Combined Microsatellite Instability, MLH1 Methylation Analysis, and Immunohistochemistry for Lynch Syndrome Screening in Endometrial Cancers From GOG210: An NRG Oncology and Gynecologic Oncology Group Study

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ABSTRACT

Purpose

The best screening practice for Lynch syndrome (LS) in endometrial cancer (EC) remains unknown. We sought to determine whether tumor microsatellite instability (MSI) typing along with immunohistochemistry (IHC) and MLH1 methylation analysis can help identify women with LS.

Patients and Methods

ECs from GOG210 patients were assessed for MSI, MLH1 methylation, and mismatch repair (MMR) protein expression. Each tumor was classified as having normal MMR, defective MMR associated with MLH1 methylation, or probable MMR mutation (ie, defective MMR but no methylation). Cancer family history and demographic and clinical features were compared for the three groups. Lynch mutation testing was performed for a subset of women.

Results

Analysis of 1,002 ECs suggested possible MMR mutation in 11.8% of tumors. The number of patients with a family history suggestive of LS was highest among women whose tumors were classified as probable MMR mutation (P = .001). Lynch mutations were identified in 41% of patient cases classified as probable mutation (21 of 51 tested). One of the MSH6 Lynch mutations was identified in a patient whose tumor had intact MSH6 expression. Age at diagnosis was younger for mutation carriers than noncarriers (54.3 v 62.3 years; P < .01), with five carriers diagnosed at age > 60 years.

Conclusion

Combined MSI, methylation, and IHC analysis may prove useful in Lynch screening in EC. Twenty-four percent of mutation carriers presented with ECs at age > 60 years, and one carrier had an MSI-positive tumor with no IHC defect. Restricting Lynch testing to women diagnosed at age < 60 years or to women with IHC defects could result in missing a substantial fraction of Lynch-related EC.

INTRODUCTION

Endometrial cancer (EC) is the second most common malignancy in patients with Lynch syndrome (LS). Identifying patients with EC with LS benefits both those individuals already affected with cancer and their at-risk relatives. Estimates for LS frequency among patients with EC have ranged from 2% to 6%. A majority of Lynch families have mutations in MSH2, MLH1, MSH6, PMS2, or EPCAM. Mutation penetrance and expressivity are determined by which Lynch genes are defective and the nature of the mutations. MSH6 mutation confers a particular risk for EC and a relatively lower risk for colon cancers. International collaborative studies have led to screening recommendations reflecting the risks associated with the gene responsible for disease in a given family and age of cancer onset in relatives.

The best practices for identifying LS are still being determined, with general consensus that many, if
not all, patients with colon cancer or EC should be screened for LS.10-13 Tumor immunohistochemistry (IHC) is central to screening and has been widely adopted; however, Lynch screening in patients with EC presents challenges. Somatic or epigenetic inactivation of the MLH1 gene is a frequent event, and consequently, triage based on MLH1 methylation has been recommended.15 The higher frequency of MSH6 defects in EC and the distinct clinical features associated with MSH6 mutations also need to be considered in screening for LS in patients with EC. Later age of onset for Lynch mutation carriers, lower levels of tumor microsatellite instability (MSI), and differences in MSH6 mutation penetrance and expressivity compared with other Lynch genes must be considered as part of screening efforts.

In this study, we assessed tumor IHC, MSI, and MLH1 methylation analysis in a large cohort of patients with endometrioid EC enrolled onto an NRG Oncology and Gynecologic Oncology Group (GOG) trial to determine which test or combination of tests best predicts LS. Analyses were limited to endometrioid tumors, the most common histologic type of EC seen in LS.9

Each patient was classified as having either no defect in DNA mismatch repair (MMR), a sporadic epigenetic MMR defect, or probable MMR mutation based on tumor findings. Germline mutation testing was performed for a subset of patients considered to be possible mutation carriers based on tumor testing studies. Age at diagnosis, cancer family history, tumor, and Lynch testing (as appropriate) findings were compared for the three molecularly defined groups. Our analysis of 1,002 tumors illustrated that tumor screening for LS that includes MSI analysis identifies germline mutation carriers who would have gone untested based on IHC screening alone and that as many as 24% of mutation carriers were age > 60 years at the time of EC diagnosis.

### RESULTS

#### Patient Cohort and Clinical, Demographic, and Family History Data

Patients were investigated as part of the GOG8020 protocol. They were recruited to GOG210 (Molecular Staging Study of Endometrial Carcinoma: ClinicalTrials.gov identifier NCT00340808) during the so-called unrestricted enrollment period when all stages, grades, and histologic subtypes were eligible (2003 to 2007),14 after which eligibility was restricted to poor-prognosis tumor patients who had completed chemotherapy. Patients were investigated as part of the GOG8020 protocol. They were recruited to GOG210 (Molecular Staging Study of Endometrial Carcinoma: ClinicalTrials.gov identifier NCT00340808) during the so-called unrestricted enrollment period when all stages, grades, and histologic subtypes were eligible (2003 to 2007),14 after which eligibility was restricted to poor-prognosis tumor patients who had completed chemotherapy.

The combined molecular data were used to assign tumors to one or more of the following molecularly defined categories: MSI high (MSI positive, methylation, and absent MLH1), MSI low (MSI positive, normal IHC, or absent methylation), or microsatellite stable (MSI negative, normal IHC, and PMS2 methylation positive). Tumors classified as MSI high were then further analyzed to determine the presence of MLH1 methylation, MLH1 expression, and PMS2 methylation.

