Increased Risk for Hereditary Nonpolyposis Colorectal Cancer-Associated Synchronous and Metachronous Malignancies in Patients with Microsatellite Instability-Positive Endometrial Carcinoma Lacking MLH1 Promoter Methylation

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ABSTRACT

Purpose: The aim of this study was to evaluate number and types of synchronous and metachronous malignancies in patients with endometrial carcinoma with and without microsatellite instability (MSI).

Experimental Design: From a series of 413 endometrial cancer patients, we identified 94 patients with MSI-positive (MSI+) cancers and grouped them by tumor MLH1 promoter methylation status. These 94 patients were matched by year of surgery to 94 patients with MSI-negative (MSI−) endometrial cancers from the same series. Medical records were reviewed for clinicopathologic information including rates and types of synchronous and metachronous malignancies. Hereditary nonpolyposis colorectal cancer (HNPCC)-associated second and third cancers were analyzed for MSI and MSH2, MSH6, and MLH1 expression for comparison with the corresponding endometrial cancers.

Results: The MSI+ and MSI− cohorts were similar with regard to age, race, grade, and histology. Twenty-eight MSI+ endometrial cancers (29.8%) were MLH1 unmethylated. Rates of synchronous and metachronous cancers were also similar in the MSI+ and MSI− groups at 20 and 23%, respectively. However, patients with MSI+ MLH1 unmethylated endometrial cancers had an excess of HNPCC-associated second and third cancers compared with those with MSI+ MLH1 methylated and MSI− endometrial cancers (18% versus 4.5%, P = 0.034, and 2.1%, P = 0.002). Six of seven second tumors from 5 patients with MSI+ MLH1 unmethylated endometrial cancers showed concordant MSI and mismatch repair protein expression status.

Conclusions: Our observation that patients with MSI-positive MLH1 unmethylated endometrial carcinoma are at increased risk for HNPCC-associated synchronous and metachronous malignancies suggests inherited cancer susceptibility. These patients and their families may warrant more intense cancer surveillance.

INTRODUCTION

Endometrial cancer is the most common gynecologic malignancy in the United States with 40,100 new cases expected in 2003 (1). It is also the most common extracolonic malignancy associated with the autosomal dominant cancer susceptibility syndrome hereditary nonpolyposis colorectal cancer (HNPCC). Overall, only 5% of endometrial cancer cases are thought to be hereditary (2). However, female carriers of an HNPCC-associated mutation have a 40–60% lifetime risk of developing endometrial cancer and a 50–80% lifetime risk of developing colon cancer (3, 4). Other HNPCC-associated tumors include cancers of the renal pelvis/ureter, stomach, small bowel, ovary, brain, and hepatobiliary tract, as well as benign sebaceous adenomas (5). Patients with HNPCC tend to develop cancers at a relatively young age (mean 45 years) and are at risk of multiple synchronous and metachronous malignancies (5).

To date, the only known cause of HNPCC is a defect in DNA mismatch repair (MMR) in the form of germ-line mutation in one of the MMR genes. HNPCC is usually due to mutations in MLH1 or MSH2 (6, 7). However, germ-line mutations in MSH6 have been reported in a number of classic HNPCC as well as atypical HNPCC families (8–10). Loss of DNA mismatch repair in cancer cells leads to an accumulation of replication errors and genetic instability at short tandem repeat sequences known as microsatellites. This molecular phenotype, called microsatellite instability (MSI), is found in the majority of HNPCC-associated cancers.

About 30% of all endometrial cancers exhibit MSI (11, 12). Seventy percent of MSI+ endometrial cancers are associated with MLH1 promoter hypermethylation (12). MSI+ cancers with MLH1 methylation are thought to be sporadic. We have shown previously that patients with MSI+ endometrial cancer have a moderately increased risk for familial clustering of cancers (13). Specifically, patients with MSI+ tumors that are unmethylated in the MLH1 promoter were found to have an increased relative risk for familial clustering of cancers (13),
suggesting that these women may be at risk of inherited cancer susceptibility. In this study, we set out to determine whether patients with MSI+ endometrial cancers are at increased risk for developing synchronous and metachronous malignancies, one of the cardinal features of inherited cancer susceptibility.

