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Role of the Clinical Pathology Laboratory in the Evaluation of Endometrial Carcinomas for Lynch Syndrome

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Introduction

Hereditary cancer syndromes include Li-Fraumeni syndrome, Familial Adenomatous Polyposis Syndrome, BRCA1/BRCA2 Hereditary Breast/Ovarian Cancer Syndrome, Cowden's Syndrome, Juvenile Polyposis, and Lynch Syndrome (Hereditary Non-Polyposis Colorectal Cancer Syndrome). For nearly all of these hereditary cancer syndromes, identification of affected individuals is dependent on recognition of tell-tale clinical features, such as young age of cancer onset and a striking family history of characteristic cancers, followed by germline sequencing of the suspected affected gene. Such sequencing is typically complex and carried out in only a few large reference genetic laboratories. Most clinical pathology laboratories, thus, do not play substantive roles in the diagnostic work-up of such familial cancer syndromes. For Lynch Syndrome, however, the ancillary tests of immunohistochemistry for mismatch repair proteins (MMR) and PCR-based microsatellite instability (MSI) analysis are more widely available and have emerged as key components of the clinical evaluation of this syndrome. Importantly, these tests can be performed using formalin-fixed, paraffin-embedded tissues and do not require special fixatives or frozen tissue preservation. Such ancillary tests have been shown to be a cost-effective first step in patient screening for Lynch Syndrome (1, 2). Many pathology practices are reflexively subjecting all colon carcinomas to such immunohistochemical and/or MSI testing, and testing of endometrial carcinomas is starting to increasingly occur. Because these cancer types are common, it is important for pathologists to have a good working knowledge of test interpretation and pitfalls.

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Lynch Syndrome

Lynch Syndrome occurs due to a germline mutation in one of a family of DNA *MMR* genes, with subsequent loss of associated protein expression. Mutation of *MLH1* or *MSH2* genes is most common, but other important *MMR* genes include *MSH6* and *PMS2*. The hallmark cancers for Lynch Syndrome are colorectal adenocarcinoma and endometrial carcinoma. Less common cancer types also associated with this syndrome include ovarian carcinoma, urothelial carcinomas of the ureter/renal pelvis, duodenal adenocarcinoma, and gastric adenocarcinoma. Loss of DNA MMR function typically results in high levels of DNA MSI, which can readily be measured by a PCR-based analysis. Similarly, immunohistochemical expression of *MLH1*, *MSH2*, *MSH6*, and *PMS2* can be performed by routine immunohistochemical analyses. In 15–20% of all sporadic endometrial carcinomas, *MLH1* immunohistochemical loss and MSI results from *MLH1* gene promoter methylation with subsequent transcriptional silencing (3–7). Therefore, PCR-based *MLH1* promoter methylation analysis represents the third component of tissue testing for Lynch Syndrome.

Immunohistochemistry for Mismatch Repair Proteins

Immunohistochemistry for MMR proteins *MLH1*, *MSH2*, *MSH6* and *PMS2* is carried out using commercially available antibodies which work quite reliably. Gene mutation of *MMR* genes or methylation of the *MLH1* promoter typically results in loss of immunohistochemical expression of the corresponding protein. For a tumor to be considered as having a loss of an MMR marker, complete absence of nuclear expression should be observed. Strong nuclear staining in the surrounding endometrial stroma, myometrium, lymphocytes or normal endometrium should serve as an internal positive control (Figure 1). The *MLH1* and *PMS2* proteins and the *MSH2* and *MSH6* proteins act as functional pairs (8). Loss of the *MLH1* protein (due to mutation of the *MLH1* gene or methylation of *MLH1* gene promoter) typically results in loss of immunohistochemical expression of *MLH1* and *PMS2*. Mutation of *MSH2* typically results in immunohistochemical loss of *MSH2* and *MSH6*. On the other hand, mutation of *MSH6* is associated with loss of *MSH6* protein but retention of *MSH2* by immunohistochemistry. Similarly, mutation of *PMS2* is typically associated with loss of *PMS2* protein but retained *MLH1* immunohistochemical expression.

While MMR loss and MSI in complex atypical hyperplasia of endometrium has been reported, not all endometrial cancer cases with concurrent complex atypical hyperplasia may exhibit these molecular abnormalities in the areas of hyperplasia (9–11). Although *MMR* gene mutation carriers may show higher levels of concordance between complex atypical hyperplasia and endometrial carcinoma than patients with sporadic endometrial cancers, it is recommended that tissue testing always be carried out in foci of endometrial carcinoma rather than adjacent complex atypical hyperplasia (Figure 2).

