

Screening for Lynch Syndrome (Hereditary Nonpolyposis Colorectal Cancer) among Endometrial Cancer Patients

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Abstract

Endometrial cancer is the most common cancer in women with Lynch syndrome. The identification of individuals with Lynch syndrome is desirable because they can benefit from increased cancer surveillance. The purpose of this study was to determine the feasibility and desirability of molecular screening for Lynch syndrome in all endometrial cancer patients. Unselected endometrial cancer patients ($N = 543$) were studied. All tumors underwent microsatellite instability (MSI) testing. Patients with MSI-positive tumors underwent testing for germ line mutations in *MLH1*, *MSH2*, *MSH6*, and *PMS2*. Of 543 tumors studied, 118 (21.7%) were MSI positive (98 of 118 MSI high and 20 of 118 MSI low). All 118 patients with MSI-positive tumors had mutation testing, and nine of them had deleterious germ line mutations (one *MLH1*, three *MSH2*, and five *MSH6*). In addition, one case with an MSI-negative tumor had abnormal *MSH6* immunohistochemical staining and was subsequently found to have a mutation in *MSH6*. Immunohistochemical staining was consistent with the mutation result in all seven truncating mutation-positive cases but was not consistent in two of the three missense mutation cases. We conclude that in central Ohio, at least 1.8% (95% confidence interval, 0.9-3.5%) of newly diagnosed endometrial cancer patients had Lynch syndrome. Seven of the 10 Lynch syndrome patients did not meet any published criteria for hereditary nonpolyposis colorectal cancer, and six of them were diagnosed at age >50. Studying all endometrial cancer patients for Lynch syndrome using a combination of MSI and immunohistochemistry for molecular prescreening followed by gene sequencing and deletion analysis is feasible and may be desirable. (Cancer Res 2006; 66(15): 7810-7)

Introduction

Lynch syndrome (also known as hereditary nonpolyposis colorectal cancer or HNPCC) is the most common form of hereditary colorectal cancer, accounting for 1 of every 45 cases of colorectal cancer (1). However, its frequency among endometrial cancer patients is less well studied. Although reported cancer risks for individuals with Lynch syndrome vary by population and by gene, it seems that women with this syndrome have a higher risk of endometrial cancer than of colorectal cancer (2-4). Given this risk information, it might be expected that the proportion of endometrial cancer cases accounted for by Lynch syndrome would be the same or greater than that of colorectal cancer. It is important to identify endometrial cancer patients with Lynch syndrome because they require annual colonoscopy given their high risk for developing subsequent colorectal cancers (5), whereas endometrial cancer patients who do not have Lynch syndrome can follow the American Cancer Society guidelines for colorectal cancer screening (colonoscopy every 10 years beginning at age 50). In addition, women with Lynch syndrome may have many additional family members who are also at risk for Lynch syndrome and who could benefit from genetic testing and increased cancer surveillance if they have also inherited the condition.

The true frequency of Lynch syndrome among all newly diagnosed cases of endometrial cancer is difficult to assess from most studies because they either determined the frequency of Lynch syndrome among cases with microsatellite instability (6-9) and among subsets of cases with early-onset disease, metachronous colorectal cancer, or positive family history (10-14), or they did not include complete testing (including deletion analysis) for all known mismatch repair genes (15, 16). Judging from all these previous studies, the frequency of Lynch syndrome among endometrial cancer patients could be as low as 0% and as high as 10%. Two of the largest series studied to date have found that 1.8% (8 of 441; 12 mutations were found total, but this included four variants of uncertain significance) to 2.1% (11 of 519) of endometrial cancer patients have Lynch syndrome (14, 16). One of these studies (16) did not include testing for *MLH1* mutations at all, whereas mutations in this gene accounted for 6 of 11 mutations found in the other study (14). The latter series of patients was from Finland (14), a country with two well-known founder mutations in *MLH1*, which could lead to a different frequency of Lynch syndrome.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

H. Hampel had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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To clarify the frequency of Lynch syndrome among all newly diagnosed cases of endometrial cancer, we did testing in a large series of patients from a defined geographic region unselected for age at diagnosis or family history. We compared the strategies of microsatellite instability (MSI) testing, immunohistochemical staining, and family history as methods to characterize patients with endometrial tumors caused by Lynch syndrome. This is the largest series of endometrial cancer patients studied to date and involves the most complete genetic testing (sequencing of *MLH1*, *MSH2*, and *MSH6* and testing for large deletions or rearrangements of *MLH1*, *MSH2*, *MSH6*, and *PMS2*) for the diagnosis of Lynch syndrome.

Materials and Methods

Patients. Individuals eligible for the study were those newly diagnosed with adenocarcinoma of the endometrium regardless of age or family history of cancer at three participating hospital systems. These hospitals perform the vast majority of all operations for endometrial cancer in the Columbus, OH metropolitan area (population is 1.5 million).

