Prevalence of defective DNA mismatch repair and MSH6 mutation in an unselected series of endometrial cancers

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Endometrial cancer is the most common gynecologic malignancy in the United States and the most frequent extracolonic tumor in hereditary nonpolyposis colorectal cancer (HNPCC). HNPCC patients have inherited defects in DNA mismatch repair and the microsatellite instability (MSI) tumor phenotype. Sporadic endometrial cancers also exhibit MSI, usually associated with methylation of the MLH1 promoter. Germ-line MSH6 mutations, which are rare in HNPCC, have been reported in several families with multiple members affected with endometrial carcinoma. We reasoned that MSH6 mutation might account for loss of mismatch repair in MSI-positive endometrial cancers in which the cause of MSI is unknown. We therefore investigated MSI and MLH1 promoter methylation in 441 endometrial cancer patients unselected for age or personal and family history of cancers. MSI and MLH1 promoter methylation status were associated with age of onset and tumor histology. One hundred cases (23% of the entire series) were evaluated for MSH6 defects. Inactivating germ-line MSH6 mutations were identified in seven women with MSI-positive, MLH1 promoter unmethylated cancers. Most of the MSI in these cases was seen with mononucleotide repeat markers. The MSH6 mutation carriers were significantly younger than the rest of the population (mean age 54.8 versus 64.6, $P = 0.04$). Somatic mutations were seen in 17 tumors, all of which had MSI. Our data suggest that inherited defects in MSH6 in women with endometrial cancer are relatively common. The minimum estimate of the prevalence of inherited MSH6 mutation in endometrial cancer is 1.6% (7 of 441), comparable with the predicted prevalence for patients with colorectal cancer.

The link between defective DNA mismatch repair and the development of tumors has been firmly established. Inherited mutations in DNA mismatch repair genes are associated with the autosomal dominant cancer susceptibility syndrome, hereditary nonpolyposis colorectal cancer (HNPCC). Patients with HNPCC are at high risk for colorectal and endometrial cancer and a variety of other malignancies. Tumors from HNPCC patients frequently show mutations in repetitive sequences, giving rise to a molecular phenotype referred to as microsatellite instability (MSI). Not surprisingly, a significant fraction of sporadic colorectal and endometrial cancers also show MSI.

Most families with clinically recognized HNPCC have mutations in either MLH1 or MSH2. MSH6 mutations appear to be associated with atypical HNPCC and in particular with development of endometrial carcinoma or atypical endometrial hyperplasia, the presumed precursor of endometrial cancer (1–4). The age of onset of cancers in HNPCC kindreds with MSH6 mutations is higher than in MSH2 or MLH1 mutation carriers, and it has been reported that tumors from affected family members are less likely to have high MSI than tumors from MSH2 or MLH1 mutation carriers (5).

The estimated frequency of MSH6 mutation in patients with colorectal cancer ranges between 0% based on a tumor immunohistochemistry study of a consecutive series and 1.5% based on mutation analysis of a combination of sporadic and familial cases (6, 7). To date, there have been no prospective studies to determine the frequency of germ-line MSH6 mutations in endometrial cancer patients unselected for family history or age of onset. We determined the rate of defective DNA mismatch repair in 441 endometrial cancers, assessed MLH1 promoter methylation in the 137 tumors with MSI, and evaluated a sample of 100 cases for MSH6 mutations.

Methods

Patient Population. Tumor and normal tissues from women with primary endometrial cancers were collected at the time of hysterectomy. Mixed malignant Müllerian tumors and sarcomas were excluded from the analyses. Written informed consent was obtained from all study participants. Patients treated at the University of Washington and Indiana University were recruited prospectively as part of a clinical trial. Tissues were frozen immediately and stored at −70°C. Representative portions of the tumor tissues were formalin-fixed and paraffin-embedded to assess neoplastic cellularity. The average estimated neoplastic cellularity of tumor tissues used to prepare DNA was >70%. Normal DNA was prepared from peripheral blood leukocytes or, in a small number of cases, from uninvolved myometrium. Information on personal and family history of cancers was obtained by review of medical records. Detailed medical and family histories were completed for 80 of 441 patients as part of an earlier study (8). MSH2 mutation analysis was performed for the majority of cases with MSI and no MLH1 methylation. Five germ-line mutations were identified [cases 1150 and 1232 described in Whelan *et al.* (8) and two deletion mutations and an Asn127Ser missense change (our unpublished data)]. MLH1 was investigated in a limited number of cases. The 100 specimens analyzed for MSH6 mutation were selected from the 441 tumors, based on the results of MSI and MLH1 methylation analyses (Fig. 1).

MSI Analysis. Tumor MSI analysis was performed as described (9, 10). Following the 1998 recommendations for the detection

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Abbreviations: MSI, microsatellite instability; HNPCC, hereditary nonpolyposis colorectal cancer; MSI-H, MSI high; MSI-L, MSI low; MSS, microsatellite-stable; SSCV, single-strand conformational variant.