Microsatellite Instability Analysis

MSI analysis is a PCR-based test that measures errors in DNA replication resulting from absence of MMR protein function. Microsatellites are sequences of DNA composed of repeating units of one to six base pairs in length. MSI analysis requires DNA from both the endometrial tumor and normal non-tumor tissues such as histologically normal cervix,

myometrium, or ovary. The pathologist will typically circle appropriate areas of tumor and non-tumor tissue, and DNA will be extracted from corresponding tissue blocks. A panel of 7 markers recommended by the NCI (12) (BAT25, BAT26, BAT40, D2S123, D5S346, D173250, and TGF- β R2) is used to detect changes in the number of microsatellite repeats in the tumor compared to normal tissue (Figure 3). Tumors with allelic shift in 3 or more microsatellites in the panel are designated as MSI-high, while tumors with no allelic shift in all 7 microsatellites are considered microsatellite-stable (MSS). The designation of MSI-low is applied to tumors with allelic shift in only 1 or 2 microsatellites. The clinicopathological significance of MSI-low cases will be discussed later in this chapter.

MLH1 Promoter Methylation Analysis

For MSI-high tumors with loss of MLH1 by immunohistochemistry, a PCR-based *MLH1* promoter methylation analysis should be performed. *MLH1* promoter methylation and gene silencing is the primary cause of MSI in sporadic endometrial cancers (13). 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 which can distinguish the two types of sequences (Figure 4).

Pitfalls in Test Interpretation

A number of pitfalls in the interpretation of MMR immunohistochemistry exist. Most commonly, false negative nuclear tumor staining will occur in the setting of inadequate internal positive control (Figure 5). On the other hand, immunohistochemical staining of the tumor cells may be focal or relatively weak. We have particularly noted this for MSH6. In our experience, tumors with a few foci of definite tumor cell staining in the nucleus, even if weak, are typically MSS. In these cases, the adjacent stromal cell nuclei are also staining weak. Immunohistochemistry on the tissue should be repeated with consideration of prolonging the antigen exposure time. Failing this, particularly in instances of poorly preserved or necrotic tissue, immunohistochemistry should be repeated on a different tissue block from the same specimen. Alternatively, if available, another tissue specimen, such as a preoperative tumor biopsy may be used (Figure 6). While immunohistochemistry on a specific tissue block may not work well, MSI analyses are typically not affected and yield excellent results.

In some cases, cytoplasmic staining may be present in the tumor, regardless of presence or absence of nuclear staining. We have encountered this phenomenon most commonly in tissues which have previously been frozen for the purposes of intraoperative consultation. Cytoplasmic staining should be disregarded in the evaluation of MMR immunohistochemistry (Figure 6).

Another immunohistochemistry interpretation pitfall involves endometrial stroma or tumor infiltrating lymphocytes masquerading as tumor cells. 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 these patterns and correlation with the corresponding H&E section should resolve most of these problems (Figure 7).

In rare cases, genuine unusual immunohistochemistry patterns may be seen. In our experience, we have observed several cases of MSI-high tumors with strong expression of MLH1 and MSH2, and a complete loss of PMS2 and MSH6. The patients involved did not have genetic testing and therefore the significance of these patterns is not known. Another unusual immunohistochemical pattern involves heterogeneous areas of MLH1 loss (with secondary PMS2 loss) by immunohistochemistry. In this pattern, stromal staining is retained throughout the entire tumor. However, large areas of carcinoma show retained positive expression of MLH1, while in adjacent large geographic areas of carcinoma the expression of MLH1 is lost. Thus far, *MLH1* methylation was observed in these cases, implying that these tumors are sporadic.

In terms of 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. This is in contrast to immunohistochemistry reporting for estrogen and progesterone receptor, for example. If tumor staining is negative, it should be indicated that internal control stromal cells/normal mucosa are positive. Terminology such as “focally positive”, “patchy staining,” “weakly positive”, “positive in X% of cells”, or “equivocal staining” should be avoided.

Problems in MSI analysis and *MLH1* promoter methylation testing most commonly involve situations in which there 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 situations of tumor heterogeneity or where a significant amount of non-tumor tissue accompanies tumor tissue in the material submitted for MSI and *MLH1* promoter methylation analyses, the analyses may detect both possible analytical outcomes. Generally, however, any detection of the molecular abnormality is interpreted as evidence of this abnormality being associated with the tumor. Amplification errors in MSI analysis are relatively uncommon. In a recent study of 646 colorectal, endometrial and other Lynch Syndrome associated tumors (14), 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 lead to re-classification of the MSI analysis result (ie. MSS to MSI-low or MSI-low to MSI).

