MLH3 Mutation in Endometrial Cancer


1Department of Obstetrics and Gynecology, Division of Gynecologic Oncology; Department of Pathology and Immunology and 3Surgery, Washington University School of Medicine, St. Louis, Missouri; and Laboratory Medicine and Pathology, Mayo Clinic College of Medicine, Rochester, Minnesota

Abstract

MLH3 is a recently described member of the DNA mismatch repair gene family. Based on its interaction with the MutL homologue MLH1, it was postulated that MLH3 might play a role in tumorigenesis. Germ line and somatic mutations in MLH3 have been identified in a small fraction of colorectal cancers, but the role of MLH3 in colorectal cancer tumorigenesis remains controversial. We investigated MLH3's role in endometrial tumorigenesis through analysis of tumor and germ line DNA from 57 endometrial cancer patients who were at increased risk for having inherited cancer susceptibility. Patients with known MSH2 or MSH6 mutations were excluded as well as those who had MLH1-methylated tumors. Sixteen different variants were identified by single-strand conformational variant analysis. Of the 12 missense changes identified, three were somatic mutations. One patient had a germ line missense variant and loss of heterozygosity (LOH) in her tumor specimen. There was no evidence of MLH3 promoter methylation based on combined bisulfite restriction analysis. The identification of inherited missense variants, somatic missense mutations (present in 3 of 57 tumors), and LOH in the tumor from a patient with a germ line missense change suggest a role for MLH3 in endometrial tumorigenesis. (Cancer Res 2006; 66(15): 7502-8)

Introduction

Genomic instability is an essential feature of human cancers and generally takes the form of either microsatellite instability or chromosomal instability (1-4). Defective DNA mismatch repair (DMMR) leading to a microsatellite instability (MSI) phenotype is seen in 17% to 30% of endometrial cancers (5-9). There are two possible explanations for how defects in DMMR contribute to tumorigenesis. When DMMR function is lost, the mutation rate increases dramatically. It has been suggested that tumor suppressor genes or critical cell cycle regulators may preferentially accumulate frameshift and/or single-base mismatch mutations in cells lacking DMMR (8, 10, 11). Alternatively, defective DMMR may fail to trigger apoptosis in cells with overwhelming DNA damage (12). Although considerable progress has been made in understanding the molecular basis of DMMR deficiency in endometrial tumorigenesis, a sizable fraction of sporadic MSI-positive endometrial cancers do not have identifiable mutations or epigenetic inactivation of one of the known DMMR genes (9).

MLH3 is a DMMR gene family member that has been investigated in hereditary and sporadic colorectal cancers (13, 14). The role of MLH3 mutation in colorectal tumorigenesis, however, remains controversial (15-17). MLH3 was cloned using the MLH1-PMS2 binding domain, and initial functional evaluation suggested a role for MLH3 in the DNA repair process (18). In vitro studies in yeast showed that the MLH1-MLH3 complex participates in repairing insertion/deletion type mutations and has some functional redundancy with the MLH1-PMS2 complex (19). In a Pms2 null mouse cells, Mlh1 levels were normal, suggesting that Mlh1 may bind an alternative protein, such as Mlh3, to mediate DMMR (20, 21). Mlh1 seems to interact with Mlh3 and have some DMMR function in vitro, but its role in vivo is less clear (22). Initial studies in Mlh3 null mice suggested that Mlh3 played a role in meiosis but had a limited role in tumor formation (23-25). Recent investigation, however, has shown that Mlh3 null mice have an abnormal response to DNA damage and increased cancer susceptibility (26). Taken together, it seems that MLH3 is likely to play a limited role in DMMR but may contribute to carcinogenesis through abnormal interaction with apoptotic pathways.

Studies in primary colorectal cancers indicate that MLH3 mutation plays a minor role in colorectal cancer tumorigenesis. A low frequency of pathogenic MLH3 mutations have been identified in inherited (hereditary nonpolyposis colorectal cancer or HNPCC) colorectal cancer (14-17). Sporadic colorectal cancers with MSI frequently have somatic MLH3 mutations (~25%), but it is unclear if MLH3 mutations are a cause or consequence of defective DMMR (13). We hypothesized that MLH3 mutation may be important to endometrial tumorigenesis, as cancers of the colon and endometrium have a different spectrum of DMMR defects. For example, MSH2 and MSH6 loss are observed more frequently in endometrial cancer than colorectal cancer (27), and MSH6 mutations seem to confer a high risk for endometrial cancers (28). In a series of consecutively collected endometrial cancers, germ line MSH6 mutations were found in 5.5% of MSI-high tumors (9). These MSH6 mutations were shown to be highly penetrant (29).

