MLH1 promoter methylation and gene silencing is the primary cause of microsatellite instability in sporadic endometrial cancers

Sally B. Simpkins, Tina Bocker, Elizabeth M. Swisher, David G. Mutch, Deborah J. Gersell, Albert J. Kovatich, Juan P. Palazzo, Richard Fishel and Paul J. Goodfellow*

Department of Surgery, 1Department of Obstetrics and Gynecology and 2Department of Pathology, Washington University School of Medicine, 660 South Euclid Street, St Louis, MO 63110, USA and 3Department of Microbiology and Immunology and 4Department of Pathology, Kimmel Cancer Institute, Thomas Jefferson University, 233 South 10th Street, Philadelphia, PA 19107, USA

Received November 20, 1998; Revised and Accepted January 21, 1999

Defective DNA mismatch repair in human tumors leads to genome-wide instability of microsatellite repeats and a molecular phenotype referred to as microsatellite instability (MSI). MSI has been reported in a variety of cancers and is a consistent feature of tumors from patients with hereditary non-polyposis colorectal cancer. Approximately 20% of cancers of the uterine endometrium, the fifth most common cancer of women world-wide, exhibit MSI. Although the frequency of MSI is higher in endometrial cancers than in any other common malignancy, the genetic basis of MSI in these tumors has remained elusive. We investigated the role that methylation of the MLH1 DNA mismatch repair gene plays in the genesis of MSI in a large series of sporadic endometrial cancers. The MLH1 promoter was methylated in 41 of 53 (77%) MSI-positive cancers investigated. In MSI-negative tumors on the other hand, there was evidence for limited methylation in only one of 11 tumors studied. Immunohistochemical investigation of a subset of the tumors revealed that methylation of the MLH1 promoter in MSI-positive tumors was associated with loss of MLH1 expression. Immunohistochemistry proved that two MSI-positive tumors lacking MLH1 methylation failed to express the MSH2 mismatch repair gene. Both of these cancers came from women who had family and medical histories suggestive of inherited cancer susceptibility. These observations suggest that epigenetic changes in the MLH1 locus account for MSI in most cases of sporadic endometrial cancers and provide additional evidence that the MSH2 gene may contribute substantially to inherited forms of endometrial cancer.

INTRODUCTION

Endometrial cancer is the most common gynecologic malignancy in the USA with an estimated 34,000 new cases each year (1). Although the majority of these cancers are sporadic, endometrial carcinoma is the most frequently reported extracolonic malignancy in patients with hereditary non-polyposis colorectal cancer (HNPCC) (2). Relatively little is known about the genetic events that underlie endometrial tumorigenesis. Mutations in the PTEN and TP53 tumor suppressor genes and the KRAS2 proto-oncogene are among the most frequent abnormalities reported for these cancers (3–9). Genome-wide instability of simple sequence repeats, referred to as microsatellite instability (MSI), is seen in 17–23% of endometrial cancers (10–13). MSI is associated with defective DNA mismatch repair and is a consistent feature of tumors from patients with HNPCC (14–16). Germline mutations in either the MLH1 or MSH2 mismatch repair gene account for the majority of HNPCC kindreds (17). However, mutations in mismatch repair genes in sporadic tumors with MSI are relatively infrequent. In particular, MSI-positive endometrial cancers rarely have MLH1 or MSH2 mutations (13,18–20).

There is a strong correlation between MSI and lack of detectable MLH1 and/or MSH2 protein product (21). Thibodeau et al. (21) noted that among seven MSI-positive colon cancers in which no MSH2 or MLH1 mutation could be identified, six failed to express MLH1. The absence of MLH1 expression in mutation-negative tumors suggested an alternative mechanism for DNA mismatch repair gene silencing in human cancers. Several groups have shown that methylation of the MLH1 promoter is associated with MSI in primary human cancers and