#### Family Cancer History for Lynch-Associated Tumors and Relationship With Tumor MMR Status

Family history data were available for 938 of 1,002 patient cases with molecularly characterized tumors. Clinicopathologic and demographic features are listed in Appendix Table A1 (online only). Most patients were white (90.4%) and had early-stage and low-grade disease, with a mean age of 62.1 years (range, 25 to 100 years) and body-mass index of 35 kg/m² (range, 16.6 to 82.8 kg/m²).

Thirty-eight percent of tumors had features indicative of defective DNA MMR (Table 1). MLH1 methylation and tumor MSI were evaluated using pyrosequencing and/or combined bisulfite restriction analysis (COBRA).18 Primers and conditions are available on request. Finally, MSH6, MSH2, and MLH1 IHC was performed using whole-section slides; PMS2 was evaluated in a subset of patient cases.16,19,20 IHC staining was interpreted by a gynecologic pathologist (R.R.B.).

Normal DNA from 51 patient cases of probable mutation with sufficient high-quality DNA was available for LS mutations using ColoSeq (http://tests.labmed.washington.edu/COLOSEQ).21 Two additional DNA samples failed quality control assays for mutation testing. Patients considered probable carriers of Lynch mutations for whom normal tumor DNA yield or quality was inadequate were not tested. None of the IHC-normal MSI-low patient cases were considered for mutation testing.

### Statistical Analysis

The patterns of cancer family history for the three molecularly defined patient groups were compared descriptively using contingency analyses. Ages were compared using Mann-Whitney tests. Pearson’s correlation analysis was used to assess pyrosequencing methylation data (InStat3 software; GraphPad, La Jolla, CA).

### Molecular Features of Tumors

MSI, IHC, and MLH1 methylation analysis was undertaken for 1,043 ECs. Overall, 28.4% of tumors (296 of 1,043) were MSI high, with only 29 MSI low (2.8%). Thirty-nine tumors failed MLH1 analysis, and three failed IHC (one failing both), leaving 1,002 tumors for further analysis. MLH1 methylation pyrosequencing was successful for 673 patient cases (67.2%), with COBRA used for the remainder. COBRA findings were 100% concordant for 86 tumors assessed by pyrosequencing. Methylation levels at the four CpG DNA sequences investigated were highly correlated ($r^2 = 0.98$; Pearlson’s $P < .001$; primary data available on request). Tumors with $\geq 12\%$ methylation at all four CpGs were classified as methylation positive.

Average methylation for 282 MSI patient cases was 61.2% (range, 0% to 97.2%). Mean methylation value of MSI-low tumors (17 assessed by pyrosequencing) was 10.3%, with only three classified as methylation positive. Forty-eight of 265 MSI-high tumors (18.1%) lacked methylation. Average methylation for 391 microsatellite stable (MSS) tumors assessed by pyrosequencing was 4.58% (range, 0% to 92.1%); 21 methylated tumors (mean methylation, 37.8%) expressed MLH1. COBRA confirmed methylation in 10 of 10 tumors tested. The combined molecular data were used to assign tumors to one of three molecular classes: 617 (61.6%) were classified as MMR normal (no MSI, no IHC defect), 266 (26.5%) as sporadic epigenetic MMR defective (MSI positive, methylation, and absent MLH1), and 119 (11.9%) as probable MMR mutation (absence of MLH1 methylation and MSI and/or combined MSI and IHC defect).
tumors with MMR defects considered probable mutation had MSI (MSI high, n = 79; MSI low, n = 20; MSS, n = 8). The most frequent IHC defects were combined MSH2 and MSH6 loss and MSH6 loss alone (22 and 21 instances, respectively). All 22 tumors lacking both MSH2 and MSH6, consistent with an MSH2 mutation, were MSI high. One woman had a variant of uncertain significance (VUS). Nineteen germline mutations were identified (40.4% of those tested). On the basis of the nine MSH6, six MSH2, two PMS2, and two MLH1 germline mutations identified, we estimated the rate of LS at 4.4%. However, when the frequency of each class of predicted defect was considered, the overall minimum rate for LS was 3.89% (Appendix Table A2, online only). It is noteworthy that the largest single group of predicted mutations was those with no IHC defect (n = 33; 3.5% of entire cohort; Table 1). Among these patient cases, most women had MSI-low tumors; none were tested for mutations. The single mutation identified in the no–IHC defect group was in MSH6, and one additional MSH6 mutation was detected in a patient whose tumor was MSI high but for whom IHC classification was uncertain.

For the 47 probands assessed for mutations, PREMM1,2,6 gave overall risk predictions for LS ranging from 5.3% to 45.7% (Table 4). Only 13 probands were assigned risk > 10%. Eleven of 13 had Lynch mutations, and among the 34 with risk < 10%, eight had mutations. The sensitivity of the PREMM1,2,6 prediction model was 58% and specificity 93% in this molecularly high-risk selected cohort.
Our ColoSeq mutation testing included four probands whose family history data were unavailable. Three carried germline mutations: one each in MSH2, MSH6, and PMS2; one had a PMS2 VUS (Appendix Table A3, online only). Unexpectedly, both patient cases with PMS2 variants (mutation and VUS) had IHC defects consistent with an MSH2 mutation (absent MSH2 and MSH6). On the basis of three mutations identified, we estimate approximately one in 300 patients with EC carry a PMS2 mutation, consistent with IHC predictions for colorectal cancer. With the additional MSH6 mutation (10 total), MSH6 remains the most frequent cause of LS. Mutation carriers were younger than noncarriers (54.3 vs 62.3; Mann-Whitney P < .01).

