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Application of Immunohistochemistry and Molecular Diagnostics to Clinically Relevant Problems in Endometrial Cancer Bojana Djordjevic, Shannon Westin, Russell R. Broaddus

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Synopsis

A number of different clinical scenarios are presented in which lab-based analyses beyond the usual diagnosis based on light microscopic examination of H&E stained slides – immunohistochemistry and PCR-based assays such as sequencing, mutation testing, microsatellite instability analysis, and determination of MLH1 methylation - are most helpful for guiding diagnosis and treatment of endometrial cancer.

The central goal of this information is to provide a practical guide of key current and emerging issues in diagnostic endometrial cancer pathology that require the use of ancillary laboratory techniques, such as immunohistochemistry and molecular testing. The authors present the common diagnostic problems in endometrial carcinoma pathology, types of endometrial carcinoma, description of tissue testing and markers, pathological features, and targeted therapy.

Keywords

endometrial cancer; targeted therapy; Lynch Syndrome

Introduction

Because endometrial cancer is a heterogeneous disease, its molecular pathogenesis is complex and demands a sophisticated and versatile array of diagnostic pathology tools. The central goal of this chapter is to provide a practical guide of key current and emerging issues

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in diagnostic endometrial cancer pathology that require the use of ancillary laboratory techniques, such as immunohistochemistry and molecular testing. This includes differentiation of endocervical and endometrial primaries on curettage specimens, subtyping of high-grade endometrial cancers into biologically meaningful categories, evaluation of endometrial carcinoma patients for Lynch syndrome and identifying endometrial cancer patients who may benefit from specific targeted therapies.

Common Diagnostic Problems in Endometrial Carcinoma Pathology

Endometrial versus Endocervical Primary Tumors

One of the most common situations in gynecological pathology that requires the use of immunohistochemistry is the differentiation of endometrial from endocervical adenocarcinoma in endocervical and endometrial biopsies. This distinction is clinically important, as it will help to guide the gynecological surgeons in their surgical planning and subsequent treatment approach. Premenopausal patient age and presence of concurrent cervical squamous dysplasia or adenocarcinoma-in-situ favor endocervical origin, while endometrial hyperplasia and/or squamous morules, particularly in a post menopausal patient, favor endometrial origin. However, patient age may sometimes be misleading and morphological clues are not always present, necessitating the use of ancillary testing.

The immunohistochemical panel used to distinguish endometrial from endocervical primaries includes estrogen receptor (ER), vimentin, monoclonal carcinoembryonic antigen (CEA) and p16. Endocervical adenocarcinoma is typically diffusely positive for p16 and shows diffuse membranous staining for CEA, while it is negative for ER and vimentin. Endometrial endometrioid adenocarcinoma typically shows diffuse immunohistochemical expression of ER and vimentin and patchy p16 staining. Endometrial endometrioid adenocarcinoma is usually negative for CEA; squamous morules in endometrial carcinoma should not be included in interpretation, as they usually do stain for CEA (Figures 1 and 2).

Many publications regarding the usefulness of the markers in this panel reported performance of each individual marker in a cohort of cases (1–4). However, when all four markers are examined together, up to 50% of endocervical, and as many as 70% (5) of endometrial tumors do not strictly adhere to the anticipated patterns, with aberrant positive or negative expression of at least one of the four markers in the panel. This underscores the need for judicious interpretation and correlation with tumor morphology. Some authors have suggested that type of differentiation, along with tumor site of origin, must be considered when interpreting the panel (6). For example, ER staining tends to be seen in endometrial tumors of both endometrioid or mucinous differentiation, but may also be retained in some endocervical adenocarcinomas (7). However, tumors with endometrioid differentiation, regardless of endometrial or endocervical origin, may be positive for vimentin. Finally, monoclonal CEA staining tends to occur only in mucinous adenocarcinomas from the endocervix (6).

p16 controls the G1-S checkpoint of the cell cycle via phosphorylation of the retinoblastoma protein (RB). p16 expression is in turn regulated by RB. High risk HPV E7 protein binds to RB and inactivates it, resulting in uncontrolled cell proliferation and p16 overexpression. While some level of p16 is expressed in many tumors, cervical adenocarcinoma-in-situ and invasive adenocarcinomas are characterized by diffuse and strong p16 expression, typically in greater than 75% of tumor cells (7, 8).

HPV in situ hybridization (ISH) is another test that can be used in conjunction with the 4 marker panel. However, DNA degradation can lead to false negative HPV ISH results,

which renders HPV ISH less accurate and more difficult to perform than p16. Recently, ProExC has been shown to perform comparatively to p16 (9).

A number of pitfalls in the interpretation of the 4 marker panel must be considered.

