutations can also occur. A very small percentage of chromosomal instability tumors are inherited and arise secondary to germline mutations in the APC gene (familial adenomatous polyposis; less than 1% of CRCs) or the MUTYH gene (MUTYH-associated polyposis; <1% of CRCs). About 15% of CRCs arise through the MSI pathway and most of these tumors are sporadic. A small percentage of CRCs that arise via the MSI pathway are inherited as the result of a germline mutation in one of the mismatch repair (MMR) genes (Lynch syndrome/hereditary nonpolyposis colon cancer [HNPPC]; about 2% to 5% of all cases of CRCs).

Microsatellites and Mismatch Repair

Microsatellites are simple repetitive DNA sequences scattered throughout the genome. They are composed of 2 or more base pair units that may be repeated up to 100 times. Given their repetitive nature they are liable for errors that can occur during DNA replication. Among these errors is the occurrence of DNA slippage. Such errors result in insertion/deletion loops.1 Mismatch repair genes play a critical role in the identification and correction of these errors. Failure of the mismatch repair apparatus leads to persistence of errors and an alteration in the length of a microsatellite sequence, a process described as microsatellite instability. These changes are particularly important when the microsatellites are present within critical areas of genes responsible for cell growth regulation. Persistence of such errors leads to frameshift mutations with loss of the normal function of these genes and the promotion of tumorigenesis.2,3

The DNA mismatch repair system requires the cooperation of many genes including MSH2, MSH6, MLH1, MSH3, and PMS2. The MSH2 protein recognizes and binds directly to the mismatched DNA sequence. It forms a heterodimeric complex with MSH6 if a single base-pair mismatch is recognized or with MSH3 if there is a larger, 2- to 8-nucleotide insertion or deletion.4 A second heterodimeric complex of MLH1 and PMS2 is then recruited, which subsequently directs the remainder of the MMR machinery to excise the mismatched nucleotides.5
Lynch Syndrome

Also known as hereditary nonpolyposis colorectal cancer, Lynch syndrome is a familial autosomal dominant cancer predisposition syndrome that accounts for 2% to 5% of CRCs. Patients inherit a germline mutation in one of the mismatch repair genes. About 90% of these mutations involve either MLH1 or MSH2 and less frequently can involve other MMR genes, including MSH6 and PMS2. Most of these mutations are nonsense or frameshift mutations, leading to truncated (unstable) proteins.

It is important to note that the inherited germline alteration does not result in complete loss of function of the affected gene in nonneoplastic cells. This is because these patients inherit 1 mutated allele from 1 parent, and the second nonmutated (wild-type) allele provides sufficient DNA mismatch repair protein expression. The inherited defect results in the predisposition of early-onset (mean age, 45 years) CRC and increased frequency of multiplicity for CRC. The lifetime risk for CRC for patients with Lynch syndrome is estimated to be 80%. These patients may also have an increased risk of extracolonic malignancies; endometrial cancer is the most common extracolonic cancer followed by gastric, small intestinal, hepatic, pancreaticobiliary, ovarian, ureteral, and brain tumors. The biologic basis for the organ specificity is unknown. In these tumors, the second allele of the affected DNA mismatch repair gene is somatically mutated, thereby leading to biallelic gene inactivation.

The terms Lynch syndrome and hereditary nonpolyposis colorectal cancer have largely been used interchangeably over the years. Henry Lynch used the term hereditary nonpolyposis colorectal cancer in his early reports of this syndrome to emphasize the heritable nature of an inherited cancer predisposition. The current definition of HNPCC is primarily based on family history. Some families with attenuated forms of familial adenomatous polyposis syndrome (including MUTYH-associated polyposis) might fulfill the Amsterdam criteria (see below) for having HNPCC. Without knowledge of the family history, a diagnosis of HNPCC cannot be made. Lynch syndrome, on the other hand, is defined by the documentation of inherited inactivating mutations in the DNA MMR system, that is, the genetic basis can be confidently linked to a germline mutation in a DNA MMR gene. Without genetic testing, a diagnosis of Lynch syndrome cannot be made.

It is important to note that patients with Lynch syndrome/HNPCC harbor similar numbers of adenomatous polyps as the general population. These polyps are histologically indistinguishable from conventional adenomas. Many may appear as “flat” lesions endoscopically. An important distinction, however, is that complete loss of expression of 1 of the mismatch genes has been demonstrated in up to 88% of these adenomas. These polyps are also commonly seen at a younger age and their progression to malignancy can be relatively rapid, sometimes within as short a period as 2 years.

