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Importance of PCR-Based Tumor Testing in the Evaluation of Lynch Syndrome-Associated Endometrial Cancer

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Abstract

Lynch Syndrome is a hereditary cancer syndrome caused by a germline mutation in a DNA mismatch repair gene, usually *MLH1*, *MSH2*, *MSH6*, or *PMS2*. The most common cancers associated with Lynch Syndrome are colorectal adenocarcinoma and endometrial carcinoma. Identification of women with Lynch Syndrome-associated endometrial cancer is important, as these women and their affected siblings and children are at-risk of developing these same cancers. Germline testing of all endometrial cancer patients is not cost effective, and screening using young age of cancer diagnosis and/or presence of family history of syndrome-associated is underutilized and ineffective. Therefore, most groups now advocate for tumor tissue testing to screen for Lynch Syndrome, with germline testing targeted to women with abnormal tissue testing results. Immunohistochemistry for *MLH1*, *MSH2*, *MSH6*, and *PMS2* is used in many clinical laboratories for this tumor screening step, as immunohistochemistry is relatively inexpensive and is technically more accessible for smaller clinical labs. PCR-based tissue testing, while technically more challenging, does play an important role in the identification of these patients. *MLH1* methylation analysis identifies women with tumor *MLH1* loss who likely have sporadic endometrial cancer and do not need heightened cancer prevention surveillance. High levels of microsatellite instability have been identified in tumors with retained positive expression of mismatch repair proteins. Somatic sequencing of mismatch repair genes from tumor DNA, while not currently available in most clinical laboratories, is helpful in resolution of cases in which germline sequencing fails to identify a mutation in a mismatch repair gene. The tumor tissue testing approach can help to identify most women at-risk for germline mutations in a Lynch Syndrome gene, but not all patients will be captured using this approach. Clinical suspicion can still play a pivotal role in accurately identifying a subset of these patients.

Keywords

Endometrial cancer; Lynch Syndrome; microsatellite instability; *MLH1* methylation; mismatch repair gene sequencing

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Introduction

Lynch Syndrome (LS), also known as hereditary non-polyposis colorectal cancer (HNPCC), is an autosomal dominant, hereditary cancer syndrome typified by increased prevalence of colorectal and endometrial adenocarcinomas among affected probands. In addition to these two cancers, other Lynch Syndrome associated tumors (LATs) include small intestine, hepatobiliary tract, ovarian, urinary tract, brain and skin.¹ For women with germline mutations, the lifetime risk of developing either colorectal cancer or endometrial cancer is 73.4%.² Additionally, women with germline mutations are at least equally as likely to present with a gynecologic cancer as their sentinel cancer diagnosis as they are colorectal cancer.³ Individuals with Lynch Syndrome harbor a mutation in DNA mismatch repair (MMR) genes, *MLH1*, *MSH2*, *MSH6*, *PMS2* and *EPCAM*. In an unaffected individual, the DNA MMR system identifies and repairs DNA mismatches post-replication.⁴ An *EPCAM* mutation causes secondary methylation of the *MSH2* gene, causing loss of *MSH2* protein. Identifying an individual with Lynch Syndrome at the time of his/her sentinel cancer diagnosis allows for enhanced screening for other Lynch Syndrome associated tumors and risk-reducing surgeries, such as hysterectomy for women.⁵ In addition, once a Lynch Syndrome mutation is identified, family members can be tested and undergo enhanced screening to prevent or detect cancers early.

