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Utility of *MLH1* Methylation Analysis in the Clinical Evaluation of Lynch Syndrome in Women with Endometrial Cancer

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Abstract

Clinical screening criteria, such as young age of endometrial cancer diagnosis and family history of signature cancers, have traditionally been used to identify women with Lynch Syndrome, which is caused by mutation of a DNA mismatch repair gene. Immunohistochemistry and microsatellite instability analysis have evolved as important screening tools to evaluate endometrial cancer patients for Lynch Syndrome. A complicating factor is that 15-20% of sporadic endometrial cancers have immunohistochemical loss of the DNA mismatch repair protein MLH1 and high levels of microsatellite instability due to methylation of *MLH1*. The PCR-based *MLH1* methylation assay potentially resolves this issue, yet many clinical laboratories do not perform this assay. The objective of this study was to determine if clinical and pathologic features help to distinguish sporadic endometrial carcinomas with MLH1 loss secondary to *MLH1* methylation from Lynch Syndrome-associated endometrial carcinomas with MLH1 loss and absence of *MLH1* methylation. Of 337 endometrial carcinomas examined, 54 had immunohistochemical loss of MLH1. 40/54 had *MLH1* methylation and were designated as sporadic, while 14/54 lacked *MLH1* methylation and were designated as Lynch Syndrome. Diabetes and deep myometrial invasion were associated with Lynch Syndrome; no other clinical or pathological variable distinguished the 2 groups. Combining Society of Gynecologic Oncology screening criteria with these 2 features accurately captured all Lynch Syndrome cases, but with low specificity. In summary, no single clinical/pathologic feature or screening criteria tool accurately identified all Lynch Syndrome-associated endometrial carcinomas, highlighting the importance of the *MLH1* methylation assay in the clinical evaluation of these patients.

Keywords

Lynch Syndrome; molecular diagnostics; *MLH1* methylation; immunohistochemistry; endometrial cancer

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Introduction

Lynch syndrome is an autosomal dominant, inherited cancer syndrome due to a germline mutation in a gene controlling DNA mismatch repair (MMR), such as *MLH1, MSH2, MSH6* or *PMS2*. Lynch Syndrome is thought to account for 1-6% of all endometrial cancers [1, 2]. For women with Lynch Syndrome, the lifetime risk of endometrial cancer is 60%, while the lifetime risk of colorectal cancer is 39-54% [3,4]. Endometrial cancer or ovarian cancer precede the diagnosis of colorectal cancer in approximately half of women with Lynch Syndrome, leading to the concept of these gynecological cancers being a sentinel cancer for these women [5]. Diagnosis of Lynch Syndrome at the time of sentinel cancer diagnosis provides the patient with the opportunity to undergo surveillance for other cancers associated with Lynch Syndrome, especially colorectal cancer. Identification of Lynch Syndrome at the time of sentinel cancer diagnosis also impacts family members, as they can be subsequently tested and appropriate cancer prevention surveillance initiated for affected individuals.

Sequencing germline DNA for detecting pathologic mutations in *MLH1, MSH2, MSH6*, and *PMS2* is the gold standard for diagnosing Lynch Syndrome. Universal germline testing of all patients with a diagnosis of colorectal or endometrial cancer is not a cost effective option for patient identification given that less than 5% of these cancers are thought to be due to Lynch Syndrome mutations [1,2]. To identify individuals who would most benefit from genetic testing, clinical screening tools and tumor testing have emerged as methods to assist in triaging which individuals with endometrial carcinomas would benefit from germline testing.

Clinical screening criteria have primarily been developed from information found in familial colorectal cancer registries. Frequently used clinical features include young age at diagnosis, strong family history of colorectal and/or endometrial cancer and right-sided colon cancer tumors [6]. Amsterdam II criteria, published in 1999, were intended to identify individuals with Lynch Syndrome based on clinical history alone and sacrifice sensitivity (39-72%) for a higher specificity (78-91%) [3,4]. These criteria also place heavy emphasis on colorectal cancer, with little recognition of extra-colonic tumors. The Revised Bethesda Criteria, published in 2002, were intended to determine which cancer patients warranted further screening with PCR-based microsatellite instability testing, as high levels of microsatellite instability result from defects in DNA mismatch repair [7]. Clinical criteria are less rigid than Amsterdam II criteria, and accordingly published data show higher sensitivity (82-94%) and lower specificity (25-41%) [3,4]. Tumors demonstrating high levels of microsatellite instability can then undergo further analysis with immunohistochemistry and/or genetic testing to pinpoint the probable MMR gene mutation. Within the field of gynecologic oncology, one critique of Amsterdam II criteria and Revised Bethesda criteria is their “colocentric” emphasis. The Society of Gynecologic Oncology (SGO) devised guidelines in 2007 to broaden the inclusion criteria of women that should undergo screening [8]. These criteria parallel the revised Bethesda criteria, but SGO criteria include both endometrial cancer and colorectal cancer as the cancer of reference when analyzing patient and family history.

