hMLH1 Promoter Hypermethylation Is an Early Event in Human Endometrial Tumorigenesis

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It has recently been suggested that silencing of the *bMLH1* gene by promoter hypermethylation is the mechanism underlying the presence of the microsatellite instability (MSI) phenotype in sporadic colon and endometrial carcinomas. To determine whether *bMLH1* promoter hypermethylation is a relatively early event in endometrial tumorigenesis we evaluated endometrial hyperplasia (EH) characterized as simple, complex, and atypical (the direct precursor of endometrial carcinoma) for *bMLH1* aberrant methylation. In addition, we studied the *bMLH1*, *bMSH2*, *bMSH3*, and *bMSH6* promoter methylation and MSI status of those endometrial carcinomas with synchronous hyperplasias and those without them. We found that 11 of 12 (91%) cases of endometrial carcinoma (EC) displaying MSI had *bMLH1* promoter hypermethylation, whereas aberrant methylation of any of the other mismatch repair genes was not observed. All 15 cases of EC without MSI were unmethylated at *bMLH1*. Abnormal methylation of *bMLH1* was also present in 8 of 116 (7%) cases of EH and was restricted primarily to the atypical endometrial hyperplasia (AEH) type with coexisting endometrial carcinoma. In this set, half of EH methylated at *bMLH1* displayed MSI, whereas none of the unmethylated EH had MSI. Our data suggest that hypermethylation of *bMLH1* can be an early event in the pathogenesis of EC, preceding the development of an apparent MSI phenotype in a subset of cases. (*Am J Pathol* 1999, 155:1767–1772)

Endometrial carcinoma is the most common gynecological malignancy and is the fourth most common cancer of women in the United States.¹ Uterine endometrioid carcinoma (UEC) is the most common histological type. UEC often precedes or coexists with endometrial hyperplasias, which have been proposed as possible precursor

lesions.^{2,3} Atypical endometrial hyperplasia (AEH) is most associated with progression to carcinoma, and women with AEH without concurrent invasive carcinoma have a 30% risk of developing UEC.³ UEC and AEH share cytological atypia and a monoclonal pattern,^{4,5} but UEC are distinguished by the presence of invasion.³ A spectrum of hyperplasia without atypia, including simple and complex hyperplasia, also exists. These lesions show a low rate of progression to UEC³ and a putative polyclonal pattern.^{4,5}

Very little is known about the genetic alterations underlying the biology of the precursor lesions of UEC. Only mutations in the oncogene K-ras and the tumor suppressor gene PTEN have been described in AEH.^{6,7} PTEN mutations may even precede the appearance of an evident atypia in the endometrial hyperplasia.⁸ However, a subset of AEH displays the microsatellite instability (MSI) phenotype.^{5,7,9,10} MSI was first detected in tumors from patients with hereditary non-polyposis colorectal carcinoma (HNPCC). In this setting, the cause of MSI has been attributed to germline defects in DNA mismatch repair (MMR) genes, mainly involving *hMLH1* and *hMSH2*.^{11–19} Other MMR genes identified include *hPMS1*, *hPMS2*, *hMSH3*, and *hMSH6*.^{20–21} Endometrial carcinoma (EC) is the most common noncolorectal carcinoma occurring in women with HNPCC.²² MSI has also been observed in approximately 20% of sporadic EC.^{9,23–31} It was expected that UECs with MSI+ would have mutations in one of the known DNA MMR genes. However, *hMSH2* or *hMLH1* mutations have been found in less than 10% of UECs with MSI+.^{27–30}

Several studies have described a different pathway for inactivation of *hMLH1* in colorectal tumors with MSI associated with silencing of the gene by promoter hypermethylation.^{32–35} These studies demonstrate that *hMLH1* promoter hypermethylation is associated with the loss of

Supported in part by grant from Asociacion Espanola contra el Cancer and SAF96–0363, Madrid, Spain and Telmarato-Fundacio Pi i Sunyer (29/95), Barcelona, Spain. M. E. is a recipient of a Spanish Ministerio de Educacion y Cultura Award. J. G. H. is a Valvano Foundation Scholar. S. B. B. and J. G. H. receive research funding and are entitled to sales royalties from Oncor, Inc., which is developing products related to research described in this paper. The terms of this arrangement have been reviewed and approved by The Johns Hopkins University in accordance with its conflict of interest policies.

Accepted for publication July 7, 1999.