Our efforts to define the role MLH3 mutation plays in endometrial cancers were focused on women with clinical or molecular features suggestive of inherited cancer susceptibility, including women who had early-onset endometrial cancer, synchronous or metachronous malignancies, and cases with unexplained MSI. We then screened tumor specimens from a cohort of 57 patients classified as “at risk” for MLH3 mutation using single-strand conformational variant (SSCV) analysis. Missense variants were then analyzed in silico to predict whether the observed amino acid changes were likely to have functional consequences. To our knowledge, this is the first report showing that mutations in MLH3 are likely to contribute to endometrial tumorigenesis.
Table 1. Inclusion criteria for MLH3 mutation analysis

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSI-H U</td>
<td>17</td>
</tr>
<tr>
<td>Double primary malignancies</td>
<td>23</td>
</tr>
<tr>
<td>Family history of endometrial cancer</td>
<td>15</td>
</tr>
<tr>
<td>BMI &lt;27 and age &lt;55</td>
<td>11</td>
</tr>
<tr>
<td>MSI-L</td>
<td>1</td>
</tr>
</tbody>
</table>

NOTE: n = number of patients fulfilling inclusion criterion. Tumors from 57 women, selected from 479 consecutive cases evaluated for MSI, were used for molecular analysis. The numbers listed total 67, reflecting the fact that 10 patients met more than one entry criterion. Abbreviation: MSI-H U, MSI-high, MLH1-unmethylated in tumor DNA.

Materials and Methods

Inclusion/exclusion criteria for women with endometrial cancer.

Institutional review board approval was obtained for the molecular analysis of all blood and tissue specimens. A cohort of 57 endometrial cancer patients treated in the Division of Gynecologic Oncology at Washington University School of Medicine was selected using the inclusion criteria shown in Table 1. These patients are part of a consecutive series of 479 women whose tumors were evaluated for MSI. All tumors were evaluated for MSI using five microsatellite repeats (BAT25, BAT26, D2S123, D5S346, and D17S250) as previously described (9). Tumors were classified as MSI-unmethylated (MSI-L), MSI-low (MSI-L), MSI-high (MSI-H), or MSI-HU (MSI-high, unmethylated phenotype) using five microsatellite repeats (BAT25, BAT26, D2S123, D5S346, and D17S250) as previously described (9).

Tissues and DNA preparation.

