Methylation of hMLH1 in a Population-based Series of Endometrial Carcinomas

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ABSTRACT
Microsatellite instability (MSI) is a characteristic feature of hereditary nonpolyposis colorectal cancer and is also observed in sporadic colorectal and endometrial cancers. Alterations in the mismatch repair genes hMLH1 and hMSH2 are important for the development of MSI. It has recently been demonstrated that hypermethylation of the hMLH1 promoter region is associated with MSI and appears to be a common mechanism for gene inactivation. For endometrial carcinoma, however, previous studies have been relatively small and have not been population based. We therefore wanted to assess the frequency and prognostic significance of hypermethylation of the hMLH1 and hMSH2 genes in conjunction with hMLH1 protein expression in a prospective and population-based series of endometrial carcinoma patients with known MSI status and complete follow-up. A total of 138 patients were studied, and methylation of hMLH1 was found in 23% of tumors with conclusive results, whereas methylation of hMSH2 was seen in only 1% of tumors. Methylation of hMLH1 was significantly correlated with MSI (P < 0.001). Loss of nuclear staining of hMLH1 protein was seen in 14% of the cases and was significantly correlated with hMLH1 methylation and MSI (P < 0.001). Normal expression of hMLH1 was seen in all of the unmethylated tumors (100%). Of the 14 MSI-positive tumors that were also methylated, all but 1 (93%) showed a loss of nuclear expression of hMLH1. None of the tumors with loss of hMLH1 expression or hMLH1 methylation were aneuploid (P for both ≤ 0.05), and loss of hMLH1 expression and hMLH1 methylation was significantly correlated with lack of p53 overexpression (P for both ≤ 0.05). Nuclear hMLH1 staining and hMLH1 methylation did not significantly influence survival. In conclusion, hMLH1 methylation was common and was significantly correlated with loss of hMLH1 protein expression, MSI, diploid tumors, and lack of p53 overexpression. In contrast, hMSH2 methylation was infrequent in this prospective and population-based series of endometrial carcinomas.

INTRODUCTION
Genetic instability of microsatellite repeat sequences, i.e., MSI, is commonly seen in tumors associated with the hereditary nonpolyposis colorectal cancer syndrome (1). Endometrial cancer is one of the most common types of extracolonic tumors associated with this disease (2). In patients with hereditary nonpolyposis colorectal cancer, germ-line mutations have been identified in the mismatch repair genes hMLH1 and hMSH2 (3).

In sporadic colorectal, endometrial, breast, and gastric cancers, MSI is present in a substantial proportion of cases (4–6). The frequency of MSI reported to date in sporadic endometrial cancers varies between 9% and 43% (7–10); however, mutations in the DNA mismatch repair genes have been reported to be rare (11–13).

Promoter region methylation has recently been demonstrated to be an important mechanism of gene inactivation in cancer (14–17). MSI has been associated with hypermethylation of the hMLH1 promoter region in sporadic colorectal cancer (18–21) and gastric carcinoma (6, 22). Recent studies on sporadic endometrial carcinoma have found methylation of the promoter region of hMLH1 in 71–92% of the tumors with MSI, whereas methylation of the promoter region of hMSH2 seems to be rare (13, 23, 24). However, these studies were relatively small, and most patients were selected for inclusion according to MSI status. Therefore, prospective and population-based studies have been suggested to further elucidate the role of hMLH1 methylation in endometrial cancer and its clinical significance (23, 24). This kind of study is important to reduce selection bias and problems with external validity. The specific aims of this study were to assess the pattern and prognostic impact of hMLH1 and hMSH2 promoter region methylation and hMLH1 protein expression in a large, prospective, population-based study.
series of endometrial carcinoma patients with long and complete follow-up.

