Molecular Tumor Testing for Lynch Syndrome in Patients With Colorectal Cancer

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Abstract
Lynch syndrome (LS), first recognized by the characterization of “Family G”\(^1,2\) and other “cancer families”\(^3\) in the early 20th century, is a prime illustration of how identification of familial cancer syndromes directly impacts individual risk stratification. LS accounts for an estimated 2% to 3% of all colorectal cancers (CRCs), and well-known diagnostic criteria have been established. In 1990, the International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer developed the Amsterdam criteria for the diagnosis of LS, which relied solely on family history. These criteria were modified in 1999 as the Amsterdam II criteria and are as follows: 3 or more relatives with a Lynch-associated cancer (one of whom is a first-degree relative of another and excluding familial adenomatous polyposis); Lynch-associated cancer in 2 or more generations; and at least 1 cancer diagnosed at an age younger than 50 years.

As the molecular basis of LS was elucidated, the Bethesda guidelines were developed to extend the recognition of LS by analyzing tumors for the characteristic microsatellite instability (MSI) phenotype. Bethesda criteria suggest that tumors should be tested for MSI if any of the following criteria are met: 1) CRC at an age younger than 50 years; 2) synchronous or metachronous CRC or another Lynch-associated tumor; 3) CRC with MSI-high (MSI-H) histology (presence of tumor-infiltrating lymphocytes, Crohn’s-like lymphocytic reaction, mucinous/signet ring differentiation, or medullary growth pattern) if the patient is younger than 60 years; 4) CRC in a patient with at least 1 first- or second-degree relative with a Lynch-associated tumor; 3) CRC with MSI-high, MSI-H histology (presence of tumor-infiltrating lymphocytes, Crohn’s-like lymphocytic reaction, mucinous/signet ring differentiation, or medullary growth pattern) if the patient is younger than 60 years; 4) CRC in a patient with at least 1 first- or second-degree relative with a Lynch-associated tumor if 1 of the tumors is diagnosed at younger than 50 years; and 5) CRC in a patient with at least 2 first- or second-degree relatives with Lynch-associated tumor, regardless of age.\(^4\) The strategies for identifying those with LS continue to evolve as appropriate cancer screening and surveillance programs are initiated for at-risk individuals.
Molecular Basis and Identification of LS

LS is caused by mutations in DNA base mismatch repair (MMR) genes, leading to colorectal and extracolonic malignancies. MutS homolog 2 (MSH2) and MutL homolog 1 (MLH1) were the first genes identified to cause LS, followed by postmeiotic segregation 2 (PMS2) and MutS homolog 6 (MSH6).

Initial studies determined MSI status in tumors using analysis of extracted DNA for the presence of polymorphisms in microsatellite repeats from specific markers (loci). Most studies agree that the presence of instability in 30% or more of the total number of markers tested qualifies the carcinoma as MSI-H, whereas anything less classifies the tumor as having low levels of MSI (MSI-L), with absence of instability considered microsatellite stable (MSS). Using this cutoff, approximately 8.5% to 10.0% of CRC would be labeled MSI-L, and 15.0% to 19.0% would be MSI-H. Over the years, immunohistochemical (IHC) staining of tumors for loss of expression of the common DNA MMR proteins has become a reasonable surrogate for formal MSI testing. This technique, which can be performed in most pathology laboratories, involves staining tumors with antibodies directed against MLH1, MSH2, PMS2, and MSH6 proteins to evaluate loss or retention of protein expression specifically in tumor cells, relative to nearby control normal cells. Hence, tumor cells that show absent staining in any of these proteins would be identified as having MSI.