Some endocervical adenocarcinomas may not show p16 overexpression. High-risk HPV is the key pathogenetic agent in 67 to 91% of in-situ and invasive cervical adenocarcinomas (7). However, the role of HPV in the pathogenesis of other histotypes of endocervical carcinoma is still under investigation. Mesonephric adenocarcinoma has not been reported in association with HPV, and HPV has only infrequently been associated with minimal deviation adenocarcinoma (10–13). Data on serous carcinoma of the cervix is controversial, as some investigators have found no HPV by ISH in 13 cases (9), whereas other investigators have detected HPV by PCR in 3 of 4 cases (14). Similarly, some investigators have reported HPV presence in 2 of 3 cases of cervical clear cell carcinomas, whereas others found no HPV in 4 (10) and 6 (9) cases studied. Adenoid basal cell carcinoma (10, 15), small cell carcinoma (10, 14, 16) and adenosquamous carcinoma (10) are also generally accepted as HPV-associated cervical carcinomas. Furthermore, some tumor types, such as serous carcinoma and clear cell carcinoma, may overexpress p16 (9) due to non-HPV related mechanisms. This can also lead to discrepant p16 immunohistochemistry and HPV ISH results (17).

Another common pitfall, particularly in the interpretation of small biopsies, involves uterine serous carcinomas. These tumors are typically diffusely p16 positive and ER negative (8), and as such may be mistaken for cervical primaries. Histological features including high-grade nuclei with loss of intra-epithelial polarity (with or without papillary formations) will be clues to making the diagnosis of serous carcinoma. It should be noted that endocervical and endometrial primaries of serous carcinoma cannot be distinguished using immunohistochemistry (18). Similarly, both uterine and cervical clear cell carcinoma will be ER negative and positive for hepatocyte nuclear factor 1 β (17, 19), and as such cannot be differentiated by use of immunohistochemistry.

Uncommonly, independent primary endometrial and endocervical adenocarcinomas coexist in the same patient. Such cases are evident when the endometrial and endocervical tumors have a different histological appearance and, in most cases, discordant immunohistochemical profiles. (20)

Carcinomas arising in the lower uterine segment are generally thought to be endometrial carcinomas. Experience with these tumors is limited. One study has found that adenocarcinomas of the lower uterine segment generally have the same immunohistochemical profile as conventional endometrioid adenocarcinomas arising in the uterine fundus. (21)

Synchronous ovarian and endometrial tumors

Immunohistochemistry is not useful in distinguishing endometrial from ovarian endometrioid adenocarcinoma. WT-1 and ER are often negative in uterine serous carcinoma (8, 18) and usually positive in ovarian and peritoneal high-grade serous carcinoma (22) and thus may be helpful in differentiating metastasis from synchronous primaries. However, immunohistochemistry will not be helpful in this differential in instances when uterine serous carcinoma is positive for WT-1 and/or ER (23, 24). If the ovarian and endometrial carcinomas both have loss of a DNA mismatch repair protein (MLH1, MSH2, MSH6, or PMS2) by immunohistochemistry, the PCR-based microsatellite instability analysis could potentially be used to help classify the tumors accurately as synchronous primary tumors or one primary with a metastasis. In synchronous primary ovarian and uterine tumors that are

MSI-high, the ovarian tumors typically have allelic shift in fewer microsatellites, and the pattern of allelic shift is distinct from that observed in the endometrial tumors (25). Microsatellite instability analysis is discussed in more detail under [Tissue Testing for Hereditary Non-Polypsis Colorectal Cancer Syndrome](#). [COMP, PLEASE LINK TO THIS SECTION THAT COMES LATER, CURRENTLY ON PAGE 14 OF THIS MANUSCRIPT]

Extragenital Metastases to the Endometrium

Endometrial involvement by extragenital metastatic tumors is rare. Breast carcinoma is the most common, followed by colon carcinoma (26). Breast carcinoma with metastasis to the endometrium should be easily distinguishable from an endometrial primary in a hysterectomy specimen, as it usually does not form a mass and it diffusely involves the lymphatics. However, diagnostic confusion may arise in endometrial curettage or biopsy specimens. (27) Both breast and endometrial endometrioid carcinomas can be positive for ER. Gross cystic disease fluid protein-15 is the preferred marker to distinguish breast from an endometrial primary, as mammoglobin is expressed in the majority of endometrial tumors.(28) The most useful markers to distinguish metastatic colorectal carcinoma from primary endometrial carcinoma are ER, vimentin and CK7 (positive in endometrial carcinoma) and CDX2 and CK20 (positive in colorectal carcinoma) (Figure 3). Pax 8 is a novel marker that will stain endometrial carcinoma, but not breast nor colon carcinoma.(29)

Subtyping of Endometrial Carcinoma

Endometrial cancer is a heterogeneous disease. The first attempt at endometrial carcinoma classification recognized two broad categories (30). The Bokhman type I tumors were well-to-moderately differentiated adenocarcinomas that arose in women with hyperestrogenism and/or disturbances in carbohydrate metabolism. The tumors arose in the background of endometrial hyperplasia, had superficial myometrial invasion, were responsive to progestin therapy, and had an excellent prognosis. The Bokhman Type II tumors, however, had no apparent precursor lesion, were poorly differentiated adenocarcinomas with deep myometrial invasion and metastasis to lymph nodes, and were associated with poor prognosis. While this model was informative, it is now recognized that there is an appreciable number of tumors that cannot be easily categorized in either of the two groups. For example, some low grade and low stage endometrioid carcinomas recur or metastasize to lymph nodes. Furthermore, classification of some endometrial carcinomas, particularly those that are high grade according to the World Health Organization system (31), is poorly reproducible and does not accurately predict tumor behavior.(32)