**Molecular Features of Tumors and MMR Germline Mutations**

MSH6 was the most frequently mutated Lynch gene in our cohort (Table 4). Tumors from nine MSH6 mutation carriers were MSI high; the number of MSI events in MSH6 MSI-high tumors was, however, fewer than that for tumors from women with MSH2 and MSH6. On the basis of three mutations identified, we estimate approximately one in 300 patients with EC carry a PMS2 mutation, consistent with IHC predictions for colorectal cancer.

| Relative | No. (%) | Colon | Endometrial | Ovarian | Other |
|----------|---------|-------|-------------|---------|-------|
| Mother† | 854 (13) | 56 (3) | 38 (10) | 23 (7) | 28 (1) |
| Father† | 760 (11) | 40 (4) | — | — | 38 (5) |
| Sister§ | 1,473 (22) | 19 (6) | 25 (11) | 10 (4) | 12 (9) |
| Brother¶ | 1,468 (22) | 24 (7) | — | — | 15 (3) |
| Daughter¶ | 1,009 (15) | 3 (2) | 7 (7) | 3 (3) | 4 (2) |
| Son | 1,053 (16) | 0 | — | — | 2 |

NOTE. No data for: 85 mothers, 180 fathers, 29 sisters, 41 brothers, 19 daughters, and 45 sons.

*Other Lynch-associated cancers included stomach, hepatobiliary system, small bowel, renal pelvis or ureter, glioblastoma or brain, pancreas, and female reproductive tract.
†Seven mothers with ≥ two cancers.
‡One father with ≥ two cancers.
§Three sisters with two cancers.
¶One brother with two cancers.
†One daughter with two cancers.

Our analysis of endometrioid ECs from GOG210 provides an estimate of 3.89% frequency for LS, consistent with other large population-based series. The frequency of LS may be higher because of the fact that only 5% of the cohort (51 of 1,002) had germline mutation testing, and some women with prior colorectal cancers would have been excluded from GOG210. The GOG210 protocol was, however, amended on September 18, 2006, to allow for patients with prior malignancies. Given that metachronous cancers are a hallmark of LS, and EC is a second malignancy in approximately 50% of patients with LS, it is probable some Lynch patient cases were excluded.

Combined, IHC and MLH1 methylation of tumors identified Lynch patient cases that would not have been considered for mutation testing if only IHC and methylation analysis were used for initial screening for referral for genetic testing. One patient, G25, had an MSI-high tumor that expressed all four MMR proteins and carried a germline MSH6 mutation. IHC findings were inconclusive for a second MSH6 carrier, G1063. MSH2 and MSH6 staining was uncertain for both and reported as “favor positive,” but on the basis of tumor MSI status, we undertook mutation analysis. Considering the testing was limited to < 50% of the patients with probable MMR mutation, we estimate approximately one in 150 women with ECs have LS with

**DISCUSSION**

Our ColoSeq mutation testing included four probands whose family history data were unavailable. Three carried germline mutations: one each in MSH2, MSH6, and PMS2; one had a PMS2 VUS (Appendix Table A3, online only). Unexpectedly, both patient cases with PMS2 variants (mutation and VUS) had IHC defects consistent with an MSH2 mutation (absent MSH2 and MSH6). On the basis of three mutations identified, we estimate approximately one in 300 patients with EC carry a PMS2 mutation, consistent with IHC predictions for colorectal cancer. With the additional MSH6 mutation (10 total), MSH6 remains the most frequent cause of LS. Mutation carriers were younger than noncarriers (54.3 vs 62.3; Mann-Whitney P < .01).

**Molecular Features of Tumors and MMR Germline Mutations**

MSH6 was the most frequently mutated Lynch gene in our cohort (Table 4). Tumors from nine MSH6 mutation carriers were MSI high; the number of MSI events in MSH6 MSI-high tumors was, however, fewer than that for tumors from women with MSH2, MLH1, and PMS2 mutations (P < .001; Appendix Table A4, online only). Mononucleotide repeats (BAT26 and BAT25) accounted for most MSI events, with only four of nine MSH6 carriers’ tumors showing a dinucleotide change. It was noteworthy that for the 19 MSI-low tumors with no IHC defect, 16 had dinucleotide, and only three had mononucleotide repeat MSI.

### Table 2. Lynch-Associated Cancers Reported in First-Degree Relatives of Probands With Endometrial Cancer (n = 938)

| Relative       | No. (%) | Colon | Endometrial | Ovarian | Other |
|----------------|---------|-------|-------------|---------|-------|
| Mother†        | 854 (13) | 56 (3) | 38 (10)     | 23 (7)  | 28 (1) |
| Father†       | 760 (11) | 40 (4) | —           | —       | 38 (5) |
| Sister§        | 1,473 (22) | 19 (6) | 25 (11)    | 10 (4)  | 12 (9) |
| Brother¶      | 1,468 (22) | 24 (7) | —           | —       | 15 (3) |
| Daughter¶     | 1,009 (15) | 3 (2)  | 7 (7)       | 3 (3)   | 4 (2)  |
| Son           | 1,053 (16) | 0     | —           | —       | 2      |

NOTE. No data for: 85 mothers, 180 fathers, 29 sisters, 41 brothers, 19 daughters, and 45 sons.

*Other Lynch-associated cancers included stomach, hepatobiliary system, small bowel, renal pelvis or ureter, glioblastoma or brain, pancreas, and female reproductive tract.
†Seven mothers with ≥ two cancers.
‡One father with ≥ two cancers.
§Three sisters with two cancers.
¶One brother with two cancers.
†One daughter with two cancers.

