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Laboratory Assays in Evaluation of Lynch Syndrome in Patients with Endometrial Carcinoma

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Lynch Syndrome

Lynch Syndrome occurs due to a germline mutation in a gene corresponding to a family of DNA mismatch repair (MMR) proteins, MLH1, MSH2, MSH6 and PMS2. The hallmark cancers for Lynch Syndrome are colorectal adenocarcinoma and endometrial carcinoma, while less common cancer types include ovarian carcinoma, urothelial carcinomas of the ureter/renal pelvis, duodenal adenocarcinoma, and gastric adenocarcinoma. Loss of DNA MMR protein function typically results in high levels of DNA microsatellite instability (MSI). In 15–20% of all sporadic endometrial carcinomas, MLH1 immunohistochemical loss and MSI result from *MLH1* gene promoter methylation with subsequent transcriptional silencing^{1–5}.

For most hereditary cancer syndromes, affected individuals are identified on the basis of recognition of a constellation of clinical features, such as young age of cancer onset and a strong family history of characteristic cancers, with subsequent germline sequencing of the suspected affected gene. However, tumor tissue testing in the pathology laboratory is a key component of Lynch Syndrome diagnosis, involving MMR immunohistochemistry, MSI analysis and *MLH1* methylation analysis.

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The Authors have nothing to disclose

Tumor testing and pitfalls in test interpretation

Immunohistochemistry

Immunohistochemistry for MMR proteins is carried out using commercially available antibodies that work quite reliably. Gene mutation of *MMR* genes or methylation of the *MLH1* gene promoter typically results in loss of immunohistochemical expression of the corresponding protein. Complete absence of nuclear expression should be observed in order for a tumor to be considered as having a loss of an MMR marker. Strong nuclear staining in the surrounding endometrial stroma, myometrium, lymphocytes or normal endometrium should serve as an internal positive control (Figure 1). The MSH2 and MSH6 proteins and the MLH1 and PMS2 proteins act as functional pairs⁶. Therefore, when MLH1 protein expression is lost (due to mutation of the *MLH1* gene or methylation of *MLH1* gene promoter), typically there is secondary loss of PMS2 protein expression. Mutation of the PMS2 gene is associated with loss of PMS2 protein but retained MLH1 immunohistochemical expression. Similarly, mutation of the *MSH2* gene typically results in immunohistochemical loss of MSH2 and MSH6 proteins. On the other hand, mutation of *MSH6* gene results only in MSH6 protein loss, while MSH2 protein expression remains intact.

Regarding MMR immunohistochemistry reporting recommendations, it is important to note that, for the vast majority of cases, the percentage or intensity of staining is not relevant, and the interpretation result should be either positive or negative. Terminology such as “focally positive”, “patchy staining,” “weakly positive”, “positive in X% of cells”, or “equivocal staining” should be avoided. If tumor staining is negative, it should be indicated that internal control stromal cells/normal endometrium are positive.

Several pitfalls in the interpretation of MMR immunohistochemistry exist (Figure 2). Most commonly, false negative nuclear tumor staining will occur in the setting of an inadequate internal positive control. On the other hand, immunohistochemical staining of the tumor cells may be focal or relatively weak. In most cases, this represents genuine nuclear staining. Both of these problems may be resolved by repeating the immunohistochemistry with consideration of prolonging the antigen exposure time or using a different tissue block from the same specimen. Another immunohistochemical issue involves cytoplasmic tumor staining, regardless of the presence or the absence of nuclear staining, especially when the tissue has previously been frozen for the purposes of intraoperative consultation. Cytoplasmic staining should be disregarded in the evaluation of MMR immunohistochemistry. Finally, endometrial stroma or tumor infiltrating lymphocytes may cause difficulties in MMR immunohistochemistry interpretation. Typically, however, these cells will be relatively focal and will appear within a background of immunohistochemically negative nests and sheets of tumor cells. Awareness of this pattern and correlation with the corresponding H&E section should resolve this particular diagnostic dilemma.