Screening Criteria

Prior to the availability of tumor-based screening tests, clinically based screening criteria were developed to help identify individuals at increased risk for having Lynch Syndrome. These criteria have largely emerged from information collected via familial colorectal cancer registries and rely heavily on the presence of colon cancer among probands and their families. Young age at cancer diagnosis, significant family history of colorectal or endometrial cancer, and right-sided colon cancer tumors are key clinical traits incorporated into many of the existing criteria.⁶ The two most commonly used clinical screening criteria are Amsterdam II and the Revised Bethesda Criteria (Table 1). The Amsterdam II criteria were intended to identify individuals with Lynch Syndrome based on clinical history alone, do not fully recognize extra-colonic tumors, and maximize specificity (78–91%) at the cost of sensitivity (39–72%).^{7–10} The Revised Bethesda Criteria were developed to identify which patients would benefit from proceeding with tumor-based screening and have a higher sensitivity (82–94%) and lower specificity (25–41%) for detecting germline mutations.^{7,8,11–13} In 2007, the Society of Gynecologic Oncology (SGO) published a statement with clinical criteria for which gynecologic cancer patients would benefit from further evaluation for Lynch Syndrome, which was similar to Amsterdam II and Revised Bethesda criteria (Table 2).¹⁴ A study performed by our group evaluated 412 sequential, unselected endometrial cancer patients, comparing SGO clinical screening criteria to universal tumor testing, and found the sensitivity and specificity for detecting tumors at elevated risk for Lynch Syndrome were 32.6% and 77%, respectively.¹⁵ The clinically based screening criteria performed better at identifying women with increased risk for *MLH1* and *MSH2* mutations and performed poorly at identifying tumors with increased risk for *MSH6* and *PMS2* mutations. Other studies have also shown that individuals with germline mutations in *MSH6* and *PMS2* are more likely to have an older age at diagnosis and family

histories that are dominated by gynecologic cancer or have less prevalent colon cancers.^{15–18} In addition to the limitations of these available screening criteria, patients with concerning family histories for Lynch Syndrome are not being universally screened by providers.¹⁹

Immunohistochemistry

Immunohistochemistry (IHC) is an efficient and relatively inexpensive technique to screen carcinomas for the presence or absence of MLH1, MSH2, MSH6, and PMS2 protein expression.²⁰ Light microscopy is performed to evaluate for nuclear protein expression, with stromal cells serving as an internal positive control. Absence of mismatch repair protein expression in tumor cells with retained expression in adjacent stromal cells indicates a defect in DNA mismatch repair (Figure 1A, 1B; Figure 2A, 2B). Individuals with mutations in *MLH1* will exhibit loss of MLH1 and PMS2 protein expression, while patients with *MSH2* and *EPCAM* mutations will have IHC loss of MSH2 and MSH6, due to the dominant role of MLH1 and MSH2 in heterodimer formation of these proteins during mismatch repair. Individuals with germline mutations in *MSH6* or *PMS2* typically show only IHC loss of the corresponding MMR protein.^{21,22} The greatest strengths of the IHC approach are that it is easily accessible, cost effective, and informative results point to the gene of interest (Table 3).

The sensitivity and specificity of IHC alone for the detection of germline Lynch Syndrome mutations overall is 21% but varies based on mutation (Table 3). For tumors with IHC loss of MLH1/PMS2 and the absence of *MLH1* promoter methylation, the probability of identifying a germline mutation is 33%. For tumors exhibiting IHC loss of MSH2 with or without loss of MSH6, the probability is 67%. For tumors with IHC loss of MSH6 or PMS2, the probability of identifying a germline mutation is 24% and 62%, respectively.^{17,23}

Addition of PCR-based *MLH1* Promoter Methylation to Immunohistochemistry

An estimated 15–20% of all endometrial and colorectal adenocarcinomas will exhibit epigenetic silencing of the *MLH1* gene promoter secondary to methylation, a sporadic event in carcinogenesis.^{24–27} When using solely IHC to screen tumors for Lynch Syndrome, all patients that exhibit IHC loss of MLH1/PMS2 would be recommended referral to a genetic counselor for consideration of germline testing. The addition of the PCR-based *MLH1* promoter methylation assay to the assessment of these tumors has the potential to identify sporadic carcinomas and limit the number of patients referred for germline testing.²⁷ To identify the presence of *MLH1* promoter methylation, DNA is isolated from formalin-fixed, paraffin-embedded tissue sections, microdissected with a scalpel blade, and treated with bisulfite to convert methylated cytosine to uracil. DNA is then amplified using fluorescently labeled PCR primers specific for methylated (M) or the unmethylated (U) versions of *MLH1* and amplified PCR products are then detected using capillary electrophoresis and GeneScan software. Chromatograms for the tumor are then compared to known positive and negative control cell lines (Figure 2C).²⁷