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hMLH1 expression and the MSI phenotype in colorectal cancer cell lines and sporadic colorectal carcinomas. Recent data also strongly suggest that the epigenetic silencing of *hMLH1* is associated with MSI in gastric cancer.^{36,37} In addition, the demethylation of the *hMLH1* gene in cell lines, including one endometrial carcinoma cell line, using the agent 5-azacytidine results in reactivation of *hMLH1* expression and restoration of the MMR activity.³³ We have also recently described the strong association between *hMLH1* promoter hypermethylation and the presence of a MSI phenotype in sporadic cases of UEC.³⁸ This finding has also been corroborated lately in two independent studies.^{39,40} Methylation of normally unmethylated CpG islands in the promoter regions of tumor suppressor genes including *p16*, *p15*, *VHL*, and E-cadherin correlates with loss of transcription and suggests an alternative mechanism for tumor suppressor gene inactivation.⁴¹

All of the above findings led us to investigate promoter methylation status in relation to the MSI phenotype in a large series of endometrial hyperplasias and carcinomas. Our results suggest that *hMLH1* promoter hypermethylation is an early event in endometrial tumorigenesis, being present in AEH, and preceding in some cases a detectable MSI phenotype.

Materials and Methods

Tissue Samples and DNA Isolation

Twenty-seven endometrial carcinomas and 116 endometrial hyperplasias were obtained from the Departments of Pathology of Hospital de la Santa Creu i Sant Pau (Barcelona, Spain) and Brigham and Women's Hospital (Boston, MA). All tumors and hyperplasias were examined and classified according to the criteria of the World Health Organization. Concerning our series of hyperplasias, 62 cases of simple endometrial hyperplasia without atypia, 33 cases of complex endometrial hyperplasia without atypia, and 21 cases of complex hyperplasia with atypia were included in the study. No cases of simple endometrial hyperplasia with atypia were found in this set of samples. DNA was isolated from tumors and hyperplasias and matching normal DNA by standard methods from fresh frozen biopsies, paraffin-embedded material, and peripheral blood. Microdissection was performed on some sections to separate neoplastic and nonneoplastic tissues.

Microsatellite Analysis

For MSI analysis five dinucleotide microsatellite DNA sequences (D5S107, D10S197, D12S79, D12S95, and D18S58) on chromosomes 5, 10, 12, and 18 were amplified by the PCR using MapPairs (Research Genetics Inc.) according to the same conditions previously described.³¹ Reconfirmation of MSI in endometrial cancers and determination of MSI in hyperplasias were performed using primer BAT-26. BAT-26 is a repeat of 26 deoxyadenosines localized within intron 5 of the *MSH2* gene.

BAT-26 was amplified by polymerase chain reaction (PCR) and analyzed by single-strand conformation polymorphism (SSCP) analysis as described.⁴² DNA bands were visualized by silver staining method.

Methylation-Specific PCR (MSP)

DNA methylation patterns in the CpG islands of *hMLH1*, *hMSH2*, *hMSH6*, and *hMSH3* genes were determined by MSP.⁴³ MSP distinguishes unmethylated from methylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated, but not methylated, cytosines to uracil, and subsequent PCR using primers designed for either methylated or unmethylated DNA.⁴³ Primer sequences of *hMLH1* and *MSH2* for the MSP analysis have been previously described.^{33,38} Primer sequences of *hMSH6* for the unmethylated reaction were 5'-TTT GGG TTT TTT TGG TGG AGT GT-3' (sense) and 5'-CTT AAA AAA AAA ACT ATA CAA AAT ACT CTA TCA CA-3' (antisense) and for the methylated reaction 5'-TTT TTT CGG CGG AGC GC-3' (sense) and 5'-AAA AAA AAA CTA TAC AAA ATA CTC TAT CGC-3' (antisense). Primer sequences of *hMSH3* for the unmethylated reaction were 5'-GGT TTG TGT TTT TTG TTA GGT TTT GTT-3' (sense) and 5'-CTA AAA ACA ACA AAA CCA CCC AAC A-3' (antisense) and for the methylated reaction 5'-CGT TTT TCG TTA GGT TTT GTC GTC-3' (sense) and 5'-AAC GAA ACC GCC CGA CG-3' (antisense). SW48 DNA, which has previously been characterized as methylated at the *hMLH1* locus,^{32,33} was used as a positive control for methylated alleles of *hMLH1*. Because there are no previous reports for cell lines with hypermethylation of *hMSH2*, *hMSH6*, and *hMSH3*, placental DNA treated *in vitro* with Sss I methyltransferase was used as a positive control for methylated alleles of the three genes. DNA from normal lymphocytes was used as negative control for methylated genes. Ten microliters of each PCR reaction were loaded directly onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Restriction Enzyme Analysis after Bisulfite Modification