The research protocol and consent form were approved by full review of the Institutional Review Board in accord with an assurance filed with and approved by the U.S. Department of Health and Human Services at all participating hospitals in 1999. From January 1, 1999 to December 4, 2003, 1,154 patients were diagnosed with endometrial cancer at the three hospital systems. Eligible patients were approached by their physician and a protocol nurse, and written informed consent was obtained allowing MSI testing of their tumor. If their tumor was found to be MSI positive, this initial consent also permitted additional genetic testing of their tumor and blood sample to determine whether or not they had Lynch syndrome. In total, 588 of 1,154 (50.9%) eligible patients consented to the study and provided family history information, a 10- to 20-mL EDTA blood sample, and released a paraffin-embedded tumor block. Twenty-four patients were removed from the study because they had insufficient tumor samples, leaving 564 patients on the study. This report is based on the completed analyses of 543 patients (mean age = 60.9 years; range, 17-94 years). The remaining 21 patients had not completed testing at the time of this report. Race information was self-reported by the study participants for National Cancer Institute reporting purposes. The patients were largely Caucasian (95%); thus, minorities were underrepresented in this study based on the 2000 U.S. census report for Ohio.⁷

Patients with endometrial sarcomas or squamous cell carcinomas were not eligible for this study. The vast majority of patients had endometrioid adenocarcinoma (421 of 543, 77.5%) followed by adenosquamous and adenocarcinoma with squamous metaplasia (49 of 543, 9.0%). There were 27 patients with serous or papillary serous histology (5.0%), 24 with mixed histology (4.4%), 15 with clear cell histology (2.8%), 5 with mucinous adenocarcinomas (0.9%), and 2 with adenocarcinoma NOS (0.4%).

Patients found to have Lynch syndrome received full genetic counseling and signed a second informed consent allowing clinical confirmation of their mutation on a new blood sample.

Samples. DNA and RNA were extracted from the blood by standard methods. The histology of the tumor was reevaluated, an area containing tumor cells marked on the block, and the percentage of tumor cells was determined. The proportion of tumor cells in material used for DNA extraction exceeded 40% in all cases and was generally >80%. Additionally, an area containing no tumor cells (i.e., normal tissue) was marked on the block. Material from tumor and normal tissue was obtained by microdissection.

Analytic strategy. All 543 tumors were analyzed for MSI because more than two thirds of endometrial cancers among Lynch syndrome patients exhibit this characteristic (17). All 118 tumors with MSI and a subset of 211 MSI-negative tumors underwent immunohistochemical staining and evaluation. If one of the mismatch repair proteins is absent in a tumor, this indicates that the corresponding gene is not producing functional or

stable protein. The 118 MSI-positive cases and 9 MSI-negative cases with abnormal immunohistochemical staining underwent mutation testing (Fig. 1). Mutations in *MLH1*, *MSH2*, and *MSH6* account for >95% of known Lynch syndrome-associated mutations (18). All MSI-positive cases underwent (a) sequencing of *MLH1*, *MSH2*, and *MSH6*; (b) multiplex ligation-dependent probe assay (MLPA) of *MLH1*, *MSH2*, *MSH6*, and *PMS2* in search of deletions and duplications (19, 20); (c) immunohistochemical staining for the four mismatch repair proteins; and (d) methylation analysis of the *MLH1* promoter (21). The MSI-negative cases with abnormal immunohistochemical staining received testing only for the gene(s) corresponding to the protein(s) absent in the tumor. See Supplementary Table S1 for the overall results of the 118 MSI-positive cases.

In an effort to identify mismatch repair-deficient tumors missed by MSI, two subsets of MSI-negative tumors were studied by immunohistochemical staining for the mismatch repair proteins. First, among the first 401 cases accrued, a subset of MSI-negative cases ($n = 46$) was chosen meeting one of the following criteria: (a) diagnosis under age 50, (b) synchronous or metachronous endometrial and colorectal cancer primaries, or (c) a first-degree relative with colorectal or endometrial cancer diagnosed at any age. This group was intentionally biased to be more likely to represent cases of Lynch syndrome that were missed by MSI. In addition, after all patients had been accrued to the study, we made a concerted effort to perform immunohistochemical staining on as many MSI-negative cases as possible. Due to logistics, this included all of the cases diagnosed at the main study site (Ohio State University) with enough available tissue (we could re-obtain these tumor blocks) and any of the outside cases that were processed after June 2004. These cases received immunohistochemical staining for all four proteins (with the exception of *PMS2* in the cohort of 46). In the end, this heterogeneous group of 211 MSI-negative patients who received immunohistochemical staining included 46 patients selected because they were more likely to have Lynch syndrome, 159 OSU patients, and 6 patients from the other two study sites. Only nine patients (9 of 211, 4.3%) were found to have an abnormal immunohistochemical staining result and were subjected to sequencing and MLPA of the gene(s) corresponding to the protein(s) that were absent in the tumor if possible. A germ line mutation was found in one of these cases; this case we describe as being missed by MSI. This patient was among the 46 selected based on the criteria listed above.

Molecular methods. All assay reactions described below that required thermal cycling (PCR and PCR product purification) and electrophoresis of fluorescently labeled PCR fragments (MSI analysis and sequencing, MLPA) were done with the 96-well GeneAmp PCR System 9700 and with the ABI Prism 3700 or with the Applied Biosystems 3730 capillary DNA analyzers, respectively (Applied Biosystems, Foster City, CA).