Table 2. PCR primers used for SSCV and sequence analysis of MLH3

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward primer (5’ → 3’)</th>
<th>Reverse primer (5’ → 3’)</th>
<th>Amplicon length (bp)</th>
<th>Optimized T_m (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A</td>
<td>CAGTGGCTGGCATTCTTCCAC</td>
<td>GAAATGGAAGGGTGTCATGAG</td>
<td>592</td>
<td>62</td>
</tr>
<tr>
<td>2B</td>
<td>AAGCCCTGAAGCCCTTGGTGA</td>
<td>GACCATTCTTGGCTTGCTAT</td>
<td>472</td>
<td>60</td>
</tr>
<tr>
<td>2C</td>
<td>CAAGAATATCGAGTTTTTGTTT</td>
<td>TGGTACGTCTTCAATCTCTCTAGA</td>
<td>557</td>
<td>57</td>
</tr>
<tr>
<td>2D</td>
<td>ACTCTCGTACGAGCAGAAGCAGA</td>
<td>TGAGCTATGATCTTCCTCAAT</td>
<td>587</td>
<td>62</td>
</tr>
<tr>
<td>2E</td>
<td>ATGTCTACGCGTGAGGTCG</td>
<td>AAAGACTTCAGTGGTGACTCAAA</td>
<td>595</td>
<td>65</td>
</tr>
<tr>
<td>2F</td>
<td>TGAGTACAGACGTGTTCAATGGA</td>
<td>CGGAACCTTTCGACTGTTCAATT</td>
<td>496</td>
<td>61</td>
</tr>
<tr>
<td>2G</td>
<td>CTTTGGGACCTTGAAGATGCA</td>
<td>CATTTGACGTCGGAGATCAAG</td>
<td>426</td>
<td>60</td>
</tr>
<tr>
<td>2H</td>
<td>GTTGCAATACCACTATGCA</td>
<td>TAAAAGCATCTCTATTGG</td>
<td>523</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>TGTCGCAAAAGGTTTATGTC</td>
<td>TTTTTGATTAGTTGTAAGTCA</td>
<td>429</td>
<td>59</td>
</tr>
<tr>
<td>4</td>
<td>TGGTGGTCTGGATGAATCACT</td>
<td>CATGGATCTGGATTGATGATT</td>
<td>241</td>
<td>62</td>
</tr>
<tr>
<td>5</td>
<td>TCAATTATATTTGCTGGTATTTCT</td>
<td>TGGCAAGGGTGTCATATGCT</td>
<td>250</td>
<td>56</td>
</tr>
<tr>
<td>6</td>
<td>TCGAATTAAAGCCCTTGGTAAATT</td>
<td>GTTCTCCTAAATACATCCTCA</td>
<td>249</td>
<td>57</td>
</tr>
<tr>
<td>7</td>
<td>CATGATTGTGTTGCTGCTTTG</td>
<td>GGTTGACTGTATTGCTGTTGGA</td>
<td>185</td>
<td>60</td>
</tr>
<tr>
<td>8</td>
<td>TGTCCCTCTCTCCTCTATC</td>
<td>TGTAACCGTCTCTGCTGTCATTG</td>
<td>265</td>
<td>59</td>
</tr>
<tr>
<td>9</td>
<td>GGAACCTACGATGTGAGAATCGA</td>
<td>TGAAGGTACAGATCAATGCA</td>
<td>280</td>
<td>59</td>
</tr>
<tr>
<td>10</td>
<td>GCTAGATTTAACAGGGTTTTTC</td>
<td>TGCACTGACAGGGTGTGTTT</td>
<td>283</td>
<td>58</td>
</tr>
<tr>
<td>11</td>
<td>GCTAGATTTAACAGGGTTTTTC</td>
<td>AAATTTTGCTCCTCTGCTT</td>
<td>251</td>
<td>60</td>
</tr>
<tr>
<td>12</td>
<td>GCCAGCCTGATGCTACTC</td>
<td>CAGTGAAACATTCCCTCTGCT</td>
<td>226</td>
<td>61</td>
</tr>
</tbody>
</table>

*Exon 2 was broken into eight overlapping PCR assays; All eight amplicons were digested with a restriction endonuclease to generate two fragments of approximately equal size for SSCV analysis. 

An additional forward primer was used to sequence this exon (described in text).
Table 3. Intrinsic variants in MLH3

<table>
<thead>
<tr>
<th>Exon</th>
<th>Variant and position</th>
<th>Frequency</th>
<th>SNP designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>−67 T/C</td>
<td>1/57</td>
<td>Undescribed</td>
</tr>
<tr>
<td>4</td>
<td>+27 delT</td>
<td>5/57</td>
<td>Liu et al. (15)</td>
</tr>
<tr>
<td>5</td>
<td>+28 del (AT),n</td>
<td>Many</td>
<td>rs8019155</td>
</tr>
<tr>
<td>6</td>
<td>+27 C/T</td>
<td>4/57</td>
<td>rs175075</td>
</tr>
<tr>
<td>6</td>
<td>+35 C/G</td>
<td>2/57</td>
<td>rs3742780</td>
</tr>
<tr>
<td>10</td>
<td>+66 G/A</td>
<td>2/57</td>
<td>rs175057</td>
</tr>
</tbody>
</table>

NOTE: Position is given relative to the coding sequence. A minus sign indicates the variant is upstream (5') of the exon; a plus sign indicates a downstream (3') variant.

Only tumors with >65% neoplastic cellularity were used. DNA preparation from tumor tissue and matched normal bloods was done as previously described (35).