In addition to the clinical screening criteria summarized above, tumor tissue testing using immunohistochemistry (IHC) for MLH1, MSH2, MSH6, and PMS2 proteins and microsatellite instability analysis can provide useful triage steps in the evaluation for Lynch Syndrome. This approach can be further streamlined by omitting microsatellite instability analysis, as concordance with IHC is 88-95% [9]. Tissue testing can be used after patients have been identified by clinical screening criteria, or it can be applied reflexively on all colorectal and endometrial cancer specimens. Advantages of up-front tissue testing include

that IHC and MSI analysis can be performed relatively quickly, they are less expensive than germline MMR gene sequencing, and they do not rely on physician acquisition of complete and accurate family history information. Family history acquisition in the healthcare setting can be inconsistent. For example, one study investigating genetic counselor referral for *BRCA* testing among women presenting with ovarian cancer showed that only 23.8% of patients with increased risk for a *BRCA* mutation were actually referred to a genetic counselor [10].

Informative IHC results provide a gene of interest for directed germline testing. IHC loss of MSH2, MSH6, or PMS2 is generally thought to be pathognomonic for Lynch Syndrome and can point to a specific MMR gene for sequencing. IHC loss of MLH1, however, entails further investigation to delineate a sporadic tumor from a probable Lynch Syndrome tumor. Approximately 15-20% of sporadic colorectal adenocarcinomas and endometrial carcinomas have immunohistochemical loss of MLH1 due to epigenetic methylation of the *MLH1* gene promoter [11-14]. Thus, tumors exhibiting immunohistochemical loss of MLH1 in the absence of *MLH1* promoter methylation are presumed to be associated with Lynch Syndrome, prompting sequencing of the *MLH1* gene to detect a mutation. The addition of *MLH1* promoter methylation analysis to the tissue testing approach has the potential to significantly decrease the number of individuals who would need to be referred for germline *MLH1* testing. Unfortunately, this PCR-based test is not universally performed prior to referral to a genetic counselor when MLH1 IHC loss is detected in a tumor [2, 7, 15, 16]. The reasons for this are unclear. Some clinical pathology laboratories do not have access to PCR-based testing, so only IHC is performed. The purpose of this study was to determine if existing clinical screening criteria for Lynch Syndrome could accurately distinguish MLH1-negative, *MLH1* methylated (sporadic) endometrial carcinomas from MLH1-negative, *MLH1* unmethylated (Lynch Syndrome) endometrial carcinomas.

MATERIALS AND METHODS

Patient Population and Study Design

After obtaining Institutional Review Board approval, we identified 350 endometrial carcinoma patients for this study. Endometrial cancer cases were included if the patient was 18 years of age or older, and surgery was performed at our institution. Patient characteristics, including age at diagnosis, body mass index (BMI), medical history of diabetes or hypertension, and family history of cancer were derived from the patient's electronic medical record. Tumor characteristics such as histologic tumor type, grade, stage, depth of myometrial invasion, lymphatic/vascular space invasion and tumor size were derived from pathology reports generated by 6 gynecologic pathologists. Of the initial 350 patients, 13 were excluded for further analysis because of insufficient clinical or pathologic data. For the remaining 337 patients, MLH1 immunohistochemistry and *MLH1* methylation analysis were performed according to the study design summarized in Figure 1.

Immunohistochemistry

Sections from formalin-fixed, paraffin-embedded endometrial carcinomas were used for immunohistochemical analysis (IHC). These analyses were conducted in Clinical Laboratory Improvement Amendments (CLIA) – and College of American Pathology – approved laboratories. Immunohistochemistry for MLH1 (G168-15, 1:25; BD Biosciences Pharmingen) was performed using standard techniques [9, 17]. Tumor with loss of MLH1 protein as visualized by light microscopy were classified as MLH1 negative. Stromal and normal tissues adjacent to tumor cells served as internal positive controls. Figure 2 shows examples of endometrial carcinomas with intact (positive) and absent (negative) MLH1 protein expression.

MLH1 Methylation Analysis

For cases in which there was immunohistochemical loss of MLH1 protein expression, PCR-based *MLH1* promoter methylation analysis was performed. DNA was isolated from formalin-fixed, paraffin-embedded tissue sections that were microdissected with a scalpel blade to provide relatively pure tumor samples for analysis. Isolated DNA was treated with bisulfite to convert methylated cytosine to uracil. The treated DNA was then amplified using fluorescently labeled PCR primers that were specific for methylated (M) or the unmethylated (U) versions of *MLH1* (*MLH1*-M forward, 5_-gatagcgatttttaacgc-3_ and *MLH1*-M reverse, 5_-tctataaatactaaatctcttcg-3_; *MLH1*-U forward, 5_-agagtggatagtgatttttaatgt-3_ and MLH1-U reverse, 5_-actctataaattactaaatctcttca-3_). Amplified PCR products were then detected using capillary electrophoresis and GeneScan software. Chromatograms for tumor were compared to those generated for the RKO colon carcinoma cell line (positive control known to have loss of MLH1 protein due to *MLH1* promoter methylation) and the leukemia cell line K562 (negative control with no *MLH1* methylation) [17]. Figure 3 shows an example of an *MLH1* methylation chromatogram.