It can often be difficult to distinguish higher grade endometrioid carcinomas from serous carcinomas. Use of immunohistochemistry as an adjunct has been the subject of a number of recent studies. In one study, investigators have found nuclear expression of beta-catenin in 47% of International Federation of Gynecology and Obstetrics (FIGO) grade 3 endometrioid carcinomas, but no expression in serous carcinomas. On the other hand, E-cadherin was expressed in 41% of serous carcinomas compared to only 6% endometrioid carcinomas in the study cohort. This expression of E-cadherin in uterine serous carcinomas is in contrast to the lower expression of E-cadherin typically seen in aggressive carcinomas from other organ systems. (33) In another study, FIGO grade 3 endometrioid carcinoma and serous carcinoma were found to be best distinguished from one another by an immunohistochemical panel of PTEN (scored positive with any amount of tumor cells with cytoplasmic staining), p16 (scored positive if greater than 75% of tumor cells with nuclear and cytoplasmic staining) and IMP3 (scored positive if greater than 1% of tumor cells with cytoplasmic staining), with the three markers being expressed in serous carcinoma much more frequently than in FIGO grade 3 endometrioid carcinoma. In fact, a combination of only p16 and PTEN could

identify serous carcinomas with 90% sensitivity and 96.8% specificity. Clear cell carcinomas in this study were typically PTEN and IMP3 positive, but p16 negative. Notably, p53 (scored positive if greater than 50% of tumor cells with nuclear staining) was positive in 25.0% of clear cell carcinomas, 39.4% of FIGO grade 3 endometrioid carcinomas and 69.2% of serous carcinomas, and could not successfully differentiate between these three histotypes. (24) Two different examples of uterine serous carcinoma with differing immunohistochemical expression of p53, ER, p16, and WT-1 are presented in Figures 4 and 5.

Following in the footsteps of ovarian carcinoma investigators (34, 35), there have been recent efforts to use immunohistochemistry to help better classify high grade endometrial carcinomas into biologically meaningful categories. In a study of 200 endometrial carcinomas, using a panel of 12 immunohistochemical markers, three separate groups of tumors were defined through unsupervised hierarchical clustering analysis. These groups showed a strong correlation with histological and clinical parameters. The first group consisted of predominantly low grade endometrioid carcinomas (FIGO grades 1 and 2) that were ER positive, and had a low frequency of cases that were positive for PTEN, p53 and cell cycle regulatory proteins such as p27, p21 and E2F-1. This group had the most favorable outcome and was most reminiscent of type I Bokhman tumors. The third group consisted of a high proportion of non-endometrioid tumors, including serous, clear cell and mixed (serous and endometrioid) tumors. All tumors in this group were ER negative, expressed either PTEN and/or p53 and had variable rates of expression of cell cycle regulatory proteins. This group had the worst prognosis and fit the profile of type II Bokhman tumors. The second group contained predominantly FIGO grade 2 and 3 endometrioid carcinomas, some mixed tumors, and infrequent endometrioid FIGO grade 1 and serous cases. Tumors in this group were ER positive and p53 positive, and showed the highest rates of cell cycle regulatory protein expression. This group had intermediate clinical prognosis. The authors interpreted this group to represent type I Bokhman tumors with transformation to higher grade tumors through accumulation of additional mutations. (36)

Another recent study examined a cohort of carcinomas with overlapping features of endometrioid and serous histotypes. For example, tumors in this group showed architectural and cytologic dys-synchrony in the form of glands containing cells with nuclei with threefold variation in size and shape and prominent nucleoli. Other tumors were predominantly papillary, but lacked significant nuclear atypia. p53 was assessed by immunohistochemistry, and tumors showing strong nuclear expression in greater than 80% of the cells were considered as having p53 overexpression. Such tumors were associated with a significantly shorter progression-free survival and disease-specific survival. (37)

Immunohistochemistry by itself may not be an ideal technique to help in the pathological distinction of endometrioid from non-endometrioid carcinomas. Furthermore, immunohistochemistry has not proven to be useful in the identification of patients with lower grade endometrioid carcinomas who are at highest risk for recurrence. PCR-based or qRT-PCR based assays addressing these important clinical issues have not been incorporated into routine clinical practice. However, such assays may be feasible. For example, it has been shown that the qRT-PCR assessment of a small panel of estrogen-induced genes can help to identify low grade endometrioid carcinomas that recur (38). Using a genomic approach, one cDNA microarray study of different histotypes of endometrial cancer identified the expressed sequence tag (EST) KIAA1324 to be one of the best molecular signatures to distinguish endometrioid from non-endometrioid carcinomas(39). A separate microarray study identified this same EST to be induced 3-fold in the human endometrium by estrogen-based hormone replacement therapy (40). It was subsequently shown that this EST (termed *Estrogen Induced Gene 121; EIG121*) was significantly up-

regulated 21-fold in grade 1 endometrioid endometrial carcinomas compared to benign endometrium. Furthermore, *EIG121* expression was significantly suppressed in the non-endometrioid tumors uterine serous carcinoma and uterine malignant mixed mullerian tumor (carcinosarcoma). The level of *EIG121* mRNA in these non-endometrioid carcinomas was less than only 5% of that of the benign endometrium (40). Therefore, these separate studies have identified *EIG121* to be an excellent single gene biomarker to distinguish endometrioid from non-endometrioid endometrial carcinomas. It should be noted, however, that all of these studies were carried out using frozen tissues. Therefore, the possible applicability of these findings to clinical practice is still unknown.