### Table 3. Familial Risk, Proband Age, and Tumor MMR Status for Patient Cases of Endometrioid Endometrial Cancer (n = 938)

| Tumor MMR Status | Low | Baseline | Moderate† | High | P     | Age of Proband Median (range) | P     |
|------------------|-----|----------|-----------|------|-------|------------------------------|-------|
| MMR normal       | 427 | 99       | 36        | 16   | < .001 | 60 (25-91)                   | < .001 |
| Sporadic epigenetic | 169 | 58       | 20        | 6    |       | 65 (36-100)                  |       |
| Probable mutation | 62  | 24       | 11        | 10   |       | 59 (35-87)                   |       |

Abbreviations: LAC, Lynch-associated cancer; MMR, mismatch repair.
†Familial risk classification: low, no relative with LAC; baseline, single relative with one LAC diagnosed at age ≥ 50 years; moderate, one relative with two LACs and/or diagnosed at young age; high, ≥ two relatives with LACs and/or diagnosed at young age.
‡Mean age (range) of four risk groups: low, 62 (25-91); baseline, 63 (37-89); moderate, 61 (30-81); and high, 62 years (43-100).
§Fifty-six had a single relative who either had early-onset cancer (n = 46) or double primary LACs (n = 10).
$\chi^2$ test.
|Kruskal-Wallis test.
Fig 1. Two-generation pedigrees representative of familial risk group for women whose tumors classified as mismatch repair (MMR) normal, sporadic epigenetic MMR defect, or probable MMR mutation. Blue symbols indicate histologically confirmed endometrioid endometrial cancer. Gold symbols represent reported cancers. Age at diagnosis and at death (d) given when known. CRC, colorectal cancer.
Table 4. Tumor and ColoSeq Findings for Women With Tumors Classified As Having Probable Genetic MMR Defects

| Predicted Gene Defect* | Mutation Identified | Proband Age (years) | Risk Category | MSI Status | PREMM1,2,6 Risk Score (%) |
|------------------------|---------------------|---------------------|--------------|-----------|-------------------------|
|                        |                     |                     |              | Overall   | MLH1 | MSH2 | MSH6 |
| MSH2                   |                     |                     |              |           |           |           |       |
| G494 T                 | MSH2 c.1853delC, p.P618Hfs*17 | 52 | High | High | 27.8 | 8.8 | 14.5 | 4.5 |
| G839 T                 | MSH2 c.1861C>T, p.R621* | 53 | Moderate | High | 22.7 | 7.3 | 12.5 | 3.0 |
| G194 T                 | MSH2 del ex11 | 35 | Low | High | 5.9 | 1.1 | 2.7 | 2.1 |
| G930 T                 | MSH2 c.229_230delAG, p.S77Cfs*4 | 57 | Low | High | 5.4 | 1.1 | 1.7 | 2.6 |
| G1116 T                | MSH2 del ex 1-6 | 56 | Baseline | High | 13.9 | 2.6 | 3.0 | 8.4 |
| G734 T                 | MSH2 c.1226_1227delAG, p.Q409Rfs*7 | 46 | High | High | 33.4 | 10.7 | 18.8 | 4.0 |
| G119 T                 |                     | 54 | Baseline | High | 8.0 | 1.2 | 2.5 | 4.3 |
| G800 T                 |                     | 83 | Low | High | 5.3 | 1.1 | 1.0 | 3.2 |
| G838 T                 |                     | 53 | Low | High | 5.4 | 1.1 | 1.8 | 2.5 |
| G669 T                 |                     | 69 | Low | High | 5.3 | 1.1 | 1.3 | 2.9 |
| G1148 T                |                     | 54 | Baseline | High | 8.0 | 1.2 | 2.5 | 4.3 |
| G1166 T                |                     | 55 | Low | High | 5.4 | 1.1 | 1.8 | 2.6 |
| G531 T                 |                     | 61 | Low | High | 5.3 | 1.1 | 1.5 | 2.7 |
| G209 T                 |                     | 54 | Baseline | High | 5.4 | 1.1 | 1.8 | 2.5 |
| G878 T                 | MSH6 c.3768T>G, p.Y1256* | 51 | Low | High | 5.5 | 1.1 | 1.9 | 2.5 |
| G783 T                 | MSH6 c.892C>T, p.R288* | 53 | High | High | 20.1 | 2.8 | 5.8 | 11.5 |
| G852 T                 | MSH6 c.3332_3335dup, p.D1112Efs*2 | 54 | Low | High | 5.4 | 1.1 | 1.8 | 2.5 |
| G573 T                 | MSH6 c.3939_3957dupTCAAAGGGACATAGAAAA, p.A1320Sfs*5 | 55 | Baseline | High | 5.4 | 1.1 | 1.7 | 2.6 |
| G31 T                  | MSH6 c.3013C>T, p.R1005* | 45 | Low | High | 5.6 | 1.1 | 2.2 | 2.3 |
| G1064 T                | MSH6 c.3991C>T, p.R1331* | 61 | Moderate | High | 13.8 | 4.7 | 6.0 | 3.1 |
| G697 T                 | MSH6 c.3332_3335dup, p.D1112Efs*2 | 55 | Moderate | High | 21.0 | 5.3 | 6.8 | 8.9 |
| G705 T                 |                     | 59 | Low | High | 5.4 | 1.1 | 1.6 | 2.7 |
| G1171 T                |                     | 68 | Low | MSS | 5.3 | 1.1 | 1.3 | 2.9 |
| G116 T                 |                     | 65 | Low | High | 5.3 | 1.1 | 1.4 | 2.8 |
| G117 T                 |                     | 74 | Baseline | MSS | 7.9 | 1.2 | 1.6 | 5.1 |
| G562 T                 |                     | 84 | Low | MSS | 5.3 | 1.1 | 1.0 | 3.2 |
| PMS2                   |                     |               |              |           |           |           |       |
| G480 T                 | PMS2 c.736741delCCCTTnsTGTGTGAAAG, p.P246_P247Fs*7 | 57 | Baseline | High | 14.0 | 2.6 | 4.0 | 7.4 |
| G212 T                 | PMS2 del ex8 | 85 | High | High | 37.0 | 11.1 | 9.1 | 16.7 |
| G236 T                 | MLH1 c.191A>G, p.N64S | 61 | Low | High | 5.3 | 1.1 | 1.5 | 2.7 |
| G717 T                 |                     | 70 | Low | High | 5.3 | 1.1 | 1.3 | 2.9 |
| G174 T                 |                     | 59 | Low | High | 5.4 | 1.1 | 1.6 | 2.7 |
| G262 T                 |                     | 54 | Low | High | 5.4 | 1.1 | 1.8 | 2.5 |
| G206 T                 |                     | 64 | Moderate | High | 7.9 | 1.2 | 2.0 | 4.7 |
| No IHC defect or epitope stable |                 |               |              |           |           |           |       |
| G25 T                  | MSH6 c.393delAC, p.V131fs*2 | 56 | Low | High | 5.4 | 1.1 | 1.7 | 2.6 |
| G894 T                 |                     | 55 | Baseline | High | 14.0 | 2.6 | 3.8 | 7.6 |
| G920 T                 |                     | 50 | Low | High | 5.5 | 1.1 | 1.9 | 2.4 |
| G983 T                 |                     | 76 | Low | High | 5.3 | 1.1 | 1.1 | 3.1 |
| G234 T                 |                     | 67 | Low | High | 5.3 | 1.1 | 1.4 | 2.8 |
| MLH1                   |                     |               |              |           |           |           |       |
| G146 T                 | MLH1 c.34insG, p.G12fs*17 | 46 | Moderate | High | 19.8 | 6.6 | 10.5 | 2.6 |
| G805 T                 |                     | 62 | Low | High | 5.3 | 1.1 | 1.5 | 2.7 |
| G345 T                 |                     | 60 | Low | High | 5.4 | 1.1 | 1.6 | 2.7 |
| G1117 T                |                     | 50 | Low | High | 5.5 | 1.1 | 1.9 | 2.4 |
| G118 T                 |                     | 58 | Baseline | High | 7.9 | 1.2 | 2.3 | 4.4 |
| G510 T                 |                     | 65 | High | High | 45.7 | 21.5 | 21.5 | 2.7 |
| Uncertain staining     |                     |               |              |           |           |           |       |
| G1063 T                | MSH6 c.3261delC, p.F1088Sfs*2 | 55 | Moderate | High | 21.6 | 8.3 | 10.5 | 2.9 |
| G359 T†                | Variant of uncertain significance MSH6 c.2057G>A, p.G686D | 53 | Moderate | High | 8.0 | 1.2 | 2.5 | 4.3 |
| G677 T                 |                     | 57 | Baseline | High | 7.1 | 1.5 | 2.7 | 2.9 |