Recently, a heterogeneous pattern of MSH6 expression was described in rare (0.17%) cases of colorectal, endometrial and sebaceous tumors⁷. In this pattern, areas of strong MSH6 expression are juxtaposed with areas of complete loss of expression. In some of these cases, MSH2 expression was subtly diminished as well. The cases showed somatic instability

within the C8 repeat region of the *MSH6* gene along with a normal *MSH6* in the germline. In addition, some of the cases exhibited MLH1/PMS2 loss without evidence of *MLH1* promoter methylation. Therefore, heterogeneous expression of MSH6 is not a typical feature of Lynch Syndrome, but it does not exclude the possibility of germline mutations in other *MMR* genes. The authors have noted similar heterogeneity of MLH1/PMS2 immunohistochemistry, but at this time there is inadequate follow-up information regarding the outcomes of these cases (Figure 3).

Microsatellite instability and MLH1 methylation analyses

Microsatellites are sequences of DNA composed of repeating units of one to six base pairs in length. MSI arises as a result of uncorrected errors in DNA replication due to faulty MMR protein function. MSI analysis is a PCR-based test that requires DNA from both the endometrial tumor and normal non-tumor tissues, such as histologically normal cervix, myometrium, or ovary. Appropriate areas of tumor and non-tumor tissue are typically circled on a slide by a pathologist, and DNA from tissue in the corresponding paraffin blocks will be extracted. A panel of 7 markers recommended by the NCI⁸ (BAT25, BAT26, BAT40, D2S123, D5S346, D17S250, and TGF- β R2) is used to detect changes in the number of microsatellite repeats in the tumor compared to normal tissue (Figure 4). Tumors with an allelic shift in 3 or more microsatellites in the panel are considered MSI-high, while tumors with no allelic shift in all 7 microsatellites are designated as microsatellite-stable (MSS). Tumors with allelic shift in only 1 or 2 microsatellites are considered MSI-low.

For MSI-high tumors with loss of MLH1 protein by immunohistochemistry, a PCR-based *MLH1* promoter methylation analysis should be performed (Figure 5). In this assay, extracted tissue DNA (typically from the same sample used in the MSI assay) is exposed to a bisulfite compound, which converts unmethylated cytosines to uracil, whereas methylated cytosines are resistant to this conversion. This in turn allows the design of different PCR primers that can distinguish the two types of sequences. *MLH1* promoter methylation and gene silencing is the primary cause of MSI in sporadic endometrial cancers⁹.

While immunohistochemistry on a specific tissue block may not work well due to sub-optimal fixation or tissue processing, MSI and *MLH1* methylation analyses are typically not affected as much by and can still yield excellent results. The most common problem involving these analyses is an insufficient amount of tumor in the specimen, so that MSI or *MLH1* promoter methylation is not detected. By recording the approximate amount of tumor present in a section submitted for molecular testing, cases at risk for a false negative result of this type may be identified upfront. In instances of tumor heterogeneity or where a significant amount of non-tumor tissue accompanies tumor tissue in the material submitted for analysis, detection of the molecular abnormality is generally interpreted as evidence of this abnormality being associated with the tumor. Amplification errors in MSI analysis are relatively uncommon. In a large study of colorectal, endometrial and other Lynch Syndrome associated tumors¹⁰, only 0.6% microsatellites amplified suboptimally, with failure at no more than one microsatellite per tumor. In only a few of these cases would successful amplification of the missing microsatellite have led to re-classification of the MSI analysis result (ie. MSS to MSI-low or MSI-low to MSI-high).