Microsatellite instability. MSI testing was done by genotyping two quasimonomorphic markers (BAT25 and BAT26) and three to four polymorphic markers (D2S123, D5S346, D18S69, and/or D17S250) in tumor and unaffected tissue (blood or endometrium). A consensus panel has recommended the use of a five-marker panel (all of the markers listed above except D18S69; ref. 22). The genotypes were amplified in multiplex fashion with 4 μ L HotStarTaq Master Mix (Qiagen GmbH, Hilden, Germany), 10 pmol per primer, and 6 ng genomic DNA in an 8- μ L reaction. The thermal cycling conditions were as follows: 11-minute hold at 95°C, 1-minute hold at 96°C, 10 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 70°C for 1 minute followed by 20 cycles at 90°C for 30 seconds, 56°C for 30 seconds, 70°C for 1 minute, a 30-minute hold at 60°C and a final hold at 4°C indefinitely. The resulting fluorescently labeled fragments were electrophoresed, and the markers' stability status was analyzed with the Genotyper or GeneMapper software (Applied Biosystems).

The markers were considered unstable when an allele was present in the tumor but not in unaffected tissue. Tumors with two or more unstable markers are MSI high; tumors with one unstable marker are MSI low; tumors with no unstable markers are MSI negative (22). It is important to distinguish MSI-high from MSI-low tumors because this may be indicative of the gene in which the germ line mutation occurs (with MSI-low tumors more common among *MSH6* gene mutation carriers; ref. 17).

Immunohistochemical staining. Immunoperoxidase staining was done on formalin-fixed, paraffin-embedded tissue as previously described (23).

⁷ <http://quickfacts.census.gov/qfd/states/39000.html>.

The primary antibodies were MLH1 (1:10; BD Biosciences PharMingen, San Diego, CA), MSH2 (1:200; EMD Biosciences, La Jolla, CA), MSH6/GTBP (1:300; BD Biosciences PharMingen), and PMS2 (C20, 1:400; Santa Cruz Biotechnology, Santa Cruz, CA and later 1:50; BD Biosciences PharMingen). Positive and negative controls stained appropriately, and any convincing nuclear staining was considered positive.

Methylation of the MLH1 promoter. The majority of all mismatch repair-deficient sporadic endometrial carcinomas are caused by hypermethylation of the *MLH1* promoter (14). We studied the methylation of the *MLH1* promoter with the aim of determining whether methylation analysis might be used routinely to triage Lynch syndrome from non-Lynch syndrome tumors that exhibit loss of MLH1 protein on immunohistochemical staining.

First, tumor DNA (2 µg) was treated with sodium bisulfite according to published protocols. Using the bisulfite-treated DNA, in which only the unmethylated cytosines were converted into uracil, we assessed methylation at two different areas of the promoter referred to as the H and D regions.

The H region, located -710 to -576 bp upstream of the translation start site, was studied by methylation-specific PCR (24). We used the primers 5'-ACGTTTTATTAGGGTCGCGC-3' (sense) and 5'-AAACCCTATACCTAATC-TATCGCCG-3' (antisense). A 134-bp and/or 130-bp PCR fragment was generated in a 25-µL reaction using 2 µL of bisulfite-treated DNA with a mixture containing 67 mmol/L Tris-HCl, 16.6 mmol/L (NH₄)₂SO₄, 0.0175 µL 2-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA), 1 mmol/L of each deoxynucleotide triphosphate (dNTP; Roche Diagnostics, Indianapolis, IN), 1.25 µL of DMSO (Fisher Scientific, Fair Lawn, NJ), 500 µmol/L of each

primer, 1 unit of Taq polymerase (Roche Diagnostics), and 6.7 mmol/L of fresh MgCl₂ (Applied Biosystems). PCR conditions were as follows: 95°C for 3 minutes; then 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; and finally, 3 minutes at 72°C. The PCR products were visualized on a 3% agarose gel. If a band was seen at 130 bp, the sample was considered methylated in the H region.

The D region, located -377 to -156 bp upstream of the translation start site, was analyzed by the combined bisulfite restriction analysis (25). The target of the analysis was a *Bst*UI restriction endonuclease cleavage site that consists of two tandemly organized CpG sites (CGCG). We used primers 5'-GTTAGATTATTTTAGTAGAGGTATATAAGTT-3' (sense) and 5'-ACCAATCAAATTTCTCAACTCTATAA-3' (antisense). With unmethylated DNA, a 221-bp PCR fragment was generated in a 50-µL reaction using 2 µL of bisulfite-treated DNA with a mixture containing a 1 × dilution of 10× PCR buffer (Applied Biosystems), 0.2 mmol/L of each dNTP (Roche Diagnostics), 500 µmol/L of each primer, 2 units of Ampliqa Gold DNA Polymerase (Applied Biosystems), and 2 mmol/L of fresh MgCl₂ (Applied Biosystems). PCR conditions were as follows: 95°C for 10 minutes; then 45 cycles of 95°C for 30 seconds, 53°C for 30 seconds, and 72°C for 60 seconds; and finally, 5 minutes at 72°C. The PCR products were digested by *Bst*UI (New England Biolabs, Ipswich, MA) for 4 hours at 60°C and separated on a 3% agarose gel. If bands were seen at 120 and 100 bp, the sample was considered methylated in the D region.