Abbreviations: IHC, immunohistochemistry; MMR, mismatch repair; MSI, microsatellite instability.
*Based on IHC and MSI findings; all tumors unmethylated for MLH1 except for G838 T.
†Variant of uncertain significance not considered mutation.
tumors that do not have IHC defects (Appendix Table A4). We note that some tumors with IHC defects lacked MSI (Appendix Table A4).

Another important and clinically relevant finding is that Lynch mutations are seen at appreciable frequency in patients with EC diagnosed at age \( \geq 60 \) years. Five mutation carriers (\( MSH6, n = 3; MLH1, n = 1; PMS2, n = 1 \)) were identified among the 17 women age \( \geq 60 \) years tested for germline mutations (Table 4; Appendix Table A4). Thirty-two women with tumors that had IHC defects or were MSI high but lacked \( MLH1 \) methylation were diagnosed at age \( \geq 60 \) years (3.2\% of cohort; 938 had family history data; 64 lacked family data). On the basis of these data, we estimate 0.94\% of women diagnosed with EC at age \( \geq 60 \) years have LS. Overall, this represents 24\% of Lynch patient cases presenting with EC.

\( MSH6 \) mutations accounted for half of Lynch patient cases in our series, confirming earlier reports that \( MSH6 \) is a major cause of LS among families ascertained through EC probands. Among relatives of the 938 probands with family history data, ECs were almost as frequent as colon cancers among female relatives (Table 2), which could reflect genetic and nongenetic risk factors. It is noteworthy that 11\% of probands whose tumors were classified as having probable MMR mutation reported one relative with EC, compared with 6.7\% for the rest of the cohort (Appendix Table A5, online only).

Cancer family risk (our categories or PREMM1,2,6 scores) did not reliably predict germline mutation, and several mutation carriers had no history of LACs in relatives (Table 4), confirming reports that family history fails to identify Lynch carriers. As noted, some women with a previous history of cancer were excluded from the GOG210 study.