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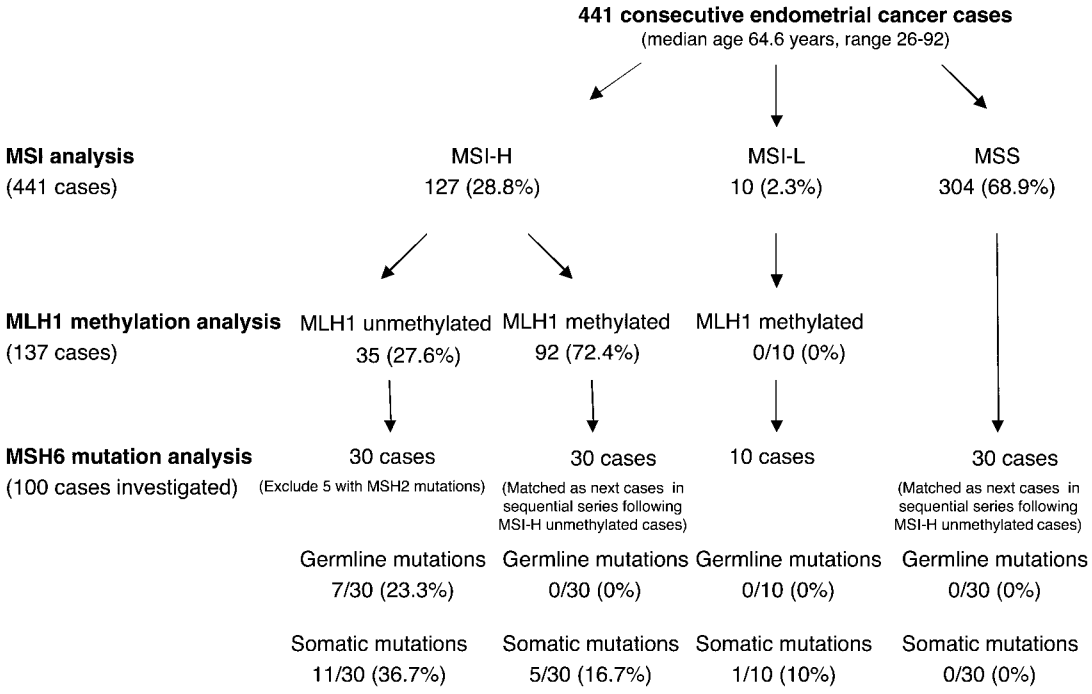

Fig. 1. Flow diagram for results of testing for defects in DNA mismatch repair in 441 unselected patients with endometrial cancer. All tumors were assessed for MSI, and those with MSI were evaluated for MLH1 promoter methylation. Among the 35 cases lacking MLH1 methylation, five were previously shown to have germ-line MSH2 mutations (ref. 8 and our unpublished data). The remaining 30 cases, classified as MSI-H unmethylated, were assessed for MSH6 mutations along with 10 MSI-L, 30 MSI-H methylated, and 30 MSS cases.

of MSI in colorectal cancer (11), we have analyzed the BAT25, BAT26, D5S346, D2S123, and D17S250 markers to assess MSI in our endometrial cancers. A sample was classified as MSI-high (MSI-H) if two or more markers showed instability, MS-stable (MSS) if no instability was noted, and MSI-low (MSI-L) if a single marker revealed novel bands.

MLH1 Methylation Analysis. Methylation was assessed in MSI-positive tumors by COBRA of cytosines −229, −231, and −241 as described (8).

Single-Strand Conformational Variant (SSCV) and Direct Sequence Analysis. The 10 MSH6 exons were evaluated for sequence alterations by SSCV analysis as described (10). Eighteen PCR assays were devised by using the PRIMER3 program (12). Information on MSH6 organization was taken from the Ensembl Human Genome Browser (ID NSG00000116062). A total of 5,683 base pairs of nonoverlapping sequence, including 5′ and 3′ untranslated and intronic sequences, were evaluated. The primers, amplicon sizes, and restriction digests used were as described in Table 3, which is published as supporting information on the PNAS web site, www.pnas.org. Two primers for exon 1 assays were as described (13).

All variants were confirmed by repeat SSCV analysis of the normal and tumor DNA by direct sequencing of conformers or amplified genomic DNA as described (14).

MSH6 Methylation Analysis. Three COBRA assays (15) were devised to assess methylation of the MSH6 CpG island (16). Bisulfite conversion of tumor DNA was performed by using two rounds of PCR (second round with nested primers) for a total of 70 cycles (8). The PCR primers, annealing temperatures, and restriction analyses performed are described in Table 4, which is published as supporting information on the PNAS web site.

Statistical Analysis. Fisher's exact tests were used to evaluate pairwise differences in the proportions of women with early onset disease and histologic subtypes among subgroups and for the numbers of MSI events seen with mono- and dinucleotide repeats among cases with MSH6 germ-line mutation and other MSI-H cases. Linear regression analyses were used to evaluate differences in the mean and median ages of patient populations stratified according to the molecular characteristics of the tumors. Logistic regression analyses were used to evaluate differences in rates of early onset disease and occurrence of variant histologic subtypes among subgroups of women with different molecular tumor characteristics. We used STATA 7.0 (Stata, College Station, TX) for all analyses.