**MATERIALS AND METHODS**

**Patient Population and Study Design.** After appropriate consent was obtained, patients diagnosed with endometrial cancer and treated in the Division of Gynecologic Oncology at Washington University School of Medicine between 1993 and September 2002 were prospectively accrued and entered into a computerized database. All of the histological diagnoses were confirmed by a gynecologic pathologist (P. H.). Tumor as well as normal blood or normal tissue was collected from each patient for DNA studies. Tumors from 413 patients were analyzed for MSI, and MLH1 promoter methylation status was assessed in all of the MSI-positive samples. One hundred and eleven (27%) cases were found to be MSI-positive (MSI+/H11001 assessed in all of the MSI-positive samples. One hundred and one hundred and forty-three (35%) cases were MSI-negative (MSI−/H11002), and 32 (29%) were unmethylated at the MLH1 promoter (MSI+/U).

Medical records were available for review on 44 patients with MSI+ endometrial cancers (66 MSI+M and 28 MSI+U). These patients were matched by year of surgery to 94 patients with MSI-negative (MSI−) endometrial cancers. Patients with MSI-low cancers were excluded from the study as these cancers are quite rare (there were only 11 cases in the series), and they are regarded as pathophysiologically distinct from both MSI+ and MSI− cancers (12, 14). Data abstracted from medical records were analyzed for patient demographics, tumor grade, stage, and histology, as well as follow-up, recurrence, and mortality rates. In addition, numbers and types of synchronous and metachronous cancers were recorded. The combined approach for molecular and clinical correlative studies is presented in Fig. 1. All of the reported cancers with the exceptions of 1 melanoma, 1 basal cell carcinoma, and 1 skin cancer of unknown histology were confirmed by review of medical records and/or pathology reports.

Archival tissue specimens were requested for HNPCC-associated synchronous and metachronous cancers including 1 rectal adenoma/carcinoma, 1 gastric, 1 duodenal carcinoma, 3 colon cancers, 4 ovarian cancers, and 1 renal cell carcinoma. All of these specimens except for 1 colon cancer were received and analyzed for MSI and MLH1, MSH2, and MSH6 protein expression. Results were compared with MSI status and MLH1, MSH2, and MSH6 expression in the corresponding endometrial cancers of the patients.

**Tissue Specimens.** Endometrial cancer tissue and peripheral blood were obtained from study participants at the time of surgery and immediately frozen at −70°C. Tumors were assessed histologically to ensure high neoplastic cellularity for the tissues used for DNA preparation. Normal DNA was prepared from peripheral blood leukocytes or from normal myometrium.

**DNA Extraction from Archival Specimens.** Archival specimens of second and third HNPCC-associated malignancies were obtained when available. H&E-stained slides were examined by one pathologist (P. H.) to confirm the diagnosis and to mark areas of >70% neoplastic cellularity. Tumor tissues were scraped from unstained 5 μm slides, placed in a 50 μl volume of extraction solution [0.04% proteinase K in 10 mM Tris, 1 mM EDTA, and 1% Tween 20 (pH 8.0)], and digested overnight at 37°C. After digestion, the proteinase K was inactivated at 95°C for 8 min. The DNA stock solution was then used to prepare working dilutions (usually 1:6) as templates for PCR amplification.

**MSI Analysis.** Tumor MSI analysis was performed as described previously by our group (15, 16) using a panel of five microsatellite loci described by Boland et al. (17) in the National Cancer Institute Consensus Conference on MSI in colorectal cancer. Tumors were designated as MSI+ if MSI was observed in two or more of the five consensus markers, MSI− if a single marker showed MSI, and MSI− if MSI was absent at all five of the markers.

**MLH1 Promoter Methylation Analysis.** MLH1 promoter methylation was assessed using bisulfite conversion of tumor DNA with PCR amplification and restriction analysis of critical residues as described previously (13).

