Absence of Mutations in DNA Mismatch Repair Genes in Sporadic Endometrial Tumors with Microsatellite Instability

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ABSTRACT

DNA mismatch repair genes have been reported to play a role in the pathogenesis of hereditary nonpolyposis colorectal cancer (HNPCC). Mutations of DNA mismatch repair genes have accounted for 90% of HNPCC-related colon and endometrial tumors. These mutations have been associated with microsatellite instability (MIN). Because endometrial cancer (EC) is the most common extracolonic malignancy associated with HNPCC, we hypothesized that similar molecular alterations may occur in sporadic endometrial tumors exhibiting MIN. Mutational analysis of the *MSH2* and *MLH1* genes was undertaken in sporadic EC that demonstrate MIN to determine the role of these genes in the pathogenesis of sporadic ECs. Established microsatellite markers were used to determine the incidence of MIN from 28 patients with sporadic EC. MIN was observed in 32% (9 of 28) of the tumor specimens analyzed. Mutational analysis of *MSH2* and *MLH1* genes was performed by immunohistochemical analysis and direct sequencing of tumor specimens that exhibited MIN. All 28 tumor specimens exhibited strong nuclear staining with both MSH2 and MLH1 antibodies, suggesting the absence of mutations. Sequencing of all exons of both the *MSH2* and *MLH1* genes in the nine MIN-positive tumor specimens demonstrated no mutations. We conclude that the *MSH2* and *MLH1* genes do not play a role in the pathogenesis of sporadic endometrial cancer.

INTRODUCTION

EC[2] is the most frequently diagnosed gynecological cancer in the United States, with approximately 31,000 new cases and 5,900 deaths anticipated during 1994 (1). It is known that excess estrogen exposure, obesity, hypertension, and diabetes enhance the risk of developing EC. At the molecular level, mutations in several proto-oncogenes (*e.g.*, c-*myc*, c-*erb*-2, and K-*ras*) have been reported to play a role in the tumorigenesis of EC (2–4). Despite these findings, the molecular basis of the tumorigenesis for EC is not well understood.

A novel genetic alteration known as MIN has been described in some sporadic colon cancers (5), some sporadic ECs, and in the majority of both colon and endometrial tumors associated with HNPCC (6–8). HNPCC has recently been shown to result from germline mutations in DNA mismatch repair genes. At least five different genes, *MSH2*, *MLH1*, *PMS1*, *PMS2*, and *GTBP*, involved in human DNA mismatch repair pathway have been identified (9–14). These genes are the human homologues of the *mutS* and *mutL* genes involved in bacterial DNA mismatch repair (15). Recently, it has been estimated that ~90% of HNPCC kindreds have germline mutations of *MSH2* and *MLH1* genes, suggesting that mutations in these DNA repair genes may be the cause of HNPCC (15, 16). Likewise, somatic mutations in DNA mismatch repair genes have been hypothesized to be responsible for the MIN exhibited by apparently sporadic tumors (17, 18).

Because EC is the most commonly associated extracolonic malignancy with HNPCC (19), we hypothesized that similar molecular alterations may occur in sporadic endometrial tumors exhibiting MIN. Therefore, we examined sporadic endometrial tumors that demonstrate MIN for mutations in the *MSH2* and *MLH1* genes.

MATERIALS AND METHODS

Fresh endometrial tumor specimens were obtained from 28 patients at the time of primary surgery for endometrial carcinoma at the Mayo Clinic. The tumor was promptly frozen at −70°C and stored until the time of DNA extraction. Either blood or fresh myometrial tissue was also obtained from each patient as a source of normal DNA.

All tumors were assigned a histopathological subtype and grade by a pathologist. A modified Broder's classification was used to grade the tumors. Patients were staged by following the current International Federation of Gynecology and Obstetrics classifications (20). DNA from both tumor and normal tissue was prepared as described previously (21). Absorbance at a 260/280 wavelength using a Shimadzu 1201 UV spectrophotometer was utilized to determine the DNA concentration.

