Loss of hMSH2 and hMSH6 Expression Is Frequent in Sporadic Endometrial Carcinomas with Microsatellite Instability: A Population-based Study

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ABSTRACT

Microsatellite instability (MSI) seems to be important in the development of various human cancers including sporadic endometrial cancer. It has previously been shown that alterations in the mismatch repair gene hMLH1 seem to be important for the development of MSI in these tumors. The role of the other mismatch repair genes hMSH2 and hMSH6 has been less well studied, but investigations on patients with hereditary nonpolyposis colorectal cancer indicate that these genes also may be involved. We therefore wanted to investigate the pattern of hMSH2 and hMSH6 expression in a prospective and population-based series of endometrial carcinomas with known hMLH1 expression and MSI status. A total of 138 patients were studied, and pathological staining was seen in 19 cases (14%) for hMLH1, 26 cases (19%) for hMSH2, and 17 cases (12.3%) for hMSH6. Pathological hMLH1 expression was more frequent among tumors with high MSI (those positive for four to five of five markers), whereas pathological expression of hMSH2 and hMSH6 was more frequent among tumors with intermediate MSI (those positive for two to three of five markers). MSI was significantly correlated with pathological expression of hMLH1 (P < 0.001), hMSH2 (P = 0.04), and hMSH6 (P = 0.001). In the group with high MSI, 14 of 16 tumors (88%) showed pathological expression for at least one of the markers. The expression of hMLH1, hMSH2, or hMSH6 did not significantly influence survival. In conclusion, pathological expression of hMLH1 does not seem to account for all tumors with a MSI-positive phenotype in this population-based series of endometrial carcinomas. Our data indicate that the other mismatch repair genes hMSH2 and hMSH6 are also involved, especially in cases with intermediate MSI.

INTRODUCTION

MSI is characterized by small deletions or expansions in tumor DNA, manifested as shifts in allelic electrophoretic mobility. Genetic instability is considered to be responsible for a rapid accumulation of somatic mutations in various tumor suppressor genes and oncogenes, thus playing an important role in the initiation and progression of malignant tumors (1).

MSI was first detected in tumors from patients with HNPCC (2). Germ-line mutations in mismatch repair genes have been reported in these HNPCC families, and hMLH1, hMSH2, and hMSH6 account for the mismatch repair defect in a majority of the patients (3–5). Later studies have also demonstrated that MSI is present in about 20% of sporadic colorectal tumors (6). Endometrial cancer is one of the most common extracolonic tumors associated with HNPCC (7), and MSI has been reported to be present in 9–45% of sporadic cases (8–18).

Previous studies have indicated that loss of hMLH1 expression can account for a large proportion of sporadic endometrial tumors (17, 19, 20). Recently, in a population-based study, we found loss of hMLH1 expression in the majority of endometrial tumors with high MSI (those positive for four to five of five markers), whereas loss of expression was less frequent in tumors with intermediate MSI (those positive for two to three of five markers; Ref. 21). Thus, pathological expression of hMLH1 does not seem to account for all tumors with a MSI-positive phenotype, indicating that other mismatch repair genes might also be involved. With this background, the aim of our study was to investigate the pattern of hMSH2 and hMSH6 expression in a prospective and population-based series of endometrial carcinomas with known hMLH1 expression and MSI status. We also wanted to study hMSH2 and hMSH6 expression in relation to clinicopathological variables, other

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3 The abbreviations used are: MSI, microsatellite instability; HNPCC, hereditary nonpolyposis colorectal cancer; FIGO, International Federation of Gynecology and Obstetrics.
tumor markers, and prognosis in this series with long and complete follow-up.

MATERIALS AND METHODS

Patient Sample. 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 a similar age-adjusted incidence rate of endometrial cancer. The distribution of patient characteristics and the treatment protocol for this period have been reported previously (18, 21, 22). We had fresh tumor tissue from 138 patients available for molecular studies, and these patients were included in further analyses. To investigate the potential selection bias in this patient population from whom frozen tumor tissue had been 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 during the same period but from whom fresh tumor tissue was not available, as reported previously [n = 161 (21, 22)]. 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, who 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.