Mutation detection. To search for germ line mutations, DNA (from blood or occasionally from normal endometrium) was directly sequenced using primers described previously (15, 16, 26) and/or alternative primers (shown in Supplementary Table S2). Common polymorphisms are described

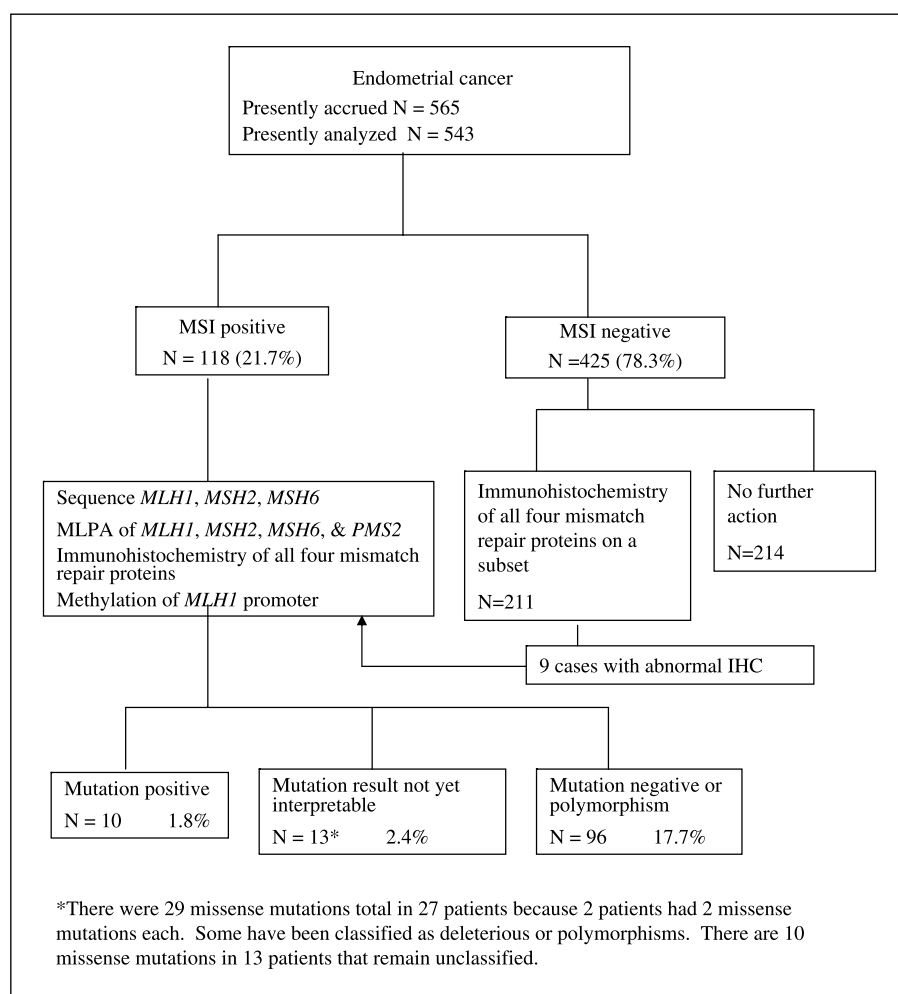


Figure 1. Flow diagram of the analytical strategy and main results of the study.

in numerous sequence variation databases, such as the University of Utah Genome Center SNP database,⁸ that are present in various annealing sites of the established primers (15, 16, 26). The alternative primers were designed and implemented to avoid preferentially amplifying a single allele, thus potentially missing mutations. The sequencing of *MLH1*, *MSH2*, and *MSH6* covered the promoter regions (*MLH1* and *MSH2* only), exons, and intronic regions adjacent to all splice sites in all 118 patients with MSI-positive tumors. The fluorescently labeled sequencing products were purified and electrophoresed. The sequences were analyzed for variations with the Sequencher (Gene Codes Corp., Ann Arbor, MI) software.

MLPA was used to search for large deletions in the *MLH1* and *MSH2* genes and in the *MSH6* and *PMS2* genes as described by the kits' manufacturer (Medical Research Council Holland, Amsterdam, the Netherlands; refs. 20, 27) and analyzed with GeneMapper software. The *MLH1* and *MSH2* MLPA test was completed for 101 of 118 patients with MSI-positive tumors (11 failed and 6 were not completed due to insufficient DNA). The *MSH6* and *PMS2* MLPA test was completed for 97 of 118 patients with MSI-positive tumors (14 failed and 7 were not completed due to insufficient DNA).

Mutations leading to a truncated or unstable protein are considered clearly deleterious and are diagnostic of the Lynch syndrome; these include frameshift, nonsense, and splice site mutations as well as large deletions and rearrangements. Mutations that result in a full-length protein with one amino acid substituted for another are known as missense mutations and are a priori considered variants of uncertain significance. Missense changes in the mismatch repair genes are common, and it is challenging to assess their pathogenicity (28, 29).⁹ As published elsewhere, we conducted extensive functional analyses of missense mutations in *MSH2*,¹⁰ *MLH1* (30), and *MSH6* (31). The clinical significance of all missense mutations identified was assessed using these results and principles described previously (1), and families were counseled about the uncertain significance of these results.

Statistical methods. Statistical analysis was conducted using R¹¹ software. χ^2 tests with Yates' continuity correction were applied to compare the frequency of Lynch syndrome and *MSH6* mutations between the endometrial and colorectal cancers. Whenever the expected count was <5, Fisher's exact test was applied instead of the χ^2 test. Two-sided 95% confidence intervals (95% CI) of the proportions were estimated using Wilson score method with continuity correction, as described by Newcombe (32).

