

Causes and Consequences of Microsatellite Instability in Endometrial Carcinoma¹

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ABSTRACT

Genetic instability of microsatellite repeat sequences [microsatellite instability (MI)] is commonly seen in tumors associated with the hereditary nonpolyposis colorectal cancer syndrome and is a result of inactivating mutations in any of several genes involved in a particular pathway of DNA mismatch repair. Sporadic (*i.e.*, nonhereditary) manifestations of several tumor types, including colorectal, gastric, and endometrial carcinomas, also exhibit MI in a significant fraction of cases. Many MI+ sporadic colorectal carcinomas are associated with somatic mutations of mismatch repair genes, and several genes with coding region microsatellites are frequently mutated as a result in these cancers. The molecular causes and consequences of MI in sporadic endometrial carcinomas remain obscure, however. The aims of this study were: (a) to identify a series of sporadic endometrial carcinomas with clear evidence of MI; (b) to determine the extent to which somatic alterations in mismatch repair genes are associated with this MI; and (c) to establish whether the genes containing coding region microsatellite repeats that are known to be disrupted in MI+ gastrointestinal cancers are also disrupted in MI+ endometrial carcinomas. Matched pairs of normal and tumor DNA from 57 consecutive cases of endometrial carcinoma were examined for evidence of MI using a consensus panel of microsatellite markers. Fourteen cases (25%) displayed unequivocal evidence of MI, consistent with previously published estimates of the incidence of MI+ sporadic endometrial carcinoma. These cases were subjected to a mutation screen of the coding regions and exon-intron boundaries of the mismatch repair genes *MSH2* and *MLH1*. Although several polymorphisms were detected, no clearly deleterious mutations were found in either of these genes. Notably, however, hypermethylation of the *MLH1* promoter region was identified in 10 of 14 (71%) MI+ cases. Somatic mutations in coding region microsatellite repeats in the *TGFβIR*, *IGFIIR*, *BAX*, *E2F4*, *MSH3*, *MSH6*, *BRCA1*, and *BRCA2* genes were generally rare. Four MI+ tumors (29%) contained somatic mutations in the *PTEN* gene, only one of which was likely the result of MI. These data indicate that somatic mutational inactivation of known mismatch repair genes does not account for the great majority of sporadic endometrial carcinomas with MI and that a significant fraction of these cases may instead be causally associated with hypermethylation of the *MLH1* promoter. Furthermore, genes with coding region microsatellites that are frequently mutated in MI+ gastrointestinal cancers are rarely mutated in MI+ endometrial cancers, implying the existence of alternative molecular targets for the tumorigenic effects of MI in this tumor type.

INTRODUCTION

Progress in elucidating the molecular genetic basis of the HNPCC³ syndrome has established that an inherited mutation in one of several genes encoding components of a family of DNA mismatch repair proteins confers predisposition to colorectal and other cancers. The great majority of HNPCC kindreds for which a specific genetic

alteration has been identified are linked to one of two genes, *MSH2* or *MLH1* (1). Following somatic inactivation of the homologous wild-type allele, the resulting defect in postreplicative DNA mismatch repair leads to MI, believed to represent the molecular phenomenon responsible for tumorigenesis in the affected tissue(s) (2, 3). Sporadic, *i.e.*, nonhereditary, manifestations of several HNPCC component tumor types also exhibit MI in a substantial fraction of cases, especially colorectal (4–6), gastric (7–10), and endometrial (11–14) carcinomas. The underlying molecular genetic defect in sporadic MI+ colorectal cancers appears to be somatic mutational inactivation of either *MSH2* or *MLH1* in some cases (15–17), perhaps in as many as 65% (18) of cases, with hypermethylation of the *MLH1* promoter and attenuation of *MLH1* expression evident in some cases (19). In contrast, the molecular basis for MI in sporadic endometrial cancers remains obscure, with somatic mutations in *MSH2* or *MLH1* having been described in only four MI+ endometrial cancers to date (20–22).