Discrepancies between Tissue Testing and Genetic Testing

In some patients with known mutations in the *MMR* genes, the results of MSI analysis and/or MMR immunohistochemistry may not always be typical of Lynch Syndrome tumors. For example, missense mutations in *MMR* genes may generate full-length but non-functional MMR proteins, resulting in 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 MSS. This may particularly be the case for endometrial carcinomas, which, in comparison colon carcinoma, exhibit a lower proportion of unstable microsatellite markers (0.27 for endometrium versus 0.45 for

colon per average tumor), a greater proportion of tumors which are MSS (23% of endometrial versus 11% of colon cancer cases), as well as have shorter allelic shifts in BAT loci (15). Furthermore, MSI analysis of paired synchronous ovarian and endometrial carcinomas reveals 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 immunohistochemistry (16). 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 types (17). From the tissue testing perspective, these biological phenomena may generate discrepancies between immunohistochemistry, MSI analysis and mutation status, particularly when non-colorectal tumors are concerned. Endometrial tumors associated with MSI-low or MSS tend to occur particularly disproportionately in patients with *MSH6* germline mutations (18–20).

Instances where MSI and MMR immunohistochemistry are both suggestive of Lynch Syndrome, but genetic analysis does not reveal a pathologic mutation in the *MMR* genes may occur due to mutations in less common genes which regulate the *MMR* genes, or in the setting of *MMR* gene variants of undetermined significance. The latter may be eventually reclassified as pathologic mutations with accumulation of experience. Of note, in a recent large study of colorectal cancer (21), mutation negative patients with MSI-high tumors and loss of MMR proteins by immunohistochemistry (and without MLH1 promoter hypermethylation) formed a distinct group that, compared to sporadic colorectal cancer patients, presented at a younger age, showed greater rates of fulfillment of Revised Bethesda Criteria (22) 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 suggested that such mutation negative and tissue testing positive patients should be included in heightened cancer prevention surveillance programs offered to patients with known deleterious mutations in *MMR* genes.

Approaches to Screening for Lynch Syndrome in Endometrial Carcinoma

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, it has been shown that in endometrial cancer clinical screening tools such as PREMM, MMRpredict, and MMRpro miss a significant proportion of patients with Lynch Syndrome (23). A population-based study of 543 women with endometrial cancer (24) identified 10 Lynch Syndrome mutation carriers. Four of the 10 women were younger than age 50 years, but six were older than 50 years of age. In a multi-institutional, retrospective analysis, the mean age of diagnosis for women with Lynch syndrome and endometrial cancer was 46.8 years, with 16 of the 50 women studied being older than 50 years of age (7). In a multi-institutional prospective study involving 8 centers, out of 179 tumors tested, 11 were found to have possible Lynch Syndrome based on MMR immunohistochemistry and MSI testing. 10 of these patients were older than age 50 years and 7 were confirmed to have *MMR* gene mutations (25). Therefore, in endometrial cancer, a significant population of endometrial cancer patients who develop tumors after the age of 50 is at risk of having Lynch Syndrome.

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 (26–28). 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 MSS (14). Based on these results, for optimal detection of all patients with Lynch Syndrome, both MSI and immunohistochemistry testing should be performed. As also suggested by others (29–32), 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.

As outlined earlier in this chapter, discordances between MSI and immunohistochemistry analyses are thought to have biological underpinnings. Furthermore, due to the predilection of endometrial carcinoma towards a lower frequency of unstable microsatellite markers (15, 33), it is felt that the number of these discrepancies would be greater in endometrial than colorectal cancers. In particular, *MSH6* mutation carriers are older, do not tend to have first degree relatives with Lynch Syndrome associated cancers and have endometrial tumors that are not typically MSI-high (18–20, 24, 34). Our limited preliminary evidence suggests that *PMS2* mutation carriers may be phenotypically similar. Therefore, we recommend concurrent MSI and MMR immunohistochemistry testing for all endometrial tumors. 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.

Pathological features of Lynch Syndrome associated Endometrial Cancer

At the present time, the practices of tumor testing and Lynch Syndrome identification are highly heterogeneous across various centers. In some centers, universal reflex tissue testing of colorectal and endometrial cancers using MMR immunohistochemistry, MSI analysis and *MLH1* promoter methylation testing (where required) has been adopted. However, there are substantial challenges in widespread implementation of universal tumor testing (35, 36) ranging from logistical to financial constraints, and in the majority of institutions, tumor tissue testing may be requested by clinicians or initiated by pathologists on a case-by-case basis.

When universal tissue testing is not possible, 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, including tumor histotype, histological tumor characteristics and tumor topography, to trigger tumor testing. It is our opinion, however, that microscopic features of endometrial cancer are not sufficiently sensitive or specific to be used alone in the clinical setting as accurate predictors of the presence of high levels of MSI.