SSCV analysis of tumor specimens. The entire coding region and flanking intronic sequences of MLH3 (Ensembl #ENSG00000119684) were analyzed. MLH3 consists of 13 exons, the first of which is untranslated. The second exon comprises the majority of the transcript (3,280 of 5,216 bp total) and was assessed using eight overlapping PCR amplicons. All eight amplicons were restriction endonuclease digested to generate fragment sizes that would optimize SSCV detection (see Table 2 for a complete list of primers used for SSCV). Exons 10 and 11, separated by a 102-bp intron, were analyzed in the same amplicon. Each PCR reaction contained 20 ng of template DNA, 0.4 unit of Taq polymerase (Perkin-Elmer, Branchburg, NJ), 0.16 \( \mu \)mol of each primer, 0.1 mmol each nucleotide triphosphate, 1.5 mmol MgCl2, and 0.1 \( \mu \)Ci \(^{32}\)P-labeled dCTP (Amersham, Arlington Heights, IL) in a 10-\( \mu \)l final volume. All reactions had a 5-minute denaturation at 95°C followed by 30 cycles of PCR. Each PCR cycle included a 1-minute denaturation at 95°C, annealing for 1 minute (see Table 2 for optimized \( T_{a} \)), and elongation at 72°C for 1 minute. PCR products were denatured at 95°C for 2 minutes, cooled on ice, and then applied to mutation detection enhancement gels (Cambrex, Rockland, ME) with and without 5% glycerol. Gels were run at 4 W for 16 to 20 hours. PCR product was detected by autoradiography, and any variants observed were directly sequenced.

Distinguishing germ line variants and somatic mutations. SSCV variants identified in the tumor specimens were defined as germ line variants or somatic mutations based on comparison with corresponding DNA extracted from blood (normal DNA). For each missense variant, the normal DNA was assessed for mutation by direct sequencing or by restriction digestion if the sequence involved a restriction site. The carrier frequencies for previously undescribed missense variants or those predicted to affect protein function were estimated in our cohort and/or in a control population (described above) by restriction digestion or SSCV. If SSCV was used to screen a control group, variants identified were sequenced to confirm the presence of the mutation and rule out a false-positive SSCV.

Sequencing. The same primers used for SSCV analysis (Table 2) were used for sequencing. An additional nested forward sequencing primer was required (5'-TCCCTGAATTTAAACCCACCTC-3') for exon 5 because an insertion/deletion variant in the 3' intronic repeated sequences complicated sequence interpretation in heterozygotes. Amplification products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced using the ABI Prism BigDye Terminator chemistry versions 1.1 and/or 3.1 (Applied Biosystems, Foster City, CA).

Assessing the potential functional significance of MLH3 sequence alterations. All missense variants were analyzed in silico for putative functional effects using the sorting intolerant from tolerant (SIFT) algorithm. SIFT is a web-based algorithm that predicts how amino acid substitutions will affect protein function (36). Calculations are based on amino acid homology. In silico analysis using the SIFT algorithm was done on the entire MLH3 protein sequence (including variants) and on the MLH3

5 http://blocks.fhcrc.org/sift/SIFT.html

Table 4. Missense changes in MLH3

<table>
<thead>
<tr>
<th>Amino acid change</th>
<th>SIFT prediction</th>
<th>SIFT score</th>
<th>No. carriers observed</th>
<th>Tumor phenotypes</th>
<th>Previously described</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germ line variants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R647C</td>
<td>Affect function</td>
<td>0.02</td>
<td>1/57 (1.8%)</td>
<td>MSI-H U</td>
<td>Wu et al. (14)*</td>
</tr>
<tr>
<td>T942I</td>
<td>Affect function</td>
<td>0.07</td>
<td>1/57 (1.8%)</td>
<td>MSS</td>
<td>rs17102999</td>
</tr>
<tr>
<td>P844L</td>
<td>Affect function</td>
<td>0.00</td>
<td>33/54 (61%)</td>
<td>All</td>
<td>de Jong et al. (38)</td>
</tr>
<tr>
<td>K231Q</td>
<td>Tolerant</td>
<td>0.47</td>
<td>1/57 (1.8%)</td>
<td>MSI-H</td>
<td>Liu et al. (15)</td>
</tr>
<tr>
<td>F390I</td>
<td>Tolerant</td>
<td>0.10</td>
<td>1/57 (1.8%)</td>
<td>MSS</td>
<td>Heimonen et al. (17)</td>
</tr>
<tr>
<td>V420I</td>
<td>Tolerant</td>
<td>0.51</td>
<td>3/57 (5.3%)</td>
<td>MSS, MSI-H M, MSI-H U</td>
<td>Heimonen et al. (17)</td>
</tr>
<tr>
<td>P551S</td>
<td>Tolerant</td>
<td>0.08</td>
<td>1/57 (1.8%)</td>
<td>MSI-H U</td>
<td>No(^1)</td>
</tr>
<tr>
<td>R797H</td>
<td>Tolerant</td>
<td>0.59</td>
<td>1/57 (1.8%)</td>
<td>MSS</td>
<td>No(^1)</td>
</tr>
<tr>
<td>N826D</td>
<td>Tolerant</td>
<td>1.00</td>
<td>5/57 (8.8%)</td>
<td>MSS, MSS, MSS, MSI-H U</td>
<td>rs175081</td>
</tr>
<tr>
<td>Somatic mutations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E823D</td>
<td>Affect function</td>
<td>0.00</td>
<td>1/57 (1.8%)</td>
<td>MSS</td>
<td>No</td>
</tr>
<tr>
<td>Y720C</td>
<td>Tolerant</td>
<td>0.21</td>
<td>1/57 (1.8%)</td>
<td>MSS</td>
<td>No</td>
</tr>
<tr>
<td>H823Y</td>
<td>Tolerant</td>
<td>0.09</td>
<td>1/57 (1.8%)</td>
<td>MSI-H U</td>
<td>No</td>
</tr>
</tbody>
</table>