Discrepancies between tissue testing and genetic testing

Known MMR gene mutations and ambiguous or normal tissue testing results

In some patients with known mutations in the *MMR* genes, the results of MSI analysis and/or MMR immunohistochemistry may be conflicting. For example, full-length but non-functional MMR proteins resulting from missense mutations in *MMR* genes may yield MSI-high tumors with intact expression of MMR proteins by immunohistochemistry. Tumors of other patients with known *MMR* gene mutations may have MMR loss by immunohistochemistry, but be MSI-low or even MSS. In comparison to colorectal carcinoma, endometrial tumors exhibit a lower proportion of unstable microsatellite markers (0.27 for endometrium versus 0.45 for colon per average tumor), have shorter allelic shifts in BAT loci and a greater proportion of tumors which are MSS (23% of endometrial versus 11% of colon cancer cases)¹¹. In particular, patients with *MSH6* germline mutations tend to have tumors which are disproportionately MSI-low or MSS^{12–14}. Furthermore, MSI analysis of paired synchronous ovarian and endometrial carcinomas revealed differing patterns of microsatellite allelic shift, despite the fact that the ovarian and endometrial tumors in each pair have lost expression of the same DNA MMR protein by immunohistochemistry¹⁵. It has been suggested that target genes of MSI are tissue-specific, and that this may be the source for differing patterns of MSI across different tumor types¹⁶. From a tissue testing perspective, these biological phenomena may generate discrepancies between immunohistochemistry, MSI analysis and mutation status, particularly when non-colorectal tumors are concerned.

Abnormal tissue testing results and no detectable MMR gene mutations

Cases may occur where MSI and MMR immunohistochemistry are both suggestive of Lynch Syndrome, but the genetic analysis does not reveal a pathologic mutation in the *MMR* genes. In fact, several population based studies using tumor tissue testing methods, including immunohistochemistry and MSI analysis, have demonstrated that the proportion of endometrial cancer patients identified as having possible Lynch Syndrome is as high as 6–10%^{17–19}. This is significantly higher than the value of 1–2%, which represents the proportion of patients with detected germline mutations^{4,20,21}.

While there are *MMR* gene variants of undetermined significance, which may in the future be reclassified as pathologic mutations with accumulation of experience, there are several other explanations for discrepancies between tissue and genetic testing results that have come to light from recent studies. For example, 5% and 20–25% of cases with *MSH2/MSH6* immunohistochemical expression defects and a non-detectable germline mutation in the *MSH2* and *MSH6* genes can be accounted by the presence of *MSH2* inversions (exons 1–7) and *EPCAM* germline mutations, respectively^{22,23}. In the latter instance, mutations in the 3' end of the epithelial cell adhesion (*EPCAM*) gene, which is located upstream of the *MSH2* gene, results in hypermethylation of the *MSH2* promoter and thus transcriptional silencing of an otherwise normal *MSH2* gene²³. Bi-allelic somatic (tumor) DNA mutations in MMR genes have also recently been uncovered in 52–69% of colorectal and endometrial tumors suspected for Lynch Syndrome based on tissue testing but without detectable germline mutations^{24–26}. Interestingly, one of these studies found the majority of somatic mutations

not to be the same as typical germline mutations, all tumors to have a hypermutator phenotype (>12 mutations/Mb), and 23% of the cases to have associated mutations in the catalytic subunit of the DNA polymerase epsilon (*POLE*) gene.²⁵ It is not clear at this time whether the *POLE* gene mutations precede the *MMR* gene mutations or vice versa, and which molecular defect will have a more dominant effect on the clinical behaviour of these tumors. It has been suggested that testing for somatic *MMR* gene mutations should be part of a diagnostic workup of patients with unresolved abnormal tissue screening results.

In a recent large study of colorectal cancer²⁷, mutation negative patients with MSI-high tumors and loss of *MMR* proteins by immunohistochemistry (and without *MLH1* promoter hypermethylation) formed a distinct group, which, compared to sporadic colorectal cancer patients, presented at a younger age, showed greater rates of fulfillment of Revised Bethesda Criteria²⁸ and greater rates of right sided tumors. In addition, their families exhibited a higher incidence of colorectal and non-colorectal Lynch Syndrome tumors. Therefore, it is rational that patients, with what has been referred to as “Lynch-like Syndrome”^{29,30}, should be offered to join cancer prevention surveillance programs available to patients with known deleterious mutations in *MMR* genes.