65–96.9% of endometrial tumors exhibiting IHC loss of MLH1 protein expression will have methylation at the *MLH1* promoter region.^{27–31} Prior studies have shown that IHC loss of MLH1 is a poor predictor for a germline mutation, and clinically based risk factors such as young age at diagnosis and family history of Lynch Syndrome associated tumors cannot reliably predict which tumors with IHC loss of MLH1 will have *MLH1* promoter methylation.^{27,32} In a retrospective study of 337 endometrial cancer patients, 54 (16%) were found to have IHC loss of protein expression for MLH1, and 45/54 (74%) of these had methylation of the *MLH1* promoter.²⁷ Comparing the individuals with and without *MLH1* methylation did not reveal statistically significant differences amongst histology, FIGO stage, endometrioid grade, lymphatic/vascular space invasion, tumor location, tumor size, or those meeting SGO clinical criteria. Specifically, when using SGO clinical criteria alone for triaging tumors with IHC loss of MLH1, 22/54 (41%) would require genetic counseling referral, subjecting 12 women to unnecessary referrals and germline testing; an additional 4 women with tumors lacking *MLH1* methylation would not be referred to genetic counseling. Costly genetic testing can be avoided by incorporating this PCR-based assay as part of a tumor-based Lynch Syndrome screening program, but it is not yet a universally available technique.^{33–36} The reasons for this are not entirely clear, but one reason may be that many community hospitals do not have clinical laboratories with access to PCR-based tumor testing.

Microsatellite Instability Analysis

DNA microsatellites consist of multiple, tandem repeats of mono-, di-, and tri- nucleotides that are susceptible to errors during DNA replication resulting in a change in this number of tandem repeats. A change in the number of tandem repeats is termed microsatellite instability (MSI).³⁷ The Bethesda Panel is a set of microsatellite sites recommended for PCR-based MSI analysis which includes BAT25, BAT26, D5S346, D2S123 and D17S250, BAT40 and TGF- β 2.¹³ Similar to *MLH1* methylation analysis, formalin-fixed, paraffin-embedded tumor sections are microdissected, and extracted DNA is amplified and analyzed with an ABI genetic analyzer. DNA from normal tissue is also needed for this analysis. Any tissue source can be used for this normal, including the buffy coat from a peripheral blood draw. A tumor exhibiting allelic shift in 3 or more markers is defined as MSI-high (MSI-H), 1–2 markers as MSI-low (MSI-L), and no allelic shift as microsatellite stable (MSS) (Figure 1C). Lynch Syndrome associated cancers are typically MSI-H, while sporadic tumors with no defects in DNA MMR are typically MSS. MSI-L represents somewhat of a clinical conundrum.³⁸ Nearly all MSI-L colorectal carcinomas are sporadic; however, women with endometrial carcinomas and known Lynch Syndrome, often with germline *MSH6* mutations, have been found to have MSI-L or MSS tumors.³⁸ The optimal management strategy for tumors with MSI-L is not clear in the endometrial cancer population.

PCR-based Screening for *BRAF* mutations

In colorectal cancer, up to 10% of MSI-H tumors will be associated with a somatic mutation in the *BRAF* gene, a gene involved in the MAPK pathway.³⁹ For tumors with IHC loss of MLH1 and a *BRAF* mutation, no further Lynch Syndrome screening needs to be performed as *BRAF* mutation is associated with sporadic MSI-H. The underlying reason for this

interesting association is unknown. *BRAF* mutations are exceedingly rare in endometrial cancer, so this test is of minimal utility in the pathologic algorithms for LS evaluation in this population.^{29,34,40} *BRAF* mutation analysis may therefore be useful in patients with colorectal cancers with IHC loss of MLH1, when an *MLH1* methylation assay is not available.