MIN Assay. Eight established microsatellite markers mapping to chromosomes 2, 3, 5, 17, and 18 were used for MIN assay (5–8): *D2S119*, *D2S123*, *D3S1259*, *D3S1295*, *D5S107*, *D17S261*, *D18S34*, and *DCC* (Research Genetics, Huntsville, AL). After DNA extraction, 25 ng of DNA were suspended in the following PCR buffer solution: 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 1 μM of each primer, and 0.5 units of AmpliTaq polymerase in a 15-μl reaction. All reagents were obtained from Promega (Madison, WI). The PCR reaction was carried out as

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[2] The abbreviations used are: EC, endometrial cancer; MIN, microsatellite instability; HNPCC, hereditary nonpolyposis colon cancer; LOH, loss of heterozygosity.

described previously (21) on an ERICOMP thermocycler (San Diego, CA). The PCR products were then processed by diluting 1:1 with a loading buffer containing 95% formamide, denatured for 10 min at 95°C, and then quenched at 4°C. Typically, 3 μl of the mixture were subjected to electrophoresis on a 6% denaturing polyacrylamide gel containing 25% formamide. The gel was then transferred to nitrocellulose paper, dried, and exposed to X-ray film (Kodak) at −70°C. All autoradiographic results were then interpreted by the two authors (P. C. L. and R. B. J.). MIN assay was performed three times to ensure reproducible data.

Sequencing of *MSH2* and *MLH1* Genes. Mutational analysis for both *MSH2* and *MLH1* genes was performed by first amplifying the individual exon fragments with the appropriate PCR primers as described (16, 18). All PCR products were analyzed on a 1.5% agarose gel to ensure that the appropriate fragment was amplified. The Life Technologies (Gaithersburg, MD) double-stranded DNA cycle sequencing system was used for direct sequencing of the individual exons using the amplified PCR product.

Immunohistochemical Analysis. Immunohistochemical analysis of the expression of MLH1 and MSH2 proteins was done on formalin-fixed, paraffin-embedded sections of MIN-positive tumors. Six-μm sections were deparaffinized through xylene and graded alcohol. Endogenous peroxidase activity was blocked by placing in 50% methanol/3% H_2O_2. The sections were subjected to antigen retrieval by heating in a steamer with 10 mM citrate buffer (pH 6.0) for 30 min and allowed to cool. Nonspecific protein binding was eliminated by application of 1% goat serum/PBS/Tween 20 for 10 min. Anti-MLH1 (clone G168-728, PharMingen, San Diego, CA) at a final concentration of 1 μg/ml was added to the sections and incubated overnight at 4°C. Sections were treated with biotinylated goat anti-mouse IgG followed by a subsequent application of horseradish peroxidase conjugated-streptavidin. Specific binding sites were visualized with diaminobenzidine, and tissue was counterstained with hematoxylin. Because its sensitivity in paraffin-embedded tissue is much less than MLH1, anti-MSH2 (clone FE11, Oncogene Research Products/Calbiochem) was added at 0.5 μg/ml for 20 min and further analyzed with the method described by the DAKO Catalyzed Signal Amplification System kit (22). An additional slide was cut for each tumor specimen and stained in the absence of both anti-MSH2 and anti-MLH1 as a negative control.

Statistical Analysis. Fischer's exact test was used to determine the significance of the differences in the frequency of MIN occurring in different tumor stages and grades. Differences of $P < 0.05$ were considered significant.

RESULTS

A MIN assay was performed on DNA extracted from the 28 endometrial tumors and paired normal tissue. Fig. 1 illustrates typical tumors with and without MIN. Table 1 demonstrates the results of MIN with respect to microsatellite markers we tested. Nine of 28 (32%) tumor specimens demonstrated MIN. Six of 28 (21%) tumor specimens exhibited MIN with at least three different markers.

Despite the numerous informative chromosomal markers, the incidence for LOH was low (5 of 28, or 17.8%), as shown in Table 1. The losses were of markers *D2S119*, *D5S107*, *D17S261*, *D18S34*, and *DCC*. There was no apparent correlation between LOH and MIN in our series of EC.

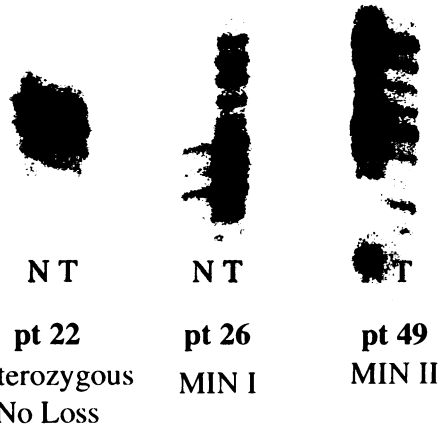

Fig. 1 Microsatellite analysis of the *DCC* locus on chromosome 18 in endometrial tumor DNA from three patients. *N*, normal DNA; *T*, tumor DNA; *MIN I*, MIN with significant expansion or deletion in allele size; *MIN II*, MIN with minor alteration in allele size.

