Hypermethylation of the hMLH1 Promoter in Colon Cancer with Microsatellite Instability

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ABSTRACT

Recent studies have demonstrated the presence of microsatellite instability (MSI) in tumors from patients with hereditary nonpolyposis colon cancer and in a subset of patients with sporadic colorectal cancer (CRC). In sporadic CRC, three tumor phenotypes have been defined: microsatellite stable (MSS), low-frequency MSI, and high-frequency MSI (MSI-H). Although defective mismatch repair, consisting primarily of alterations in hMSH2 and hMLH1, is believed to be responsible for the MSI phenotype in the majority of patients with hereditary nonpolyposis colon cancer, the genetic defect responsible for this phenotype in sporadic CRC has yet to be clearly delineated. Somatic or germ-line alterations in these two genes have been identified in only a minority of these cases. Analysis of the protein expression patterns of hMSH2 and hMLH1 in unselected CRC, however, suggests that alterations in hMLH1 may account for a majority of the MSI-H cases. In an effort to explore the underlying molecular basis for these findings, we have examined the methylation status of the presumptive hMLH1 promoter region in 31 tumors that vary in regard to their MSI status (MSI-H or MSS), their hMLH1 protein expression (MLH− or MLH+), and their gene mutation (Mut+ or Mut−) status. Hypermethylation of the hMLH1 promoter occurred in all 13 MSI-H/MLH− tumors that did not have a detectable mutation within the hMLH1 gene. Of those MSI-H tumors containing germ-line or somatic alterations in hMLH1 (n = 7, including 3 frameshift, 1 nonsense, 2 missense mutations, and 1 tumor containing multiple mutations: missense, splice-site alteration, and a frameshift), four had a normal methylation pattern, whereas three others demonstrated hypermethylation of the hMLH1 promoter region. Two of these cases had a missense alteration, the other a frameshift alteration. The single MSI-H/Mut+ tumor that had normal hMLH1 and hMSH2 expression, as well as 9 of the 10 MSS cases, lacked methylation of the hMLH1 promoter. Hypermethylation of the hMSH2 promoter was not observed for any of the cases. These results suggest that hypermethylation of the hMLH1 promoter may be the principal mechanism of gene inactivation in sporadic CRC characterized by widespread MSI.

INTRODUCTION

CRC3 is one of the three leading causes of cancer mortality worldwide, with an incidence of ~1 million cases and a mortality of 500,000 annually (1). Although the underlying etiology of colon cancer is still not well understood, genetic susceptibility is known to be an important factor in this disease. In several different studies, the proportion of hereditary CRC has been estimated to be 0.5−13% (2−5). Hereditary CRC can be subdivided into the polyposis and the nonpolyposis syndromes. Only a small proportion of colorectal malignancies are caused by the polyposis syndromes, of which familial adenomatous polyposis is the most frequent. HNPCC, on the other hand, accounts for a much larger proportion of inherited CRC (1−5%) and, until recently, was defined merely by family history (4).

In the past several years, significant progress has been made in elucidating the underlying molecular basis of HNPCC. This genetic disorder can now be explained, in part, by the presence of germ-line mutations in any one of several genes participating in DNA mismatch repair (6−9). Mutations in hMSH2 and hMLH1 are thought to account for ~60% of HNPCC kindreds, whereas only a few patients have demonstrated mutations in the other genes involved in mismatch repair (8−10). Initial clues that DNA mismatch repair played a role in HNPCC came from the observations that tumors from patients with HNPCC (11) and a subgroup of patients with sporadic CRC (12, 13) demonstrated a novel type of genetic instability characterized by alterations within microsatellites, called MSI. In sporadic CRC, three tumor phenotypes have now been defined: MSS, MSI-L (low-frequency MSI), and MSI-H (high-frequency MSI).4 Although defective mismatch repair is thought to be responsible for the MSI phenotype in tumors from patients with HNPCC, the genetic defect responsible for the MSI phenotype in sporadic colon cancer has yet to be clearly delineated. Several studies have not detected germ-line or somatic mutations for either hMSH2 or hMLH1 in a substantial number of MSI-H sporadic colon cancers (14−17).