Immunohistochemistry vs. Microsatellite Instability Analysis

Because IHC is relatively inexpensive and less technically challenging to perform, IHC assessment of the mismatch repair proteins MLH1, MSH2, MSH6, and PMS2 has become the major tissue testing strategy to screen patients for Lynch Syndrome. Furthermore, many interpret this IHC assessment as interchangeable with PCR-based MSI testing, rationalizing that both tests are unnecessary because they are presumed to be equivalent. Several large studies, however, have effectively demonstrated that there are clinically significant discordances between IHC and MSI testing. In a retrospective analysis of 591 colorectal and endometrial cancer patients who had undergone tissue testing, it was noted that amongst 102 MSI-high cancers, 12 (12%) had intact positive expression of the four mismatch repair proteins.²⁰ Eight of these patients subsequently had germline testing performed, with germline mutations detected in Lynch Syndrome genes in four patients. These four patients would not have been captured if the tissue testing approach only employed IHC for mismatch repair proteins.²⁰ In the Gynecologic Oncology Group's large retrospective study of 938 women with endometrioid-type endometrial cancer, 33 non-MSS tumors (14 MSI-high, 19 MSI-Low) had intact positive IHC expression of the four mismatch repair proteins. Tumors with intact positive expression of mismatch repair proteins by IHC represented 6% of the women with MSI-high endometrial cancers.¹⁶ From their data, they estimated that 1/150 women with endometrial cancer-associated Lynch Syndrome have tumors with intact positive IHC expression of mismatch repair proteins.¹⁶ A small subset of patients with MSI-high tumors with retained positive expression of mismatch repair proteins may have germline mutations in *MLH1* that result in expression of a defective protein, but explanations for most of these have remained elusive. The advantages and disadvantages of both IHC and MSI testing are summarized in Table 3.

Microsatellite instability panels in use in most clinical laboratories are derived from years of experience and validation of Lynch Syndrome testing in colorectal cancer patients. It has been long assumed that these same panels would be useful in the Lynch Syndrome tissue testing evaluation of women with endometrial cancer and other cancer types. There is some evidence, however, that the incidence and pattern of MSI-high in cancer types outside of the colon may be very different. Using an extended panel of 12 microsatellites, including 4 in our current panel (BAT25, BAT26, BAT40, and TGF- β R2), MSI was analyzed in 44 colorectal cancers and 57 endometrial cancers from 8 families with known germline mutations in *MLH1* or *MSH2*.³⁸ The incidence of MSS was higher in the endometrial cancers (23%) than in the colorectal cancers (11%). The colorectal cancers frequently displayed instability at the TGF- β R2 locus, but this was infrequent in the endometrial cancers. This is also observed in our clinical practice (*Broadus, R.R. unpublished data*). Alternatively, the endometrial cancers frequently displayed instability at the PTEN locus, but this microsatellite locus is not present in the panels employed by most clinical labs.

Amongst the MSI-high tumors, the endometrial cancers had a significantly lower proportion of unstable loci.³⁸ We have seen a MSS adrenocortical carcinoma with MSH2 IHC loss from a patient who also had a MSI-high colorectal cancer with MSH2 IHC loss; the family had a known germline *MSH2* mutation.⁴¹ Also, we have seen members of a family with germline mutation in *MSH2* and endometrial cancer, colon cancer, and anaplastic carcinoma of the thyroid. All three cancers displayed IHC loss of MSH2. The colon and endometrial cancers were MSI-high, while the thyroid anaplastic carcinoma was MSS.⁴¹