In tumors affected by MI, the mechanism of tumorigenesis is believed to involve frameshift mutations of microsatellite repeats within coding regions of genes, the inactivation of which contributes to tumor development. Genes commonly affected through this mechanism in MI+ gastrointestinal cancers include *TGFβRII* (23, 24), *IGFIIR* (25), *BAX* (26), *MSH3* and *MSH6* (27), *E2F4* (28), and *BRCA1* and *BRCA2* (29). Limited analyses of MI+ endometrial cancers suggest that mutations of *TGFβRII* (30) and *IGFIIR* (31) may be much less common than in MI+ gastrointestinal cancers, but the other loci have yet to be examined in endometrial cancers. Of interest in this context is the recent demonstration that *PTEN* tumor suppressor gene mutations are found in 30–50% of all endometrial carcinomas (32–34) and are more common in MI+ than in MI– tumors (32, 33). *PTEN* mutations are rare in MI+ gastrointestinal cancers (33), suggesting that *PTEN* may represent a mutational target of MI specific to endometrial tumorigenesis.

The purpose of this study was to perform a comprehensive analysis of the molecular genetic causes and consequences of MI in a series of sporadic endometrial carcinomas. The MI+ tumors were so classified using a panel of microsatellite markers developed together with a consensus recommendation for the definition of MI (35, 36). To determine the extent to which somatic mutations in known mismatch repair genes contribute to MI in sporadic endometrial cancers, complete mutational analyses were performed on the mismatch repair genes *MSH2* and *MLH1*. The same tumors were then examined for evidence of somatic frameshift mutations presumed to result from defective mismatch repair and included analyses of coding region microsatellites within the genes *TGFβIR*, *IGF2R*, *BAX*, *E2F4*, *MSH3*, *MSH6*, *BRCA1*, and *BRCA2*. A complete mutational analysis of the *PTEN* gene was also performed in all MI+ tumors.

MATERIALS AND METHODS

Tumor Acquisition and DNA Preparation. This study was approved by the Institutional Review Board of the Memorial Sloan-Kettering Cancer Center. All tumors and corresponding normal tissues were from patients that underwent primary endometrial cancer surgery at this institution, prior to chemotherapy or radiation therapy, and were obtained as frozen specimens from the Department of Pathology. Pathological review was performed on every case to confirm a diagnosis of endometrial carcinoma. Genomic DNA

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³ The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; MI, microsatellite instability; SSCP, single-strand conformational polymorphism; TBE, Tris-borate EDTA.

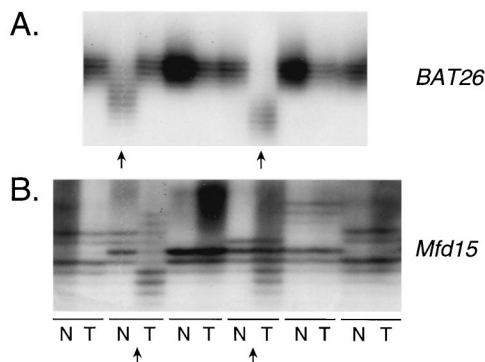


Fig. 1. Representative examples of MI in sporadic endometrial carcinomas. *A*, analysis of the nonpolymorphic *BAT26* marker using tumor DNA samples only revealed MI in two tumors (arrows). *B*, analysis of the polymorphic *Mfd15* marker in matched pairs of normal (N) and tumor (T) DNA samples revealed MI in two tumors (arrows).

was isolated using standard procedures (37). All molecular genetic analyses were performed on the same DNA sample from each tumor.