To check for a specific cytosine methylation within the *hMLH1* promoter region studied, we digested with the restriction enzyme BstU I following bisulfite modification as described.^{33,44} The BstU I recognition site, CGCG, will remain CGCG if both C's are methylated after bisulfite treatment and amplification, but will become TGTG if they are unmethylated. The colorectal carcinoma cell lines RKO and SW480 were used as positive and negative controls for *hMLH1* promoter hypermethylation.³³

Screening of Allelic Loss in *hMLH1* Gene

To determine whether the locus containing the *hMLH1* gene was deleted in tumors, LOH was assessed. Five

different (CA)_n repeats (Research Genetics, Inc., Huntsville, AL) localized on chromosome 3 (D3S1314, D3S1286, D3S1283, D3S1298, and D3S1611) were used to screen endometrial cancers for allelic loss in *hMLH1* gene. D3S1611 is actually located within an intron of *hMLH1*.¹⁸ PCR amplification of marker loci was carried out on paired normal-tumor DNAs in 25- μ l reaction volume containing 100 ng template DNA, 10 pmol each forward and reverse primers, 0.2 mmol/L each dNTP, 1.5 units of *Taq* DNA polymerase (Ecotag, Ecogen, Langhorne, PA) and 1.5 to 3 mmol/L MgCl₂, dependent on primer. Thirty PCR cycles were used, with each cycle consisting of 30 seconds at 95 °C, 30 seconds at 58 °C, and 1 minute at 72 °C. The denatured samples were loaded onto a 8% denaturing polyacrylamide gels containing 8.3 Mol/L urea, and run at 1800 V for 4.5 hours at room temperature. PCR products were capillary-blotted onto Hybond-N membrane (Amersham, Buckinghamshire, UK) and DNA was fixed by alkali treatment. The membranes were analyzed using the ECL Gene Detection System (ECL Amersham) according to the specifications of the Amersham kit. Allelic loss of heterozygosity (LOH) was defined as a visible reduction of 50% or more in the band intensity of the tumor sample when compared to the corresponding normal band.

Results

We investigated 27 UECs previously characterized for the MSI phenotype^{10,31} for promoter hypermethylation of *hMLH1* using MSP.^{33,38} Methylation of the promoter region studied for *hMLH1* correlates with the loss of *hMLH1* expression in primary sporadic colorectal cancer using MSP³³ and another PCR-based approach.^{32,34,35} Hypermethylation of the *hMLH1* promoter has also been correlated with *hMLH1* gene silencing in primary gastric carcinomas with MSI.^{36,37}

We found that 11 of 27 (40%) UECs had promoter hypermethylation of the *hMLH1* gene (Figure 1, Table 1). Moreover, when the EC samples were decoded for their MSI phenotype, *hMLH1* was hypermethylated in 11 of 12 (91%) MSI+ tumors, whereas none of 15 MSI- tumors demonstrated *hMLH1* promoter hypermethylation (Fischer's exact test, $P < 0.0001$; Table 1). These findings, added to our early independent report,³⁸ corroborate the strong linkage between this epigenetic inactivation of *hMLH1* and the MSI phenotype in EC. Thus, only one sample known to have the MSI phenotype did not show aberrant hypermethylation of *hMLH1*. No *hMLH1* promoter hypermethylation was found in any of the normal endometrium or adjacent myometrium samples studied (Figure 1). The 12 MSI+ tumors were also studied for promoter hypermethylation in three additional MMR genes: *hMSH2*, *hMSH3*, and *hMSH6*. None of these primary tumors had promoter hypermethylation of any of the mentioned MMR genes (Figure 1), confirming that *hMLH1* is the main target of inactivation in MSI+ EC.