MATERIALS AND METHODS

Patient Samples. All 316 patients diagnosed with endometrial carcinoma in Hordaland County, Norway, in the 10-year period from 1981 to 1990 have been studied. Hordaland County has approximately 400,000 inhabitants, representing about 10% of the total Norwegian population, and has a similar age-adjusted incidence rate of endometrial cancer (25). The distribution of patient characteristics and the treatment protocol for this period have been reported previously (26–28). The patients were retrospectively staged according to the 1988 FIGO criteria (6), and all microscopic slides were reclassified and graded by one pathologist (L. A. A.) according to the 1994 WHO criteria (28). Twelve cases were excluded due to changed diagnosis at reclassification. In five cases, the diagnosis was based on cytological examination only, with no histological specimens available. Paraffin blocks were available for further investigations in 286 of the remaining 299 (96%) patients. Additional tumor tissue from 138 of these patients was prospectively collected and frozen during the primary operation from the site considered macroscopically to be representative for the tumor, and histological examination confirmed that this tissue contained at least 50% tumor cells (27, 29).

To investigate the potential selection bias in the patient population from whom frozen tumor tissue was collected prospectively, patient age, FIGO stage, histological type and grade, treatment, and survival for these patients \((n = 138)\) were compared with those of the rest of the patients from Hordaland County treated for endometrial carcinoma in the same period, but from whom fresh tumor tissue was not available \((n = 161)\). Curative surgical treatment was more often possible in the group with fresh tumor tissue available \((92\%)\) than for the rest of the population \((77\%; P < 0.001, \chi^2\) test). This is in accordance with the current practice in the area; women ineligible for curative treatment due to either high age or serious intercurrent or extensive disease are less often referred to the University Hospital where the fresh tissue was prospectively collected during primary surgery. In the group of patients with fresh tumor tissue available, there was a somewhat lower frequency of MSI of 33% (36 of 110 tumors with results available for all five markers) compared with the whole population, which had a MSI frequency of 41% (92 of 225 tumors with results available for all five markers). There were no other significant differences in the patient characteristics (27).

Immunohistochemistry. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded specimens according to the standard avidin-biotin method (DAKO, Copenhagen, Denmark) as reported previously (27, 28, 30). Positive controls were sections known to express the investigated antibodies. Negative controls were obtained by omitting the primary antibodies.

For estimation of nuclear hMLH1 protein expression, the sections were subjected to microwave epitope retrieval \([750\text{ W for 7.5 min and 500 W for 30 min in EDTA buffer (pH 8)}]\) before incubation for 3 h at room temperature with the hMLH1 monoclonal antibody, clone G168-15, code 13271A at 1:50 dilution (PharMingen, San Diego, CA). For estimation of nuclear hMSH2 protein expression, the sections were subjected to microwave epitope retrieval \([750\text{ W for 7.5 min and 500 W for 30 min in EDTA buffer (pH 8)}]\) before overnight incubation at room temperature with the hMSH2 polyclonal antibody, Ab-3, PC57 (Oncogene, Cambridge, MA) diluted 1:50. DAKO TechMate 500 slide-processing equipment was used. The normal staining pattern for hMLH1 and hMSH2 is nuclear, and nuclei in stromal cells were used as internal positive controls. A few cases showed weak cytoplasmic staining, but only nuclear staining was recorded systematically. Staining intensity was graded on a scale of 0 (no staining) to 3 (strong staining), and the percentage of nuclear staining area was graded as 0 (no tumor cells stained positive), 1 (positive staining in <10% of the tumor cells), 2 (positive staining in 10–50% of the tumor cells), or 3 (positive staining in >50% of the tumor cells). A staining index was calculated as the product of nuclear staining intensity and staining area. Tumors with a nuclear staining index \(\geq 4\) were categorized as having normal expression, whereas those with a nuclear staining index \(< 4\) were categorized as having loss of expression.

The immunohistochemical staining procedures and results for microvessel density counts and Ki-67, p53, p21, and p16 expression have been described previously (27, 28, 30, 31).

DNA Ploidy, S-phase Fraction, and Steroid Receptor Analyses. In a subset of the patient population, DNA-ploidy, S-phase fraction, and steroid receptor concentrations in tumor tissue were estimated. Technical details and findings have been reported previously (27, 29) and were available for comparison with the hMLH1 methylation and immunohistochemical results.