MI Analysis. The assessment of MI was based on the use of a panel of microsatellite markers developed by a multicenter consortium for diagnostic reliability and the standardization of criteria for definition of MI (35, 36). Tumors were classified as MI+ if at least three of five (60%) markers in the primary panel (*BAT26*, *BAT40*, *APC*, *Mfd15*, and *D2S123*) displayed evidence of mutant alleles in tumor DNA compared to corresponding normal tissue DNA. Reactions for PCR amplification were carried out in a volume of 20 μ l containing 50 ng of genomic DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μ M each dNTP, 0.8 μ M each primer, and 0.75 units of Taq polymerase (Perkin-Elmer Corp., Foster City, CA). One primer was end-labeled with [γ - ^{32}P]ATP by polynucleotide kinase using the KinAce-It kit (Stratagene, La Jolla, CA) and column purified prior to PCR. Thirty PCR cycles were performed, consisting of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, followed by a 7-min extension at 72°C. The PCR products were processed by diluting 1:1 in denaturing loading buffer (95% formamide, 10 mM NaOH, 0.05% xylene cyanol FF, and 0.05% bromphenol blue), heated at 90°C for 2 min, and 5 μ l were electrophoresed in 6% polyacrylamide gels containing 8.3 M urea for 2–3 h at 70 W. The gels were fixed in 10% methanol-10% acetic acid, dried, and exposed to Hyperfilm MP autoradiography film (Amersham) for 3–24 h at room temperature.

Mismatch Repair Gene Mutation Analysis. The complete coding regions and exon-intron junctions of *MSH2* and *MLH1* were screened for mutations in all MI+ tumors by SSCP analysis followed by sequence analysis of all potential variants. Intron-based primers for PCR amplification of the 16 exons of *MSH2* and 19 exons of *MLH1* were as described previously (38, 39). Each PCR was carried out in a volume of 20 μ l containing: 50 ng of cDNA; 1.5 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 200 μ M each dATP, dGTP, and dTTP; 20 μ M dCTP; 1 μ Ci of [α - ^{32}P]dCTP (6,000 Ci/mmol, NEN); 0.8 μ M each primer; and 1 unit of Taq polymerase (Perkin-Elmer Corp.). Amplification was performed in a Perkin-Elmer 9600 thermal cycler for 35 cycles consisting of 20 s at 94°C, 20 s at 55°C, and 30 s at 72°C. PCR products were diluted 1:10 in denaturing loading buffer [95% formamide, 10 mM EDTA (pH 8.0), 0.02% xylene cyanol FF, and 0.02% bromphenol blue], heated at 94°C for 5 min, and placed on ice, and 4 μ l were loaded for electrophoresis. Gels for SSCP analysis consisted of 0.5 \times MDE solution (FMC BioProducts) and 0.6 \times TBE buffer, and were run in 0.6 \times TBE buffer at 6–8 W for 16 h at room temperature. Gels were dried and exposed to Hyperfilm MP autoradiography film (Amersham) for 4–16 h.

For sequence analysis, variant and wild-type bands were excised from SSCP gels following autoradiography and suspended in 50 μ l of TE buffer for 1 h at room temperature. One μ l of the eluted DNA sample was used as a template for PCR amplification under conditions identical to those described above for SSCP analysis, except that all dNTPs were at 200 μ M and radiolabeled dCTP was omitted. Each PCR product was electrophoresed in its entirety in NuSieve 3:1 agarose (FMC BioProducts), visualized with ethidium bromide, excised from the gel, and purified using the Qiaex II gel extraction kit (Qiagen). Two ng of each DNA template were sequenced using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham). All products were

sequenced in both directions, using one or the other PCR primer. Following the sequencing reactions, 6 μ l of each termination reaction were added to 4 μ l of stop solution and heated to 70°C for 10 min, and 4 μ l were loaded into each gel lane. Sequencing gels consisted of 6% polyacrylamide and 7 M urea in TBE buffer. After electrophoresis at 70 W for 2–3 h, gels were fixed in 10% methanol-10% acetic acid, dried, and subjected to autoradiography as above for 16 h.

MLH1 Promoter Methylation Assay. Methylation of the *MLH1* promoter region was examined using the procedure described previously (19), with the following modifications. Following restriction endonuclease digestion, 12.5 ng of DNA were used as template for PCR amplification in a volume of 20 μ l. One PCR primer (25266) was radiolabeled with [γ - ^{32}P]ATP as described above, and half of each PCR product (10 μ l) was electrophoresed in a 10% polyacrylamide/TBE gel (Bio-Rad). Gels were dried and subjected to autoradiography as above.