The availability of antibodies to the various proteins involved in mismatch repair, in particular, hMSH2 and hMLH1, has allowed for the identification of defective mismatch repair by direct examination of tumor specimens (18−22). Using this technique, the presence of tumor MSI, principally MSI-H, has been shown to correlate well with the absence of protein expression for either hMLH1 or hMSH2 (18, 19, 22). However, a more detailed analysis of a small number of MSI tumors lacking expression of the hMLH1 protein has shown an absence of a detectable mutation in a significant number of these cases (22). Furthermore, recent analysis of the protein expression patterns of hMSH2 and hMLH1 in unselected CRC suggests that alterations in hMLH1 may account for a majority of the MSI-H cases (18, 19). Cumulatively, these data suggest that altered expression of hMLH1 may be due to a mechanism other than gene mutation. An alternative mechanism for gene inactivation in these cases is promoter hypermethylation. There is accumulating evidence that this epigenetic mechanism plays an important role in cancer evolution (23). Kane et al. (24) has recently demonstrated the presence of hMLH1 promoter hypermethylation in four CRCs that had loss of hMLH1 expression but no detectable mutations, suggesting that methylation of the hMLH1 promoter plays an important role in the pathogenesis of colon cancer. Here, we sought to further explore the relationship between the presence of somatic or germ-line mutation in hMLH1, hMLH1 protein expression, and tumor MSI status and the methylation status of the hMLH1 promoter region in a group of 31 colorectal carcinomas.

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3 The abbreviations used are: CRC, colorectal cancer; HNPCC, hereditary nonpolyposis colon cancer; MSI, microsatellite instability; MSS, microsatellite stable; MSI-H, high-frequency MSI.

METHYLATION OF THE hMLH1 PROMOTER

Fig. 1. HpaII methylation assay. Examples of the gel images and respective densitometry graphs of three normal (Lanes N)/tumor (Lanes T) pairs: negative (case 29); partial (case 16); and positive (case 8). The first peak in each graph represents calcitonin, the second peak is hMLH1. +, HpaII digest; —, mock digest.

MATERIALS AND METHODS

Patient Population. The methylation status of the hMLH1 promoter was studied in 31 tumors from patients with CRC. MSI, expression of hMLH1 and hMSH2, and the presence hMLH1 and hMSH2 mutations in 14 of these tumors have been reported previously (15, 22). As part of a prospective study of CRC, MSI-H tumors from an additional 17 patients were selected for further analysis.

DNA Extraction. DNA from tumor was extracted by a standard phenol/chloroform procedure following microdissection of frozen tissue. Tumor tissue was cut into 10-μm-thick sections and mounted onto charged glass slides. One reference slide was stained with H&E, and all other slides were stained with toluidine blue. Only those areas containing >70% tumor cells were used for DNA extraction. The corresponding normal control DNA for each patient was derived from peripheral blood. For these specimens, DNA was extracted using the Puregene nucleic acid isolation kit (Gentra).

MSI. Paired normal and tumor DNA were analyzed for MSI with a minimum of six microsatellite markers. PCR and gel electrophoresis were carried out as described by Thibodeau et al. (13). Tumors were classified as MSI-H if >30% of the markers demonstrated instability in tumor-derived DNA when compared to DNA from the corresponding normal tissue (18).

Sequencing. Exons 1–19 of the hMLH1 gene were sequenced in all of the MSI-H tumors using the ThermoSequenase kit (Amersham), essentially as described by Moslein et al. (15).

Immunohistochemical Analysis. Immunohistochemical staining was performed essentially as described by Thibodeau et al. (22).