Screening Criteria

Clinical and pathologic data were collected for all cases exhibiting immunohistochemical loss of MLH1 protein. Amsterdam II, Society of Gynecologic Oncology (SGO) and Clinical History and Pathology (CHiP) criteria were applied to these cases. Amsterdam II criteria require at least 3 family members with a Lynch Syndrome associated tumor in which one is a first degree relative of the other two, two successive generations affected with one tumor occurring before the age of 50 and a diagnosis of familial adenomatous polyposis should have been ruled out [18]. SGO criteria consist of two tiers, one which parallels the Amsterdam criteria and one which parallels the Bethesda guidelines. For the purposes of this study, we utilized the criteria that parallel the Bethesda guidelines, which is associated with a 5-10% chance of detecting a MMR gene mutation among those who meet criteria. This includes women with a diagnosis of endometrial cancer or colorectal cancer prior to the age of 50, presence of synchronous endometrial cancer or ovarian cancer with colorectal cancer, first degree relatives diagnosed with Lynch Syndrome associated tumors prior to age 50 or a family history with multiple Lynch Syndrome associated tumors among extended family [8]. Based on a retrospective evaluation of Lynch Syndrome cases among endometrial cancer patients at our institution, we developed a third clinical screening tool, CHiP criteria. These criteria include endometrial cancer patients who meet any of the following: diagnosis of endometrial cancer between the ages of 30-50, a first degree relative diagnosed with endometrial cancer or colorectal cancer prior to the age of 50, 2 or more relatives diagnosed with endometrial cancer or colorectal cancer at any age, or tumor arising from the lower uterine segment (LUS) at the time of hysterectomy. These criteria were based, in part, on published data showing the association between Lynch Syndrome and endometrial cancer among young patients and those with LUS tumors [19-21].

Statistical Analysis

Statistical analyses were conducted using SAS software, version 9.2 (SAS, Inc. Cary, NC). Differences in clinical and demographic characteristics between patients with sporadic endometrial cancer (MLH1 IHC negative, presence of *MLH1* methylation) and those with presumed Lynch syndrome (MLH1 IHC negative, absence of *MLH1* methylation) were examined using the Wilcoxon rank sums test or Fisher's exact test in the case of categorical data. A P-value of <0.05 was considered statistically significant. Sensitivity was calculated as the proportion of patients who screened positive for Lynch Syndrome among those patients who were positive for it. Specificity was calculated as the proportion of patients who screened negative for Lynch Syndrome among those patients with sporadic disease.

Additionally, we used Cartesian and regression tree (CART) analysis to determine whether a particular subset of traits was more associated with Lynch syndrome. We also approached this from the opposite perspective. We used agglomerative hierarchical clustering techniques to group patients based upon similarity of traits and then examined those clusters for a difference in distribution of Lynch syndrome. Distance was calculated as Euclidean distance squared, and average linkage was used to join clusters.

RESULTS

Patients

MLH1 IHC was performed on 337 of the 350 endometrial cancer cases in which complete clinical and pathologic data were available (Figure 1). Immunohistochemical loss of MLH1 nuclear expression was detected in 54/337 (16%) of endometrial tumors. Of these, 40/54 (74.1%) endometrial tumors demonstrated *MLH1* promoter methylation and were designated as sporadic endometrial carcinomas. The remaining 14/54 (25.9%) lacked MLH1 methylation and were designated as presumed Lynch Syndrome. The proportion of *MLH1* methylated tumors is comparable to that seen in several other studies consisting of unselected endometrial cancer patients with a range of 65-96.9% [14, 22, 23-24]. The data summarized in Tables 1 and 2 are for these 54 patients with IHC loss of MLH1 in their tumors.

Evaluation of Clinical and Pathologic Characteristics

Clinical and pathologic characteristics for the endometrial cancer patients with and without *MLH1* promoter methylation are shown in Table 1. There was no statistical difference between median age of diagnosis, median body mass index (BMI), or family history of colorectal cancer or endometrial cancer between the *MLH1* methylated and unmethylated promoter groups. The median age of diagnosis for both groups was younger than the median age of diagnosis of endometrial cancer in the general population, 61 years [25]. A personal history of diabetes was statistically more common in the unmethylated group. The presence of diabetes among patients with and without *MLH1* methylation has not been previously reported for endometrial or colorectal cancer patients, so no comparisons can be made to other published studies.

Investigation of tumor-specific characteristics revealed no statistical differences between the two groups with respect to histology, FIGO stage, endometrioid grade, lymphatic/vascular space invasion, tumor location or tumor size (Table 1). Depth of myometrial invasion was the only pathologic characteristic that was statistically different between the two groups. 37.5% of *MLH1* methylated tumors had deep myometrial invasion, whereas 71.4% of the *MLH1* unmethylated tumors had myometrial invasion greater than or equal to 50% myometrial thickness. In the current study, 25/54 of the patients had endometrial carcinomas with depth of myometrial invasion greater than or equal to 50% total myometrial thickness. 10/25 of these lacked *MLH1* methylation, so this pathologic characteristic alone would have poor specificity in predicting Lynch Syndrome.

It is possible that a combination of clinical and pathological features may better distinguish the 2 groups with IHC loss of MLH1 rather than any single feature. Cartesian and regression tree (CART) analysis of all clinical and pathologic factors summarized in Table 1 was performed to further investigate these potential combinations. This analysis yielded no further statistically significant associations between different combinations of clinical and pathologic factors to differentiate between tumors with methylated and unmethylated *MLH1* (data not shown).