Upon review of patients' medical history, no obvious history of familial colon or EC was noted in our patient population. Of the 28 tumors, 27 (96.4%) were endometroid adenocarcinoma and 1 (3.4%) was papillary serous adenocarcinoma. Table 1 also summarizes the patients' clinical features. In this series, MIN was observed only in stage I tumors. However, because of the small number of advanced stage patients, no significant correlation between MIN and tumor stage ($P = 0.136$) was demonstrated (Table 2). There was no correlation between MIN and tumor grade ($P = 1.00$). It is interesting that there appears to be a correlation between the presence of LOH and a more advanced tumor stage ($P = 0.050$; Table 2).

Immunohistochemical analysis was performed on all 28 tumors, including 9 MIN-positive tumors, to screen for mutations in the *MSH2* and *MLH1* genes. Positive nuclear staining with established MSH2 and MLH1 antibodies suggests that wild-type MSH2 and MLH1 proteins are expressed, whereas an absence of nuclear staining suggests a lack of protein expression or expression of mutant proteins with reduced antibody affinity. Recently, Thibodeau *et al.* (32) showed that in sporadic colon cancer, an absence of immunostaining for *MSH2* and *MLH1* genes correlates with the presence of mutations. All 28 tumors, including the 9 MIN-positive tumors, exhibited strong nuclear staining with both MSH2 and MLH1 antibodies (data not shown).

However, because minor genetic alterations that affect protein function without disrupting antibody recognition would not be detected with the immunostaining technique, we also performed DNA sequencing. All 16 and 19 exons of the *MSH2* and *MLH1* genes, respectively, were sequenced from the nine MIN-positive tumor specimens. Normal tissue and tumor specimens without MIN were used as control materials. In our study, there were no alterations in the coding regions of the *MSH2* and

Table 1 Summary of 28 sporadic endometrial tumors with respect to clinical stage, grade, and types of MIN and LOH

Patient	Histopathological type[a]	Stage	Grade	No. of informative markers	No. of markers with MIN I[b]	No. of markers with MIN II[b]	No. of markers with LOH
22	PSACa	4	3	3	0	0	2
23	EACa	1	3	5	0	3	0
24	EACa	1	4	5	0	1	0
25	EACa	1	3	4	0	0	0
26	EACa	1	2	8	0	8	0
27	EACa	4	3	4	0	0	0
28	EACa	3	4	6	0	0	1
29	EACa	1	2	6	0	0	0
30	EACa	1	4	7	0	0	2
32	EACa	1	2	5	0	0	0
33	EACa	1	1	6	0	0	0
34	EACa	3	2	6	0	0	0
35	EACa	1	2	3	0	0	0
36	EACa	4	2	5	0	0	0
37	EACa	1	2	8	1	3	0
38	EACa	2	2	5	0	0	1
39	EACa	1	2	8	0	0	0
40	EACa	1	2	8	4	2	0
42	EACa	1	2	8	5	3	0
43	EACa	1	2	6	0	0	0
44	EACa	1	1	7	0	1	0
45	EACa	1	3	6	0	0	1
46	EACa	1	2	7	0	0	0
48	EACa	1	2	6	0	1	0
49	EACa	1	2	8	3	4	0
50	EACa	1	2	6	0	0	0
51	EACa	1	2	6	0	0	0
52	EACa	1	2	6	0	0	0

[a] PSACa, papillary serous adenocarcinoma; EACa, endometroid adenocarcinoma.
[b] *MIN I*, MIN with significant expansion or deletion in allele size (5); *MIN II*, MIN with minor alteration in allele size (5).

Table 2 A comparison of the incidence of patients with MIN and LOH with respect to sporadic endometrial tumor stage

	Stage I	Stage II–IV	*P*
MIN	9/22	0/6	0.136
LOH	2/22	3/6	0.0504

MLH1 genes. However, the previously reported polymorphism in intron 14 of the MLH1 was detected at −19 bp from the start of exon 15. Five and 7 of 12 chromosomes were observed to have a G and an A, respectively, at this position. In summary, the absence of MSH2 and MLH1 mutations correlated with the positive immunostaining results.

DISCUSSION

MIN is attributed to errors in DNA repair during replication (23). Such errors lead to somatic changes in the form of loss or gain in the number of microsatellite repeat units (di, tri, or tetra nucleotides) at multiple loci throughout the genome. MIN is thought to result in neoplasia when the accumulation of such genetic alterations in the course of cellular proliferation eventually leads to mutations in important genes (24–27). The occurrence of MIN in tumor cells may be indicative of mutations in postreplication DNA mismatch repair genes.