*To whom correspondence should be addressed. Tel: + 1 314 362 8106; Fax: + 1 314 362 8620; Email: goodfellowp@msnotes.wustl.edu
in tumor cell lines. Restriction digestion and bisulfite treatment of tumor DNA, coupled with PCR-based assays, to investigate the promoter regions of the MLH1 and MSH2 genes have revealed a link between the MSI-positive phenotype and MLH1 promoter methylation (22–25). In primary colon cancers, MSI and MLH1 promoter methylation correlate with a lack of MLH1 expression as assessed by immunohistochemistry (22,23). Although MLH1 promoter methylation has been noted in endometrial cancers with MSI (26), protein expression studies were not performed and the relationship between methylation, MSI and MLH1 gene expression in these cancers has not been determined. We undertook studies to assess the role that MLH1 promoter methylation plays in MSI-positive endometrial tumors. As is the case in colorectal carcinomas, MLH1 methylation coupled with loss of expression is a frequent event in these cancers. In our series of tumors unselected for genetic disease, MSH2 defects seem to be more common in women with an inherited susceptibility to endometrial cancer, as has been suggested by Millar et al. (27).

RESULTS

Methylation studies

The MLH1 promoter region was methylated in 41 of 53 (77%) MSI-positive tumors. Tumors with MSI were recognized by typing mono- and dinucleotide repeats (Fig. 1). In those 41 tumors with MLH1 methylation, HpaII failed to cleave the DNA at all four CCGG recognition sites within the MLH1 promoter region. These HpaII-digested DNAs gave rise to the expected 608 bp PCR products. Nine MSI-negative tumor specimens were unmethylated. Of the 11 MSI-negative tumor specimens studied, 10 were unmethylated. For these tumor specimens, the HpaII-digested DNAs failed to yield PCR products using the MLH1 primers. The single normal DNA investigated was unmethylated (Table 1). Representative examples of the MLH1 methylation analyses are shown in Figure 2. The correlation between MLH1 promoter methylation and the MSI-positive tumor phenotype is highly significant (P < 0.0001, Fisher’s exact test).

Table 1. Comparative analysis of tumor MSI, MLH1 promoter methylation and DNA mismatch repair gene expression in endometrial cancers

<table>
<thead>
<tr>
<th>Tumor specimen</th>
<th>MSI status</th>
<th>Immunohistochemistry</th>
<th>MLH1 promoter methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MLH1</td>
<td>MSH2</td>
</tr>
<tr>
<td>1137</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1141</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>1144</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>1145</td>
<td>+</td>
<td>– (+)(^a)</td>
<td>+</td>
</tr>
<tr>
<td>1155</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>1158</td>
<td>+</td>
<td>– (+)(^b)</td>
<td>+</td>
</tr>
<tr>
<td>1173</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>1174</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>1216</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>1220</td>
<td>+</td>
<td>– (+)</td>
<td>+</td>
</tr>
<tr>
<td>1231</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>1235</td>
<td>+</td>
<td>– (+)(^b)</td>
<td>+</td>
</tr>
<tr>
<td>1241</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>1248</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1172</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1217</td>
<td>–</td>
<td>+</td>
<td>Partial</td>
</tr>
<tr>
<td>1219</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1239</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1243</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1247</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1249</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1251</td>
<td>–</td>
<td>ND(^*)</td>
<td>ND(^*)</td>
</tr>
<tr>
<td>1254</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1256</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

+, positive; –, negative; – (+), heterogeneous staining with some cancer cells showing MLH1 expression. ND, not determined; *, not suitable for evaluation because of fixation artifacts.

\(^a\)Mixed histology with endometrioid and clear cell types.

\(^b\)Poorly differentiated carcinoma with areas of well-differentiated cancer with squamous features.
The results of the HpaII digestion and PCR assay for MLH1 promoter methylation were unclear for three MSI-positive tumors (1027, 1062, and 1150). In these three tumors there appeared to be partial methylation of the HpaII sites in the region. Tumor 1062 gave weak positive amplification for one reaction and failed to yield a product on a repeat assay using the same template. Repeated HpaII digestion gave similar ambiguous results. Tumors 1027 and 1150 both gave weakly positive amplification products in one experiment and were both negative for amplification in a repeat assay. The single MSI-negative tumor (1217) in which there was evidence of methylation yielded a weak PCR product, suggestive of methylation in a fraction of the DNA molecules. We categorized tumors 1027, 1062, 1150, and 1217 as having only partial methylation.