Evaluation of Screening Criteria

The sensitivity and specificity of various clinical screening criteria and selected patient characteristics are presented in Table 2. Young age of endometrial cancer diagnosis was included here, as this is a common single feature included in many different clinical screening criteria for Lynch Syndrome. BMI less than 30 was also included, because it has been previously reported that endometrial cancer patients with Lynch Syndrome have a significantly lower BMI than patients with sporadic endometrial cancer [20]. Single factors such as young age, BMI less than 30, family history of colorectal cancer, and family history of endometrial cancer showed poor overall sensitivity and specificity in ability to predict *MLH1* methylation status accurately. The SGO criteria had a moderate sensitivity (71.4%) and specificity (69.2%). Amsterdam II criteria had a high specificity, 94.9%, at the expense of sensitivity, only 14.3%. CHiP criteria, despite the inclusion of lower uterine segment tumors, did not perform as well as the SGO criteria. When the statistically significant factors from Table 1, deep myometrial invasion and patient history of diabetes, were added to SGO criteria, sensitivity increased to 100%, but specificity was low at 35.9%.

Overall, SGO criteria had the best sensitivity and specificity profile of the screening criteria evaluated (Table 2). Table 3 shows patient clinical and pathologic characteristics of the 4 endometrial cancer cases lacking *MLH1* promoter methylation (presumed Lynch Syndrome) not captured by SGO criteria (in other words, 4 patients designated as sporadic endometrial cancer rather than Lynch Syndrome associated endometrial cancer). In each case, patients are older than age 50 years, have a body mass index greater than 30, and there is no family history of colorectal cancer. One patient has a tumor arising from the lower uterine segment, an uncommon tumor location associated with a 29% risk of Lynch Syndrome [19]. All but one of the patients had deep myometrial invasion, a characteristic we found to be associated with unmethylated *MLH1* tumors (Table 1).

DISCUSSION

The current study was designed to determine if any clinical, pathological or clinical screening tool could effectively replace performance of the PCR-based *MLH1* methylation analysis in the evaluation of endometrial cancer patients for possible Lynch Syndrome. Therefore, this study was limited to endometrial carcinomas with immunohistochemical loss of MLH1. We found that clinical characteristics, with the exception of diabetes, were poor predictors of *MLH1* methylation status. Presence of diabetes had low sensitivity, limiting its potential usefulness as a screen. Furthermore, presence/absence of diabetes was typically self-reported and not formally tested for in this study. Therefore, we cannot exclude the possibility that other patients in our study had subclinical, previously undetected diabetes. Interestingly, age of onset of endometrial cancer did not distinguish between the *MLH1* methylated (sporadic) and *MLH1* unmethylated (Lynch Syndrome) groups. Several studies have shown that the prevalence of Lynch Syndrome due to defects in all DNA MMR genes is increased in women diagnosed with endometrial cancer at age younger than 50 years [20, 26]. In the study by Lu et al. [20], 9% (9/100) of women presenting with endometrial cancer at age younger than 50 years had an identifiable germline mutation confirming the diagnosis of Lynch Syndrome. Of these 9 women, 7 had an *MSH2* mutation, 1 had an *MLH1* mutation, and 1 had an *MSH6* mutation. In a similar study performed by Walsh et al. [26], 18% (26/146) of women with endometrial cancer diagnosed at less than age 50 years had molecular diagnostics testing (IHC, MSI, and *MLH1* methylation) consistent with Lynch Syndrome. In their study, there were 6 expected mutations in *MLH1*, 13 expected mutations in *MSH2*, and 7 expected mutations for *MSH6*. In both of these studies, *MSH2* mutation carriers were the most likely to present with Lynch Syndrome associated endometrial cancer at a younger age. Another previous investigation, examining 40 endometrial carcinomas

with immunohistochemical loss of MLH1, found a significant difference between the age of endometrial cancer diagnosis in *MLH1* methylated (mean age 56.1 years) versus unmethylated (mean age of 65.4 years) cases [23]. Note that in both of these groups, the mean ages were greater than 50 years.

It has been shown that women who are overweight (BMI 24.1-29.9) or obese (BMI ≥ 30.0) are 2-3.5 times more likely to develop endometrial cancer than their normal weight counterparts [27]. The relationship between BMI and Lynch Syndrome-associated endometrial cancer has been investigated previously in patients younger than age 50 [20, 28]. In the study by Schmeler et al. [28], 56% of 188 patients under the age of 50 with endometrial cancer were obese (BMI ≥ 30.0), but all six patients with Lynch Syndrome were either normal weight or overweight (BMI 25.0-29.9). Lu et al. [20] found that a median BMI of 27.6 among Lynch Syndrome endometrial cancer cases was significantly lower than the median BMI of 37.5 among sporadic cases. The sensitivity and specificity of BMI ≤ 30 for predicting Lynch Syndrome was 56% and 65%, respectively [20]. McCourt et al. evaluated microsatellite instability in a series of 473 sequential endometrial carcinomas and found that patients with MSI-high tumors had a significantly lower BMI (30.3) than those with microsatellite-stable tumors (32.7) [22]. It is possible that the association between Lynch Syndrome associated endometrial cancers and lower BMI is only applicable for patients with defects in *MSH2, MSH6, and PMS2*. Alternatively, the increasing incidence of obesity in the United States may obscure the associations with BMI that were previously reported in older publications.