Results and Discussion

Endometrial Cancer Patient Population. Four hundred forty-one endometrial cancers were assessed for MSI. One hundred twenty-seven tumors (28.8%) were classified as MSI-H, and of these, 92 (72.4%) had MLH1 promoter methylation. Ten cases (2.3%) were classified as MSI-L. None of the MSI-L cases showed methylation of the MLH1 promoter (Fig. 1, Table 1).

The median age at diagnosis for the patient population as a whole was 64.6 years, with a range of 26–92 years. The median and mean ages of the women with MSI-H and MSS tumors were similar (63.3 and 64.8 vs. 65.1 and 64.2 years, respectively). A significant association between age at diagnosis and MSI status was observed. Women with MSI-L tumors were older than those in the MSS and MSI-H groups (median and mean age at diagnosis 74.6 years and 73.2 years, $P = 0.03$ and $P = 0.01$, respectively). Women with MSI-H MLH1 unmethylated tumors (referred to as MSI-H U) were significantly younger than those in the other patient groups. The mean and median ages of women with MSI-H U tumors were 56.8 and 56.0 compared with 66.2 and 67.5 years for the MSI-H methylated (MSI-H M) group

Table 1. Characteristics of endometrial cancer patients

Patient group (*n*, % of total)	Median age (range)	Age at diagnosis,* *n* (% group)		Histology,† *n* (% of group)	
		<50	>50	Endometrioid	Others‡
All cases (441)	64.6 (26–92)	55 (12.5)	386 (87.5)	380 (86.2)	61 (13.8)
MSI-H (127, 28.8)	63.3 (36–89)	13 (10.2)	114 (89.8)	118 (92.9)	9 (7.1)
MLH1 methylated (92, 20.9)	66.2 (41–89)	4 (4.3)	88 (95.7)	89 (96.7)	3 (3.3)
MLH1 unmethylated (35, 7.9)	56.8 (36–73)	9 (25.7)	26 (74.3)	29 (82.9)	6 (17.1)
MSI-L (10, 2.3)	74.6 (58–85)	0 (0)	10 (100)	6 (60)	4 (40)
MSS (304, 68.9)	65.1 (26–92)	42 (13.8)	262 (86.2)	256 (84.2)	48 (15.8)

*$P = 0.004$ across the MLH1 methylated, MLH1 unmethylated, MSI-L, and MSS groups (Fisher's exact test).
†$P < 0.001$ across the MLH1 methylated, MLH1 unmethylated, MSI-L, and MSS groups (Fisher's exact test).
‡"Others" refers to uterine papillary serous, clear cell, and mixed histologies.

($P < 0.0001$ and $P < 0.0001$) and 65.1 and 64.2 for the MSS group ($P < 0.0001$ and $P = 0.0002$) (Fig. 2*A*).

The clinical diagnosis of HNPCC requires at least one family member with onset of cancer before age 50. More than a quarter of women with MSI-H U tumors were diagnosed before age 50 (25.7%, 9 of 35), whereas only 4 of 92 (4.4%) of the women with MSI-H M tumors were <50 years old at the time of diagnosis ($P = 0.002$; odds ratio, 7.62; 95% confidence interval, 2.17–26.75). Although the fraction of women diagnosed before age 50 was greater in the MSI-H U group than it was for women with MSS tumors, the difference was not statistically significant ($P = 0.078$, Table 1, Fig. 2*B*).

MSI was associated with histologic subtype. In our series, 380 women (86.2%) had endometrioid endometrial adenocarcinomas, and 61 (13.8%) had tumors of other histologic subtypes (papillary serous, clear cell, or mixed histologies) (Table 1, Fig. 2*C*). The majority of MSI-H tumors were endometrioid adenocarcinomas (118 of 127, 92.9%). There was an excess of nonendometrioid cancers in the MSI-L group. Four of 10 MSI-L cases (40%) were nonendometrioid cancers. The rate of nonendometrioid cancers was significantly higher in the MSI-L population than in the MSI-H group as a whole ($P = 0.008$, Fisher's exact test). Among the MSI-H group, there was an association between MLH1 methylation and histology. The MSI-H U tumors were more often nonendometrioid cancers (6 of 35, 17.1%) than MSI-H M tumors (3 of 92, 3.3%) ($P = 0.01$; odds ratio, 6.14; 95% confidence interval, 1.44–26.11). The excess of nonendometriod tumors among the MSI-H U group is surprising in that nonendometriod cancers are more common in older patients, and the MSI-H U group is made up of younger women.

MSH6 Mutation Analysis. A total of 100 endometrial cancer patients (23% of our series) were evaluated for MSH6. Sixty women had MSI-H endometrial cancers (30 MSI-H U and 30 MSI-H M), 10 had MSI-L cancers, and 30 had MSS tumors (Fig. 1). Our population included five additional MSI-H U cases that had previously been shown to have germ-line MSH2 mutations (ref. 8 and our unpublished data). Because the MSH2 mutations are believed to be the cause of MSI in these cases, they were not included in the MSH6 analysis. The MSI-H M and MSS tumors studied were the next cases with these molecular features after the MSI-H U cases in our series.