The study of LOH at loci adjoining the *hMLH1* gene located in 3p21.3 in the UEC with *hMLH1* promoter hy-

Figure 1. Methylation-specific PCR (MSP) of MMR genes in endometrial carcinomas. PBR322/Msp digest are shown at left as molecular weight markers. The presence of a visible PCR product in those lanes marked "U" indicates the presence of unmethylated genes; the presence of product in those lanes marked "M" indicates the presence of methylated genes. SW48 DNA is used as positive control for *hMLH1* promoter hypermethylation, *in vitro* methylated DNA (IVD) as positive control for *hMSH2*, *hMSH3*, and *hMSH6* promoter hypermethylation and normal lymphocytes as negative control for methylation. Water controls for PCR reactions are also shown. **A:** MSP of *hMLH1* in endometrial carcinomas (EC) and matched normal endometrium (NE). **B:** MSP of *hMSH2* (B), *hMSH3* (C), and *hMSH6* (D) in primary endometrial carcinomas (EC).

permethylation demonstrated retention of heterozygosity in the 11 cases studied. This finding suggests that the inactivation of *hMLH1* in these tumors is due to biallelic epigenetic inactivation by the promoter hypermethylation described, a phenomena reported in half of primary gastric carcinomas with MSI associated with *hMLH1* promoter hypermethylation.³⁶ This conclusion is also supported by the demonstration of *hMLH1* silencing by promoter hypermethylation affecting both maternal and paternal alleles in a study of MSI+ colorectal carcinoma cell lines.³⁵

Putative precursor lesions of UEC include different degrees of EH,^{2,3} and MSI has been found in a few cases of AEH.^{5,7,9,10} We studied more than 100 EH for the presence of *hMLH1* promoter hypermethylation, and found that 8 of 116 (7%) EH had promoter hypermethylation of the *hMLH1* gene. When the EH were subdivided in the progressive pathologies of simple endometrial hyperplasia (SEH), complex endometrial hyperplasia (CEH), and AEH, *hMLH1* abnormal methylation was absent in all of the cases of simple hyperplasia ($n = 62$), but present in 1 of 33 (3%) cases of CEH and 7 of 21 (33%)

Table 1. Summary of *hMLH1* Promoter Hypermethylation in Endometrial Tumorigenesis According to the Pathology and the Microsatellite Status

<i>hMLH1</i> Promoter	EC		EH			EH			
						MSI+		MSI–	
	MSI+	MSI–	SEH	CEH	AEH	AEC	NAEC	AEC	NAEC
Hypermethylated	11	0	0	1	7	4	0	2	2
Unmethylated	1	15	62	32	14	0	0	2	30

EC, endometrial carcinoma; EH, endometrial hyperplasia; MSI, microsatellite instability-positive (+) and -negative (–); SEH, simple endometrial hyperplasia; CEH, complex endometrial hyperplasia; AEH, atypical endometrial hyperplasia; AEC, endometrial hyperplasia associated with an endometrial carcinoma; NAEC, endometrial hyperplasia not associated with an endometrial carcinoma.

cases of AEH (Table 1). Of these 8 EH hypermethylated at *hMLH1*, 6 (75%) were associated with an UEC in the same patient. *hMLH1* promoter hypermethylation was observed in the 4 MSI+ EH cases and all EH unmethylated at *hMLH1* were MSI– ($n = 32$) showing a strong correlation between both events (Fischer's exact test, $P = 0.0008$; Table 1).

All four MSI+ EH cases with *hMLH1* methylation were AEH and coexisted with a MSI+ UEC in the same patient (Table 1), which also demonstrated *hMLH1* promoter hypermethylation (Figure 2). Interestingly, we also found aberrant *hMLH1* hypermethylation in 4 EH (3 AEH and 1 CEH) not showing an apparent MSI phenotype. In this last set, the CEH and one AEH were diagnosed in the absence of any coexistent UEC (Table 1). Of the two remaining AEH, one coexisted with a MSI+ UEC also with *hMLH1* hypermethylation and the other with a MSI– UEC that did not show *hMLH1* methylation. In this last case, another AEH studied in the same patient was unmethylated at *hMLH1* suggesting that the UEC may arise from this AEH. In two other cases of EH unmethylated at the *hMLH1* locus, coexistent UEC was available, and these invasive tumors also were unmethylated at *hMLH1* and MSI–.

Discussion

In our previous study, we have shown that *hMLH1* promoter methylation is tightly associated with the presence of the MSI phenotype in endometrial tumors.³⁸ Previously, lack of *hMLH1* expression was shown to correlate with cytosine methylation of the *hMLH1* promoter in a small series of colorectal tumors and cell lines with the MSI phenotype.³² More extensive studies in sporadic colorectal and gastric carcinomas have corroborated the strong correlation between *hMLH1* promoter hypermethylation, loss of hMLH1 protein, and the presence of MSI.^{33,34,36,37} Now, our findings expand our initial observations of the involvement of aberrant methylation of *hMLH1* in UEC with MSI by excluding epigenetic alteration of most important mismatch repair genes, and we show that *hMLH1* promoter hypermethylation is an early event in endometrial tumorigenesis, as a feature of a subset of precursor lesions.