Immunohistochemistry. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded specimens using the standard avidin-biotin method (Dako, Copenhagen, Denmark; Refs. 21–23). Positive controls were sections known to express the investigated antibodies. Negative controls were obtained by omitting the primary antibodies. The method used for estimation of nuclear hMLH1 protein expression has been described previously (21). For estimation of nuclear hMSH2 protein expression, the sections were subjected to microwave epitope retrieval [750 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 1:500. For estimation of nuclear hMSH6 protein expression, the sections were subjected to microwave epitope retrieval [750 W for 7.5 min and 500 W for 30 min in EDTA buffer (pH 8)] before incubation for 1 h at room temperature with the hMSH6 monoclonal antibody clone 44 (Transduction Laboratories, Lexington, KY) diluted 1:100. DAKO TechMate 500 slide processing equipment was used. The normal staining pattern for hMLH1, hMSH2, and hMSH6 is nuclear, and nuclei in stromal cells were used as internal positive controls. Evaluatable staining was available from all 138 cases for hMLH1 and hMSH6 and from 137 cases for hMSH2 (difference in availability was due to technical reasons). Some cases showed weak cytoplasmic staining, but only the nuclear staining was recorded. Staining intensity was recorded as a score ranging from 0 (no staining) to 3 (strong staining), and the percentage of nuclear staining area was graded as 0 (no tumor cells 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. Thus, a tumor showing strong staining in 10–50% of the tumor cells would have a staining index of 6. This approach was chosen a priori based on previous studies (21–24).

To investigate the observer reproducibility of the hMSH2 staining, a subset of 20 cases of the sections was investigated two times by the same observer (I. S.) and by two different observers (I. S. and H. B. S.). The exact intraobserver agreement for nuclear staining index = 0 versus nuclear staining index > 0 was 95% ($k = 0.77$). The corresponding exact interobserver agreement was 90% ($k = 0.71$).

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 (18)]. Tumors that exhibited shifts in electrophoretic mobility at two or more of the five loci analyzed were classified as microsatellite unstable [MSI positive (18, 25, 26)]. Because several studies have shown that the number of markers showing instability varies considerably (25, 27), we also wanted to investigate the correlation between the number of positive microsatellite markers and expression.

Results from previous studies of other tumor markers were available for comparisons. The methods applied for estimation of DNA ploidy, S-phase fraction, estrogen and progesterone receptor status, microvessels density, and expression of Ki-67, p53, p21, and p16 have been described in detail previously (22–24, 28).

Statistics. Comparisons of groups were performed using the $\chi^2$ test. Only cases with conclusive 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 (23, 24, 29). 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 (23).

RESULTS

The distribution of the staining indices for hMLH1, hMSH2, and hMSH6 is shown in three groups, index $\geq$ 4, index = 1–3, and index = 0 in Tables 1 and 2. Thirteen cases showed complete loss of hMLH1 expression, whereas six cases showed index = 1 (weak staining in <10% of the nuclei). Based on the data distribution and previous studies, all 19 of these cases (14%) were considered to have pathological expression of...
Table 1  Expression of hMLH1, hMSH2, and hMSH6 related to MSI, DNA index (diploid versus nondiploid), and p16 expression in a population-based endometrial carcinoma study