In our series of sporadic EC, the prevalence of MIN is between 21 and 32%, depending on how MIN is defined. The broad range results from the ambiguous definition of MIN in the literature (28). There were three patients who exhibited MIN in only one marker. Whether these three tumor specimens truly demonstrate the replication error-prone phenotype is unknown. The remaining six tumors exhibited MIN in at least three markers. These six tumor specimens very likely have a replication error-prone phenotype. This is consistent with previous reports of a prevalence of MIN in ECs of 17–23% (6–8).

In reviewing the stage and histopathological diagnosis of all nine tumors that exhibited MIN, we noted that all nine tumors exhibiting MIN were stage I. This finding is consistent with the report of Risinger *et al.* (8), in which six of six stage I sporadic endometrial tumors exhibited MIN. In our series, there was no apparent correlation between the type of MIN and the grade of tumor, which is consistent with previous published reports (7, 8). It is interesting that, as summarized in Table 2, we found widespread MIN only in early-stage tumors, whereas LOH was noted to be more prevalent in the advanced-stage tumors. The significance of this observation is unclear. However, our data suggest that early-stage tumors may have a different underlying pathogenesis than advanced-stage tumors.

It has been hypothesized that MIN at multiple loci is a result of mutations in genes that are responsible for DNA mismatch repair and that defective mismatch repair genes give rise to a mutator phenotype that may ultimately initiate tumorgenesis. Germline mutations in *MSH2* and *MLH1* genes are thought to account for the majority of HNPCC kindreds. Evidence to support the role of mutations in DNA mismatch repair genes in the pathogenesis of EC was suggested in a report

describing a mutation in the *MSH2* gene in an EC cell line with MIN (29).

Because EC is the most common extracolonic malignancy associated with Lynch Syndrome II, we hypothesized that a similar molecular mechanism that underlies the pathogenesis HNPCC is likewise applicable to sporadic endometrial tumors with MIN. Recently, four DNA mismatch repair genes (*MSH2*, *MLH1*, *PMS1*, and *PMS2*) were analyzed for mutations in MIN-positive sporadic endometrial tumors (30). Mutations were detected only for the MSH2 protein and at a low frequency (2 of 12). This result was confirmed by direct sequencing of the *MSH2* gene in the MIN-positive tumor specimens. Furthermore, Katabuchi *et al.* (30) reported that the two mutations in the *MSH2* gene correlated with absence of nuclear immunostaining for the MSH2 protein in two tumor specimens. However, they did not report MSH2 immunostaining results for the tumors without MIN, nor did they analyze the *MLH1* gene by immunohistochemical staining.

In our study, mutational analysis of the *MSH2* and *MLH1* genes was performed using both immunostaining and DNA sequencing techniques. All 28 tumors, including the 9 MIN-positive tumors, exhibited positive nuclear staining, suggesting that wild-type proteins were expressed; the lack of *MSH2* and *MLH1* gene mutations was confirmed by our DNA sequencing data. The absence of MLH1 mutations is in agreement with Katabuchi *et al.* (30), but we failed to detect the low rate of MSH2 mutations that they reported. This was not due to normal tissue DNA contaminating the tumor tissue DNA in our study, because the immunostaining results also showed that all nine MIN-positive tumor specimens expressed normal proteins. We conclude that there is a minimal role for *MSH2* and *MLH1* gene mutations in MIN-positive sporadic endometrial tumors. Our results do not preclude the possibility that deleterious mutations are present in other DNA mismatch repair genes. Three other genes, *PMS1*, *PMS2*, and *GTBP*, have been reported to be involved in the DNA mismatch repair mechanism (31). However, mutations in *PMS1* and *PMS2* were not observed by Katabuchi *et al.* (30). This suggests that mutations in the known DNA mismatch repair genes do not play a major role in the pathogenesis of sporadic endometrial tumors. Thus, the instability of microsatellite DNA sequences is a reflection of a mutator phenotype and likely is caused by alterations in other genes involved in DNA synthesis and repair.

In conclusion, 9 of 28 sporadic ECs demonstrated MIN in at least 1 of 8 microsatellite loci examined. Six of nine MIN-positive tumors demonstrated MIN in at least three of eight loci. However, MSH2 and MLH1 mutations were not detected in any of the nine MIN-positive tumors. These results suggest that the two genes primarily responsible for HNPCC are not involved in sporadic EC with MIN.

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