Sporadic MSI-CRC

Most MSI-CRCs are not due to Lynch syndrome, but are “sporadic” (Figure 1). With the discovery of MSI in Lynch syndrome, it is now known that this phenomenon occurs in approximately 10% to 15% of “sporadic” CRCs. However, unlike Lynch syndrome, the loss of the normal MMR function in sporadic cases is due to biallelic transcriptional silencing of the MLH1 gene secondary to promoter hypermethylation. Although sporadic MSI-CRCs share many phenotypic features with Lynch syndrome (discussed below), they have the tendency to develop in older patients and are relatively more common in women.

Sporadic MSI-CRC typically develops through the serrated pathway with the precursor lesion being the sessile serrated adenoma. An important molecular feature of this pathway is methylation of multiple regions of cytosine-guanosine dinucleotides, or CpG islands, within the promoter regions of genes and subsequent down-regulation of these genes. This is known as the CpG island methylator phenotype. Another important molecular feature of sporadic MSI-CRCs is the presence of BRAF mutations in 40% to 50% of cases.

Histologic Features of MSI-CRC

Given their closely related molecular changes, both inherited and sporadic MSI-CRCs share a number of phenotypic features. Many studies have identified histologic features that are more commonly seen in MSI-CRCs, such as the tendency to occur in the right side of the colon, medullary carcinoma phenotype, the presence of mucinous or signet-ring component, the presence of tumor-infiltrating and peritumoral lymphocytes, a “Crohn-like” inflammatory response, and a “pushing”
tumor border. Based on these features, various scoring systems have been published, such as the “MsPath score,” to help predict the probability of MSI-CRC occurrence.19

**METHODS OF TESTING FOR MSI-CRC**

**Molecular Testing**

Molecular testing relies on the evaluation of certain loci within the human genome that are known to harbor microsatellites. Given the variability in size for many of the microsatellites from one individual to another, the identification of the normal (germline) size of a particular microsatellite in the patient tested is usually needed. Hence, DNA is typically extracted from both normal and tumor tissue, which can be obtained from fresh tissue as well as formalin-fixed, paraffin-embedded tissue blocks. After polymerase chain reaction (PCR) amplification of the selected microsatellite, the size of PCR products obtained with normal DNA is considered the normal (germline) size of that microsatellite, and the PCR product obtained from the tumor tissue is compared to it. Microsatellite instability is defined as a change of any length due to either insertion or deletion of repeating units in a microsatellite within a tumor when compared to normal tissue. A panel of microsatellites validated by a National Cancer Institute (NCI) workshop, commonly referred to as the Bethesda panel, is considered the reference panel for clinical and research testing.30

Examples of MSI in 2 mononucleotide markers are shown in Figure 2, A (BAT25) and Figure 2, B (BAT26). In 2002, another NCI workshop added more to these guidelines, recommending the testing of additional mononucleotide markers in tumors with instability at only dinucleotide loci, as mononucleotide markers are more reliable in the identification of MSI-H tumors.31 The clinical significance of MSI-L and its distinction from MSS cancer is not clear. For practical purposes, in this article MSI-CRC and MSI-H CRC will be used synonymously.

**Immunohistochemistry**

There is typically complete loss of protein expression of at least 1 of the MMR genes in MSI-CRC. Most mutations involved in Lynch syndrome lead to unstable MMR proteins, and in sporadic MSI-CRC there is silencing of MLH1 with loss of protein expression. Since the DNA mismatch repair proteins are normally expressed in normal human tissues, particularly proliferating tissues, normal nuclear expression in crypt epithelium and lymphocytes serves as an internal positive control for stain quality. These features have resulted in the clinical diagnostic utility of conventional immunohistochemistry (IHC) testing with antibodies to the protein product of the 4 genes most commonly involved in MSI-CRC: MLH1, MSH2, MSH6, and PMS2.32–34

An abnormal result is the complete absence (loss) of nuclear immunoreactivity for 1 or more of those proteins
in the tumor. Understanding the interaction of these proteins is essential for the interpretation of the results. MLH1 recruits its binding partner PMS2 to the site of DNA repair. If the normal expression of MLH1 is lost (Figure 3, A), then PMS2 will also be lost (Figure 3, B) (while MSH2 [Figure 3, C] and MSH6 [Figure 3, D] will be preserved). The same interaction is seen for MSH2 and its binding partner, MSH6 (when MSH2 is lost, MSH6 will also be lost). In contrast, in the rare cases of germline mutations of either PMS2 or MSH6, only the protein affected is lost.\(^{35,36}\)