Tissue Testing for Hereditary Non-Polyposis Colorectal Cancer Syndrome (Lynch Syndrome)

The Hereditary Non-Polyposis Colorectal Cancer Syndrome (HNPCC or Lynch Syndrome) is associated with germline mutations of *MLH1*, *MSH2*, *MSH6*, *PMS2*, and other less common genes. Mutations in these DNA mismatch repair genes result in high levels of microsatellite instability (MSI) in endometrial tumor DNA as compared to DNA from normal tissues. Sporadic endometrial carcinoma can also have high levels of MSI due to methylation of the *MLH1* gene promoter with subsequent transcriptional silencing. Such tumors have been well-described in the literature and represent 15 to 20% of all sporadic endometrial and colon carcinomas (41–45). Much of what is known regarding colon and endometrial carcinoma with MSI has been derived from studies of sporadic tumors; the relevance to the HNPCC-associated tumors is not clear.

Tissue testing to detect microsatellite instability

Tissue testing (immunohistochemistry, MSI analysis, and *MLH1* methylation analysis) has emerged as a practical first step in the evaluation of women thought to be at risk for having Lynch Syndrome. Importantly, each of these tests can be performed using formalin-fixed, paraffin-embedded tissues and commercially available antibodies. In many institutions, MSI analysis is performed in parallel with immunohistochemistry for *MLH1*, *MSH2*, *MSH6*, and *PMS2*. It requires both tumor and normal non-tumor tissues. For endometrial cancer, histologically normal cervix, myometrium, or ovary could be used from the hysterectomy surgical specimen. A panel of 7 markers recommended by the NCI (46) (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. Tumors with allelic shift in 2 or more microsatellites in the panel are considered MSI-high (Figure 6). Tumors with no allelic shift in all 7 microsatellites are considered microsatellite-stable. Tumors with allelic shift in only 1 microsatellite are considered MSI-low. The clinicopathological significance, if any, of MSI-low endometrial carcinoma is not known. For MSI-high tumors with loss of *MLH1* by immunohistochemistry, a PCR-based assay to detect for possible methylation of the *MLH1* promoter can be performed (Figure 7). If methylation is present, it is much more likely that the patient has a sporadic endometrial carcinoma rather than a Lynch Syndrome-associated tumor.

The *MLH1* and *PMS2* proteins and the *MSH2* and *MSH6* proteins act as functional pairs (47). Mutation of *MLH1* or methylation of *MLH1* typically results in loss of immunohistochemical expression of *MLH1* and *PMS2*. Mutation of *MSH2* usually results in immunohistochemical loss of *MSH2* and *MSH6*. However, mutation of *MSH6* usually 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. For each of these antibodies, adjacent stromal

cells, lymphocytes, myometrium, and normal endometrium serve as useful internal positive controls (Figure 8).

Colon carcinomas with a defect in DNA mismatch repair, either due to mutation or *MLH1* methylation, are typically associated with very high levels of tumor microsatellite instability, with 6 to 7 markers in the 7 marker panel demonstrating allelic shift. However, a subset of extra-colonic carcinomas may not exhibit the usual high levels of microsatellite instability (48, 49). There are case reports of microsatellite stable thyroid anaplastic carcinoma and adrenal cortical carcinoma, both with loss of *MSH2* expression by immunohistochemistry, from 2 different individuals with known pathogenic mutations in *MSH2* (78). In one large study, 23% of the endometrial carcinomas demonstrated no microsatellite instability, even when an extended panel of 12 markers was used (49). Such MS-stable or MSI-low tumors can occur even with documented immunohistochemical loss of a DNA mismatch repair gene product. In addition, MSI analysis of paired synchronous ovarian and endometrial carcinomas revealed differing patterns of microsatellite allelic shift, despite the fact that the ovarian and endometrial tumor in each pair had lost expression of the same DNA mismatch repair protein by immunohistochemistry (25). The reason for the differing patterns in microsatellite instability between colon carcinomas and endometrial carcinomas and endometrial and ovarian carcinomas is not clear. It has been suggested that target genes of MSI are tissue-specific, and this may be the source for differing patterns of microsatellite instability across different tumor types (50).