Abbreviations: MSI-H U: MSI-high, MLH1 - unmethylated; MSI-H M: MSI-high, MLH1 - methylated; MSI-H: MSI high, no MLH1 methylation data available.
*Identified in one patient specimen and not in 185 Dutch controls; R647C was not seen in 91 race matched (Caucasian) controls.
†This tumor also has LOH; we additionally genotyped 65 African Americans with endometrial cancer and found the SNP T942I in a single case (a patient with a known mismatch repair defect).
\(^1\)SIFT scores were generated using the entire protein sequence; When the highly conserved MutL domains (amino acids 439-1188) were removed, the T942I variant was scored as affecting function.
\(^2\)P551S was not seen in 91 race matched (Caucasian) controls.
\(^3\)R797H was seen in 6 of 72 (8.3%) of African Americans with endometrial cancer.
protein sequence without the MutL domains (amino acids 439-1188). Amino acid substitutions are reported as “tolerated” or as “affect function.”

**MLH3 promoter methylation analysis.** A putative CpG island was identified upstream of the first translated exon (660 bp, 1397 bp upstream of the initiation codon). Eleven CpG sites within a 243-bp amplicon (−1,890 to −1,647 bp, ENSG00000119684) were surveyed in 27 tumors for methylation using combined bisulfite conversion and restriction analysis (COBRA), essentially as previously described (9, 30). Briefly, 1 μg of tumor DNA was bisulfite converted using the CpGenome DNA Modification kit (Chemicon International, Temecula, CA). One micro liter of converted DNA was then used as a template for a nested PCR reaction (total of 64 cycles). The first PCR reaction (Tan = 54°C for 32 cycles) used the following primers (shown 5′ to 3′) to generate a 373-bp amplicon containing the CpG-rich region: GTTTATTTGATTTGGGATG (forward) and AACCCCTCTCAAC-CAAC (reverse). The second-round (nested) PCR reaction was done at a Tan of 55°C also for 32 cycles. A 243-bp product was generated using the primers (shown 5′ to 3′): ATTTTTGGTTGGAATAATTGG (forward) and CAACCATATCATACAAACCCT (reverse). The 243-bp product was digested with BstUI (CGCG) and TaqI (TCTG; New England Biolabs, Beverly, MA) to test for methylated cytosines. PCR products were size separated on 10% polyacrylamide gels, stained with ethidium bromide, and visualized with UV fluorescence.

**Immunohistochemistry.** Immunohistochemistry was used to assess DNA mismatch repair protein expression in selected tumors. In brief, formalin-fixed paraffin sections were stained with antibody to MLH1, MSH2, MSH6, and PMS2 as previously described (37).

**Results**

**Characteristics of the cohort.** The distribution of histologic subtypes of endometrial cancers in our cohort of 57 patients approximates that of the general population. The majority (82.5%) of women had endometrioid endometrial adenocarcinoma; 8.8% had mixed cell types present; and 5.3% had clear cell histology. The racial makeup of the study population was also similar to that of general endometrial cancer populations (38) with Caucasians representing 84.2% and African Americans representing 14% of the cohort. The MSI status of the patients’ tumors was also comparable with sporadic endometrial cancer cohorts (9). Eighteen of the 57 patients (31.6%) had MSI-positive cancers (17 MSI-high and 1 MSI-low). The remainder (68.4%) were microsatellite stable (MSS).