Mutation Analysis of MI Target Genes. Mutations were detected in genes containing coding region microsatellites by PCR amplification of the appropriate segment of each gene followed by gel electrophoresis as described for the MI analysis above. Primers for PCRs were as described previously for *TGF β R2* (30), *IGF1R* (25), *BAX* (26), *E2F4* (28), *MSH3* and *MSH6* (27), and *BRCA1* and *BRCA2* (29). Mutation analysis of the *PTEN* gene was performed by screening the entire coding region and exon-intron junctions by SSCP and sequencing analyses as described above for the mismatch repair gene mutation analysis. Eleven sets of intron-based PCR primers were used to amplify the nine exons of *PTEN* as described (34).

RESULTS

A consecutive series of 57 endometrial carcinomas were examined for the presence of MI using the diagnostic criteria described in “Materials and Methods.” Fourteen (25%) tumors displayed unambiguous evidence of MI, as shown in Fig. 1. These tumors were examined further for the molecular etiology and downstream molecular effects of this phenomenon. A complete mutation screen of *MSH2* and *MLH1* failed to reveal a clearly deleterious mutation in any tumor. Two sequence alterations of unknown significance were identified in *MLH1*: a somatic missense mutation at codon 718, CAC(His) \rightarrow TAC(Tyr) (Fig. 2) and a 2-bp insertion at position -13 (relative to exon 11) of intron 10 that did not obviously affect the splice junction.

To determine whether methylation of the *MLH1* promoter region was associated with MI in these tumors, paired DNA samples from each of the 14 MI+ endometrial cancers and their corresponding normal reproductive tract tissues were examined. Methylation of the *MLH1* promoter was not observed in any of the normal tissues but was seen in 10 of 14 (71%) of the MI+ cancers (Fig. 3). Adequate clinical material was not available to perform an expression analysis of *MLH1* in these tissues; thus, 28 MI- cancers were examined to determine the extent of correlation of *MLH1* promoter methylation with the MI phenotype. Only two (7%) of these tumors displayed bands of weak autoradiographic intensity corresponding to a partially methylated *MLH1* promoter. Thus, there was a highly significant

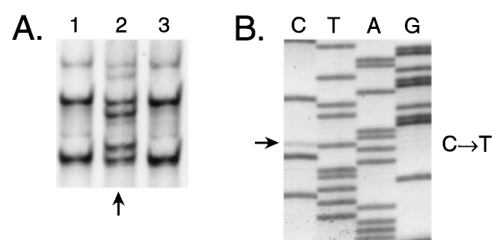


Fig. 2. Detection and characterization of a somatic missense mutation in *MLH1*. *A*, mutation screening by SSCP analysis indicated a sequence variant in DNA from tumor in Lane 2. *B*, sequence analysis of the variant PCR product confirmed a C \rightarrow T transition mutation in codon 718 of *MLH1*.

correlation of *MLH1* promoter methylation with the presence of MI in the corresponding tumor ($P = 0.01$, Fisher's exact test, two-sided).

The MI+ tumors were next examined for the presence of frameshift inactivating mutations in microsatellite-containing genes believed to be the result of MI (Fig. 4). Generally, mutations in these genes were uncommon in MI+ endometrial cancers, with a range of 0–29% of tumors containing such alterations (Table 1). Microsatellite repeats within the *TGFβRII*, *MSH3*, *MSH6*, *BRCA1*, and *BRCA2* genes appeared rarely, if ever, mutated, whereas mutations in the *BAX* gene were most common. A mutation analysis of the complete *PTEN* coding region was also performed (Fig. 5). Four of 14 (29%) MI+