All tumor DNAs were successfully amplified using primers from a polymorphic region of MLH1 intron 11/exon 12 that does not contain the HpaII enzyme recognition site (Fig. 2). None of the Msp I-digested (methylation-insensitive isoschizomer) gave rise to promoter region amplification products.

**MLH1 and MSH2 expression**

Immunohistochemical investigations to assess MLH1 and MSH2 expression were undertaken for 24 tumors. Of these, 14 were MSI-positive and 10 were MSI-negative. All MSI-negative tumors expressed both MLH1 and MSH2 and all of the MSI-positive specimens failed to express either MLH1 or MSH2. Of the 14 MSI-positive tumors, all but two (1137 and 1248) had detectable MSH2 protein. Twelve of 14 (86%) MSI-positive tumors showed abnormal MLH1 expression. In four tumors MLH1 expression was heterogeneous, with the majority of the cancer cells lacking immunodetectable MLH1 protein (Table 1). Representative examples of expression analysis are shown in Figure 3.

**DISCUSSION**

Our DNA and gene expression studies link MLH1 promoter methylation, the absence of MLH1 protein and the MSI phenotype in endometrial cancers. The correlation between MSI, promoter methylation and lack of detectable MLH1 protein suggests that loss of MLH1 expression is an important event in endometrial tumorigenesis. We and others have previously reported that mutation in the MLH1 or MSH2 genes is infrequent in endometrial cancers with MSI (13,18–20). In a series of 25 MSI-positive endometrial cancers, our group found a single germ-line MLH1 mutation and one somatic MSH2 mutation (13). In 41 of 53 MSI-positive tumors investigated in the current study there was substantial methylation of the MLH1 promoter and in the 14 MSI-positive tumors investigated for MLH1 and MSH2 expression, there was perfect concordance between MLH1 methylation and absence of immunodetectable MLH1 gene product in the majority of tumor cells (Table 1). We conclude that methylation of the MLH1 promoter is causally associated with the MSI phenotype in primary endometrial cancers. The finding that MLH1 expression was absent in 12 of 14 MSI-positive tumors in our study is in contrast to an earlier report by Lim et al. (21) in which all nine MSI-positive tumors investigated expressed MLH1.

Methylation of the MLH1 promoter in established cell lines has been shown to result in gene silencing. In a study of MSI-positive cell lines that included the endometrial cell line AN3CA, Veigel et al. (25) demonstrated that methylation of the MLH1 promoter silenced the MLH1 locus and caused MSI. They were able to reactivate MLH1 expression by treating the cell lines with the demethylating agent 5-azacytidine (25). Herman et al. (23) performed a similar methylation/gene expression study in sporadic colon cancers and tumor cell lines. The AN3CA line was among those investigated. Again, treatment of the cell lines that did not express MLH1 with 5-azacytidine resulted in restored MLH1 expression and mismatch repair activity.

The studies in cell lines and primary colorectal cancers support our conclusion that MLH1 methylation causes MSI in primary human endometrial cancers. Based on the large numbers of tumors we studied (53 MSI-positive specimens), we believe that epigenetic gene silencing is likely to account for defective DNA mismatch repair in >75% of endometrial cancers. This estimate is based on the frequency of methylation we observed and the published reports that mutations have been identified in MLH1 or MSH2 in >20% of these cancers (13,18–20). Epigenetic changes are therefore likely to account for MSI in the majority of mutation-negative cases.