### Which Test to Use?

Regarding their performance as a screening tool for Lynch syndrome, both IHC and molecular MSI testing are considered quite sensitive and produce concordant results. In a study by Lindor et al.,\(^{32}\) combined use of MLH1 and MSH2 IHC achieved a sensitivity of 92.3% and a specificity of 100% for the identification of MSI-H tumors. The addition of MSH6 and PMS2 to the panel led to increased sensitivity.\(^{37}\) More recently, based on the heterodimer paring properties of the MMR proteins, a 2-antibody panel of PMS2 and MSH6 has been shown to be as effective as a 4-antibody panel in detecting DNA mismatch repair protein abnormalities.\(^{38,39}\) Although the sensitivity of molecular MSI testing has been reported to be as low as 58%, partially owing to the ineffectiveness of detecting MSH6 mutation carriers, more recent studies using mononucleotide markers show the high sensitivities, reaching up to 100%, with increased detection of MSH6 mutation carriers.\(^{40-44}\) Although very sensitive, both IHC and molecular MSI testing have their limitations; therefore, if questionable results are obtained by either method, they should be confirmed with alternative tests.

From a practical point of view, IHC is readily available in most diagnostic anatomic pathology laboratories, while molecular testing is not. A key difference between the use of IHC for MMR proteins and MSI molecular testing is the ability of IHC to evaluate individual gene defects. Molecular testing revealing an MSI-H result may indicate either sporadic MSI-CRC or Lynch syndrome. The same is true if IHC testing shows loss of expression of MLH1. If the patient wishes to find out whether or not he or she has Lynch syndrome, then additional testing is needed (discussed below). However, IHC testing showing loss of MSH2 or individual loss of PMS2 or MSH6 are exclusively described for patients with Lynch syndrome.\(^{35,36}\) Based on these observations, there is ongoing

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**Figure 3.** Immunohistochemical staining for the mismatch repair proteins (A) MLH1, (B) PMS2, (C) MSH2, and (D) MSH6. In this example, the colorectal cancer (right side of each panel) shows loss of MLH1 and PMS2, while both stains show intact nuclear staining in stromal lymphocytes and normal colonic crypts (left side of each panel). MSH2 and MSH6 show intact staining in tumor and normal tissue. A stain should not be interpreted as lost if the internal control is absent or suboptimal (original magnifications ×200).
controversy surrounding whether or not IHC testing should be regarded as “genetic testing.” Some clinicians/pathologists do not consider it a genetic test, but rather a test of tumor phenotype, and as such, would not require informed consent before initiating MMR IHC testing. For those who do consider IHC for MMR proteins a genetic test, informed consent would be required before testing. At this point this is an ongoing debate and the type of testing offered, as well as the issue of consent, is variable from one institution to another.

CLINICAL APPLICATION

Value for Identifying MSI in CRC

Most authors agree that the primary value for the identification of MSI-CRC is its role as a screening tool for identification of probands with Lynch syndrome. This has important management implications for the patients as well as their families. For patients with confirmed Lynch syndrome–related CRC, owing to the high risk of a second CRC, the option of extensive resection should be discussed. After a subtotal colectomy, intensive surveillance of MSI on response to therapy. Although the verdict is still out, MSI-H/MMR protein–deficient CRC may be associated with better survival rates in subsets of patients with colon cancer. This effect may be related to the CpG island methylator phenotype (widespread promoter methylation). MSI-H tumors may also respond less favorably to 5-fluorouracil–based chemotherapy. However, the evidence in this regard is still conflicting and further studies are needed with more current therapeutic regimens to better determine the effect of MSI on response to therapy.

Targeted Screening

With targeted screening, patients with clinical or pathologic features that are considered high risk for Lynch syndrome are selected for testing. Since Lynch syndrome is the most common hereditary syndrome that predisposes patients to CRC, guidelines were created to detect families with this condition. The first major criteria were set by the International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer after their meeting in Amsterdam in the summer of 1990. These criteria (now known as Amsterdam criteria I), addressed only personal and family histories of CRC. Acknowledging the significance of extracolonic tumors in this syndrome led to an update in these criteria at the 1998 group meeting, now known as Amsterdam criteria II. However, with the rapid accumulation of the understanding of the molecular basis of Lynch syndrome, it was clear that the sensitivity of these criteria for detection of patients with Lynch syndrome was too low (as low as 40%). This observation, together with the identification of pathologic features related to MSI-CRC (discussed above), led to NC I workshops that resulted in the “Bethesda guidelines” to identify patients who would benefit from clinical testing for Lynch syndrome. The application of the Bethesda guidelines has limitations in daily practice owing to the lack of consistency in documenting a reliable family cancer history. Subsequently, several prediction models, such as the MSPath score, have been developed to further refine the selection of cases for genetic testing.