Table 1 Coding region microsatellite mutations in MI+ endometrial carcinomas

Tumor no.	<i>TGFβRII</i>	<i>IGFIIR</i>	<i>BAX</i>	<i>E2F4</i>	<i>MSH3</i>	<i>MSH6</i>	<i>BRCA1</i>	<i>BRCA2</i>
4	○ ^a	●	○	○	○	●	○	○
5	○	○	○	○	○	○	○	○
12	○	○	○	○	○	○	○	○
17	○	○	○	●	○	○	○	○
19	○	○	○	○	○	○	○	○
23	○	○	●	○	○	○	○	○
28	○	○	●	●	○	○	○	○
30	○	○	○	○	○	○	○	○
40	○	○	○	○	○	○	○	○
45	○	○	○	○	○	○	○	○
105	○	○	●	●	○	○	○	○
118	○	○	○	○	○	○	○	○
119	○	○	○	○	○	○	○	○
120	○	●	●	○	○	○	○	○
Total ^b	0	2 (14)	4 (29)	3 (21)	0	1 (7)	0	1 (7)

^a ●, mutation; ○, no mutation.

^b Total number of MI+ tumors with mutation (percentage of total).

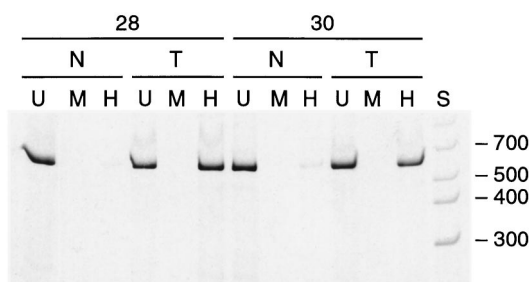


Fig. 3. Detection of *MLH1* promoter hypermethylation in tumors 28 and 30. Shown is the 604-bp PCR product, corresponding to the *MLH1* promoter region, obtained following restriction enzyme digestion under the conditions indicated. Lanes U, undigested; Lanes M, *MspI*; Lanes H, *HpaII*; Lanes N, DNA from normal uterus; Lanes T, DNA from corresponding tumor tissue; Lane S, standards. A PCR product is obtained following *HpaII* digestion only when all four restriction sites within this region are methylated. No PCR product is obtained following digestion with the isoschizomer *MspI*, which is not sensitive to restriction site methylation.

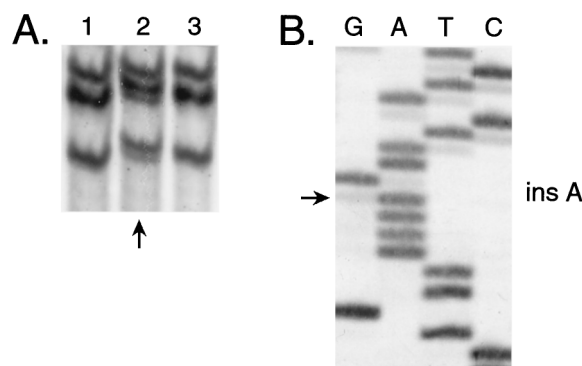


Fig. 5. Characterization of a somatic inactivating mutation in the *PTEN* gene. A, mutation screening by SSCP analysis indicated a sequence variant in DNA from tumor in Lane 2. B, sequence analysis of the variant PCR product confirmed an insertion A frameshift mutation in codons 182–183 of *PTEN*.