Methylation Analysis. To determine whether the hMSH2 and hMLH1 presumptive promoter regions were hypermethylated, a PCR-based HpaII restriction enzyme assay was used. Both tumor and normal DNA were digested with the methyl sensitive restriction endonuclease HpaII prior to the PCR. The restriction enzyme digest contained 20 units of HpaII and 0.5 μg of DNA in a 20-μl reaction. A “mock” digest without enzyme was also performed on normal and tumor DNA samples. All samples were incubated overnight at 37°C. The restricted DNA was then included in a PCR with three different primer pairs: the first was directed to the hMLH1 promoter containing two HpaII sites (GenBank accession no. U26559; −430 to −317, sense, 5′-ccgcaagggagaggag-3′, and antisense, 5′-cagccaataggagcagaga-3′); a second to a region within the hMSH2 gene containing a single HpaII site (GenBank accession no. U27346; −422 to −316, sense, 5′-gtttccttctgatgttactcc-3′, and antisense, 5′-cctgggtggggtgtatgc-3′); and a third to a region within the calcitonin gene devoid of HpaII sites (GenBank accession no. X15943; 2952 to 3096, sense, 5′-gcaggtggtttatctcattc-3′, and antisense, 5′-tggcttcaggctctgttac-3′).

For the PCR, the restriction endonuclease-treated samples were diluted 1:20 in 10 mM Tris (pH 8.0)–1 mM EDTA buffer. Multiplex PCR was performed using [32P]dCTP and primer pairs for calcitonin and either hMLH1 or hMSH2 in a 25-μl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 μM of dNTPs, 12.5 pmol of primers, 2 μl of DNA from the digest, and 0.5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer). PCR was performed with a 10-min preheat at 95°C and then 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by extension at 72°C for 10 min. Resultant products were analyzed on 8% polyacrylamide gels, which
RESULTS

For this study, a PCR-based assay was designed to probe the methylation status of the hMLH1 and hMSH2 promoter regions. This assay is based on the ability of the restriction endonuclease, HpaII, to distinguish CpG sites that are methylated versus those that are nonmethylated. Following endonuclease digestion, primers flanking the HpaII sites are used to test the HpaII-treated DNA for PCR amplification. A PCR amplicon is detected only when the original target DNA contains methylated HpaII restriction sites. For each case, normal and tumor DNA both with and without HpaII pretreatment were subjected to two multiplex PCRs. The first multiplex contained primer pairs directed to a region within the hMLH1 promoter (two HpaII sites) and to a region within the calcitonin gene devoid of HpaII sites. The second multiplex contained primer pairs to a region within the hMSH2 promoter (single HpaII site) and to the same region within the calcitonin gene. The calcitonin primer pair served as a control for the PCR. Following gel electrophoresis, the intensity of the band fragments was determined by densitometry (Fig. 1), and the ratio of the hMLH1 or hMSH2 to calcitonin for each of the reactions was then used for subsequent analyses.

Using this assay, we examined the methylation status for both the hMLH1 and hMSH2 promoter regions in a group of 31 tumors that were characterized with regard to their MSI, protein expression, and gene mutation status (Table 1). Of these 31 cases, 13 tumors were MSI-H/Mut−/MLH−. That is, these tumors demonstrated MSI, showed an absence of hMLH1 protein expression, and an absence of a detectable mutation within the hMLH1 gene. Seven cases were also MSI-H/MLH−, but each had either a germ-line or somatic alteration (3 frameshift, 1 nonsense, 2 missense, and 1 multiple mutation) within the hMLH1 gene. One other case was MSI-H/Mut+/MLH+, having a missense mutation within hMLH1. The remaining 10 cases lacked MSI, and all expressed normal protein levels for both hMSH2 and hMLH1.