Universal germline Lynch testing for patients with EC is cost prohibitive, given the low incidence of Lynch mutations in the general population, and despite nearly two decades of research, best approaches in triage for Lynch testing remains uncertain. Personal and family histories of cancer lack sensitivity because of variable penetrance and expressivity of the different LS genes and alleles and because of the lack of informativity for patients with EC from small families or for those women with limited knowledge of their biologic relatives. IHC screening identifies many ECs with MMR defects associated with epigenetic silencing of \( MLH1 \) that are not the result of inherited Lynch mutations. Buchanan et al highlighted the importance of \( MLH1 \) methylation analysis in tumors to triage patient cases for Lynch screening in EC. Whereas colon cancers with somatic or epigenetic inactivation of \( MLH1 \) frequently have \( BRAF \) mutations, and presence of \( BRAF \) mutation is used clinically in triage, no such marker exists for EC. Our study confirms the high frequency of epigenetic silencing of \( MLH1 \) (sporadic epigenetic MMR defect), with 27\% of cancers having \( MLH1 \) methylation and MSI (Table 1).

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Combined Microsatellite Instability, MLH1 Methylation Analysis, and Immunohistochemistry for Lynch Syndrome Screening in Endometrial Cancers From GOG210: An NRG Oncology and Gynecologic Oncology Group Study

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Appendix

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Table A1. Clinicopathologic and Demographic Characteristics of GOG210 Endometrioid Endometrial Cancers Investigated

| Characteristic          | No. (%) |
|-------------------------|---------|
| Race                    |         |
| White                   | 848 (90.4) |
| African American        | 55 (5.9) |
| Asian                   | 17 (1.8)  |
| Other                   | 7 (0.7)  |
| Unknown/not specified   | 11 (1.2) |
| Grade                   |         |
| 1                       | 383 (40.8) |
| 2                       | 408 (43.5) |
| 3                       | 147 (15.7) |
| Stage                   |         |
| I                       | 702 (74.8) |
| II                      | 88 (9.4)  |
| III                     | 129 (13.8) |
| IV                      | 19 (2.0)  |
| Age (mean, range)*      | 62 (25-100) |
| BMI (mean, range)       | 35 (16.6-82.8) |

Abbreviation: BMI, body-mass index.
*At time of hysterectomy.

Table A2. Estimated Frequencies of Germline Mutations

| Predicted Gene Defect* | No. (%) | No. Tested | No. Mutation Positive (%) | Predicted Mutation Frequency (%) |
|------------------------|---------|------------|---------------------------|---------------------------------|
| MSH6                   | 21 (2.2) | 12         | 7 (68.3)                  | 1.31                            |
| MSH2                   | 22 (2.3) | 14         | 6 (42.9)                  | 1.01                            |
| PMS2                   | 9 (1.0)  | 7          | 3 (42.9)†                 | 0.41†                           |
| MLH1                   | 18 (1.9) | 6          | 1 (16.7)                  | 0.32                            |
| Unknown (no IHC defect)†| 33 (3.5) | 5          | 1 (20)§                   | 0.70                            |
| Uncertain              | 4 (0.4)  | 3          | 1 (33.3)§                 | 0.14                            |

Abbreviation: IHC, immunohistochemistry; MSI, microsatellite instability.
*Based on MSI, IHC, and MLH1 methylation.
†Two PMS2 mutations and one MLH1 mutation.
‡Only MSI-high patient cases were tested, and as such, we cannot accurately predict mutation rate for this group. MSH6 mutation.
### Table A3. Tumor and ColoSeq Findings for Additional Women With Tumors Classified As Having Probable Genetic MMR Defects But No Family History

| Predicted Gene Defect | Mutation Identified | Proband Age (years) | MSI Status |
|-----------------------|---------------------|---------------------|------------|
| **MSH2**              |                     |                     |            |
| G979 T                | MSH2 del ex 1-6     | 61                  | High (four of five markers) |
| G199 T                | PMS2 p.Arg153Glufs*48 | 28                  | High (four of five markers) |
| **G728 T**            | Variant of uncertain significance PMS2 c.241G>A, p.E81K | 44 | High (five of five markers) |
| **MSH6**              |                     |                     |            |
| G1051 T               | MSH6 c.1969delC, p.Q657Rfs*6 | 62 | Low (BAT26 only) |

Abbreviations: MMR, mismatch repair; MSI, microsatellite instability.

*Variants of uncertain significance not considered mutations.