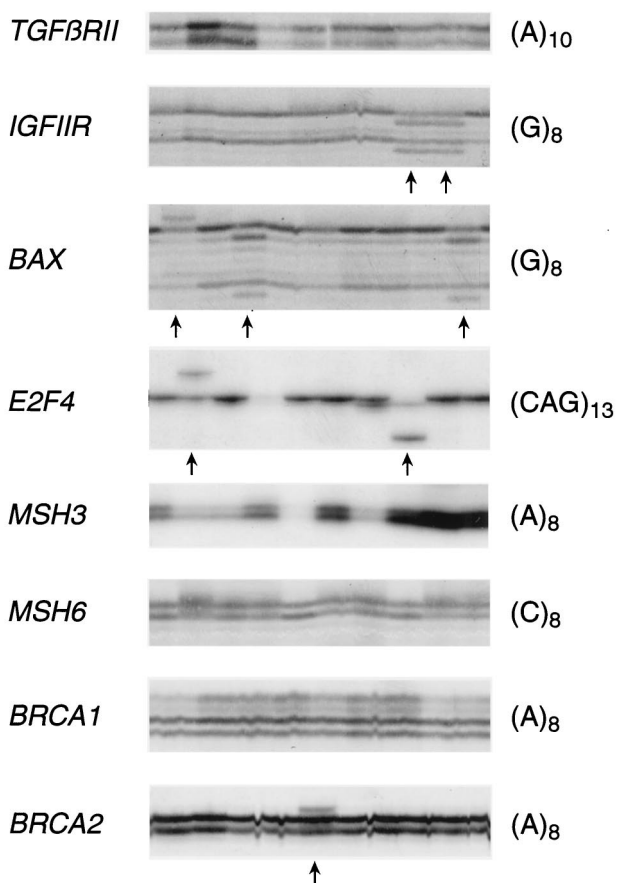


Fig. 4. Frameshift mutations in coding region microsatellites. Arrows, PCR products containing frameshift mutations in the microsatellite sequences indicated.

endometrial cancers were affected by inactivating mutations of *PTEN*: two of these four mutations were frameshift alterations, one of which occurred in a coding region microsatellite; one was a nonsense mutation; and one was a nucleotide substitution at the splice acceptor site of intron 6 (Table 2).

DISCUSSION

The finding that 25% of sporadic endometrial carcinomas display MI is consistent with previous estimates of this phenomenon, which have ranged from 17 to 23% (11–14). A comprehensive analysis of these MI+ tumors for the underlying molecular defect responsible for MI revealed that somatic mutations in the mismatch repair genes known to sustain somatic alterations in MI+ gastrointestinal tumors, *MSH2* and *MLH1*, are likely to be very rare. These findings are also consistent with previous studies in which *MSH2* and *MLH1* were screened for mutations in MI+ endometrial cancers; only four somatic mutations, two in *MSH2* and two in *MLH1*, have been identified in a total of 51 MI+ endometrial cancers (20–22). Collectively, these data imply strongly that the molecular etiology of MI in sporadic endometrial cancers involves an alternative mechanism(s) to that operative in HNPCC and sporadic gastrointestinal cancers.

Inactivation of the *MLH1* gene through promoter hypermethylation has been proposed as a mechanism for the induction of MI in sporadic colorectal cancers exhibiting attenuated expression of *MLH1* (19). A recent detailed analysis of this phenomenon demonstrates that *MLH1* promoter methylation is common in colorectal cancer cell lines and

Table 2 Mutations of *PTEN* in MI+ endometrial carcinomas

Tumor no.	Exon	Codon	Mutation	Type
12	Intron 6	-1	agATCC → atATCC	Splice acceptor
28	7	214	CAG(Arg) → TAG(stop)	Nonsense
30	8	321-323	AAAAAA → AAAAAA	Frameshift
45	6	182-183	AAA → AAAA	Frameshift

primary tumors with mismatch repair deficiencies, that the promoter methylation is correlated with decreased expression of *MLH1* at both the mRNA and protein levels, and that demethylation of the *MLH1* promoter results in the reexpression of *MLH1* in cell lines (40). This phenomenon has also been observed in the endometrial carcinoma cell line AN3CA (19, 41). Our data indicate that the *MLH1* promoter is extensively methylated in the majority (71%) of sporadic MI+ endometrial cancers. Although we were unable to examine *MLH1* expression in these tumors because of inadequate clinical material, the *MLH1* promoter methylation was highly correlated with the presence of MI because only 7% of MI- tumors displayed *MLH1* methylation. Furthermore, only a fraction of cells in these two MI- cases appeared to be affected by this phenomenon. Results from the colorectal carcinoma analysis are very similar, in that 2 of 21 (10%) sporadic MI-tumors exhibit methylation of *MLH1* (40).