**Immunohistochemistry.** Immunohistochemistry for MSH2, MSH6, and MLH1 was performed using 5 μm-thick paraffin sections mounted on charged slides, essentially as described previously for MLH1 and MSH2 (18). The concentration for the antibody against MLH1 (Clone G168–728; BD PharMingen, San Diego, CA) was 1:200, for MSH2 (Clone FE11; Zymed Laboratories, San Francisco, CA) 1:400, and for MSH6 (Clone 44; BD Transduction Laboratories, San Diego, CA) 1:600. Nuclear staining with MSH2, MLH1, and MSH6 was read as positive and absence of nuclear staining of any of these was read as negative.
Statistical Methods. Differences in proportions of HNPCC-associated (International Collaborative Group on HNPCC definition of Lynch Syndrome, Ref. 5) as well as other synchronous and metachronous malignancies among women with MSI+/H11001, MSI+/H11001, and MSI+/H11002 endometrial cancers were compared using Fisher’s exact test and chi-squared test, as well as logistic regression analysis. ANOVA was used to evaluate differences in mean ages at diagnosis of cancers.

RESULTS

Clinical characteristics of the 188 endometrial cancer patients grouped according to MSI and MLH1 promoter methylation status are presented in Table 1. Median follow-up time for the entire cohort was 24 months (mean, 30.4; range, 0.23–112.7). One hundred thirty-three patients (71%) had stage I disease, 21 patients (11%) had stage II, 29 patients (15%) had stage III, and 5 patients (3%) had stage IV disease. The majority of patients (163 or 87%) were white, and the remainder (25 or 13%) was black. One hundred sixty-three patients (87%) had cancers of endometrioid histology, whereas 25 (13%) had cancers of other histologies, including 9 mixed endometrioid/uterine papillary serous carcinomas, 14 mixed endometrioid/clear cell carcinomas, and 2 carcinosarcomas. Twenty-four patients (12.8%) recurred, and 12 patients (6.4%) died of disease during the study period, whereas 3 patients (1.6%) died of other causes. Twenty-eight patients (30% of MSI+ cases) had MSI+ endometrial cancers unmethylated at the MLH1 promoter (MSI+/U), whereas 66 patients had MSI+ cancers methylated at MLH1 (MSI+/M).

No statistically significant differences were found between the 94 patients with MSI+ and the 94 patients with MSI− endometrial cancers with regard to any of the parameters listed in Table 1 (data not shown). However, when the MSI+ cohort was divided into two subgroups according to MLH1 promoter methylation status (MSI+/U and MSI+/M), as noted previously (12), a significant difference in age at diagnosis of endometrial cancer as well as in histological subtypes was noted (Table 1). The mean age at diagnosis of endometrial cancer in patients with MSI+/U tumors was 55.4 years. This was 11.3 years younger than the mean age in the MSI+/M group (66.7) and 8 years younger than the mean age in the MSI− group (63.4; \( P = 0.001 \) and \( P = 0.002 \), respectively). With regard to histological subtypes, the MSI+/U as well as the MSI− group had lower proportions of endometrioid endometrial cancers than the MSI+/M group (78.6% and 83% versus 95.5%; \( P = 0.019 \) and \( P = 0.024 \), respectively).

Patients with MSI+/M cancers showed a trend toward higher recurrence rates and higher cancer-related death rates.
compared with the MSI+U and MSI− groups: 13 (19.7%) patients in the MSI+M group recurred compared with 2 (7.1%) and 9 (9.6%) in the MSI+U and MSI− groups, respectively. Similarly, 7 (10.6%) patients in the MSI+M group died of their cancer compared with 1 (3.6%) and 4 (4.3%) in the other 2 groups. However, these differences were not statistically significant.

Synchronous and Metachronous Malignancies in Patients with Endometrial Cancer. As is shown in Table 1, the overall rates of synchronous and metachronous malignancies were similar in the MSI+U, MSI+M, and MSI− groups at 21.4% (6 of 28), 19.7% (13 of 66), and 23.4% (22 of 94), respectively. However, the types of cancers found in each group were remarkably different.