### Table A4. MSI Events in Patient Cases Classified As Probable Genetic Disease (n = 107)

| Predicted Gene Defect | Mutation Identified | D17S250 Status | BAT25 Status | DSS346 Status | BAT26 Status | D2S123 Status | Total No. of MSI Events |
|-----------------------|---------------------|----------------|--------------|---------------|--------------|--------------|-------------------------|
| **MSH2**              |                     |                |              |               |              |              |                         |
| G494 T                | MSH2 c.1853delC, p.P618Hfs*17 | MSI | MSI | MSI | MSI | MSI | 5 |
| G839 T                | MSH2 c.1861C>T, p.R621* | MSI | MSI | MSI | MSI | MSI | 5 |
| G194 T                | MSH2 del ex11        | MSI | MSI | MSI | MSI | MSI | 5 |
| G930 T                | MSH2 c.229_230delAG, p.S77Cfs*4 | MSI | MSI | AI | MSI | 4 |
| G1116 T               | MSH2 del ex 1-6      | NI | MSI | MSI | MSI | 4 |
| G734 T                | MSH2 c.1226_1227delAG, p.Q409Rfs*7 | MSI | MSI | MSI | MSI | MSI | 5 |
| G119 T                | —                   | MSI | MSI | MSI | MSI | 5 |
| G800 T                | —                   | MSI | MSI | MSI | MSI | 5 |
| G838 T                | —                   | NI | MSI | MSI | MSI | 4 |
| G869 T                | —                   | MSI | MSI | MSI | MSI | 5 |
| G1148 T               | —                   | MSI | MSI | MSI | MSI | 4 |
| G1166 T               | —                   | MSI | MSI | MSI | MSI | 5 |
| G531 T                | —                   | MSI | MSI | MSI | MSI | 4 |
| G209 T                | —                   | ND | MSI | MSI | AI | 4 |
| Not tested            |                     | MSI | MSI | MSI | MSI | 5 |
| G71 T                 | —                   | MSI | MSI | MSI | MSI | 5 |
| G78 T                 | —                   | MSI | MSI | MSI | MSI | 5 |
| G170 T                | —                   | MSI | MSI | MSI | MSI | 5 |
| G351 T                | —                   | MSI | MSI | MSI | MSI | 5 |
| G485 T                | —                   | MSI | MSI | MSI | MSI | 5 |
| G820 T                | —                   | MSI | MSI | MSI | MSI | 5 |
| G850 T                | —                   | MSI | MSI | MSI | NL | 4 |
| G1210 T               | —                   | MSI | MSI | MSI | MSI | 5 |
| **MSH6**              |                     |                |              |               |              |              |                         |
| G778 T                | MSH6 c.3768T>G, p.Y1256* | LOH | MSI | NL | MSI | LOH | 2 |
| G783 T                | MSH6 c.892C>T, p.R298* | NL | MSI | NI | MSI | NL | 2 |
| G852 T                | MSH6 c.3332_3335dup, p.D1112Efs*2 | NL | MSI | NI | MSI | NL | 2 |
| G573 T                | MSH6 c.3939_3957dupTCACAAAGGGACATAGAAAA, p.A1320Sfs*5 | MSI | MSI | MSI | MSI | NL | 4 |
| G31 T                 | MSH6 c.3013C>T, p.Arg1005* | NL | NI | MSI | MSI | MSI | 3 |
| G1064 T               | MSH6 c.3991C>T, p.R1331* | MSI | MSI | NL | MSI | NL | 2 |
| G697 T                | MSH6 c.3202C>T, p.R1668* | NL | MSI | NL | MSI | AI | 2 |
| G705 T                | —                   | NL | MSI | NI | MSI | MSI | 3 |
| G116 T                | —                   | NI | NI | NL | MSI | MSI | 2 |
| G1171 T               | —                   | NL | NI | NL | NI | NI | 0 |
| G117 T                | —                   | LOH | NI | LOH | NI | NL | 0 |
| G562 T                | —                   | NL | NI | NL | NI | NL | 0 |

(continued on following page)
Table A4. MSI Events in Patient Cases Classified As Probable Genetic Disease (n = 107) (continued)

| Predicted Gene Defect* | Mutation Identified | D17S250 Status | BAT25 Status | DSS346 Status | BAT26 Status | D2S123 Status | Total No. of MSI Events |
|------------------------|---------------------|----------------|--------------|---------------|--------------|---------------|------------------------|
| Not tested             |                     |                |              |               |              |               |                        |
| G429 T                 | NI                  | MSI            | NL           | MSI           | MSI          | 3             |                        |
| G703 T                 | NI                  | MSI            | NI           | MSI           | NI           | 2             |                        |
| G868 T                 | MSI                 | NI             | MSI         | MSI           | NL           | 3             |                        |
| G968 T                 | MSI                 | NI             | MSI         | NL            | MSI          | 3             |                        |
| G993 T                 | NI                  | MSI            | NL           | MSI           | NI           | 3             |                        |
| G1093 T                | NL                  | MSI            | NI           | MSI           | NI           | 2             |                        |
| G1126 T                | NI                  | MSI            | NI           | NI            | NL           | 1             |                        |
| G257 T                 | NL                  | NI             | NI           | NI            | NL           | 0             |                        |
| G766 T                 | NL                  | NI             | NI           | NI            | NL           | 0             |                        |
| PMS2                   |                     |                |              |               |              |               |                        |
| G480 T                 | PMS2                | c.736_741delCCCCCTinsTGTGTGTAAG, p.P246_P247Ffs7 | MSI          | MSI          | MSI          | MSI          | 5             |
| G212 T                 | PMS2                | delex8         | NL           | MSI          | MSI          | MSI          | 4             |
| G236 T                 | MLH1                | c.191A>G, p.Asn64Ser | MSI          | MSI          | MSI          | MSI          | 5             |
| G717 T                 |                     |                | MSI          | MSI          | NL           | MSI          | 4             |
| G174 T                 |                     |                | MSI          | MSI          | MSI          | MSI          | 5             |
| G262 T                 |                     |                | MSI          | MSI          | MSI          | MSI          | 5             |
| G206 T                 |                     |                | MSI          | MSI          | MSI          | NL           | 4             |
| Not tested             |                     |                |              |              |              |               |                           |
| G184 T                 |                     | MSI            | MSI          | MSI          | MSI          | 5             |                           |
| G890 T                 |                     | NI             | NI           | MSI          | MSI          | 3             |                           |
| No IHC defect/epitope stable |                 |                |              |              |              |               |                           |
| G25 T                   |                     | MSI            | NI           | NL           | MSI          | NL           | 2             |
| G894 T                 |                     | MSI            | MSI          | MSI          | MSI          | 5             |                           |
| G920 T                 |                     | ND             | MSI          | MSI          | NI           | MSI          | 4             |
| G983 T                 |                     | MSI            | MSI          | NI           | MSI          | 3             |                           |
| G234 T                 |                     | MSI            | MSI          | NL           | LOH          | 2             |                           |
| Not tested             |                     |                |              |              |              |               |                           |
| G3 T                   |                     | MSI            | MSI          | MSI          | AI or MSI    | 5             |                           |
| G52 T                   |                     | MSI            | MSI          | MSI          | MSI          | 5             |                           |
| G182 T                 |                     | NL             | MSI          | MSI          | NI           | NL           | 2             |
| G233 T                 |                     | MSI            | MSI          | NL           | MSI          | 3             |                           |
| G388 T                 |                     | MSI            | MSI          | MSI          | MSI          | 5             |                           |
| G647 T                 |                     | MSI            | MSI          | MSI          | NL           | 4             |
| G893 T                 |                     | MSI            | NI           | NI           | MSI          | 2             |                           |
| G908 T                 |                     | MSI            | MSI          | NL           | MSI          | 3             |                           |
| G1182 T                |                     | NI             | MSI          | MSI          | NI           | NL           | 2             |                           |
| G13 T                  |                     | MSI            | NI           | NL           | NL           | 1             |                           |
| G20 T                  |                     | MSI            | NI           | NL           | NL           | 1             |                           |
| G64 T                  |                     | MSI            | NI           | NI/AI        | NL           | 1             |                           |
| G122 T                 |                     | NL             | MSI          | NI           | NL           | 1             |                           |
| G216 T                 |                     | MSI            | NL           | NL           | NL           | 1             |                           |
| G380 T                 |                     | MSI            | NL           | NI           | NI           | 1             |                           |
| G466 T                 |                     | MSI            | NL           | NI           | NI           | 1             |                           |
| G478 T                 |                     | NL             | NI           | NL           | NI           | 1             |                           |
| G507 T                 |                     | MSI            | NI           | NL           | 1             |                           |
| G522 T                 |                     | NL             | NI           | MSI          | NI           | 1             |                           |
| G569 T                 |                     | NL             | NL           | NI           | MSI          | 1             |                           |
| G720 T                 |                     | NI             | NI           | MSI          | NI           | 1             |                           |
| G933 T                 |                     | MSI            | NI           | NL           | NI           | 1             |                           |
| G957 T                 |                     | NI             | MSI          | NI           | NI           | 1             |                           |
| G970 T                 |                     | NI             | NL           | NI           | MSI          | 1             |                           |
| G1030 T                |                     | NI             | MSI          | NI           | NI           | 1             |                           |
| G1042 T                |                     | NL             | MSI          | NI           | NI           | 1             |                           |
| G1160 T                |                     | MSI            | NL           | NI           | NI           | 1             |                           |
| G1211 T                |                     | NI             | NI           | MSI          | NI           | 1             |                           |