Pathological features of MSI-high endometrial cancer

Sporadic endometrial carcinomas that are MSI-high due to *MLH1* methylation are known to be predominantly endometrioid, especially FIGO grades 2 and 3, with percentage endometrioid histotype reaching 96% (45). Endometrial carcinomas associated with Lynch Syndrome, however, tend to be more histologically diverse and can include endometrioid and non-endometrioid histotypes. Clear cell carcinoma, uterine serous carcinoma, undifferentiated carcinoma and malignant mixed mullerian tumors (carcinosarcoma) have been identified as non-endometrioid tumors in Lynch Syndrome (45, 51, 52). In the general population, non-endometrioid endometrial carcinoma is typically diagnosed in older women with a mean age of 65 to 68 years (53–57). However, 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) (45). In their study of 23 Italian women with HNPCC-associated endometrial cancer, one group of investigators (51) found an extraordinarily high percentage (43%) of non-endometrioid carcinomas; 50% of these non-endometrioid tumors were clear cell carcinoma. From the published literature, non-endometrioid tumors do not typically constitute such a high percentage of the Lynch Syndrome-associated endometrial cancer population. Interestingly, one study found that all of the non-endometrioid tumors arose in women with *MSH2* mutations.(45) In a population-based study of endometrial carcinoma (58)with subsequent follow-up(59), two Lynch Syndrome-associated non-endometrioid endometrial carcinomas were identified, both in women with *MSH6* mutations. This suggests that there may be a genotype-phenotype relationship in which microsatellite instability due to loss of *MLH1* by methylation of the promoter is almost exclusively associated with higher grade endometrioid tumors, while microsatellite instability due to defects in the *MSH2/MSH6* pair can result in a more varied spectrum of endometrial carcinoma histology. More studies including non-endometrioid tumors will be needed to verify this possible genotype-phenotype relationship.

There is considerable literature on the presence or absence of distinctive microscopic features in MSI-high colorectal carcinoma. Some of the microscopic features that have been associated with the presence of MSI-high 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.(60) Most of these studies have not distinguished between sporadic MSI-high due to *MLH1* methylation vs. MSI-high due to germline mutation of a DNA mismatch repair gene. It is therefore unclear if there are microscopic differences between these two MSI-high groups. 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 distinguishing microscopic characteristics.(60) Therefore, microscopic features alone cannot be used to determine which colorectal cancer patients should be evaluated further for Lynch Syndrome.

Microscopic features of MSI-high endometrial carcinoma have also been studied, but not to the extent of that for MSI-high colorectal carcinoma (52, 61, 62). Again, as is the case for colorectal cancer, the source of the microsatellite instability (*MLH1* methylation vs. germline mutation of a DNA mismatch repair gene) was not delineated in these studies. One study found that MSI-high endometrial cancers were associated with higher tumor grade, presence of squamous metaplasia, deeper myometrial invasion, presence of lymphatic/vascular invasion, and extra-uterine spread.(62) High numbers of tumor infiltrating lymphocytes and the presence of peritumoral lymphocytes have been associated with MSI-high.(61) 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%. Although the published data for endometrial cancer is more limited, it is our opinion that microscopic features of endometrial cancer are not sufficiently sensitive and specific to be used in the clinical setting as accurate predictors of the presence of high levels of microsatellite instability.

MSI-high endometrial cancer arising in the lower uterine segment

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 (LUS) are much less common. In a large series, only 3.5% of endometrial carcinomas were derived from the LUS (21). Interestingly, the mean age of the women with LUS tumors was significantly younger (54.2 years) compared to the women with uterine corpus endometrial carcinoma (63.0 years) (21). A relatively high percentage (34.2%) of the LUS carcinomas was MSI-high; 28.6% of the LUS carcinomas were confirmed to be from women with Lynch Syndrome (21). 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 to 2%) (44, 63, 64) or in young women (50 years of age or younger) with endometrial cancer (9 to 11%) (45, 58).

Which endometrial cancer patients should be evaluated for Lynch Syndrome?

Young age at endometrial cancer diagnosis and/or the presence of a family history of a Lynch Syndrome-related cancer are typically used clinical criteria to pursue further evaluation for Lynch Syndrome. However, the possibility of Lynch syndrome should not necessarily be excluded by later age of endometrial cancer diagnosis. In the population-based study of 543 women with endometrial cancer (58), 10 Lynch Syndrome mutation carriers were identified. Four of the 10 women were younger than age 50 years, but six were older than age 50 years. 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 older than age 50 years (45). A recent study concluded that immunohistochemistry testing of *MLH1*, *MSH2*, *MSH6*, and *PMS2* in an endometrial cancer patient of any age with at least one first-degree relative with a Lynch Syndrome associated cancer would be a cost-effective approach for identifying women with Lynch Syndrome (65). In our opinion, this approach seems too restrictive, as many *MSH6* mutation