Results

Accrual. During the period January 1, 1999 through December 4, 2003, 588 patients were accrued to the study; 21 were removed for insufficient tumor material, leaving 564 patients on study. At the time of this data analysis, 543 of them had completed molecular analysis. The study included patients seen by the eight gynecologic oncologists in the three major hospital systems in Columbus, OH. Only 39 patients declined the study, with decliners numbering between 1 and 28 at the various sites. The main reasons for declining the study were lack of interest, no at-risk family members, not wanting to worry about genetics or involve family, and concerns about insurance risks.

MSI. MSI high was seen in 98 of 543 (18.1%), and MSI low was seen in another 20 of 543 (3.7%) tumors. In contrast to our experience with colorectal cancer where no mutations were found among patients with MSI-low tumors (1), mutations were found in two patients whose tumors were MSI low. Of the six *MSH6* mutation carriers' tumors, one was a MSI-negative case ("missed by MSI"); two were MSI low; and three were MSI high. All four carriers of mutations in *MLH1* or *MSH2* had MSI-high tumors at five of five

markers tested. All results of testing for the 118 MSI-positive cases are included in Supplementary Table S1.

Immunohistochemical analysis. Immunohistochemical staining had an excellent sensitivity to detect MSI high in that 90 of 96 tumors showing high MSI (two cases did not have immunohistochemical staining) also showed abnormal immunohistochemical staining for at least one of the four proteins (93.8% sensitivity). In contrast to MSI high, as expected, abnormal immunohistochemical staining was less prevalent among MSI-low cases: 13 of 20 (65%) tumors had an absent protein. The sensitivity of immunohistochemical staining to pinpoint the affected gene in cases with deleterious mutations was high: two of three for *MSH2*, one of one for *MLH1*, and five of six for *MSH6*. The two cases in which immunohistochemical staining did not correctly identify the gene in which a germ line mutation was found were both from patients with missense mutations.

To address the clinical utility of MSI versus immunohistochemical staining as a primary screening method, we did immunohistochemical staining on 211 MSI-negative cases (described in Materials and Methods). The vast majority (202 of 211, 95.7%) had all successful stains present in the tumor as expected. Only nine MSI-negative cases had any stain absent on immunohistochemical staining, including two cases with *MLH1* and *PMS2* absent, two cases with *MSH2* and *MSH6* absent, two cases with *MSH6* only absent, two cases with *MLH1* only absent, and one with *MSH2* only absent. Eight of the nine cases received full sequencing of the suspect gene(s), and seven of the nine cases received MLPA of the suspect gene(s) (see Supplementary Table S1). One germ line *MSH6* mutation was identified (case 140); this patient's tumor had absence of *MSH6* only on immunohistochemical staining. It is possible that the two cases with incomplete testing also have germ line mutations diagnostic of Lynch syndrome. The cases with absence of protein but without germ line mutations may be due to mutations missed by the detection methods, missed promoter methylation, the presence of biallelic somatic mutations in the tumors, or a false immunohistochemical staining result.

We cannot fully address the sensitivity and specificity of MSI and immunohistochemical staining to detect Lynch syndrome because we did not perform mutation testing in the 202 cases that were MSI negative and had normal immunohistochemical staining. We can, however, assess the clinical utility of MSI and immunohistochemical staining based on the 327 cases that received both tests. Abnormal MSI results led to mutation testing for 118 cases, and these cases were tested for three mismatch repair genes (354 genes sequenced and subjected to MLPA). Using MSI alone, we would have detected 9 of the 10 cases known to have Lynch syndrome in this cohort. Abnormal immunohistochemical staining results would have led to genetic testing for 112 cases; however, at most, two genes would have needed tested (224 genes sequenced and subjected to MLPA). Eight of the 10 Lynch syndrome cases would have been detected using immunohistochemical staining as the initial screen, and 130 less genes would have been sequenced and subjected to MLPA.

Methylation of the *MLH1* promoter. The results are shown in Supplementary Table S1. Methylation testing was successful at the H region in 118 of 118 and at the D region in 116 of 118 MSI-positive tumors. Among 84 tumors that did not stain for *MLH1*

⁹ <http://www.insight-group.org>.

¹⁰ S. Ollila et al. Pathogenicity of *MSH2* missense mutations is typically associated with impaired repair capability of the mutated protein. 2006, submitted for publication.

¹¹ <http://cran.r-project.org/>.

⁸ <http://www.genome.utah.edu/genesnps>.

Table 1. Deleterious mutations detected

Case no.	Age	Race	Histology	Amsterdam*	Bethesda [†]	Family history of cancer and age at diagnosis (FDR and SDR from side of family with mutation if known)
59092	39	White	ASM	N	N	Mom; skin <50
862	50	White	E	Y	N	Mom; CRCx3 45-74, Sister; Gyn d.28, Mat half-sister; CRC and endo 50, Mat uncle CRC and bladder d.60, Mat uncle CSU 70s, Mat grandma CSU d. 50s
1020	44	Black	AS	Y	Y	Proband, CRC 46; Mom, CRC 49 and 62; Mat uncle, gastric and CRC 40s
537	45	White	ASM	N	N	Mom, Endo 54
140	47	White	E	N	N	Brother, thyroid 28; Pat aunt, CRC or gastric 82; Pat grandma, gastric?; Pat grandpa, Hodgkin 60s
55251	68	White	E	N	N	Mom, endo 80; Mat grandma, breast 70s
475	69	White	ASM	Y	Y	Proband, CRC 40; Daughter, Endo 48; Sister, endo 60; Sister, CRC 47; Brother, bladder 58; Dad, gastric 51; Pat uncle, CSU?
345	60	White	E	N	N	Mom, mouth?; Mat aunt, lung 50s, Pat uncle, CSU 60s
501	60	White	E	N	N	Brother, skin 60; Niece, bone 20s
553	64	White	M	N	N	Dad, lymphoma 67; Pat uncle, CSU 20s, Pat aunt, breast 70s, Mat aunt, CML 80s; Mat aunt, breast 60s; Mat uncle, CSU (?CRC) 70s
Mean age	54.6					

NOTE: —, not applicable.