<table>
<thead>
<tr>
<th>Variable</th>
<th>HMLH1 index</th>
<th>hMSH2 index</th>
<th>hMSH6 index</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>0</td>
<td>1–3</td>
<td>≥4</td>
</tr>
<tr>
<td>MSI, no. of positive</td>
<td>0.001</td>
<td>0.17</td>
<td>0.01</td>
</tr>
<tr>
<td>0–1 of 5</td>
<td>74</td>
<td>2 (3%)</td>
<td>0</td>
</tr>
<tr>
<td>2–3 of 5</td>
<td>19</td>
<td>2 (11%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>3–4 of 5</td>
<td>17</td>
<td>7 (41%)</td>
<td>3 (18%)</td>
</tr>
<tr>
<td>DNA index&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1.00 (diploid)</td>
<td>72</td>
<td>7 (10%)</td>
<td>4 (5%)</td>
</tr>
<tr>
<td>&gt;1.00 (nondiploid)</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p16 expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index &lt; 4</td>
<td>24</td>
<td>3 (13%)</td>
<td>2 (8%)</td>
</tr>
<tr>
<td>Index ≥ 4</td>
<td>114</td>
<td>10 (9%)</td>
<td>4 (4%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Missing data for hMSH2 in 1 case and DNA index in 44 cases.

<sup>b</sup>Staining index = 1 for all cases.

<sup>c</sup>Only cases with results available for all five MSI markers are included.

<sup>d</sup>hMSH2 index 0 versus >0; P = 0.04.

Table 2  Expression of hMLH1, hMSH2, and hMSH6 related to survival (Kaplan-Meier estimates)

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of patients</th>
<th>No. of deaths</th>
<th>% survival at 5 years</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMLH1 expression</td>
<td></td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>Index = 0</td>
<td>13</td>
<td>1</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Index = 1–3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index ≥ 4</td>
<td>119</td>
<td>22</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>hMSH2 expression&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Index = 0</td>
<td>26</td>
<td>5</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Index = 1–3</td>
<td>76</td>
<td>10</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>Index ≥ 4</td>
<td>35</td>
<td>8</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>hMSH6 expression</td>
<td></td>
<td></td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Index = 0</td>
<td>17</td>
<td>4</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Index = 1–3</td>
<td>47</td>
<td>5</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>Index ≥ 4</td>
<td>74</td>
<td>14</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Staining index = 1 for all cases.

<sup>b</sup>Missing data for hMSH2 in one case.

hMLH1 (21). Loss of expression was seen in 26 cases (19%) for hMSH2 and 17 cases (12.3%) for hMSH6, and these cases were considered to show pathological expression based on previous studies (30).

Of the 19 cases with pathological hMLH1 staining, additional loss of hMSH2 expression was seen in 1 case, and additional loss of hMSH6 expression was seen in 1 case. The majority of the 17 cases with loss of hMLH1 expression also showed loss of hMSH2 expression (n = 14), whereas 11 of 26 cases showed isolated loss of hMSH2 expression. MSI, defined as shifts in electrophoretic mobility at two or more of the five loci analyzed, was significantly correlated with pathological staining for hMLH1 (P < 0.001), hMSH2 (P = 0.04), and hMSH6 (P = 0.001). The different levels of expression of hMLH1, hMSH2, and hMSH6 are correlated with the various levels of MSI in Table 1. Only the 110 cases with results available for all five MSI markers are included. We did additional analyses categorizing the nuclear staining into smaller groups according to each level of the index, and the pattern shown in Table 1 persisted. This was also the case when separate analyses for staining intensity and staining area were performed.

Pathological hMLH1 expression was more frequent among tumors with high MSI (those positive for four to five of five markers), whereas loss of hMSH2 and hMSH6 expression was more frequent among tumors with intermediate MSI (those positive for two to three of five markers), as illustrated in Fig. 1. In the group with high MSI, 14 of 16 tumors (88%) showed pathological expression of at least one of the markers (9 tumors with isolated pathological hMLH1 expression, 1 tumor with both pathological hMLH1 and hMSH6 expression, and 4 tumors with loss of both hMSH6 and hMSH2 expression; Fig. 2). In the group with intermediate MSI, pathological expression of at least one of the markers was seen in 12 of 19 tumors (63%); 4 tumors with isolated pathological hMLH1 expression, 1 tumor with isolated hMSH6 expression, 2 tumors with isolated hMSH2 expression, and 5 tumors with loss of both hMSH6 and hMSH2 expression). In the group with low MSI, only 12 of 74 tumors (16%) showed pathological expression for at least one of the markers. One of these tumors showed isolated pathological hMLH1 expression, one tumor showed pathological hMLH1 and hMSH2 expression, one tumor...
showed isolated pathological hMSH6 expression, six tumors showed isolated pathological hMSH2 expression, and three tumors showed pathological hMSH6 and hMSH2 expression. By combining the data from all three markers, a larger proportion of the MSI-positive tumors could be accounted for than by using hMLH1 alone (Fig. 3).