Previous investigators have demonstrated that there are macroscopic and microscopic pathologic features of endometrial carcinomas that correlate with a diagnosis of Lynch Syndrome. Westin et al. examined endometrial carcinomas arising from the lower uterine segment (LUS) and found that 29% (10/35) were Lynch Syndrome-associated [19]. Among these 10 cases, 9/10 had loss of MSH2 by IHC and 1 had loss of MLH1 without *MLH1* methylation. In our current series, LUS tumor location did not distinguish between the methylated and unmethylated *MLH1* tumors with IHC loss of MLH1. Thus, as with young age of endometrial cancer diagnosis and lower BMI, tumor location in the LUS may not be a prominent feature of MLH1-associated Lynch Syndrome endometrial carcinomas. The presence of increased peritumoral lymphocytes and tumor-infiltrating lymphocytes has been associated with MSI-high endometrial carcinomas, although not specifically with Lynch Syndrome [29, 30]. Although not specifically examined in the current study, we retrospectively did not note any qualitative difference in the lymphocytic infiltrate between the *MLH1* methylated and unmethylated groups (data not shown).

In the current study, patients with tumor with deep myometrial invasion were more likely to have unmethylated *MLH1*. Previously, it was shown that presumptive Lynch Syndrome endometrial carcinomas had deeper myometrial invasion than sporadic tumors, but these results were presented as mean mm of myometrial invasion with no myometrial thickness presented [26]. Traditionally, it is the ratio of mm myometrial invasion to mm myometrial thickness which is most predictive of advanced endometrial cancer stage and lymph node metastasis. Endometrial carcinomas with greater than 50% myometrial invasion are associated with the highest risk of lymph node spread. In our study, there was no statistical difference in median depth of myometrial invasion independent of total myometrial thickness (*MLH1* methylated, 9 mm; *MLH1* unmethylated, 9.5 mm).

Multiple Lynch Syndrome screening algorithms exist which incorporate various clinical and/or molecular diagnostic criteria. Amsterdam II and SGO criteria are models of clinical criteria which emphasize young age and prominent family histories of colorectal and endometrial cancer. In the past decade, the PREMM, MMRpredict and MMRpro prediction

models have emerged as validated Lynch Syndrome prediction models among colorectal cancer patients [31-33]. A study by Mercado et al. investigated the efficacy of these models among endometrial cancer patients and found that these models performed poorly in predicting germline mutations in this patient population [34].

When immunohistochemistry for presence of expression of DNA mismatch repair proteins is used as part of the analysis for Lynch Syndrome in endometrial or colorectal cancers, absence of MLH1 immunohistochemical protein expression is a poor predictor for a germline mutation [35], as most of these tumors will also have somatic methylation of the *MLH1* gene (sporadic carcinoma), rather than germline mutation (Lynch Syndrome). For colorectal cancer, the presence of *BRAF* mutation is also indicative of a sporadic tumor, as *BRAF* mutation is present in 40-50% of tumors with *MLH1* methylation, [36-38] , but is virtually absent in Lynch Syndrome associated tumors [39]. *BRAF* mutation analysis is a relatively simple PCR-based assay, as mutations can be assayed in one hotspot. *BRAF* mutations are exceedingly rare among sporadic endometrial carcinomas, so this analysis does not aid in the triage of endometrial tumors as sporadic or hereditary in nature [14, 40]. Perhaps when The Cancer Genome Atlas has completed its genomic analyses for endometrial cancer, mutation in an alternative gene or set of genes will be identified to be associated exclusively with sporadic endometrial cancer.

Our study suggests that clinical and pathologic screening criteria poorly predict which endometrial cancers with IHC loss of MLH1 are likely to have presence or absence of *MLH1* methylation. It is well known that many patients who have MSI-high endometrial or colorectal cancers with IHC loss of MLH1/MSH2/MSH6/PMS2 and lack of *MLH1* methylation will not have a germline mutation in a mismatch repair gene detected. This is due to current technology missing many insertions and deletions of these genes. Even without a germline mutation detected, it is important to emphasize that most gastroenterologists and gastrointestinal surgeons will offer these patients the same colorectal cancer screening guidelines they offer patients who do have germline mutations detected. Thus, these tissue testing strategies (immunohistochemistry, *MLH1* methylation) become centrally important in identifying endometrial cancer patients who are at potentially higher risk for subsequently developing colorectal cancer. In our series of 54 endometrial cancer cases with immunohistochemical loss of MLH1, 14/54 cases would be candidates for germline *MLH1* testing. SGO criteria correctly identifies 10/14 unmethylated tumors. If SGO criteria were solely used without the *MLH1* methylation assay, 22/54 patients would undergo germline testing for *MLH1*, thereby subjecting 12 women to unnecessary and expensive germline testing. *MLH1* promoter methylation testing should therefore be a standard component of clinical laboratory tumor testing for Lynch Syndrome evaluation among patients with endometrial cancers exhibiting immunohistochemical loss of MLH1.