HNPCC-associated malignancies were most common in the MSI+U group. Five of 6 women (83%) with synchronous or metachronous malignancies among 28 patients with MSI+U endometrial cancer had HNPCC-associated second cancers (18% of this patient group, see Fig. 2A). In comparison, only 3 of 13 (23%) and 2 of 22 women (9%) with synchronous or metachronous malignancies in the MSI+M and MSI− groups had HNPCC-associated second cancers (4.5% of 66 and 2.1% of 94 patients, respectively). This difference in proportions between the MSI+U and MSI+M groups is statistically significant ($P = 0.002$) as is the difference between the MSI+U and the MSI− groups ($P = 0.034$).

A trend toward a difference in proportions of synchronous and metachronous breast cancers between the three MSI groups was also noted (see Fig. 2B). Sixteen of 22 women with synchronous and metachronous cancers in the MSI− group had breast cancers (17% of 94 patients). This proportion compares with 1 breast cancer among 6 women with synchronous and metachronous malignancies found in the MSI+U patient group (3.6% of 28) and 5 among 13 in the MSI+M group (7.6% of 66). The differences in proportions did not reach statistical significance.

The remaining 9 synchronous and metachronous malignancies were neither HNPCC-associated nor breast cancers (see Fig. 2B). Five of these cancers were found in the MSI+M group and included 1 unconfirmed skin cancer, 1 case of Hodgkin’s lymphoma, 1 squamous cell skin cancer, 1 osteosarcoma, and 1 case of chronic lymphocytic leukemia. The other 4 were found in the MSI− group and included 2 unconfirmed cases of skin cancer (reported as a melanoma and a basal cell carcinoma), 1 confirmed basal cell carcinoma, and 1 case of multiple myeloma. The proportions of these cancers were not significantly different between the groups.

Endometrial Cancer Patients with HNPCC-Associated Synchronous and Metachronous Malignancies. Characteristics of the 10 endometrial cancer patients with HNPCC-associated synchronous and metachronous malignancies are presented in Table 2. The majority of these cancers were synchronous or diagnosed within 3 years of the endometrial cancer. Two patients (1137 and 1064) had 2 HNPCC-associated lesions in addition to their endometrial cancers. Overall, the mean age at diagnosis of endometrial cancer in this patient group was 55.4 years compared with 63.8 years in all of the other patients ($P = 0.037$).

The results of MSI analysis and immunohistochemistry (IHC) to assess MLH1, MSH2, and MSH6 expression are presented in Table 2 for 11 synchronous and metachronous HNPCC-associated tumors from 9 patients and for their corresponding endometrial cancers. The second cancer for 1 patient (1376) was not available for analysis. MSI status for the synchronous or metachronous cancers and their corresponding endometrial carcinomas was concordant in 7 of 9 patients for whom tissues were available. This concordant MSI status was seen in 6 of 7 patients with MSI+ endometrial cancers (5 of 5 MSI+U and 1 of 2 available MSI+M) and in 1 of 2 MSI− endometrial cancer patients. Results of IHC analyses were overall consistent with expected findings for 9 of 11 tumors. The exceptions include patient 1145 whose MSI+U endometrial cancer lacks MLH1 expression, whereas her MSI+ colon cancer stains positive for all three of the mismatch repair proteins. The second exception is patient 1102. Her endometrial cancer has
MLH1 methylation, a characteristic of sporadic disease. However, both her endometrial and ovarian cancer fail to express MLH1. Representative examples of MSI analysis and IHC are shown in Figs. 3 and 4.