carriers have uninformative family histories. Furthermore, the nature of *PMS2* mutation-associated endometrial cancer has not yet been elucidated. Limited preliminary evidence from our studies suggests that women with endometrial cancers with *PMS2* immunohistochemical loss are older and do not tend to have first degree relatives with Lynch Syndrome-associated cancers. Thus, *PMS2* mutation may be phenotypically similar to *MSH6* mutation, and the incidence of *PMS2* mutation may be seriously underestimated using younger age and family history as determinants for further testing. At this time, for most effective screening for Lynch Syndrome, we recommend immunohistochemistry for MLH1, MSH2, MSH6, and *PMS2*, MSI analysis, and, when the tumor shows loss of MLH1 by immunohistochemistry, *MLH1* methylation analysis for all women diagnosed with endometrial cancer. It is currently controversial whether performing both immunohistochemistry and MSI analysis 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 microsatellite instability (66–68). However, here a cautionary note must be raised. It has been shown in a large study of more than 1,400 colorectal carcinoma patients that occasional patients have MSI-high tumors that show retained positive expression for DNA mismatch repair proteins. In the patients who were felt to be moderate-to-high risk for having Lynch Syndrome, this MSI-immunohistochemistry discordance is seen in approximately 6% of MSI-high cases(69). The reasons for such discordances are not currently clear. It is possible that some of these patients have missense mutations of a DNA mismatch repair gene that result in translation of a non-functional protein. Indeed, in our personal experiences with tissue testing, we have encountered several cases in which *MLH1* mutations were identified in patients with MSI-high tumors with intact immunohistochemical expression of MLH1, MSH2, MSH6, and *PMS2*. Because of the possibility of such discordances, we currently advocate that MSI analysis be performed in tandem with immunohistochemistry. If MSI testing is not possible, it should be recognized that a small percentage of MSI-high carcinomas will not be captured by performing immunohistochemistry alone.

Targeted Therapy

Evaluation of Estrogen and Progesterone Receptors for Therapeutic Purposes

Hormonal therapy in endometrial carcinoma has several roles, including progestin treatment of patients with endometrial hyperplasia or early stage grade 1 endometrioid adenocarcinoma who desire to preserve fertility or who are not optimal surgical candidates because of comorbid conditions, such as obesity, hypertension, or insulin-resistant diabetes (70–73). In these situations, testing endometrial tissue for immunohistochemical expression of hormone receptors is likely not necessary, as it is widely known that endometrial hyperplasia and grade 1 endometrioid carcinomas have good expression of these receptors.

Progestins can also be used to treat patients with recurrent or advanced endometrial cancer, especially the endometrioid type. In these patients, the overall rate of response was 25% and was shown to directly correlate with levels of serum progesterone (74). Selective ER modulators, such as tamoxifen, and aromatase inhibitors are other hormonal therapies used for recurrent or advanced endometrial cancer, with overall rates of response of 22% and 9.4% respectively as single therapy agents. (75, 76) In current practice, in the setting of advanced or recurrent endometrial cancer, pathologists are often asked to assess ER and PR expression levels in tumor tissue, which are commonly reported as a percentage of cells with nuclear expression. However, despite numerous previous clinical trials, there are no established guidelines for ER and PR assessment. In fact, it is not at all established that immunohistochemical expression of these hormone receptors is significantly related to treatment response (77).

PI3K –AKT-mTOR pathway

PTEN protein, and the PI3K –AKT-mTOR pathway it regulates, play a major role in the pathogenesis of endometrial carcinoma, particularly of the endometrioid histotype. Loss of *PTEN* tumor suppressor activity or activating mutations in the key molecules of the pathway lead to uncontrolled activation of mammalian target of rapamycin (mTOR) and, subsequently, protein S6 kinase, resulting in dysregulated control of protein translation and cell cycle progression (78).

AKT mutations are infrequent (2% to 4%) in endometrial carcinoma (79, 80). However, *PIK3CA* (the gene coding for the catalytic subunit of PI3K) (24 to 39%) (81–83) and *PTEN* sequence abnormalities (34 to 55%) (84, 85) commonly occur in endometrial tumors, particularly in the endometrioid histotype. As targeted therapeutics against the PI3K –AKT-mTOR pathway enter the clinic (86, 87), accurate tests to detect functional abnormalities of its components will be crucial for trial design and assessment of benefit of novel therapeutic agents. This is a complex task, as recent studies have shown that aberrations in multiple pathway components are common (88, 89). Furthermore, while *AKT* (80) and *PIK3CA* (88, 90) are altered primarily by mutation and can be detected by targeted sequencing of mutational hotspots, loss of *PTEN* protein occurs from a variety of causes including somatic mutations, abnormalities in *PTEN* transcriptional and post-transcriptional regulation, actions of micro RNAs, as well as due to aberrant mechanisms of *PTEN* protein stability and degradation (91). Therefore, clinically validated testing of endometrial tumors for aberrations in the PI3K –AKT-mTOR pathway will require the use of multimodal assays and an algorithmic approach.