Detection of Somatic Mutations in Lynch Syndrome-Associated Cancers

Immunohistochemistry for DNA mismatch repair proteins and PCR-based MSI analysis and *MLH1* methylation analysis have proven to be excellent techniques to identify endometrial and colorectal cancers with defects in DNA mismatch repair. There are numerous publications in the peer-reviewed literature that attest to the sensitivity and specificity of this tissue testing approach.^{16,38,42–44} It is well-known, however, that not all MSI-high tumors with loss of immunohistochemical expression of a mismatch repair protein and free of *MLH1* methylation will have germline mutations in a mismatch repair gene identified. In a large retrospective study, 365 endometrial cancer patients underwent tissue testing, with 51/365 (14%) of patients having tissue testing results suggestive of Lynch Syndrome.⁴⁵ A germline mutation in a mismatch repair gene was only identified in 6%, however, indicating that less than 50% of tissue testing positive cases have an associated germline mutation.⁴⁵ In a larger GOG cooperative group retrospective study examining tissue testing in 938 women with endometrioid-type endometrial carcinomas, tissue testing results suggestive of Lynch Syndrome were identified in 11% of patients, but germline testing identified a deleterious mutation in approximately 43% of these, again indicating that less than half of endometrial cancer patients with tissue testing results suggestive of Lynch Syndrome will have a germline mutation identified.¹⁶

Patients with positive tissue testing results, yet no mismatch repair gene mutation identified in the germline, represent a clinical conundrum and genetic counseling challenge.⁴⁶ Some authorities believe that these patients do have Lynch Syndrome, but they have complex mismatch repair gene alterations that cannot be detected by the usual methods. For example, a novel inversion of exons 1–7 of the *MSH2* gene has been reported in patients with tumors with MSH2 immunohistochemical loss but no germline *MSH2* mutation identified by conventional sequencing.⁴⁷ Screening recommendations for tissue testing positive/germline testing negative patients and their family members is complex. Should all siblings and children receive heightened cancer prevention screening? Some advocate that at least the index patient should receive heightened colon cancer screening, if they have not yet developed this malignancy.

Recent data suggest another explanation for at least a subset of these patients. Different groups have identified the presence of somatic mutations in mismatch repair genes for endometrial and colorectal carcinomas that had tissue testing results (IHC loss of a DNA mismatch repair protein, no *MLH1* methylation detected, and MSI-high) suggestive of Lynch Syndrome, but no germline mutation was identified.^{48–51} Some of these cases are associated with an ultra-mutated genotype and germline or somatic mutations in the genes

POLE or *POLD1*. The identification of such somatic mutations is important, as it eliminates the need for cancer screening surveillance in these patients and helps to decrease patient anxiety about the possibility of having a hereditary cancer syndrome. Unfortunately, these sequencing assays are not yet available in most clinical laboratories. Many clinical laboratories, especially those supporting large cancer patient populations, do have next-generation sequencing (NGS) capabilities, but the mismatch repair genes, *POLE*, and *POLD1*, are not present on most of these panels, which are typically designed with cancer therapeutics in mind. Currently, these genes are not considered therapeutic targets for the cancer clinical trials employing targeted therapy approaches.

The Clinical Lab Does not Have all the Answers

IHC, MSI analysis, and *MLH1* methylation analysis have proven to be highly specific and sensitive in their abilities to detect endometrial cancers with defective DNA mismatch repair. Adding somatic testing for *MLH1*, *MSH2*, *MSH6*, *PMS2*, *POLE*, and *POLD1* would help to close the loop on at least a subset of cases in which a germline mutation is not identified despite tissue testing results suggestive of Lynch Syndrome.