Finally, we examined the molecular consequences of MI in this series of endometrial cancers by quantitating the extent of frameshift mutations in microsatellite-containing genes known to be altered in gastrointestinal cancers. None of the genes examined were found to be commonly mutated. The *TGFβRII* gene is altered in the majority of MI+ colorectal and gastric carcinomas, 90% and 71%, respectively (24, 30), but we failed to identify any MI+ endometrial cancer with this alteration. A previous study reported a low incidence of *TGFβRII* mutations in MI+ endometrial compared to MI+ colorectal cancers (30). Together, these data indicate that mutational inactivation of *TGFβRII* does not provide a significant contribution to the tumorigenic process in the endometrium.

The mismatch repair genes *MSH3* and *MSH6*, which both contain coding region microsatellites, are inactivated in 30–40% of MI+ colorectal (27) and 50–60% of MI+ gastric (29) carcinomas. These observations suggest a progressive model in which “primary” mutations in mismatch repair genes such as *MSH2* or *MLH1* lead to MI, a result of which is then “secondary” mutation of additional mismatch repair genes, such as *MSH3* and *MSH6*, with the acceleration of genomic instability and the accumulation of additional mutations in cancer-related genes (27). Interestingly, however, we observed only one *MSH6* mutation and no *MSH3* mutations in MI+ endometrial cancers, again suggesting that discrete pathways exist for tumorigenesis in association with MI in the gastrointestinal tract and endometrium.

Somewhat more common were frameshift mutations of the regulator of apoptosis *BAX*, detected in 29% of MI+ endometrial cancers compared to 50% of MI+ colorectal (26) and 67% of MI+ gastric (29) carcinomas. The growth factor receptor *IGF1R* appears to be mutated at a similarly low frequency in MI+ endometrial (15%) and gastrointestinal (6–25%) cancers (25, 31). Mutations of the transcription factor *E2F4* are less common in MI+ endometrial (20%) than gastrointestinal (31–65%) cancers (28, 42), and frameshift mutations of microsatellites within the tumor suppressors *BRCA1* and *BRCA2* are equally rare in endometrial (0–7%) and gastrointestinal (0–12%) cancers (29). These data imply that a significant proportion of the molecular targets of MI in endometrial cancer remains to be identified.

The *PTEN* tumor suppressor gene is a plausible candidate for such

a target of MI in endometrial carcinoma. The gene is frequently inactivated in endometrial cancers (32–34), and most tumors with mutations display the MI phenotype (32, 33). Furthermore, the coding region of *PTEN* contains two [A]₆ tracts (43, 44), which, although of shorter length than the typical microsatellite, represent potential mutational targets in tumors with defective mismatch repair. Only one of the MI+ endometrial cancers in our series displayed a frameshift mutation in one of these poly(A) tracts, however. An additional two MI+ tumors contained nucleotide substitution mutations, and one MI+ tumor contained an insertion mutation that are not obviously related to the MI phenotype. Overall, our data suggest that the majority of MI+ endometrial carcinomas do not sustain *PTEN* mutations during tumor development. Furthermore, the mutations that occur are not obviously attributable to MI; this view is supported by a previous observation that frameshift mutations in a poly(A) tract of *PTEN* are found in MI- endometrial cancers as well (33).

In summary, these and previous published data support a model in which ~25% of all sporadic endometrial carcinomas are characterized by MI, and in which the great majority of these tumors are not causally associated with somatic mutations in the mismatch repair genes responsible for HNPCC and some sporadic gastrointestinal cancers. Instead, our data suggest that MI in most of these cancers develops as a result of *MLH1* inactivation through promoter hypermethylation. Further studies will be required to establish a cause-and-effect relationship between *MLH1* hypermethylation and MI in endometrial carcinoma, but the strong statistical correlation between these two phenomena and the demonstration of a direct mechanistic relationship in colorectal cancer cells (40) suggest that such a direct relationship in endometrial cancer is likely. Finally, our data imply that the majority of molecular genetic targets for MI in endometrial tumorigenesis remain to be identified.

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