For 49 of the 53 tumor specimens we investigated, our restriction digestion and PCR amplification-based assay for MLH1 promoter methylation gave clear positive or negative results. However, in one MSI-negative tumor (1217) we saw weak and inconsistent amplification of the 608 bp product that arises when all four HpaII sites in the promoter region are unrestricted. We concluded that in specimen 1217, a fraction of the DNA was methylated and we categorized this tumor as having partial methylation (Table 1). Immunohistochemical studies, however, proved that tumor 1217 expressed MLH1. There are two possible explanations for this apparent inconsistency. If one allele was methylated in the cancer cells and the other unmethylated, the methylation assay would give a positive signal and the MLH1 gene would still be expressed. The second explanation relates to the differences in the sensitivities of the MLH1 methylation, MSI and expression assays we used. The PCR assay to detect methylation would be expected to give a positive signal even when a limited number of cancer cells had methylation of the MLH1 promoter. Immunohistochemistry, on the other hand,
is much less sensitive and isolated cells that failed to express MLH1 might go undetected. Similarly, if a small fraction of tumor cells had MSI, the relative proportion of abnormal sized bands seen in marker typing studies would be small and could go unnoticed.

Herman et al. (23) reported a similar discordancy for MLH1 promoter methylation and tumor MSI phenotype in primary colorectal cancers. They noted MLH1 methylation in a small subset of MSI-negative colorectal cancers. In the two MSI-negative, MLH1 methylation-positive tumors they investigated by immunohistochemistry they saw heterogeneous staining, with most cancer cells expressing MLH1 (23). It is possible that like colorectal cancers, some endometrial cancers without detectable MSI fail to express MLH1 in a subpopulation of the tumor cells. A similar situation might exist for the three MSI-positive tumor specimens in our series (1027, 1062 and 1150) which were categorized as having partial methylation of the MLH1 promoter. Immunohistochemical studies have not been performed with these three tumors. If heterogeneous staining for MLH1 was seen in these specimens, it would support a model for clonal differences in methylation and MLH1 expression within a tumor population. It is of note that in four MSI-positive tumors with promoter methylation (1145, 1158, 1220 and 1235) there was heterogeneous staining for MLH1. Two of these cases had
endometrial and colorectal cancer, Millar increased risk for genetic disease because they had both to endometrial cancer. In a study of 40 women who were at the other hand, may play a larger role in inherited susceptibility positive phenotype in most sporadic endometrial cancers. MSH2, link between tumor methylation status and inherited disease. The other had two previous gastrointestinal malignancies. Aous colon carcinoma and a family history of colorectal cancers. susceptibility to the development of tumors. One had a synchron-ious colon carcinoma and a family history of colorectal cancers. The other had two previous gastrointestinal malignancies. A search for MSH2 mutation in these tumors and the corresponding normal cellular DNA is underway. We are also collecting family histories for the remaining seven women in whose tumors the MLH1 promoter region was unmethylated to further assess the link between tumor methylation status and inherited disease. Our studies prove that MLH1 defects account for the MSI-positive phenotype in most sporadic endometrial cancers. MSH2, on the other hand, may play a larger role in inherited susceptibility to endometrial cancer. In a study of 40 women who were at increased risk for genetic disease because they had both endometrial and colorectal cancer, Millar et al. (27) found six germline MSH2 mutations (15%) and a single example of MLH1 mutation. Only a subset of the tumors from these patients were evaluated for MSI and as such it is difficult to speculate as to the overall role that inherited MSH2 defects play in endometrial cancers with MSI. In a study of HNPCC kindreds, Vasen et al. (28) compared the risk of endometrial cancer in patients with either MLH1 or MSH2 mutation. Although there was a marked increase in the number of endometrial cancers in the MSH2 mutation carriers compared with MLH1 mutation carriers (11 of 38 versus 9 of 59), the difference was not statistically significant. Many of the families included in the study of Vasen et al. (28) were selected because they met the Amsterdam criteria for the diagnosis of HNPCC, which does not include endometrial cancer (29). There may have been an unintentional bias against families with endometrial cancers by inclusion of very large families that present predominantly with colorectal cancers. Conversely, small families with as few as three members affected with colon cancer would be included, but small families with multiple cases of endometrial cancer and fewer than three colon cancers would be excluded. Our studies provide compelling evidence that MLH1 methylation and gene silencing account for most sporadic cases of endometrial cancer. Prospective population-based studies are required to assess the relative role that the MSH2 and MLH1 genes play in inherited susceptibility to endometrial cancer.