Despite the implementation of these excellent clinical tests, there still remain a few patients who defy the best efforts of the clinical laboratory. In our previous study of 365 endometrial cancer patients with both tissue testing and germline analyses performed, 2/20 (10%) women with germline mutations in a Lynch Syndrome gene had intact positive expression of the four DNA mismatch repair proteins. Each of these women had tumors that were too small to support MSI analyses, so it is uncertain if the detection of MSI-high would have captured these patients. These patients had germline mutations in *PMS2* and *MSH6*, each was older than 50 years of age, and each had no family history of a Lynch-associated cancer.⁴⁵ Outside of a study, we have recently identified an endometrial cancer patient older than age 50 with no family history of a Lynch-associated cancer whose tumor had intact positive expression of mismatch repair proteins and was MSI-low. The patient's gynecologic oncologist was suspicious of the possibility of Lynch Syndrome due to her tumor arising from the lower uterine segment. Tumors with this anatomic location represent 3% of all endometrial cancers but nearly 30% are associated with Lynch Syndrome.⁵² Subsequent germline testing indeed identified a deleterious *MSH6* mutation. Therefore, while laboratory testing can help to identify most women at-risk for germline mutations in a Lynch gene, it is important to realize that not all patients will be captured using this approach, and clinical suspicion stills plays a pivotal role in identifying these cases.

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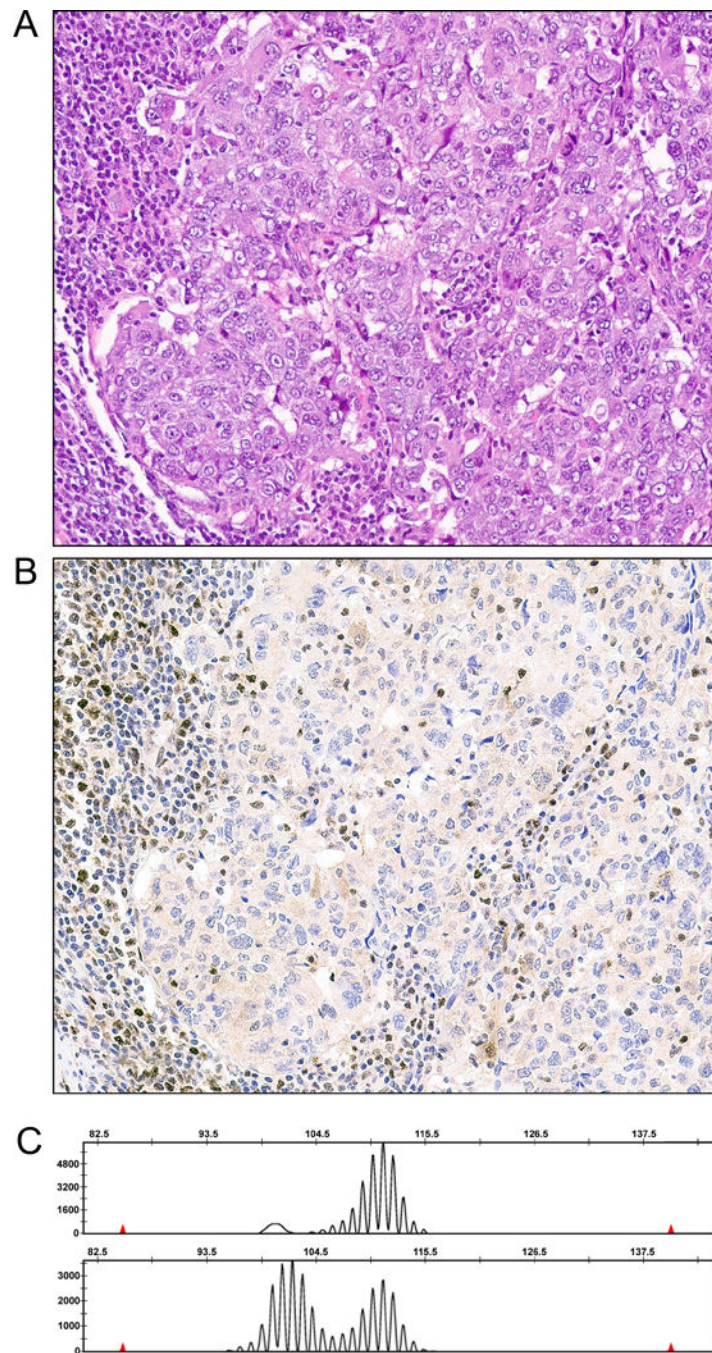