(continued on following page)
### Table A4. MSI Events in Patient Cases Classified As Probable Genetic Disease (n = 107) (continued)

| Predicted Gene Defect* | Mutation Identified | D17S250 Status | BAT25 Status | DSS346 Status | BAT26 Status | D2S123 Status | Total No. of MSI Events |
|------------------------|---------------------|----------------|--------------|---------------|--------------|---------------|------------------------|
| MLH1                   |                     |                |              |               |              |               |                        |
| G146 T                 | MLH1 g.34insG,p.Gly12fsX17 | LOH MSI MSI MSI NI | 3            |               |              |               |                        |
| G805 T                 | —                   | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G345 T                 | —                   | MSI NI MSI NI MSI | 3            |              |              |               |                        |
| G1117 T                | —                   | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G118 T                 | —                   | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G510 T                 | —                   | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| Not tested             |                     | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G88 T                  |                     | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G465 T                 |                     | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G683 T                 |                     | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G769 T                 |                     | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G823 T                 |                     | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G854 T                 |                     | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G878 T                 |                     | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G917T                  |                     | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G926 T                 |                     | MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G139 T                 |                     | NL NI NL NI NL NL | 0            |              |              |               |                        |
| G354 T                 |                     | NL NI NL NI NL NL | 0            |              |              |               |                        |
| G708 T                 |                     | NL NI NL NI NL NL | 0            |              |              |               |                        |

**Uncertain staining**

| G1063 T                | MSH6 g.3261delC, p.F1088Sfs2 | NL MSI NL MSI MSI MSI | 3            |               |              |               |                        |
| G359 T†                | Variant of uncertain significance MSH6 g.2057G>A, p.Gly686Asp | MSI MSI MSI MSI MSI MSI | 5            |              |              |               |                        |
| G677 T                 | —                               | MSI NI NI MSI MSI MSI | 3            |              |              |               |                        |
| Not tested             | G369 T                          | NI MSI NL NL MSI MSI | 3            |              |              |               |                        |

**Abbreviations:** AI, allelic imbalance; IHC, immunohistochemistry; LOH, loss of heterozygosity; MSI, microsatellite instability; NI, not informative and no evidence of MSI; NL, no loss (informative).

*Based on IHC and MSI findings; all tumors unmethylated for MLH1 except for G838 T.

†Variant of uncertain significance not considered mutation.

### Table A5. Lynch-Associated Cancers Reported in First-Degree Relatives by Molecular Group

| Molecular Tumor Classification | No. of Probands | Colon | Endometrial | Ovarian | Other | None |
|-------------------------------|-----------------|-------|-------------|---------|-------|------|
| Probable MMR mutation         | 107             | 28    | 12          | 4       | 16    | 62   |
| Sporadic                      | 253             | 37    | 21          | 11      | 36    | 169  |
| MMR normal                    | 578             | 70    | 35          | 21      | 53    | 427  |

**Abbreviation:** MMR, mismatch repair.