When DNA from normal control tissue was examined, the hMLH1: calcitonin and hMSH2:calcitonin ratios were less than 0.23 for all cases examined. When DNA from tumor tissue was examined, the hMLH1/calcitonin ratio was less than 0.23 in 14 cases, and these were considered to be nonmethylated (Fig. 2, top; Table 1). The hMLH1: calcitonin ratio was greater than 0.8 in all but four of the remaining samples, suggesting hypermethylation of this region within the hMLH1 gene. The remaining four values were intermediate between the clearly negative (≤0.23) and clearly positive (≥0.8) cases. These tumors appear to exhibit partial hypermethylation at these particular sites. Of interest, the hMSH2:calcitonin ratio was less than 0.23 for all tumors tested (Fig. 2, bottom), indicating that hypermethylation does not occur within this region of the hMSH2 gene.

Of the 21 MSI-H cases, 16 demonstrated hypermethylation of the hMLH1 promoter region (Fig. 2 and Table 1). Of significance, this was the case for all 13 cases in the MSI-H/Mut−/MLH− category. That is, the MSI-H tumors that had altered protein expression but no apparent hMLH1 mutation demonstrated hypermethylation of the hMLH1 promoter. On the other hand, all but one of the five tumors containing frameshift or nonsense alterations in hMLH1, which would be expected to result in truncated protein products had a normal

Table 1 Comparison of MSI, mutation, and protein expression status to methylation status of the hMLH1 promoter

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Category</th>
<th>Gene</th>
<th>Exon</th>
<th>Codon</th>
<th>Base changes</th>
<th>Consequence</th>
<th>MSI status</th>
<th>hMLH1</th>
<th>hMSH2</th>
<th>hMLH1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H+/MLH− (n = 13)</td>
<td>MLH1(So)</td>
<td>7</td>
<td>195</td>
<td>del A Frameshift</td>
<td>+ (6/7)</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>H+/MLH− (n = 7)</td>
<td>MLH1(G)</td>
<td>12</td>
<td>441</td>
<td>G → A Missense, Ala → Thr</td>
<td>+ (6/7)</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>H+/MLH− (n = 7)</td>
<td>MLH1(G)</td>
<td>18</td>
<td>667</td>
<td>A → G Missense, Asp → Gly</td>
<td>+ (4/7)</td>
<td>−</td>
<td>+</td>
<td>Partial</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>H+/MLH− (n = 7)</td>
<td>MLH1(So)</td>
<td>13</td>
<td>499</td>
<td>del G Frameshift</td>
<td>+ (7/8)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>H+/MLH− (n = 7)</td>
<td>MLH1(So)</td>
<td>17</td>
<td>638</td>
<td>G → T Nonsense, Gly → stop</td>
<td>+ (3/7)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>H+/MLH− (n = 7)</td>
<td>MLH1(So)</td>
<td>7</td>
<td>195</td>
<td>del A Frameshift</td>
<td>+ (5/6)</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>H+/MLH− (n = 7)</td>
<td>MLH1(So)</td>
<td>(G)</td>
<td>546−1</td>
<td>g → a Splice acceptor</td>
<td>+ (3/4)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>H+/MLH− (n = 7)</td>
<td>MLH1(G)</td>
<td>19</td>
<td>716</td>
<td>G → A Missense, Val → Met</td>
<td>+ (28/33)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>MSS/ND/MLH− (n = 10)</td>
<td>MLH1(G)</td>
<td>13</td>
<td>492</td>
<td>G → A Missense, Ala → Thr</td>
<td>+ (30/34)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>MSS/ND/MLH− (n = 10)</td>
<td>MLH1(G)</td>
<td>(G)</td>
<td>546−1</td>
<td>g → a Splice acceptor</td>
<td>+ (28/33)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>MSS/ND/MLH− (n = 10)</td>
<td>MLH1(G)</td>
<td>(G)</td>
<td>546−1</td>
<td>g → a Splice acceptor</td>
<td>+ (28/33)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>MSS/ND/MLH− (n = 10)</td>
<td>MLH1(G)</td>
<td>(G)</td>
<td>546−1</td>
<td>g → a Splice acceptor</td>
<td>+ (28/33)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>MSS/ND/MLH− (n = 10)</td>
<td>MLH1(G)</td>
<td>(G)</td>
<td>546−1</td>
<td>g → a Splice acceptor</td>
<td>+ (28/33)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
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<tr>
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<td>MSS/ND/MLH− (n = 10)</td>
<td>MLH1(G)</td>
<td>(G)</td>
<td>546−1</td>
<td>g → a Splice acceptor</td>
<td>+ (28/33)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
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<td>MSS/ND/MLH− (n = 10)</td>
<td>MLH1(G)</td>
<td>(G)</td>
<td>546−1</td>
<td>g → a Splice acceptor</td>
<td>+ (28/33)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>MSS/ND/MLH− (n = 10)</td>
<td>MLH1(G)</td>
<td>(G)</td>
<td>546−1</td>
<td>g → a Splice acceptor</td>
<td>+ (28/33)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>MSS/ND/MLH− (n = 10)</td>
<td>MLH1(G)</td>
<td>(G)</td>
<td>546−1</td>
<td>g → a Splice acceptor</td>
<td>+ (28/33)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>MSS/ND/MLH− (n = 10)</td>
<td>MLH1(G)</td>
<td>(G)</td>
<td>546−1</td>
<td>g → a Splice acceptor</td>
<td>+ (28/33)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>MSS/ND/MLH− (n = 10)</td>
<td>MLH1(G)</td>
<td>(G)</td>
<td>546−1</td>
<td>g → a Splice acceptor</td>
<td>+ (28/33)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>MSS/ND/MLH− (n = 10)</td>
<td>MLH1(G)</td>
<td>(G)</td>
<td>546−1</td>
<td>g → a Splice acceptor</td>
<td>+ (28/33)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
</tbody>
</table>