MSI. Tumor DNA and corresponding normal DNA were analyzed using a panel of five microsatellite markers for mononucleotide and dinucleotide repeat sequences (BAT 26, BAT 40, D10S187, D18S55, and D18S58). The markers on chromosome 18 and the mononucleotide markers were chosen because they had previously been analyzed in studies of MSI in colorectal or endometrial carcinomas (32–36). BAT 26 lies within the hMSH2 gene on chromosome 2. D10S187 was analyzed because it lies within the area of the PTEN gene that has been shown to be mutated in a high proportion of endometrial cancers with MSI (37). According to a generally accepted categorization, tumors that exhibited shifts in electrophoretic mobility at two or more of the five loci analyzed were classified as microsatellite unstable [MSI positive (32, 35, and 38)].

Methylation Analysis. The methylation status of the promoter regions of the hMLH1 and hMSH2 genes in the tumors was determined by the M-PCR method described by Herman et al. (39). Briefly, tumor genomic DNA was treated with sodium bisulfite, which converts all unmethylated cytosine residues to uracil, which is then converted to thymidine in the subsequent PCR. Primer sets specific for the methylated and unmethylated versions of the hMLH1 and hMSH2 genes were designed to the CpG islands at the 5'-untranslated regions of these genes. For the hMLH1 gene, one primer set specific for the unmethylated sequence (hMLH1-U) and two primer sets specific for the methylated sequence (hMLH1-M1 and hMLH1-M2) were used. The primers specific for the unmethylated hMLH1 sequence have been described previously (18). The hMLH1-M1 primer set
Fig. 1 M-PCR with bisulfite-treated tumor samples. U, reactions using the hMLH1-U primer set specific for the unmethylated CpG sites giving rise to a 124-bp band. M, reactions using the hMLH1-M1 primer set specific for the methylated CpG sites giving rise to an 86-bp band. CI, the positive control for the unmethylated primer set; C2, the positive control for the methylated primer set (see “Materials and Methods”). Because the tumors also contain normal cells, amplification of the unmethylated sequence is seen for all tumors, whereas amplification of the methylated sequence is seen for four tumors (T27, T31, T45, and T53). Reactions containing untreated DNA (C3) and no DNA (C4) were included as negative controls for both primer sets. A 100-bp DNA ladder (Life Technologies, Inc., Gaithersburg, MD) was used for sizing.

The 5'-end of the primers comprising the hMLH1-M1 primer set are located at 865 and 1045 bp, respectively, of the hMLH1 gene (GenBank accession number U83845), whereas in the hMLH1-M2 primer set, the positions are 865 and 1045 bp, respectively. The primers used for the methylated and unmethylated versions of the hMSH2 gene have been described previously (18). We chose to investigate all samples with two different primer sets specific for hMLH1 promoter methylation to allow the analysis of additional CpG sites in the promoter region. All of the tumors were investigated with the methylated primer sets at least twice.

The PCRs were carried out in a 25-μl volume containing 1× PCR buffer II (Perkin-Elmer, Foster City, CA), 2.5 mM MgCl$_2$, 200 μM deoxynucleotide triphosphate, 0.5 μM of each PCR primer, 0.5 unit of AmpliTaq Gold (Perkin-Elmer), and approximately 40 ng of sodium bisulfite-modified DNA. Amplification was carried out in a Perkin-Elmer model 9600 thermocycler at 95°C for 10 min, followed by cycling at 94°C for 45 s, 30 s at the annealing temperature (hMLH1-U, 64°C; hMLH1-M1, 60°C; hMLH1-M2 and hMSH2-M, 62°C; hMSH2-U, 66°C), and 30 s at 72°C (35 cycles for hMLH1-M1, hMSH2-U, and hMSH2-M; 40 cycles for hMLH1-U and hMLH1-M2), followed by a 10-min extension at 72°C. The positive control used for the methylated primer sets for hMLH1 was the SW480 cell line, which lacks hMLH1 expression and has previously been found to be hypermethylated at the 5’ CpG island (19). The positive control for hMSH2-M consisted of lymphocyte DNA treated in vitro with excess SsoI methyltransferase (New England Biolabs, Beverly, MA), generating DNA completely methylated at all CpG sites, followed by treatment with sodium bisulfite. The positive control for the unmethylated primer sets consisted of sodium bisulfite-treated lymphocyte DNA from individuals without cancer. These DNAs were also used as a negative control for the methylated primer sets to exclude non-specific cross-reaction. Reactions containing untreated DNA and no DNA were included as negative controls for all primer sets. The amplification products were separated on a 3% agarose gel and visualized by ethidium bromide staining and UV illumination.