Strategies and challenges of screening for Lynch Syndrome in endometrial carcinoma

It is not universally accepted that performing both immunohistochemistry and MSI analysis in tumor screening for Lynch Syndrome is necessary. A number of different groups have demonstrated that immunohistochemistry alone has a high sensitivity and specificity in identifying endometrial carcinomas with high levels of MSI^{31–35}. However, in a recent study of consecutive 646 colorectal (88%), endometrial (7%) and other Lynch Syndrome associated tumors (5%), which underwent *MMR* immunohistochemistry and MSI testing, it was identified that 12 of 102 (11.8%) MSI-high tumors had intact *MMR* expression. In contrast, only 1 of 91 (1%) cases with *MMR* loss was MSS¹⁰. Based on these results, for optimal detection of all patients with Lynch Syndrome, both MSI and immunohistochemistry testing should be carried out. As also suggested by others^{36–39}, an argument can be made that MSI analysis in colon carcinoma could be used as an initial screen, followed by immunohistochemistry. However, because Lynch Syndrome associated endometrial carcinomas are more often MSI-low or MSS, this strategy would likely not work well for screening endometrial cancer patients. Therefore, concurrent MSI and *MMR* immunohistochemistry testing is desirable. If MSI testing is not possible and only *MMR* immunohistochemistry can be performed, it should be recognized that a small percentage of MSI-high carcinomas will not be captured by performing immunohistochemistry alone.

While young age at endometrial cancer diagnosis and/or a family history of a Lynch Syndrome-related cancer are criteria typically used to pursue further evaluation for Lynch Syndrome in a patient, it has been shown that in endometrial cancer clinical screening tools such as PREMM (93% sensitivity, 5% specificity), *MMR*Predict proximal (71% sensitivity, 64% specificity), *MMR*Predict distal (57% sensitivity, 85% specificity) and *MMR*Pro (57% sensitivity, 85% specificity) miss a significant proportion of patients with Lynch

Syndrome⁴⁰. This is in part due to the fact that some endometrial cancer patients with Lynch Syndrome, and particularly those with *MSH6* mutations, do not tend to have first degree relatives with Lynch Syndrome associated cancers, have endometrial tumors that are not typically MSI-high and present at an older age^{5,12–14,41,42}. In addition, as family sizes decrease, the probability of detecting a family history of cancers also decreases. Therefore, universal testing of endometrial cancer patients will detect Lynch Syndrome in a significant proportion of patients that would otherwise have been missed on the basis of age and/or family history¹⁷.

Early identification of Lynch Syndrome in a patient with endometrial cancer is essential not only to identify other family members with the syndrome, but also in order to proactively manage the patient's own risk of developing subsequent cancers. In particular, for women with Lynch Syndrome, endometrial cancer is considered to be a "sentinel cancer" which precedes colorectal cancer by approximately one decade⁴³. It is currently recommended that all newly diagnosed colorectal cancer patients undergo tissue testing for Lynch Syndrome regardless of their family or personal history⁴⁴. For endometrial cancer, the Society of Gynecologic Oncology has released a clinical practice statement in March of 2014⁴⁵, as follows: "All women who are diagnosed with endometrial cancer should undergo systematic clinical screening for Lynch Syndrome (review of personal and family history) and/or molecular screening. Molecular screening of endometrial cancers for Lynch Syndrome is the preferred strategy when resources are available." A group of European experts (The Mallorca Group) in 2013 recommended testing of all endometrial cancer patients under the age of 70 years by immunohistochemistry or MSI analysis.⁴⁶

At the present time, the practice of tumor testing for the purpose of Lynch Syndrome identification is highly variable across different centers and countries. Universal tissue testing permits the diagnosis of Lynch Syndrome in the highest number of patients compared to other screening strategies and has been implemented in some centers. For the majority of others, however, this entails a significant logistical and financial challenge^{47–49}.

Interestingly, a study from the United States demonstrated that, although the overall cost of universal testing of patients with endometrial cancer is higher than the overall cost of testing only patients with a 5% to 10% probability of having Lynch Syndrome (as defined by Society of Gynecologic Oncology criteria⁵⁰), the cost of testing per every probable Lynch Syndrome patient identified is actually comparable between the two testing strategies. An Australian group has recently proposed a testing approach involving testing of all endometrial carcinoma patients 60 years of age or younger, as well as testing of older patients with suggestive personal or family history of Lynch Syndrome using *MSH6* and *PMS2* immunohistochemistry and *MLH1* promoter methylation analysis. This strategy maximizes specificity (93.5%) and sensitivity (100%) of testing, while minimizing cost relative to universal tumor testing using all four MMR immunomarkers and *MLH1* methylation analysis³⁵. A recent Canadian study has corroborated that immunohistochemical testing of all patients younger than 60 years of age yielded high specificity (86.1%) and sensitivity (100%)³⁴. Screening strategies employing immunohistochemistry alone are of particular interest in terms of achieving widespread acceptance, given that MMR immunostains are inexpensive, reliable, accessible, and

reproducibly interpretable, while MSI and *MLH1* methylation testing is more costly and requires access to a clinical laboratory with expertise in PCR-based analyses.