PTEN gene sequence abnormalities are highly variable in type (frameshifts, point mutations) and can occur throughout all 9 exons (84, 85, 92). This necessitates the use of full length sequencing in order to detect *PTEN* mutations and subsequent loss of *PTEN* protein function. We recently compared the utility of sequence analysis with immunohistochemistry as a method for assessment of functional *PTEN* loss. In a large cohort of endometrioid and non-endometrioid endometrial carcinomas, we demonstrated that *PTEN* immunohistochemistry can identify 89% of cases with a *PTEN* sequence abnormality. Interestingly, however, among cases classified as *PTEN* wildtype by sequencing, as many as 44% exhibited *PTEN* protein loss by immunohistochemistry (93). In this study, we employed a 3-tiered *PTEN* immunohistochemical scoring system, based on intrinsic staining patterns which we observed empirically. *PTEN* expression was designated as positive (cytoplasmic and nuclear staining in greater than 90% of cells), negative (no or only occasional tumor cells staining), and heterogeneous (distinct positive and negative foci within the tumor) (Figure 9). The biological nature of the *PTEN* heterogeneous endometrial carcinomas is presently not known. The *PTEN*-positive and *PTEN*-negative tumor cells may represent different subclones within the same tumor. Additional studies are needed to determine whether patients with this pattern of *PTEN* immunohistochemical expression may derive some benefit from PI3K-AKT pathway inhibitors. In addition to immunohistochemistry being less expensive and a more accessible laboratory technique compared to gene sequencing, we have shown that this scoring system is highly reproducible for endometrial carcinoma (94).

In a recent study of patients with advanced solid tumors, including endometrial carcinomas, patients with *PIK3CA* tumor mutations had a better response to PI3K –AKT-mTOR inhibitors than patients with tumors with wild-type *PIK3CA* (87). Interestingly, the presence of a *KRAS* mutation in the same tumor acted as a “dominant negative;” in other words, *KRAS* mutation is associated with resistance to pathway inhibition, no matter the *PIK3CA* mutation status (88). Similar results with *KRAS* mutation have been demonstrated in cell lines (95–97). Therefore, analysis of *KRAS* mutational status may be necessary for

optimally defining biomarker directed eligibility to targeted therapies in endometrial cancer patients. The incidence of *KRAS* mutations in endometrial carcinoma is 14 to 36%.(98–101) As part of the RAS-RAF-MEK-MAP kinase pathway, RAS recruits RAF to the plasma membrane, triggering pathway activation and eventual translocation of MAP kinase to the nucleus, where it promotes transcription of genes involved in cell proliferation.(102) RAS can also bind to and activate PI3K, resulting in stimulation of the AKT-mTOR pathway. (103) *KRAS* activating mutations occur almost exclusively in codon 12 as single point mutations, which can be detected by PCR-based assays. (99–101, 104) Activating mutations in *BRAF* have been shown to induce sensitivity to MEK inhibitors in cancer cells lines and early clinical trials of melanoma (105). However, *BRAF* mutations are rare in endometrial carcinoma (1 to 4%)(106, 107).

Challenges

Targeted Therapy

At this time, there are many more clinical trials of novel targeted therapy agents than there are clinically ready pathology assays to assess for tissue targets or indicators of treatment response. In the past, clinical trials testing new chemotherapeutic agents did not involve the pathology laboratory. However, as the treatment of endometrial cancer patients, especially those who have recurrent or metastatic disease, becomes more individualized, it will be a challenge for pathologists to provide clinically useful assays, either immunohistochemical or molecular, to help identify the endometrial cancer patients who would benefit most from these therapies. Current targeted agents being used in endometrial cancer clinical trials with no companion pathology assays include metformin, MEK inhibitors, IGF-IR inhibitors, bevacizumab and other angiogenesis inhibitors, fibroblast growth factor inhibitors, poly (ADP-ribose) polymerase (PARP) inhibitors, EGFR inhibitors, and multi-kinase inhibitors. Many of the novel targeted therapies target growth factor related cellular pathways that require activation by protein phosphorylation. Currently, pathologists do not have clinically-ready assays to assess for important phosphorylated proteins such as pERK or pAKT. Many antibodies directed against phosphorylated proteins are useful in Western blots of frozen tissues, but do not work well in formalin-fixed, paraffin-embedded tissues.

Gene Sequencing

PCR-based molecular assays are typically developed using frozen cancer tissues, especially from primary tumors from which ample tumor is typically available. However, the routine use of frozen tissues is usually not clinically feasible. The adaptation of select molecular assays for use in formalin-fixed, paraffin-embedded tissues can be achieved, especially if these assays involve detection of point mutations in single exons or a small number of exons. Such assays include mutational analysis of *BRAF*, *KRAS*, and *PIK3CA*. However, for many genes, hotspot mutations either do not exist, or they have not yet been identified, making sequencing the entire gene necessary. Sequencing of large genes, such as *PTEN*, may not be feasible using formalin-fixed, paraffin-embedded tissues. In the case of *PTEN*, the commercially available immunohistochemical antibodies can be used as a surrogate method to determine *PTEN* functional loss in an endometrial cancer. However, for many gene products, such clinically useful antibodies do not exist.

At this time, for most molecular assays, it is uncertain whether the primary tumor from the hysterectomy surgical specimen or the recurrence/metastasis should be examined. One advantage of using the primary surgical specimen is that more tumor is available for analysis than in the typical fine needle aspirate or needle core biopsy of a recurrence. However, it is quite possible that the recurrence/metastasis may have disparate molecular alterations. This has not been adequately explored in endometrial cancer or in other solid cancer types.