Screening All Patients With CRC

One weakness to Bethesda guidelines and most prediction models is the use of age as a decision point. There is evidence in the literature that patients with Lynch syndrome can have cancer when they are older than 50 or 60 years. Several recent studies have demonstrated the importance of screening all patients with CRC. Among the larger studies addressing this issue is the Columbus area Lynch syndrome study. This population-based study included 1566 patients with CRC. All patients underwent MSI analysis with a modified “Bethesda panel” of 5 or 6 microsatellites. For the first 1066 patients, IHC for 4 MMR proteins was performed on all MSI-H and MSI-L CRCs and an additional set of 109 MSS cases with “high-risk” clinic features. For the final 500 patients, MMR IHC was performed on all cases with available tumor. Abnormal results were followed by additional specific testing for the identification of Lynch syndrome. Forty-four patients with Lynch syndrome (2.8%) were identified. Fifty percent were older than 50 years and 28% failed to meet revised Bethesda criteria. These results were supported by a French study of 216 patients with CRC. These and other studies led the Evaluation of Genomic Applications in Practice and Prevention Working Group to recommend “offering genetic testing for Lynch syndrome to individuals with newly diagnosed CRC to reduce morbidity and mortality in relatives.”

DIAGNOSING LYNCH SYNDROME

Most inherited cases of CRC are detected on the basis of a strong family history of the disease. In familial adenomatous polyposis, index cases can be detected because they typically present at an early age with numerous (hundreds) of adenomatous polyps. Patients with Lynch syndrome tend to develop cancer at a younger age, but the cancers are not associated with a polyposis syndrome. Hence, in Lynch syndrome, detection of index cases is challenging and requires the use of specific testing.

The demonstration of a germline mutation is the gold standard for the diagnosis of Lynch syndrome. Germline testing is typically offered after one of the screening tests described above flags the patient as being at high risk for Lynch syndrome. Although loss of expression by IHC of MSH2 or isolated loss for MSH6 or PMS2 are almost exclusively seen in patients with Lynch syndrome, further germline testing of the suspected gene must be performed to identify the specific mutation. On the other hand, molecular testing showing MSI-H by PCR or IHC showing loss of MLH1 expression could be seen in either sporadic MSI-CRC or Lynch syndrome. In these cases, an additional layer of testing may be performed on the tumor before proceeding to germline analysis. These tests include MLH1 promoter methylation and/or BRAF mutation analysis. Given the technical difficulty related to methylation analysis, testing for BRAF mutations is more commonly performed. Examples of tests for BRAF
mutations are shown in Figure 4, A (melting curve analysis of a BRAF wild-type CRC); Figure 4, B (melting curve analysis of a BRAF-mutated CRC); and Figure 4, C (sequence analysis showing a point mutation in the BRAF gene). BRAF mutations are present in about 40% to 50% of sporadic MSI-H tumors and are absent in Lynch syndrome. Hence, a “positive” BRAF mutation virtually excludes the possibility of Lynch syndrome. Patients with absent BRAF mutations may then proceed to germline testing if the clinical suspicion for an inherited tumor remains high.

Germline testing is performed on DNA isolated from peripheral blood mononuclear cells. There are no well-described mutational “hot spots.” Hence, full gene sequencing is usually required. More than 90% of Lynch syndrome cases that have been genetically characterized show germline mutations in MSH2 or MLH1. However, the selection of which gene to test is dependent on the abnormality identified on the initial screening test. It is also important to recognize that failure to identify a mutation does not completely rule out Lynch syndrome. If a specific germline mutation is identified in the patient, then the close family members can proceed with testing.

**KRAS, BRAF, AND ANTI–EPIDERMAL GROWTH FACTOR RECEPTOR THERAPY**

The use of targeted therapy was first applied to CRC in 2004 when it was reported that cetuximab, an anti–epidermal growth factor receptor (EGFR) monoclonal antibody, had activity in advanced disease. Since it was known that EGFR is often upregulated in colorectal cancer, positive expression of the EGFR protein, as assessed by IHC, was initially required before cetuximab was administered. It was soon discovered, however, that there was no correlation between EGFR protein expression in the tumor and response to therapy. Cetuximab was therefore administered as indicated (to patients with metastatic disease refractory to first-line therapy) without a companion theranostic marker. By 2008, several studies reported the predictive value of a KRAS mutation for patients with colorectal cancer treated with anti-EGFR monoclonal antibodies, including cetuximab (Erbitux,
Bristol-Myers Squibb, New York, New York) and panitumumab (Vectibix, Amgen, Thousand Oaks, California). Mutations at codon 12/13 of the KRAS gene were found to be associated with resistance to cetuximab therapy, worse prognosis, and poor survival of patients with metastatic CRC.\textsuperscript{60–65} A diagram of the normal EGFR signaling pathway is shown in Figures 5, A; the pathway blocked by anti-EGFR monoclonal antibodies is shown in Figure 5, B; and the constitutively activated pathway due to a mutation in KRAS or BRAF is depicted in Figure 5, C.