Detailed family histories were available for 4 of the patients with double primary HNPCC-associated cancers (1064, 1145, 1248, and 1102) as described previously (13). Patient 1145 had a son with lymphoma (diagnosed at age 34), a brother with a non-small cell lung cancer at age 76, and an otherwise unremarkable family history. One patient (1248) was clinically diagnosed with HNPCC (Amsterdam II criteria: paternal uncle with synchronous colon and rectal cancers at age 46, paternal grandfather synchronous colon cancers age 75, and colonic polyps in her father at age 60) but had no identifiable mutation in MLH1, MSH2, or MSH6. The father of patient 1102 had colon cancer at age 75 and an adenomatous polyp at 78. Her mother was diagnosed with multiple myeloma at age 60, her paternal uncle had lung cancer at age 71, her paternal grandmother also had lung cancer at age 76, and maternal uncle had lung cancer at age 61. Patient 1064 had a brother with squamous cell cancer of the tongue at age 60, a maternal uncle with colon cancer at age 65, a nephew with glioblastoma at age 3, and her mother had lung cancer at age 66. Two patients have germ-line MSH6 mutations (1064 and 1497), and 1 (1137) has a germ-line mutation in MSH2 (12). Mutation analysis (MLH1, MSH2, and MSH6), however, was performed for only a subset of the cases included in theses analyses.

**Endometrial Cancer Patients with Other Types of Synchronous and Metachronous Malignancies.** Table 3 describes MSI status, age of diagnosis, and history of Tamoxifen use among 22 endometrial cancer patients with synchronous and metachronous breast cancers. The mean age at diagnosis of endometrial cancer (71 years) was significantly higher than the mean age at diagnosis of breast cancer in these patients (64.9 years; *P* = 0.04) reflecting the fact that breast cancer preceded endometrial cancer in 17 of the 22 cases. Furthermore, 12 of these 17 patients were on Tamoxifen before developing their endometrial cancer. The majority of breast cancer patients (16 of 22 or 73%) had MSI− endometrial cancers.

Table 4 lists characteristics of the 9 endometrial cancer patients with synchronous and metachronous cancers other than breast and HNPCC-associated malignancies. In 3 of the cases, cancer diagnoses could not be confirmed. Two of these unconfirmed cancers occurred at young ages patients were unable to recall exactly. The mean ages at diagnosis of endometrial cancer versus the other cancer (60.5 versus 50.1 years, respectively) were not significantly different in this patient group.

**DISCUSSION**

This study shows that in our cohort of 188 patients with MSI+ and MSI− endometrial cancers unselected for family history or age of first cancer diagnosis, a high number (41 or 22%) of patients developed synchronous and metachronous malignancies. This finding substantiates the results of a large population-based case series of patients with endometrial cancer from Orange County, California, in which a similar rate of synchronous and metachronous cancers is reported (19).

We anticipated that patients with MSI+ endometrial cancer might have a higher rate of second malignancies. However, we found no difference in proportions of synchronous and metachronous cancers between patients with MSI+ and those with MSI− endometrial cancers. Even on subdividing patients with MSI+ endometrial cancers into those with and without

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**Table 2** HNPCCa associated synchronous and metachronous tumors in endometrial cancer patients grouped by MSI/MLH1 promoter methylation status

<table>
<thead>
<tr>
<th>Patient ID #</th>
<th>MSI/MLH1 methyl methylation status</th>
<th>IHC results (endometrial cancer)</th>
<th>Age at diagnosis (endometrial cancer)</th>
<th>Type of synchronous or metachronous tumor</th>
<th>MSI status</th>
<th>IHC results (other tumor)</th>
<th>Age at diagnosis (other tumor)</th>
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<tr>
<td>1064</td>
<td>MSI+</td>
<td>+ + +</td>
<td>53</td>
<td>Rectal adenoma</td>
<td>MSI+</td>
<td>– + ND</td>
<td>61</td>
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<td>1137a</td>
<td>MSI+</td>
<td>– – +</td>
<td>57</td>
<td>Rectal carcinoma</td>
<td>MSI-L</td>
<td>– + ND</td>
<td>61</td>
</tr>
<tr>
<td>1145</td>
<td>MSI+</td>
<td>+ + –</td>
<td>73</td>
<td>Colon carcinoma</td>
<td>MSI+</td>
<td>– – +</td>
<td>74</td>
</tr>
<tr>
<td>1248</td>
<td>MSI+</td>
<td>– – –</td>
<td>39</td>
<td>Colon carcinoma</td>
<td>MSI+</td>
<td>– – +</td>
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<td>1497</td>
<td>MSI+</td>
<td>– + +</td>
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<td>Ovarian carcinoma</td>
<td>MSI−L</td>
<td>– + +</td>
<td>52</td>
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<td>1102</td>
<td>MSI+</td>
<td>+ + +</td>
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<td>Ovarian carcinoma</td>
<td>MSI+</td>
<td>– + –</td>
<td>52</td>
</tr>
<tr>
<td>1137</td>
<td>MSI−</td>
<td>+ + –</td>
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<td>Colon carcinoma</td>
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Mean 55.4