Using IHC as a screening test, fewer than 2.7% to 3.0% of tumors are estimated to have discordant MSI results (IHC showing retention of protein expression, but DNA analysis showing MSI-H). Germline mutations or MLH1 promoter hypermethylation were eventually discovered in only approximately half of these discordant cases, even though these patients are often clinically treated as having a tumor with MSI. Although the loss of the MSH2/MSH6 pair strongly suggests a germline mutation in MSH2, and thus a diagnosis of LS, loss of MLH1/PMS2 is more often seen in the sporadic setting. Therefore, if IHC reveals a loss of MLH1/PMS2, BRAF V600E mutation testing is warranted: presence of this missense mutation indicates a sporadic case of epigenetic MLH1 silencing through promoter hypermethylation, whereas absence of the BRAF mutation suggests a germline mutation in MLH1, and thus LS.

Depending on the population and study design, the percentage of carcinomas that show loss of DNA MMR protein expression is approximately 15% (and reported to be as high as 30%–40%), with most of those (50%–75%) showing MLH1/PMS2 loss, and a smaller proportion (15%–40%) showing MSH2/MSH6 loss. A small group (3%–8%) may show loss of MSH6 alone (with retention of MSH2 expression), and even fewer show loss of PMS2 expression alone. The latter possibilities have prompted some authors to recommend that a 2-antibody panel (ie, PMS2 and MSH6 only), rather than the full 4-antibody panel, may be more efficient and cost-effective in identifying tumors with loss of DNA MMR protein expression, but quality control issues have precluded that recommendation from taking hold (PMS2 and MSH6 are invariably weaker stains than MLH1 and MSH2). Specifically, it has been reported that approximately 4% of MSI-H tumors that arise in the setting of an MSH6 gene mutation may retain IHC expression of the MSH6 protein (along with the other 3 MMR proteins), and thus remain undetected if only IHC is used as a screening method.

For all of these reasons, most authors recommend using a panel containing all 4 MMR proteins during IHC testing as a surrogate for MSI. Overall, IHC is faster and easier than DNA testing for MSI, and has quickly replaced it during screening of CRC specimens. The revised Bethesda guidelines establish clinicopathologic criteria that, given their presence, should prompt pathologists to test for MMR deficiency. However, studies have indicated that using only these criteria for testing would have missed diagnosing up to 70% of tumors with MSI and up to half of patients with LS, whereas others have found the histologic features to be only 67% sensitive in correctly predicting tumor MSI status. This shows how many individuals with MMR-deficient CRCs do not meet Bethesda guidelines, which has prompted many investigators to institute testing for MSI using IHC on all CRC, which is also known as universal testing.

Importance of Identifying LS

Once the molecular basis of LS was defined, it became clear that many families who met Amsterdam and/or Bethesda criteria did not have LS, and that LS could occur in families that did not meet these criteria. In addition, not all tumors that dem-
Because colonoscopic screening for CRC has recently pooled data from the EPICOLON II cohort, albeit only including MLH1 and MSH2 IHC. The investigators concluded that universal MMR testing should be implemented to improve detection of LS.

Moreira et al11 recently pooled data from the EPICOLON cohort with those from 3 other large cohorts to compare universal screening with alternate selected strategies. In addition to the EPICOLON cohort, data from the Cancer Family Registry, Clinical Cancer Genetics Program of the Ohio State University, and The Department of Medical Genetics at the University of Helsinki, Finland were included in the study sample. Results of tumor MMR testing for all patients with CRCs were compared with results of testing based on the presence of at least 1 Bethesda criterion, Jerusalem recommendations (MMR screening in all CRC occurring in individuals <70 years of age),22 or a specific multivariate model. Predictably, universal testing had the highest sensitivity for detecting LS (100%), whereas 12% to 15% of cases of LS were missed using the Bethesda guidelines or Jerusalem recommendations. However, the specificity of universal testing was lower (93.0%) than that of the strategies using the Jerusalem guidelines (96.7%) and Bethesda criteria (97.5%), with a positive predictive value of 24.5% and a 7.0% false-positive rate (vs 32.6% and 4.5%, respectively, for tumors meeting Jerusalem guidelines or ≥1 Bethesda criterion). The authors suggest that the increased sensitivity of universal testing justifies its use as a screening program for LS.

The Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group also recommended that all patients with newly diagnosed CRC tumors should be offered laboratory testing, independent of family history or age of diagnosis, because collection of family history information is onerous and unreliable in clinical practice.32 Their evidence-based review and recommendations assumed 99.50% sensitivity and 99.96% specificity for sequencing tests for MMR genes, but only a sensitivity of 83.0% and specificity of 88.8% for IHC, and the combined IHC/MSI approach compared with the Bethesda guidelines, and additionally noted that 14.3% of patients with LS did not fulfill the Bethesda guidelines. Results of this study were somewhat at odds with those of a previous study from the same group,10 which indicated similar sensitivity for the Bethesda guidelines and IHC/MSI analysis in the EPICOLON I cohort.

Clinical Applications of Reflex Testing

To compare the sensitivity of the Bethesda criteria with that of universal testing for determining MMR status, Pèrez-Carbonell et al28 used a combination of IHC and MSI testing in the EPICOLON I and EPICOLON II29 cohorts that had a low prevalence of LS. In this study, IHC was followed by directed germline analysis based on IHC results, and MSI-positive tumors were analyzed for all 4 MMR genes. Additional testing for EpCAM rearrangements was performed if loss of MSH2 IHC was noted without a corresponding MSH2 mutation. The investigators showed improved sensitivity for detecting LS with
sensitivity of 89.0% and specificity of 90.2% for MSI testing.\textsuperscript{33} Based on these recommendations, Mvunudura et al\textsuperscript{34} performed a cost-effectiveness analysis of 4 reflex genetic testing strategies for LS: 1) IHC for 4 MMR genes, followed by MMR gene sequencing or rearrangement testing if 1 or more proteins were absent; 2) IHC for 4 MMR genes as in strategy 1, but to include BRAF V600E mutation testing if MLH1 was absent, and only proceed with MMR sequencing if BRAF mutation testing was negative (all others would proceed as in the first strategy); 3) MSI testing followed by MMR sequencing/rearrangement testing if MSI-H; and 4) direct sequencing and rearrangement studies of MMR genes on all CRC. The strategy using IHC and BRAF testing was the most cost-effective, with a cost of $25,000 or less per life-year saved relative to no testing, and $40,000 or less per life-year saved relative to testing only patients younger than 50 years. They concluded that, if available in the local laboratory, IHC testing (+/− BRAF testing) is the most cost-effective strategy from a national health perspective. They further suggested that their estimates of uptake among family members were very conservative, which would make universal testing an even more cost-effective approach.

A subsequent cost-effectiveness analysis of universal testing methods in new diagnoses of CRC by Ladabaum et al\textsuperscript{35} used a Markov model, and incorporated differential risk for men and women, and risk for endometrial, ovarian, and colorectal cancers. They found that the overall number of relatives tested per proband identified from universal testing was crucial; meeting a threshold of $50,000 per life-year gained required testing of 3 to 4 relatives for each proband identified. The preferred method was IHC followed by BRAF V600E testing; improvements in cost-effectiveness were demonstrated with upper-limit of age cutoffs, but overall strategies that incorporated IHC performed better than those with universal sequencing or MSI testing. However, MSI testing may be an alternative form of screening in areas where specially trained pathologists are not available.\textsuperscript{36}

The cost-effectiveness of a molecular tumor testing strategy is critical to determine general uptake of the testing strategy. A recent study showed that some form of molecular tumor testing (either in select CRC specimens or universally) is being used at 71% of NCI-designated cancer centers and 15% of community-based hospitals.\textsuperscript{25} This suggests that molecular tumor testing for LS is likely to expand across all centers, necessitating an infrastructure designed to support the clinical volume. By approaching positive results systematically, the number of patients (and thus families) evaluated will be maximized. In the absence of this process, many cases will likely remain undetected.