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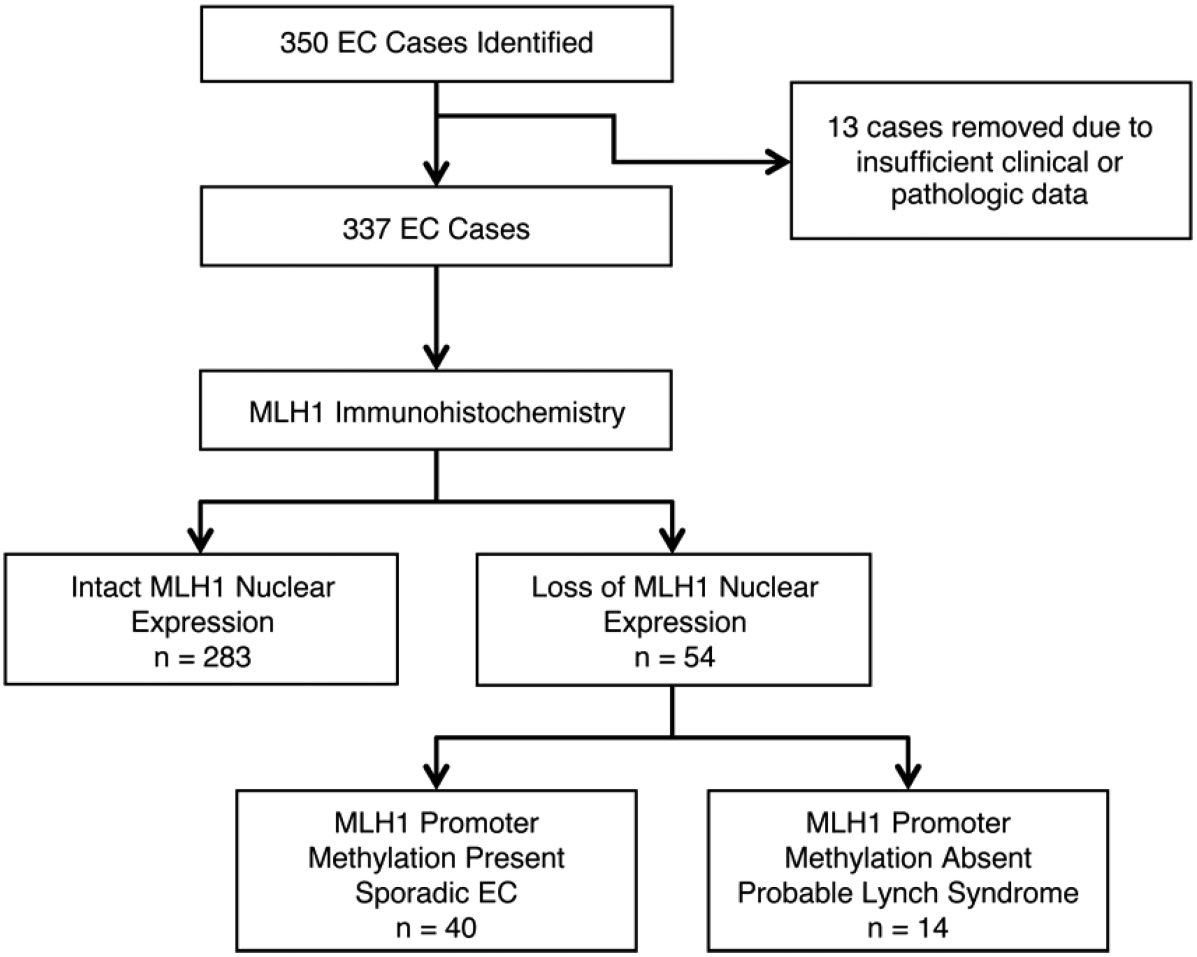

Figure 1.
Flowchart summarizing research design. Endometrial carcinomas with loss of MLH1 by immunohistochemistry were subjected to PCR-based *MLH1* methylation.