Abbreviations: E, endometrioid adenocarcinoma; AS, adenosquamous carcinoma; ASM, adenocarcinoma with squamous metaplasia; M, mixed endometrioid and clear cell adenocarcinoma; FDR, first-degree relatives; SDR, second-degree relatives; CRC, colorectal cancer; Gyn, gynecologic cancer; Endo, endometrial cancer; CSU, cancer site unknown; CML, chronic myelogenous leukemia; N, no; Y, yes; d., died.

*Indicates whether or not the patient met Amsterdam II criteria (43).

[†]Indicates whether or not the patient met revised Bethesda guidelines (45).

‡Number of microsatellite markers unstable out of total number tested.

§Reference sequence: NT_022184.14.

protein, which were tested successfully for methylation at both regions, 79 displayed methylation in at least one of the two regions (71 H⁺D⁺ and 8 H⁺D⁻). This confirms that epigenetic silencing of the *MLH1* gene through acquired methylation was likely the cause of the absent MLH1 immunohistochemical staining. Totally, only five tumors with absent MLH1 staining were not methylated; one of these had a germ line mutation in *MLH1*.

Conversely, when assessing the correlation of methylation status at the two regions with the presence or absence of MLH1 on immunohistochemical staining, we found 5 of 13 (38.5%) H⁺D⁻ tumors had the presence of MLH1 protein on immunohistochemical staining compared with only 6 of 73 (8.2%) H⁺D⁺ tumors with presence of MLH1 protein on immunohistochemical staining.

Deleterious mutations. There were 10 probands found to have a deleterious mutation causative of Lynch syndrome whose essential characteristics are shown in Table 1. None of these patients had previously been diagnosed with Lynch syndrome; however, one of the patients (case 1020) was also among our case series of colorectal cancer (1) because she was diagnosed with both cancers during the enrollment period for these studies. Nine patients are Caucasian, and one is African American. Mutations in *MSH6* ($n = 6$) were more common than mutations in *MLH1* ($n = 1$) and *MSH2* ($n = 3$) combined. Of note, 3 of 10 probands had large gene deletions underscoring the importance of deletion/rearrangement testing in this patient population. Two missense mutations (Thr³³Pro in

MSH2 and Glu¹¹⁹³Lys in *MSH6*) were deemed deleterious based on extensive functional studies (31)⁷, and the three patients who had one of these mutations were diagnosed with Lynch syndrome.

Missense mutations. Twenty-nine missense mutations were identified in 27 (27 of 543, 5.0%) of the endometrial cancer patients (Table 2). Eight missense mutations (accounting for 12 patients: *MLH1* Val213Met, Lys618Ala, Arg659Gln, and Val716Met; *MSH2* Gly322Asp; and *MSH6* Arg128Leu, Pro623Leu, and Gly881Lys+Ser mutations) are considered polymorphisms based on their frequency in controls and other functional evidence (30, 31).⁷ Three of the patients with missense mutations were also found to have a deleterious mutation. Two missense mutations (accounting for three patients) were determined to be deleterious as noted above. There are 13 patients with missense mutations for which the clinical significance is still unknown.

Of interest, the Val878Ala *MSH6* missense mutation was found in four of the 118 MSI positive endometrial cancer patients (3.4%). We also found this mutation in one of 140 population controls (0.7%). This mutation has been reported multiple times in the literature with some suggesting it could be deleterious (33–35), some uncertain (12, 36–39), and some that it is a polymorphism (40, 41). The largest study involving this mutation reported this mutation in more controls (46/1315; 3.5%) than in colorectal cancer patients (25/1192; 2.1%) and suggested that it is not associated with colorectal cancer risk (40). Although this does not rule out the possibility that it is

Table 1. Deleterious mutations detected (Cont'd)

MSI [†]	Gene	Mutation	Nucleotide Change	Large deletion breakpoints
5/5	<i>MLH1</i>	p.Gln562X	c.1684C>T	—
2/5	<i>MSH2</i>	Large deletion	Exon 1-6 Deletion	g.26445441_26465485del20045 [§]
5/5	<i>MSH2</i>	Large deletion	Exon 1-11 Deletion	g.26370866_26515842del144977 [§]
2/5	<i>MSH2</i>	p.Thr33Pro	c.97A>T	—
0/5	<i>MSH6</i>	p.E546GfsX16	c.1637_1638delAG	—
3/5	<i>MSH6</i>	p.Arg911X	c.2731C>T	—
1/5	<i>MSH6</i>	p.Tyr1256X	c.3768 T>G	—
1/5	<i>MSH6</i>	Large deletion	Exon 5-6 Deletion	c.3173-446_3556+215del2269; g.26846046_26848314del2269 [§]
3/5	<i>MSH6</i>	p.Glu1193Lys	c.3577G>A	—
2/5	<i>MSH6</i>	p.Glu1193Lys	c.3577G>A	—

associated with endometrial cancer risk, it certainly puts it in doubt. The prediction programs SIFT, PolyPhen, and ESEfinder all predict that Val878Ala does not damage the MSH6 protein and is unlikely to be pathogenic.