Statistics. Comparisons of groups were performed using the χ² test. Only the cases with conclusive hMLH1 methylation results and results available from all five MSI markers were included in the correlation studies. The median follow-up period for the survivors was 9 years (range, 4–15 years). None of the patients was lost due to insufficient follow-up data (26–28). Univariate survival analyses of time to death due to endometrial carcinoma (cause-specific death) were performed using the product-limit procedure (Kaplan-Meier method), with the time of primary operation as the entry date. Patients who died of other causes were censored at the date of death. The Mantel-Cox test was used to compare the survival curves for groups of patients defined by categories of each variable. Data were analyzed using the SPSS software package.

RESULTS

All of the 138 prospectively collected endometrial tumors were initially investigated with primers specific for the unmethylated hMLH1 and hMSH2 sequence (hMLH1-U and hMSH2-U). As expected, because the samples also contain normal cells, all tumors showed amplification of the unmethylated sequence, as illustrated in Figs. 1 and 2. The 138 tumors were studied for hypermethylation of the hMLH1 gene by M-PCR using two sets of primers specific for the methylated sequence (hMLH1-M1 and hMLH1-M2), allowing the investigation of a higher number of CpG sites in the promoter region. The targeted gene region has been studied previously for methylation in primary colorectal cancer tumors and cell lines (18, 19). Twenty-five of these tumors showed strong amplification for both hMLH1-M1 and hMLH1-M2 primer sets (18), which is indicative of promoter region methylation, whereas 85 cases showed no amplification for both primer sets. The results for a subset of the tumors with the hMLH1-M1 primer set are shown in Fig. 1. Some cases showed amplification with only one of the two sets (n = 8) or inconsistently weak amplification with both primer sets (n = 20), suggesting partial methylation. These 28 tumors were not included in the correlation studies. The results from the 110 conclusive cases are correlated with FIGO stage, histopathological variables, MSI, ploidy, and p53 expression in Table 1. Because previous studies have shown that the number of markers showing instability varies considerably (32, 34), we also wanted to investigate the correlation between hMLH1 methylation and the number of positive microsatellite markers. We therefore divided the MSI-positive tumor group into tumors with high MSI and those with intermediate MSI. Among the tumors...
with high MSI (those positive for 4–5 of 5 markers), 69% showed methylation of \textit{hMLH1} compared with only 10% of the MSI-negative tumors (those positive for 0–1 of 5 markers), whereas in the group of tumors with an intermediate number of microsatellite-unstable loci (those positive for 2–3 of 5 markers), 29% showed methylation of \textit{hMLH1} (\(P, 0.001\)). None of the aneuploid tumors were methylated for \textit{hMLH1} compared with 25% of the diploid tumors (\(P, 0.03\); Table 1). When the aggressive serous papillary/clear cell histological types were excluded from the analyses, the same pattern persisted, with no methylated tumors in the aneuploid group compared with 26% in the diploid group of tumors. Only one of the tumors methylated for \textit{hMLH1} showed overexpression of \textit{p53} protein (6%), whereas 28% of the tumors that did not overexpress \textit{p53} were methylated (\(P, 0.04\); Table 1). This pattern also persisted when the aggressive serous papillary/clear cell histological types were excluded from the analyses, with only one of the methylated tumors showing \textit{p53} overexpression (8%) compared with 30% of the tumors not overexpressing \textit{p53}. Only one methylated tumor was of the clear cell histological type, whereas the others were of the less aggressive histological types; however, the correlation with histological type did not reach statistical significance (\(P, 0.2\)). Methylation of \textit{hMLH1} was not significantly correlated with patient age, FIGO stage, histological grade (Table 1), S-phase fraction, hormone receptor concentration, expression of the proliferation marker Ki-67, \textit{p21} expression, \textit{p16} expression, or microvessel density.