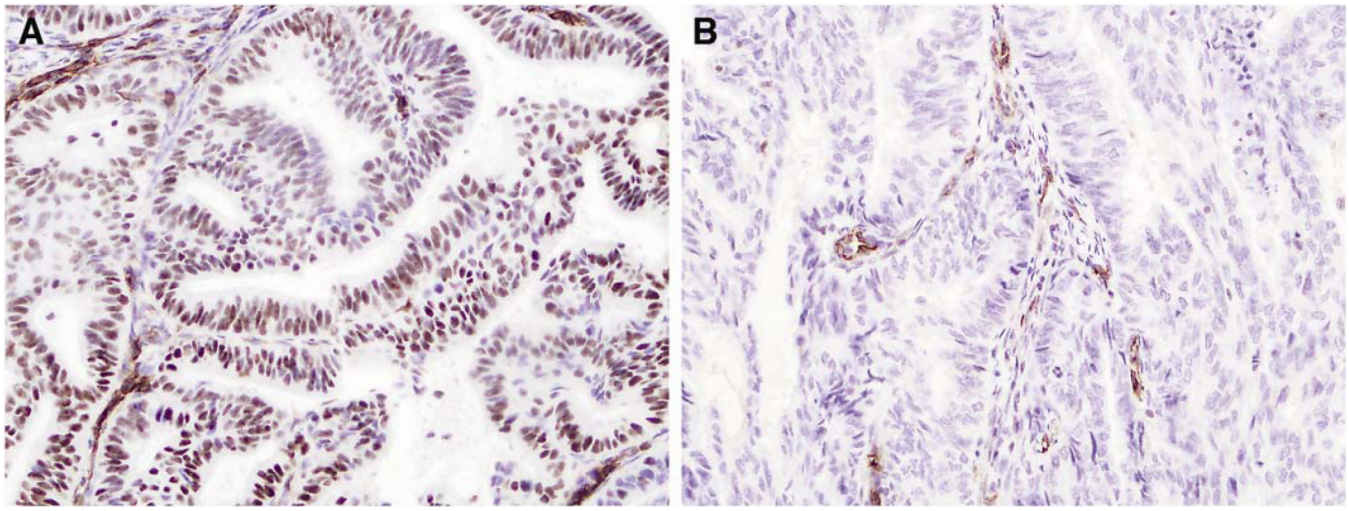

Figure 2.

Immunohistochemistry for MLH1 in endometrial carcinoma. A. This tumor has strong, positive nuclear expression of MLH1 in a majority of tumor cells, which typically corresponds to lack of *MLH1* mutation or methylation. B. Tumor cells exhibit loss of nuclear MLH1 protein expression. Adjacent normal stromal cells have positive expression of MLH1, indicating that the immunohistochemistry reaction is working.

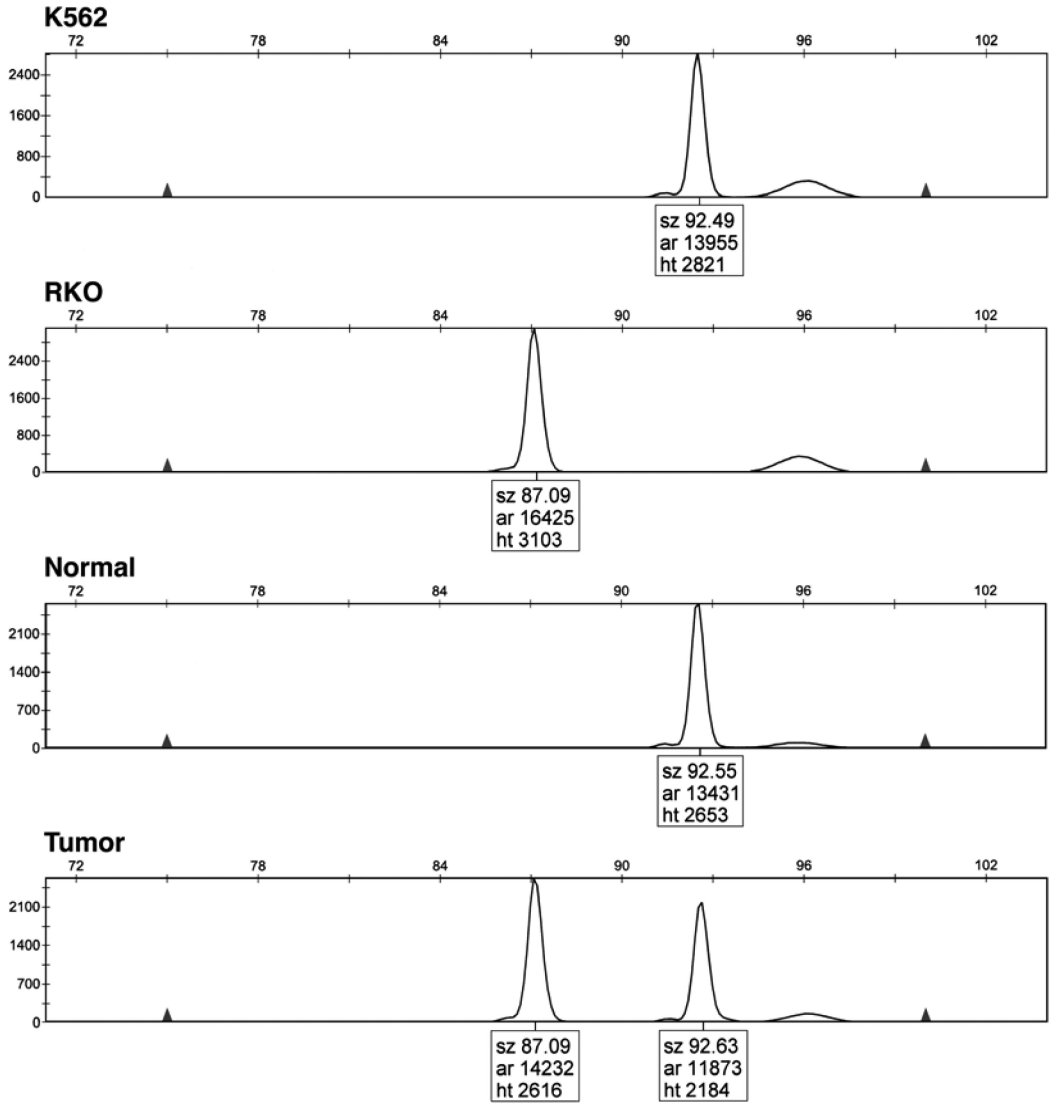

Figure 3.