Diagnostic criteria met by the Lynch syndrome probands.

The mean age of diagnosis among the 10 probands was 54.6 years (range, 39-69 years). Only three fulfilled the Amsterdam criteria for the diagnosis of Lynch syndrome based on assessment of the family history information the patients provided (42, 43). Seven of the 10 patients with Lynch syndrome did not meet either Amsterdam criteria or Bethesda guidelines (44, 45). Notably, if testing had been limited to those under age 50 (a commonly applied criterion), 6 of the 10 probands would not have been detected.

Testing of the probands' relatives. All 10 endometrial cancer probands with deleterious mutations have received cancer genetic counseling. As part of this counseling, the proband was offered confirmation of the research result on a new blood sample in a Clinical Laboratory Improvement Act–approved laboratory. This is standard practice to ensure that there were no sample mix-ups or quality control issues in the research laboratory. All 10 patients with Lynch syndrome elected to have their results confirmed and the clinical laboratory confirmed each of the results. In addition, free genetic counseling and testing was offered to at-risk relatives. Out of a total of 21 relatives counseled and tested thus far, 10 had the familial mutation, and 11 tested negative. One of the 10 mutation-positive relatives had had an HNPCC associated cancer (the mother of patient 537 who had endometrial cancer at age 50). Nine mutation carriers reported no cancer.

Statistical analysis of the mutation spectrum. The frequency of Lynch syndrome among newly diagnosed endometrial cancer patients (1.8% or 10 of 543) was compared with the frequency among newly diagnosed colorectal cancer patients in the same Ohio population (2.2% or 23 of 1,066; ref. 1) using the χ^2 test. The difference was not significant with a $P = 0.81$ and overlapping CIs. The 95% CIs for the 1.8% frequency among endometrial cancer

patients were from 0.9% to 3.5%. The 95% CI for the 2.2% frequency among colorectal cancer patients ranged from 1.4% to 3.3%.

The number of *MSH6* mutations found among the endometrial cancer patients was compared with the number of *MSH6* mutations found among colorectal cancer patients (1). The difference in the frequency of deleterious *MSH6* mutations found in endometrial cancer patients (8 of 543) compared with colorectal cancer patients (3 of 1,066) was significant ($P = 0.009$, Fisher's exact test).

Discussion

There were several important findings in this study. First, the frequency of Lynch syndrome seems to be similar to that seen in colorectal cancer patients from the same geographic area. At least 1.8% (95% CI, 0.9-3.5%) of all endometrial cancer patients have Lynch syndrome versus 2.2% (95% CI, 1.4-3.3%) of colorectal cancer patients (1). Two other large studies of endometrial cancer patients have found that 1.8% and 2.1% of unselected endometrial cancer patients have Lynch syndrome (14, 16), nearly identical to our findings in both endometrial cancer (this study) and colorectal cancer (1). It is important to consider that our finding of 1.8% must represent a minimum. Some mutation carriers may have been overlooked for a variety of reasons. In addition, because we only counted two of the missense mutations identified in this study as deleterious, there is a high likelihood that we have actually underestimated the frequency of Lynch syndrome. Missense mutations pose an extreme clinical challenge. Even with excellent research resources, which are not typically available clinically, the significance of these mutations often remains uncertain.

It needs to be determined whether or not this frequency should warrant routine screening of all endometrial cancer patients for Lynch syndrome. These women are at increased risk for developing other Lynch syndrome malignancies, most notably colorectal cancer, and would benefit from increased colonoscopic surveillance