In addition to the financial challenges of systematic screening of endometrial cancer patients for Lynch Syndrome, it is important to note that success of screening programs also relies on close communication between clinicians, pathologists, geneticists and patients. For example, in a large cancer center with universal screening for Lynch Syndrome through tissue testing, when test results of colorectal cancer patients were made available to surgeons and genetic counselors, patients who were contacted directly by the genetic counselors on behalf of the treating surgeon had higher rates of referral to genetic testing (100%) compared to those who were not (55%)⁵¹. Finally, beyond the logistical obstacles to systematic tumor screening, a number of patient factors limit the proportion of patients who decide to proceed with genetic testing after having been identified by tumor tissue testing as having possible Lynch Syndrome. Some of these factors include limited health literacy, perceived lack of relevance, being physically and emotionally overwhelmed by diagnosis of cancer, limited insurance coverage (where applicable) and requirement for patient consent^{49,52}.

Pathological features of Lynch Syndrome associated endometrial cancer

When systematic tissue testing is not possible, tumor tissue testing may be initiated by pathologists or requested by clinicians on a case-by-case basis. In addition to clinical patient information such as patient age of less than 50 years and/or family history of Lynch Syndrome associated tumors, the pathologist may use a number of endometrial tumor features to trigger tumor testing.

Microscopic features in MSI-high colorectal carcinoma have been extensively studied and include poor differentiation, mucinous features, signet ring cell differentiation, mixed tumor histology, tumor cells growing in a medullary-type pattern, increased tumor infiltrating lymphocytes, and a Crohn's like inflammatory infiltrate at the tumor periphery. Up to 40% of colorectal carcinomas, however, do not have such unique microscopic characteristics⁵³. The experience with the microscopic features of MSI-high endometrial carcinoma is less than with those of MSI-high colorectal carcinoma^{54–56}. In one study, MSI-high endometrial cancers exhibited higher tumor grade, presence of squamous metaplasia, deeper myometrial invasion, presence of lymphatic/vascular invasion, and extra-uterine spread⁵⁵. High numbers of tumor infiltrating lymphocytes and the presence of peritumoral lymphocytes have been associated with MSI-high⁵⁴. At the higher numbers of tumor infiltrating lymphocytes (40 lymphocytes per 10 high power fields), these counts had a sensitivity of 85% in predicting MSI-high status, but a specificity of only 46%.

Sporadic endometrial carcinomas that are MSI-high due to *MLH1* promoter methylation are predominantly endometrioid, especially FIGO grades 2 and 3, and comprise the majority (96%) of such tumors⁵. Endometrial carcinomas associated with Lynch Syndrome, however, tend to be more histologically diverse and include a much greater proportion of non-endometrioid histotypes ranging from clear cell, undifferentiated, uterine serous and mixed carcinoma and carcinosarcoma^{5,56–59}. In addition, patients with Lynch Syndrome and non-endometrioid tumors tend to be similar in mean age at the time of diagnosis (46.4 years) as

those with Lynch Syndrome and endometrioid tumors (46.8 years)⁵. The patients are considerably younger than the average age of women with non-endometrioid endometrial carcinoma (65–68 years) in the general population^{56,60–64}.

Tumor topography may be another clue to the presence of Lynch Syndrome. A large series of endometrial carcinomas described a very strong association of tumors derived from the lower uterine segment and Lynch Syndrome. 34% of lower uterine segment tumors were MSI high and 29% had *MMR* gene mutations⁶⁵.