Recently, it was reported that a small subset of colorectal cancer patients have discordant *KRAS* mutation results when comparing the primary tumor to the metastasis (108). Furthermore, metastases may also be heterogeneous, harboring mutant and wild-type clones. Therefore, if a mutation analysis from a metastasis/recurrence initially yields a negative result, does this mean the entire tumor lacks this mutation? Should a different metastatic site be biopsied and re-tested? The answers to these questions are currently unknown.

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Key Points

- Differentiation of endometrial from endocervical adenocarcinoma in endocervical and endometrial biopsies requires use of immunohistochemistry; the distinction is clinically important because it will help to guide the gynecological surgeons in their surgical planning and subsequent treatment approach.
- Judicious interpretation and correlation with tumor morphology is essential; when all four markers are examined together (estrogen receptor, vimentin, monoclonal carcinoembryonic antigen, and p16), a high percentage of endocervical and endometrial tumors do not strictly adhere to the anticipated patterns.
- Immunohistochemistry is not useful in distinguishing endometrial from ovarian endometrioid adenocarcinoma.

Figure 1. Typical immunohistochemical expression pattern for an endometrial endometrioid adenocarcinoma

Endometrioid-type endometrial adenocarcinoma (A, representative H&E stain) is usually strongly and diffusely positive for vimentin (B), negative for monoclonal CEA (C), positive for ER (D), and has patchy positive expression of p16 (E). Higher grade endometrioid tumors may lose some nuclear ER expression, but even the grade 3 endometrioid tumors will have at least some ER expression.

Figure 2. Typical immunohistochemical expression pattern for an endocervical adenocarcinoma In biopsies, it can frequently be difficult to distinguish an endocervical adenocarcinoma from an endometrial adenocarcinoma. This distinction is important, as the surgical approaches can differ between the two tumor types. Endocervical adenocarcinoma (A, representative H&E) is usually negative for vimentin (B; strong staining in left portion of figure is stroma), strongly positive for monoclonal CEA (C), negative for ER (D), and diffusely positive for p16 (E).

Figure 3. Hysterectomy specimen with mucinous adenocarcinoma involving the endocervix and lower uterine segment

Unfortunately, immunohistochemistry cannot always resolve difficult pathology diagnostic problems. Based on the gross examination and examination of H&E stained slides (A), the differential diagnosis of this tumor includes endocervical adenocarcinoma, endometrial endometrioid adenocarcinoma with mucinous differentiation, and metastatic colorectal adenocarcinoma. The tumor was negative for cytokeratin 20 (B) and strongly positive for cytokeratin 7(C). While this profile favors a gynecological origin for this tumor, it must be remembered that a subset of MSI-High colorectal adenocarcinomas can express CK 7(109). The tumor was negative for vimentin and ER and had weak and patchy nuclear expression of CDX2, patchy expression of p16, and strong expression for CEA (data not shown). The immunohistochemistry profile therefore could not definitively establish a primary site for this malignancy. A subsequent colonoscopy was negative, so this tumor most likely represents a primary endocervical or endometrial adenocarcinoma. An endocervical primary may be favored because of the lack of ER and vimentin expression.

Figure 4. Uterine serous carcinoma

This serous carcinoma (representative H&E, A) shows a typical immunohistochemical expression pattern. The tumor has strong and diffuse nuclear expression of p53 (B) and p16 (C). WT-1 is positive (nuclear) in most ovarian high grade serous carcinomas. However, uterine serous carcinoma is usually negative for WT-1 (D). This tumor is ER positive (E).

Figure 5. Uterine serous carcinoma with atypical immunohistochemical expression pattern

By routine H&E staining (A), this tumor has features typical of uterine serous carcinoma, specifically high nuclear grade and loss of nuclear intra-epithelial polarity. However, the tumor is negative for p53 (B). The tumor did show strong and diffuse expression of p16 (C) and was negative for WT-1 (D). Unlike the serous carcinoma presented in Figure 4, this example is negative for ER (E).

Figure 6. MSI analysis chromatogram

In this example, the tumor has allelic shift for the microsatellites BAT26 and D17S250, as demonstrated by the extra peaks in the tumor tracings compared to that of normal tissue. The five other microsatellites in the panel had similar allelic shift, so this is an MSI-High endometrial carcinoma.

Figure 7. *MLH1* methylation analysis

Extracted 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. Concurrent analyses of control cell lines (top tracing, negative control K562; middle tracing, positive control RKO) are important. The endometrial tumor (bottom tracing) has both unmethylated and methylated *MLH1*. The unmethylated peak is likely due to the presence of contaminating normal stromal cells.

Figure 8. Immunohistochemical expression of DNA mismatch repair proteins in endometrial carcinoma

This tumor is negative for MSH2 (A) and has positive nuclear expression for MLH1 (B). In (A) note the presence of positive staining stromal cells. Such cells are a useful internal positive control.

Figure 9. Patterns of PTEN immunohistochemical expression seen in endometrial carcinoma Positive (A), heterogeneous (B), and negative (C) examples are provided. In cases with PTEN negative staining, the presence of the positive staining stromal cells serves as a useful internal positive control.