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<tr>
<th>Patient ID #</th>
<th>MSI/MLH1 methyl methylation status</th>
<th>IHC results (other tumor)</th>
<th>Age at diagnosis (other tumor)</th>
</tr>
</thead>
</table>

Mean 55.8

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*HNPCC, hereditary nonpolyposis colorectal cancer; MSI, microsatellite instability; ND, not determined; +M, methylated; +U, unmethylated; L, low; IHC, immunohistochemistry.

a Patient 1137 had two synchronous HNPCC-associated cancers prior to her endometrial cancer.
MLH1 promoter methylation, the proportion of synchronous and metachronous malignancies remained the same.

This finding would suggest that synchronous and metachronous malignancies as they occur in conjunction with endometrial cancer are not necessarily a hallmark of inherited cancer susceptibility. Rather, these second cancers may simply reflect the multitude of shared etiologic pathways between endometrial cancer and other types of cancers. Alternatively, some of these synchronous and metachronous malignancies may arise as a result of cancer treatment or by chance alone. However, our results suggest that only HNPCC-associated synchronous and metachronous cancers are associated with an increased risk for inherited cancer susceptibility as an excess of these types of second tumors was found in a molecular subgroup of patients (MSI+U) shown previously to have increased familial cancer risk (13).

Many studies have addressed multiple primary cancers of the endometrium and colorectum. Epidemiological studies have shown a 1.5–3-fold increased relative risk for the development of colorectal cancer after a diagnosis of endometrial cancer (20–22) as well as for endometrial cancer after a diagnosis of colorectal cancer (23). A recent retrospective cohort analysis of the Surveillance, Epidemiology, and End Results data by Weinberg et al. (24) found that the elevated risk for developing colorectal cancer after endometrial cancer is largely confined to women with endometrial cancer diagnosed before age 50. This observation suggests an association between double primary endometrial cancers and inherited cancer susceptibility, bearing in mind that double primary cancers and early age of onset are features of inherited disease.

Several investigators have studied population-based cohorts of patients with colon-colon and colon-endometrial double primary cancers (25–29). All found that these synchronous and metachronous cancers are highly correlated with risk for inherited cancer susceptibility. For example, first-degree relatives of patients with these cancers have a 2-fold elevated relative risk for developing colon, rectal, and uterine cancers (26, 29). Wijnen et al. (30) showed that the likelihood of finding a germ-line mutation in MSH2 or MLH1 increased from 45% in a family meeting the Amsterdam criteria to 90% if the family includes one member with colon-endometrial cancer or two colon cancers. Similarly, Millar et al. (27) found that double primary endometrial and colon cancers are a strong predictor of HNPCC in a population-based cohort of patients.

Molecular analysis of double primary colon-endometrial cancers substantiates the association with inherited cancer susceptibility. Several studies found that 30–60% of these double primary tumors are MSI+ with the highest percentage found in tumors of patients diagnosed with their first cancer under the age of 50 (28, 29). Millar et al. (27) demonstrated that 18% of 40 unselected patients with double primary colon-endometrial cancers harbored germ-line mutations in MLH1 or MSH2. Furthermore, immunohistochemical studies of MMR gene protein expression showed a high rate of concordant absence of MSH2, MSH6, or MLH1 expression in double primary colon-colon or colon-endometrial cancers as well as good correlation with MSI status (28). All of these features point to an inherited defect in DNA mismatch repair as a frequent etiology of these double primary cancers.