Germ-Line Mutations. Seven germ-line MSH6 mutations were identified (Fig. 3, Table 2). All germ-line mutations were in women with MSI-positive cancers in which the MLH1 promoter was unmethylated. Six of the seven mutations predict truncated proteins, and the seventh (case 1064) is an insertion of a single amino acid in the conserved ATPase domain of the protein (Fig. 3*C*).

The fact that all of the germ-line mutations we identified occurred in women with MSI-H tumors was unexpected. Previous studies of endometrial cancer patients with medical or family histories consistent with inherited cancer susceptibility suggest that germ-line MSH6 mutations may be more common among women whose tumors are MSI-L or MSS than among women with MSI-H tumors (2, 3, 5). A population-based study of mismatch repair gene expression in endometrial cancers indicated that absent or reduced MSH6 expression was more frequent among tumors with intermediate levels of MSI (tumors positive for two or three of five markers evaluated) than among tumors with high levels of MSI (positive for four or five markers)

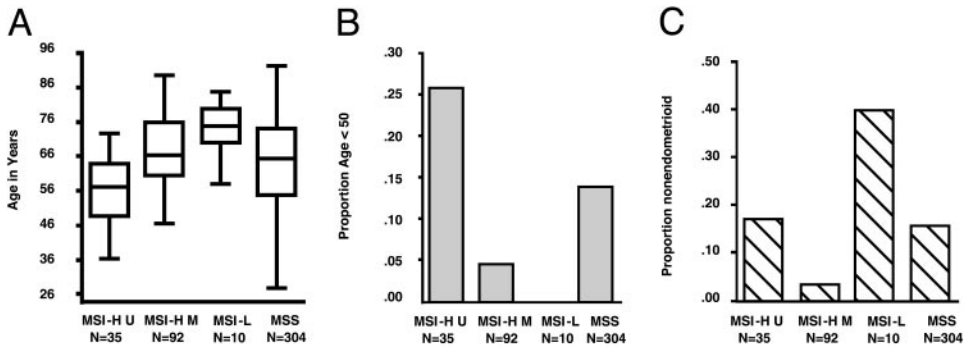

Fig. 2. Association between MSI status and MLH1 promoter methylation and age at diagnosis and tumor type. (*A*) Box plot showing the distribution of ages among women with MSI-H unmethylated, MSI-H methylated, MSI-L, and MSS tumors. Boxes include the 25th through 75th centile for each group, and the median for each is shown with a horizontal bar. (*B*) Proportions of each group with onset of endometrial cancer before age 50. (*C*) Proportion of each group with nonendometrioid tumors (papillary serous, clear cell, or mixed histologies). MSI-H U, MSI-H MLH1 promoter unmethylated; MSI-H M, MSI-H MLH1 promoter methylated.

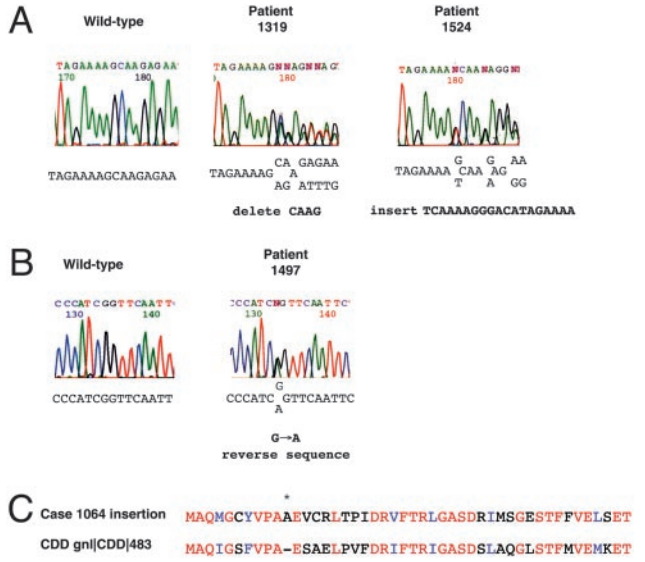

Fig. 3. Germ-line mutations in endometrial cancer patients. (*A*) Representative DNA sequence analyses reveal mutations in exon 9. The overlapping sequences reflect a deletion in patient 1319 and an insertion in patient 1524. (*B*) Single base C → T substitution at codon 911 in patient 1497. Reverse sequence is shown (G → A). (*C*) Alignment of MSH6 peptide sequence with the Ala insertion in case 1064 within the ATPase domain of DNA mismatch repair MUTS family. Conserved amino acids are shown in red. The Ala insertion (A), marked with an asterisk, disrupts at the conserved VPAE sequence.