Loss of nuclear \textit{hMLH1} protein expression was seen in 19 of the 138 cases studied (14%). Among the tumors with high MSI, 59% had loss of nuclear \textit{hMLH1} expression compared with only 3% of the MSI-negative tumors (\(P, 0.001\)). The tumors also contain normal cells; amplification of the unmethylated sequence is seen for all tumors, whereas amplification of the methylated sequence is seen for two tumors (T132 and T145). Reactions containing untreated DNA (C3) and no DNA (C4) were included as negative controls for both primer sets. A 100-bp DNA ladder (Life Technologies, Inc.) was used for sizing.

Table 1  Methylation of the \textit{hMLH1} promoter region and \textit{hMLH1} nuclear protein expression related to FIGO stage, histological type, and grade, DNA ploidy, and p53 index in a prospective and population-based endometrial carcinoma study

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>hMLH1 methylation</th>
<th>hMLH1 protein expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II</td>
<td>117</td>
<td>73 (78%)</td>
<td>21 (22%)</td>
</tr>
<tr>
<td>III/IV</td>
<td>21</td>
<td>12 (75%)</td>
<td>4 (25%)</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrioid/adenocanthoma-</td>
<td>123</td>
<td>74 (76%)</td>
<td>24 (25%)</td>
</tr>
<tr>
<td>adenosquamous Serous papillary/clear cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High (grade 1)</td>
<td>24</td>
<td>15 (83%)</td>
<td>3 (17%)</td>
</tr>
<tr>
<td>Moderate (grade 2)</td>
<td>89</td>
<td>56 (75%)</td>
<td>19 (25%)</td>
</tr>
<tr>
<td>Poor (grade 3)</td>
<td>25</td>
<td>14 (82%)</td>
<td>3 (18%)</td>
</tr>
<tr>
<td>MSI, no. of positive markers(c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–1 of 5</td>
<td>74</td>
<td>54 (90%)</td>
<td>6 (10%)</td>
</tr>
<tr>
<td>2–3 of 5</td>
<td>19</td>
<td>12 (71%)</td>
<td>5 (29%)</td>
</tr>
<tr>
<td>4–5 of 5</td>
<td>17</td>
<td>4 (31%)</td>
<td>9 (69%)</td>
</tr>
<tr>
<td>DNA index(d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\geq1.00)</td>
<td>72</td>
<td>44 (75%)</td>
<td>15 (25%)</td>
</tr>
<tr>
<td>(&gt;1.00)</td>
<td>22</td>
<td>14 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>p53 expression(d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index (\leq4)</td>
<td>80</td>
<td>43 (72%)</td>
<td>17 (28%)</td>
</tr>
<tr>
<td>Index (&gt;4)</td>
<td>21</td>
<td>17 (94%)</td>
<td>1 (6%)</td>
</tr>
</tbody>
</table>

\(a\) Twenty-eight cases with inconclusive data for \textit{hMLH1} methylation are not included.

\(b\) \(\chi^2\) test. NS, not significant.

\(c\) Only cases with results available from all five MSI markers are included.

\(d\) Data are not available for DNA index in 44 cases and p53 expression in 37 cases.