Other HNPCC-associated cancers in conjunction with endometrial cancer have been studied less extensively. Wijnen et al. (30) found no significant association between concomitant colorectal cancer/other HNPCC-associated cancers and the presence of MLH1 or MSH2 germ-line mutations. Studies of double primary endometrial-ovarian cancers have revealed contradictory results. Two small series report concordant MSI rates of 24–29% in the ovarian and endometrial cancers suggesting that defective DNA mismatch repair is a common shared etiology among these synchronous cancers (31, 32). On the other hand, Shannon et al. (33) found that only 2–4% of 45 patients with double primary endometrial and ovarian cancers had MSI+ tumors. Many of these studies suffer from lack of central pathology review making it difficult to compare results.

In our study, the increased rate of HNPCC-associated synchronous and metachronous malignancies in patients with MSI+U endometrial cancers is additional evidence that these
patients are at high risk for inherited cancer susceptibility. This was suggested by our previous findings of a younger age at diagnosis and an increased relative risk of familial clustering of cancers in these patients. The observation that 8 of 10 patients with HNPCC-associated second cancers have MSI+ endometrial cancers, coupled with the fact that in that group of women 6 of 7 available second malignancies also showed MSI, suggests that the cancers in these patients likely arise due to a shared defect in DNA mismatch repair.

The results of immunostaining are biologically plausible given what we know about the individual patient index cancers in all but 2 of the cases (1145 and 1102). For example, the IHC data are consistent with the germ-line mutation status of the women in which the cancers arose. Cancers and precancers in the two MSH6 germ-line mutation carriers (1064 and 1497) lack MSH6 protein expression and show evidence of MSI (both MSI-low and MSI-H). For the MSH2 germ-line mutation carrier (1137), all three of the cancers are MSI-H and show absence of

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**Fig. 4** Examples of immunohistochemistry for hereditary nonpolyposis colorectal cancer-associated cancers. **A**, normal staining for MSH6, MSH2, and MLH1 in metachronous ovarian cancer and endometrial cancer from case 1465. **B**, absence of MSH6 staining in both synchronous ovarian and endometrial cancer from case 1497. **C**, absence of MLH1 staining in both synchronous ovarian and endometrial cancer from case 1102. **Left**, ovarian carcinoma; **right**, endometrial carcinoma.
MSI-Positive MLH1 Unmethylated Endometrial Cancers

Although the specimen was unmethylated at MLH1 does not reveal absent MLH1 expression. Furthermore, MSH6 staining is absent in all three of the cancers for patient 1137 as is often seen in conjunction with MSH2 germ-line mutations. Patient 1145 has absence of MLH1 staining in her endometrial cancer only. Although the specimen was unmethylated at MLH1 using the accepted critical residues, absence of immunodetectable protein may reflect methylation at other sites. Because the colon cancer does not reveal absent MLH1 staining, an underlying germ-line defect in MLH1 is less likely. Patient 1102, on the other hand, has a MSI+M endometrial cancer and has absence of MLH1 staining in both her endometrial and her ovarian cancer. This finding could be reflective of an unrecognized germ-line defect in MLH1, or it could be seen as evidence that the ovarian cancer is clonally related to the endometrial cancer. However, this was not apparent on pathology review. In general, IHC results correlate well with germ-line mutation when MSH2 and/or MSH6 is absent in tumors (33, 34). The absence of immunodetectable MLH1 is usually associated with MLH1 promoter methylation, and relatively few cases with methylated tumors have been evaluated for germ-line mutations.

Several reports suggest that family history alone is not adequate for diagnosing patients with an inherited cancer syndrome, especially those with atypical HNPCC caused by MSH6 germ-line mutations (8–10). Molecular classification of endometrial cancer in a patient as MSI+U could prove useful as a screening tool for inherited cancer susceptibility and may give clinicians enough reason to obtain a detailed family history, initiate more intense cancer surveillance, and potentially, MMR gene mutation testing. It has been suggested that a combination of MSI and IHC analysis can predict a particular MMR gene germ-line mutation in patients with HNPCC (34). Further study may show that a combination of these screening modalities could also be used to predict which endometrial cancer patients deserve genetic counseling, mutation testing, and early cancer screening.