a IHC, immunohistochemistry; ND, not determined
b So, somatic mutation; G, germ-line mutation.
c +, positive; −, negative. Numbers in parentheses represent numbers of markers showing instability/total number tested.
methylation pattern. Two of the three remaining MSI+ tumors, bearing missense mutations, exhibited hypermethylation of hMLH1. One of these, however, demonstrated only partial methylations. All but one of the MSS tumors (n = 10) lacked methylation.

DISCUSSION

Current data suggest that the vast majority (in excess of 90%) of tumors with MSI-H have an absence of either hMLH1 or hMSH2 protein expression (18, 19). However, in many sporadic MSI-H CRCs lacking protein expression, gene mutations have not been identified. These data suggest that other mismatch repair genes are likely to play a minor role in the etiology of the MSI-H tumor phenotype in sporadic CRC and that other mechanisms of gene inactivation for hMLH1 and hMSH2 need to be examined. In this regard, Kane et al. (24) recently reported that methylation of the hMLH1 promoter region correlated with lack of hMLH1 expression in four CRCs and several cell lines that did not possess hMLH1 mutations.

Here, we have confirmed and extended these initial observations by examining a variety of CRC that vary with respect to their MSI, protein, and gene mutation status. Of 13 MSI-H cases that lacked both hMLH1 protein expression and a detectable gene mutation, all showed some degree of promoter hypermethylation. Conversely, all but 1 of 10 MSS colon cancer cases with normal protein expression showed an absence of methylation within the presumptive hMLH1 gene promoter region. The presence of hypermethylation in tumors that did not have mutations within hMLH1 yet exhibited phenotypic evidence of defective mismatch repair, as well as an absence of hMLH1 protein expression, supports the hypothesis that hypermethylation can abrogate hMLH1 function. It is important to note that the aberrant methylation pattern was detected only for hMLH1. At least with the assay used, the upstream region of hMSH2 was not affected.