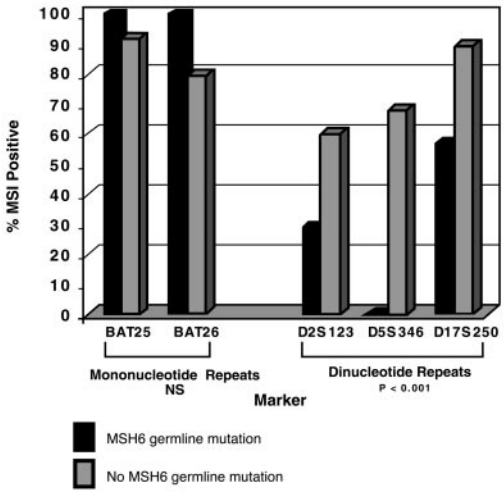

Fig. 4. Reduced numbers of MSI events involving dinucleotide repeats in tumors from women with inherited MSH6 mutations. The rates of MSI at each of the five markers tested are compared for MSH6 mutation carriers ($n = 7$) and the 53 other cases classified as MSI-H. The combined number of MSI events seen with dinucleotide repeat markers (D2S123, D5S346, and D17S250) is significantly less in MSH6 mutation carriers than in the other MSI-H cases ($P < 0.001$, Fisher's exact test). NS, not significant.

(17). In our patient population we saw a similar association between MSH6 mutations and MSI. Tumors from MSH6 germline mutation carriers were significantly more likely to have what has been referred to as "intermediate" MSI (evident with only two or three of five markers evaluated, ref. 17) than the remaining 53 MSI high cases. Seven of 7 (100%) of the tumors from MSH6 mutation carriers showed intermediate MSI, whereas 16 of 53 (30%) of the remaining tumors had intermediate MSI ($P = 0.001$, Fisher's exact test).

The lower level of MSI seen in tumors from MSH6 mutation carriers reflects a significant reduction in the number of events seen with the dinucleotide repeat markers (Fig. 4 and Table 5, which is published as supporting information on the PNAS web site). MSH6 recognizes base–base and single nucleotide insertion–deletion mismatches (18). The high rate of MSI seen with the BAT25 and BAT26 mononucleotide repeats coupled with the lower rate of MSI with dinucleotide markers is consistent with loss of MSH6 function.

In addition to the 30 MSI-H U tumors, we evaluated 10 MSI-L, 30 MSI-H M, and 30 MSS tumors. No germ-line mutations were found in these groups. The absence of germ-line mutations in the MSI-L group is not surprising, given that only 10 cases were investigated. Similarly, the absence of germ-line MSH6 mutation in women with tumors with MLH1 methylation is expected. MLH1 methylation is associated with MSI and the absence of immunodetectable MLH1 (19, 20). No mutations were identified in 30 MSS cases, consistent with the MSS tumor phenotype and normal DNA mismatch repair. MSS endometrial cancers have been reported in women with MSH6 mutations (2, 3, 5). Given that we investigated only 30 of 304 (<10%) of MSS cases, we cannot exclude the possibility that there are MSH6 mutation carriers in this group. MSS cases with early onset disease or those women with personal or family histories of cancer represent a cohort in which disease may be associated with inherited susceptibility.

The tumors in the seven cases with germ-line MSH6 mutations were all of the endometrioid histologic subtype. Limited information on family history of cancers was available for the probands. None of the seven mutation carriers have a history suspicious for HNPCC. Two women had a second HNPCC-associated malignancy (Table 2). Studies have been initiated to determine whether additional family members have malignan-

Table 2. Germ-line MSH6 mutations

Patient identifier	Age*	Exon	Codon	Nucleotide	Consequence
1401	58	4	654	Delete TG at 1901,1902	Leu→Stop
1335	45	4	911	C→T at 2731	Arg→Stop
1497†	52	4	911	C→T at 2731	Arg→Stop
1389	58	5	1066	Insert TA after 3195	Frameshift
1064†	53	6	1163/1164	Insert CTG after 3488	Insert Ala
1319	71	9	1320/1321	Delete CAAG 3959–3962	Frameshift
1524	47	9	1320	Insert TCAAAAGGGACATAGAAAA after 3957‡	Frameshift

*Median age at diagnosis, 53.6 years (45–71 years); mean age, 54.8 years.
†Patient with a second HNPCC-associated malignancy. Patient 1497 had a synchronous ovarian carcinoma, and patient 1064 had a rectal cancer 8 years after the diagnosis of her endometrial cancer.
‡Direct duplication of 19 base pairs.

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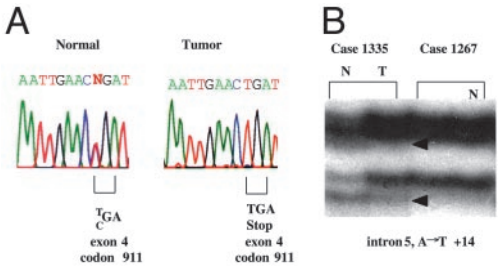

Fig. 5. Somatic MSH6 deletion in tumor 1335. (*A*) Germ-line C → T mutation at codon 911 (Arg911Stop) is heterozygous in the normal cellular DNA. (*B*) Allelic deletion in tumor 1335. Patient 1335 is heterozygous for the intron 5 variant detected by SSCV. Arrowheads indicate bands that reveal loss of heterozygosity.

cies and whether the mutations segregate with cancers in these families.