Fig. 2  M-PCR with bisulfite-treated tumor samples. \(U\), reactions using the hMSH2-U primer set specific for the unmethylated CpG sites giving rise to a 143-bp band. \(M\), reactions using the hMSH2-M primer set specific for the methylated CpG sites giving rise to a 132-bp band. \(C1\), the positive control for the unmethylated primer set; \(C2\), the positive control for the methylated primer set (see “Materials and Methods”). Because the tumors also contain normal cells, amplification of the unmethylated sequence is seen for all tumors, whereas amplification of the methylated sequence is seen for two tumors (T132 and T145). Reactions containing untreated DNA (C3) and no DNA (C4) were included as negative controls for both primer sets.

A 100-bp DNA ladder (Life Technologies, Inc.) was used for sizing.
Table 2  Correlations between MSI and methylation of the hMLH1 promoter region and hMLH1 nuclear protein expression in primary endometrial carcinomas

<table>
<thead>
<tr>
<th>hMLH1 expression</th>
<th>Normal expression</th>
<th>Loss of expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSI-negative tumors (n = 60)</td>
<td>Methylation – (n = 54)</td>
<td>54</td>
</tr>
<tr>
<td>MSI-positive tumors (n = 30)</td>
<td>Methylation + (n = 16)</td>
<td>16</td>
</tr>
</tbody>
</table>

* Data include only cases with conclusive results for hMLH1 promoter region methylation and all five MSI markers.

DISCUSSION

Our results are in line with previous studies indicating that hMLH1 promoter methylation is a common feature in endometrial carcinomas with MSI (13, 23, 24). However, this study is, to our knowledge, the first to address this issue in a prospective and population-based setting. Esteller et al. (24) selected 29 endometrial carcinomas according to MSI phenotype and found that 12 of 13 MSI-positive cases (92%) were methylated for hMLH1 compared with 1 of 16 MSI-negative tumors (6%). Gurin et al. (13) selected 14 tumors displaying unambiguous evidence of MSI (tumors positive for ≥3 of 5 markers) for further methylation studies and found 10 of 14 MSI-positive cases (71%) to be methylated for hMLH1, whereas 2 of 28 MSI-negative cases (7%) showed a weak band interpreted as partial methylation. Simpkins et al. (23) selected 53 MSI-positive endometrial tumors with ≥2 of 7 markers showing instability and found hMLH1 hypermethylation in 41 cases (77%), whereas methylation was seen in only 1 of 11 MSI-negative cases (9%) studied. Our finding that only 10% of the MSI-negative tumors were methylated for hMLH1 compared with 69% of tumors with high MSI is in line with previous reports. However, we found methylation in 29% of tumors with an intermediate number of microsatellite-unstable loci, indicating that although hMLH1 promoter region hypermethylation plays a more significant role in tumors that are highly microsatellite unstable, it is also a common mechanism of inactivation in this less unstable group as well. The apparently stronger correlations between MSI and hMLH1 methylation reported in previous studies could be due to smaller sample sizes and to the fact that the cases studied were selected according to MSI phenotype (13, 23, 24), which differs from the population-based approach in our study.

Of the 138 tumors we studied for hMLH1 promoter region methylation, 28 (20%) showed results suggestive of partial methylation. Simpkins et al. (23) used HpaII digestion to study the methylation of the hMLH1 promoter region (23) and reported that 3 of 53 cases (6%) had findings indicative of partial methylation. In our study, the relatively large number of cases with results suggesting partial methylation could be due to the different methods applied. Furthermore, in our study, all samples were investigated for methylation with two different primer sets to allow the analysis of a larger number of CpG sites in the hMLH1 promoter, which could also contribute to the difference. Our results suggest that not all CpG sites in the hMLH1 promoter region are equally methylated. This is in line with a recent study of hMLH1 promoter region methylation in colon cancer cell lines by Deng et al. (40) showing that different regions of the hMLH1 promoter tend to have different levels of methylation.