The study of this new series of UEC shows again that the vast majority of endometrial tumors with MSI have *hMLH1* promoter hypermethylation. Only 1 of 12 (8%) UEC with MSI was unmethylated at the *hMLH1* locus. In

the unmethylated case, a somatic mutation in any of the MMR genes seems the most likely mechanism to account for the MSI phenotype. This low percentage is not surprising and, in fact, only three somatic mutations in

Figure 2. *hMLH1* promoter hypermethylation analysis in endometrial tumorigenesis. **A:** MSP of *hMLH1* in matched endometrial carcinomas (EC) and endometrial hyperplasias (EH) from two patients. The presence of a visible PCR product in those lanes marked "U" indicates the presence of unmethylated genes; the presence of product in those lanes marked "M" indicates the presence of methylated genes. **B:** Endometrioid carcinoma (right) coexisting with atypical endometrial hyperplasia (left), both methylated at *hMLH1* and MSI+. **C:** Restriction enzyme analysis following bisulfite modification in matched endometrial carcinomas (EC) and endometrial hyperplasias (EH). The colorectal cancer cell lines RKO and SW480 are used as positive methylated and negative unmethylated control for *hMLH1*, respectively. The colorectal carcinoma cell line HT-29 shows a partial methylation pattern as previously described.³³

hMSH2 and one mutation in *hMLH1* of 61 MSI+ sporadic endometrial carcinomas (less than 7%) have been reported to date.²⁷⁻³⁰ In our cases, we presume that hypermethylation affects both alleles of *hMLH1* due to the absence of LOH. The same phenomena of *hMLH1* biallelic inactivation by promoter hypermethylation has been observed in colorectal carcinoma cell lines with MSI³⁵ and in a subset of sporadic gastric carcinomas with MSI.³⁶ In all cases studied, *hMLH1* methylation has not been observed in normal endometrium. In addition, in endometrial carcinomas, *hMLH1* promoter hypermethylation is not associated with the abnormal methylation of other tumor suppressor and DNA repair genes such as *p16*, *GSTP1*, or *MGMT*.^{38,45,46}

We have also found that *hMLH1* promoter hypermethylation is present in a subgroup of endometrial precancers. Studying a group of more than 100 endometrial hyperplasias subdivided into simple, complex, and atypical revealed that *hMLH1* aberrant methylation is restricted almost exclusively to the AEH type. AEH is the most immediate precursor of UEC³ and, of all of the precursor lesions, is the only one that shows a monoclonal pattern.^{5,6} Strikingly, the AEH methylated at *hMLH1* that show a MSI phenotype are those associated with a coexistent UEC also with MSI and *hMLH1* methylation. In some cases we have observed the presence of aberrant methylation of *hMLH1*, but the MSI phenotype is still not present or apparent. Most of these EH were without a coexisting carcinoma. This may suggest that these lesions are deficient in mismatch repair, but have not yet accumulated a large number of microsatellite alterations and do not yet have an apparent MSI+ phenotype. Data that may support this explanation are the presence of cancer cell lines defective in mismatch repair not showing MSI⁴⁷ and the presence of *hMLH1* promoter hypermethylation in gastric carcinomas showing only a low rate of MSI.³⁶ In our series, an example of progression through this pathway would be a case in which we observed *hMLH1* methylation and MSI in the UEC, but the AEH contiguous to this cancer does not show an apparent MSI phenotype, although it is methylated at *hMLH1*. Alternatively, study of a larger number of microsatellite markers may reveal a low grade of MSI in the subset of hyperplasias methylated at *hMLH1* without obvious MSI. Finally, another explanation in these few cases would be that methylation affects only one allele as, for example, in the colorectal carcinoma cell line HT-29, which is only partially methylated at *hMLH1* and is not mismatch repair-deficient.³³

For the cyclin-dependent kinase inhibitor *p16*, the silencing of the gene mediated by promoter hypermethylation seems to be an early event in the tumorigenic process.⁴⁸ Our results suggest that *hMLH1* promoter methylation is also an early event in the chain of alterations leading ultimately to the invasive carcinoma, which can precede, in some cases, the detection of an apparent MSI phenotype. However, when the endometrial carcinoma is established, *hMLH1* promoter hypermethylation and MSI are linked in the vast majority of cases.

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