Universal IHC testing was implemented in 2009 at the authors’ institution. A recent preliminary (unpublished) survey from this institution revealed that many physicians who had received an MSI-positive result were unaware of ever seeing such a report. Even those who acknowledged receiving a pathology report of a tumor with MMR deficiency had a variety of responses regarding how to act on the findings.\textsuperscript{37} This suggests that implementation of reflex testing alone might be insufficient to identify high-risk individuals. Thus, any formalized process should specifically include the following steps: 1) identifying positive results; 2) contacting the physician who is responsible for the affected individuals; 3) genetic counseling and testing if available and desired; and 4) if LS confirmed, evaluating for extracolonic tumors and contacting family members to counsel and risk stratify.

**Management of Positive Results**

Although the sequence of events conducted after positive molecular tumor testing results is not based on concrete guidelines, and different approaches could certainly be reasonable, developing a centralized process is often most helpful. One approach is to appoint a specific individual or individuals who can streamline the process to ensure that all patients are followed appropriately, because relying on individual providers may lead to missed opportunities for tracking patients and making referrals to genetics counselors as appropriate. In a survey conducted by Beamer et al\textsuperscript{38} among NCI-comprehensive cancer centers, 64.7% relied on the result recipient to initiate genetics referral, and 52.9% of these programs reported problems with patient follow-up of these referrals.

Typically, the health care provider who submits a pathology specimen is the one who receives the result. This provider may be the only one who sees a report suggesting an MMR-deficient cancer. These providers are generally gastroenterologists and surgeons, and their understanding of the implication of
MSI can vary. Heald et al\(^8\) reviewed the process of universal screening for MSI/IHC at a major academic institution and compared 3 different approaches to follow-up of abnormal results: 1) genetics referral initiated by the result recipient; 2) notification of the result recipient by a genetics counselor, who received an alert of all abnormal results; and 3) referral directly from the geneticist. More patients underwent genetics counseling with the third approach compared with the first (72% vs 32%), suggesting that standardizing the follow-up and review process to include automatic inclusion of selected experts may increase the number of patients who undergo appropriate evaluation. Centralizing this process will also aid in data collection and provide verification that all patients are being followed.

Although this approach may increase the number of referrals to genetic consultation, some drawbacks may limit the applicability of this strategy across all institutions. This strategy relies on access to qualified genetics counselors, because germline testing in the absence of these experts may lead to an inadequate understanding of results, and possibly misuse of this testing.\(^39\) In settings where genetics counselors are not readily available, gastroenterologists, oncologists, and other providers will need to be educated and relied on to act on positive results. Alternatively, patients can be referred to cancer centers where genetic counselors are available. Rigorous protocols as seen in the above studies are unlikely to be initiated in most health care settings. In addition, centralizing the process of follow-up might require the use of a sophisticated electronic medical record, which may not be available at all health care settings. Although the applicability of this strategy might remain limited, the institutions that have already implemented reflex testing would likely benefit from defining a standardized process of following up abnormal results.

One critique of molecular tumor testing for LS is the undefined role of informed consent, because this could be considered a type of genetic testing (which does require consent).\(^60\) Although it is accepted that germline testing requires informed consent, testing for somatic mutations has not been explicitly addressed. Currently, it is generally accepted that because the evaluation of a colon cancer specimen for somatic mutations can have a direct impact on treatment, this testing does not require informed consent.\(^\text{18,19,25,41}\)

Future Directions

Universal molecular testing of CRC tumors to identify individuals with LS is gaining popularity, likely because of the unreliability of traditional diagnostic criteria. Although this strategy offers hope that more patients and family members will be recognized at a stage when appropriate surveillance can be implemented, further data are required to fully determine whether this strategy is cost-effective when applied across all health care settings. A successful approach for molecular tumor testing of all CRCs for LS will rely on an infrastructure that is capable of handling positive results, followed by rigorous implementation of recommended surveillance and management strategies.

References

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