**MLH3 variants.** A total of 22 variants were identified. Twelve of the 22 sequence alterations were missense changes. There were four silent and six intronic variants. No frameshift or nonsense mutations were identified. Five of the six intronic variants are single nucleotide polymorphisms (SNP), and one is an insertion/deletion polymorphism in a TA repeat (Table 3). Of the 12 missense variants, three were somatic mutations (Table 4). One tumor specimen had both a germ line missense variant (T942I) and loss of heterozygosity (LOH). Figure 1 illustrates the distribution and type of the variants in relation to the conserved structural domains. The majority of the missense changes occurred in a region of MLH3 that has no homology with other known proteins (Table 4). Four variants are predicted to affect protein function by in silico analysis using the SIFT algorithm (36). These are:

- **R647C.** The R647C change was identified in a poorly differentiated endometrioid adenocarcinoma tumor specimen from a 54-year-old Caucasian patient. This patient met two of the criteria for inclusion in our study: (a) MSI-high, MLH1-unmethylated tumor phenotype and (b) BMI ≤ 27 and age ≤ 55. She did not have a family history suggestive of inherited cancer susceptibility. The R647C variant was identified at low frequency (carrier frequency 1.8%) in our study population. The R647C has been previously described in a study of colorectal cancer patients with genetic cancer susceptibility, and in that study, the missense change was not identified in a population-matched control group (14). We analyzed DNA from 91 race-matched (Caucasian) cancer-free controls from our institution. None of these carried the R647C variant.

- **P844L.** P844L is a common germ line missense variant that has been investigated previously in a large colorectal cancer case-control study (39). Thirty-three of 54 (61%) patients in our cohort were heterozygous. This common polymorphism is predicted to affect protein function (Table 4).

- **E828D.** The E828D somatic mutation (Fig. 2A) is predicted to affect protein function based on SIFT analysis (36). The E828D mutation was identified in a MSS tumor from a 46-year-old Caucasian patient with an early-stage endometrioid adenocarcinoma. The patient was included in our study because she had a second primary malignancy (multiple myeloma). She also gives an extensive family history of unconfirmed HNPCC-related malignancies (confirmation pending). It is noteworthy that this patient also carried the germ line P844L variant in cis with the somatic E828D mutation (data not shown).

- **T942I in a tumor with LOH.** The T942I germ line variant (predicted to affect protein function) was seen in a tumor that exhibited LOH at the MLH3 locus (Fig. 2B). The patient was a 64-year-old African American who presented with an early-stage endometrioid adenocarcinoma. She had a second primary malignancy (non–small cell lung cancer). The patient expired, and it has not been possible to further assess her family history for cancers. Interestingly, the T942I variant is reported in the National Center for Biotechnology Information SNP database (rs#17102999). The minor allele, however, has only been
identified among Chinese Americans. Although no family history is available for this patient, the fact that the variant was not identified in 64 additional African Americans indicates that the minor allele is rare in this population. Immunohistochemistry for the endometrial cancer revealed normal expression for MLH1, MSH2, MSH6, and PMS2 (data not shown).

**Other somatic mutations and inherited variants.** In addition to the LOH and the E828D mutation described above, two additional somatic mutations not predicted to affect protein function were discovered (Table 4). The Y720C somatic mutation was identified in an MSS tumor specimen from a 71-year-old African American patient included in the study because she had a second primary malignancy, gastric cancer (an HNPCC-type). The H823Y mutation was identified in a 49-year-old Caucasian patient with an MSI-high, cancer. The H823Y mutation was included in the study because the patient was otherwise unremarkable for cancers. Immunohistochemistry for her tumor revealed normal expression of MLH1, MSH2, MSH6, and PMS2 (data not shown).

A novel germ line variant (P551S) was identified in a 57-year-old Caucasian patient with an MSI-high, MLH1 unmethylated endometrioid adenocarcinoma. Although P551S was not predicted to alter protein function (SIFT analysis), the variant was not seen in 91 race-matched controls. Interestingly, this patient has a significant family history with a confirmed esophageal malignancy in a first-degree relative and a confirmed uterine cancer in a second-degree relative.