Sample *MLH1* methylation chromatogram. Panel 1 shows analysis of the negative control leukemia cell line K562, which is known to lack *MLH1* methylation, while panel 2 demonstrates a different peak for the colon cancer cell line RKO, which is known to have loss of MLH1 protein expression secondary to *MLH1* methylation. Panel 3 is for normal tissue from an endometrial cancer, and panel 4 is for an endometrial carcinoma. Note in panel 4 the presence of 2 peaks, methylated and unmethylated. The presence of the unmethylated peak is likely due to the presence of a minor population of normal stromal cells or inflammatory cells which will not have methylation of *MLH1*.

Table 1

Patient and tumor characteristics for endometrial carcinomas with immunohistochemical loss of MLH1, stratified by presence or absence of *MLH1* promoter methylation

	MLH1 Immunohistochemical Loss		
	Methylated *MLH1* n (%)	**Unmethylated *MLH1* n (%)**	**P - value**
Patient Characteristics			
Age			
Median age at diagnosis	57	52	0.4295
Age range	31-92	42-79	
Median Body Mass Index	32.9	30.9	> 0.999
<30	13 (33.3)	5 (35.7)	
≥ 30	26 (66.7)	9(64.3)	
Family History of EC[1]	4 (10.5)	3 (21.4)	0.370
Family History of CRC[2]	7 (18.4)	3 (21.4)	0.999
Diabetes	4 (10)	6 (42.9)	0.013
Hypertension	23 (57.5)	6 (42.9)	0.371
Tumor Characteristics			
Histology			
Endometrioid	35 (87.5)	11(78.6)	0.413
Non-Endometrioid	5 (12.5)	3(21.4)	
FIGO Stage[3]			
I & II	27 (67.5)	11 (78.6)	0.515
III & IV	13 (32.5)	3 (21.4)	
Endometrioid Tumor Grade			
1 or 2	26 (74.3)	9 (81.8)	
3	9 (25.7)	2 (18.1)	> 0.999
Median depth of myometrial invasion (mm)[4]	9.0	9.5	0.487
< 50% myometrial invasion	25 (62.5)	4 (28.5)	0.035
≥ 50% myometrial invasion	15 (37.5)	10 (71.4)	
Lymphatic/vascular space invasion	24 (60.0)	8 (57.1)	> 0.999
Tumor location			
Corpus	37 (92.5)	11 (78.6)	0.173
Lower uterine segment	3 (7.5)	3 (21.4)	
Tumor Size			
< 4 cm	21 (52.5)	8 (57.1)	> 0.999

	MLH1 Immunohistochemical Loss		
	Methylated *MLH1* n (%)	**Unmethylated *MLH1* n (%)**	**P - value**
≥ 4 cm	19 (47.5)	6 (42.9)	

[1] EC, endometrial cancer

[2] CRC, colorectal cancer

[3] FIGO stage I and II denote endometrial carcinomas confined to the uterus. FIGO stages III and IV represent extra-uterine spread of tumor.

[4] Depth of myometrial invasion ≥ 50% total myometrial thickness is associated with increased risk of lymph node metastasis.

Table 2

Sensitivity and specificity of selected clinical characteristics and screening criteria in predicting presence or absence of *MLH1* methylation in endometrial carcinomas with MLH1 loss by immunohistochemistry

	Sensitivity	Specificity
Age < 50	50.0	77.5
Body mass index < 30	33.3	35.7
History of diabetes	42.8	90.0
Myometrial invasion > 50%	71.4	62.5
Family history colorectal cancer	21.4	81.6
Family history endometrial cancer	21.4	89.5
Amsterdam II Criteria	14.3	94.9
SGO Criteria	71.4	69.2
CHiP Criteria	64.3	48.7
SGO Criteria or ≥ 50% myometrial invasion or diabetes	100	35.9

Table 3

Characteristics of endometrial cancer cases with immunohistochemical loss of MLH1 and absence of *MLH1* methylation (presumed Lynch Syndrome) that were incorrectly designated as sporadic by SGO criteria

Case	Age at EC Diagnosis[1]	BMI	DM[2]	Family History of EC	Family History of CRC[3]	Meets Amsterdam II Criteria	Tumor Location (Corpus or LUS[4])	FIGO Stage	Tumor Grade	Tumor Size (cm)	Histology[5]	Depth of Uterine Wall Invasion
1	61	51.8	No	Cousin age > 50	No	No	Corpus	II	1	13	E	> 50%
2	64	30.9	No	No	No	No	LUS	II	1	3	C	> 50%
3	71	38	Yes	Mother unknown gyn cancer age > 50	No	No	Corpus	IA	2	5.5	M	< 50%
4	79	31	Yes	No	No	No	Corpus	IIIC2	2	1.9	E	> 50%

[1] EC, endometrial cancer

[2] DM, diabetes mellitus

[3] CRC, colorectal cancer

[4] LUS, lower uterine segment

[5] E, endometrioid carcinoma; C, clear cell carcinoma; M, mixed endometrioid and sarcomatoid carcinoma