On the basis of these and other studies, The American Society of Clinical Oncology (ASCO) released its provisional clinical opinion on the use of KRAS gene mutation testing in patients with metastatic colorectal cancer to guide treatment with the anti-EGFR monoclonal antibodies cetuximab and panitumumab. The ASCO provisional clinical opinion states: “Based on systematic reviews of the relevant literature, all patients with metastatic colorectal carcinoma who are candidates for anti-EGFR antibody therapy should have their tumor tested for KRAS mutations in a CLIA-accredited laboratory. If a KRAS mutation in codon 12 or 13 is detected, then patients with metastatic colorectal carcinoma should not receive anti-EGFR antibody therapy as part of their treatment.”\textsuperscript{66} Similarly, the National Comprehensive Cancer Network (NCCN) now states that KRAS mutations occur in 9\% to 22\% of CRCs\textsuperscript{77,78} and are mutually exclusive with KRAS mutations. Of metastatic CRCs that are found to be KRAS wild type at codons 12/13, approximately 15\% may harbor BRAF mutations and show resistance to anti-EGFR therapy.\textsuperscript{72} Although not required, the NCCN now states that BRAF testing in KRAS nonmutated cases should be considered. Testing for PI3KCA/PTEN deregulation appears to be on the horizon; further study into the impact of these genes on anti-EGFR therapy is ongoing.

CONCLUSION

Molecular diagnostic testing is currently used in cases of CRC both to aid in the diagnosis of an inherited cancer predisposition syndrome (Lynch syndrome) and to predict response of targeted anti-EGFR therapy in cases of advanced (metastatic) disease. Since CRC can arise through different molecular pathways, including the chromosomal instability pathway, the MSI pathway, or the CpG island methylator phenotype pathway, CRC should no longer be viewed as one disease. Today, when a pathologist reviews a case of colorectal cancer, a cascade of events begin. After histologic diagnosis and appropriate staging of the tumor comes selection of a formalin-fixed, paraffin-embedded tumor section for molecular...
testing. The molecular diagnostic tests that follow will depend upon each individual institution’s algorithm (Figure 6). For MSI screening, testing of all tumors, regardless of patient age or family history, is on the horizon. To exclude sporadic MSI cases and to detect potential cases of Lynch syndrome, tumors may be tested for either MSI by molecular methods or MMR protein deficiency by immunohistochemistry, followed by BRAF mutational analysis and/or MLH1 methylation testing. On the basis of these test results, if the possibility of Lynch syndrome exists, the patient should be referred for genetic counseling and confirmatory germline mutational analysis should be performed, as indicated. If the CRC is at an advanced state (metastatic disease), the tumor will also be tested for KRAS mutations, and if found to be wild type, then for BRAF mutations. If either KRAS or BRAF genes are mutated, anti-EGFR (epidermal growth factor receptor) therapy is currently not indicated. Abbreviations: MSS, microsatellite stable; mut, mutated; PCR, polymerase chain reaction; wt, wild type.

Figure 6. Molecular testing algorithm. In this example, primary colorectal cancers (CRCs) (resection or biopsy specimens) are first tested for microsatellite instability (MSI) by polymerase chain reaction. If found to be MSI-high (MSI-H), BRAF mutational analysis is performed. If BRAF is wild type, the clinician or genetics counselor can request immunohistochemistry (IHC) for the mismatch repair proteins. Immunohistochemistry may be used as the initial screening test instead, and MLH1 methylation analysis can be added if the tumor is MSI-H, BRAF wild type, and shows loss of MLH1 by IHC; if the tumor lacks MLH1 hypermethylation, there is likely a germline mutation of MLH1. Advanced CRCs can be first tested for KRAS mutations, and if wild type, then for BRAF mutations. If either KRAS or BRAF genes are mutated, anti-EGFR therapy is currently not indicated. Abbreviations: MSS, microsatellite stable; mut, mutated; PCR, polymerase chain reaction; wt, wild type.

References
The genetics of HNPCC: screening in colorectal adenocarcinoma.

- 2008;66(8):374–381.
- 2008;66(8):374–381.