Synchronous carcinomas of the ovary and endometrium tend to occur in younger patients, have lower stage and grade, endometrioid histology and favorable prognosis^{66–71}. Although some investigators have suggested that the presence of such tumor pairs may be indicative of Lynch Syndrome⁵⁶, two studies of 45⁷² and 59¹⁵ synchronous ovarian and endometrial tumors revealed that only 3.3% to 7% of cases had the molecular abnormalities suggestive of Lynch Syndrome. Furthermore, in one of these studies¹⁵ all tumors identified originated from patients with a prior history or a first-degree relative with a Lynch Syndrome associated cancer. On the other hand, in a recent study, 21% of all ovarian non-serous tumors over a two year period (with or without concurrent endometrial tumors) in a single institution were found to have MMR immunohistochemical abnormalities. All of the tumors were of endometrioid or clear cell histology.

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KEY POINTS

1. Immunohistochemistry, microsatellite instability analysis and *MLH1* promoter methylation analysis constitute the cornerstone of laboratory molecular testing of endometrial tumors for Lynch Syndrome
2. Discrepancies between genetic, immunohistochemistry and microsatellite instability analyses may arise and can often be explained as a reflection of the underlying tumor biology
3. While universal endometrial tumor screening is recommended in order to identify the most patients with possible Lynch syndrome, the current practice of screening is highly variable across different centers and countries
4. Lynch Syndrome associated endometrial tumors are associated with a number of pathological characteristics, which may be used by the pathologist to trigger tissue testing

SYNOPSIS

Endometrial tumor tissue testing in the pathology laboratory is a key component of evaluation for Lynch Syndrome. This article represents an overview of the main tissue testing modalities such as immunohistochemistry and PCR based analyses, their routine application, interpretation pitfalls, and troubleshooting of some of the common technical performance issues. Patients identified by tissue testing as having probable Lynch Syndrome continue onto genetic testing, but discrepancies between tissue testing and genetic testing commonly arise. Novel insights into the complexity of molecular abnormalities associated with Lynch Syndrome, which form the basis for some of these discrepancies, are discussed. The merits of targeted versus universal screening strategies in a changing healthcare climate are addressed. In the absence of universal screening programs, specific tumor topography and histological features that may prompt pathologist-initiated molecular tumor testing are outlined.

Figure 1.

Application of immunohistochemistry in endometrial carcinoma tissue testing for Lynch Syndrome. A) Nuclear expression of mismatch repair proteins (MLH-1, MSH-2, MSH-6 or PMS-2) B) Good internal positive control in the surrounding endometrial stroma ensures that the interpretation of mismatch repair protein expression loss is accurate.

Figure 2.

Common pitfalls in the interpretation of mismatch protein repair immunohistochemistry A) Absence of mismatch expression in both the epithelium and the stroma suggests that the immunohistochemical reaction did not work and should be repeated under different conditions or on a different block. B) Cytoplasmic tumor staining should be disregarded. C) Some Lynch-associated endometrial tumors exhibit tumor infiltrating lymphocytes. D) Tumor infiltrating lymphocytes mimic tumor cells with retained mismatch repair protein expression, which appear within otherwise immunonegative islands of tumor.

Figure 3. Heterogeneous pattern of mismatch repair protein expression. In this pattern, A) low power B) high power. In this pattern, geographic areas of retained expression are present adjacent to geographic areas of expression loss.

Figure 4.

Microsatellite instability analysis. In this example of an MSI-high endometrial carcinoma, the tumor has allelic shift for the mononucleotide microsatellites BAT25 and BAT40 and the dinucleotide microsatellite D2S123. There are extra peaks in the tumor tracings compared to that of normal tissue for each of the microsatellites.

Figure 5.

MLH1 promoter methylation analysis. In this assay, tumor DNA is treated with bisulfite, which converts cytosine to uracil; methylated cytosines are resistant to such conversion, allowing for creation of different PCR reaction primers which can differentiate the two types of sequences. Tumor DNA is analyzed concurrently with DNA from control cell lines (first tracing, negative control K562 with no *MLH1* methylation; second tracing, positive control RKO with presence of *MLH1* methylation). The endometrial carcinoma in the third tracing has both unmethylated and methylated *MLH1*. The unmethylated peak is likely due to the presence of contaminating normal stromal cells.