Somatic Mutations. Twenty-two coding sequence mutations and one case of allelic deletion were identified in 17 cancers (Table 6, which is published as supporting information on the PNAS web site). All 17 cases were MSI-positive: 16 were MSI-H, and 1 was MSI-L. Among the MSI-H cases, 11 were MSI-H U, and 5 were MSI-H M. The MSI-L tumor (1269) lacked MLH1 methylation.

The majority of the somatic defects identified involved the C_8 repeat in exon 5 (14 of 23, 60.9%). Among the 12 cases in which the 14 somatic C_8 repeat mutations were seen, 4 were MSI-H M, and 8 were MSI-H MLH1 (Table 6).

One tumor (1335) showed allelic deletion (Fig. 5). Eight single base substitutions were identified in seven tumors. One of the coding sequence mutations was a silent change (C→A at nucleotide 241, Gly → Gly codon 141) in the MSI-H M tumor 1257. Among the remaining substitution mutations, two were nonsense and five were missense changes. One case, 1269, was an MSI-L tumor that harbored two somatic mutations: a Glu → Stop at codon 946 and a Glu → Ala change at codon 198. Two of the MSI-H U tumors with misssense changes, 1401 and 1524, arose in women with germ-line defects (Tables 2 and 6). The Arg1024Gln substitution in tumor 1401 (basic charged polar to polar change) occurred within the DNA binding domain. The Arg772Trp mutation in case 1524 was also within the DNA binding domain, involving an evolutionarily conserved residue. Tumor 1442 had two somatic mutations: one resulted in a Lys → Stop at codon 804 and the other in a Val → Leu change at codon 480. Case 1532 had a single mutation leading to a Val → Ala change at amino acid 474.

Nine of 17 tumors with somatic defects had two mutations, and eight had a single mutation. Six of the seven cases with germ-line mutations (1064, 1335, 1389, 1401, 1497, and 1524) also had somatic defects. In women with germ-line mutations, it seems likely that the somatic mutations inactivated the wild-type allele. The other three cancers with two MSH6 somatic mutations were also MSI-H U: Two tumors were MSI-H (1331 and 1442) and one was MSI-L (1269). The MSI and MLH1 methylation status correlated with the number of MSH6 mutations in a tumor. The nine cases with two mutations were all unmethylated at the MLH1 promoter. It is noteworthy that none of the 30 MSI-H M cases had two MSH6 defects. Among the nine cases with only one identifiable mutation (eight somatic and one germ line, Tables 2 and 6), seven were informative for one or more intragenic polymorphisms. None showed loss of heterozygosity, making allelic deletion improbable (data not shown). In MSI-H M tumors, particularly those with a single mutation, somatic MSH6 defects could be seen as a consequence of the MSI-H phenotype rather than a cause. Immunohistochemistry to assess MSH6 expression could shed light on the functional importance of the mutations seen in tumors. It has, however, been shown that endometrial cancers that fail to express MSH6 are also frequently deficient in MSH2 (17), and in such cases, making a link between MSH6 mutation and function could prove difficult.

Although C_8 somatic mutation is frequent in MSI-H endometrial cancers, its role in the development of MSI is unclear. In three tumors that arose in women with germ-line mutations (cases 1401, 1524, and 1389), there were two somatic mutations. In two of these three cases, one somatic mutation involved the C_8 repeat in exon 5, whereas in the remaining case both somatic mutations were in the C_8 repeat. The majority of tumors with a single mutation (six of eight, 75%) had deletions within the C_8 repeat in exon 5.

Intronic somatic mutations involving mononucleotide repeats were also seen in MSI-H tumors. The T_{14} repeat in intron 6 (−4 to −17) and T_{17} repeats in intron 9 (−10 to −26) were subject to frequent deletion or insertion mutation in MSI-H tumors. The intronic repeat sequence is polymorphic (21, 22), and the somatic mutations are not expected to affect MSH6 function.

MSH6 Promoter Methylation. MSI-H U tumors were assessed for MSH6 methylation by using three COBRA assays spanning the MSH6 CpG island (Table 4). Twenty-five CpG residues were evaluated. One tumor (1110) showed methylation of cytosines at positions +39, +37, −10, −12, and −27, but it had no or undetectable methylation at the additional residues evaluated. No methylation was seen in the remaining 29 MSI-H U cases evaluated and additional MSI-H M cases and normal controls (data not shown).

Prevalence of MSH6 Mutations. Most HNPCC kindreds that fulfill the Amsterdam diagnostic criteria (23) have mutations in either MSH2 or MLH1. Although germ-line MSH6 defects are relatively uncommon in HNPCC, a modest number of mutations have been identified in families with atypical HNPCC or in individuals with suspected inherited cancer susceptibility (1, 4, 5, 7, 24–26). MSH6 mutations appear to confer a particular risk for endometrial cancer (1, 2, 4). The rate of MSH6 mutation in endometrial cancer patients in general has not been evaluated previously.