As for the other synchronous and metachronous cancers encountered in our cohort of patients, we thought it noteworthy to highlight the trend toward an excess in metachronous breast cancers in the MSI− patient group, although the numbers did not reach statistical significance. Breast cancer is believed by most investigators not to be part of the HNPCC spectrum. Therefore, as one might expect, only one metachronous breast cancer was found in patients with MSI+U endometrial cancers. Most of the breast cancers (17 of 22 or 77%) preceded the diagnosis of endometrial cancer and, of those, 12 were on Tamoxifen. Although we are unable to show a causal association in this case, we can hypothesize that endometrial cancers arising as a consequence of Tamoxifen use may be etiologically distinct from those related to defective DNA mismatch repair.

<table>
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<tr>
<th>Patient ID #</th>
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<th>Age at diagnosis (breast cancer)</th>
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<td>MSI+M</td>
<td>65</td>
<td>63</td>
<td>CLL</td>
</tr>
<tr>
<td>1072</td>
<td>MSI−</td>
<td>73</td>
<td>20'</td>
<td>Melanoma</td>
</tr>
<tr>
<td>1274</td>
<td>MSI−</td>
<td>58</td>
<td>53</td>
<td>Basal cell carcinoma</td>
</tr>
<tr>
<td>1299</td>
<td>MSI−</td>
<td>47</td>
<td>48</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>1456</td>
<td>MSI−</td>
<td>54</td>
<td>54</td>
<td>Basal cell carcinoma</td>
</tr>
</tbody>
</table>

Mean 60.5  Mean 50.1

a MSI, microsatellite instability; M, methylated; U, unmethylated.
'b' Unconfirmed.
'c' Age, approximation of patients' best recall.
Besides breast cancer, a number of other synchronous and metachronous cancers were found in both the MSI+M and the MSI− groups. Some of these cancers (all skin cancers) were not verified due to the long time interval since the patient diagnosis and the benign nature of the cancers obviating the need for close follow-up and surveillance. Therefore, although the mean age at diagnosis of these cancers is younger than the mean age at diagnosis of endometrial cancer, we assume that this discrepancy is not a reflection of inherited cancer susceptibility.

In summary, we have shown that patients with MSI+U endometrial cancers are at increased risk for developing synchronous and metachronous HNPCC-associated malignancies. Together with our previous observation that these patients are also younger at first cancer diagnosis and have an increased risk for familial clustering of cancers (13), this finding confirms the hypothesis that MSI+U endometrial cancers frequently are a manifestation of inherited cancer susceptibility. As the role of \textit{MSH6} germ-line mutations in the development of atypical HNPCC continues to be elucidated, there is heightened awareness of the need for alternative diagnostic tools to help identify cancer patients who may harbor disease-causing mutations but would not be recognized as such by conventional screening methods. Further study is needed to determine whether a combination of MSI typing with \textit{MLH1} promoter methylation analysis and IHC may be a sensitive and cost-effective screening tool for detecting patients at high risk for inherited cancer susceptibility.

ACKNOWLEDGMENTS

We thank Erin Ball for assistance in procuring tissue specimens and pathology reports, Ming-Yu Fan for help with the statistical analysis, Rhonda Walters for help with immunohistochemistry, and Sharyn Lewin for help in reviewing charts. We also thank the Alvin J. Siteman Cancer Center at Washington University School of Medicine and Barnes-Jewish Hospital in St. Louis for the use of the Hereditary Cancer Core and Biostatistics Core.

REFERENCES


Increased Risk for Hereditary Nonpolyposis Colorectal Cancer-Associated Synchronous and Metachronous Malignancies in Patients with Microsatellite Instability-Positive Endometrial Carcinoma Lacking MLH1 Promoter Methylation

Barbara M. Buttin, Matthew A. Powell, David G. Mutch, et al.