All of the 85 tumors found to be unmethylated in our series showed normal hMLH1 protein expression. This agrees with the findings reported by Simpkins et al. (23), who investigated 24 endometrial tumors for hMLH1 protein expression and found that 10 unmethylated tumors all showed normal protein expression, and supports a strong correlation between hMLH1 promoter region methylation and hMLH1 expression. In our study, we found that 13 of 14 (93%) MSI-positive tumors methylated for hMLH1 showed loss of expression. The one hMLH1-methylated MSI-positive tumor that still expressed hMLH1 was reinvestigated and was found to have a heterogeneous staining pattern. We did not systematically record heterogeneity in the staining pattern for all 138 cases; however, one possible explanation for this finding could be that the M-PCR assay is likely to be more sensitive in detecting small clones of tumors with hMLH1 methylation than the immunohistochemical method. Five of the six MSI-negative tumors that were found to be methylated for hMLH1 showed normal expression of hMLH1.

None of the aneuploid tumors showed methylation of the
hMLH1 promoter region or loss of hMLH1 expression. To our knowledge, this is the first study reporting a significant correlation between hMLH1 methylation and DNA ploidy. However, a previous study on colon cancer found a correlation between MSI and DNA ploidy (34). These findings are in line with the hypothesis of two separate pathways in endometrial tumorigenesis, one through nucleotide instability and mismatch repair deficiency and the other through aneuploidy, as hypothesized by Lengauer et al. (41) for colorectal cancers.

The p53 system has also been identified as a major tumor suppressor pathway in tumorigenesis (42, 43). None of the cases with loss of hMLH1 expression and only one of the tumors with hMLH1 methylation was found to overexpress p53 protein. Increased expression of p53 protein is indicative of p53 alterations, with the concordance between immunohistochemical nuclear p53 overexpression and mutations being reported as 76% in endometrial carcinoma (44). Mismatch repair deficiency leads to mutations that contribute to the oncogenic process. The p53 gene, however, does not appear to be a target for mutations induced by defects in the mismatch repair system because p53 mutation rates have been reported to be equal in MSI-negative and MSI-positive endometrial tumors (45). Our finding that tumors with hMLH1 methylation and loss of hMLH1 protein expression do not overexpress the p53 protein appears to be in line with this finding.

It is important to note that although the correlation between hMLH1 methylation and histological type did not reach statistical significance, only one methylated tumor was of the clear cell histological type, whereas the others were of less aggressive histological types. p53 overexpression has been found to be significantly correlated with aggressive histological types of endometrial carcinoma such as clear cell and serous papillary carcinomas (46), and the possibility that the correlation between hMLH1 methylation and p53 expression found in this study might also be related to histological type should be kept in mind. Overexpression of p53 protein has also been reported to be significantly correlated with aneuploidy (47). Taken together, these data imply that MSI and hMLH1 methylation might represent a separate pathway in endometrial carcinogenesis, distinct from those involving aneuploidy or p53 disturbances. The tendency toward a survival benefit seen for the group of patients with hMLH1 promoter region methylation could be explained by the correlation with diploid tumors and the absence of aberrant p53 protein expression, both of which were previously found to be strong prognostic markers in endometrial carcinoma (28, 29).

Methylation of the hMSH2 promoter region was found in only 2 of the 138 cases studied (1.4%) in our population-based setting. This is in line with one previous study failing to show methylation of the hMSH2 promoter region in 29 tumors investigated (24) and supports available evidence that methylation of the hMSH2 promoter region seems to be infrequent in endometrial carcinoma. The two tumors methylated for hMSH2 in the present study were both MSI negative. They also showed expression of nuclear hMSH2 protein, indicating that these tumors still have one functioning hMSH2 allele.

In conclusion, this prospective and population-based study reinforces the significant correlation between hMLH1 methylation, MSI, and loss of hMLH1 protein expression in sporadic endometrial carcinomas, as well as the lack of hMSH2 methylation in this group of tumors. However, nuclear hMLH1 staining and hMLH1 methylation did not significantly influence survival. Methylation of hMLH1 does not seem to account for all tumors with the MSI-positive phenotype in our population-based setting, indicating that other mismatch repair genes might also be involved and need to be studied further. Our data also suggest that hMLH1 methylation is involved in a distinct pathway in endometrial carcinogenesis, separate from pathways associated with aneuploidy or p53 disturbances.

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