**Methylation analysis.** None of the 27 tumors analyzed for promoter methylation using COBRA were methylated.

**Discussion**

It has been well established that defective DMMR is an important feature of endometrial tumorigenesis. MSH2 and MSH6 defects play a significant role in the development of hereditary and sporadic endometrial cancers (9, 27, 28, 40). Approximately 30% of MSI-positive endometrial cancers have a DMMR defect of unknown origin (9) in that they have neither MSH2 or MSH6 mutations (germ line or somatic) nor epigenetic inactivation of MLH1. Although this represents a small fraction (~4%) of all endometrial cancers, identification of causal germ line variants in this group could lead to significant clinical benefit for these women and their families through intensified cancer surveillance.

MLH3, a relatively new member of the MMR gene family, was proposed to participate in DNA error correction (based on *in vitro* studies) with some functional redundancy with PMS2 (18–21). Recent studies suggested functional interaction with MLH1 *in vitro* and limited participation in DMMR function (22). Additional evidence has implicated MLH3 as a putative tumor suppressor, as *Mlh3* null mice have increased cancer susceptibility and significantly shorter life spans than wild-type mice (26). In primary MSI-negative tumors, MLH3 mutations are infrequent (16), perhaps because MLH3 and PMS2 have some functional redundancy (20), or possibly MLH3 plays a role in tumorigenesis distinct from DMMR. In fact, *Mlh3* null mouse embryonic fibroblasts display decreased apoptosis when exposed to an alkylating agent (26).

In human cancer, an inherited frameshift mutation in MLH3 was identified in a single representative of a cohort of colorectal cancer patients with suspected HNPCC (14). A study of 70 probands with apparent genetic predisposition to colorectal cancer revealed a relatively high frequency of germ line MLH3 mutations (23%), although the mutations identified were believed to be of low penetrance (15). Although MLH3's role in colorectal cancer remains controversial (17), we sought to assess whether MLH3 mutation contributes to the development of endometrial cancer. The frequency and spectrum of other DMMR gene defects differ in endometrial and colorectal cancers, and we hypothesized that MLH3 might account for cancer susceptibility in a subset of endometrial cancer patients with molecular and clinical features suggestive of inherited endometrial cancer.

Our analysis of a cohort of 57 endometrial cancer patients enriched for genetic susceptibility (see Table 1 for a complete list of the inclusion criteria) revealed both germ line and somatic MLH3 mutations. The mutations identified suggest a role for MLH3 in the initiation and/or progression of endometrial cancers. Although the cohort investigated was selected to enrich for genetic disease, tumor histology, rate of MSI positivity, and patient race were not different from our larger endometrial cancer patient population (9).

Of the 16 coding sequence variants identified, the majority (12 or 75%), were missense changes. Three were somatic changes: two occurring in MSS tumors and one in an MSI-high tumor. SIFT analysis suggested that three germ line variants (R647C, P844L, and T942I) and one somatic mutation (E828D) would affect protein function (see Table 4). The prediction accuracy of the SIFT analysis has been determined experimentally as ranging from 63% to 68%.

Because MLH3 is not well conserved, and because SIFT predictions vary among mammalian species.
were based on closely related sequences, it is not possible to know how reliable SIFT predictions are and how the sensitivity and specificity of this method are uncertain. Of the variants predicted to affect function, only one, the germ line T942I alteration was seen along with a second mutation, LOH. The two somatic mutations (Y720C and H823Y) as well as one germ line variant (P551S), both not predicted to alter function, were not seen in a race-matched control population. A common polymorphism in our endometrial cancer population (P844L) was predicted to be functionally significant (Table 4). The SIFT algorithm makes predictions, in part, based on homology with known proteins. As shown in Fig. 1, all the sequence changes predicted to alter function fall outside of conserved domains, and as such, SIFT algorithm may be giving both false-positive and false-negative results. It is noteworthy that three of the four silent changes were in the MutL-conserved NH2 terminus of the protein (Fig. 1).