Table 2. Sequence changes of unknown significance detected

Case no.	Age	Race	Amsterdam*	Bethesda [†]	MSI [‡]	Gene	Nucleotide	Mutation	Impression	Population frequency [§]
1020	44	Black	N	Y	5/5	<i>MLH1</i>	c.637G>A	p.Val213Met	P (31)	0/280; 3/114
904	68	White	N	Y	3/5	<i>MLH1</i>	c.977T>C	p.Val326Ala	UV	ND
55251	68	White	N	Y	3/5	<i>MLH1</i>	c.1852_1853delAAinsGC	p.Lys618Ala	P (31)	0/280
132 [¶]	47	White	Y	Y	5/5	<i>MLH1</i>	c.1852_1853delAAinsGC	p.Lys618Ala	P (31)	0/280
132 [¶]	47	White	Y	Y	5/5	<i>MLH1</i>	c.1976G>A	p.Arg659Gln	P (31)	1/280
541	59	White	N	N	3/5	<i>MLH1</i>	c.2066A>G	p.Gln689Arg	UV	0/280
74	39	White	N	Y	5/5	<i>MLH1</i>	c.2146G>A	p.Val716Met	P (31)	0/280
537	45	White	N	Y	2/5	<i>MSH2</i>	c.97A>C	p.Thr33Pro	D (30)	0/280
57	77	White	N	N	5/6	<i>MSH2</i>	c.965G>A	p.Gly322Asp	P	5/212
1509	56	White	N	N	5/5	<i>MSH2</i>	c.965G>A	p.Gly322Asp	P	5/212
1715	83	White	N	N	3/5	<i>MSH2</i>	c.965G>A	p.Gly322Asp	P	5/212
59092	39	White	N	Y	5/5	<i>MSH2</i>	c.965G>A	p.Gly322Asp	P	5/212
1544 [¶]	57	White	N	N	1/5	<i>MSH2</i>	c.965G>A	p.Gly322Asp	P	5/212
1544 [¶]	57	White	N	N	1/5	<i>MSH2</i>	c.1461C>G	p.Asp487Glu	UV	0/280
1261	70	White	N	N	3/5	<i>MSH2</i>	c.1730T>C	p.Ile577Thr	UV	0/280
192	38	White	N	N	2/5**	<i>MSH2</i>	c.2503A>C	p.Asn835His	UV	ND
313	68	White	N	N	2/5	<i>MSH6</i>	c.383G>T	p.Arg128Leu	P (32)	0/280
1493	59	White	N	N	3/5	<i>MSH6</i>	c.1304T>C	p.Leu435Pro	UV	ND
266	58	White	N	N	4/5	<i>MSH6</i>	c.1868C>T	p.Pro623Leu	P (32)	0/280
1123	60	White	N	N	3/5	<i>MSH6</i>	c.2633T>C	p.Val878Ala	UV	1/280
1228	54	White	N	N	2/5	<i>MSH6</i>	c.2633T>C	p.Val878Ala	UV	1/280
1493	59	White	N	N	3/5	<i>MSH6</i>	c.2633T>C	p.Val878Ala	UV	1/280
56591	83	White	N	N	5/5	<i>MSH6</i>	c.2633T>C	p.Val878Ala	UV	1/280
509	83	White	N	N	3/5	<i>MSH6</i>	c.2641delGinsAAAA	p.Gly881delinsLysSer	P (32)	0/280
1167	69	White	N	N	3/5	<i>MSH6</i>	c.3442G>C	p.Gly1148Arg	UV	0/280
501	60	White	N	N	3/5	<i>MSH6</i>	c.3577G>A	p.Glu1193Lys	D (32)	0/280
553	59	White	N	N	2/5	<i>MSH6</i>	c.3577G>A	p.Glu1193Lys	D (32)	0/280
1038	61	White	N	N	3/5	<i>MSH6</i>	c.3674C>T	p.Thr1225Met	UV	ND
702	67	White	N	N	2/5	<i>MSH6</i>	c.4001+10_4001+13delTAAC	—	UV	ND
Mean age	59.7									

Abbreviations: UV, unclassified variant; P, polymorphism; D, deleterious; ND, population frequency was not studied among controls; N, no; Y, yes.

*Indicates whether or not the patient met Amsterdam II criteria (43).

[†]Indicates whether or not the patient met revised Bethesda guidelines (45).

[‡]Number of microsatellite markers unstable out of total number tested.

[§]Number of alleles with the mutation from a set of 106 or 140 Caucasian controls except where noted.

^{||}Frequency of mutation among a set of 57 African-American controls.

[¶]Patient with two missense mutations.

**Patient was originally MSI negative, but repeat MSI testing following an abnormal immunohistochemical staining result was positive.

(46). Furthermore, their relatives who are found to have Lynch syndrome could also benefit from colorectal and endometrial cancer surveillance. There is evidence that endometrial cancer surveillance using transvaginal ultrasound and endometrial biopsy is effective in postmenopausal women, but its efficacy in younger, asymptomatic women has not yet been studied (5).

A common practice is to screen for Lynch syndrome among cancer patients diagnosed under age 50. In our study, this would have only required genetic testing for 13 patients (an 89% reduction in effort). Of the 81 patients diagnosed under age 50, 4 (4.9%; 95% CI, 1.6-12.8%) have Lynch syndrome. Another recent study found that 8.6% of all endometrial cancer patients diagnosed under age 50 have Lynch syndrome (11). Nevertheless, we reject the idea of testing only young patients based on our data, showing that 6 of 10 patients would not have been detected using this strategy.

Cost-benefit analysis data will be necessary to consider these screening options on a large, public health scale. It would certainly reduce costs if mutation analysis was not necessary in cases exhibiting methylation of the *MLH1* promoter. However, our data are not extensive enough to favor or rule out methylation of the *MLH1* promoter as a routine test to exclude Lynch syndrome. It seems that methylation at the D region may be more predictive of the loss of MLH1 protein than the H region.

Another important finding is the lower level of MSI in endometrial cancer patients with Lynch syndrome when compared with colorectal cancer patients with Lynch syndrome as has been published previously (47). In the present study, there were one MSI-negative case and two MSI-low cases among the endometrial cancer patients diagnosed with Lynch syndrome. This is different from the MSI findings in our colon cancer series, where there were

no mutation carriers with MSI-low tumors. This finding may explain how MSI missed one mutation carrier in this study.

The differences in MSI levels between the endometrial cancer patients with Lynch syndrome may be directly owing to the larger number of *MSH6* mutations found in this population. There was a 5-fold increased likelihood of finding a deleterious mutation in *MSH6* among endometrial cancer patients when compared with colorectal cancer patients, and this difference was statistically significant. This supports previous findings suggesting a higher risk for endometrial cancer for *MSH6* mutation carriers than is found for *MLH1* and *MSH2* mutation carriers (4) and a lower level of MSI among endometrial cancers from *MSH6* mutation carriers (17).

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