Figure 1. MSI-high endometrial carcinoma with PMS2 loss by immunohistochemistry. (A) H&E of tumor, demonstrating a poorly differentiated endometrial carcinoma with adjacent tumor infiltrating lymphocytes. (B) PMS2 immunohistochemistry, showing that tumor infiltrating lymphocytes and stromal cells adjacent to the tumor have retained nuclear expression for PMS2. Tumor cells have faint cytoplasmic expression of PMS2, but no nuclear expression for this mismatch repair protein. (C) A representative microsatellite tracing for BAT40 from this MSI analysis. Note that tumor tracing has more peaks than the tracing for normal. In

this case, normal ovary was used as a source of normal. This tumor had allelic shift in 4 other microsatellites as well (not shown).

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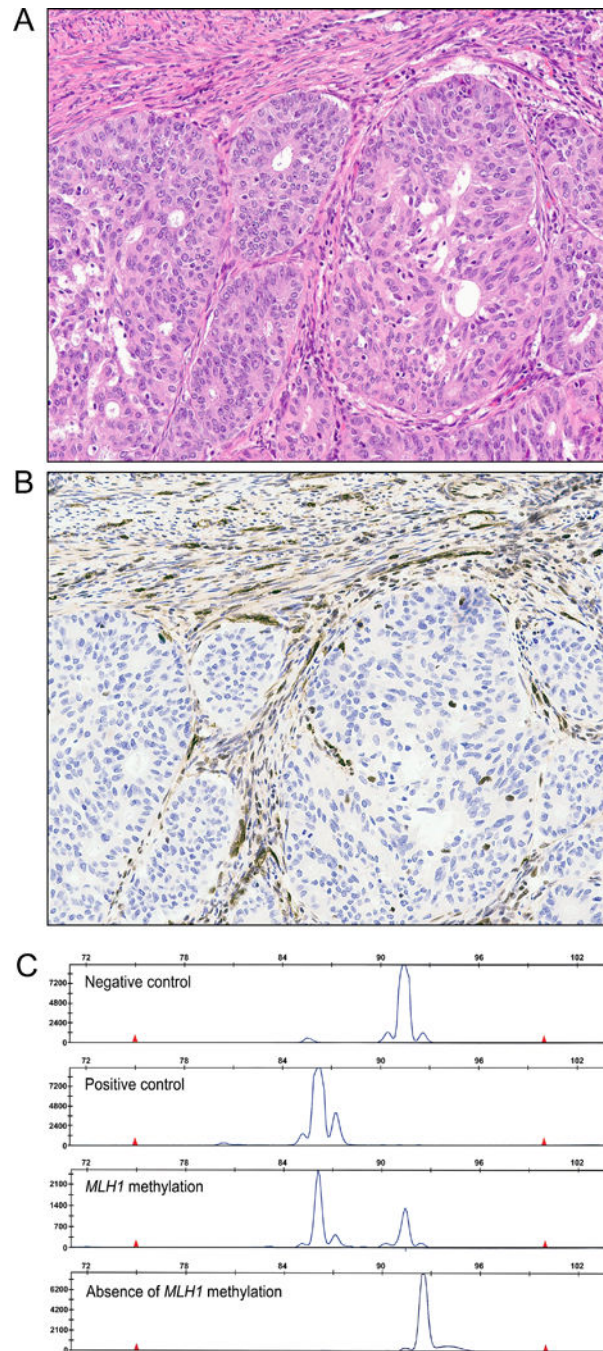


Figure 2. *MLH1* methylation analysis. (A) H&E for an endometrial carcinoma subsequently shown to have *MLH1* protein loss due to methylation of the *MLH1* gene. This tumor is typical in that routine examination of H&E stained slides would not have identified that this tumor would have *MLH1* protein loss. (B) *MLH1* immunohistochemistry, demonstrating that this endometrial carcinoma has IHC loss of *MLH1*. Adjacent myometrial cells and stromal cells have intact positive expression for *MLH1*. (C). This tumor had presence of *MLH1* gene methylation (third panel) and was therefore considered sporadic. For each analysis, negative

control and positive control cell lines are used as controls. Most tumors with *MLH1* methylation will have two peaks detected, one corresponding to a methylated peak and one corresponding to an unmethylated peak. The presence of the unmethylated peak is due to contamination by stromal cells and inflammatory cells that lack *MLH1* methylation.