It is interesting to note that one of the MSS tumors with no evidence of defective mismatch repair also exhibited some degree of hypermethylation. A similar observation has recently been reported in an endometrial cancer (25). One possible explanation for such an observation is the presence of hemimethylation. This idea is supported by the finding of a reduced level of methylation in the specimen analyzed in this study compared to the majority of other cases showing methylation (Fig. 2). Under these circumstances, the remaining nonmethylated allele would be expected to produce a sufficient amount of hMLH1 protein, resulting in proficient mismatch repair. Alternatively, it may be that methylation of other CpG sites are important in silencing the hMLH1 gene. Methylation of the specific sites examined may be important but may not be sufficient for gene inactivation. Clearly, it will be important to examine the methylation status of all

Fig. 2. HpaII methylation assay. Peak area hMLH1:calcitonin (top) and hMSH2:calcitonin (bottom) ratios for the 31 tumor samples, divided according to MSI status, hMLH1 expression, and hMLH1 mutation status.
CpG sites within a much larger region to define more precisely the sites critical for gene silencing. In this regard, we are in the process of mapping the methylation sites within this region by sequencing bisulfite-treated DNA (26). Our preliminary data shows that the aberrant methylation extends over a 1-kb region and is consistent with the HpaII assay described here.

Of the eight cases harboring either a germ-line or somatic mutation, five did not show hypermethylation of the hMLH1 promoter. The majority of these (four of five) were frameshift or nonsense mutation. These data suggest that germ-line or somatic mutations in hMLH1, along with either allelic loss or the presence of a mutation in the remaining allele, are sufficient for loss of function. Promoter hypermethylation would not be required under these circumstances. The presence of a gene mutation, however, is not sufficient to exclude methylation, because this was observed in three such cases. For these cases, the most likely explanation is that methylation is important in silencing the wild-type allele. A recent study has reported selective silencing of wild-type but not mutant alleles of p16

The mechanism(s) responsible for the aberrant methylation remain unknown. An association between MSI and CpG island methylation at other loci (p16, IGFB3, TSP-1, and HIC-1 genes) has also been reported in CRC (28). Another study (29) reported that methylation of exogenuously introduced retroviral sequences was normal in tumors with MSI but deficient in MSS tumors. These investigators also indicated that the de novo methylation of the introduced sequences was not due to the absence of mismatch repair activity (29). For some loci, such as the estrogen receptor, methylation of CpG islands increase as a function of age in normal cells (30). Methylation at the loci described above (including hMLH1), however, are the result of de novo methylation. There are several possible mechanisms that may alter patterns of methylation, including an increased level of methyltransferase activity (31). Although there is only one known human methyltransferase, there may be modifiers of methylation that may prove important in carcinogenesis (32). Other means by which methylation can be altered include various types of DNA damage incurred by carcinogenic exposure, chemotherapeutic regimens, and other mechanisms (33). Another possibility is a change in chromatin structure that increases the accessibility of specific promoter regions (34). Whatever the mechanism that results in hypermethylation of the hMLH1 promoter region, the close association between hypermethylation and MSI, the loss of hMLH1 protein expression, and the inverse association with specific gene mutations strongly implicates methylation as a means of regulation hMLH1 function.

On the basis of these and previous results (13, 18, 35), defective mismatch repair may account for up to ~20% of the colon cancer burden observed today. The vast majority of these cases appear to be due to a defect in hMLH1, with aberrant methylation being the most common mechanism of gene silencing. The aberrant methylation is likely to be one of the earliest tumorigenic events and may provide an important clue to the cause of cancer in the proximal colon. It is noteworthy that hypermethylation is infrequent in tumors with specific hMLH1 gene mutations. Altered hMLH1 promoter methylation, therefore, may provide a means of helping to distinguish hereditary from sporadic cases of CRC. The absence of promoter hypermethylation in the setting of MSI and an absence of protein expression would suggest the presence of either a germ-line or somatic mutation within the hMLH1 gene.

NOTE ADDED IN PROOF

Following the submission of this work, Herman et al. (36) also reported on the increased frequency of hMLH1 promoter hypermethylmation in CRC.

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