None of the aneuploid tumors showed pathological hMLH1 expression, and when the group with pathological expression was compared with the rest, this difference was significant (\( P = 0.05 \)). hMSH2 expression was also significantly correlated with DNA index (Table 1).

Loss of p16 expression was correlated with hMSH6 expression (\( P = 0.02 \)) and hMSH2 expression, although the latter did not reach statistical significance (\( P = 0.09 \); Table 1).

None of the studied markers (hMLH1, hMSH2, or hMSH6) was significantly correlated with patient age, FIGO stage, histological type or grade, estrogen or progesterone receptor concentration, S-phase fraction, microvessel density, or expression of Ki-67, p21, or p53. The expression of hMLH1, hMSH2, and hMSH6 did not influence survival significantly in univariate survival analysis, as shown in Table 2 and Fig. 4.

DISCUSSION

Previous studies have shown that MSI is a common feature in endometrial carcinoma (18). This has been linked to loss of hMLH1 function, and in sporadic cases, promoter region hypermethylation seems to be the major mechanism involved (17, 19, 20). In our recent study of this population, however, pathological expression of hMLH1 did not seem to account for all tumors with MSI-positive phenotype, especially the tumors with intermediate MSI (21). This indication that other mismatch repair genes might be involved in sporadic endometrial carcinoma is supported by the present study. We find that pathological expression of hMLH1 seems to account for the majority of the cases with high MSI, but in the group with intermediate MSI, both hMSH2 and hMSH6 seem to play a more important role. This is in line with a recent study by Staebler et al. (31) reporting loss of expression of hMSH2 in 25% of the MSI-positive endometrial carcinomas and loss of expression of hMLH1 in 41% of the MSI-positive endometrial carcinomas. Another recent but smaller study of endometrial carcinomas among young patients found loss of expression for hMLH1 in 12 of 21 MSI-positive cases and loss of hMSH2 in 4 of 21 MSI-positive cases, indicating that loss of hMLH1 function cannot account for all MSI-positive endometrial carcinomas (32).

The role of the DNA mismatch repair protein hMSH6 in relation to different cancer types has been less clear. In HNPCC families, germ-line mutations of hMSH6 have been reported (5). For HNPCC families, data have suggested that hMSH6 may be involved in a substantial proportion of the patients with lower MSI compared with the patients with defects in hMLH1 (33). Our finding that a relatively high proportion of the tumors with intermediate MSI showed loss of hMSH6 expression seems to be in line with this finding. A recent study of endometrial tumors from HNPCC families by de Leeuw et al. (30) also reported that hMSH6 mutation carriers showed a lower MSI phenotype compared with hMLH1 mutation carriers. Interestingly, this study also revealed that pathological expression of both hMSH2 and hMSH6 was observed in a high proportion of the endometrial carcinomas studied. In a study by Guerrette et al. (34), it was suggested that hMSH2 and hMSH6 exist as heterodimeric proteins, and Acharya et al. (35) demonstrated a protein-protein cross-linking between hMSH2 and hMSH6. This might be in accordance with our findings because we found that the majority of the 17 cases with loss of hMSH6 expression also showed loss of hMSH2 expression.

In conclusion, pathological expression of hMLH1 does not seem to account for all tumors with a MSI-positive phenotype in this population-based series of endometrial carcinomas. Our data indicate that the other mismatch repair genes hMSH2 and hMSH6 are involved, especially in the cases showing intermediate MSI.
The expression of hMLH1, hMSH2, and hMSH6 related to survival. Survival curves are estimated according to the Kaplan-Meier method.

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