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Table 1Summary of Amsterdam II and Revised Bethesda Criteria for the Evaluation of Lynch Syndrome.¹³

Amsterdam II (All criteria must be met)	Revised Bethesda
At least 3 relatives with a Lynch Syndrome associated cancer; one should be a first-degree relative of the other 2	Colorectal cancer diagnosed at age < 50
Familial adenomatous polyposis has been ruled out	Synchronous, metachronous or other Lynch Syndrome related tumors
Two affected generations	MSI-H colorectal cancer in a pt <60
At least one Lynch Syndrome associated cancer should be diagnosed < age 50	Colorectal cancer in 1 first-degree relatives with a Lynch Syndrome related tumor, one occurring at age <50
	Colorectal cancer in 2 first- or second-degree relatives with Lynch Syndrome related tumors

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Table 2

Society of Gynecologic Oncology (SGO) Criteria for those at 5–10% and 20–25% risk of having a germline mutation in *MLH1*, *MSH2*, *MSH6*, or *PMS2*.¹⁴

SGO 5–10% Criteria	SGO 20–25% Criteria
Endometrial or colorectal cancer diagnosed before age 50	Probands meeting Amsterdam II Criteria
Endometrial or ovarian cancer with a synchronous or metachronous colorectal cancer or other Lynch Syndrome associated tumor at any age	Synchronous or metachronous endometrial or colorectal cancer first cancer occurring before age 50
Endometrial or colorectal cancer and a first degree relative with Lynch Syndrome associated tumor diagnosed before age 50	Synchronous or metachronous ovarian cancer or colorectal cancer, first cancer occurring before age 50
Endometrial or colorectal cancer and 2 first or second degree relatives with Lynch Syndrome associated tumors	Endometrial or colorectal cancer with tumor testing suggestive of Lynch Syndrome (IHC or MSI-H)
Proband with first- or second- degree relatives who meet these criteria	First or second degree relative with a known germline mutation

Table 3

Probability of identifying a germline Lynch Syndrome mutation based on microsatellite instability testing or immunohistochemistry result.^{14,17,23,32}

Mircosatellite Instability/Immunohistochemistry Result	Probability of identifying a germline mutation	Advantages	Disadvantages
MSI-High	21%	<ol style="list-style-type: none"> 1 MSI-high when mutations results in expression of non-functional mismatch repair protein 2 MSI-High when mismatch repair proteins other than MLH1/MSH2/MSH6/PMS2 are mutated 	<ol style="list-style-type: none"> 1 Requires more tissue and a source of normal 2 Requires tissue microdissection prior to PCR 3 Decreased sensitivity in tissues outside the colon 4 MSI-L in endometrial cancer is of uncertain significance 5 Mutations in <i>MSH6</i> are more often associated with MSI-L or MSS
Any type of IHC loss	21%	<ol style="list-style-type: none"> 1 Lower cost 2 Loss of protein expression points to specific gene affected 3 Technique is more widely available 4 Requires less tissue 	<ol style="list-style-type: none"> 1 Mutations resulting in expression of non-functional proteins result in intact IHC 2 Expression of some proteins, especially MSH6, can be focal 3 Requires individual pathologist interpretation 4 Loss of MLH1 requires subsequent PCR-based <i>MLH1</i> promoter methylation analysis
IHC loss of MLH1/PMS2 (and absence of <i>MLH1</i> promoter methylation)	33%		
IHC loss of MSH2/MSH6	67%		
IHC loss of MSH6	24%		
IHC loss of PMS2	62%		