MATERIALS AND METHODS

Patient materials

Sixty-four endometrial cancer and one normal blood DNA specimens were studied. Tumor DNAs were prepared from portions of flash frozen cancer tissues in which the neoplastic cellularity exceeded 70%. The tumor DNAs were investigated for MSI as described previously by our group (13,30) and categorized as MSI-positive if novel sized alleles were seen with two or more of seven markers studied. Fifty-three MSI-positive and 11 MSI-negative tumors from our consecutive series of >250 endometrial cancers were included in these MLH1 methylation analyses.

Sections (5 μm) from 14 MSI-positive and 10 MSI-negative formalin-fixed, paraffin-embedded pathology specimens were prepared for immunohistochemical analyses. All patient materials were obtained with appropriate HSC/IRB approval (Washington University School of Medicine HSC Protocol 93-0828).

Promoter methylation assays

An aliquot of 160 ng of each DNA was digested overnight with a 60-fold excess of HpaII enzyme (New England Biolabs, Beverly, MA). A subset of the specimens was digested with the methylation-insensitive isoschizomer, MspI (Boehringer Mannheim, Indianapolis, IN). Approximately 20 ng of DNA was then amplified using: (i) primers from the MLH1 promoter region that flank the four HpaII sites upstream of the ATG initiator codon [forward primer 5′-CGCTCGTAGTTATTCGTC-3′ (22) and reverse primer 5′-ACCTCAAGTCCTGCTACGTTC-3′ (adapted from ref. 22)]; (ii) primers from intron 11/exon 12 of MLH1 (13) that give rise to an amplicon containing no HpaII sites. The 10 μl PCR reactions consisted of 0.4 μM each primer, 0.125 mM each dNTP, 0.4 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) and 1.5 mM MgCl2 buffer containing 10 mM Tris–HCl (pH 8.3) and 50 mM KCl. Thirty rounds of PCR were performed using the following conditions: 94°C, 60 s; 55°C, 60 s; 72°C, 60 s. PCR products were size sorted by electrophoresis on 10% acrylamide non-denaturing gels and visualized by staining with ethidium bromide. All restriction digestions and PCR reactions were repeated to verify results.

Immunohistochemistry to assess MLH1 and MSH2 expression

Paraffin sections (5 μm) were mounted on Neoprene-coated slides (Aldrich, Milwaukie, WI). Following routine deparaffinization and rehydration, antigen retrieval was accomplished by microwaving slides in 200 ml ChemMate HIER buffer of pH 5.5–7.5 (Ventana Medical Systems, Tucson, AZ) for 5 min at 800 W. Microwaving was repeated after replenishing with 50 ml of water and samples were then cooled at room temperature for 20 min.

Staining was performed using a Techmate 1000 system (Ventana Medical Systems). Slides were washed in water and placed in ChemMate Buffer 1 (phosphate-buffered saline con-
taining carrier protein and sodium azide) and incubated for 20 min in normal serum block. Slides were incubated overnight in primary antibody hMLH1 (monoclonal Ab, clone G168-728; PharMingen) diluted 1:200 or hMSH2 (polyclonal Ab, ‘AB-3’; serum purified by protein A column purification; Oncogene) diluted 1:2000 followed by a 30 min incubation in biotinylated secondary antibody and 30 min incubation in ABC complex with each incubation followed by a phosphate-buffered saline wash. Finally, DAB/peroxide was applied for 3 × 7 min and the slides were counterstained with hematoxylin. The slides were assessed independently by four investigators (T.B., R.F., J.P. and A.K.) for MLH1 and MSH2 staining.

**ACKNOWLEDGEMENTS**

We thank Drs T. Herzog, J. Rader and A. Elbendary for access to tumor specimens, and Dr A. Whelan for her important help in obtaining family histories for some of the patients for whom tumors were studied. We are grateful to Ms T. Lane for expert assistance in preparing this manuscript. This work was supported by NIH grant CA71754.

**REFERENCES**