Sporadic endometrial carcinomas that are MSI-high due to *MLH1* methylation are predominantly endometrioid, especially FIGO grades 2 and 3, and comprise as many as 96% of such tumors (7). 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 (37), undifferentiated (38), uterine serous (7) and mixed carcinoma (39, 40) and carcinosarcoma (41). While non-endometrioid endometrial carcinoma is typically diagnosed in older women with a mean age of 65–68 years in the general population (42–46), in Lynch Syndrome, the mean age of diagnosis of these non-endometrioid tumors is 46.4 years, similar to the mean age of endometrial cancer diagnosis in the Lynch Syndrome group overall (46.8 years)(7). These data suggest that there may be a genotype-phenotype relationship between *MMR* gene loss and endometrial tumor histotype, however, more studies of Lynch Syndrome associated endometrial tumors are required in order to substantiate this claim.

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. It must be noted, however, that these distinctive microscopic features may not be present in a substantial subset of colorectal carcinoma. Up to 40% of colorectal carcinomas do not have such unique microscopic characteristics (47). Furthermore, most of these studies have not distinguished between sporadic MSI-high due to *MLH1* methylation and MSI-high due to germline mutation of a DNA *MMR* gene. It is therefore unclear if there are microscopic differences between these two MSI-high groups.

Microscopic features of MSI-high endometrial carcinoma have also been studied, but to a far lesser extent than MSI-high colorectal carcinoma (38, 48, 49). 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 spread (49). High numbers of tumor infiltrating lymphocytes and the presence of peritumoral lymphocytes have been associated with MSI-high (48). 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%.

Endometrial carcinoma most commonly arises in the mucosa of the uterine corpus, which includes the body and fundus of the uterus. Carcinomas derived from the lower uterine segment are much less common. In a large series, only 3.5% of endometrial carcinomas were derived from the lower uterine segment (50). However, women with lower uterine segment tumors were significantly younger (54.2 years) than women with tumors in the uterine corpus (63.0 years) and had a high percentage (34.2%) MSI high tumors and *MMR* gene mutations (28.6%) (50). This percentage of Lynch Syndrome associated endometrial cancers is extremely high when compared to the incidence of endometrial cancer in the general endometrial cancer patient population (1– 2%) (6, 51, 52) or in young women (50 years of age or younger) with endometrial cancer (9– 11%) (7, 24).

Synchronous carcinomas of the ovary and endometrium tend to occur in younger patients, have lower stage and grade, endometrioid histology and favorable prognosis (53–58). Although some investigators have suggested that the presence of such tumor pairs may be indicative of Lynch Syndrome (38), two studies of 45 (59) and 59 (16) 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 studies (16) all tumors identified originated from patients with a prior history or a first-degree relative with a Lynch Syndrome associated cancer. Therefore, without a positive personal or family history, routine tissue testing of patients with synchronous ovarian and endometrial carcinomas is unlikely to uncover a large number of patients with possible Lynch Syndrome.

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Figure 1.

- a) Nuclear PMS2 expression with good internal positive control in the stroma, 20 $\times$.
- b) Loss of nuclear MSH2 expression, with a good internal positive control in the stroma, 20 $\times$.

Figure 2.

- a) Nuclear MLH1 expression is retained in complex endometrial hyperplasia, 20 $\times$.
- b) Loss of nuclear MLH1 expression in adjacent endometrioid endometrial adenocarcinoma, 20 $\times$.

Figure 3. Microsatellite instability analysis. In this example of an MSI-high endometrial carcinoma, the tumor has allelic shift for the mononucleotide microsatellites BAT25, BAT26, and BAT40 and the dinucleotide microsatellites D2S123 and D17S250. For each microsatellite, there are extra peaks in the tumor tracings compared to that of normal tissue.

Figure 4.

MLH1 promoter methylation analysis. Tumor DNA is treated with bisulfite, which converts cytosine to uracil; methylated cytosines are resistant to such conversion, and this forms the basis of this assay. 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. The endometrial carcinoma in the fourth tracing lacks *MLH1* methylation.

Figure 5. Absence of nuclear staining for MSH6 in endometrial adenocarcinoma with concurrent absence of staining of the tumor stroma. The immunohistochemical reaction did not work and should be repeated either after altering the reaction conditions or by using a different tissue block, 20 $\times$.

Figure 6.

- a) Carcinoma derived from an autopsy with cytoplasmic staining for MSH2, but no nuclear staining, 20 $\times$. Nuclear MSH6 expression was retained in the same tissue.
- b) Immunohistochemistry is repeated in a tumor biopsy from the same patient and reveals retained nuclear MSH2 expression in the tumor cells with background cytoplasmic staining, 20 $\times$. Cytoplasmic tumor staining should be disregarded in interpretation of mismatch repair protein immunohistochemistry.

Figure 7.

a) Endometrial tumor with numerous tumor infiltrating lymphocytes and unusual “syncytial” morphology, H&E, 10×. b) The tumor exhibits immunohistochemical nuclear MLH1 loss. However, MLH1 expression is retained in tumor infiltrating lymphocytes which may mimic tumor cells.