Only one tumor specimen in our series had sequence alterations involving both alleles (“double hit” T942I + LOH). If MLH3’s role in endometrial tumorigenesis is similar to that of other DMMR family members (e.g., MSH2, MSH6, or MLH1), then loss of function mutations would be expected. The fact that we saw a single example of LOH plus an inherited missense mutation in our patient population does not exclude a cellular recessive mode of action for MLH3 in endometrial carcinoma. We identified several variants predicted to alter function, but in all cases other than the case carrying the T942I variant, the second allele seemed to be wild type. The missense changes we observed (both germ line and somatic) could act as dominant negatives. Alternatively, in those cases, there may have been other “second” mutations in MLH3 that we failed to detect. The SSCV method that was used to screen for mutations may have failed to reveal some sequence alterations. Mutations in regulatory sequences, deletions, or rearrangements cannot be excluded. Although Cannavo et al. (22) saw MLH3 promoter methylation in cancer cell lines, epigenetic silencing of this gene is unlikely based on COBRA of 27 cancers. Functional studies for the variants seen in our cancer population could help verify the in silico analysis, but it is unclear whether MLH3 plays a primary role in DNA error correction or in cellular apoptosis (18, 22, 26). The functional assessment of mismatch repair activity using reporter plasmids that are frequently applied in the analysis of MSH2, MSH6, MLH1, and PMS2 alterations would provide information only on mismatch repair function. Cavano et al. (22) suggest that MLH3 is unlikely to play a role in mismatch repair in vivo, but other cancer-relevant functions are yet to be determined. Interestingly, the missense mutations predicted to affect protein function occurred within a domain that is not evolutionarily conserved. Of the three somatic mutations (E828D, Y720C, and H823Y), two were identified in microsatellite stable tumors (MSS), and as such, it is unlikely the somatic mutations are secondary to existing DMMR defects. The H823Y somatic defect was identified in an MSI-positive tumor that expressed MLH1, MSH2, and MSH6 but had no immunodetectable PMS2. PMS2 mutation analysis has not been undertaken but seems likely given the specific defect seen in both the proband’s endometrial cancer and her sister’s colon cancer. A germ line PMS2 mutation and loss of mismatch repair in the tumor could have led to a secondary mutation in MLH3. The partial redundancy MLH3 has with PMS2 (19, 22) could lead to selection for mutation/inactivation of both MLH3 and PMS2 in tumors. Of the two germ line variants predicted to affect protein function, one (T942I + LOH) was identified in an MSS tumor that had normal expression of the MLH1, PMS2, MSH6, and MSH2 mismatch repair proteins. If the missense mutation coupled with deletion of the wild-type allele contributes to the tumor phenotype, it seems likely selection is for loss of a function distinct from mismatch repair.

The location of the missense variants identified in endometrial cancers, as well as the fact that a significant number of variants were identified in MSS tumors, supports the knockout mouse and primary tumor data that suggest MLH3 may contribute to tumorigenesis through a mechanism other than DNA error correction. As MMR proteins have been shown to induce apoptosis in response to DNA damage (12), it is possible that MLH3 also acts to promote endometrial cancer tumorigenesis through disruption of apoptotic pathways.

Our analysis provides evidence that MLH3 mutation plays a role in endometrial cancers. In this selected cohort of 57 endometrial cancers, six putative mutations were identified (10.5%). Three lines of evidence suggest these variants are likely to be mutations. First, the germ line variants R647C, T942I, and P551S were not observed in race-matched controls. However, the number of controls evaluated was modest and, as such, does not rule out the possibility that these are rare polymorphisms. Second, in one case (the patient with the T942I substitution), the tumor also had LOH at the MLH3 locus. Finally, somatic MLH3 mutations were observed in 3 of 57 tumors. Based on these observations, we conclude that MLH3 mutations are likely to play a role in a subset of endometrial cancers. Further studies are needed to better understand what role inherited MLH3 variants play in cancer susceptibility.

Acknowledgments

Received 1/23/2006; revised 5/4/2006; accepted 5/31/2006.

Grant support: NIH grant R01 CA071754, Barnes-Jewish Hospital Foundation grant 00161-0265, and National Cancer Institute Cancer Center Support grant P30 CA91842 (Siteman Cancer Center).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Ken Walls, Mary Ann Mullon, and Amy Schmidt for providing excellent technical support and the Alvin J. Siteman Cancer Center at Washington University School of Medicine and Barnes-Jewish Hospital Hereditary Cancer and Tissue Procurement Coves for providing cancer-free control DNAs.

References

4. Draviam VM, Xie S, Sorger PK. Chromosome segrega-


8. Garin CC, Federici MG, Kang L, Boyd J. Causes and con-

sequences of microsatellite instability in endometri-


www.aacrjournals.org

7507


Downloaded from cancerres.aacrjournals.org on July 22, 2017. © 2006 American Association for Cancer Research.


MLH3 Mutation in Endometrial Cancer


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/15/7502

Cited articles
This article cites 40 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/15/7502.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/66/15/7502.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.