In our series of 441 cases of endometrial cancer unselected for age at diagnosis or personal or family history of cancers, we observed seven germ-line MSH6 mutations among 100 cases evaluated. From this series we estimate that the minimum frequency of MSH6 mutation in endometrial cancer patients is 1.6%. The actual frequency of mutation may be higher. We investigated only 100 of the 441 cases in our patient population, and the methods used to assess mutation may not have detected all sequence variants. SSCV analysis is reported to have a sensitivity of >90% (27). Up to 10% of single-base changes or small insertions or deletions could have gone undetected in our analyses. Direct sequencing could identify additional mutations not recognized in the SSCV analyses. Deletion of entire exons or portions of exons, including the primer sequences that we used, would not be detected.

Germ-line MSH6 mutations appear to be associated with a later age of onset of malignancies than MSH2 or MLH1 mutations (1, 2, 4). There have been at least three reports on endometrial cancers or precancers in MSH6 mutation carriers (2–4). The mean age at diagnosis for the 27 published carriers of MSH6 mutations is 54.8 years. All of these women were from kindreds with known or suspected HNPCC, and as such were selected from larger populations based on early onset and familial disease. The mean age at diagnosis for our MSH6 mutation cases, unselected for age or medical or family history is the same (54.8 years, Table 2) and is significantly less than

the age of the rest of the patient population (mean age 54.8 vs. 64.6 years, $P = 0.04$).

Somatic MSH6 mutations have been described in MSI-positive endometrial cancers (28, 29). Most mutations in sporadic MSI-positive cancers involve the exon 5 C_8 coding repeat, and as noted it is unclear whether such mutations are a cause or consequence of defective DNA mismatch repair. In tumors with mutations outside the C_8 repeat and in those cases with two identifiable mutations (one germ-line and one somatic or two somatic), it is likely that mutation is associated with loss of MSH6 function. In our series, the fraction of cases with two MSH6 mutations was greater among MSI-H U than MSI-H M tumors. Eight of 30 MSI-H U compared with 0 of 30 MSI-H M tumors had two mutations ($P = 0.005$, Fisher's exact test). Our findings are consistent with somatic MSH6 mutations contributing to the MSI phenotype in a substantial fraction of endometrial cancers.

In summary, we have shown that germ-line MSH6 mutations occur in an estimated 1.6% of women with endometrial carcinoma. This frequency of MSH6 mutation is comparable with what has been predicted for colorectal cancer patients based on a study of probands with suspected familial disease (7) and greater than the estimate that came from an analysis of an unselected cohort (6). In our population, 7 of 35 women (20%) with MSI-H tumors in which the MLH1 promoter was unmethylated had germ-line MSH6 mutations. MSI typing coupled with MLH1 promoter methylation analysis may prove useful in identifying endometrial cancer patients who are at increased risk for HNPCC because of inherited mutations in MSH6 or MSH2. Furthermore, consideration of the relative number of MSI events in mono- and dinucleotide repeats could help direct mutation analysis, given the observation that tumors from MSH6 mutation carriers have fewer MSI events involving dinucleotide repeat sequences. The true rate of germ-line mutation in DNA mismatch repair genes for endometrial cancer patients is unknown. Comprehensive mutation analysis of MSH6, MSH2, MLH1, and potentially other DNA mistmatch repair genes in a large patient series will be required to determine the overall mutation rate and relative contribution that MSH6 plays in endometrial cancer susceptibility.

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1. Wijnen, J., De Leeuw, W., Vasen, H., van der Klift, H., Møller, P. & Stormorken, A. (1999) *Nat. Genet.* **23,** 142–144.
2. Wagner, A., Hendriks, Y., Meijers-Heijboer, E. J., de Leeuw, W. J., Morreau, H., Hofstra, R., Tops, C., Bik, E., Brocker-Vriends, A. H., van Der Meer, C., *et al.* (2001) *J. Med. Genet.* **38,** 318–322.
3. Berends, M. J., Wu, Y., Sijmons, R. H., Mensink, R. G., van der Sluis, T., Hordijk-Hos, J. M., de Vries, E. G., Hollema, H., Karrenbeld, A., Buys, C. H., *et al.* (2002) *Am. J. Hum. Genet.* **70,** 26–37.
4. Miyaki, M., Konishi, M., Tanaka, K., Kikuchi-Yanoshita, R., Muraoka, M., Yasuno, M., Igari, T., Koike, M., Chiba, M. & Mori, T. (1997) *Nat. Genet.* **17,** 271–272.
5. Wu, Y., Berends, M. J. W., Mensink, R. G. J., Kempinga, C., Sijmons, R. H., van der Zee, A. G. J., Hollema, H., Kleibeuker, J. H., Buys, C. H. & Hofstra, R. M. W. (1999) *Am. J. Hum. Genet.* **65,** 1291–1298.
6. Cunningham, J. M., Kim, C. Y., Christensen, E. R., Tester, D. J., Parc, Y., Burgart, L. J., Halling, K. C., McDonnell, S. K., Schaid, D. J., Walsh Vockley, C., *et al.* (2001) *Am. J. Hum. Genet.* **69,** 780–790.
7. Kolodner, R. D., Tytell, J. D., Schmeits, J. L., Kane, M. F., Gupta, R. D., Weger, J., Wahlberg, S., Fox, E. A., Peel, D., Ziogas, A., *et al.* (1999) *Cancer Res.* **59,** 5068–5074.
8. Whelan, A., Babb, S., Mutch, D., Rader, J., Herzog, T., Todd, C., Ivanovich, J. & Goodfellow, P. (2002) *Int. J. Cancer* **99,** 697–704.
9. Peiffer, S. L., Herzog, T. J., Tribune, D. J., Mutch, D. G., Gersell, D. J. & Goodfellow, P. J. (1995) *Cancer Res.* **55,** 1922–1926.
10. Kowalski, L. D., Mutch, D. G., Herzog, T. J., Rader, J. S. & Goodfellow, P. J. (1997) *Genes Chromosomes Cancer* **18,** 219–227.
11. Boland, C. R., Thibodeau, S. N., Hamilton, S. R., Sidransky, D., Eshleman, J. R., Burt, R. W., Meltzer, S. J., Rodriguez-Bigas, M. A., Fodde, R., Ranzani, G. N. & Srivastava, S. (1998) *Cancer Res.* **58,** 5248–5257.
12. Rozen, S. & Skaletsky, H. (2000) *Methods Mol. Biol.* **132,** 365–386.
13. Charbonnier, F., Olschwang, S., Wang, Q., Boisson, C., Martin, C., Buisine, M. P., Puisieux, A. & Frebourg, T. (2002) *Cancer Res.* **62,** 848–853.
14. Cohn, D. E., Mutch, D. G., Herzog, T. J., Rader, J. S., Dintzis, S. M., Gersell, D. J., Todd, C. R. & Goodfellow, P. J. (2001) *Genes Chromosomes Cancer* **32,** 295–301.
15. Xiong, Z. & Laird, P. W. (1997) *Nucleic Acids Res.* **25,** 2532–2534.
16. Szadkowski, M. & Jiricny, J. (2002) *Genes Chromosomes Cancer* **33,** 36–46.
17. Stefansson, I., Akslen, L. A., MacDonald, N., Ryan, A., Das, S., Jacobs, I. J. & Salvesen, H. B. (2002) *Clin. Cancer Res.* **8,** 138–143.
18. Drummond, J. T., Li, G.-M., Longley, M. J. & Modrich, P. (1995) *Science* **268,** 1909–1912.
19. Simpkins, S. B., Bocker, T., Swisher, E. M., Mutch, D. G., Gersell, D. J., Kovatich, A. J., Palazzo, J. P., Fishel, R. & Goodfellow, P. J. (1999) *Hum. Mol. Genet*. **8,** 661–666.
20. Esteller, M., Catasus, L., Matias-Guiu, X., Mutter, G. L., Prat, J., Baylin, S. B. & Herman, J. G. (1999) *Am. J. Pathol.* **155,** 1767–1772.
21. Verma, L., Kane, M. F., Brassett, C., Schmeits, J., Evans, D. G., Kolodner, R. D. & Maher, E. R. (1999) *J. Med. Genet.* **36,** 678–682.
22. Charames, G. S., Millar, A. L., Pal, T., Narod, S. & Bapat, B. (2000) *Hum. Genet.* **107,** 623–629.
23. Vasen, H. F. A., Mecklin, J.-P., Meera Khan, P. & Lynch, H. T. (1991) *Dis. Colon Rectum* **34,** 424–425.
24. Akiyama, Y., Sato, H., Yamada, T., Nagasaki, H., Tsuchiya, A., Abe, R. & Yuasa, Y. (1997) *Cancer Res.* **57,** 3920–3923.
25. Plaschke, J., Kruppa, C., Tischler, R., Bocker, T., Pistorius, S., Dralle, H., Ruschoff, J., Saeger, H. D., Fishel, R. & Schackert, H. K. (2000) *Int. J. Cancer* **85,** 606–613.
26. Huang, J., Kuismanen, S. A., Liu, T., Chadwick, R. B., Johnson, C. K., Stevens, M. W., Richards, S. K., Meek, J. E., Gao, X., Wright, F. A., *et al.* (2001) *Cancer Res.* **61,** 1619–1623.
27. Michaud, J., Brody, L. C., Steel, G., Fontaine, G., Martin, L. S., Valle, D. & Mitchell, G. (1992) *Genomics* **13,** 389–394.
28. Gurin, C. C., Federici, M. G., Kang, L. & Boyd, J. (1999) *Cancer Res.* **59,** 462–466.
29. Kuismanen, S. A., Moisio, A. L., Schweizer, P., Truninger, K., Salovaara, R., Arola, J., Butzow, R., Jiricny, J., Nystrom-Lahti, M. & Peltomaki, P. (2002) *Am. J. Pathol.* **160,** 1953–1958.